

Neue Methoden zur Bestimmung von NMR

Projektionsrestraints:

Bestimmung der Struktur und Dynamik von nativen und denaturierten Proteinen

Dissertation

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1 Einleitung:

Die Entschlüsselung des menschlichen und anderer Genome hat nicht nur in der wissenschaftlichen Gemeinschaft für großes Aufsehen gesorgt, sondern auch großes Interesse in Politik und Gesellschaft ausgelöst. Genau genommen stehen wir aber mit der Entschlüsselung des menschlichen Genoms (Nature: <http://www.nature.com/genomics>; Nature 15 Februar 2001, 409, 745-964 (No.6822); Science: <http://www.sciencemag.org/content/vol291/issue5507>) erst am Anfang einer viel wichtigeren Aufgabe: Alle neuen Informationen müssen verarbeitet und, was sehr komplex ist, verstanden werden^{1,2}. Einer der wichtigen Punkte wird es sein die Funktion vieler Proteine zu verstehen und diese, in weiterer Folge, in ihrer Funktion unterstützen oder inhibieren zu können. Dies wird es wahrscheinlich ermöglichen viele Krankheiten therapieren oder vielleicht sogar schon vorbeugend behandeln zu können. Um die Funktion eines Proteins zu verstehen ist es notwendig die 3-dimensionale Struktur zu kennen^{3,4}. In den letzten Jahren haben sich weltweit „structural genomic“ Zentren gebildet, deren Aufgabe es ist möglichst viel Strukturen zu lösen und daraus die Funktion dieser abzuleiten. Beispiele für diese Zentren sind: Joint Center for Structural Genomics (<http://www.jcsg.org>), Northeast Structural Genomics Consortium (<http://www.nesg.org>) und das Riken Genomics Science Center (<http://www.gsc.riken.go.jp>). Die zwei wichtigsten Methoden zur Strukturaufklärung von Biomakromolekülen sind Röntgenkristallographie und die Kernresonanzspektroskopie (nuclear magnetic resonance, NMR). Der wichtigste Unterschied zwischen diesen zwei Methoden ist, dass bei der Röntgenkristallographie ein Festkörper (Proteinkristall) und bei der NMR Spektroskopie das Protein in seiner natürlichen Umgebung, in wässriger Lösung, vermessen wird.

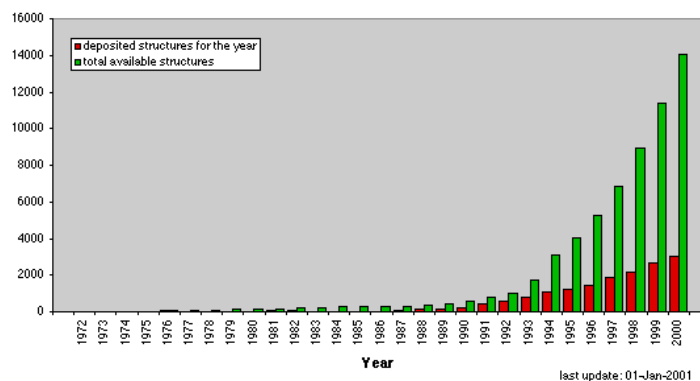


Abbildung 1.1: Anzahl der abgelegten Strukturen in der PDB Datenbank⁵ (Protein-Datenbank; <http://www.rcsb.org/pdb>). Am 3.Juli 2001 waren insgesamt 15531 Protein-, RNA-, DNA- und Kohlenhydratstrukturen gespeichert.

Ungefähr 80% aller aufgeklärten Proteinstrukturen wurden mit Röntgenkristallographie gelöst. Ist ein Proteinkristall gezüchtet, dauert die Aufklärung der 3-dimensionalen Struktur nur wenige Wochen. Das Problem ist jedoch, dass man nicht alle Proteine kristallisieren kann. Dies gilt besonders für membranständige Proteine⁶. Neben der eigentlichen Strukturaufklärung erlaubt die NMR-Spektroskopie auch die Bewegung von Biomakromolekülen auf den verschiedensten Zeitskalen zu beschreiben⁷⁻¹⁰. Dies ist besonders wichtig, wenn man den Mechanismus und die Funktion von Enzymen verstehen will, oder die Wechselwirkung zwischen verschiedenen Proteinen oder Proteinen und Nukleinsäuren erklären möchte.

Es existieren eine Anzahl von Experimenten, welche es erlauben mittels NMR-Spektroskopie Abstands- und Winkelinformationen zu bestimmen¹¹⁻¹³. Diese Experimente ermöglichen somit die Analyse von Konstitution, relativer Konfiguration und Konformation von Molekülen. Zum Beispiel kann man über die quantitative Auswertung von NOESY- und ROESY-Spektren Abstände und mittels skalarer ³J-Kopplungskonstanten Winkel bestimmen.

In den letzten Jahren gab es vier neue Messmethoden in der NMR Spektroskopie, welche die Genauigkeit der Strukturaufklärung erhöhen, gleichzeitig aber auch den zeitlichen Rahmen dieser reduzieren können:

1. Direkte Bestimmung von Wasserstoffbrücken über J-Kopplungskonstanten.
2. Kreuzkorrelierte Relaxation.
3. TROSY (transversal relaxation optimized spectroscopy).
4. Dipolare Kopplungen.

Bis auf die direkte Bestimmung von Wasserstoffbrücken in Proteinen¹⁴ und Nukleinsäuren¹⁵ beruhen diese neuen Messparameter auf anisotropen Wechselwirkungen. Diese anisotropen Wechselwirkungen bewirken entweder Relaxationseffekte, welche durch die Bewegung des Biomakromoleküls hervorgerufenen Modulationen der Energieniveaus durch die Anisotropieeffekte verursacht werden, oder durch eine Frequenzverschiebung aufgrund einer globalen Orientierung des Moleküls entstehen.

Die kreuzkorrelierte Relaxation gestattet es Aussagen über Projektionswinkel und über Dynamik in Molekülen zu machen¹⁶⁻¹⁸. Diese Information kann direkt gemessen werden und es bedarf keiner Kalibrierung der Messwerte wie im Falle von skalaren ³J-Kopplungskonstanten.

Die TROSY-Technik ermöglicht es die zwei wichtigsten Relaxationswechselwirkungen, die Dipol-Dipol-Relaxation und die chemische Verschiebungsrelaxation (chemical shift anisotropy, CSA) gegeneinander auszuspielen^{19,20}. Diese Technik erlaubt somit erstmals den Zugang zu sehr großen Proteinen, Protein-Protein und Protein-Nukleinsäure Komplexen. Es existieren Spektren von Proteinen, die 800 kDa groß sind. Die Zuordnung des Proteinrückgrats, welches die Grundlage für alle weiteren Experimente darstellt, gelang bei einem Protein mit einem Molekulargewicht von 110 kDa²¹. Es soll aber nicht verschwiegen werden, dass es sich hier um multimere Proteine handelt. Monomere, 100 kDa schwere Proteine würden massive Probleme - durch Überlagerung von Signalen - in den NMR Spektren hervorrufen. Aber auch hier gibt es schon Ansätze, diese Probleme durch Blockmarkierung einzelner Domänen mittels Protein-Splicing zu umgehen²². So ist anzunehmen, dass in naher Zukunft Biomakromoleküle im Bereich von 100 kDa untersucht werden können. Ein weiterer wichtiger Punkt ist in diesem Zusammenhang die stetige Weiterentwicklung der Spektrometer, genauer der Magnettechnologie. Seit dem Jahr 2000 stehen erstmals Magnete mit einer Protonenresonanzfrequenz von 900 MHz zur Verfügung, die eine Homogenität von mehr als 10^{-6} Tesla besitzen. Dadurch wird nicht nur eine höhere Dispersion der Signale möglich, sondern der vorher erwähnte TROSY-Effekt wird noch besser nutzbar. Die Dipol-Dipol-Relaxation ist feldunabhängig, jedoch skaliert die CSA mit der Feldstärke. Bei ca. 950 MHz sollte eine maximale Auslöschung der beiden Relaxationskomponenten von H^N -N Paaren auftreten^{19,23}. Allerdings haben diese hohen Dispersionen der chemischen Verschiebungen nicht nur Vorteile. Besonders die ^{13}C -Chemische Verschiebungsdispersion erreicht bei einer ^{13}C -Resonanzfrequenz von 225 MHz einen Frequenzbereich von ca. 45000 Hz. Deshalb benötigt man für solche Spektrometer auch spezielle Pulssequenzen, die es erlauben solche extremen Frequenzbereiche abzudecken. Dafür wurde in der vorliegenden Arbeit eine TOCSY (total correlated spectroscopy) Sequenz entwickelt²⁴, welche adiabatische Pulse zu der Erzeugung einer Hartmann Hahn Bedingung benutzt (Kapitel 2).

Einen wichtigen Beitrag zur Bestimmung der 3-dimensionalen Struktur von Biomakromolekülen liefern dipolare Kopplungen²⁵⁻²⁸. Diese sind erstmals weitreichende globale NMR Strukturparameter, die nicht nur die genaue Definition von lokaler Struktur erlauben, sondern auch die Bestimmung der relativen Orientierung von räumlich weiter als ca. 5 Å entfernten Domänen von Proteinen und Nukleinsäuren. Da die Bestimmung und das

bessere Verständnis der dipolaren Kopplungen ein überwiegender Inhalt dieser Arbeit ist, werde ich diese im Kapitel 3 ausführlich beschreiben.

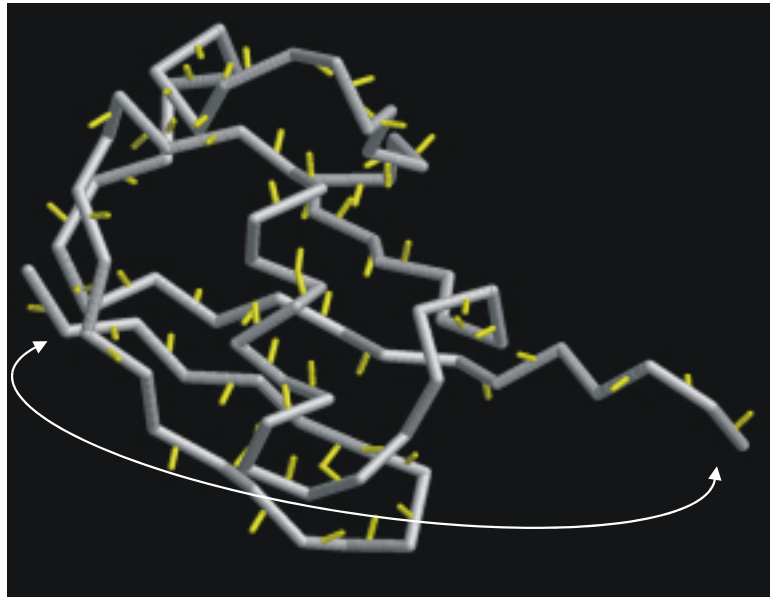


Abbildung 1.2: C_{α} Draht Darstellung von Ubiquitin. In dieser Darstellung ist jeder NH Vektor gelb hervorgehoben. Dipolare Kopplungen sind deshalb so wichtig, da der NH Vektor von Aminosäure Gly 76 auf den NH Vektor der Aminosäure Gln 2 projiziert werden kann.

Wie schon erwähnt gestattet die NMR-Spektroskopie auch die Betrachtung der Dynamik von Biomakromolekülen. Oft kommen Proteine auch in hochdynamischen Zuständen vor: dies ist zum Beispiel für vollkommen oder teilentfaltete Proteine der Fall. Es gibt eine große Anzahl von Beweisen, dass viele Proteine, zum Beispiel Proteine welche in die Signal-Übertragung involviert sind, unter physiologischen Bedingungen größtenteils oder teilweise unstrukturiert vorliegen²⁹⁻³². Viele dieser Proteine bilden erst eine definierte tertiäre Struktur aus, wenn ein Bindungspartner an das Protein andockt. Die NMR-Spektroskopie ist hervorragend geeignet, um diese hochdynamischen Zustände zu charakterisieren³³⁻³⁵. Dies ist besonders deshalb von großem Interesse, da diese Proteine nicht kristallisieren und so nur mittels NMR Spektroskopie auf atomarer Ebene untersucht werden können. Im Rahmen der vorliegenden Arbeit wurden Untersuchungen an denaturiertem Ubiquitin durchgeführt. Dabei sollten Methoden entwickelt werden, welche es erlauben diese Zufallsstrukturen möglichst genau zu charakterisieren und durch ein Modell dieses Random Coil Zustandes (Zufallszustandes) zu beschreiben (Kapitel 4).

Da viele Ergebnisse dieser Doktorarbeit in Publikationen dargelegt sind, werde ich im Hauptteil der Arbeit viele praktische Anleitungen geben, welche ich aus meiner Erfahrung während der Arbeit gewonnen habe. Ich werde auf allzu lange theoretische Abhandlungen

verzichten, da diese in vielen im Anhang zitierten Publikationen nachlesbar sind. Auch werde ich nicht auf Details aus schon publizierten Arbeiten eingehen.

Die meisten Experimente wurden an dem Protein Ubiquitin getestet. Ubiquitin ist ein essentielles Protein, welches in allen Eukaryonten gefunden wird und dort eine entscheidende Rolle als eine Kontrollkomponente im Proteinumsatz spielt. Viel wichtiger für die Etablierung von Experimenten aber ist, dass Ubiquitin wohl eines der am besten NMR spektroskopisch untersuchten Systeme darstellt. Homonukleare NOE's, homonukleare und heteronukleare skalare Kopplungskonstanten^{12,36-38} und auch dipolare Kopplungen^{27,39-44} wurden an Ubiquitin mit höchster Genauigkeit gemessen. Gemessene auto- und kreuzkorrelierte Raten erlauben einen genauen Einblick in die Dynamik von Ubiquitin^{23,45-51}. Außerdem gestattete eine Kooperation mit VLI-Research, einer Firma, welche markiertes Ubiquitin herstellt, das Protein in großen Mengen zu beziehen (<http://www.vli-research.com>). Deshalb hat sich Ubiquitin als ideales Testmolekül für viele NMR Arbeitskreise bewährt.

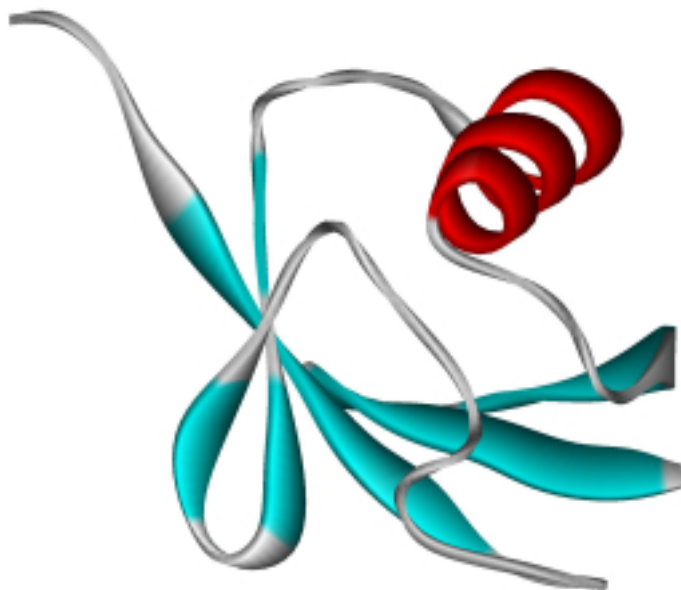


Abbildung 1.3: Ubiquitin ist aus 76 Aminosäuren aufgebaut mit einem Molekulargewicht von 8184.88 Da (bei natürlicher Isotopenverteilung; 8668.31 Da 100% ¹³C und ¹⁵N markiert, 9291.2 Da 100% ¹³C, ¹⁵N und ²H Markierung). Die Sekundärstruktur von Ubiquitin setzt sich aus einer langen zentralen Helix (Aminosäure 23-34), 2 kleinen ³¹⁰ Helices und fünf β -Faltblättern zusammen. Die letzten fünf Aminosäuren am C-Terminus des Proteins zeigen sehr große Flexibilität.

Am Ende der Einleitung möchte ich eine kurze Übersicht über die weitere Gliederung in der vorliegenden Arbeit darstellen. Die Philosophie der Arbeitsgruppe ist es neue NMR Methoden zu finden, welche die Strukturaufklärung von Biomolekülen

erleichtern. Diese neuen Methoden sollen dann an einem biologisch interessanten Zielmolekül angewandt werden. Wie in der folgenden Abbildung dargestellt, gibt es zwei wichtige Parameter für Proteine, welche man untersuchen muss, bevor man Aussagen über die Funktion eines Proteins machen kann.

In Kapitel 2 beschäftige ich mich mit der Strukturaufklärung von Proteinen mittels NMR Spektroskopie. Ein Schwerpunkt darin ist das neu entwickelte adiabatische TOCSY Element, welches z.B. in HCCH-TOCSY Spektren wichtige Hilfe bei der Zuordnung von Seitenketten geben kann. Auch stelle ich kurz Arbeiten am Sensorprotein DcuS vor, in welchen auch das adiabatische TOCSY bei der Seitenkettenzuordnung verwendet wurde.

Das Kapitel 3 beschreibt meine Arbeit auf dem Gebiet der dipolaren Kopplungen. Das Kapitel ist in vier Abschnitte unterteilt:

1. Beschreibung von flüssigkristallinen Phasen zur Messung von dipolaren Kopplungen
2. Messverfahren:
 - dipolare Kopplungen von direkt aneinander gebundenen Atompaaaren
 - dipolare Kopplungen in CH₂ Gruppen
 - „long range“ ¹H-¹H dipolare Kopplungen
3. Auswertung von dipolaren Kopplungen und deren Verwendung in der Strukturrechnung:
 - Spektrenanforderung und Spektrenauswertung
 - Ausrichtungstensorberechnung
 - Beispiele: Neue Wege für die Strukturaufklärung von Polysacchariden in Lösung
Triggerfaktor
Ubiquitin
 - Interpretation der Verteilung der Orientierungen von NH dipolaren Kopplungen

Die genauere NMR Charakterisierung von denaturierten Proteinen beschreibe ich in Kapitel 4. Hierzu wurden denaturiertes Ubiquitin, Lysozym und all-ALA- α -Lactalbumin als Modelle verwendet. Kopplungskonstanten und kreuzkorrelierte Raten, welche die Winkel ϕ und ψ beschreiben, chemische Verschiebungen und autokorrelierte Raten werden dazu verwendet.

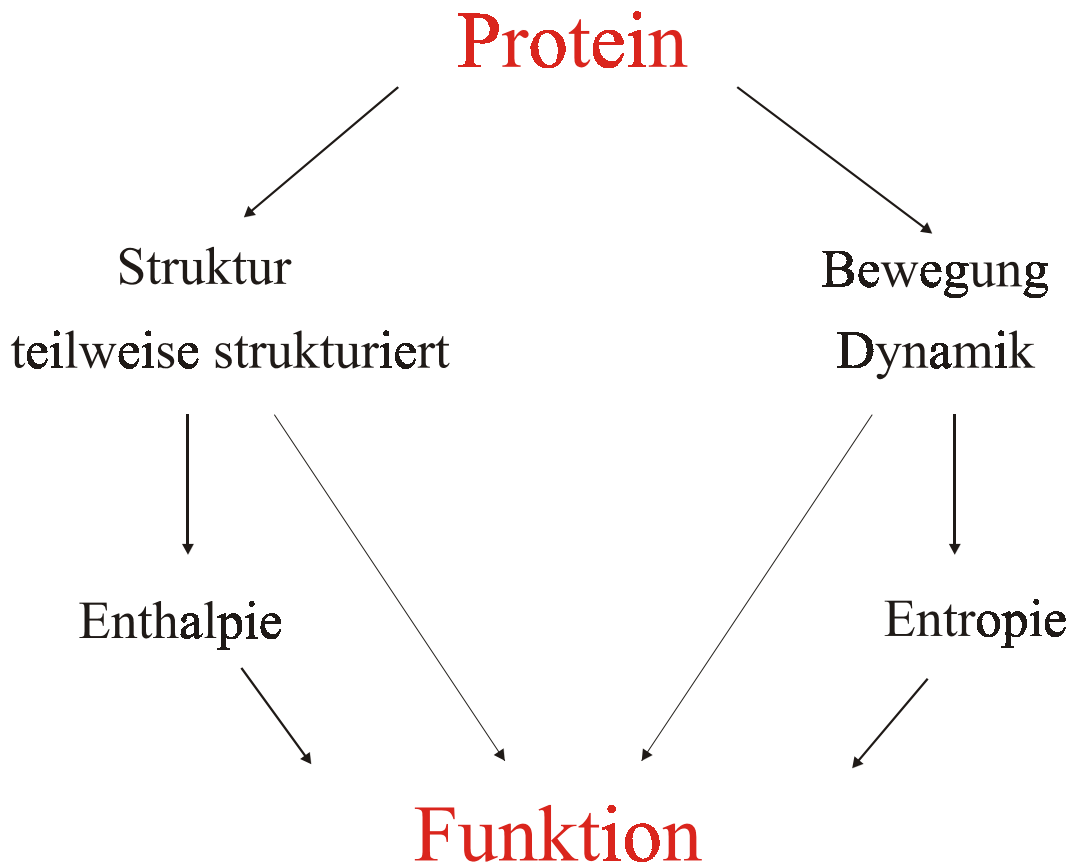


Abbildung 1.4: Die NMR Spektroskopie kann auf zwei Arten wichtige Details bezüglich der Funktionsweise von Proteinen liefern. Über die 3D Struktur und die Dynamik von Proteinen kann man Aussagen über die mögliche Funktionsweise von dem untersuchten Protein machen. Aus den dynamischen Daten ist es gelungen Aussagen über die Entropie eines Proteins zu machen^{52,53}. Ein weiteres Ziel wird es sein auch Aussagen über die Enthalpie aus der Struktur machen zu können.

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2 Strukturaufklärung von Proteinen

Die Strukturaufklärung von Proteinen mittels hochauflösender NMR Spektroskopie ist in den letzten 15 Jahren zu einem wichtigen Werkzeug für alle Naturwissenschaftler geworden¹. Durch die Möglichkeit Proteine vollständig isotonenangereichert biochemisch herzustellen, haben sich vollständig neue Wege für die Strukturbestimmung von Proteinen bis ungefähr 25kDa ergeben². Dadurch wurde es auch möglich mehrdimensionale Experimente einzusetzen, welche die Zuordnung der stark überlappenden Regionen in den NMR-Spektren aufzulösen erlauben. Die Empfindlichkeit dieser Experimente wird zu einem großen Teil durch die Relaxationseigenschaften der aliphatischen Kohlenstoffe bestimmt. Durch die Möglichkeit die an diese Kohlenstoffe gebundenen ¹H durch ²H zu ersetzen, konnte man die größte Relaxationsquelle (Dipol-Dipol-Wechselwirkung) eliminieren^{3,4}. Weitere signifikante Empfindlichkeitssteigerungen konnten durch die TROSY⁵-Technik, welche verschiedene Relaxationswege, wie Dipol-Dipol- und CSA-Wechselwirkungen (chemische Verschiebungsanisotropie, chemical shift anisotropy) bei geeigneten Feldstärken gegeneinander ausschaltet, erreicht werden. Durch diese rasante methodische Weiterentwicklung ist die NMR-Spektroskopie, neben der Röntgenstrukturanalyse, zur wichtigsten Methode der Strukturaufklärung von Biomakromolekülen geworden.

Der Weg der Strukturaufklärung von allen Molekülen ist gleich⁶. Zuerst werden durch COSY und TOCSY-artige Experimente die Spinsysteme zugeordnet. Danach sollte jede einzelne Resonanzfrequenz zugeordnet sein (Zuordnung des Proteins DcuS im Anhang dieser Arbeit). Danach können Abstands-, Winkel- und Orientierungsparameter gemessen werden. Diese werden dann in einer Molekular-Dynamik-Simulation verwendet, um die Struktur der Moleküle zu erhalten. Deshalb bekommt man im Gegensatz zur Röntgenstrukturanalyse nicht eine Struktur, sondern ein Ensemble von Strukturen, welche die experimentellen Parameter am besten erfüllen.

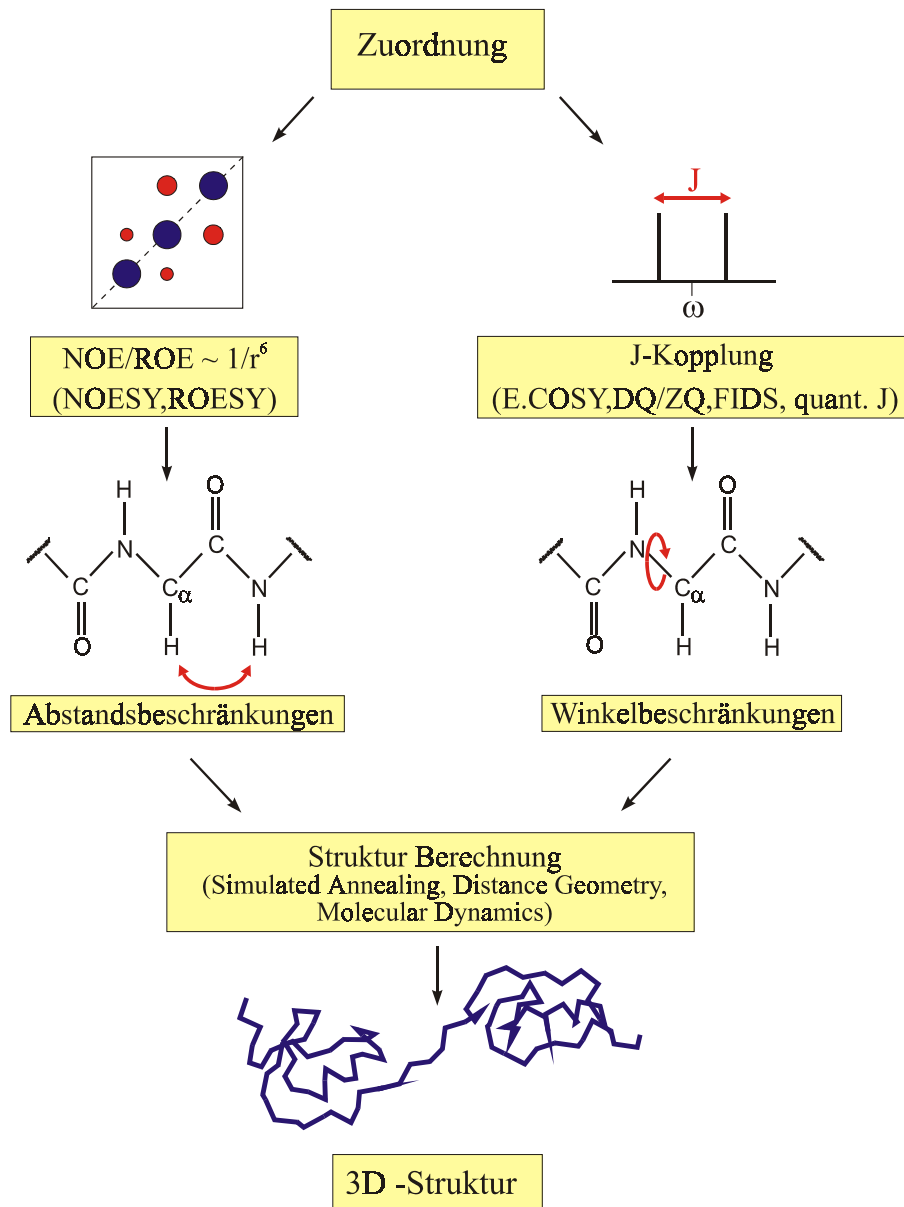


Abbildung 2.1: Strukturaufklärung mittels NMR-Parametern: Nach der Zuordnung können mittels Abstandsbeschränkungen aus NOESY Spektren und Winkelbeschränkungen aus skalaren Kopplungsexperimenten Information über die Tertiärstruktur des Proteins gewonnen werden. Diese können in der Strukturrechnung (meist Simulated annealing (simuliertes „Glühen“ des Proteins) verarbeitet werden.

Die Abweichung der Strukturen untereinander beschreibt der RMSD-Wert. Um die Qualität der Strukturen mit anderen und untereinander zu vergleichen gibt es seit dem

einfachen Zugang zu dipolaren Kopplungen auch den Q-Wert⁷
$$Q = \frac{\sum_{i=1}^n (D_{\text{exp}}^i - D_{\text{calc}}^i)^2}{\sum_{i=1}^n (D_{\text{exp}}^i)^2}$$

oder den R-Wert⁸, welcher allerdings nur ein leicht modifizierter Q-Wert ist: $R = \frac{Q}{\sqrt{2}}$. Der

R-Wert wurde früher auch schon für NOE-Intensitäten benutzt, stellte sich aber als problematisch heraus und fand daher nur wenig Verbreitung. Dies ist darauf zurückzuführen, dass ein direktes Refinement auf NOE-Intensitäten nicht durchgeführt wird.

Die klassische sequenzielle Zuordnung von unmarkierten Proteinen basiert auf der Auswertung von folgenden zweidimensionalen homonuklearen ^1H - ^1H Korrelationsexperimenten: COSY⁹, TOCSY¹⁰, NOESY^{11,12} und ROESY¹³. Die zwei zuletzt genannten Experimente erlauben es Abstandsinformation zu bekommen. Zusammen mit ^3J -Kopplungskonstanten aus COSY Spektren bilden sie so die Grundlage der Strukturaufklärung⁶. Mit Hilfe dieser ^1H - ^1H Korrelationsexperimente kann man Proteine bis ungefähr 6-10 kDa, abhängig von der Sekundärstruktur des zu untersuchenden Proteins, aufklären. Da die Überlagerung der Resonanzen und die Linienbreiten aber schnell mit dem Molekulargewicht zunehmen, kann man als nächsten Schritt ^{15}N -markierte Proteine verwenden, welche den Zugang zu 3-dimensionalen heteronuklearen Spektren ermöglichen. Bei ^{15}N - und bei ^{15}N -, ^{13}C -markierten Proteinen bildet das ^1H , ^{15}N -HSQC¹⁴ das wichtigste Spektrum für die Strukturaufklärung. Es ist wie ein Fingerabdruck des Proteins. Verändert sich das ^1H , ^{15}N -HSQC-Spektrum während den vielen Messungen, die für eine vollständige Strukturaufklärung notwendig sind, so ist dies ein deutlicher Hinweis, dass sich die Probe verändert hat. Deshalb sollte man vor jeder multidimensionalen Messung ein ^1H , ^{15}N -HSQC aufnehmen, um so die Probe zu überprüfen, denn das ^1H , ^{15}N -HSQC darf sich über den gesamten Zeitraum der Spektrenaufnahme nicht verändern. Sieht man jedoch Veränderungen in den chemischen Verschiebungen der Peaks ist schnell klar, dass die Probe sich verändert hat. In Abbildung 2 sind verschiedene ^1H , ^{15}N -HSQC Spektren von unterschiedlichen Proteinen gezeigt. Die meisten 3- und 4-dimensionalen Methoden bauen auch in zwei Dimensionen auf das ^1H , ^{15}N -HSQC-Spektrum auf. So kann man bei ^{15}N -markierten Proteinen TOCSY-HSQC¹⁵, NOESY-HSQC¹⁶⁻¹⁸ oder HSQC-NOESY-HSQC¹⁹ Spektren aufnehmen. Man erzielt so einen drastischen Auflösungsgewinn gegenüber den zweidimensionalen Methoden. Auch sind die Experimente alle relativ empfindlich, da die $^1\text{J}_{\text{HN}}$ -Kopplung fast immer uniform 93.5Hz beträgt, wodurch die Zeit für den Magnetisierungstransfer sehr kurz ausfällt.

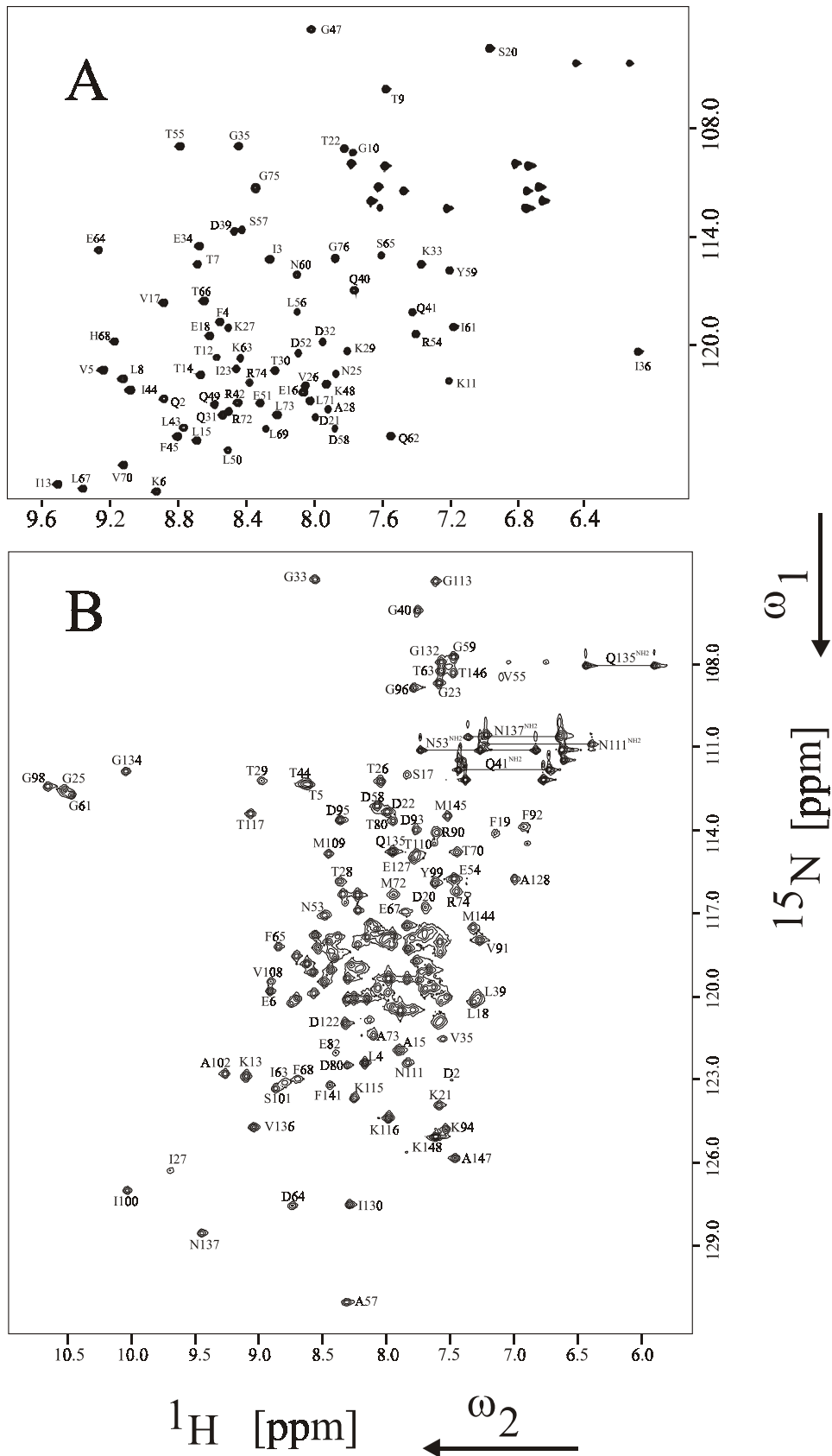


Abbildung 2.2: $^1\text{H},^{15}\text{N}$ HSQC Spektren: (A) Ubiquitin: 76 Aminosäuren, 1 α -Helix und 5 β -Faltblätter; (B) Calmodulin/C20W²⁰: 148 Aminosäuren, ausschließlich α -helikal.

Da in den letzten Jahren die Preise für $^{15}\text{N-NH}_4\text{Cl}$ ($^{15}\text{N}-(\text{NH}_4)_2\text{SO}_4$) und ^{13}C -Glucose, welche bei der Proteinexpression als Isotopenquelle verwendet werden, immens gefallen sind, werden fast alle Proteine ^{13}C - und ^{15}N -markiert überproduziert (Preise Frühjahr 2001: 1g ^{15}N NH_4Cl 70DM, 1g ^{13}C -Glucose 450DM). Dies ermöglicht nun auch Magnetisierung über kohlenstoffgebundene Atome zu transferieren und hier Informationen wie die chemische Verschiebung abzufragen. Schnell wurde klar, dass besonders die C_α und C_β chemischen Verschiebungen eindeutig sind für die Erkennung von Spinsystemen; für deren sequenzielle Verknüpfung liefern sie wertvolle Informationen. Deshalb werden heute folgende Sequenzen routinemäßig für die Zuordnung von Proteinen verwendet: HNCO^{21-23} , HNCACB^{24} , CBCACONH^{25} . Nur noch von untergeordneter Bedeutung sind Sequenzen, welche nur Information über die C_α chemische Verschiebung, wie $\text{HNCA}^{21,22}$ und $\text{HNCOCA}^{22,26}$ liefern. In der Literatur kann man viele Übersichtsartikel über diese heteronuklearen 3D-Experimente finden. Einer der aktuellsten ist von Michael Sattler et al.²⁷.

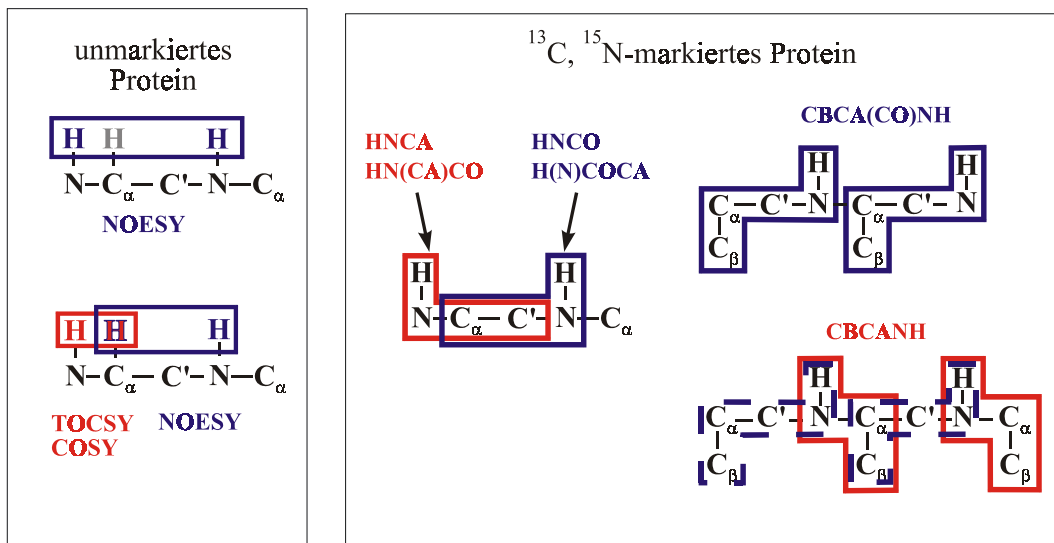


Abbildung 2.3: Zuordnungsstrategien: Im linken Rahmen ist die interresiduale und sequenzielle Zuordnungsstrategie in unmarkierten Proteinen dargestellt. Im rechten Fenster sind die verschiedenen Zuordnungsstrategien für doppelt markierte Proteine gezeigt. Da die chemische Verschiebungsinformation des C_β Atoms oft sehr eindeutig, besonders zusammen mit der C_α chemischen Verschiebung, für eine Aminosäure ist, hat sich der HNCACB und CBCACONH Weg als der einfachste herauskristallisiert.

Viele der Experimente sind unterschiedlich empfindlich, abhängig im Besonderen von der Länge der Delays für den Magnetisierungstransfer (invers proportional zu der Kopplungskonstanten der zwei verknüpften Atome, s.a. Abbildung 4).

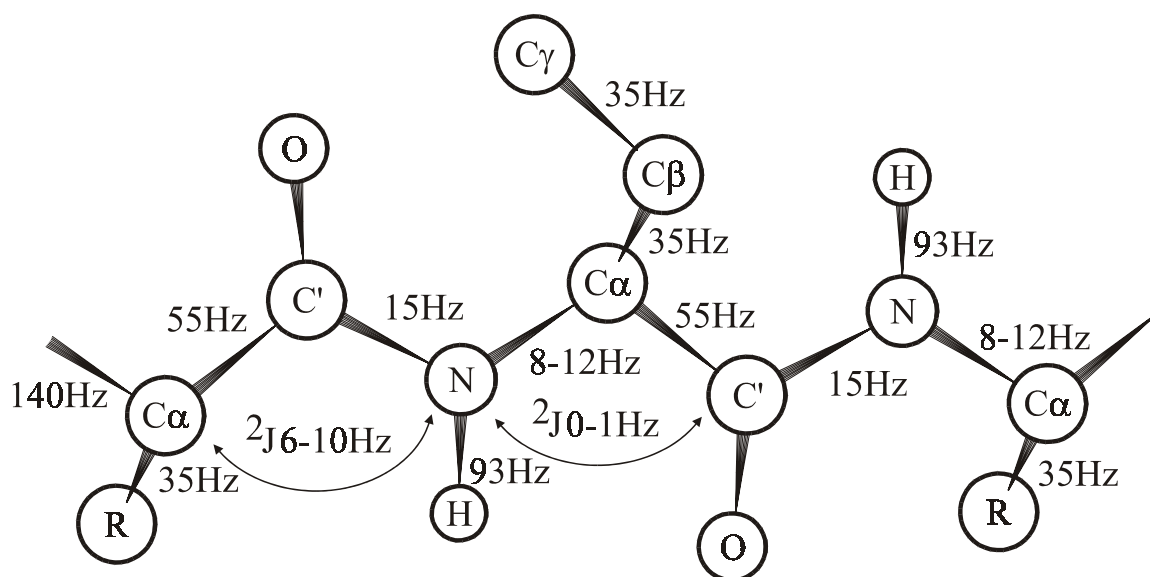


Abbildung 2.4: Kopplungskonstanten in Proteinen: Die wichtigsten Kopplungskonstanten im Proteinrückgrat.

Allgemein ist die Empfindlichkeit der Experimente, seit der Entwicklung der Kryokopf-Technologie²⁸⁻³¹ von Bruker, Varian und Nalorac, und der Möglichkeit solche Köpfe zu bauen, nicht mehr ganz so limitierend. Bei der Kryoprobenkopf-Technologie werden alle elektronischen Teile mit Helium gekühlt, so dass die leitenden Teile bei 20°K operieren. Damit kann der Widerstand erheblich verringert werden und so die Empfindlichkeit etwa um einen Faktor 3 bis 4 gesteigert werden. Am Beginn meiner Doktorarbeit haben wir einen neuen Langspul- (dies war eine neue Technik, welche es ermöglichte mehr aktives Volumen, also mehr Probe zu messen) TXI HCN z-Gradienten Probenkopf an einem DRX 600 MHz Spektrometer erhalten. Dieser zeigte bei einem Test auf der Standard Ethylbenzol Probe eine Empfindlichkeit von ungefähr 1000:1, was damals (1998!) als außergewöhnlich gut galt. Heute erreicht man an einem DRX 600 MHz Spektrometer mit einem TXI HCN z-Gradienten Kryoprobenkopf eine Empfindlichkeit von über 4500:1, bei einem DRX 800 MHz Spektrometer von mindestens 7500:1. Dies bedeutet, dass die Messzeit um den Faktor 55 verringert werden kann (z.B. statt 3 Tagen Messzeit für ein typisches 3D Spektrum nur noch 90 Minuten!), oder aber die Konzentration der Proben drastisch verkleinert werden kann (nun kann man eine 200 μ M Probe in der Zeit messen, die früher für eine 1.5mM konzentrierte Probe gebraucht wurde).

Nachdem man nun das Proteinrückgrat zugeordnet hat, fehlt noch die wichtige und oft sehr zeitaufwendige Zuordnung der Kohlenstoff- und Protonen-Seitenketten Resonanzen. Hierzu kann man HBHACONH³², HCC(CO)NH oder ¹⁵N-TOCSY-HSQC Experimente verwenden. Eine weitere wichtige Gruppe von Experimenten stellen die

HCCH-COSY³³ und HCCH-TOCSY³⁴ Sequenzen dar. Diese bauen nicht auf ein ¹H,¹⁵N-HSQC-Spektrum auf. Deshalb ist es essentiell, zumindest die C_α und H_α chemischen Verschiebungen zu kennen. Sind die C_α und H_α chemischen Verschiebungen bekannt, ist es möglich mittels der Spektren des HCCH Typs die Seitenketten vollständig zuzuordnen. Es ist sehr wichtig, eine möglichst vollständige Zuordnung der Seitenkettenprotonen zu haben, weil nur so NOE's zwischen den Protonen eindeutig zugeordnet werden können.

Der Fortschritt der NMR Spektroskopie geht in den letzten Jahren immer Hand in Hand mit einer ständigen Steigerung der Magnetfeldstärke (Resonanzfrequenz) einher. Dies führt zu einer immer besseren Auflösung der Signale und einer höheren Empfindlichkeit. Letztes Jahr haben beide großen Hersteller von NMR Spektrometern (Bruker, Rheinstetten, Deutschland; Oxford Magnet/Varian, Palo Alto, USA) geschafft langzeitstabile Instrumente mit einer Protonenresonanzfrequenz von 900 MHz auf den Markt zu bringen. Die Auslieferung der ersten Instrumente wurde gerade begonnen. Der Schritt von 800 zu 900 MHz bringt eine Steigerung der Auflösung im 1D um 12.5%, im 4D aber schon um 50% und in der Empfindlichkeit um ungefähr 20% und dies ohne den TROSY Effekt zu berücksichtigen. Dies erlaubt besonders Proben mit noch geringeren Probenkonzentrationen zu messen. Auch wird die zuvor schon vorgestellte TROSY Technik hier, durch die noch größere ¹⁵N-CSA, einen noch größeren Effekt auf die Linienbreite bei großen Proteinen haben. Dies erlaubt mit der gleichzeitig herausragenden Auflösung, die Struktur immer größerer Proteine und Proteinkomplexe aufzuklären.

2.1 *Adiabatische TOCSY-Sequenzen*

Natürlich darf man nicht verschweigen, dass immer größere Feldstärken auch Probleme aufwerfen. In diesem großen Frequenzbereich wird es immer problematischer die gesamte spektrale Weite homogen anzuregen. Das gleichzeitige homogene Anregen eines breiten Frequenzbereiches setzt voraus, dass die 90° Anregungspulse sehr kurz sind. Dadurch werden auch Inversions- und Entkopplungspulse immer kürzer und brauchen deshalb mehr Leistung. Diese kann aber nicht mehr von den Probenköpfen verarbeitet werden, was besonders kritisch bei dem Kohlenstofffrequenzbereich ist: bei 900 MHz Protonenfrequenz beträgt die Resonanzfrequenz ca. 225 MHz. ¹³C Resonanzsignale von Proteinen erfassen einen Bereich von ungefähr 200 ppm also 45000 Hz.

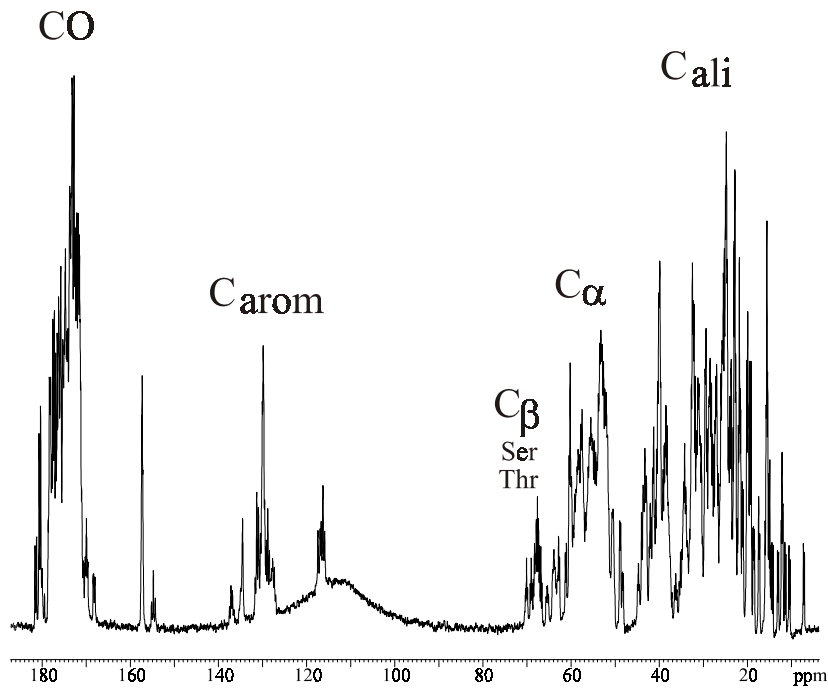


Abbildung 2.5: ^{13}C 1D Spektrum von denaturiertem Ubiquitin; Bruker DRX 600, TXI-HCN-z-Grad Probenkopf, 5mg ^{13}C , ^{15}N Ubiquitin in 250 μl Shigemi Röhren, 303K, pH 2.

Deshalb verwendet man bandselektive Gaußkaskaden^{35,36} als Anregungs- (G4 und Q5) und Inversionspulse (G3 und Q3) für bestimmte Typen von Kohlenstoffatomen (z.B. C', oder den aliphaten Bereich). Natürlich kann man alternativ auch selektive Rechteckpulse mit den Feldstärken von γB_1 (90° Puls) = $\pm \left(\frac{\Omega}{\sqrt{15}} \right)$ bzw. γB_1 (180° Puls) = $\pm \left(\frac{\Omega}{\sqrt{3}} \right)$ einsetzen,

welche einen Nulldurchgang im Anregungsprofil bei einem Offset Ω besitzen. Neben diesen Modifikationen kann man besonders zur Inversion und zur Entkopplung frequenzmodulierte Pulse unter Ausnutzung der sogenannten „adiabatic fast passage“ verwenden^{37,38} (Adiabate: Kurve im pV Diagramm mit gleichbleibender Entropie

$\left(\Delta S = \frac{\Delta Q}{T} \right)$; Da die Entropieänderung proportional zur Wärmeänderung ($\Delta S \propto \Delta Q$) ist,

gilt für einen adiabatischen Zustand $\Delta Q = 0$). Adiabatische Pulse sind aber bei genauerer Betrachtung nicht Adiabatisch. Wie gerade erwähnt gilt für einen adiabatischen Zustand $\Delta S = 0$. Es gilt aber für alle Pulse und Evolution unter Vernachlässigung der Relaxation: $S = -\text{trace}(\rho \ln \rho)$. Diese Tatsache ist gültig für alle Transformationen. Die Namensgebung „Adiabatischer Puls“ stammt von Felix Bloch^{39,40}. Anscheinend hat Felix Bloch diesen Fakt nicht gleich realisiert^{39,40} und so hat sich die Nomenklatur adiabatischer Puls seit dieser Zeit gehalten. Adiabatische Pulse sind Pulse bei denen die Dichtematrix

immer proportional zum Hamiltonian ist $(\rho \sim H(t))^{41}$. Bei der Verwendung von Rechteckpulsen bleibt die Trägerfrequenz während der Dauer des Pulses konstant. Die Trägerfrequenz sitzt üblicherweise in der Mitte des anzuregenden spektralen Bereichs. Bei adiabatischen Pulsen ändert man während der Dauer des Pulses die Trägerfrequenz. Der gesamte Frequenzbereich eines Spektrums wird überstrichen. Die Geschwindigkeit der Frequenzänderung ist bei adiabatischen Pulsen in der hochauflösenden-NMR-Spektroskopie sehr schnell. Adiabatische Pulse werden bevorzugt als Inversionspulse verwendet. Sobald ein Spin mehr oder weniger on resonance zur Radiofrequenz ist, wird er invertiert, so dass jeder Spin abhängig von seiner zu verschiedenen Zeitpunkten invertiert wird. Ein adiabatischer Puls kann sehr große Bandbreiten mit geringer Leistung invertieren. Durch den Sweep der Radiofrequenzpuls-Frequenz von einer Seite des Resonanzspektrums zur anderen Seite ist die Gesamtrotation der Magnetisierung praktisch unempfindlich gegen Änderungen der B_1 Feld Amplitude.

1998 konnte Eriks Kupce⁴², zusammen mit der Gruppe von Gerhard Wagner, erstmals zeigen, dass es möglich ist auch einen homonuklearen H,H-TOCSY-Transfer mittels adiabatischen Pulsen (ca-Wurst) zu erzeugen. Dies initiierte unsere Untersuchung ob dies nicht auch für den viel größeren aliphatischen Kohlenstofffrequenzbereich möglich ist. Derzeit wird für C-C Transfer die DIPSI-3⁴³ Sequenz als Standard verwendet. Durch das Verwenden von tanh/tan frequenzmodulierten und BIR-4 adiabatischen Pulsen, welche in besonderer Weise phasengezykelt werden, ist es uns gelungen eine adiabatische TOCSY Sequenz vorzustellen⁴⁴, deren Bandbreite die von DIPSI-3 übertrifft. Die benutzten Phasenzyklen haben bisher noch wenig Verwendung in der hochauflösenden NMR Spektroskopie gefunden. Die Grundelemente der Phasenzyklen wurden von Alexander Pines (Universität Berkeley) durch die Verwendung von Iterativen „Maps“ (Abbildungen), welche den Weg zum Propagator weisen, gefunden^{45,46}. Es wurden Abbildungen gesucht, welche im Lie Raum der Gruppe (SO(3)) einen Fixpunkt mit hoher Stabilität besitzen. Der (SO(3)) Raum steht für eine orthogonale Gruppe in 3 Dimensionen mit folgenden Eigenschaften: alle reellen orthonormalen 3x3 Matrizen deren Determinante +1 ist, sind enthalten („S“ steht für speziell, hiermit sind gerade diejenigen Rotationen mit $\det(R_{ij})=+1$ gemeint). Der Lie Raum ist ausreichend für die Beschreibung alle Rotationen von Spins $([I_x, I_y] = iI_z)$. Um Spins zu drehen, braucht man einen Propagator, welcher eine Rotation um einen bestimmten Winkel beschreibt. Die für unsere Zwecke

beste Sequenz war bistabil ($1, \pi$), hatte also ihr Stabilitätsmaximum für 0° und 180° Pulse. Dass 0° Pulse stabile Abbildungen ergeben ist eine triviale Forderung. Für eine bistabile Sequenz sind jedoch mindestens 7 Phasen notwendig. Allerdings zeigt erst die Sequenz mit 9 Phasen ausreichende Stabilität für Magnetisierungen auch außerhalb der x-y Ebene. Deshalb liefert der Phasenzklus $\{0,15,180,165,270,165,180,15,0\}$ den best möglichen Inversionspuls (P_9 -Zyklus)⁴⁶. Natürlich könnte man noch weitere bistabile Punkte im Lie-Raum finden, welche aber noch komplexer wären. Dies würde automatisch einen längeren Phasenzklus bedeuten, was wiederum in unserem Fall die Flexibilität der TOCSY-Mischzeit stark begrenzen würde. Allerdings sind besonders bei Proteinen bestimmte Mischzeiten vorgegeben, da die relativ kurzen Relaxationszeiten und die Spintopologie diese vorgeben. Um das Breitbandverhalten zu erhöhen wurde der P_9 -Puls noch MLEV-16 gezykelt.

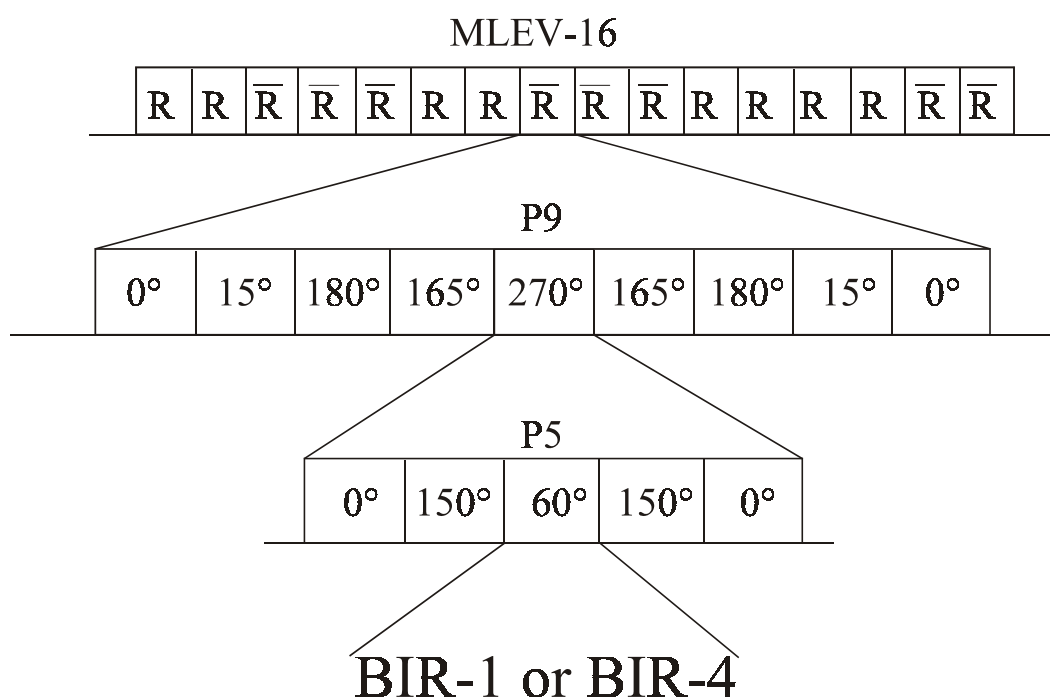


Abbildung 2.6: Schematischer Aufbau der Phasenzyklen für H,H adiabatische TOCSY Sequenzen. Die grundlegende Idee ist es einen immer perfekteren 180° Puls zu erzeugen. Wie von Alexander Pines und seiner Gruppe gezeigt ist es besser verschiedene Zyklen ineinander zu bauen als einen Zyklus öfter zu wiederholen. Der P_5 Zyklus $(0,150,60,150,0)$ hat sich als stabiler Zyklus schon bei Entkopplungssequenzen etabliert und wurde hier auch für TOCSY verwendet.

Diese Sequenz bringt eine ca. 15-20%ige Intensitätssteigerung bei gleicher oder besserer Bandbreite im Vergleich zu den schon etablierten Sequenzen. Auch die Feldstärke (γB_1) ist etwas geringer als bei Sequenzen mit harten Rechteckpulsen. Eine besondere Eigenschaft

von adiabatischen Pulsen ist deren große Robustheit gegenüber B_1 -Feld-Inhomogenitäten und falscher Pulsabstimmung. Diese Eigenschaften zeigen die adiabatischen Pulse auch, wenn sie in einer TOCSY-Sequenz als Inversionspulse eingesetzt werden. So haben sich unsere adiabatischen TOCSY-Sequenzen auch als sehr robust gegen falsche Kalibrierung der Pulse herausgestellt. Sogar bei Pulsen, welche um 4 dB falsch kalibriert waren (dies entspricht einem 55° Puls statt eines 90° Pulses), konnte man noch einen Transfer erzielen. Deshalb können unsere Sequenzen auch optimal in automatisierter Umgebung eingesetzt werden. Erfreulicherweise war es möglich H,H, adiabatische TOCSYs auch bei 900 MHz zu messen (Magnet des Scripps Research Institutes Prof. P. Wright). Diese zeigen, dass wie vorhergesagt die Sequenzen bei höherem Feld noch bessere Ergebnisse bringen und die DIPSI Sequenzen deutlich überholen.

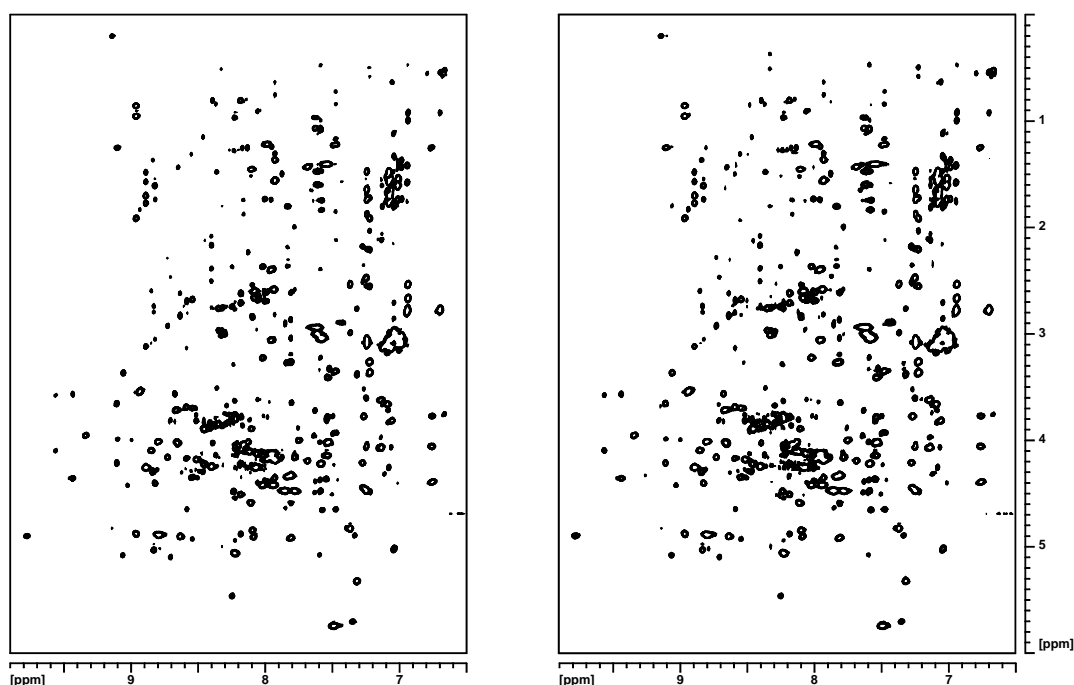


Abbildung 2.7: 900 MHz H,H-TOCSY-Spektren gemessen an 2mM Lysozym: links: DIPSI-2 10 kHz; rechts: tanh/tan (300 kHz, 50 μ s, 10.6 kHz, $\zeta = 10$, $\tan \kappa = 20$) mit P5P9M16 Superzyklus. Beide Spektren wurden mit einer Mischzeit von 72 ms gemessen. Es ist deutlich zu erkennen, dass die adiabatische TOCSY Sequenz eine größere Bandbreite innehat.

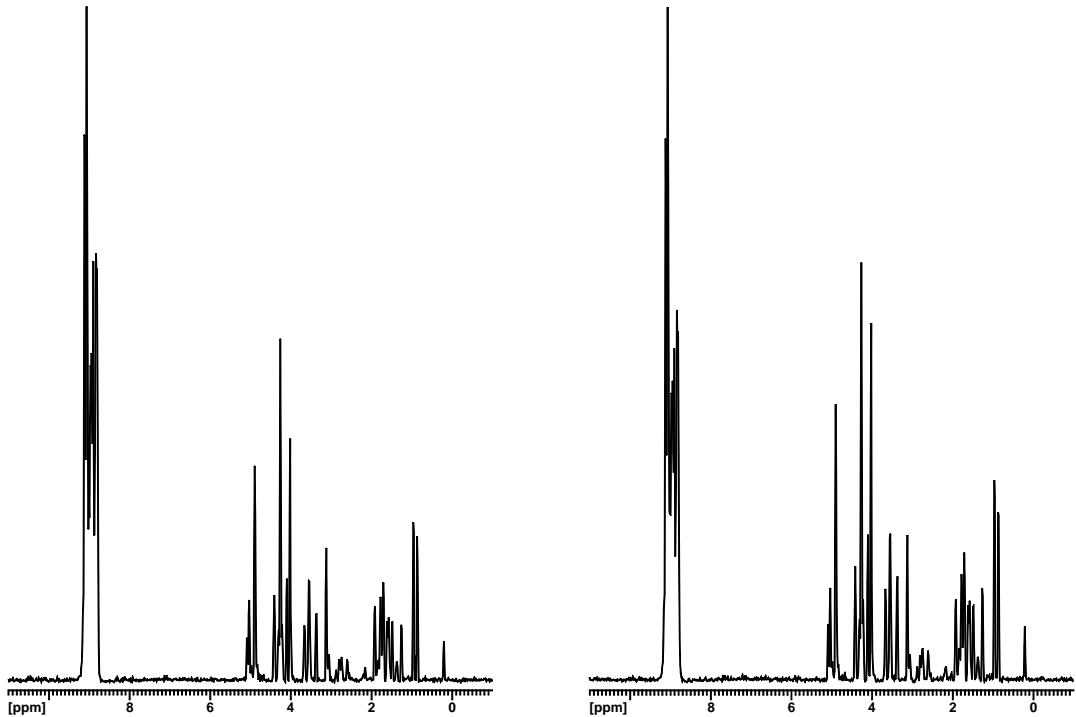


Abbildung 2.8: Spuren aus den 2D-Spektren in Abbildung 2.7; links: DIPS1-2 und rechts: P5P9M16. In diesen Spuren erkennt man, dass die adiabatische Sequenz auch eine ca. 20-25% höhere Intensität besitzt (Diagonalsignale zwischen 8-9 ppm).

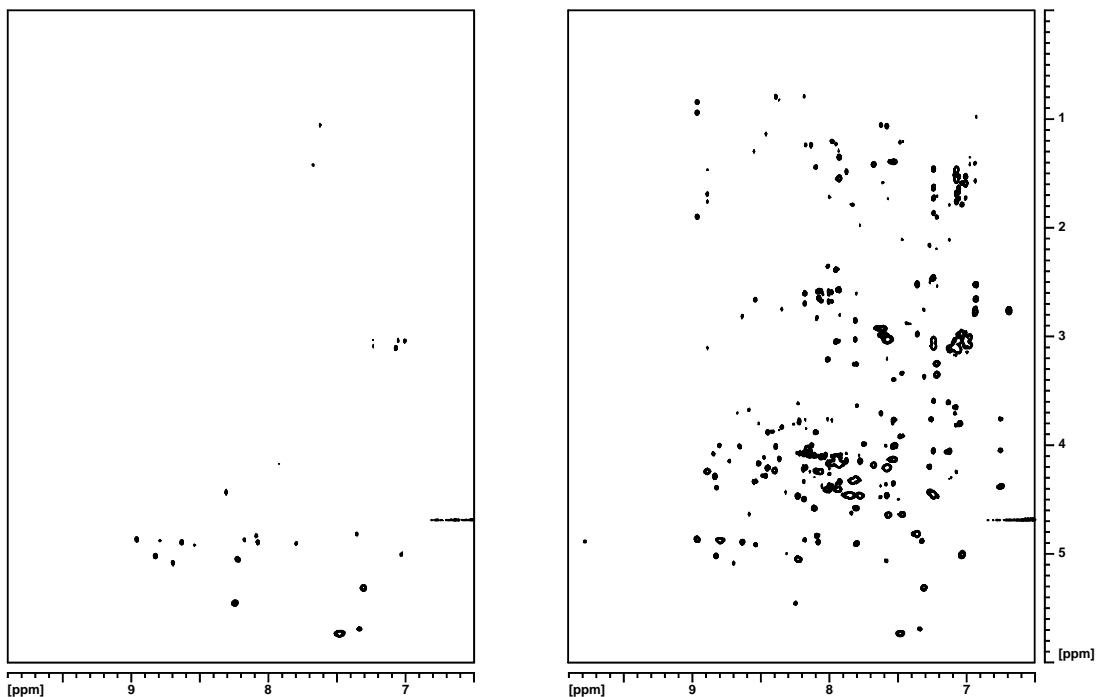


Abbildung 2.9: Die gleichen H,H adiabatischen TOCSY Spektren wie in Abbildung 2.7 (links: DIPS1-2 und rechts: P5P9M16), jedoch wurde hier die Leistung des TOCSY Mischpulses um +4 dB falsch gesetzt. Hier ist die hervorragende Robustheit gegen Pulsmiskalibrierung und B_1 -Inhomogenität des adiabatischen Pulses zu erkennen. Diese Eigenschaft kann wichtig sein, wenn man einen hohen Grad an Automatisierung erreichen will, z.B. in Zusammenhang mit „Structural Genomics“ Strukturaufklärung.

Natürlich wollen wir unsere neuen adiabatische TOCSY- und alle anderen Sequenzen nicht nur an Ubiquitin, sondern auch an einem biologisch interessanten Protein zum Einsatz bringen. In einer Zusammenarbeit mit Evelyne Zientz und Prof. Gottfried Unden aus dem Institut für Mikrobiologie und Weinforschung der Universität Mainz wollen wir die Struktur des Sensorproteins DcuS aufklären.

In Bakterien wird eine große Anzahl von Signalübertragungsproteinen gebraucht um alle verschiedenen Arten für die Übertragung eines Signals zu ermöglichen⁴⁷. Viele dieser verschiedenen Sensortypen erhalten das Signal auf der periplasmatischen Seite der Membran. Durch die Übertragung des Signals über die Membran wird das Signal zu einer zellulären Information. Dieses Signal kann nun zur Regelung von allen möglichen Prozessen genutzt werden.

Die wichtigsten Signalübertragungssysteme bestehen aus zwei Proteinen, der membranständigen Histidin Kinase und einem cytoplasmatischen Regulator Protein. Der Signaltransfer zwischen dem Sensor und dem Antwortprotein ist durch einen Phosphorylierungsschritt gesteuert.

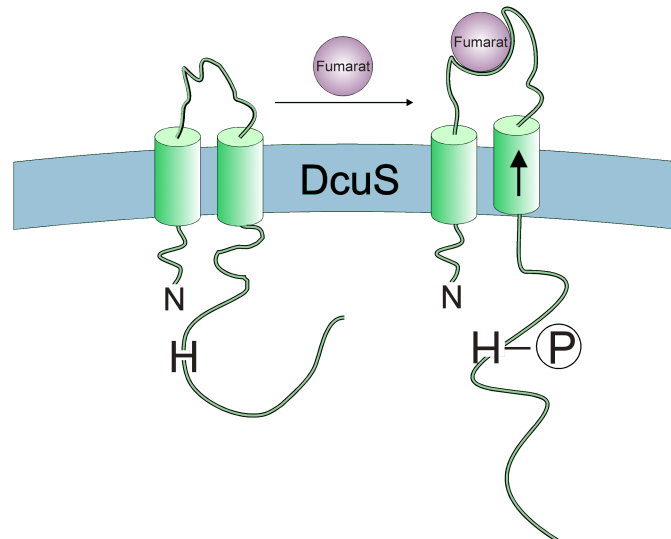


Abbildung 2.10: Eines der möglichen Modelle zur Signalübertragung in DcuS: durch die Bindung des Liganden Fumarat erfährt die periplasmatische Domäne von DcuS eine Konformationsänderung. Diese Konformationsänderung wird über die Transmembranhelices in die cytoplasmatische Transmitterdomäne übertragen. Durch eine erneute Konformationsänderung in der Transmitterdomäne wird die Phosphorylierung des konservierten Histidinrests ermöglicht (Abbildung von Evelyne Zientz zur Verfügung gestellt).

Im Rahmen meiner Arbeit war es das Ziel, zusammen mit Tatjana Parac, Boris Coligaev, Evelyne Zientz und Gottfried Uden die Struktur des Sensorproteins DcuS aus dem DcuSR 2-Komponenten-Regulationssystem aufzuklären^{48,49}. Dcu steht für dicarboxylate uptake, weil die Genexpression der anaeroben Fumarat Respiration durch C₄-Dicarboxylate (Fumarat) gesteuert wird. Das System besteht aus dem Sensor DcuS, welcher in der cytoplasmatischen Membran liegt und dem Antwortprotein DcuR. DcuS selbst besteht aus 2 transmembranen Helices, einer periplasmatischen Domäne, der C-Terminalen Kinase und einer Transmitter Domäne. Zuerst sollte die Struktur der periplasmatischen Domäne aufgeklärt werden, da an diese verschiedene C₄-Dicarboxylate binden⁴⁸. Ein wichtiger Punkt war es auch die genaue Bindungsstelle der C₄-Dicarboxylate (im speziellen Fumarat) mittels chemischen Verschiebungsänderungsexperimenten⁵⁰ aufzuklären. So sollte man auch in der Lage sein wichtige Strukturveränderungen nach der Bindung zu sehen. In weiterer Folge wurde begonnen die Struktur der transmembranen Helices mittels Flüssig- und gleichzeitig mit Festkörper-NMR Spektroskopie zu untersuchen. Auch theoretische Untersuchungen mittels Monte Carlo Rechnungen⁵¹ wurden für die Membran Helices von der Arbeitsgruppe Arseniev durchgeführt. Das wichtigste, nun experimentell zu bestätigende Ergebnis ist, dass der höchst wahrscheinlichste Zustand der beiden Helices eine spezifische Bindung derer ist. Es ist von großem Interesse die Struktur dieser Domäne zu kennen, da in der Literatur nur wenige Strukturen von Sensorproteinen bekannt sind^{52,53}. Mit den zuvor beschriebenen Methoden konnte eine vollständige Zuordnung des Proteins erreicht werden⁵⁴. Auch hier leistete ein adiabatisches HCCH-TOCSY Experiment⁴⁴ große Hilfe bei der Zuordnung der Seitenkettenatome.

Leider war es bis jetzt nicht möglich die endgültige Struktur zu ermitteln. In der Abbildung 11 habe ich die Sekundärstruktur, welche aus chemischer Verschiebungsinformation⁵⁵⁻⁵⁸, ³J_{HNHA}-Kopplungskonstanten und sequenzieller NOESY-Information gewonnen wurde, zusammen mit der vorläufigen 3D-Struktur des Proteins, dargestellt.

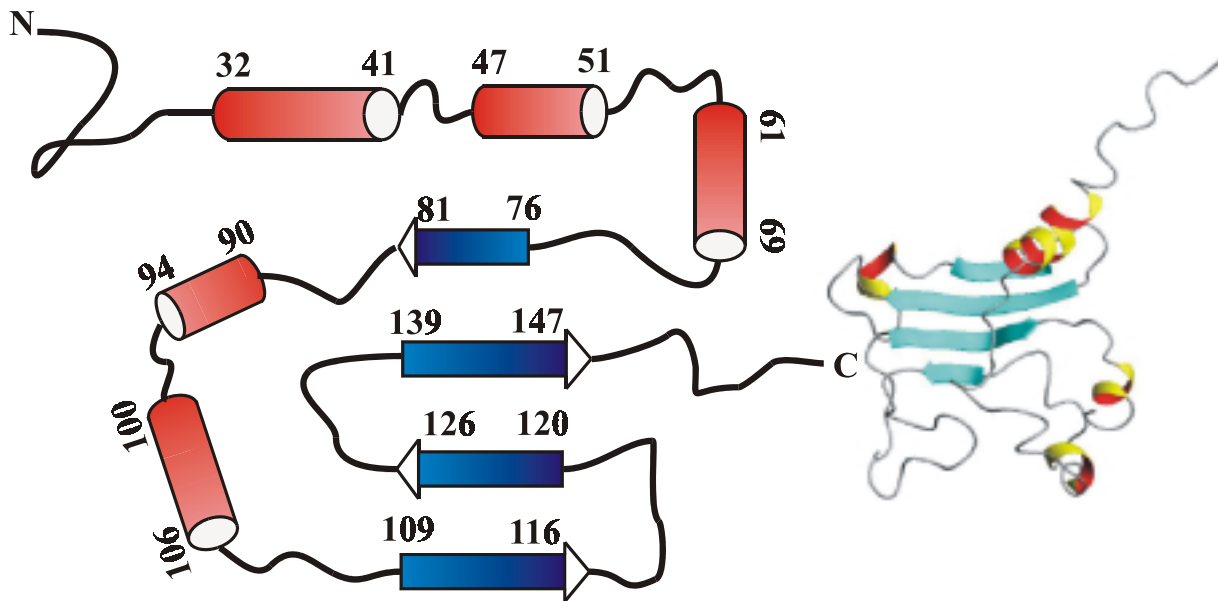


Abbildung 2.11: Links: Sekundärstruktur aus chemischer Verschiebungsinformation, Kopplungskonstanten und sequenzieller NOESY Information. Rechts: vorläufige 3D Struktur DcuS.

Wie schon zuvor erwähnt konnte durch chemische Verschiebungsanalyse auch die Bindungsstelle näher charakterisiert werden. Fumarat bindet mit einer sich nur im millimolaren Bereich befindenden Bindungskonstante an DcuS. Drei Arginine haben sich als Bindungsstelle für Fumarat herauskristallisiert. Arginin 107, 139 und 147 und auch die umgebenden Aminosäuren haben nach Zugabe von Fumarat eine deutliche Steigerung der Signalintensität gezeigt. Auch zeigten die chemischen Verschiebungen der Arginine eine Änderung bei Zugabe von Fumarat. Die K_d Werte für Maleinat (2.5mM), Citrat (6mM) und Fumarat (1mM) wurden auch *in vivo* bestimmt und zeigten eine gute Übereinstimmung mit unseren gemessenen Werten.

Es wurden zwei Mutanten hergestellt: Arginin 139 und 147 wurden jeweils durch Isoleucine ersetzt. Bei der Mutante R147I stellte sich heraus, dass diese biochemisch vollkommen inaktiv ist und keine Fumaratatmung mehr möglich ist. Die Mutante R139I zeigt diesen Effekt nicht, allerdings zeigt sie im $^1\text{H},^{15}\text{N}$ -HSQC-Spektrum deutliche Anzeichen für Aggregation. Die gleichen Bindungsstellen wurden an der Sensorkinase CitA aus *Klebsiella pneumoniae* gefunden⁵⁹. Hier wurden alle Aminosäuren durchmutiert und geschaut, wo es keine Aktivität mehr gibt. Große Übereinstimmungen mit DcuS konnten gefunden werden. Die Mutante von R150 in CitA (vergleichbar mit R147 in DcuS) zeigt überhaupt keine Aktivität mehr. Auch bei der Mutante von R107 in CitA (R109 in DcuS) gab es einen deutlichen Abfall der Aktivität. Diese Ergebnisse stimmen

mit unseren überein. In CitA hat man aber noch eine weitere wichtige Bindungsstelle gefunden: R152. In DcuS korreliert dies mit einem Phenylalaninrest. Allerdings bindet an CitA bevorzugt Citrat, welches 3 Carboxygruppen enthält und an DcuS Fumarat mit nur 2 Carboxygruppen. Nun ist man gerade dabei viele wichtige Strukturparameter zu sammeln und so die Struktur weiter zu verbessern.

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3 Dipolare Kopplungen, ein neuer „long-range“ Parameter in der NMR-Spektroskopie

Auf Grund der schnellen Rotationsdiffusion zeigen gelöste Moleküle in isotroper Lösung keine dipolare Kopplung. Bei einer nicht isotropen Verteilung der Orientierung eines Moleküls in Lösung werden dipolare Kopplungen beobachtbar. Eine isotrope Verteilung bedeutet, dass das Molekül in Lösung keine Vorzugsorientierung in bezug auf ein angelegtes Magnetfeld besitzt. Die Rotationsdiffusion, welche im übrigen anisotrop sein kann, muss schneller als der Frequenzbereich des dipolaren und des chemischen Verschiebungstensors sein, damit die dipolaren Kopplungen der verschiedenen Orientierungen sich um die Vorzugsorientierung mitteln. Die H,C und die H,N dipolaren Tensoren sind 24.21 kHz beziehungsweise 11.49 kHz breit, die chemische Verschiebungsanisotropie von Amidstickstoffen und Carbonylkohlenstoffen ist 160 bis 200 ppm. Daraus ergibt sich für Feldstärken von 600 MHz, dass die Rotationsdiffusion schneller als ungefähr 30 kHz sein muss, damit die anisotropen Wechselwirkungen ausgemittelt sind. Diese Korrelationszeit definiert die Grenze zwischen Flüssig- und Festkörper NMR. Im Folgenden möchte ich die wichtigsten Methoden, mit denen eine anisotrope Orientierungsverteilung erreicht werden kann, beschreiben. Diese anisotrope Verteilung erlaubt es dipolare Kopplungen auch in Flüssig-NMR zu messen:

Joel R. Tolman und James H. Prestegard¹ haben 1995 erstmalig dipolare Kopplungen in Biomakromolekülen gemessen. In einem 17.62 T (¹H Frequenz: 750 MHz) supraleitenden Magneten ist das magnetische Feld so stark, dass die eigene magnetische Suszeptibilität ausreicht, um das Molekül ganz schwach auszurichten und so dipolare Kopplungen additiv zu skalaren Kopplungen zu messen. Natürlich war es von großem Vorteil, dass Tolman ein paramagnetisches Protein (Cyanometmyoglobin; ein Protein, welches aus 8-Helices besteht) verwendete, bei dem die magnetische Suszeptibilität so groß ist, dass damit auch die Größe der dipolaren Kopplung leichter messbar wird. Dieser Vorgang kann verglichen werden mit dem Einbringen eines kleinen Stabmagneten in ein starkes Magnetfeld. Deshalb wurden im darauf folgenden Jahr Methoden entwickelt, welche es erlauben die kleinen dipolaren Kopplungen mit hoher Genauigkeit zu messen^{2,3}. Ein Jahr später zeigten Nico Tjandra und Ad Bax, dass sich auch das Protein Ubiquitin in Lösung orientiert⁴. Dieses Protein ist nicht paramagnetisch, sondern die Orientierung wird allein durch die erhöhte magnetische Suszeptibilität der drei aromatischen Gruppen hervorgerufen. Die dipolaren Kopplungen sind sehr klein (ca. 0.3 Hz), deshalb wurde bei

der Messung eine Pulssequenz verwendet, die die Kopplung auf die Intensität der Peaks moduliert und so die Genauigkeit der Messung steigert⁴. Größer sind die Effekte in Nukleinsäuren^{5,6}, welche einen relativ großen Suszeptibilitätstensor besitzen. Dieser wird durch die regelmäßige Anordnung der Basen und des Phosphatrückgrats hervorgerufen.

Natürlich war man intensiv auf der Suche nach geeigneten Methoden, um möglichst alle Biomakromoleküle zu orientieren und dabei die dipolaren Kopplungen in einen für die Messgenauigkeit sinnvollen Bereich zu bekommen. Dies wurde durch die schon Anfang der 90er Jahre vorgestellten verdünnten, flüssigkristallinen Lösungen^{7,8} ermöglicht. Nico Tjandra konnte in verdünnten nematischen, flüssigkristallinen Phasen von Phosphorlipiden das erste Mal H^N -N dipolare Kopplungen in Ubiquitin^{9,10} messen. Die flüssigkristallinen Phasen aus Phosphorlipiden werden als Bicellen bezeichnet¹¹. Bicellen (bilayered discoidal mixed micelles) wurden zuerst in viel stärker konzentrierter Form verwendet um Membranproteine in diesen rekonstituieren zu können. Die Bicellen orientieren sich im Magnetfeld. Verwendet man stark verdünnte Bicellenlösungen haben die Moleküle inmitten der Bicellen genügend Platz um sich zu reorientieren; allerdings kommt es zu gelegentlichen Stößen und so zu einer ganz schwachen Vorzugsorientierung.

In den letzten Jahren ist eine Vielzahl von verschiedenen flüssigkristallinen Phasen gefunden worden, welche es erlauben unterschiedliche Moleküle bei vielen divergenten Bedingungen (Temperatur, pH-Wert, etc.) zu messen. Alle Methoden haben gemeinsam, dass nur eine ganz schwache Vorzugsorientierung erzeugt wird. Da die dipolare Kopplung sehr groß ist - z.B. 11.49 kHz für NH und 24.21 kHz für CH dipolare Kopplungen - reicht eine Orientierung von nur 0.4% aus um eine $^1H^{15}N$ dipolare Kopplung von 40Hz zu induzieren. Dies ist auch der große Unterschied zu früheren Untersuchungen, in denen konzentrierte flüssigkristalline Materialien zur Orientierung eingesetzt wurden und so eine 10 bis zu 50%ige Orientierung hervorgerufen wurde. Der geringe Grad der Orientierung bewirkt, dass die Spektren wie bekannte hochaufgelöste NMR Spektren aussehen und so die Auswertung recht einfach vonstatten geht. Generell kann man die flüssigkristallinen Phasen in zwei Gruppen teilen:

1. Die, die nur räumliche Wechselwirkung hervorrufen, so dass der Ausrichtungstensor größtenteils nur von der Form des Biomakromoleküls abhängig ist¹².
2. Flüssigkristalline Phasen, welche neben der räumlichen Blockade auch elektrostatische Wechselwirkungen hervorrufen.

Die elektrostatischen Wechselwirkungen können durch Zugabe von bestimmten Chemikalien (CTAB, SDS etc.) zu den neutralen, verdünnten flüssigkristallinen Phasen erzeugt werden, oder aber sie werden auf Grund der eingesetzten Stoffe selbst hervorgerufen. Diese verschiedenen Möglichkeiten sind wichtig für den Einsatz der dipolaren Kopplungen in der Strukturaufklärung und bei der Extraktion von dynamischer Information¹³, da die Richtung von Tensoren 2ten Rangs (dipolare Kopplung) nicht von dem inversen Tensor unterschieden werden kann (180° Inversion). Deshalb beschreibt die dipolare Kopplung nicht eine, sondern zwei kegelartige Flächen im axialsymmetrischen Fall, entsprechend der verschiedenen Vektororientierungen, in entgegengesetzter Richtung. $D = D_{zz} (3 \cos^2 \theta - 1)$ ist die Formel für die axialsymmetrische dipolare Kopplung, wobei D_{zz} die axiale Größe des Ausrichtungstensors ist. So ist es unmöglich lokale Information aus dipolaren Kopplungen zu extrahieren, da es ein Kontinuum von möglichen Orientierungen gibt. Diese Entartung kann man durch die zuvor erwähnten Modulationen des Tensors verringern, oder mit vielen sehr unterschiedlichen Orientierungen auch aufheben. Somit ist es möglich eine Art 3-dimensionales Bild des Vektors zu liefern.

Die exakte Wirkungsweise der flüssigkristallinen Phasen ist noch nicht bekannt. Allerdings kann man sich die Bicellen-Lösungen und auch andere flüssigkristalline Phasen als Wände in der Lösung vorstellen, welche die Biomakromoleküle daran hindern sich vollständig frei zu reorientieren.

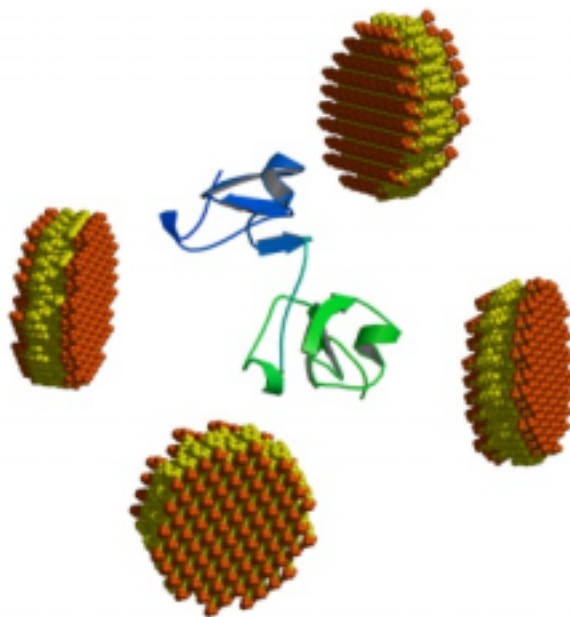


Abbildung 3.1: Schematische Darstellung der flüssigkristallinen Phasen bestehen aus Bicellen. Das Protein kann noch immer frei rotieren. Die schwache Vorzugsorientierung geschieht durch schwache Stöße an die Bicellenwände.

Die Orientierung beruht nur auf einer Oberflächenwechselwirkung und nicht auf einer Bindung zwischen Biomakromolekül und den flüssigkristallinen Phasen¹⁴. Dies bedeutet, dass man sich als einfachstes Modell das schwache Kollisionsmodell vorstellen kann. Experimentell kann man dafür auch Beweise finden. Die effektiven transversalen Relaxationszeiten (T_2^*) verändern sich nicht, gleichgültig ob diese in isotroper Lösung oder in flüssigkristallinen Phasen gemessen werden, dies gilt allerdings nur für ungeladene Phasen. Daten gemessen an Ubiquitin und an *HgiCIC*-Protein¹⁵⁻¹⁷, dessen genauere Charakterisierung auch im Rahmen dieser Arbeit vorgenommen wurde, belegen dies. Die mittleren gemessenen T_2^* -Zeiten waren in isotroper Lösung 160ms und in CHAPSO/DLPC (1:5; 5%) Bicellen 155ms. Der Fehler der Messung liegt bei ca. 5%, sodass es zu keinen Änderungen der Relaxationszeiten kommt und damit auch zu keiner Bindung zwischen Protein und den Bicellen, was bedingt, dass die τ_c -Zeit unverändert bleibt. Würde es zu einer Bindung kommen, sanken die gemessenen T_2^* -Zeiten dramatisch ab, da die mittlere Bicellenmasse ca. 500 kDa beträgt (Abschätzung auf Grund der mittleren Größe der Bicellen, gemessen mit Streuexperimenten¹⁸) und so die Korrelationszeit τ_c des Komplexes sehr groß würde. Allerdings gibt es in der vorhandenen Literatur schon erwähnte Orientierungsmedien, bei welchen die T_2^* -Zeiten deutlich kleiner wurden, was doch auf eine starke Wechselwirkung schließen lässt^{19,20}. Dies sieht man auch in den Spektren: die Linienbreite, besonders der hochfeldigen Dublett Peaks in einem gekoppelten $^1\text{H}, ^{15}\text{N}$ -HSQC-Spektrum (CSA und Dipol-Dipol Relaxation verhalten sich hier additiv), wird wesentlich größer²¹. Deshalb ist die Messung in solchen Medien nicht immer mit jedem Biomakromolekül möglich und sinnvoll, da die dipolaren Kopplungen zu ungenau wären. Auch könnte diese starke Wechselwirkung eine leichte Veränderung der Proteinstruktur bewirken. Dies will man natürlich ausschließen.

Durch Zugabe von Lanthaniden ist es möglich die Bicellen um 90° zu drehen²². Hierzu eignen sich die Lanthanide Eu^{3+} , Er^{3+} , Tm^{3+} und Yb^{3+} . Eine Drehung um 90° bewirkt eine Reduktion der dipolaren Kopplungen um den Faktor -2 . Dies konnte auch experimentell bewiesen werden.

In den nachfolgenden Ausführungen möchte ich die flüssigkristallinen Phasen im Einzelnen detailliert darstellen (geordnet nach den verschiedenen Materialien, welche die flüssigkristallinen Phasen darstellen) und dabei auch persönliche Beobachtungen und Kunstgriffe für die Herstellung dieser Phasen erläutern.

Allgemein gilt zu sagen, dass es viele Faktoren gibt um frühzeitig abschätzen zu können in welcher flüssigkristallinen Phase sich ein Biomakromolekül orientiert und es nicht zu starker Wechselwirkung mit den flüssigkristallinen Phasen kommt. Einer der wichtigsten Parameter ist der pI-Wert eines Stoffes. Dieser ist ein Maß für die Ladung des Moleküls. Details möchte ich bei der Beschreibung der Phasen geben.

3.1 Anisotrope Medien

3.1.1 Bicellen²³ basierend auf Phosphocholinen

Phosphocholine waren die ersten verwendeten Typen von flüssigkristallinen Stoffen, die für die Orientierung von Proteinen verwendet wurden^{7,8}. Allgemein bestehen diese Bicellen aus einem langkettigen Phosphocholin z.B.: DMPC oder DLPC, welches das Zentrum der Bicelle bildet. Die langkettigen Alkanketten bilden das hydrophobe Zentrum. Die Ränder der Bicellen werden von kurzkettigen Phosphocholinen wie DHPC⁷ oder von Derivaten der Gallensäure - CHAPSO^{24,25} - abgedeckt. Diese sind stärker hydrophil.

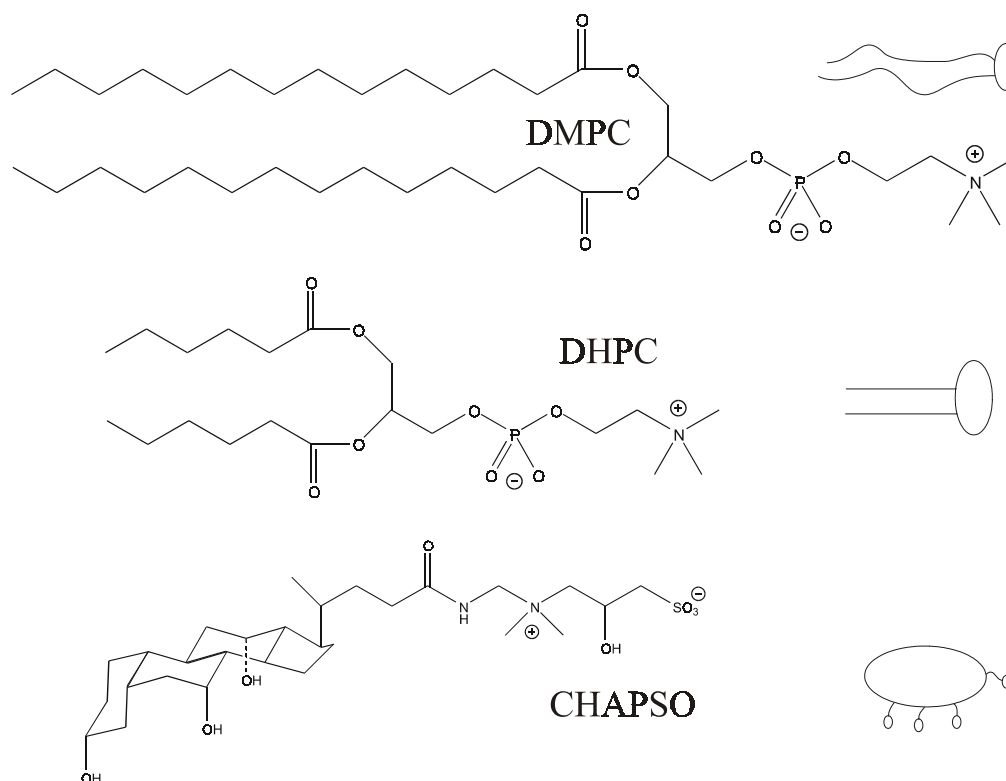
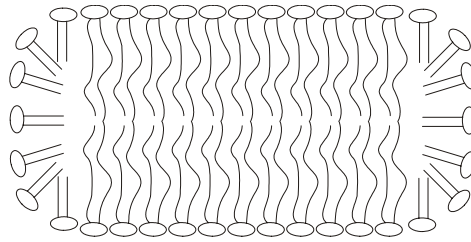


Abbildung 3.2: DMPC (*L*- α -Phosphatidylcholinedimyristoyl) (C14); DHPC (*L*- α -Phosphatidylcholinedicaproyl) und CHAPSO (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propansulfonat) bilden die Grundbausteine für die Bicellen.

DMPC/DHPC



DLPC/CHAPSO

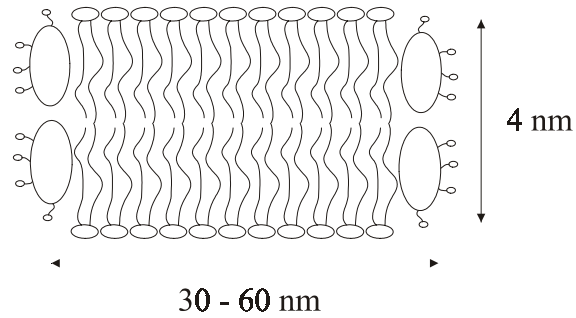


Abbildung 3.3: Schematische Darstellung der Bicellen. Die Größe variiert mit dem Verhältnis der eingesetzten Stoffe.

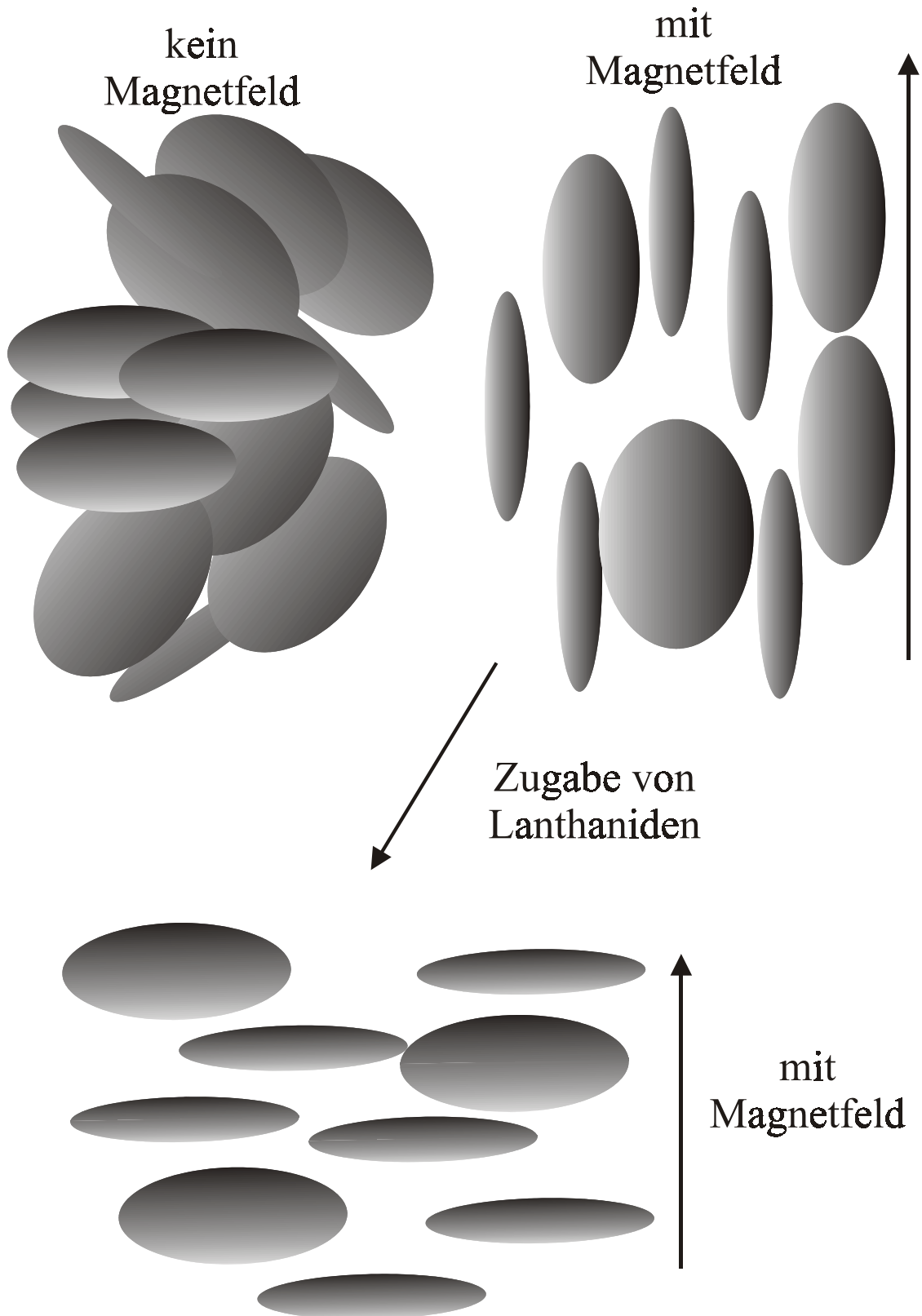


Abbildung 3.4: Unterschiedliche Zustände von flüssigkristallinen Lösungen aus Bicellen; Ohne starkem äußeren Magnetfeld gibt es keine Vorzugsorientierung nur Bezirke gleicher Orientierung. Bringt man die flüssigkristalline Lösung jedoch in ein Magnetfeld ein (z.B. ein NMR Spektrometer) orientieren sich die Bicellen entlang des Magnetfelds. Durch Zugabe von Lanthaniden kann die Orientierung zum Magnetfeld um 90° verändert werden.

Für diese Arten von Bicellen wurden wohl die meisten Untersuchungen gemacht, wobei sie auch einen direkten spektroskopischen Nachweis für die Orientierung über ^{31}P -NMR-Spektren erlauben. Die Änderung der chemischen Verschiebungsanisotropie von ^{31}P reflektiert direkt den Grad der Ausrichtung der Bicellen im Magnetfeld. Die chemische Verschiebung von ^{31}P kann man auch leicht rechnerisch nachvollziehen: Nimmt man einen mittleren Ordnungsparameter der Bicellen (S_{Bicelle}) mit ca. 0.7-0.8 an (0.8 sind die höchsten beobachteten S-Werte in nematischen Flüssigkeiten), dann ergibt sich aus

$$\delta_{\text{bic}} = \delta_{\text{iso}} + S_{\text{Bicelle}} * \frac{(\delta_{\parallel} - \delta_{\perp})}{3}, \text{ mit } (\delta_{\parallel} - \delta_{\perp}) = 45 \text{ ppm}, \text{ eine chemische Verschiebung von}$$

ca. $\delta_{\text{bic}} = -12 \text{ ppm}$, wie man sie auch in experimentellen ^{31}P -NMR-Spektren wiederfindet

(δ_{iso} : isotrope chemische Verschiebung, $\delta_{\perp}, \delta_{\parallel}$ senkrechter und paralleler Anteil des chemischen Verschiebungstensors). Diese direkte Nachweismethode ist bei vielen anderen flüssigkristallinen Phasen nicht möglich. Dort kann der Nachweis nur mittels ^2H Spektroskopie (Quatropol Deuteriumsplitting) der gebundenen Fraktion des $\text{H}_2\text{O}/\text{D}_2\text{O}$ an der Oberfläche der Bicellen gemacht werden. Das derzeitige Modell für die Bicellen geht von einer leicht ovalen diskusförmigen Form mit den Maßen von ca. $300 \text{ \AA} \times 20 \text{ \AA}^{18}$ aus. Ausschlaggebend für die Größe der Bicellen ist das Verhältnis q der verwendeten lang und

kurzkettigen Phosphocholine $\left(q = \frac{[\text{DLPC}]}{[\text{CHAPSO}]} \right)$. Typische q -Werte für die Systeme sind:

$q=5$ für CHAPSO/DLPC und $q=3$ für DHPC/DMPC. Je größer das Verhältnis wird, desto stärker nimmt auch die Größe der Bicellen zu. Bei den extremen Verdünnungen der flüssigkristallinen Phasen muss man allerdings davon ausgehen, dass nicht alles CHAPSO oder DHPC gebunden sein wird, sondern auch ein Austausch mit freiem CHAPSO erfolgt

$\left(q_{\text{eff}} = \frac{[\text{DLPC}]}{[\text{CHAPSO}] - [\text{CHAPSO}]_{\text{frei}}} \right)$. Dieser Umstand vergrößert die Bicellen weiter. Da

die Lösungen relativ stabil sind und sich viele Biomakromoleküle darin orientieren lassen, gibt es eine größere Anzahl von Publikationen mit für verschiedene Temperatur- und pH-Bereiche adaptierten Versionen von Phosphocholinbicellen. Das CHAPSO/DLPC-System erlaubt das Messen bei niedrigen Temperaturen bis ca. 285 K^{26} ; dieses System ist stabil von 288 bis 328 K. Der pH-Bereich von CHAPSO/DLPC und DHPC/DMPC liegt optimal bei ca. 6.5-7.0. Niedrige pH-Werte beschleunigen die Spaltung der Esterbindung und führen demzufolge zu einer Instabilität der flüssigkristallinen Phase.

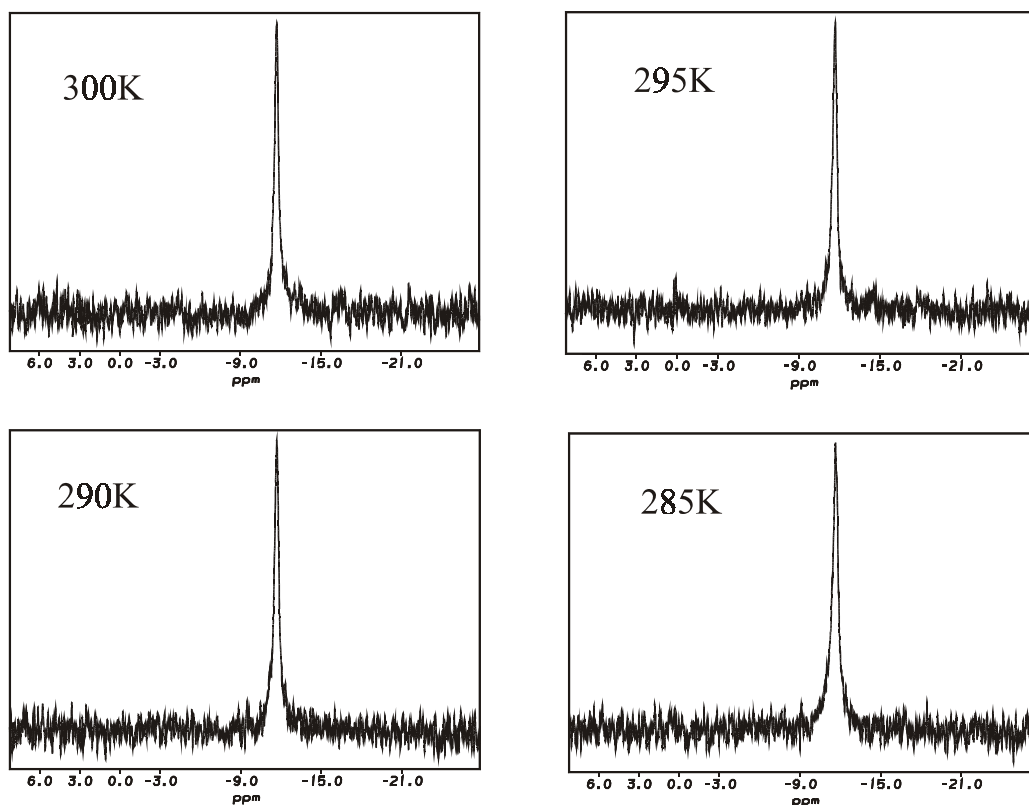


Abbildung 3.5: Temperaturreihe für ein CHAPSO/DLPC (q:1/5; 5% Gesamtlipid) Bicellensystem. Die chemische Verschiebung von ca. -12 ppm stimmt mit der zuvor beschriebenen Orientierung von ca. 0.7-0.8 überein. In CHAPSO/DLPC Systemen ist es möglich dipolare Kopplungen bis ca. 285K zu messen.

Andere Systeme für das Messen bei anderen Temperaturen und pH-Werten sind:

- Cavagnero et al.²⁷ haben ein System aus DIODPC/CHAPSO (4.3:1) verwendet, welches über einen pH Bereich von 1.0 bis 6.5 stabil ist (hier wurde die säurelabile Esterfunktion gegen eine säurestabile Etherfunktion ausgetauscht).
- Ottiger et al.²⁸ haben auch die Esterfunktionen durch Etherfunktionen ersetzt und so eine Stabilität der Bicellen sogar von pH 2.3 – pH 10.4 erreicht. Ottiger hat Ditetradecyl-phosphatidylcholine, Didodecyl-phosphatidylcholine oder Dihexyl-phosphatidylcholine als Material für die flüssigkristalline Phase verwendet.

Untersuchungen²⁹ an DHPC/DMPC-Bicellen haben gezeigt, dass die kleinstmögliche Konzentration an Bicellen ca. $1.5 \pm 0.5\%$ betragen muss. Unterhalb dieser Konzentration konnte kein Deuteriumsplitting mehr gemessen werden.

Wie schon erwähnt, kann man durch Zugabe von CTAB und SDS die Bicellen positiv (CTAB) oder negativ (SDS) laden^{13,30,31}. Dies erlaubte es erstmals verschiedene Ausrichtungstensoren an einem Molekül zu messen und so die Mehrdeutigkeit der dipolaren Kopplungsinformation zu verringern.

Praxis der Bicellen-Präparation:

Im Folgenden möchte ich einige praktische Hinweise zur Herstellung der Bicellen-Lösungen geben: Es ist sinnvoll größere Mengen an Bicellen herzustellen, welche - bei Lagerung bei -20°C - mehrere Monate haltbar sind. Wichtig ist, dass die Lösungen vor dem Einfrieren in die isotrope Phase überführt werden, d.h. erst auf ca. 5°C und dann schnell auf -20°C abgekühlt werden, dies stabilisiert die Bicellen weiter. Bei der Verwendung von CHAPSO/DLPC- oder CHAPSO/DMPC-Bicellen können die beiden Stoffe direkt im richtigen molaren Verhältnis eingewogen werden. DHPC ist hingegen sehr hygroskopisch und es ist vorteilhafter sich zu Beginn eine Stammlösung zu erzeugen und diese dann bei -20°C zu lagern. Nachdem man H_2O (Puffer, etc.) und D_2O zugesetzt hat, sollte man die Lösung kräftig vortexen und auf ca. 45° bis 50°C erwärmen. Nachdem die Bicellenlösung nach einer Stunde milchig geworden ist, wird sie nochmals gevortext und dann für ca. 1 Stunde in Eis gekühlt. Hierauf wird die Lösung wieder durchsichtig isotrop. Dieser Vorgang sollte ca. 10 mal wiederholt werden, damit sichergestellt ist, dass sich die Bicellen stabil gebildet haben und sie auch beim wiederholten Messen über längere Zeiträume (bis zu einer Woche) im NMR-Magneten bei normalen Messbedingungen stabil bleiben.

Während der Promotion war es mehrmals nötig, das teilweise ^{15}N oder ^{15}N und ^{13}C markierte Protein aus den Bicellen wiederzugewinnen. Dies ist mit relativ wenig Aufwand verbunden. Die Probe sollte mit H_2O auf ca. 50ml verdünnt werden. Da DLPC und DMPC relativ schwer wasserlöslich sind, wird die Lösung schnell trüb. Über Nacht kann man DLPC und DMPC abzentrifugieren. Der Überstand enthält nun nur noch das Protein und CHAPSO. Zentrifugiert man die Lösung über einer 3000-Da-Membran, kann man das relativ gut wasserlösliche CHAPSO (603.9 g/mol) leicht vom Protein trennen. Über diesen Weg ist es mir gelungen ca. 80% des Proteins aus den flüssigkristallinen Lösungen wiederzugewinnen.

3.1.2 Flüssigkristalline Phase aus Phagen

Phagen ist eine aus dem Griechischen abgeleitete Bezeichnung von Viren, welche Bakterien spezifisch anfallen. Phagen bestehen normalerweise aus einem Kopf und einem länglichen Schwanz. Der Kopf besteht aus Nukleinsäuren, welche von Proteinen umgeben sind. Manche Viren besitzen auch noch Schwänze, welche meist hohl sind und zur

Injektion des genetischen Materials dienen. Der Kopf mit den langen Nukleinsäureketten orientiert sich im magnetischem Feld und bildet so Wände wie die zuvor beschrieben Bicellen.

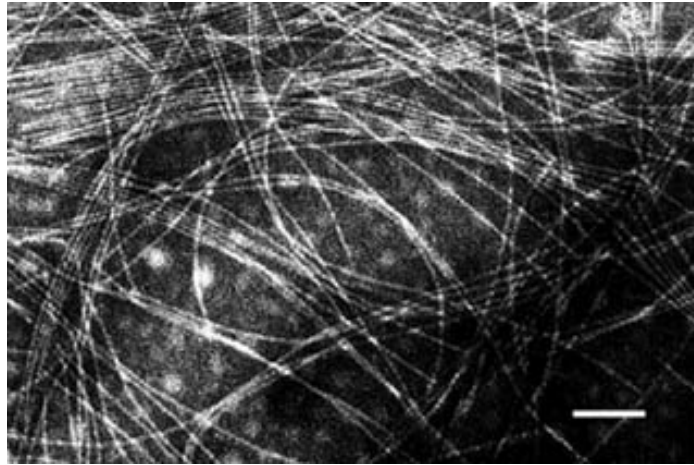


Abbildung 3.6: Mikroskopische Aufnahme von Pf-1 Phagen. Nach dem Anlegen des Magnetfeldes orientieren sich die Phagen mit ihrer Hauptachse entlang des Magnetfelds.

Die Arbeitsgruppen von Marius Clore und Arthur Pardi erkannten dies fast gleichzeitig³²⁻³⁴. Sowohl der Tobacco Mosaic Virus (TMV) als auch Pf-1 - und *fd* - Phagen³⁵ eignen sich um Nukleinsäuren, Proteine und Polysaccharide zu orientieren. Ein großer Nachteil der Phagen ist, dass sie durch die langen Nukleinsäureketten negativ geladen sind (pI-Wert: *fd*: 4, Pf-1: 4.2). Deshalb sind Phagen oft für Proteine ungeeignet, da Proteine häufig einen neutralen oder positiven pI-Wert haben und so starke elektrostatische Wechselwirkungen zwischen den Proteinen und den Phagen vorkommen können. Es kann sogar eine Bindung des Proteins an die Phagen auftreten. Da das Molekulargewicht von Phagen im Megadalton-Bereich liegt, kann man die Protein-Resonanzen im NMR-Spektrum nicht mehr sehen. Die T_2 -Zeiten werden zu kurz, wodurch die Linien zu breit werden¹⁹. Manchmal ist es möglich die Konzentration der Phagen so stark zu verringern und die elektrostatischen Wechselwirkungen so stark durch zusätzliche Ionen in der Lösung zu verkleinern, dass es wieder möglich wird auch Proteine in diesen Phasen zu orientieren. Dies war zum Beispiel für Ubiquitin der Fall, welches einen pI-Wert von 7.6 hat. Die Konzentration der Phagen war nun 5mg/ml (normalerweise liegt die Konzentration bei 20-50 g/ml) und die Ionenstärke wurde durch Steigerung des Salzgehaltes drastisch erhöht (100mM NaCl). Somit konnte auch Ubiquitin orientiert werden³⁶. Im Gegensatz hierzu lassen sich Nukleinsäuren, welche selbst einen pI-Wert deutlich kleiner als 6 haben, mittels Phagen sehr gut orientieren³⁷⁻³⁹. Durch den relativ hohen elektrostatischen Anteil an der

Orientierung sind Phagen sehr gut geeignet stark unterschiedliche Ausrichtungstensoren im Vergleich zu den zuvor beschriebenen Bicellen zu liefern. Dies ist wie schon angedeutet wichtig für das bessere lokale Verständnis der Vektoren und der Dynamik. Pf-1 Phagen können von der Firma ASLA (Riga, Lettland; <http://www.asla-biotech.com>) erworben werden und sind dann sofort einsetzbar.

Interessant ist auch, dass die verschiedenen Phagen-Typen eine unterschiedliche Länge besitzen. Der Durchmesser (6.6 nm) und die Masse pro Längeneinheit (ca. 18500 Da/nm) von *fd*, Pf-1 und *fdK48A* sind sehr ähnlich. *fdK48A* ist eine *fd*-Phagen-Mutante, aus der eine einzelne Aminosäure des Gen-VII-Proteins des *fd*-Phagens mutiert wurde. Durch diese kleine Veränderung ist die Mutante um ca. 35% länger. Der Pf-1-Phage ist ungefähr doppelt so lang wie ein *fd* Phage. Es wurden an einem Protein dipolare Kopplungen in allen drei Medien gemessen. Hat man die Konzentrationen der Phagen relativ um die Größe der Länge der Phagen verkleinert $\left(\frac{c_{Phage}}{L_{Phage}} = konst. \right)$, waren die gemessenen dipolaren Kopplungen alle von gleicher Größe. Dies lässt sich mit dem theoretischen Modell von Khoklov-Semenov-Onsager vergleichen, wonach die Größe der dipolaren Kopplung von der Länge des Phagen (L) abhängig $\left(\frac{1}{L} \right)^{35}$ ist. Auch konnte gezeigt werden, dass bei niedrigen pH-Werten (3.0) das Vorzeichen der NH^N dipolaren Kopplungen invertiert wird. Dies ist dadurch begründet, dass Phagen bei pH Werten, niedriger als ihr eigener pI-Wert (pI-Wert: *fd*-Phage: 4, Pf-1-Phage: 4.2), anders geladen sind (positiv)⁴⁰. Dieses Resultat ist ein weiterer Beweis dafür, dass die Orientierung mittels Phagen sehr stark durch elektrostatische Effekte beeinflusst wird.

3.1.3 Flüssigkristalline Phase aus n-Alkyl-Polyethylenglykol/n-Alkylalkohol/H₂O und Oberflächenlipiden (Helfrich Phasen)

Erst kürzlich haben Markus Rückert und Gottfried Otting⁴¹ eine flüssigkristalline Phase vorgestellt, deren großer Vorteil es ist, dass die Orientierung nur von der Form der Biomakromoleküle abhängt und es keine elektrostatischen Wechselwirkungen gibt. Die Linienbreiten der Biomakromoleküle in diesen n-Alkyl-Polyethylenglykol/n-Alkylalkohol/H₂O Phasen sind deshalb nicht durch elektrostatische Wechselwirkungen beeinflusst und deshalb sehr schmal. Daher eignet sich diese Phase auch hervorragend um

eine Vielzahl von heteronuklearen dipolaren Kopplungen wie NC' , $H^N C'$, $NC\alpha$, $C\alpha C'$, $C\alpha H\alpha$ zu messen. Verschieden lange n-Alkyl-Polyethylenglykole und n-Alkylalkohole ermöglichen es in verschiedenen Temperaturbereichen zu messen. Allgemein war aber zu beobachten, dass diese Phasen bei Temperaturen unter 30°C am stabilsten waren. Einige dieser Phasen gestatten sogar Messungen bei Temperaturen um 0°C , was für spezielle Probleme interessant sein könnte. Da die Phasen ungeladen sind, sind sie gegen unterschiedliche pH-Werte, Salzkonzentrationen und Proteinkonzentrationen sehr tolerant. In der Praxis sind diese Phasen auch sehr einfach herzustellen: Man legt ein n-Alkylpolyethylenglykole/ H_2O Gemisch vor (z.B: n-Lauryl-polyethylenglykole C12E5) und titriert unter starkem Vortexen n-Alkylalkohol (z.B: n-Hexanol oder n-Oktanol) mit einem bestimmten Verhältnis zu, bis die Phase klar wird. Danach kann man die flüssigkristalline Phase sofort verwenden. Titriert man über wird die Phase wieder trüb. Diese Phase trennt sich relativ rasch und ist somit nicht mehr für NMR Messungen zu gebrauchen. Ein weiterer Vorteil ist, dass sich die Phase spontan im Magnetfeld orientiert und die Messung sofort gestartet werden kann. Bei den anderen Phasen hat es sich als gut erwiesen, zwischen 1 und 12 Stunden vor dem Messen zu warten, um einen stabilen Zustand zu erreichen.

Helfrich-Phasen sind flüssigkristalline Phasen (Oberflächenlipid-Phasen), welche sich im Magnetfeld orientieren und somit Molekülen eine Vorzugsorientierung zu geben^{42,43}.

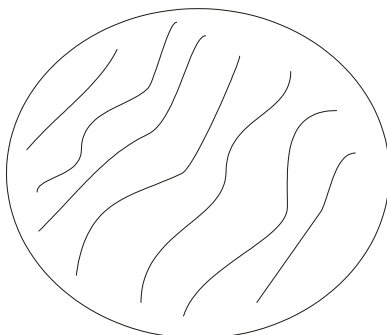


Abbildung 3.7: Lamellare Helfrich Phasen verhindern die gleichmäßige Diffusion in alle Richtungen. Die rotatorische Diffusion wird aber nicht zu stark behindert. Helfrich Phasen sind stark elektrostatisch geladen und zeigen oft eine sehr starke Ausrichtung.

Im Gegensatz zu den vorher erwähnten Polyethylenglykol/Alkohol-Phasen sind die Oberflächenlipid-Phasen geladen und können so elektrostatisch mit den Biomakromolekülen wechselwirken. Aus diesem Grund ist es wichtig immer zu testen, ob das Biomakromolekül noch in seiner stabilen nativen Form in der flüssigkristallinen Phase

vorliegt. Bei uns hat sich herausgestellt, dass dies nicht immer der Fall war. Selbst stabile Proteine, wie Triggerfaktor und Cyclophilin A, sind in den Helfrich-Phasen ausgefallen. Die Helfrich-Phasen (in den 70er Jahren hat Helfrich diese laminierten Phasen vorhergesagt, daher der Name) bestehen aus Cetylpyridiniumbromid, Hexanol und einer Natriumbromid Lösung. Vorteil dieses Systems ist die schnelle Erzeugung der flüssigkristallinen Lösung (man mischt die drei Komponenten im richtigen Verhältnis, vortext die Phase, erhitzt diese für eine Stunde auf 70°C und lässt die Phase dann abkühlen) und die große Toleranz gegen pH-Wert und Temperatur. Helfrich-Phasen sind lamellare Phasen welche lagenförmig vorliegen (siehe Abbildung 7). Die verschiedenen Lagen sind vom Lösungsmittel gequollen. Der Raum zwischen den einzelnen Lagen ist genügend groß, dass sich Biomakromoleküle problemlos bewegen können. Im Gegensatz zu n-Alkyl-Polyethylenglykol/n-Alkylalkohol/H₂O Phasen, welche keine elektrostatische Wechselwirkung zwischen Protein und der Phase hat, zeigen T₂-Relaxationszeiten (Abbildung 8), dass in Helfrich Phasen große elektrostatische Wechselwirkungen vorkommen.

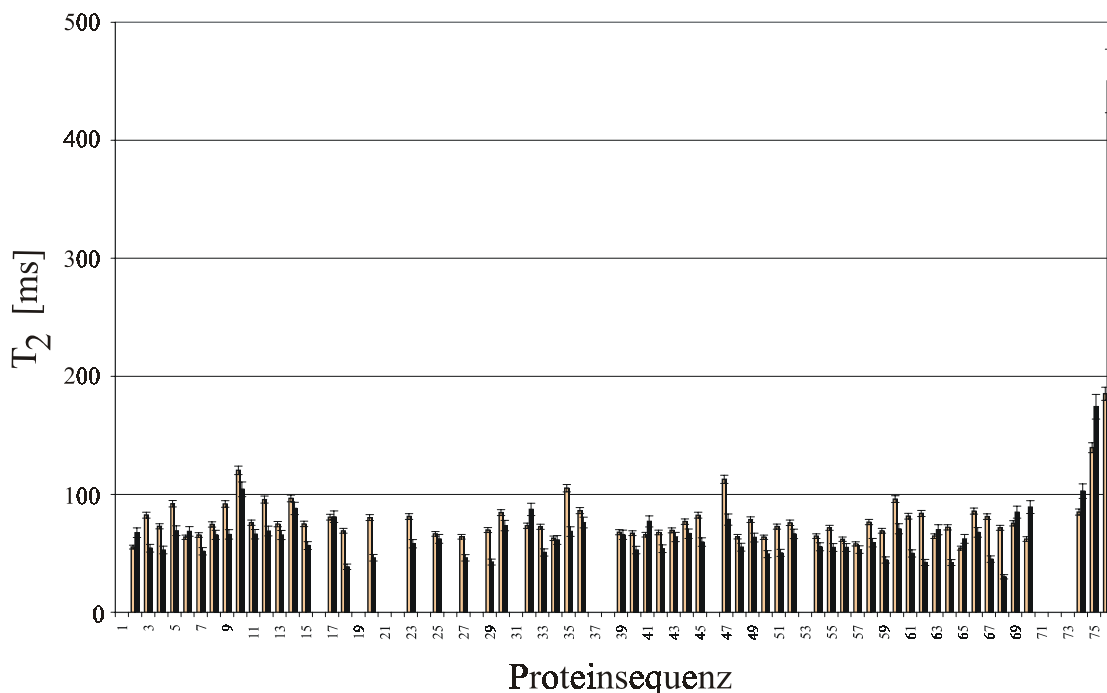


Abbildung 3.8: T₂-Relaxationszeiten gemessen in isotroper Lösung (hell, pH 6.5, 10% D₂O) und in Helfrich Phasen (schwarz, pH 6.5, 10% D₂O, Cetylpyridiniumbromid-Hexanol = 1.33:1 und 25mM NaBr, 3.5% Gesamtlipid). Die T₂-Relaxationszeiten sind in den Sekundärstrukturelementen im Mittel um 20% kleiner in der Helfrich Phase als in isotroper Lösung. Allerdings sieht man am C-Terminus verlängerte T₂-Relaxationszeiten in den Helfrich Phasen, was auf eine erhöhte Flexibilität hindeutet.

Beide genannten Phasen kann man bei Raumtemperatur ca. 1-2 Monate stabil lagern. Allerdings ist die n-Alkyl-Polyethylenglykol/n-Alkylalkohol/H₂O weit weniger langzeitstabil, wenn man das Protein zugegeben hat. Auch lässt sich das eingesetzte Biomakromolekül sehr leicht wieder aus der Phase extrahieren. Man verdünnt die Phase auf 50ml (Zerstörung der nematischen Phase) und zentrifugiert das Protein über einer 3000Da Membran. So war es möglich ca. 75% des Proteins wiederzugewinnen.

3.1.4 Flüssigkristalline Phase aus kristalliner Zellulose

Die Idee mittels einer flüssigkristallinen Phase, bestehend aus kristalliner Zellulose, Biomakromoleküle zu orientieren wurde von der Gruppe von Steve Matthews aufgezeigt⁴⁴. Zellulose Kristallite sind ca. 200±70 nm lang, 10 nm breit und haben eine ähnliche Form wie die oben genannten Bicellen. Lösungen von ca. 8% w/v kristalliner Zellulose orientieren sich im Magnetfeld (größer 11.7T) innerhalb von ca. 4 Stunden vollständig. Großer Nachteil der kristallinen Zellulose ist die recht schwierige Herstellung der Phase. Auch sind anscheinend die kristallinen Zellulose-Partikel unterschiedlich stark geladen, je nachdem wie groß der Ansatz beim Herstellen ist. Kristalline Zellulose kann durch Hydrolyse von natürlicher Zellulose (Filterpapier) mit Schwefelsäure hergestellt werden (45°C). Zuvor muss das Filterpapier mit scharfen Messern in einer Mühle zerkleinert werden (auch dies hat sich als kritischer Parameter bei der Herstellung erwiesen). Danach muss die Lösung verdünnt, dialysiert und neutralisiert werden. Nach längerem Ruhen sollten sich zwei Phasen bilden, von denen eine die flüssigkristalline Phase ist. Diese ist aber nur durch Polarisationsgläser erkennbar.

Die Phase ist überdies nur bei Salzkonzentrationen kleiner als 100mM stabil, sodass man davon ausgehen kann, dass die Phase eine weniger starke Verbreitung finden wird.

3.1.5 Flüssigkristalline Phase aus Purpur-Membran-Fragmenten (Bacteriorhodopsin⁴⁵)

Purpur-Membran-Fragmente (Bacteriorhodopsin) werden durch die extrem hohe Anisotropie der eigenen magnetischen Suszeptibilität in Magnetfeldern größer als 10T (ein 600MHz ¹H-Frequenz Gerät hat 14.1T) zu fast 100% orientiert^{20,46}. Die magnetische Anisotropie wird durch die Helices in Bacteriorhodopsin dominiert⁴⁷. Anders als bei

Bicellen gibt es hier keine kritische untere Konzentration. Nur das Magnetfeld muss genügend stark sein. Bevor man die Purpur-Membran-Fragmente verwendet, sollte man diese noch im Ultraschallbad für ca. 5 Minuten beschallen. Dies zerbricht die größten Fragmente, wodurch man eine homogenere Lösung erhält. Bacteriorhodopsin und die Organisation in Purpur-Membran-Fragmenten ist stabil bei pH-Werten von 2.5 bis 10, Ionenkonzentrationen bis zu 5M und Temperaturen von -269°C bis 69°C . Ein großer Nachteil ist die stark negative Ladung der Purpur-Membran-Fragmente. Deshalb ist auch die Wechselwirkung mit dem auszurichtenden Biomakromolekül auf eine starke elektrostatische Wechselwirkung zurückzuführen. Dies kann genauso wie bei der Orientierung mittels Phagen durch geringe Konzentration von Bacteriorhodopsin ($<2\text{mg/ml}$) und durch Zugabe von Salz verringert werden. Allerdings zeigen alle bisher gemessenen Systeme starke Linienverbreiterung, welche auf stark verringerte T_2 -Zeiten hinweisen. Dies bedeutet, dass die Proteine starke Wechselwirkungen mit dem Bacteriorhodopsin eingehen. Allerdings haben die Ausrichtungssensoren deutlich andere Orientierungen als die Ausrichtungssensoren von Bicellen und liefern dadurch zusätzliche Informationen. Die Normale der Purpur-Membran-Fragmente richtet sich parallel zum Magnetfeld aus. Dies ist 90° anders als Bicellen-Phasen und gleich wie mit Lanthaniden beladene Bicellen. Eigentlich sollte dies nur eine Skalierung der dipolaren Kopplungen um einen Faktor -2 bedeuten. Dies ist aber auf Grund der schon erwähnten starken elektrostatischen Wechselwirkungen nicht der Fall. Die Proben sind bei niedrigen Ionenkonzentrationen über 1-2 Monate stabil. Jedoch muss man bei den Messungen oft die Ionenkonzentration erhöhen, was allerdings auch die Lebensdauer der Proben verkürzt. Bei höheren Ionenkonzentrationen klumpen die Purpur-Membran-Fragmente zusammen, wodurch die Ausrichtungseigenschaften verringert und der Ausrichtungstensor verändert werden.

3.1.6 Flüssigkristalline Phase aus Polyacrylamidgelen

Unabhängig voneinander haben zwei Gruppen^{48,49} (R. Tycko (NIH) und S. Grzesiek (Universität Basel)) beschrieben, wie man mit Polyacrylamidgelen, welche entweder mechanischen Druck ausgesetzt sind oder magnetisch orientierendes Bacteriorhodopsin eingebettet haben, eine Vorzugsorientierung von Biomakromolekülen zu Wege bringen kann. Allein schon durch die Wechselwirkung von Proteinen mit den

Polyacrylamidgel-Phasen kommt es zu einer schwachen Orientierung der Moleküle. Die Wechselwirkung ist so stark, dass sich bei einem 10% Gel die Korrelationszeit um das ungefähr 1.5-fache erhöht. Allerdings kann man die Konzentration bei größeren Proteinen bis auf ca. 4% verringern (darunter werden die Gele mechanisch zu schwach und zerreißen). Dies erlaubt somit auch die Messung von größeren Proteinen bei denen sonst durch die zu kleinen T_2 -Zeiten die Linien zu breit werden würden.

Man kann die Orientierung in diesen Gelen aber noch vergrößern, wenn man diese unter mechanischen Druck stellt. Ein Beispiel hierfür ist die Druckausübung mittels eines Shigemi-Stempels auf das gerade aufquellende Gel vor der Messung, was eine nicht isotrope vertikale Spannung hervorruft. Natürlich kann man auch axiale Spannungen erzeugen, indem man das Gel noch stärker komprimiert oder indem man ein zu breites Gel in ein schmäleres NMR Röhrchen gibt und so den Durchmesser verringert. Durch den Vergleich der so gefundenen dipolaren Kopplungen mit den dipolaren Kopplungen in DHPC/DMPC-Bicellen wurde festgestellt, dass die orientierenden Kräfte ähnlich sein müssen, denn die Ausrichtungstensoren sind sehr ähnlich.

Durch das Einbetten von Bacteriorhodopsin, welches im Magnetfeld selbst orientiert wird (s.a. Kapitel oben), in Polyacrylamidgele, kann man auch eine Vorzugsorientierung einstellen. Die Orientierung von Bacteriorhodopsin in Magnetfeldern ($>10T$) kann permanent gemacht werden, wenn diese in Polyacrylamidgele eingebaut werden, wobei die Polymerisierung im Magneten stattfindet. Der Nachteil scheint zu sein, dass das Protein nur sehr langsam in das Gel eindiffundiert und so das Ansetzen einer Probe sehr lange (2 Wochen oder mehr) dauern kann. Natürlich kann man das Protein auch schon beim Polymerisieren im Magneten zusetzen wobei aber ca. 20-30% des Proteins mitimmobilisiert werden. Allerdings kommt es auch in diesen Gelen zu sehr starken elektrostatischen Wechselwirkungen zwischen Bacteriorhodopsin und den Proteinen, sodass die T_2 -Zeiten drastisch verkürzt werden. Zukünftig könnte man jedoch auch andere Stoffe in Polyacrylamidgele einlagern, welche vollständig in Magnetfeldern orientiert werden, aber nicht oder viel schwächer mit Proteinen wechselwirken.

3.1.7 Orientierung von Molekülen mittels elektrischer Felder

Mittels elektrischer Felder ist es möglich dipolare und quadrupolare Kopplungen in NMR-Spektren zu messen. Diese Polarisierung von verschiedensten Substanzen mit einem

permanenten, elektrischen Dipolmoment mittels elektrischer Felder wird schon lange untersucht. Eigentlich wäre es eine optimale Methode um verschieden starke Ausrichtungen einfach mittels elektrischen Feldern zu induzieren. Ein zusätzlicher großer Vorteil wäre, dass man einfach die isotrope Probe dafür verwenden könnte und man keine weitere teure isotoopenmarkierte Probe herstellen müsste. Die ersten Erfolge wurden im Arbeitskreis von MacLean gemacht^{50,51}. Diesem gelang es mittels Gleichspannung dipolare Wechselwirkungen in Nitrobenzolen zu messen^{52,53}. Da Biomoleküle oft viel größere Dipolmomente als Nitrobenzole aufweisen (z.B. Dipolmoment von Lysozym 407 D), welche ihren Ursprung in dem Dipolmoment der Peptidgruppe und den Seitenkettenladungen haben, sollten sie sich eigentlich viel besser zur Orientierung im elektrischen Feld eignen. Die dazu benötigte Spannung sollte ca. 10^4 - 10^5 V/m sein. Das große Problem stellt allerdings das Lösungsmittel Wasser bei Proteinen dar. Gleichspannung würde in einer wässrigen Lösung zur einer Wanderung der Ionen und dadurch zu einer vollständigen Kompensation des elektrischen Feldes führen. Durch Anlegen von Wechselfeldern mit Frequenzen größer als 15kHz können Ionen, dem nun rasch wechselnden Feld nicht mehr folgen. Die Ionenwanderung kann so unterbunden werden. Im Arbeitskreis Griesinger wurde von Ariane Tüchelmann⁵⁴ ein NMR Probenkopf entworfen, welcher den genannten Erfordernissen genügt. Tests an Cytochrom C und Ribonuclease A zeigten jedoch, dass die Effekte, sehr klein und nicht einfach reproduzierbar sind.

Ähnliche Versuche wurden auch in den Labors von Ann McDermott und Matthew Augustine unternommen^{55,56}. McDermott hat gepulste Wechselfeldern mit bis zu 70 kV/m verwendet um Nitrobenzol-d₅ auszurichten. Durch diese Methode war es möglich eine Deuterium Quadrupolare Aufspaltung in Nitrobenzol-d₅ zu messen. Die molekularen Ordnungsparameter waren in der Größe von bis zu 0.1%.

3.1.8 Anorganische Medien

Kürzlich wurde auch eine anorganische flüssigkristalline Phase vorgestellt, welche Polysaccharide ausrichtet⁵⁷. Diese MLCs (mineral liquid crystals) bestehen in diesem Fall aus wässrigen Suspensionen von V₂O₅ und zeigen im magnetischen Feld eine Konzentrationsdomäne ($0.13 < c < 0.2$ M), welche eine nematische Phase ist. Im Moment ist die Phase nur bei pH 1-3 stabil und deshalb noch ungeeignet für Proteine und

Ribonukleinsäuren. Aber sie zeigt einen weiteren Weg zu neuen orientierenden Medien auf.

Alle diese jetzt vorgestellten Methoden induzieren eine schwache Ausrichtung, welche die Messung von physikalischen Größen zugänglich macht, die bisher nur mittels Festkörper-NMR zugänglich waren. Dies sind dipolare Kopplungen, chemische Verschiebungs-Anisotropien^{31,58,59} und quadrupolare Kopplungen⁶⁰ um nur Einige zu nennen.

Nachfolgend möchte ich mich nun auf die Messung von dipolaren Kopplung und deren Anwendung für die Strukturbestimmung von Biomakromolekülen beschränken.

3.2 ¹J-Kopplungskonstanten^{61,62}

In isotropen Lösungen liefert die Größe der ³J-Kopplungskonstanten, also Kopplungskonstanten über drei Bindungen, Information über den dihedralen eingeschlossenen Winkel^{63,64}. Die Größe der ³J-Kopplung in Hz, von z. B. ¹H-C-C-¹H-Kopplungskonstanten, kann über die empirische Karplus-Beziehung^{65,66} in Winkelinformation übersetzt werden: $J = A + B \cos \theta + C \cos^2 \theta$.

A, B und C sind Konstanten, welche von der Elektronegativität der Substituenten abhängen. Mit Hilfe der ³J-Kopplungskonstanten ist es möglich die Winkel ϕ , φ , χ_1 und χ_2 in Proteinen zu bestimmen. Noch wichtiger ist die Bestimmung möglichst vieler ³J-Kopplungskonstanten in Nukleinsäuren⁶⁷, da in diesen die Dichte an Protonen viel niedriger ist⁶⁸ und hier andere Parameter erst die genauere Bestimmung der Struktur der nicht Sekundärstrukturelemente ermöglichen. In den letzten Jahren wurden die theoretischen Verfahren zur Berechnung von Kopplungskonstanten, Hand in Hand gehend mit immer leistungsfähigeren Computern, drastisch verbessert⁶⁹. Dies erlaubt es nun experimentelle Kopplungen immer öfter mit theoretisch berechneten Kopplungen zu vergleichen und daraus mehr Informationen über Konfiguration, Konformation und intermolekulare Wechselwirkungen zu erlangen^{70,71}.

Auf Grund dessen hat man der Messung von ¹J-Kopplungskonstanten nur relativ wenig Aufmerksamkeit geschenkt. 1992 berichteten Vuister et al.^{72,73} über die Abhängigkeit von ψ von der ¹J_{C α H α} -Kopplung in Proteinen. Sie konnten einen empirischen Zusammenhang zwischen der Kopplung und dem Rückgratwinkel finden. Erst kürzlich

wurde auch gezeigt, dass die Variationen der $^1J_{C\alpha C\beta}$ -Kopplungskonstante in Ubiquitin mit der Rückgratkonformation des Proteins korrelierbar sind⁷⁴. Vergleichend bestätigt wurde dieses auch durch DFT Rechnungen.

3.3 Messung von dipolaren Kopplungen

In den nachfolgenden Ausführungen möchte ich einen Überblick über die wichtigsten Methoden zur Messung von dipolaren Kopplungen geben. Diese werde ich nur für die $^1J_{NH}$ -Kopplung beschreiben, analog kann man aber die meisten beschriebenen Methoden - nur leicht modifiziert - zur Bestimmung von fast allen Kopplungen verwenden. Die einfachste Methode $^1J_{HN}$ -Kopplungen zu messen ist die Bestimmung aus einem gekoppelten 2D 1H - ^{15}N -HSQC-Korrelations-Spektrum^{4,75}. Sie wird im isotropen Fall die skalare $^1J_{NH}$ -Kopplung sein und im anisotropen Medium die Summe aus $^1J_{NH}$ - und $^1D_{NH}$ -Kopplung.

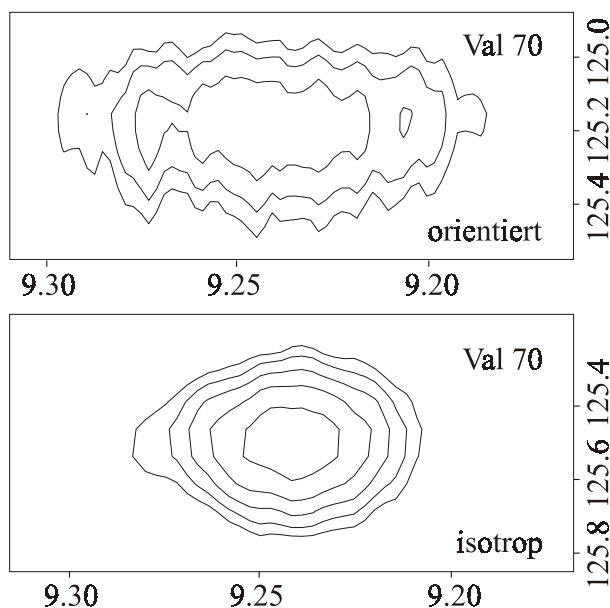


Abbildung 3.9: NH Korrelationspeak von Aminosäure Valin 70 in Ubiquitin; unten: isotrope Lösung (pH 6.5, 10% D₂O, 10mM NaCl) der Peak ist eine scharfe Linie. Oben: Ubiquitin gemessen in einer verdünnten flüssigkristallinen Phase (pH 6.5, 10% D₂O, Cetylpyridiniumbromid-Hexanol =1.33:1 und 25mM NaBr, 3.5% Gesamtlipid). Der Peak zeigt eine deutliche Linienverbreiterung, welche einerseits durch die Reduktion der T₂-Zeiten hervorgerufen wird, andererseits die Summe der 1H - 1H dipolaren Kopplungen. Zuiderweg und Mitarbeitern haben vorgeschlagen die 1H - 1H dipolaren Kopplungen mittels adiabatischen Protonenentkopplungen in der Evolutionszeit zu zerstören⁷⁶, was wir bis jetzt an Bruker Spektrometern nicht implementieren konnten. Bei der Auswertung der Spektren fitte ich die Peaks immer mit Lorentz Kurven an, um so den Mittelpunkt möglichst genau zu bestimmen.

Es hat sich herausgestellt, dass es sinnvoller ist die Aufspaltung in der F1-Dimension zu messen. In großen Biomolekülen ist die transversale Relaxationszeit von ^{15}N relativ zu der von $^1\text{H}^{\text{N}}$ Protonen länger, was zu einer höheren Auflösung in der F1-Dimension führt. Daten für das Protein Villin 14T (126 Aminosäuren, 14kDa) belegen dies deutlich (mittlere Relaxationszeiten): $T_1(^{15}\text{N})=595\text{ms}$ und $T_1(^1\text{H}^{\text{N}})=128\text{ms}$; $T_2(^{15}\text{N})=82\text{ms}$ und $T_2(^1\text{H}^{\text{N}})=19\text{ms}$. So ist im Mittel die transversale Relaxation der Amidprotonen ungefähr 4 mal kürzer⁷⁷. Ein Problem bei dieser Messung könnten Protonen sein, welche eine weitreichende Kopplung zu ^{15}N haben und somit nicht ^1H - ^1H J-Kopplungskonstanten erster Ordnung hervorrufen. Beispiele hierfür sind $^1\text{H}^{\beta}$ -Spins, welche an ^{15}N koppeln und eine leichte Asymmetrie in der Linienform des Dubletts hervorrufen können. Die Größe der nicht erste Ordnungs- ^1H - ^1H J-Kopplungskonstante ist abhängig von der Magnetfeldstärke, und kann kleine Fehler bei der Messung bewirken. Ein noch viel größeres Problem stellt aber die mit der Proteingröße ansteigende Überlappung der Signale dar. Deshalb war es notwendig eine neue Methode, mit besonderem Augenmerk auf hohe Präzision der Kopplung und Verringerung der Überlappung der Signale zu entwickeln.

Ein weitere Methode für die Messung war die Einführung einer weiteren $^1\text{J}_{\text{NH}}$ -Evolutionszeit in einem normalen 2D ^1H - ^{15}N -HSQC-Korrelations-Spektrum. Indem man eine Serie von 2D-Spektren aufnimmt und die Evolutionszeit verändert, kann man die $^1\text{J}_{\text{NH}}$ -Kopplung mittels eines Intensitätsfits exakt bestimmen⁴. Die großen Vorteile dieser Messmethode sind die hohe Genauigkeit und die Tatsache, dass keine zusätzliche Überlappung der Signale in den Spektren erzeugt wird. Die Nachteile sind, dass die Messdauer durch die Messung einer Serie von Spektren (in der Praxis hat sich herausgestellt, dass es sinnvoll ist mindestens 10 Spektren mit verschiedenen Evolutionszeiten aufzunehmen) extrem verlängert wird und dass sich die Methode nur für die exakte Bestimmung relativ kleiner dipolarer Kopplungen bis ca. 5 Hz eignet. Bei größeren dipolaren Kopplungen wechselt das Vorzeichen der Peaks zu rasch und die Fitting-Routine wird ungenau.

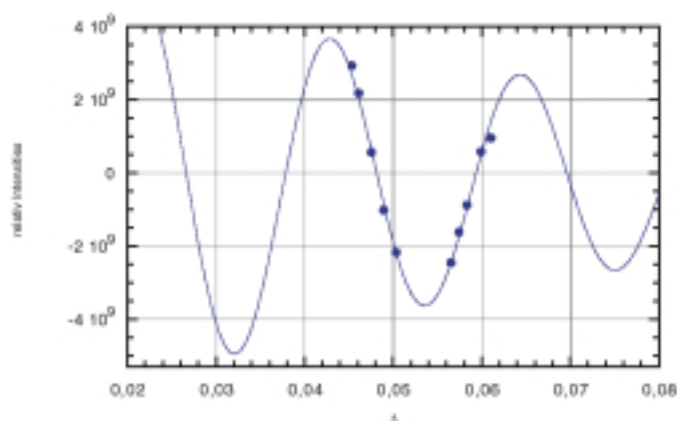


Abbildung 3.10: Bestimmung einer $^1J_{NH}$ Kopplungskonstante mittels $J\text{-}^1\text{H}\text{-}^{15}\text{N}\text{-HSQC}$ -Spektren. Die verschiedenen Kopplungsevolutionszeiten können gefittet werden:

$I(2\Delta) = C \cos(2\pi J_{NH}\Delta) \exp\left(-\frac{2\Delta}{T_2^*}\right)$, wobei I die Intensität der Peaks bei den verschiedenen inkrementierten Zeiten Δ ist. Diese Methode erlaubt eine sehr genau Bestimmung der Kopplung, wenn die Größe der Ausrichtung nicht zu verschieden von dem isotropen Durchschnittswert ist.

Die wichtigste Methode zur Messung von $^1J_{NH}$ dipolaren Kopplungen ist die sogenannte S^3E -Methode⁷⁸ (Spin State Selective Excitation, Spinzustand selektive Anregung). Ein Variation davon ist die IPAP (in Phase, anti Phase) Methode⁷⁹, welche aber eine um den Faktor 1.4 schlechtere Empfindlichkeit besitzt. Die S^3E -Methode erlaubt es den hochfeldigen und tieffeldigen Peak des Dubletts in zwei separaten Spektren darzustellen. Dies ermöglicht die schnelle und einfache Messung wie bei einem gekoppelten 2D $^1\text{H}\text{-}^{15}\text{N}$ -

HSQC aber zusätzlich mit der gleichen Einfachheit (geringer Überlapp der Peaks) wie bei einem entkoppelten 2D ^1H - ^{15}N -HSQC.

Aus diesem Grunde möchte ich diese Methode etwas genauer beschreiben. In Abbildung 11 sieht man den Anfangsweg der Magnetisierung, wobei die Pulssequenz mit einem INEPT Baustein beginnt (Übertragung der Magnetisierung vom angeregtem Proton auf den Stickstoff durch die skalare Spin-Spin Kopplung).

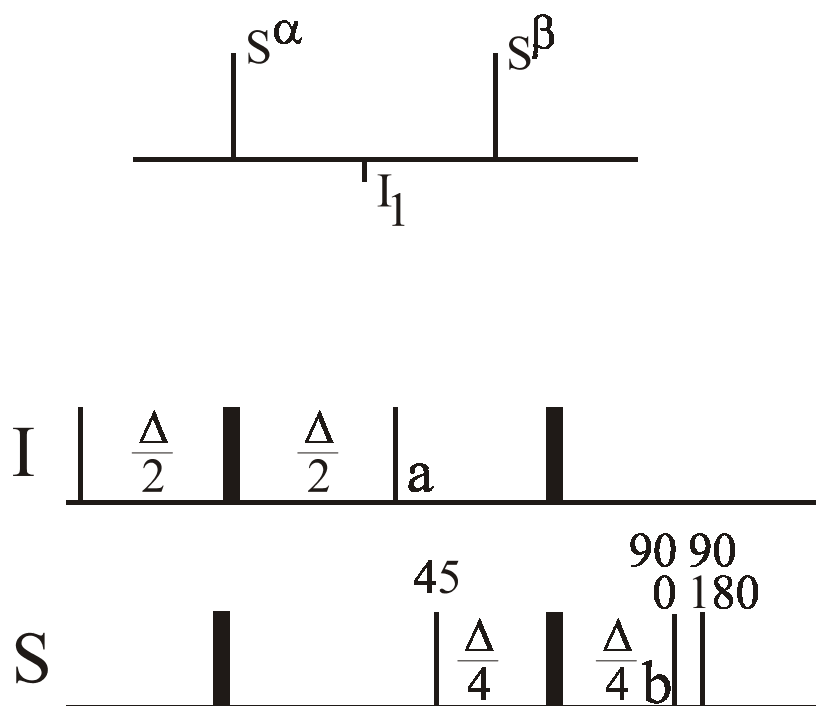


Abbildung 3.11: oben: Der Spin I koppelt zum Spin S, wobei dieser im α oder β Zustand sein kann. Unten: Schematische Darstellung des ersten Teils der S^3E Sequenz inklusive des Anfangs-INEPT-Schrittes Erklärungen im nachfolgendem Text..

Zum Zeitpunkt a lautet der Spinoperator $2I_z S_z$. Der darauffolgende Anregungspuls auf dem Heterospinkanal mit einer 45° Phase verwandelt den Operator in

$$2I_z S_z \xrightarrow{\left(\frac{\pi}{2}\right)^{45}} 2I_z (S_x \cos 45 - S_y \sin 45) \rightarrow 2I_z \frac{1}{\sqrt{2}} (S_x - S_y).$$

Im nachfolgendem Delay entwickelt sich die Kopplung über die Zeit $\frac{1}{4} J_{\text{NH}}$. Die Magnetisierung bei b lautet:

$$\begin{aligned} & \left(S_x \sin \pi J \frac{\Delta}{2} - 2S_y I_z \cos \pi J \frac{\Delta}{2} + \left(S_y \sin \pi J \frac{\Delta}{2} + 2S_x I_z \cos \pi J \frac{\Delta}{2} \right) \right) \frac{1}{\sqrt{2}} = \\ & (S_x + 2S_x I_z + S_y - 2S_y I_z) \frac{1}{2} \\ & \rightarrow S_x I^\alpha + S_y I^\beta \end{aligned}$$

Durch die zwei nachfolgenden 90° (S) Pulse, deren Phase so eingestellt wird, dass beim ersten Experiment die Phase 0° und beim zweitem Experiment die Phase 180° erhalten

wird, ergibt sich folgende Situation:

$$S_x I^\alpha + S_y I^\beta \xrightarrow{0^\circ} S_x I^\alpha + S_y I^\beta$$

$$S_x I^\alpha + S_y I^\beta \xrightarrow{180^\circ} S_x I^\alpha - S_y I^\beta$$

Addiert man nun im einen Fall die Spektren bekommt man den Operator $2S_x I^\alpha$ und bei der Subtraktion den Operator $2S_y I^\beta$. Durch diesen Kniff ist es möglich, die zwei Spinzustände zu trennen, wodurch das Spektrum „zerteilt“ wird.

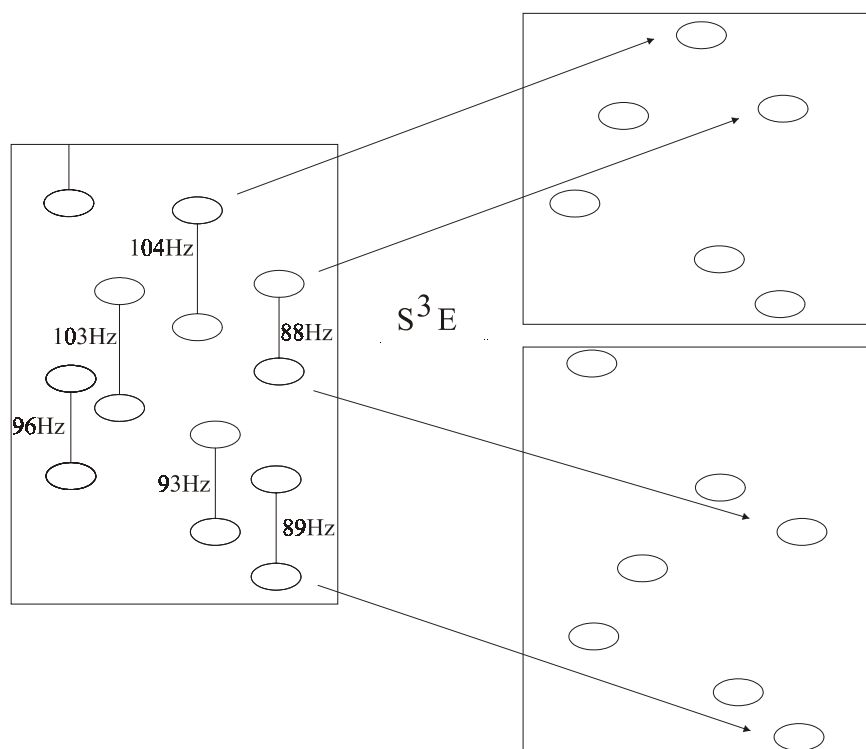


Abbildung 3.12: Schematische Darstellung des $S^3 E$ Spektrums. Der Größte Vorteil von $S^3 E$ ist die viel geringere Überlappung der Signale, und so eine viel einfachere Auswertung.

Der größte Nachteil all dieser Methoden liegt darin, dass die Delays nicht mehr optimal auf die Summe der skalaren und der dipolaren Kopplungskonstante abgestimmt werden können, denn obwohl die skalaren Kopplungskonstanten in Proteinen konstant sind, ändern sich die dipolaren Kopplungen orientierungsabhängig stark und somit die Summe der beiden Kopplungskonstanten.

3.3.1 J_{HH}-NOESY

Die vorgestellten Methoden eignen sich besonders gut um heteronukleare dipolare Kopplungen zu bestimmen. Heteronukleare dipolare Kopplungen beinhalten aber nur Orientierungsinformation und keine Abstandsinformation, da sie einen fixen Abstand haben, nämlich die Bindungslänge, z.B. 1.04 Å für N-H^{N80}. Auch sind skalare heteronukleare Kopplungen meist sehr groß, sodass der dipolare Anteil kleiner als der skalare und das Vorzeichen der dipolaren Kopplung eindeutig ist. Homonukleare ¹H-¹H dipolare Kopplungen sind wie alle dipolare Kopplungen über den Raum vermittelt ($\left(\frac{1}{r^3}\right)$ Abhängigkeit), haben hier aber keinen festen Abstand. Deshalb beinhalten diese Kopplungen nicht nur Orientierungsinformation sondern auch Abstandsinformation. ¹H-¹H dipolare Kopplungen haben jedoch meist keinen großen skalaren Kopplungsgegenpart, sodass man sich hier besondere Methoden überlegen muss um das Vorzeichen der dipolaren Kopplung zu bestimmen. Das Vorzeichen der dipolaren Kopplung reduziert die Mehrdeutigkeit der Information aus den dipolaren Kopplungen um den Faktor 2. Bildlich gesprochen kann man einen „schwarz-weißen Ball bunt machen“ und so die verschiedenen Orientierungen unterscheiden. Man sieht, dass die ¹H-¹H dipolaren Kopplungen eigentlich in jedem 2D ¹H-¹⁵N-HSQC oder anderem Spektrum vorhanden sind, und so ohne zusätzlichen Mehraufwand bestimmt werden können. Diese Voraussetzung wollten wir ausnützen. Die Methode, welche wir im April 2000 präsentierten nannten wir J_{HH}-NOESY Technik⁸¹. Die Pulssequenz ist eine modifizierte Version des J_{HH}-TOCSYs, welches von Willker⁸² vorgestellt wurde. Die Bestimmung des Vorzeichens ist möglich, da wir die ¹H-¹H dipolare Kopplung auf die Größe der J_{NH}+D_{NH}-Kopplung beziehen. Das Vorzeichen dieser Kopplung ist immer bekannt, da die skalare J_{NH}-Kopplung groß ist (ca. 93.5 Hz). Die dipolare Kopplung müsste größer als die skalare Kopplung werden um eine Bestimmung des Vorzeichens unmöglich zu machen. Dies ist aber aus praktischen Gründen beinahe nicht möglich. Spektren mit einem solch hohem Grad an Orientierung verlieren das typische Aussehen eines hochauflösenden NMR Spektrums und die einfache Auswertung ist unmöglich.

Die Technik beruht auf dem E.COSY Prinzip⁸³⁻⁸⁵. Über einen INEPT Schritt wird die Magnetisierung vom angeregten Proton auf den Stickstoff übertragen: $N_{1y}H_{1\alpha}^N$. Durch planares Mischen (BIRD, Pulse werden zur Spintopologiefilterung verwendet^{86,87}) wird die Magnetisierung dann auf das Proton zurück übertragen, der Spinzustand aber festgehalten:

$H_{1z}^N N_{1\alpha}$. Für ein leichteres Verständnis ist ein Teil einer BIRD Pulssequenz in nachfolgender Abbildung dargestellt und beschrieben.

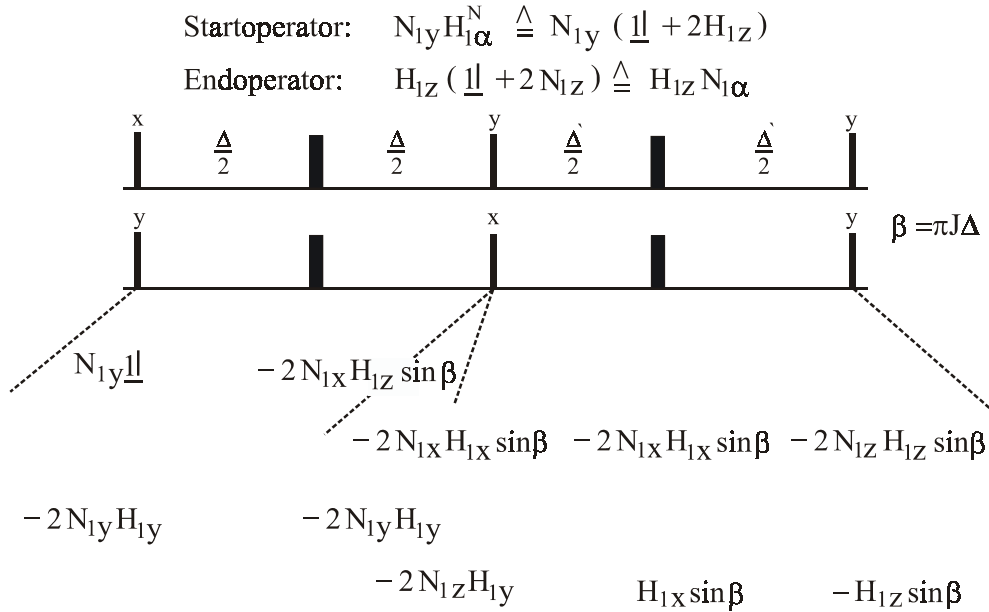


Abbildung 3.13: BIRD-Sequenz, welche in der J_{HH} -NOESY Sequenz als Spintopologiefilter verwendet wird.

Durch den nachfolgenden NOESY Schritt wird die Information von einem Proton auf ein weiteres Proton übertragen: $H_{2z} N_{1\alpha}$. So wurde erreicht, dass die Magnetisierung auf dem anderen Proton ist, der passive Spinzustand aber weiter erhalten bleibt. Fehler aus möglichen Spin-Flips, welche während der NOESY Mischzeit auftreten können, sind kleiner als 5%. Dieser Fehler wird mit zunehmender Proteingröße auch noch kleiner, weil die T_1 -Zeiten größer werden. Durch eine weitere BIRD-Spintopologiefilterung wird der Spinzustand wieder auf das Proton übertragen: $H_{2y} H_{1\alpha}^N$. Obwohl durch Wiederholen der Messung gezeigt wurde, dass der Fehler der Messung kleiner als 1Hz ist, war die Übereinstimmung der experimentell und berechneten 1H - 1H dipolaren Kopplungen nicht

überragend. Der Q-Wert³¹
$$Q = \frac{\sum_{i=1}^n (D_{\text{exp}}^i - D_{\text{calc}}^i)^2}{\sum_{i=1}^n (D_{\text{exp}}^i)^2}$$
, welcher ein Gütemaß für dipolare

Kopplungen darstellt, wurde zwar kleiner wenn die Kopplungen für eine auf NMR-Daten basierende Ubiquitin Struktur⁸⁸, welche schon mit dipolaren Kopplung verfeinert wurde, berechnet wurde, erreichte aber nie die Güte von heteronuklearen dipolaren Kopplungen. Diese großen Differenzen zwischen den verschiedenen Strukturen (es wurde die X-Ray⁸⁹-

und die schon erwähnte NMR-Struktur verwendet) zeigen eindeutig den Unterschied zwischen den Strukturen auf. Der Unterschied zwischen den experimentellen und berechneten Kopplungen führen wir auf starke Dynamik in den Seitenketten von Proteinen zurück. Die berechneten dipolaren ^1H - ^1H dipolaren Kopplungen sind größer als die experimentell bestimmten Kopplungen. Für die Berechnung wurde ein Ausrichtungstensor verwendet, welcher aus der Verteilung der D_{NH} dipolaren Kopplungen berechnet wurde. Dieser ist, wie wir später mathematisch und experimentell gezeigt haben, durch die Gesamtdynamik des Proteins verkleinert worden (ca. 25%). Deshalb sind alle berechneten ^1H - ^1H dipolaren Kopplungen zu klein.

3.3.2 Bitte einen MOCCA Herr Ober!

Um die Messungen noch genauer und mit besserem Signal zu Rausch Verhältnis durchzuführen, wäre es gut die lange NOESY Mischzeit durch eine effektive TOCSY Sequenz zu ersetzen. Die TOCSY Sequenzen sind jedoch nur optimal für skalaren J-Transfer und nicht für dipolaren Transfer. Deshalb wurden, zusammen mit Frank Kramer und Steffen Glaser (Technische Universität München), TOCSY Bausteine gesucht, welche den dipolaren Transfer verstärken⁹⁰. Die so gefundenen MOCCA-(Modified phase cycles Carr-Purcell-type)-Sequenzen, ergaben einen viel besseren Transfer als bekannte, für skalaren J-Transfer entworfene Sequenzen wie z.B. die DIPSI-Sequenz^{91,92}.

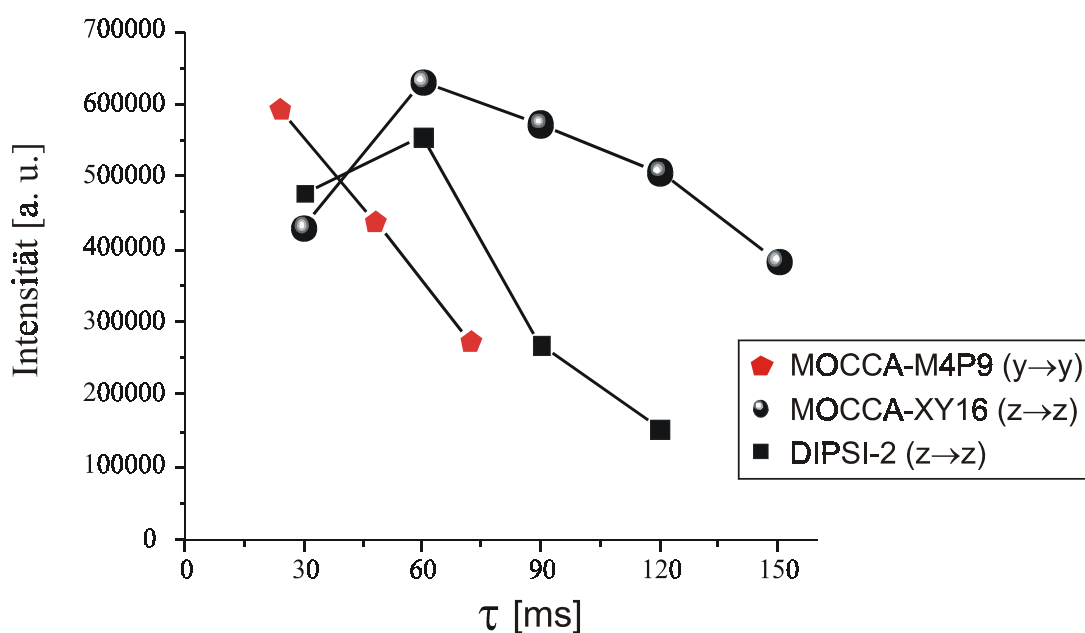


Abbildung 3.14: Experimentelle Transferkurven für 2 dipolare MOCCA Sequenzen und DIPSI-2. Die Transferintensitäten wurden für den Kreuzpeak von Leu 43 (H^N) und Gln 41 (H^N) gemessen.

In den letzten Jahren hat es noch einige andere Vorschläge für Messtechniken für ^1H - ^1H dipolare Kopplungen gegeben. Einige dieser Messtechniken beruhen auf klassischen 2D-Techniken wie COSY^{29,93} und TOCSY³³. Alle diese Vorschläge haben den Nachteil, dass sie kein Vorzeichen liefern. Auch mittels der HNHA-Technik wurden dipolare Kopplungen bestimmt^{94,95}. Constant-Time COSY Techniken erlauben es auf schnellem Weg ^1H - ^1H dipolare Kopplungen zu messen. Diese wurden sowohl in Polysacchariden als auch in Proteinen gemessen. Mit Zuhilfenahme dieser Kopplungen wurde gezeigt wie die NOE-basierende Strukturaufklärung unterstützt und beschleunigt werden kann⁹⁶⁻⁹⁸.

Andere Methoden beruhen auf der E.COSY Technik und liefern daher auch das Vorzeichen der ^1H - ^1H dipolaren Kopplung. Beispiele hierfür sind das nicht protonenkoppelte HNCA Experiment⁹⁹, oder andere auf dem HNCA Experiment basierende Sequenzen¹⁰⁰. Auch die Arbeitsgruppen von Gottfried Otting und Allen Bush hatten die Idee J_{HH} ähnliche Sequenzen zur Quantifizierung von ^1H - ^1H dipolaren Kopplungen zu verwenden^{101,102}.

3.3.3 SPITZE-HSQC

Um die starken Abweichungen zwischen gemessenen und berechneten ^1H - ^1H dipolaren Kopplungen genauer zu verstehen, war es wichtig weitere Parameter zu bestimmen. Die Messung von heteronuklearen dipolaren Kopplungen in Methylengruppen von Proteinen war der nächste Schritt. 1998 präsentierte Marcel Ottiger¹⁰³ eine Methode (intensitätsbasierende HSQC- J^4 Technik), welche es erlaubt dipolare Kopplungen von CH_2 und CH_3 Gruppen zu bestimmen. Allerdings ist es nur möglich die Summe und nicht die einzelnen Kopplungen zu bestimmen. Zusammen mit Teresa Carlomagno wurde eine Technik entwickelt, welche es ermöglicht simultan die Größe und das Vorzeichen von den zwei $^1\text{D}_{\text{CH}}$ und $^1\text{D}_{\text{HH}}$ dipolaren Kopplungen zu messen¹⁰⁴.

Die SPITZE-HSQC-Technik (SPIn sTate selective Zero overlap HSQC) möchte ich nun kurz darstellen: Die Abbildung stellt eine schematische Repräsentation der Peaks aus einer CH_2 Gruppe dar.

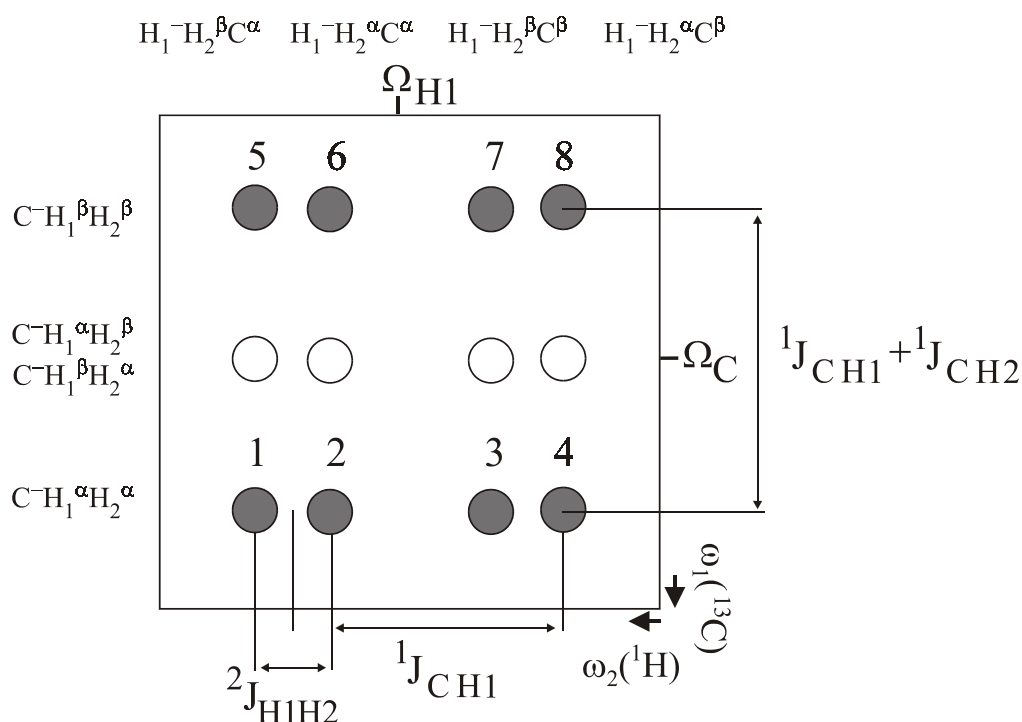
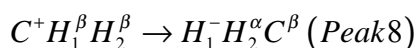
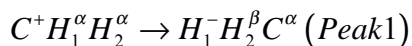


Abbildung 3.15: Schematische Darstellung eines CH₂ HSQC Spektrums. Durch die Große Linienbreite in Proteinen sind die durch die Proton, Proton Kopplung getrennten Peaks 1,2 und 3,4 meist nicht aufgespaltet und überlappen.

Das SPITZE-HSQC erreicht durch die Kombination aus einem S³E selektiven Transfer und dem Übergang zwischen den folgenden Operatoren in der CH₂ Gruppe seine hohe Selektivität:



oder:



Dies ermöglicht eine maximale Reduktion der Überlappung der Peaks (¹D_{HH} dipolare Kopplungen könnten wegen der viel zu großen Linienbreite überhaupt nicht direkt gemessen werden) und erlaubt es so alle Kopplungen zu bestimmen. Man erhält vier Spektren. In jedem Spektrum ist ein Peak der CH₂-Gruppe enthalten. Da man die Kopplung in der Protonendimension bestimmt, ist die Auflösung sehr hoch, sodass die Kopplung mit hoher Präzision bestimmt werden kann. Dies ist ein wichtiger Gegensatz zu einer kürzlich vorgestellten Methode, welche es gestattet alle Kopplungen in Größe und Vorzeichen einer NH₂ Gruppe zu bestimmen¹⁰⁵. Diese beruht auf der ZQ/DQ-HMBC-Technik, bestimmt aber die Kopplungen mit viel geringerer Auflösung. Ein weiterer

wichtiger Punkt ist, dass die Magnetisierung von den Operatoren $C^+H_1^\alpha H_2^\alpha$ und $C^+H_1^\beta H_2^\beta$ nicht auf vier Linien verteilt ist, wie in einem gekoppelten HSQC Spektrum, sondern auf einer einzelnen Linie konzentriert ist. Dies führt zu einem bessern Signal zu Rausch Verhältnis um den Faktor 4 im Vergleich zu einem gekoppelten HSQC, in dem die $J_{H_1H_2}$ -Kopplung aufgelöst ist.

Die Operatoren $C^+H_1^\alpha H_2^\alpha$ und $C^+H_1^\beta H_2^\beta$, welche die gewünschten Übergänge darstellen müssen selektiv in die Terme $H_1^-H_2^\beta C^\alpha$ und $H_1^-H_2^\alpha C^\beta$ überführt werden. Um dies zu bewerkstelligen muss zuerst der Zustand $C^\beta H_1^\beta H_2^\beta$ entweder in den $C^\alpha H_1^\beta H_2^\alpha$ - oder den $C^\alpha H_1^\alpha H_2^\beta$ -Zustand überführt werden. Der Hamiltonian $C^+H_1^+ + C^+H_2^+ = C^+F^+$ ermöglicht dies. Ebenso muss der Spinzustand $C^\alpha H_1^\alpha H_2^\alpha$ entweder in den $C^\beta H_1^\beta H_2^\alpha$ - oder den $C^\beta H_1^\alpha H_2^\beta$ -Zustand überführt werden. Dies erreicht der $C^-H_1^- + C^-H_2^- = C^-F^-$ Hamiltonian. Um die beiden selektiven Transfers gleichzeitig durchzuführen, muss eine Kombination der beiden Hamiltonians benützt werden: $C^+F^+ + C^-F^- = C_xF_x - C_yF_y$. Dieser Hamiltonian kann durch eine Hartmann-Hahn Sequenz, welche durch zwei 90° Pulse flankiert ist, verwirklicht werden. Der planare Hamiltonian $C_xF_x + C_zF_z$ wird durch eine y-DIPSI-Sequenz erhalten. Der $C_xF_x + C_zF_z$ Hamiltonian wird zum gewünschten $C_xF_x - C_yF_y$ Hamiltonian durch zwei 90° (\pm) Pulse auf dem X (^{13}C) und dem Protonen Frequenzkanal. Mit dieser Implementierung können die Peaks nun in vier Spektren aufgeteilt werden. Die endgültige Trennung von z.B. Peak 1 und Peak 8 kann nun durch ein $S^3\text{E}$ -Element erfolgen.

Kürzlich sind zwei Methoden vorgestellt worden, welche es erlauben dipolare Kopplungen in Methyl- CH_3 -Gruppen zu bestimmen. Die eine Methode verwendet ein constant time ^1H - ^{13}C HSQC Spektrum mit einem IPAP Block zum Separieren der Spektren¹⁰⁶. Ein weiterer Ansatz wurde von der Gruppe von Otting gezeigt¹⁰⁷. Die Bestimmung der dipolaren Kopplungen in Methyl- CH_3 -Gruppen kann besonders für sehr große Proteine interessant sein. Es gibt spezielle Markierungswege, welche es erlauben selektiv CH_3 -Gruppen nicht zu deuterieren. Diese zeigen scharfe Linien und sind somit immer mit guter Intensität bestimmbar.

3.4 Strukturbestimmung mittels dipolarer Kopplungen

3.4.1 Spektrenauswertung

Nach der Vorstellung der wichtigsten Methoden zur Messung der dipolaren Kopplungen möchte ich nun die Anwendung der dipolaren Kopplung in der Strukturaufklärung beschreiben. Wichtig dafür ist, dass die dipolaren Kopplungen richtig ausgewertet werden. Für die genaue Bestimmung von heteronuklearen dipolaren Kopplungen muss man darauf achten, dass die digitale Auflösung der Spektren genügend hoch ist, um die Kopplungen mit der gewünschten Präzision zu messen. Bei Biomolekülen ist es deshalb wichtig die transversalen Relaxationszeiten der betreffenden Kerne abschätzen zu können. Diese lassen sich durch einfache constant-time Experimente leicht abschätzen. Danach kann man die sinnvolle Anzahl an den zu akquirierenden Punkten festsetzen. Bei der Auswertung sollte man die Peaks an eine Lorentz-Linie fitten, um so den Mittelpunkt des Peaks zu bestimmen. Nach der Auswertung der Peaks kann man sich theoretische Daten mit dem zuvor erlangten Ausrichtungstensor berechnen. Diese kann man dann iterativ mit den experimentellen Daten vergleichen und so Fehler beheben. Dieses Vorgehen ist ähnlich der iterativen Auswertung von NOESY Spektren.

Wichtig bei der Auswertung von dipolaren Kopplungen ist es, auf das richtige Vorzeichen zu achten, z.B. auf das negative gyromagnetische Verhältnis des Stickstoffs. Die nachfolgende Tabelle, welche mir schon oft geholfen hat, darf als kleine Gedächtnisstütze für die wichtigsten dipolaren Kopplungen verstanden werden:

Dipolare Kopplung	Bestimmung des Vorzeichens
$N-H^N$	Isotrop-Ausrichtung
$CO^{(i-1)}-N^{(i)}$	Isotrop-Ausrichtung
$C\alpha-H\alpha$	Ausrichtung-Isotrop
$C\alpha-CO$	Ausrichtung-Isotrop
$CO^{(i-1)}-H^{N(i)}$	Ausrichtung-Isotrop

Tabelle 3.1: Gedächtnisstütze für die Auswertung von dipolaren Kopplungen.

Nachdem die dipolaren Kopplungen bestimmt sind, will man die Information für die globale weitreichende Strukturinformation zugänglich machen. Zuerst muss die Größe der axialen und rhombischen Komponente des Ausrichtungstensors bestimmt werden. Hier kann man sich zu Nutze machen, dass verschiedene internukleare Vektorentypen, die einen fixen Abstand haben, verschieden verteilt zum Ausrichtungstensor auftreten.

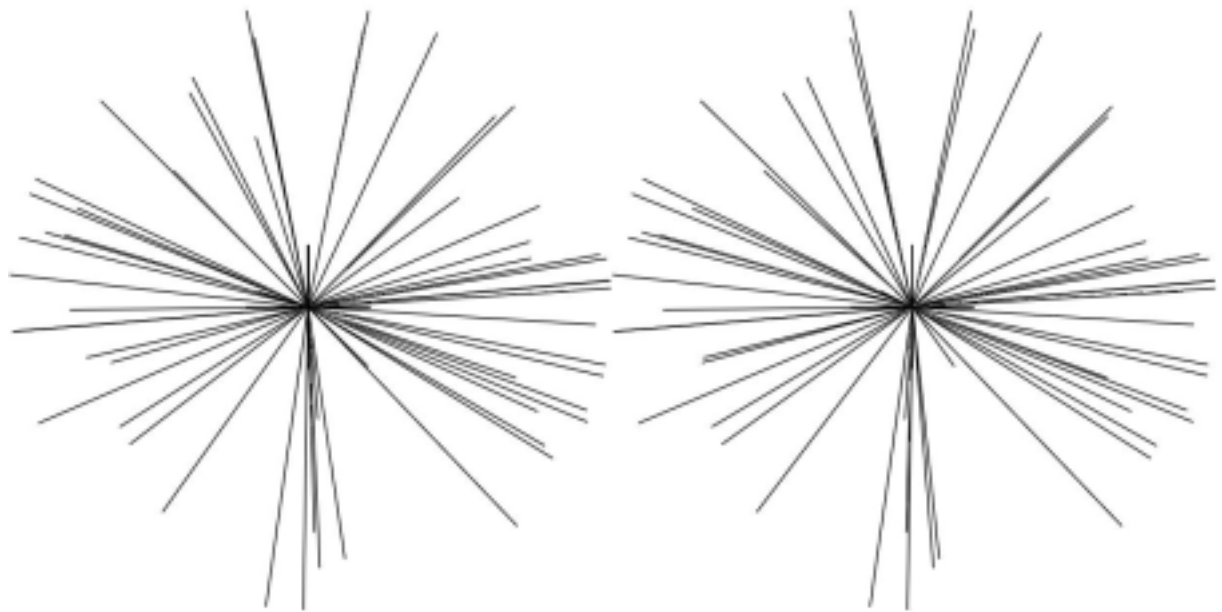


Abbildung 3.16: Stereoabbildung der Verteilung der Orientierung der NH Vektoren in Ubiquitin. Durch die relativ gute Gleichverteilung ist es möglich den axialen und den rhombischen Anteil des Ausrichtungstensors aus einem Histogramm zu bekommen.

Deshalb kann ein Histogramm, aus einem Ensemble von verschiedenen Vektortypen, wie eben erwähnt, ein Festkörper-Pulver-NMR-Spektrum approximieren¹⁰⁸. Aus einem solchen Pulverspektrum ist bekannt, wie man die Information der axialen und rhombischen Komponenten ohne jegliche Art von Strukturinformation extrahiert. Die Orientierung des Ausrichtungstensors, welche im Pulverspektrum nicht bestimmt wird, muss nicht bekannt sein, da diese als Fließvariable während der Rechnung gilt^{109,110}. In Abbildung 16 wird eine Pulvermodellverteilung gezeigt. Die Extraktion wird umso genauer, je mehr unabhängige Orientierungen der internuklearen Vektoren vorhanden sind. Deshalb wurde eine „Maximum Likelihood“ Methode (größte Wahrscheinlichkeitsmethode) präsentiert¹¹¹, welche es erlaubt die axialen und rhombischen Anteile mit größerer Genauigkeit zu extrahieren.

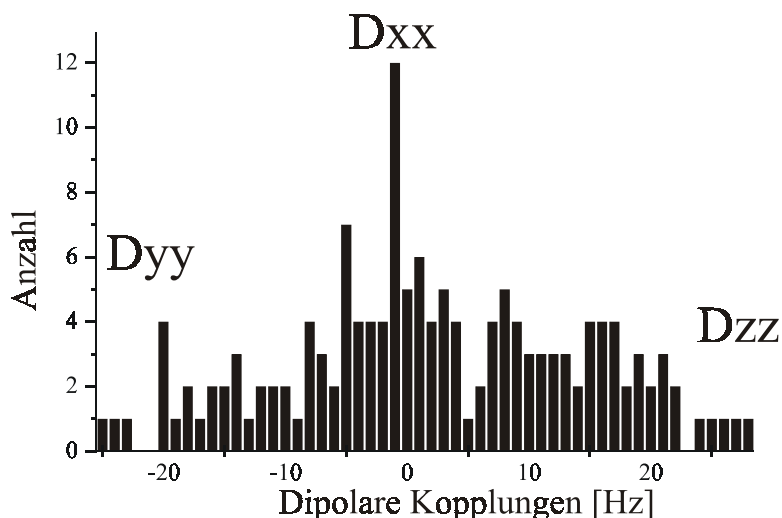


Abbildung 3.17: Histogramm aus dipolaren Kopplungen gemessen für das Protein Ubiquitin in einem anisotropen Medium. Wie eingezeichnet kann man aus dem Histogramm die Hauptachsen des Ausrichtungstensors ablesen.

3.4.2 DipoCoup

Im Rahmen der Auswertung der dipolaren Kopplung ist klar geworden, dass es bei der großen Menge an Daten wichtig wäre ein Computerprogramm zu haben, mit welchem die vielen oft benötigten Daten schnell berechnet werden können. Zusammen mit Jens Meiler wurde das Programm *DipoCoup* entwickelt¹⁷. *DipoCoup* bietet neben der Möglichkeit die wichtigsten Größen für dipolare Kopplung schnell zu berechnen auch ein Modul einen 3D Homologie Vergleich zwischen Proteinen vorzunehmen^{98,112-114}. Dieses gestattet es schnell die Topologie eines Proteins zu bestimmen und zu vergleichen. Wie eben dargestellt, ist es möglich dipolare Kopplung relativ schnell zu bestimmen. Es darf nicht verschwiegen werden, dass natürlich die chemischen Verschiebungen des Proteinerückgrats vorher zugeordnet werden müssen. Dies ist aber heute reine Routinearbeit. Die Methode erlaubt es schon in einem sehr frühen Stadium der Strukturaufklärung zu erkennen, ob die Topologie des Proteins schon einer bekannten Faltungsfamilie angehört oder nicht. Außerdem kann durch die bekannte Topologie auch das sonst sehr mühsame Zuordnen der NOESY Kreuzpeaks beschleunigt werden.

Anschließend möchte ich kurz die Arbeitsweise von *DipoCoup* erklären. Es gilt die Struktur eines unbekanntes Proteins zu bestimmen. Zuerst müssen die chemischen Resonanzen des Proteinerückgrates zugeordnet werden. Dann muss ein Medium gefunden werden in dem dipolare Kopplungen gemessen werden können und das Protein nativ vorliegt. Die dipolaren Kopplungen können dann verwendet werden um die axiale und

rhombische Komponente des Ausrichtungstensors zu bestimmen, s.o.. Mit diesem Ausrichtungstensor können nun dipolare Kopplungen für eine große Anzahl von Proteinen berechnet werden (z.B.: die gesamte Protein Datenbank: pdb). Vergleicht man die so berechneten dipolaren Kopplungen und die experimentellen Kopplungen mittels des schon bekannten Q-Wertes kann man ähnliche Topologien sehr schnell erkennen. *DipoCoup* erlaubt auch das Arbeiten mit Pseudokontaktshifts, welche beim Arbeiten mit magnetisch ausgerichteten Metalloproteinen messbar sind. Dipolare Kopplungen können aber nicht nur in Proteinen für eine schnelle Topologiesuche eingesetzt werden. Besonders in Nukleinsäuren war die nicht weitreichende Information basierend auf NOE Daten und J-Kopplungen oft der Grund für sehr schlecht definierte Strukturen. Dipolare Kopplungen erlauben es nun verschiedene Domänen von Nukleinsäuren gegeneinander zu ordnen^{37,38} oder den helikalen Anteil der Ribonukleinsäurestrukturen schnell festzustellen³⁹. Das Gleiche gilt auch für Proteine: die relative Orientierung von Proteindomänen kann eindeutig bestimmt werden^{115,116}. Früher war dies eine der besonderen Schwächen der NMR-Spektroskopie, da oft keine NOEs zwischen den Domänen messbar waren und so eine strukturelle Information nicht zugänglich war.

Im letzten Jahr gab es auch den Vorschlag aus Proteinfragmenten, welche aus der pdb-Datenbank entstanden sind und durch den Vergleich von dipolaren Kopplungen und chemischen Verschiebungen gefunden wurden, Proteine aufzubauen¹¹⁷. Auch die Strukturbestimmung von Proteinen mittels dipolaren Kopplungen wurde für Ubiquitin gezeigt¹¹⁸. Ob dies allgemein für alle Proteine möglich ist, gilt es nun zu prüfen. Des Weiteren erlaubt *DipoCoup* das Ausschreiben von dipolaren Kopplungen für die Verwendung in den Strukturrechnungsprogrammen XPLOR¹¹⁹ und CNS¹²⁰. Ermöglicht wird dadurch das schnelle Verwenden der neuen Parameter in der Strukturrechnung.

Für die Verwendung von dipolaren Kopplungen in der Strukturrechnung gibt es zwei verschiedene Ansätze, welche ich nun kurz beschreiben will. Der erste Ansatz besteht darin den Ausrichtungstensor bei der Rechnung zu verwenden¹¹⁰. Dieser wird ca. 100 Å zur Proteinstruktur in der Rechnung platziert. Damit kann die Struktur durch die dipolaren Kopplungen verfeinert werden. Nachteil dieser Methode ist, dass der Tensor festgelegt werden muss und daher möglichst genau bekannt sein sollte. Dies kann bei Proteinen mit ähnlichen Sekundärstrukturelementen, durch die gleiche Ausrichtung der internuklearen Vektoren, zu einer nicht homogenen isotropen Verteilung führen und so zu einer falschen Bestimmung des Ausrichtungstensors. Auch eignet sich die Methode nur für die

Proteinverfeinerung. Dipolare Kopplungen können erst gegen Ende der Rechnung verwendet werden. Wie schon berichtet gibt es aber neue Methoden den Ausrichtungstensor auch bei nur wenigen bestimmten Kopplungen genauer zu bestimmen¹¹¹. Dies würde für diese Methode wichtig sein.

In der zweiten Methode wird dieser Nachteil durch die Verwendung von Projektionswinkeln, welche aus dipolaren Kopplungen erzeugt werden, umgangen^{121,122}. Diese Projektionsrestraints beschreiben den eingeschlossenen Winkel zwischen zwei internuklearen Vektoren. Die großen Vorteile dieser Methode sind, dass man hier den Ausrichtungstensor nicht kennen muss und die dipolaren Kopplungen vom Beginn der molekulardynamischen Rechnung verwendet werden können. Auch führt dies zu einer besseren Konvergenz der so berechneten Strukturen.

3.5 *Beispiele für die Strukturrechnung von Molekülen mittels dipolaren Kopplungen*

3.5.1 Polysaccharide

Dipolare Kopplungen sind nicht nur wertvolle Parameter für große Biomakromoleküle. Auch für kleine Moleküle, wie zum Beispiel Polysaccharide, können dipolare Kopplungen essentiell zur Strukturbestimmung beitragen. Dies haben wir für Saccharose und Raffinose gezeigt¹²³. Polysaccharide haben oft sehr wichtige Funktionen in vielen Stoffwechselsystemen inne. Deshalb ist es auch von Interesse die 3-dimensionale Struktur zu kennen. Polysaccharide sind aber meist nicht exakt mittels X-Ray-Kristallographie bestimmbar. Durch Packungseffekte im Kristall sind oft große Unterschiede zwischen Festkörper X-Ray-Strukturen und hochaufgelösten NMR-Strukturen zu erkennen. Eine weitere wichtige Methode zur Strukturbestimmung ist die Neutronenbeugungsanalyse. Beide Festkörpermethoden zeigen aber oft unterschiedliche Strukturen. Deshalb ist es wichtig die Struktur in Lösung möglichst genau mittels NMR-Spektroskopie zu bestimmen. Dies ist jedoch schwierig, weil nur wenige NOE Abstände und Kopplungskonstanten in Polysacchariden gemessen werden können und so zum Beispiel die glykosidische Bindung (Bindung zwischen den einzelnen Ringen) oft schlecht definiert ist¹²⁴. Aus den eben genannten Gründen haben zusätzliche Parameter großen Einfluss auf die Strukturbestimmung mittels NMR-Spektroskopie. Literaturbekannt sind schon zahlreiche Studien, welche rein auf der Messung von heteronuklearen CH dipolaren Kopplungen in den einzelnen Zuckerringen beruhen¹²⁵⁻¹²⁸. Aus diesen CH dipolaren

Kopplungen wurde ein Ausrichtungstensor extrahiert, welcher dann für die Strukturrechnung verwendet wurde. Wie schon zuvor aufgezeigt, ist bei der Extraktion der axialen und rhombischen Komponenten des Ausrichtungstensors eine isotrope Verteilung der internuklearen Vektoren essentiell¹⁰⁸. Durch die Geometrie des Pyranoseringes, ist die Verteilung der CH-Vektoren nicht isotrop. Die Bestimmung des Ausrichtungstensors ist auf diese Weise also sehr ungenau.

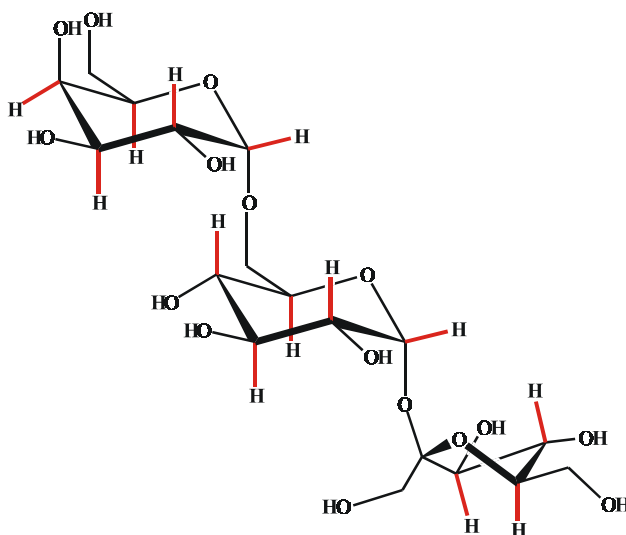


Abbildung 3.18: Raffinose ist ein Trisaccharid, welches aus Galaktose, Glukose und Fruktose aufgebaut ist. Hier sind die CH Vektoren hervorgehoben, um zu zeigen, dass die Orientierung der Vektoren im Raum gleich ist und deshalb keine Extraktion des Ausrichtungstensors, wie zuvor beschrieben, möglich ist.

Deshalb war es wichtig zusätzliche dipolare Kopplungen zu messen¹²³. ^1H - ^1H dipolare Kopplungen, welche wir ja auch schon in Proteinen zugänglich gemacht hatten, erwiesen sich für unsere Zwecke als ideal. Auch hier haben wir wieder die E.COSY Methode^{83-85,129} verwendet um ^1H - ^1H dipolare Kopplungen zu bestimmen.

Insgesamt gibt es drei verschiedene Arten von dipolaren Kopplungen:

- ^1H - ^1H dipolare Kopplungen, welche einen skalaren Gegenpart besitzen; Zur Bestimmung muss man sowohl die skalaren ^3J -Kopplungen in isotroper Lösung als auch die skalaren und dipolaren Kopplungen in orientierender Lösung messen. Die Differenz der beiden liefert wieder die dipolare Kopplung.
- Da, wie schon dargelegt, die dipolare Kopplung nicht wie die skalare Kopplung über Bindungen vermittelt wird, sondern durch den Raum, können auch $^4\text{D} - ^x\text{D}$ dipolare Kopplungen, welche nun keinen skalaren Gegenpart besitzen, gemessen werden. Die Kreuzpeaks treten nur in anisotropen Medien auf.

- Schließlich können auch dipolare Kopplungen zwischen zwei Ringen gemessen werden (Auswertung mittels der DISCO Methode).

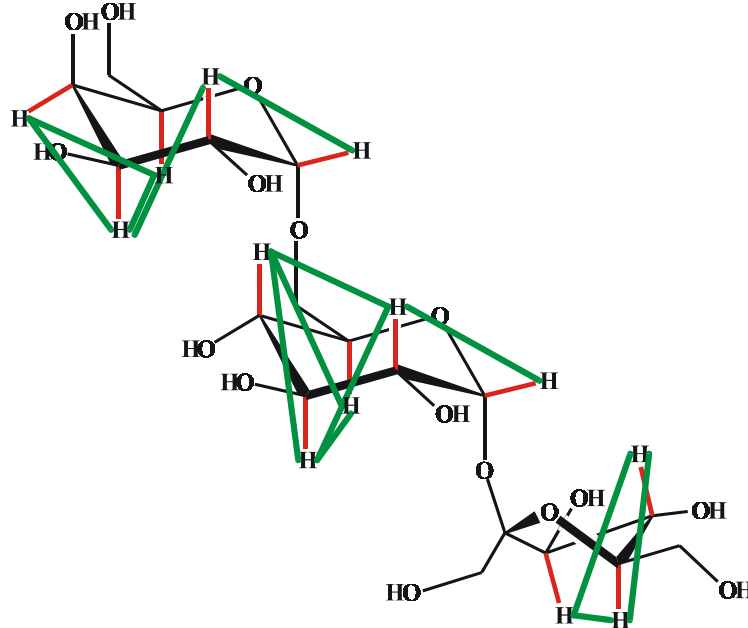


Abbildung 3.19: In dieser Darstellung von Raffinose sind auch einige der gemessenen ^1H - ^1H dipolaren Kopplungen eingezeichnet. Die Orientierung dieser Vektoren unterscheidet sich deutlich von den CH-Vektoren. Hiermit konnten wir einen Starttensor berechnen.

Durch diese große Anzahl von neuen experimentellen Parametern ist eine viel genauere Bestimmung von Polysacchariden möglich. Auch andere Gruppen haben einen ähnlichen Weg gewählt^{101,130}. Theoretische Betrachtungen haben aber gezeigt, dass die so gewonnene Anzahl an unterschiedlichen internuklearen Vektoren noch immer nicht vollständig ausreicht um den Ausrichtungstensor exakt festzulegen. Deshalb war es notwendig sich eine neue Vorgehensweise bei der Strukturrechnung zu überlegen:

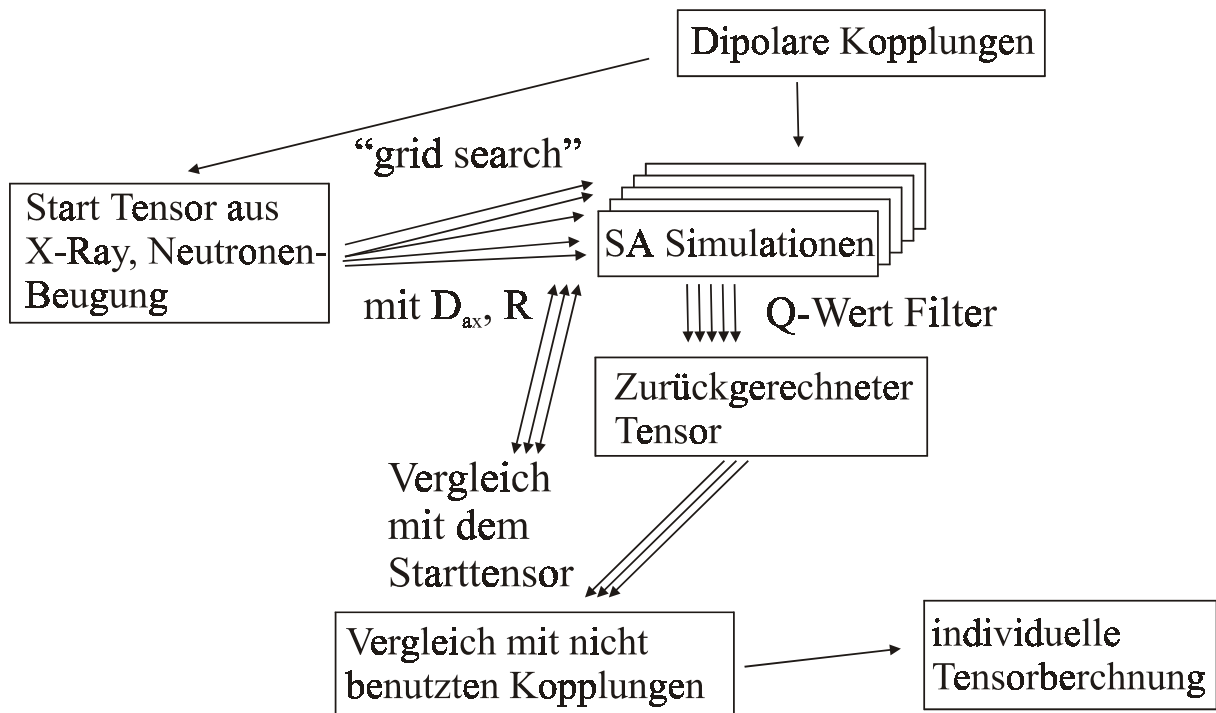


Abbildung 3.20: Übersicht über das benützte Rechenprotokoll um die NMR Lösungsstruktur von Raffinose zu beschreiben. Der experimentelle Ausrichtungstensor wurde mittels eines „Grid-Searches“ variiert. Die Ergebnisse werden mehrmals gefiltert (Q-Wert Filter, zurückgerechneter Ausrichtungstensor, nicht verwendete dipolare Kopplungen). Danach war es möglich für jedes Monosacchrid in Raffinose einen individuellen Ausrichtungstensor zu bestimmen.

Aus den experimentellen dipolaren Kopplungen wird ein vorläufiger Ausrichtungstensor bestimmt. Man weiß, dass dieser mit Fehlern behaftet ist. Aus diesem Grund macht man einen Grid-Search von ungefähr $\pm 20\%$ für den axialen und rhombischen Anteil des Ausrichtungstensors. Mit all diesen Tensoren startet man eine Strukturrechnung. Nach der Strukturrechnung filtert man die Daten nach folgenden Kriterien:

Q-Filter Ausschluss: Man vergleicht die experimentellen dipolaren Kopplungen mit den berechneten dipolaren Kopplungen. Von den Strukturen mit dem kleinsten Q-Wert berechnet man den Ausrichtungstensor und vergleicht diesen mit dem Tensor, welcher der Rechnung zugrunde gelegt wurde. Wie wir in der Praxis gesehen haben, lässt sich so die Zahl der möglichen Strukturen drastisch reduzieren. Zu Beginn der Rechnung wurden nicht alle experimentellen dipolaren Kopplungen für die Strukturrechnung verwendet. Diese restlichen Kopplungen sollten als Kreuzvalidierungshilfe am Ende der Rechnung dienen. Die endgültige wahrscheinlichste Struktur ermittelt man nun, indem nicht verwendete experimentelle dipolare Kopplungen mit den theoretischen dipolaren

Kopplungen aus dem besten Modell verglichen werden. Die so berechneten Strukturen für Saccharose und Raffinose werden in nachfolgender Abbildung gezeigt.

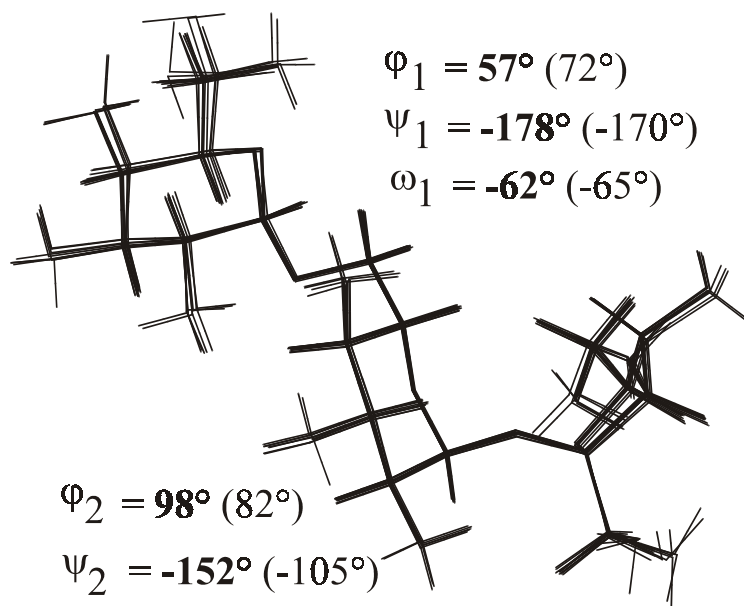


Abbildung 3.21: Ensemble der 10 energieärmsten Strukturen von Raffinose, welche mit den zuvor beschriebenen NMR-Parametern erzeugt wurden. Der Rückgrat RMSD-Wert beträgt nur 0.28Å. In der Abbildung sind auch die Winkel über die glykosidische Bindung beschrieben (fett: NMR; in Klammer: X-Ray). In diesen zeigt sich ein deutlicher Unterschied zwischen der NMR Lösungsstruktur und der Röntgenstruktur besonders um die zweite glykosidische Bindung.

In Abbildung 22 wird der Unterschied zu der nur aus NOE-Daten berechneten Raffinose Struktur gezeigt.

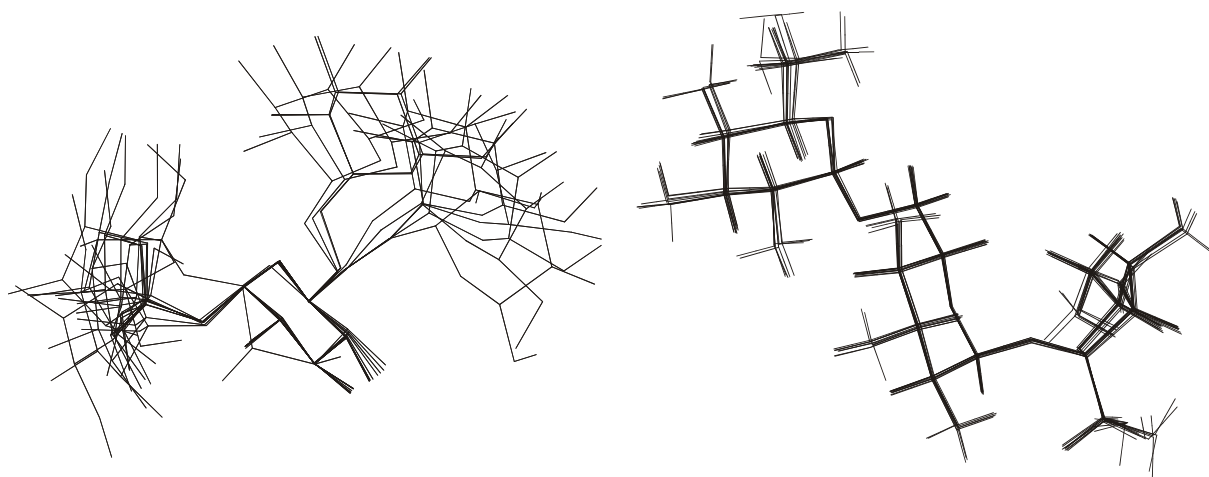


Abbildung 3.22: Vergleich der aus NOE-Daten (links, Schweratom-RMSD: 1.02Å) und der aus NOE-Daten und dipolaren Kopplungen berechneten NMR-Ensembles (rechts, Schweratom-RMSD: 0.28Å) von Raffinose.

Dies macht deutlich, dass es wichtig ist, neue Parameter nicht nur messen zu können, sondern dass neue Parameter auch neue Wege für die sinnvolle Verarbeitung benötigen. Aus den so berechneten Strukturen und der erhöhten Anzahl an internuklearen dipolaren Vektoren konnten auch erstmals Ausrichtungstensoren für die einzelnen Zuckerringe des Polysaccharides bestimmt werden. Diese gestatten einen Rückschluss auf die Dynamik von Polysacchariden. Im Fall der Raffinose ist der Galaktose Tensor ca. 40% kleiner als die beiden Ausrichtungstensoren der Glucose und der Fruktose. ^{13}C -Relaxationsmessungen haben auch unterschiedliche longitudinale Relaxationsraten von den betreffenden Zuckerringen ergeben. Interessanterweise ist diese höhere Amplitudenbewegung anisotrop, denn die zwei axialen CH-Vektoren an C_1 und C_4 zeigen eine höhere Mobilität als die äquatorialen. Dies führen wir auf eine Rotation um den Winkel ϕ_1 oder ω_1 der glykosidischen Bindung zwischen der Galaktose und der Glucose zurück. Diese dynamischen Effekte werden auch in Zukunft eine große Rolle spielen und als wichtige Parameter in die Strukturrechnung für dynamische Ensemble einfließen.

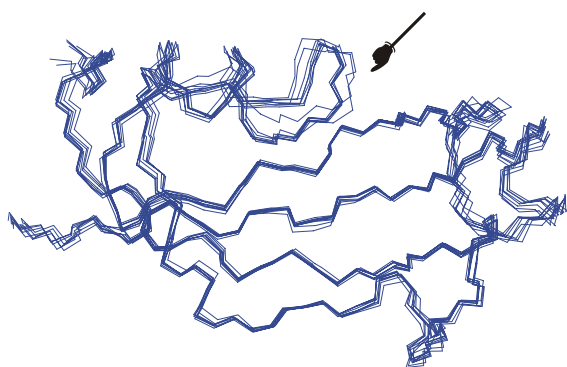
3.5.2 Proteine

Wie im vorhergehenden Text schon mehrmals erwähnt erlauben dipolare Kopplungen Proteinstrukturen mit viel größerer Genauigkeit zu bestimmen¹³¹⁻¹³³. Dies hat folgende Gründe: Dipolare Kopplungen sind globale Parameter, welche von der ersten bis zur letzten Aminosäure „miteinander sprechen“. Deshalb ist wie schon erwähnt auch die Bestimmung der relativen Domänenorientierung möglich^{115,116}. Dipolare Kopplungen sind in großer Anzahl schnell und einfach zugänglich. Deshalb war es auch von großem Interesse zu sehen, wie viele NOE Abstandsparameter man noch neben der Orientierungsinformation aus dipolaren Kopplungen benötigt. Die NOE Abstandsparameter liefern weiter die translatorische Information in der Strukturrechnung. Abhängig von der Größe und der Topologie des Proteins kann man mit nur wenigen NOE Zusatzabständen eine Struktur vom Protein meist schnell festlegen¹³⁴. Heteronukleare Kopplungen erlauben besonders das Proteinrückgrat genauer zu definieren. Dieses wurde bisher mit klassischen Parametern nur über $\text{H}^{\text{N}}-\text{H}^{\text{N}}$ und $\text{H}^{\text{N}}-\text{H}^{\alpha}$ NOE Abstände und über die J-Kopplungskonstanten ϕ und ψ definiert. Viel mehr NOE Distanzen wurden für die Seitenketten gemessen. Dies sieht man auch bei Strukturrechnungen mit heteronuklearen dipolaren Kopplungen: Die Genauigkeit des Proteinrückgrats ist stark erhöht; hingegen ist der RMSD-Wert aller Atome nur geringfügig besser.

Nachstehend möchte ich kurz den Effekt von dipolaren Kopplungen an zwei Beispielen beschreiben. Das erste Beispiel ist die PPIase-Domäne des Triggerfaktors aus *Mycoplasma genitalium*¹³⁵. In jeder Zelle gibt es Proteine, welche bei der Proteinfaltung behilflich sind.

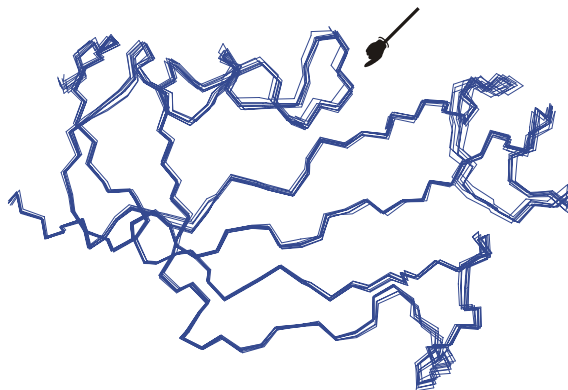
Es gibt hier zwei wichtige Klassen: Chaperone, welche die Proteinaggregation unterdrücken und ein möglichst korrekt gefaltetes Protein entlassen sollen und Isomerasen, welche wichtig für bestimmte Prozesse in der Faltung selbst sind. Es existieren zwei Klassen von Isomerasen: Disulfid Isomerasen und PPIasen (Peptidyl-Prolyl *cis/trans* Isomerasen). PPIasen wiederum können in drei Klassen unterteilt werden: Cyclophiline, FK506 Bindeproteine und Parvuline. Der Triggerfaktor ist ein Protein, welches eine hohe Affinität zu entfaltenen Proteinen zeigt. Die PPIase Aktivität der FKPB-artigen-Domäne ist selbst gering, aber der gesamte Triggerfaktor kann Proteine mit einer höheren Rate falten als bisher bekannte Proteine. Der Triggerfaktor besteht aus drei Domänen: der N-terminalen Ribosom Bindungsstelle, der FKBP-artigen-Domäne und einer C-terminalen-Domäne, deren Funktion bis jetzt noch nicht geklärt werden konnte.

Die 3-dimensionale Struktur der PPIase-Domäne wurde bei uns im Labor aufgeklärt¹³⁶. Dazu sollten auch dipolare Kopplungen gemessen werden. Die Orientierung wurde durch eine n-Alkyl-Polyethylenglykol/n-Alkylalkohol/H₂O-Phase hervorgerufen⁴¹. 67 N-H^N dipolare Kopplungen konnten mit einem Q-Wert von 0.25 gemessen werden. Dies sind 81% aller möglichen N-H^N Vektoren. Für die Strukturrechnung wurde eine Struktur mit 959 NOE Abständen, 35 Wasserstoffbrücken, 72 H^N-H^α Kopplungskonstanten und 68 weiteren dihedralen Winkelparametern berechnet. In einer zweiten Strukturrechnung wurden zusätzlich auch noch die dipolaren Kopplungen, welche als Winkelprojektionsparameter vorgegeben wurden, verwendet. Die Strukturrechnung ohne dipolare Kopplungen zeigt für die besten 15 berechneten Strukturen einen Rückgrat RMSD-Wert von 0.34Å für die strukturierten Bereiche. Hingegen konnte die Genauigkeit des Rückgrats mit den dipolaren Kopplungen noch weiter gesteigert werden: 0.21Å Rückgrat RMSD-Wert. Viel wichtiger als der reine Vergleich ist aber die Tatsache, dass der Bereich der hydrophoben Bindungstasche (Ile 67, vergleichbar mit Ile 57 aus FKBP) viel besser definiert ist. An dieser Bindungstasche NH Ile67 bindet vermutlich das CO der Peptidyl-Prolyl-Bindung und wird dadurch bei der Isomerisierung katalysiert. Diese Tatsache kann für weitere Bindungsbetrachtungen entscheidend sein.



NOE und Winkelinformation

RMSD = 0.34 Å



NOE, Winkelinformation
und dipolare Kopplungen

RMSD = 0.21 Å

Abbildung 3.23: NMR Ensemble von Triggerfaktor berechnet nur aus NOE-Daten (links) und mit NOE-Daten inkl. dipolarer Kopplungen. Der Pfeil zeigt die Bindungstasche, welche mit den dipolaren Kopplungen viel besser definiert ist.

Als ein weiteres Beispiel für die Verwendung dipolarer Kopplungen in der Strukturrechnung möchte ich Ubiquitin anführen. Während meiner Promotion konnte ich mehr als 10 verschiedene Datensätze von dipolaren Kopplungen messen. Dadurch konnte eindeutig gezeigt werden, dass nicht jede Orientierung gleich viele Informationen erhält. Auch ist der Ausrichtungstensor nicht vorhersagbar. Eindeutig festzustellen war, dass das Dopen von Bicellenmedien mit CTAB und SDS (s. oben) den Ausrichtungstensor nicht stark verändert. Deshalb ist es besonders wichtig in unterschiedlichen Medien zu messen, um den Ausrichtungstensor möglichst stark zu variieren und so divergente Informationen zu erhalten. Diese Informationen waren außerdem wichtig zur Extraktion dynamischer Information aus dipolaren Kopplungen, welche ich im Folgenden beschreiben will.

3.6 Dynamik aus dipolaren Kopplungen

Die bisher dargestellte Suche nach einer molekularen Struktur basierte auf der Annahme, dass eine rigide Struktur die gemessenen Daten genau reproduzieren kann. Dies kann für auf NOE-Daten basierenden Strukturen der Fall sein, aber für auf Orientierungsparametern basierenden Strukturen trifft dies nicht zu. Bei auf NOE-Daten basierenden Strukturen kompensieren sich Distanz- und Winkel-mittelnde Effekte. Dies ist bei Strukturen, die von Orientierungsparameter abhängen, nicht der Fall. Ein Ausweg wäre

es Daten, die auf starke Bewegungsmittlung hindeuten, durch heteronukleare NOE Messungen zu identifizieren und auszuschließen.

Im Moment stellen heteronukleare (^{15}N , ^{13}C) Messungen die populärsten Methoden zur Beschreibung der Dynamik dar¹³⁷⁻¹⁴². Longitudinale T_1 -, transversale T_2 -Zeiten und heteronuklearer NOE sind die meist gemessenen Parameter¹⁴³. Für die Interpretation der Daten stehen verschiedene Modelle zur Verfügung^{144,145}. Das vorwiegend verwendete Modell ist das von Lipari und Szabo entwickelte „Model-free Modell“^{146,147}, welches mittels einer Über-Alles-Korrelationszeit τ_c die rotatorische Diffusion und mit dem Ordnungsparameter S^2 die Amplitude des XH-Vektors beschreibt¹⁴⁸. Die Amplitudenbewegung des XH-Vektors ist unabhängig von τ_c und ist charakterisiert durch eine Zeit τ_i , welche normalerweise im ps-Bereich liegt.

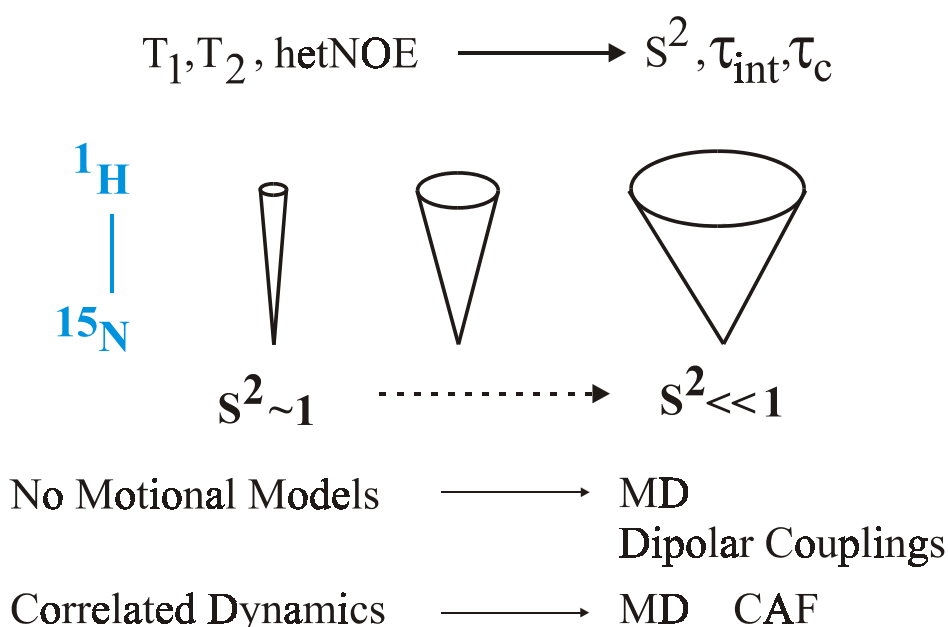


Abbildung 3.24: Vereinfachte Darstellung der Parameter, welche aus experimentellen NMR Daten aus der „Model-Free“ Analyse entnommen werden können. Der größte Nachteil ist, dass keine Bewegungsmodelle beschrieben werden können. Nur die Amplitude der Bewegung ist über den Ordnungsparameter dargestellt.

Die Art der Bewegung wird aber nicht durch den Ordnungsparameter wiedergegeben. Hierzu hat es andere Versuche gegeben, welche z.B. das 3D-GAF Modell für die Peptidebene hervorgebracht haben. Dieses nimmt an, dass es gaußförmig verteilte Bewegungen um die drei orthogonalen Achsen (α, β, γ) gibt^{149,150}. Alle diese Analysen zeigen aber nur Bewegungen welche schneller als τ_c sind auf. Um dynamische Aspekte zu messen, welche langsamer als τ_c sind, ist es üblich, $T_{1\rho}$ - und off-resonance $T_{1\rho}$ -Methoden zu verwenden. Off-resonance $T_{1\rho}$ -Methoden^{151,152} erlauben es das Zeitfenster deutlich in

den μs Bereich zu verschieben, allerdings mit immer geringer werdender Empfindlichkeit. Interessanterweise gibt es bis jetzt noch keine NMR Methode welche es erlaubt dynamische Prozesse in Proteinen im ns- bis μs - Bereich zu beobachten

Deshalb ist es von großem Interesse dynamische Größen, die langsamere Bewegungen beschreiben, z.B. Domänenbewegung, kooperative Bewegung von Helices oder β -Faltblättern miteinander zu detektieren. Dass dipolare Kopplungen das Potential dazu besitzen wurde schon sehr früh erkannt¹⁵³⁻¹⁵⁶. Auch wurde kürzlich gezeigt, dass auch Aussagen über die Seitenkettendynamik um χ_1 in Proteinen durch dipolare Kopplungen möglich sind¹⁵⁷. In Zusammenarbeit mit Jens Meiler haben wir Methoden entwickelt um dipolare Ordnungsparameter bestimmen zu können. Dies erlaubt uns die Dynamik von Proteinen mittels dipolarer Kopplungen über einen viel längeren Zeitraum als bisher möglich zu beschreiben. Dipolare Kopplungen zeigen Flexibilität auf verschiedenen Zeitskalen auf und besitzen somit die Fähigkeit Bewegungen in größerem Detail darzustellen. Dies ist auch wichtig für die Strukturrechnung, denn der eingesetzte Ausrichtungstensor wird nicht nur von Bewegungen schneller als τ_c skaliert, sondern auch von Bewegungen größer als diese ns-Bewegungen. Die Zeitskala für dipolare Kopplungen erstreckt sich über den Zeitraum solange man die Differenz der chemischen Verschiebungen der dipolaren Kopplungen der verschiedenen Konformationen detektieren kann.

Die theoretischen Grundlagen wurden in einem Paper dargestellt¹⁵⁸, in dem zur Kontrolle des Modells nur mit theoretischen und somit messfehlerfreien dipolaren Kopplungen gearbeitet wurde. Über die Zeit sehen wir keine starre Orientierung eines NH-Vektors, sondern eine Verteilung der verschiedenen Orientierungen. Diese Verteilung kann man benützen um dynamische Information aus dipolaren Kopplungen zu extrahieren. Sehr deutlich sieht man den Einfluss der Dynamik auch an der Schnittstelle der isodipolaren Kurven.

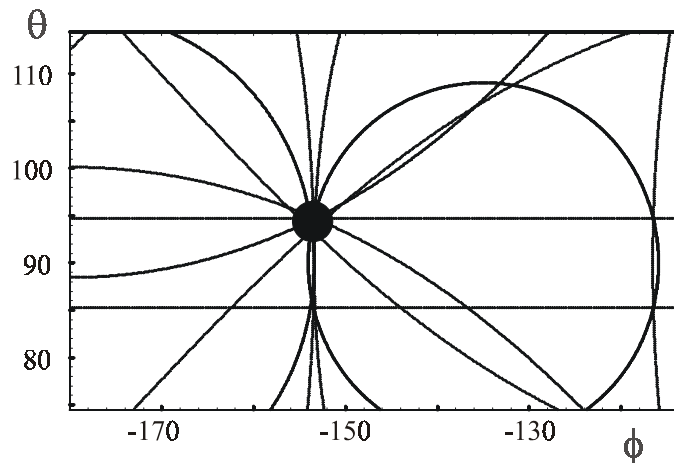


Abbildung 3.25: Nimmt man eine starre Struktur um die Ausrichtungstensoren, der hier neun verschiedenen dipolaren Kopplungen zu bestimmen, dann sieht man dass sich alle isodipolare Kurven in einen Punkt schneiden.

Bei einer Messung von dipolaren Kopplungen in einem Ausrichtungsmedium erhält man einen Ausrichtungstensor und zwei isodipolare Kurven für θ und ϕ die mit der gemessenen dipolaren Kopplung sind. Diese Mehrdeutigkeit kann man durch die Verwendung von mehreren möglichst verschiedenen Ausrichtungsmedien begrenzt werden¹³. Geht man von einer rigiden Proteinstruktur aus, so schneiden sich alle isodipolare Kurven in einem Punkt (Abbildung 25). Hat man jedoch eine Verteilung -hervorgerufen durch Bewegung-, gibt es keinen genau definierten Schnittpunkt mehr (Abbildung 26).

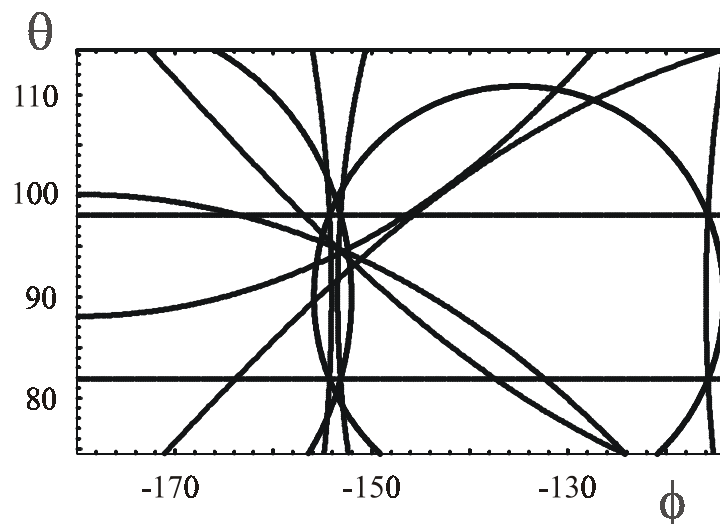


Abbildung 3.26: Nimmt man hingegen eine 10 ns Trajektorie als dynamische Modell und berecht hierfür die Ausrichtungstensoren, sieht man dass sich die isodipolaren Kurven nicht mehr in einem Punkt schneiden.

In der Folge möchte ich nun beschreiben, wie man dies mathematisch ausarbeiten kann um daraus einen dipolaren Ordnungsparameter zu berechnen, der analog dem Lipari-Szabo-Ordnungsparameter eine Amplitude der Bewegung, in einer viel größeren Zeitspanne (bis ms) beschreibt. Außerdem ist es möglich zusätzlich auch die Größe und Richtung der isotropen und der anisotropen Bewegungen aminosäurespezifisch zu beschreiben.

Dipolare Kopplungen können durch folgende Gleichung dargestellt werden:

$$D_i^{\text{exp}} = D_{i,zz} \left\{ \langle 3 \cos^2 \theta_i^{\text{at}} - 1 \rangle + \frac{3}{2} R_i \langle \sin^2 \theta_i^{\text{at}} \cos 2\phi_i^{\text{at}} \rangle \right\} \quad \text{Gl. 1}$$

Die Mittelungskammern in Gleichung 1 deuten an, dass dipolare Kopplungen üblicherweise konformationeller Mittelung ausgesetzt sind. Um dipolare Kopplungen in Hinblick auf Dynamik zu untersuchen, ist es sinnvoll Gleichung 1 durch die normalisierten sphärischen harmonischen Funktionen auszudrücken.

$$D_i^{\text{exp}} = D_{i,zz} \sqrt{\frac{4\pi}{5}} \left(\langle Y_{20}(\theta_i^{\text{at}}, \phi_i^{\text{at}}) \rangle + \sqrt{\frac{3}{8}} R_i \left(\langle Y_{22}(\theta_i^{\text{at}}, \phi_i^{\text{at}}) \rangle + \langle Y_{2-2}(\theta_i^{\text{at}}, \phi_i^{\text{at}}) \rangle \right) \right) \quad \text{Gl. 2}$$

$$\frac{D_i^{\text{exp}}}{D_{i,zz}} = \sqrt{\frac{4\pi}{5}} \left(\sum_{M=-2}^2 e^{-iM\alpha^i} d_{M^0}^2(\beta^i) \langle Y_{2,M}(\theta^{\text{mol}}, \phi^{\text{mol}}) \rangle \right) + \sqrt{\frac{4\pi}{5}} \sqrt{\frac{3}{8}} R \left(\sum_{M=-2}^2 e^{-iM\alpha^i} d_{M^2}^2(\beta^i) e^{-2i\gamma^i} \langle Y_{2,M}(\theta^{\text{mol}}, \phi^{\text{mol}}) \rangle + e^{-iM\alpha^i} d_{M-2}^2(\beta^i) e^{2i\gamma^i} \langle Y_{2,M}(\theta^{\text{mol}}, \phi^{\text{mol}}) \rangle \right) \quad \text{Gl. 3}$$

$$\frac{D_i^{\text{exp}}}{D_{i,zz}} = \sum_{M=-2}^2 F_{i,M} \langle Y_{2,M}(\theta^{\text{mol}}, \phi^{\text{mol}}) \rangle \quad \text{Gl. 4}$$

In diesen Gleichungen ist $D_{i,zz}$ die axiale Komponente und R_i die Rhombizität des Ausrichtungstensors. $\langle Y_{2,M}(\theta, \phi) \rangle$ sind die zeitlich gemittelten sphärischen harmonischen

Funktionen für einen NH Vektor $\left(Y_{20}(\theta, \phi) = \sqrt{\frac{5}{16\pi}} (3 \cos^2 \theta - 1) \right)$;

$\left(Y_{2\pm 1}(\theta, \phi) = \sqrt{\frac{15}{8\pi}} e^{\pm i\phi} \cos \theta \sin \theta \right)$; $\left(Y_{2\pm 2}(\theta, \phi) = \sqrt{\frac{15}{32\pi}} e^{\pm 2i\phi} (\sin^2 \theta) \right)$. Das hochgestellte at

beschreibt die Funktionen im Koordinatensystem des Ausrichtungstensors. Mol deutet den Wechsel des Koordinatensystems an: Der Wechsel vom Koordinatensystem des Ausrichtungstensors in das Koordinatensystem des Moleküls wird durch die 3 Eulerwinkel α_i , β_i und γ_i durchgeführt. In der Gleichung 3 werden alle Wignerrotationselemente und die axialen und rhombischen Komponenten aller gemessenen verschiedenen

Ausrichtungsmedien in der \hat{F} Matrix zusammengefasst. Die Eigenwerte und das Verhältnis des größten und des kleinsten Eigenwertes (Condition Number) der \hat{F} Matrix erlauben es auch eine Aussage über die lineare Unabhängigkeit der Ausrichtungstensoren zu machen.

Wie wir schon in dem theoretischen Paper¹⁵⁸ gezeigt haben, bekommt man beim Fitten der gemessenen dipolaren Kopplung an eine „starre“ Struktur einen zu kleinen Ausrichtungstensor (charakterisiert durch $\tilde{D}_{i,zz}$, \tilde{R}_i , $\tilde{\alpha}_i$, $\tilde{\beta}_i$, und $\tilde{\gamma}_i$). Diese Skalierung betrifft nicht die Orientierung und die Rhombizität, sondern nur eine Skalierung der Größe des Tensors: $D_{i,zz} = S_{overall} \cdot \tilde{D}_{i,zz}$.

$$\frac{D_i^{exp}}{\tilde{D}_{i,zz}} = \sum_{M=-2}^2 F_{i,M} \langle \tilde{Y}_{2,M}(\theta^{mol}, \phi^{mol}) \rangle = \sum_{M=-2}^2 F_{i,M} (\langle Y_{2,M}(\theta^{mol}, \phi^{mol}) \rangle / S_{overall}) \quad \text{Gl. 5}$$

Etwas illustrativer kann man die Formel zusammenfassen und den Sachverhalt nochmals folgendermaßen darstellen: $\vec{D} = \hat{F} \cdot \langle \vec{Y} \rangle$. Durch Inversion der \hat{F} Matrix ist es möglich die sphärischen harmonischen Funktionen zu ermitteln: $\langle \vec{Y} \rangle = \hat{F}^{-1} \cdot \vec{D}$. Aus diesen gemittelten sphärischen harmonischen Funktionen ist es, analog dem Lipari Szabo Ordnungsparameter, möglich, einen dipolaren Ordnungsparameter zu berechnen:

$$S_{rdc}^2 = \frac{4\pi}{5} \sum_{M=-2}^2 \langle Y_{2M}(\theta, \phi) \rangle \langle Y_{2M}^*(\theta, \phi) \rangle \quad \text{Gl. 6}$$

Neben der direkten Analyse der dipolaren Ordnungsparameter ist es auch möglich Bewegungen mittels der sphärischen harmonischen Funktionen zu beschreiben.

Da die Extraktion der sphärischen harmonischen Funktionen ohne $S_{overall}$ möglich ist (auch die unskalierten sphärischen harmonischen Funktionen zeigen die Bewegungen der NH-Vektoren), hat man einen modellfreien Zugang zu der Dynamik von Proteinen über einen langen Zeitraum (unskalierte Werte sind im Folgenden mit \sim gekennzeichnet). Um die Bewegungen besser erkennen zu können, benützen wir ein neues gestrichenes Koordinatensystem x', y', z' in dem die $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ Werte maximiert werden:

$$\max \langle \tilde{Y}_{20}(\theta', \phi') \rangle = \sum_{M=-2}^2 D_{M,0}(\phi_{eff}^{(1)}, \theta_{eff}^{(1)}, 0) \langle \tilde{Y}_{2M}(\theta, \phi) \rangle = \sqrt{\frac{4\pi}{5}} \sum_{M=-2}^2 Y_{2,-M}(\theta_{eff}^{(1)}, \phi_{eff}^{(1)}) \langle \tilde{Y}_{2M}(\theta, \phi) \rangle \quad \text{Gl. 7}$$

Es ist offensichtlich, dass $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ maximal ist, wenn der durchschnittliche Vektor parallel zu der z-Achse steht. Durch diese modellfreie Berechnung der sphärischen

harmonischen Funktionen ist es auch möglich, so direkt Bewegungen einzelner NH-Vektoren zu sehen. Den Anteil der axialsymmetrischen Bewegung kann man in den $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ Werten sehen. Durch die Maximierung des $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ Wertes finden wir, dass die $\langle \tilde{Y}_{21}(\theta', \phi') \rangle$ und die $\langle \tilde{Y}_{2-1}(\theta', \phi') \rangle$ Werte null sind. Maximiert man $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$

so ist die Ableitung $\left(\frac{\partial \langle \tilde{Y}_{20} \rangle}{\partial \phi_{x', y', z'}} = 0 \right)$ null. Eine unendlich kleine Rotation entspricht

mathematisch einer Anwendung des Drehimpulsoperators:

$$0 = \frac{\partial}{\partial \delta_{x'}} \langle \tilde{Y}_{20}(\theta', \phi') \rangle = \frac{L_{x'} \langle \tilde{Y}_{20}(\theta', \phi') \rangle}{i\hbar} = \frac{\langle \tilde{Y}_{21}(\theta', \phi') \rangle + \langle \tilde{Y}_{2-1}(\theta', \phi') \rangle}{i\hbar}$$

$$0 = \frac{\partial}{\partial \delta_{y'}} \langle \tilde{Y}_{20}(\theta', \phi') \rangle = \frac{L_{y'} \langle \tilde{Y}_{20}(\theta', \phi') \rangle}{i\hbar} = \frac{(\langle \tilde{Y}_{21}(\theta', \phi') \rangle - \langle \tilde{Y}_{2-1}(\theta', \phi') \rangle)}{\hbar}$$

Die Größe der anisotropen Bewegung ist in den $\langle \tilde{Y}_{22}(\theta', \phi') \rangle$ Werten ablesbar. Um den Anteil der anisotropen Bewegung noch besser sichtbar zu machen haben wir einen Anisotropieparameter η eingeführt, der folgendermaßen definiert ist:

$$\eta = \sqrt{\frac{\sum_{M=-2,2} \langle \tilde{Y}_{2M}(\theta', \phi') \rangle \langle \tilde{Y}_{2-M}(\theta', \phi') \rangle}{\sum_{M=-2}^2 \langle \tilde{Y}_{20}(\theta', \phi') \rangle \langle \tilde{Y}_{20}(\theta', \phi') \rangle}}$$

Das Verhältnis des Imaginär- und des Realteiles von $\langle \tilde{Y}_{22}(\theta', \phi') \rangle$ and $\langle \tilde{Y}_{2-2}(\theta', \phi') \rangle$ definiert die Orientierung der anisotropen Bewegung.

$$\bar{\phi}' = \frac{1}{2} \arctan \frac{\langle \tilde{Y}_{22}(\theta', \phi') \rangle - \langle \tilde{Y}_{2-2}(\theta', \phi') \rangle}{i(\langle \tilde{Y}_{22}(\theta', \phi') \rangle + \langle \tilde{Y}_{2-2}(\theta', \phi') \rangle)}$$

$\bar{\phi}'$ ist unterschiedlich von einem mittleren ϕ' einer Verteilung von Vektororientierungen $\langle \phi' \rangle$.

Somit haben wir die fünf sphärischen harmonischen Funktionen in fünf neue Parameter übersetzt, welche uns erlauben die Bewegung einzelner NH-Vektoren oder Gruppen von NH-Vektoren einfacher sichtbar zu machen. $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ beschreibt die axiale Ordnung, $\theta_{eff}^{(1)}$ und $\phi_{eff}^{(1)}$ zeigen die durchschnittliche Orientierung und η und $\bar{\phi}'$ die Anisotropie der Bewegung eines NH-Vektors.

Mit Hilfe dieser theoretischen Grundlagen habe ich versucht genügend experimentelle Daten zu sammeln und das Modell in die Praxis umzusetzen³⁶. Teile dieser Grundlagen wurden erst ersichtlich durch die Verwendung experimenteller Daten. Diese zuvor beschriebenen Zusammenhänge habe ich in der Abbildung 24 zusammengefasst. Auf diese Praxisarbeit möchte ich nun etwas genauer eingehen, da diese einen Zeitraum von ungefähr 18 Monaten betrug und so einen wichtigen Bestandteil meiner Doktorarbeit ausmacht.

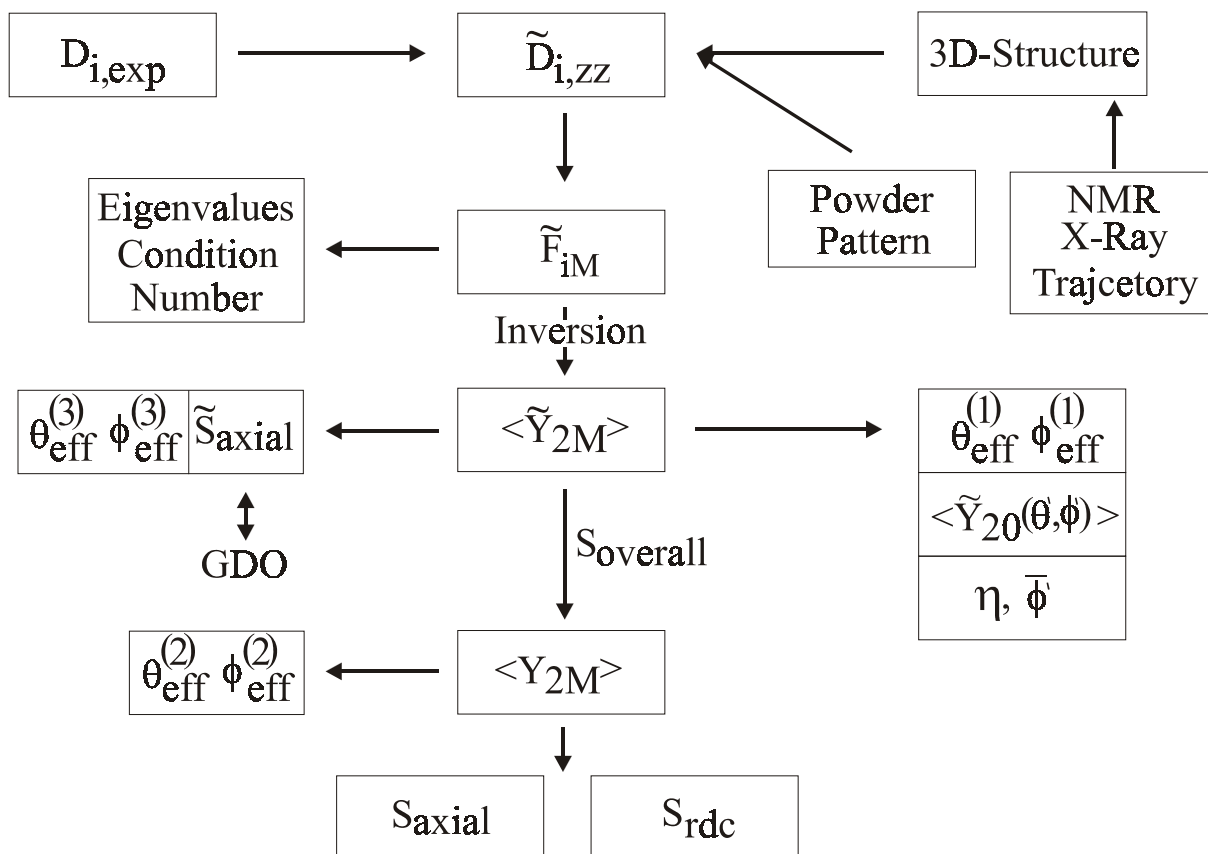


Abbildung 3.27: Übersicht über alle Parameter in der Analyse dipolarer Kopplungen. Nicht nur die Amplitude der Bewegung kann über den dipolaren Ordnungsparameter, sondern es können auch die Richtung der isotropen und anisotropen Bewegungen der NH Vektoren in einem Zeitfenster von ns bis ms entnommen werden.

Für das theoretische Modell wurden neun -maximal divergente- dipolare Datensätze generiert, welchen eine 10ns lange MD-Trajektorie als dynamisches Modell zu Grunde gelegt wurde. Hiermit konnte erzielt werden, dass der 5-dimensionale Raum der durch die \hat{F} Matrix aufgespannt wird, optimal abgetastet wird. Es ist nur sehr schwer möglich den Ausrichtungstensor für eine bestimmte flüssigkristalline Phase vorherzusagen. Hierzu wurden schon Überlegungen von Zweckstetter et al.¹² gemacht. Mit Hilfe des Programms PALES (Prediction of Alignment from Structure;

<http://spin.niddk.nih.gov/bax/>) soll es möglich sein alleine durch die 3 dimensionale Struktur des Proteins den Ausrichtungstensor zu bestimmen. In unseren Versuchen haben die vorhergesagten und die experimentell bestimmten Tensoren allerdings nicht übereingestimmt. Deshalb kann man hier als Regel gelten lassen: “Probieren geht über studieren!“. Mit dem in dieser Arbeit gewonnenen Wissen ist es jedoch möglich nun schneller vorherzusagen, welches Ausrichtungsmedium wohl das Beste für ein bestimmtes Protein ist. Mit elf verschiedenen Ausrichtungstensoren war es möglich auch experimentelle dipolare Ordnungsparameter für das Protein Ubiquitin zu bestimmen. Fast alle zuvor beschriebenen Ausrichtungsmedien wurden dazu verwendet. Für 43 aus den 76 Aminosäurenresten von Ubiquitin konnte ein Ordnungsparameter ermittelt werden. Die Ordnungsparameter bewegen sich zwischen 0.108 (Gly 76) und 1.62 (Glu 18).

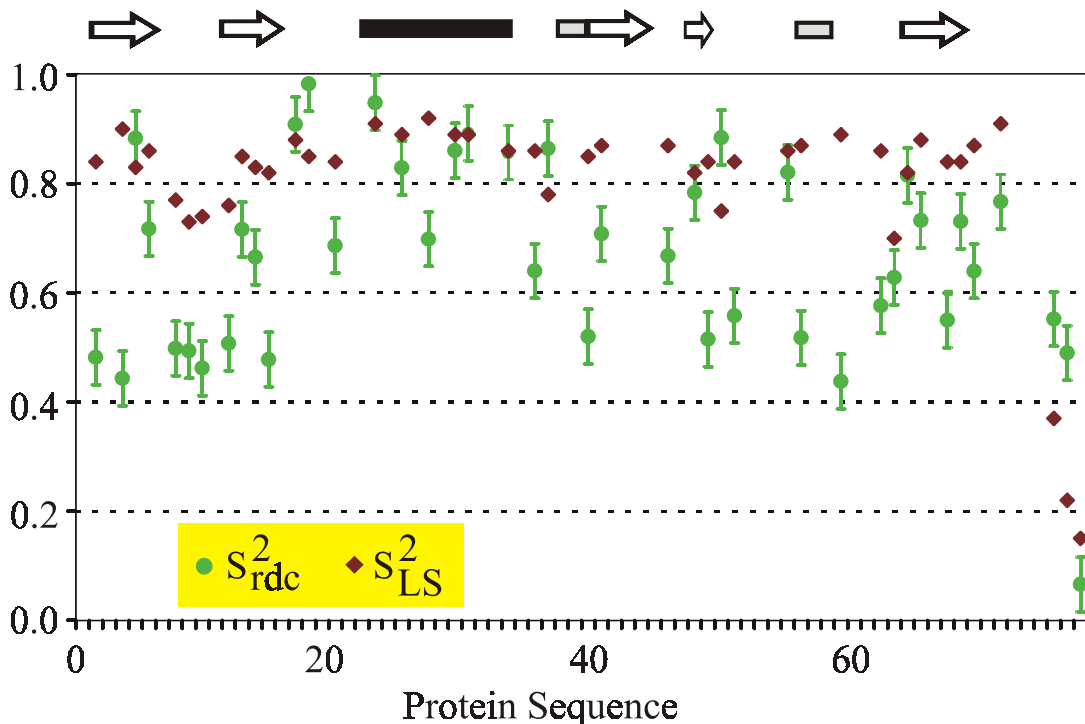


Abbildung 3.28: Vergleich des dipolaren und des Lipari-Szabo Ordnungsparameters für das Protein Ubiquitin, dessen Sekundärstruktur oberhalb des Graph dargestellt ist. Man erkennt, dass die zentrale α -Helix das starrste Sekundärstrukturelement ist. Nur wenig Bewegung langsamer als die Über-Alles-Korrelationszeit ist sichtbar in der α -Helix. Jedoch sieht man, dass in Loops und β -Faltblättern in Ubiquitin reichlich langsame Dynamik vorhanden ist.

Die dipolaren Ordnungsparameter \tilde{S}_{rdc}^2 sind teilweise größer als 1, was darauf zurückzuführen ist, dass der wahre dynamikfreie Ausrichtungstensor unbekannt ist, da -wie zuvor beschrieben- eine rigide Struktur zur Berechnung des Ausrichtungstensors verwendet wurde. Um den Skalierungsfaktor des Ausrichtungstensors zu bestimmen gibt

es mehrere Möglichkeiten: Betrachtet man ein Histogramm aller experimentell bestimmter dipolarer Ordnungsparameter, so liegt der Mittelwert bei 1 mit einem Extremwert bei 1.62 (Glu 18). Skaliert man nun diesen Extremwert auf 1 (denn Ordnungsparameter können nur maximal 1 werden), so liegt der Mittelwert bei ca. 0.78. Dies würde dann einen dipolaren Über-Alles-Skalierungsfaktor, welchen wir $S_{overall}$ genannt haben, entsprechen. Was jedoch bedeutet dieser überraschend kleine über alles Über-Alles-Skalierungsfaktor? Übersetzt man die Bewegung in das einfachste Bewegungsmodell (Wobbling in a cone, Bewegung in einem Kegel), so bedeutet dies, dass der halbe Öffnungswinkel ungefähr 21° , bei ausschließlich schneller Bewegung, welche durch die heteronuklearen Relaxationsmessungen betrachtet werden, beträgt. Der Anteil der dipolaren Kopplungen entspricht einen halben Öffnungswinkel von ungefähr 24° . Die beiden Bewegungen sind nicht korreliert.

Ein zweites Indiz, für eine Skalierung in dieser Größenordnung, sind die -schon zuvor erwähnten- ^1H - ^1H dipolaren Kopplungen, welche mittels der J_{HH} -NOESY Technik bestimmt wurden. Alle berechneten dipolaren Kopplungen waren zu groß. Deshalb war die Steigung der Korrelationsgeraden nur 0.75. Der Ausrichtungstensor, welcher für die Berechnung der ^1H - ^1H dipolaren Kopplungen verwendet wurde, ist aus experimentellen D_{NH} Kopplungen bestimmt. Die Skalierung von 0.75 entspricht wieder den zuvor schon genannten Über-Alles-Skalierungsfaktor $S_{overall}$.

Ein drittes Indiz liefern die Arbeitsgruppen von Prestegard und Bertini. Die Gruppe um Bertini hat dipolare Kopplungen und Pseudokontaktshifts an Metalloproteinen, an denen paramagnetische Metalle komplexiert waren, gemessen. Mit Hilfe der dynamisch unabhängigen Pseudokontaktshifts kann der Ausrichtungstensor bestimmt werden. Der Vergleich zwischen dem aus Pseudokontaktshifts berechneten und dem aus dipolaren Kopplungen bestimmten Ausrichtungstensor zeigte Differenzen von 5-50% (je nach eingesetztem Metall). Dies korreliert im Mittel mit unserer Skalierung von 25%.

Auch in James Prestegards Arbeitsgruppe erkannte man schon auf Grund von NH-Relaxationsmessungen, dass die Skalierung der dipolaren Kopplungen größer ist als der mittlere Lipari Szabo Ordnungsparameter ($S_{LS} = 0.9$). Joel Tolman verglich experimentelle und theoretische Kopplungen aus Cyanometmyoglobin¹⁵³. Da die dipolaren Kopplungen durch magnetische Orientierung gemessen wurden, konnte der Ausrichtungstensor sehr genau ermittelt werden auf Grund einer relativ großen Anzahl an Pseudokontaktshifts (ungefähr gleich viele wie dipolare Kopplungen). Die theoretischen

Kopplungen wurden basierend auf diesem Ausrichtungstensor und der X-Ray Struktur berechnet. Auch wenn man dipolare Kopplungen aus hoch-dynamischen Bereichen wie Loops auslässt, sieht man, dass die Korrelation nicht 1 ist. Um die Daten besser zu fitten musste auch die Steigung der Korrelationsgeraden verändert werden. Diese Änderung der Steigung spiegelt auch eine Reduktion der Aufspaltung der dipolaren Kopplungen wider, welche auf Grund von Unordnung durch Bewegung hervorgerufen wird. Zusätzlich müsste man die Steigung um mehr als 0.9 vergrößert werden. Dies ist mehr als - wie schon erwähnt - der mittlere NH Lipari Szabo Ordnungsparameter. Nur Bewegungen langsamer als NH Bewegungen könnten solch eine Reduktion der Kopplung hervorrufen.

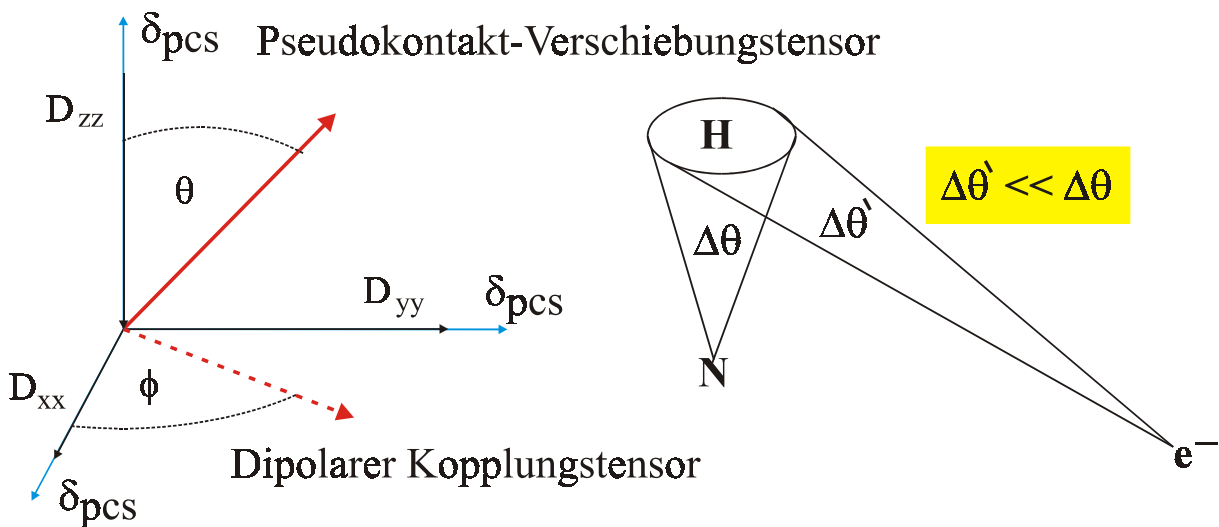


Abbildung 3.29: Eine alternative Methode den Skalierungsfaktor des dipolaren Ausrichtungstensors für dipolare Kopplungen zu finden, existiert in Metalloproteinen. Hier ist es möglich Pseudokontakt-Verschiebungen und dipolare Kopplungen zu messen. Der daraus berechnete dipolare Ausrichtungstensor enthält die gesamte Dynamik, der Pseudokontakt-Verschiebungstensor jedoch ist starr. Dies ist darin begründet, dass der Abstand zwischen Elektron und Proton viel größer ist und dadurch der Kegelöffnungswinkel klein und die Dynamik somit minimal ist. Der Unterschied zwischen den beiden Tensoren ist zwischen 5-50%, abhängig von verwendetem Metall.

Die niedrigen Ordnungsparameter würden bedeuten, dass Helices in Cyanometmyoglobin Bewegungen mit Winkeln größer als 30° ausführen. Theoretische Modellierung Analysen haben gezeigt, dass auch genügend Platz für diese Bewegungen vorhanden wäre^{153,155}. Größte Fehlerquelle für diese Analyse¹⁵⁹ könnte der berechnete χ -Tensor sein. Um dies auszuschließen wurde der Tensor mit soviel wie möglich Pseudokontaktshifts bestimmt. Diese beinhalten, da sie über sehr lange Distanzen gemessen werden und so der Bewegungskegel einen minimalen Öffnungswinkel besitzt, nur sehr wenig bis keine Dynamik. Deswegen sollte der Tensor darausfolgend nicht skaliert sein und der „starr

Wahrheit“ entsprechen. In Simulationen konnte gezeigt werden, dass diese Annahme ab einem Abstand von 2Å zu 95% erfüllt ist. Auch konnte der Suszeptibilitätstensor durch molekulare Assoziation, d.h. Aggregation verändert werden. Dies kann durch Messungen bei niedrigeren Konzentrationen ausgeschlossen werden. Die Berechnungen von Tolman et al. haben auch eine Reduktion des Tensors um ca. 25% ergeben, was wiederum im Einklang mit unseren Ergebnissen steht.

Für das Protein Ubiquitin wurden von Ad Bax und Mitarbeitern⁴ dipolare Kopplungen bei 360, 600 und 750 MHz gemessen. Diese Messungen beruhen auf der Selbstorientierung des Proteins Ubiquitin durch die magnetische Suszeptibilität. Die Differenz zwischen der Summe (J+D) der Kopplungen bei 750MHz-360MHz und 600MHz-360MHz zeigten keine große Abweichung von den theoretischen Daten, berechnet mit einem Suszeptibilitätstensor, welcher sich aus der Summe der Suszeptibilitäten im Protein zusammensetzt. Allerdings waren die gemessenen dipolaren Kopplungen sehr klein (ca. 0.3Hz), sodass hier der Fehler bei der Kopplungskonstantenbestimmung auch das Ergebnis beeinflussen könnte.

Den größten Anteil zum Suszeptibilitätstensor in Ubiquitin liefern die aromatischen Aminosäuren und die Peptid-Doppelbindung. Da es nur drei aromatische Aminosäuren (2 Phenylalanine und 1 Histidin) in Ubiquitin gibt, ist der Tensor sehr klein und deshalb die Ausrichtung selbst bei den benützten hohen Magnetfeldern (>10T) sehr gering. Außerdem wurden die dipolaren Kopplungen in nur einem Medium bestimmt, sodass eine Aussage über die Dynamik eigentlich nicht möglich ist.

Allerdings könnten auch andere mögliche Fehler bei der Analyse der dipolaren Kopplungen vorliegen: Der wichtigste Punkt ist, dass man sicherstellt, dass die Struktur des Proteins in den verschiedenen anisotropen Phasen nicht verändert wird. Eine Änderung der Struktur würde dann als Bewegung gedeutet werden. Um dieses auszuschließen habe ich NOESY Spektren gemessen deren Mischzeiten mit den mittleren T₂-Relaxationszeiten

gewichtet wurden $\left(\left(\frac{\tau_{m, isotrop}}{T_{2, isotrop}} \right) = \left(\frac{\tau_{m, aligned}}{T_{2, aligned}} \right) \right)$, um verschiedene Über-Alles-

Rotationsbewegungen auszugleichen. Man sieht, dass die NOE-Kreuzpeak-Intensitäten sich um weniger als ±6% unterscheiden. Leider ist die Qualität der Spektren auf Grund des starken Hintergrundrauschens, welches durch die anisotropen Phasen erzeugt wird, nicht überragend. Der Fehler der NOESY Spektren liegt bei ungefähr ±5% .

Im Folgenden möchte ich genauer auf die modellfreie Bewegungsanalyse eingehen. In Abbildung 29 ist deutlich sichtbar, dass die experimentell gefundene Anisotropie der Bewegung größer ist wie in der MD-Trajektorie. Speziell in den Loop-Regionen und am C-Terminus von Ubiquitin sieht man stark anisotrope Reste. Im Mittel ist η 16% für experimentelle Werte ($\eta=12\%$ für nur Sekundärstruktur Elemente) mit Extrema von 66% für die Aminosäure Glycin 76. Für die Trajektorie ist η im Mittel nur 3.8%.

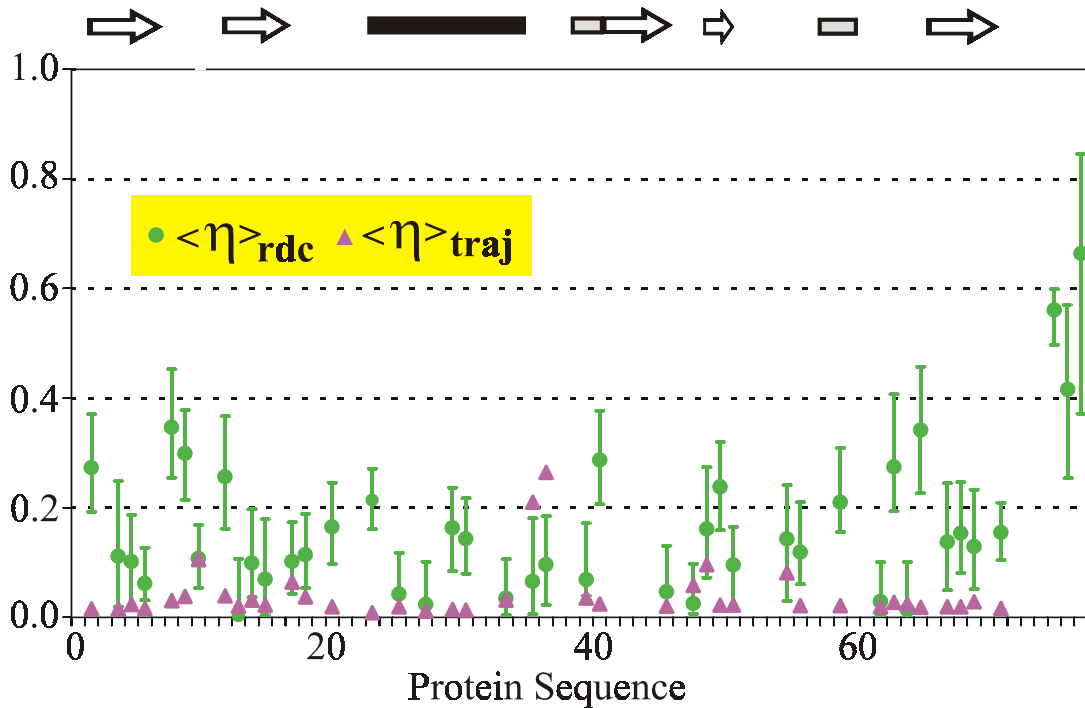


Abbildung 3.30: Größe der Amplitude der anisotropen Bewegung in Ubiquitin (Punkte: experimentell, Dreieck: entnommen aus 10ns Ubiquitin Trajektorie). Die Trajektorie kann die Größe der Bewegung nicht deutlich wiedergeben. Die anisotrope Bewegung ist deutlich im gesamten Protein verteilt zu sehen.

Die Helix in Ubiquitin ist ein relativ starres Sekundärstrukturelement. Dies deuten die experimentell höchsten $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ Werte an. Auch die Daten aus der Trajektorie zeigen dies. Trotzdem können wir anisotrope Bewegung auch in der Helix detektieren. Der durchschnittliche $\bar{\phi}'$ Winkel in der Helix ist -20° mit Extremwerten von $+20^\circ$ bis -50° . Dies deutet eine Vorzugsorientierung aller NH-Vektoren in diese Richtung an. Allerdings können wir auf Grund der unkorrelierten Natur der dipolaren Kopplung nicht sagen, ob sich die NH-Vektoren korreliert bewegen. Die β -Faltblätter hingegen haben in Ubiquitin viel kleinere $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ - und S_{rdc} -Werte als S_{LS} -Werte. Dies deutet auf Bewegungen im ns- bis μs -Bereich hin. Im Ubiquitin konnten keine $T_{1\rho}$ -Effekte gemessen werden, welche

auf noch langsamere Bewegung hindeuten würden. Die Anisotropie der Bewegung ist in den β -Faltblättern deutlicher ausgeprägt was man auch an Hand der größeren η -Werten deutlich erkennen kann.

Eine weitere interessante Gleichartigkeit zeigt Abbildung 30. Experimentelle $\bar{\phi}'$ und $\bar{\phi}'_{traj}$ Werte aus der Trajektorie zeigen eine gute Übereinstimmung in Bereichen mit ausgebildeter Sekundärstruktur. Die einzigen Ausnahmen bilden Ile30, Thr66 und His68. NH von His68 bildet eine schwache Wasserstoffbrücke mit Ile4 am Ende eines parallelen β -Faltblatts. Die NH's von Ile30 und Thr66 bilden keine internen Wasserstoffbrücken aus. Daraus kann man schließen, dass die Kraftfelder, welche in der MD-Trajektorie verwendet werden, die Richtung der Bewegung relativ gut beschreibt. Die Amplitude der Bewegung (durch η beschrieben) wird allerdings mit viel größerem Fehler wiedergegeben.

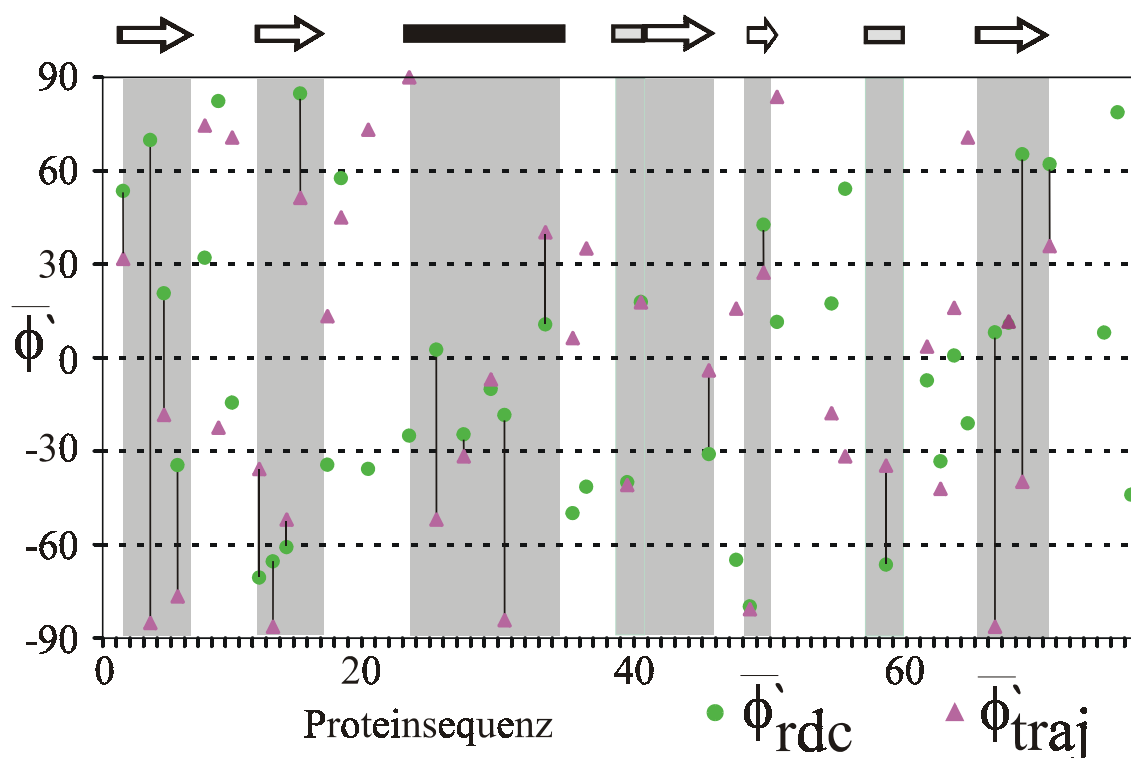


Abbildung 3.31: Richtung der anisotropen Bewegung in Ubiquitin. Einzig die Richtung der anisotropen Bewegung der NH Vektoren von Ile 30, Thr 66 und His 68 stimmen nicht mit den Richtungen der Bewegung, simuliert in der Trajektorie, überein. Der Fehler auf den experimentell bestimmten Orientierungen beträgt ungefähr $\pm 20^\circ$.

Der erste Loop, welcher die beiden ersten β -Faltblätter von Ubiquitin verbindet – Aminosäuresequenz Thr⁷-Gly⁸-Thr⁹-Gly¹⁰-Lys¹¹-Thr¹² –, zeichnet sich in allen bisher publizierten Relaxationsstudien durch eine sehr hohe Mobilität aus. Diese erhöhte Flexibilität ist auch in den dipolaren Ordnungsparametern sichtbar, welche im Mittel

$S_{rdc} = 0.7$ sind und somit deutlich kleiner als der sequenzgemittelte dipolare Ordnungsparameter von 0.78.

Vorausblickend kann man sagen, dass man eine noch detaillierte Analyse der Bewegung individueller NH-Vektoren nur schwer machen kann, da die individuellen Fehler, besonders durch die Quotientenbildung bei der Berechnung von $\bar{\phi}$, sehr groß werden. Deshalb ist es zukünftig wichtig NH-Vektoren mit gleicher Bewegung (z.B. die Helix in Ubiquitin) zu einem Element zusammenzufassen und als ein Element zu beschreiben.

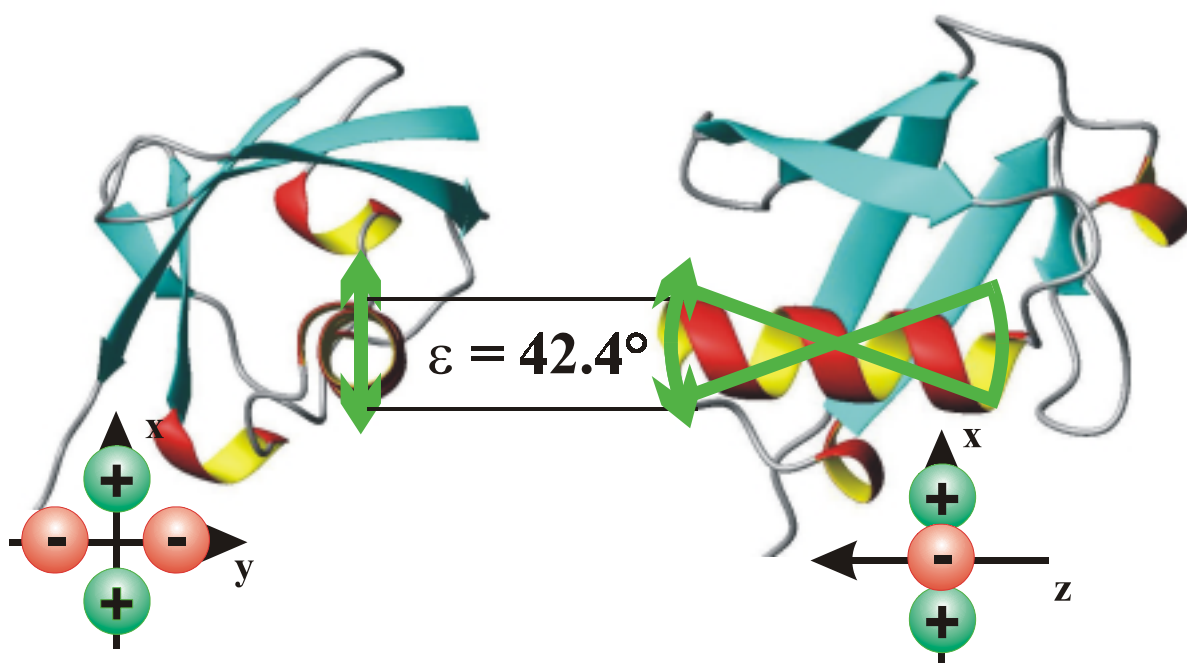


Abbildung 3.32: Alle parallelen NH-Vektoren der zentralen Helix in Ubiquitin wurden aufsummiert und eine Gesamtbewegung der Helix bestimmt. Eine genaue Analyse ist in Arbeit.

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4 Charakterisierung von denaturierten Proteinen: das Random Coil

Modell

Auf dem Weg vom Gen zum funktionellen Protein sind die Schritte der Transkription und der Translation nach der Lösung der Strukturen von Polymerase II^{1,2} und dem Ribosom^{3,4} soweit bekannt, dass sie auf atomarem Niveau untersucht werden können. Derartige Information steht im Moment noch nicht für das Splicen und die Proteinfaltung zur Verfügung. Zum Beispiel sind bis jetzt nur wenige Chaperone, welche wichtige Hilfsproteine bei der Proteinfaltung sind, mit atomarer Auflösung untersucht worden.



Abbildung 4.1: Weg des Informationsflusses in der Zelle. Die Information der DNA wird in der Transkription auf die Premessenger-RNA übertragen. Diese wird gespliced und in Messenger-RNA umgewandelt. Im Translationsschritt am Ribosom wird die Messenger-RNA in die entsprechenden Aminosäuren übersetzt und die Proteine aufgebaut. Der letzte Schritt, die Proteinfaltung ist noch nicht vollständig verstanden.

Daher ist die Proteinfaltung ein sehr aktiv untersuchtes Gebiet, insbesondere seit der Erkenntnis, dass falsch gefaltete Proteine für Krankheiten verantwortlich sind. Bei der Proteinfaltung sind sowohl der gefaltete, also biologisch aktive Zustand, als auch der entfaltete Zustand sowie der Weg zwischen den beiden von Interesse. In diesem Teil der Arbeit galt mein Interesse der Charakterisierung des denaturierten Zustands von Ubiquitin. Natürliche Proteine bestehen aus L-Aminosäuren, die mittels der Peptidbindung verknüpft sind. Die Peptidbindung entsteht unter Wasserabspaltung bei der Kondensation einer Carboxy-Gruppe (-COOH) mit einer Amino-Gruppe (-NH₂). Den deutlichsten Unterschied innerhalb der verschiedenen Aminosäuren bilden somit die individuellen Seitenketten, welche entweder basische (Lys, Arg, His), saure (Asp, Glu) hydrophobe (Ala, Val, Leu, Ile, Phe, Pro, Met) oder polare (Ser, Thr, Cys, Trp, Tyr, Asn, Gln) Eigenschaften besitzen.

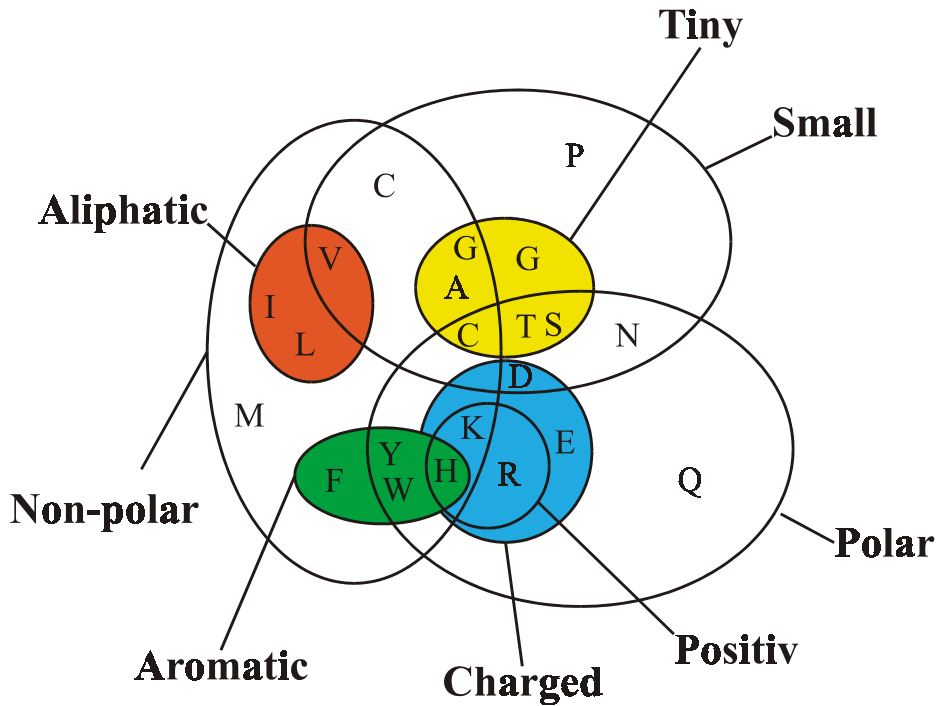


Abbildung 4.2: Unterteilung der verschiedenen Aminosäuren in Gruppen, gemäß ihrer physikalischen Eigenschaften.

Eine Ausnahme bildet Glycin, welches nur ein Proton am C_{α} (neben dem H_{α} , welches jede Aminosäure hat) trägt und nicht chiral ist. Allgemein zeigt die Peptidbindung eine eingeschränkte Drehbarkeit um die C-N Achse infolge der Delokalisierung der π -Elektronen der C'-O-Bindung mit dem freien Elektronenpaar des Stickstoffs (ca. 40% Doppelbindungscharakter)⁵. Der Winkel ω ist in Proteinen fast immer trans mit einem statistischen gemittelten Winkel etwas kleiner als 180° , was auf eine Flexibilität der Bindung hindeutet (Ausnahme sind L-Proline, wo unter Mithilfe von Peptidylprolyl-cis-trans-isomerasen cis-Peptid gebildet wird).

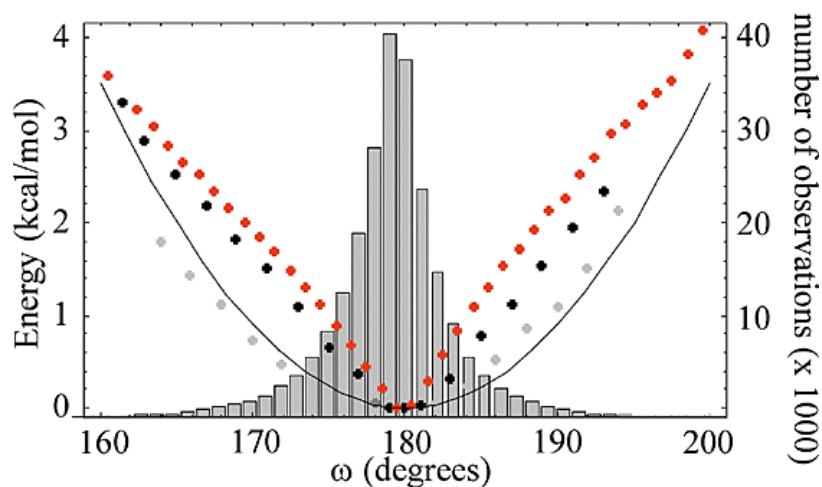


Abbildung 4.3: Verteilung des Winkels ω in der Proteindatenbank. Das Histogramm zeigt die Winkelverteilung um ω aus 237807 Winkeln aus 3938 Proteinen aus der PDB Datenbank mit einer Auflösung $>2.0\text{\AA}$ und einem R-Faktor von 20%. Die roten, grauen und schwarzen Punkte geben Energien der Peptidbindungsrotation, welche aus verschiedenen Datenbanken mittels Maxwell-Boltzmann Statistik berechnet wurden, an.

Die einzigen großen Bewegungsmöglichkeiten des Proteinrückgrats sind die Winkel ϕ ($C^{(i-1)}-N-C\alpha-C'$) und ψ ($N-C\alpha-C'-N^{(i+1)}$), die in Sekundärstrukturelementen definierte Werte annehmen. Durch die systematische Konformationsanalyse⁶ ist es möglich die ϕ - ψ Winkelkombinationen mit den geringsten Energien zu bestimmen. Geringe Pfitzer-, Newman- und 1,3-Allyl-Spannung ergeben die energieärmsten Konformationen. So bilden sich in erster Linie α -Helices, β -Faltblätter oder $^3_{10}$ -Helices aus, welche über Wasserstoffbrücken stabilisiert werden.

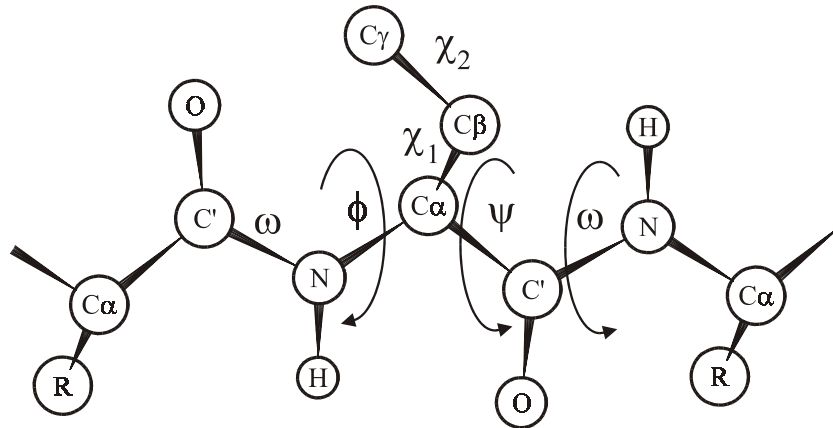


Abbildung 4.4: Darstellung des Proteinrückgrats. Die ϕ - ψ Winkelkombinationen bestimmen die Sekundärstruktur eines Proteins, welche in weiterer Folge durch Wasserstoffbrücken stabilisiert wird.

Diese Bereiche können durch ϕ / ψ Darstellungen, die sogenannten Ramachandran Plots, graphisch dargestellt werden. In Abbildung 5 ist ein Ramachandran Plot für das gefaltete Ubiquitin präsentiert. An diesem Protein wurden auch die meisten Untersuchungen in meiner Arbeit durchgeführt. Mittels NOE und Kopplungskonstanten konnte ein Modell für den entfalteten Zustand abgeleitet werden, in dem die ϕ , ψ Verteilung für jede Aminosäure im Einklang mit einer ϕ , ψ Verteilung aus einer Datenbank, welche aus nativen Proteinen aufgebaut wurde, ist. Allerdings ist die NOE-Analyse sensitiv auf ϕ und ψ Mittelung und durch die r^{-6} Mittelung und durch komplexe Dynamik nicht quantitativ interpretierbar. Deswegen war es wünschenswert die ψ Mittelung mittels Kopplungskonstanten, kreuzkorrelierten Raten und chemischen Verschiebungen zu analysieren.

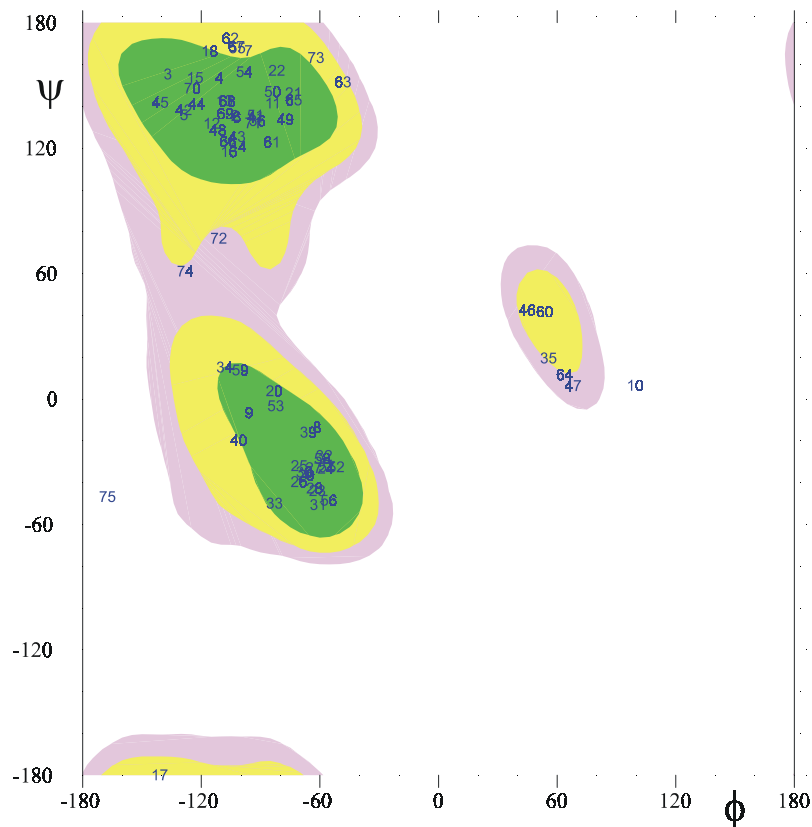


Abbildung 4.5: Ramachandran Plot für Ubiquitin.

4.1 Das Random Coil Modell

Wie schon angeführt finden Proteine erst durch geeignete Wechselwirkungen in ihre native Form. Diese ist energieärmer als die entfaltete Form. Die Konformationsanalyse hat gezeigt, dass nur bestimmte Winkelkombinationen energetisch geeignete Wege aufzeigen, auf denen die Faltung verlaufen könnte. Die ungünstigen Winkelkombinationen sollten von Faltungstrajektorien nicht stark populiert werden. Für eine Beschreibung von Faltungswegen ist es von großem Interesse herauszufinden, welche Startkonformationen vorliegen. Die energetischen Vorzugskonformationen lassen sich durch ein Random Coil Modell beschreiben^{7,8}. Es zeigt sich, dass experimentelle aus denaturierten oder entfaltenen Proteinen ermittelte Kopplungskonstanten, welche die Winkel ϕ , ψ und χ_1 beschreiben, mit vorhergesagten Kopplungskonstanten, die aus einer Datenbank gefalteter Proteine erzeugt wurden, übereinstimmen. Diese Präferenzen zeigen die bevorzugte Ausbildung bestimmter Sekundärstrukturelemente von Aminosäuren auf. In Proteinen, die in denaturierenden Medien gelöst sind, kann man keine weitreichenden Wechselwirkungen messen, sodass davon ausgegangen werden kann, dass alle diese

Präferenzen aus lokalen Vorgaben entstehen. Im Random-Coil-Modell wird angenommen, dass in der Datenbasis aus 402 nicht strukturhomologen Proteinen (Auflösung besser als 2.0 Å) sich alle langreichweitigen Wechselwirkungen vollständig herausmitteln⁹. Mit Hilfe von statistischen Verteilungsmodellen lassen sich unterschiedliche Parameter aus dieser Datenbasis vorhersagen. Die statistische Verteilung ist deshalb wichtig, da im Random Coil Zustand die konformative Dynamik im Vergleich zur Korrelationszeit langsam ist. Nachdem in den letzten Jahren schon zahlreiche Untersuchungen die Winkel ϕ ^{8,10}, χ_1 ^{9,11} und NOE¹²-Abstände beleuchtet haben, war es in dieser Arbeit unser Ziel den Winkel ψ ¹³ und die ¹⁵N chemischen Verschiebungen¹⁴ zu analysieren.

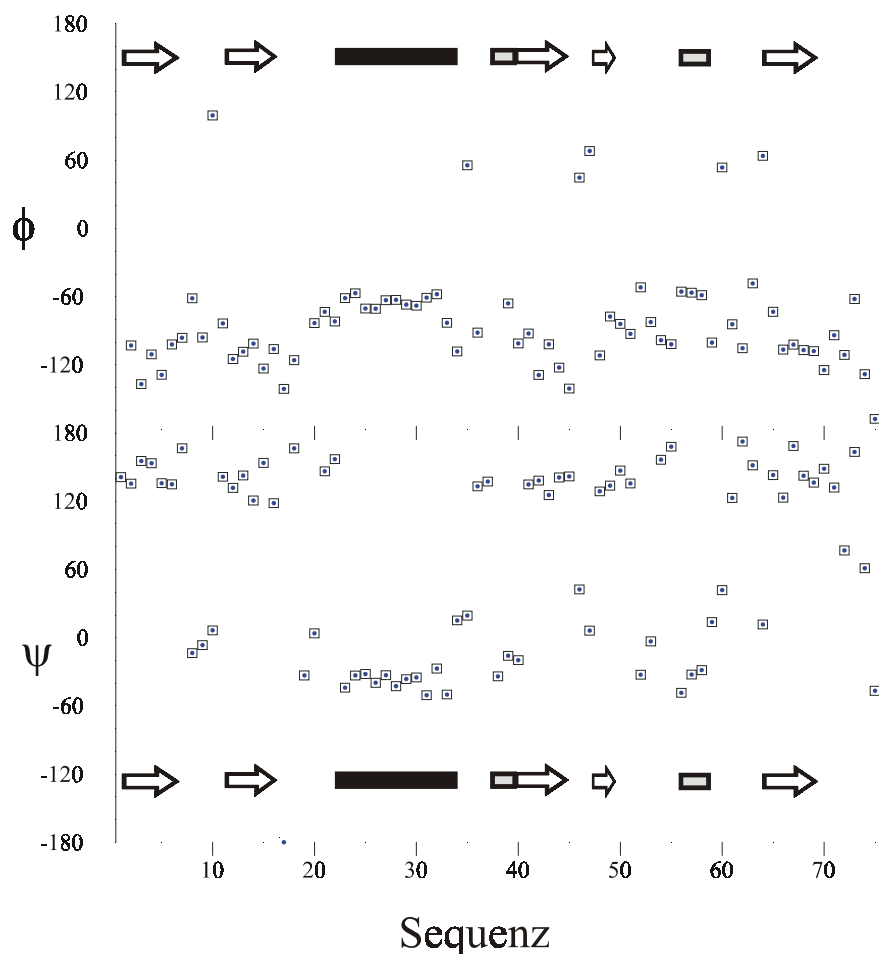


Abbildung 4.6: ϕ und ψ Verteilung in Ubiquitin. Hier ist ganz deutlich zu sehen, dass eine Sekundärstruktur Unterscheidung über den ψ Winkel viel eindeutiger ist. Der Unterschied im ϕ Winkelbereich ist nur sehr klein.

Der Winkel ψ ist NMR spektroskopisch schwer zugänglich. Dies ist besonders darin begründet, dass die für J-Kopplungen nutzbaren Kerne kleine gyromagnetische Verhältnisse haben. So wurden ³J_{NN}-Kopplungen von Löh¹⁵ und Theis¹⁶ gemessen. Die

Kopplungen waren jedoch sehr klein und schwer bestimmbar. Außerdem wurden Methoden entwickelt die $^1J_{C\alpha H\alpha}$ -Kopplung¹⁷ mit der Rückgratkonformation um ψ zu verknüpfen. Auch konnten 2 und 3 Bindungs-Deuterium Isotopen Verschiebungen auf den $^{13}C_{\alpha}$ chemischen Verschiebungen detektiert werden¹⁸. Als am besten untersucht gilt die $^1H\alpha$ - ^{15}N 3J -Kopplung¹⁹, welche mit der E.COSY Technik zugänglich ist. Allerdings ist auch diese Kopplung nicht sehr groß.

Erst kürzlich wurde nachgewiesen, dass auch die $^3J_{C\alpha C\alpha}$ Kopplung²⁰ vom Winkel ψ abhängig ist. Da bei der Messung die Magnetisierung lange transversal auf C_{α} ist, kann man diese Messungen nur an deuterierten Proteinen oder an entfalten Proteinen durchführen. In beiden Fällen ist die transversale Relaxation von C_{α} langsam genug um Magnetisierung detektieren zu können. Auch $^3J_{C\alpha HN}$ Kopplungen haben eine Abhängigkeit von ψ gezeigt. Allerdings hängt die Kopplung auch von ϕ ab, sodass eine Interpretation recht schwierig ist.

Die $^3J_{C\alpha C\alpha}$ Kopplung konnte gut in denaturiertem Ubiquitin bestimmt werden. Die experimentell bestimmten mittleren $^3J_{C\alpha C\alpha}$ Kopplungskonstanten in nativem Ubiquitin betragen für β -Faltblattbereiche 1.69 ± 0.1 Hz, jedoch konnten in α -helikalen Bereichen keine Kopplungskonstanten bestimmt werden²⁰. Im denaturierten Ubiquitin waren alle $^3J_{C\alpha C\alpha}$ Kopplungskonstanten größer als 0.7 Hz (auch im α -helikalen Bereich) mit einem Mittelwert von 0.85 ± 0.2 Hz, was einer mittleren Gleichverteilung zwischen α -helikalen und β -Faltblattbereich entspricht. Diese Mittelungsverteilung konnte auch in den $^3J_{H^N H\alpha}$ Kopplungskonstanten für den Winkel ϕ beobachtet werden, welche ungefähr 4 Hz für α -helikale und 10 Hz für β -Faltblattbereiche in nativen Proteinen ist und im Durchschnitt 7.1 ± 0.6 Hz für denaturiertes Ubiquitin beträgt.

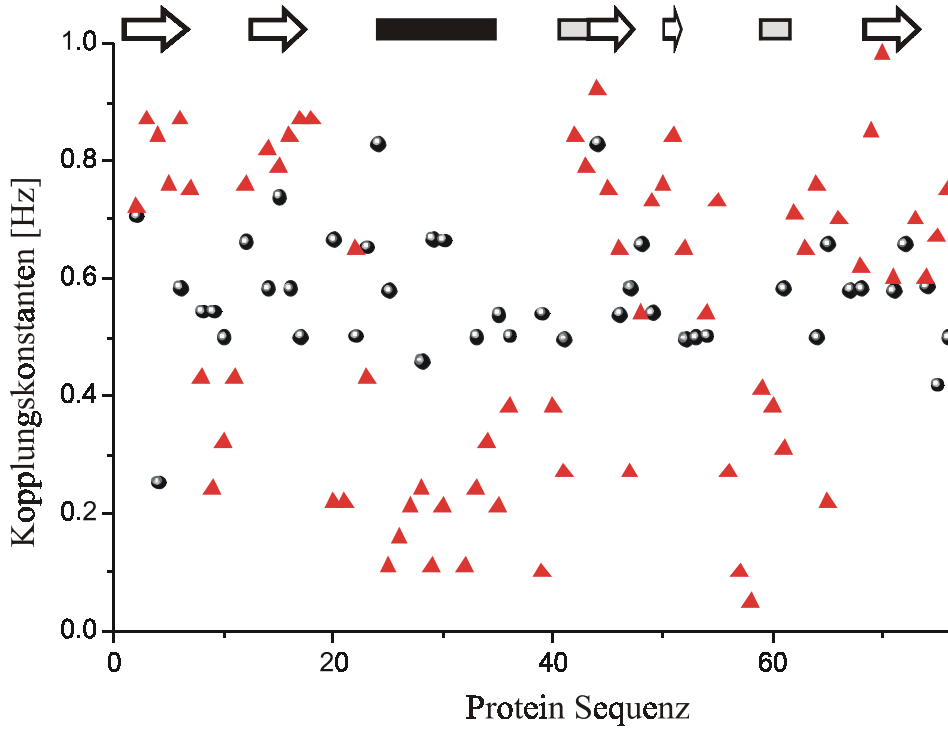


Abbildung 4.7: Vergleich der experimentell bestimmten denaturierten (Bälle) und nativen (Dreieck) ${}^3J_{C\alpha HN}$ Kopplungen.

Auch ${}^3J_{C\alpha HN}$ Kopplungen zeigen eine ψ Abhängigkeit. Auch hier ist eine qualitative Interpretation wie bei den ${}^3J_{C\alpha C\alpha}$ Kopplungskonstanten möglich (Abbildung 7). Für natives Ubiquitin konnte die Kopplung mittels einer Pachleranalyse parametrisiert werden²⁰. Deutlich sieht man, dass die Kopplung von ψ und von ϕ abhängig ist. Da nur wenige Kopplungen gemessen werden konnten, haben wir nicht versucht die gemittelten Kopplungen aus dem Random Coil Modell mit den experimentellen Kopplungen zu vergleichen.

Auch mittels gekreuzkorrelierter Raten^{21,22} ist es möglich ψ zu bestimmen. Die Messung der Dipol-Dipol gekreuzkorrelierten Rate $\Gamma_{H^N N, C\alpha H\alpha}^C$ oder der Dipol-CSA gekreuzkorrelierten Rate $\Gamma_{C\alpha H\alpha, C'}^C$ ²³ liefert Werte, welche ohne eine externe Kalibrierung in einen Winkel übersetzt werden können:

$$\Gamma_{H^N N, C\alpha H\alpha}^C = \frac{\gamma_H \gamma_N}{(r_{N, H^N})^3} \frac{\gamma_H \gamma_C}{(r_{C\alpha, H\alpha})^3} \left(\frac{\hbar \mu_0}{4\pi} \right)^2 \times \frac{1}{5} (3 \cos^2 \theta - 1) S^2 \tau_c$$

Die einzigen unbekanntenen Größen stellen der gekreuzkorrelierte Ordnungsparameter S und die „über alles Korrelationszeit“ τ_c dar (eine detaillierte Beschreibung ist in Kapitel 3 gegeben). Der gekreuzkorrelierte Ordnungsparameter S ist a priori unbekannt und kann in nativen Proteinen nahe 1 angenommen werden. τ_c ist durch heteronukleare

Relaxationsmessungen^{24,25} in nativ gefalteten Proteinen durch das Lipari Szabo Modelfree Modell^{26,27} zugänglich. Die quantitative Interpretation kreuzkorrelierter Relaxationsraten in denaturierten Proteinen ist allerdings schwieriger, da es keine eindeutige Korrelationszeit gibt. Für denaturierte Proteine wurden etliche Methoden, zum Beispiel statistisch gemittelte τ_c Zeiten²⁸, oder andere Methoden^{29,30} entwickelt, welche die Dynamik beschreiben. Diese Methoden wurden allerdings nicht für die Interpretation der kreuzkorrelierten Raten verwendet. Statt dessen wurde eine Analyse verwendet, welche auf einem konstanten T_1/T_2 Verhältnis über die Proteinsequenz (Abbildung 8), wie es experimentell gefunden wurde, beruht. Das T_1/T_2 Verhältnis, welches zumindest größtenteils unabhängig von den schnellen internen Bewegungen und der chemischen Verschiebungsanisotropie ist, ist ein gutes Maß wie schnell sich jeder einzelne NH-Vektor reorientiert²⁵. Nur am N- und am C-Terminus kann man einen starken Abfall feststellen. Aus den autokorrelierten Raten (R_1 , R_2 und hetNOE) erhält man ein τ_c berechnet für 600, 500 und 400 MHz von: 2.8, 3.1 und 2.7 ns. Der mittlere autokorrelierte Ordnungsparameter ist 0.45 ± 0.5 .

Kreuzkorrelierte Raten für natives Ubiquitin mit einer Korrelationszeit von 4.05ns sind im Mittel -12.2 ± 1.7 Hz für β -Faltblätter und im Mittel -2.8 ± 1.9 Hz für die α -Helix³¹. Für denaturiertes Ubiquitin sind die kreuzkorrelierten Raten im Durchschnitt -4.3 ± 1.8 Hz. Einsetzen in die Formel:

$$-4.3 = \left[x_\beta \cdot (-12.2) + (1 - x_\beta) \cdot (-2.8) \right] \frac{2.8}{4.05} * 0.45$$

ergibt dann eine Wahrscheinlichkeit ($x_\beta = 1$) für den β -Faltblatt Bereich. Dies ist im Widerspruch zu den bisher gefundenen Daten und zeigt auf, dass eine bessere Beschreibung der Dynamik des Random Coil Zustands notwendig ist. Im Moment ist es somit nur möglich die kreuzkorrelierten Raten qualitativ zu interpretieren und die Mittelung der kreuzkorrelierten Raten analog der Mittelung der Kopplungskonstanten zu verstehen.

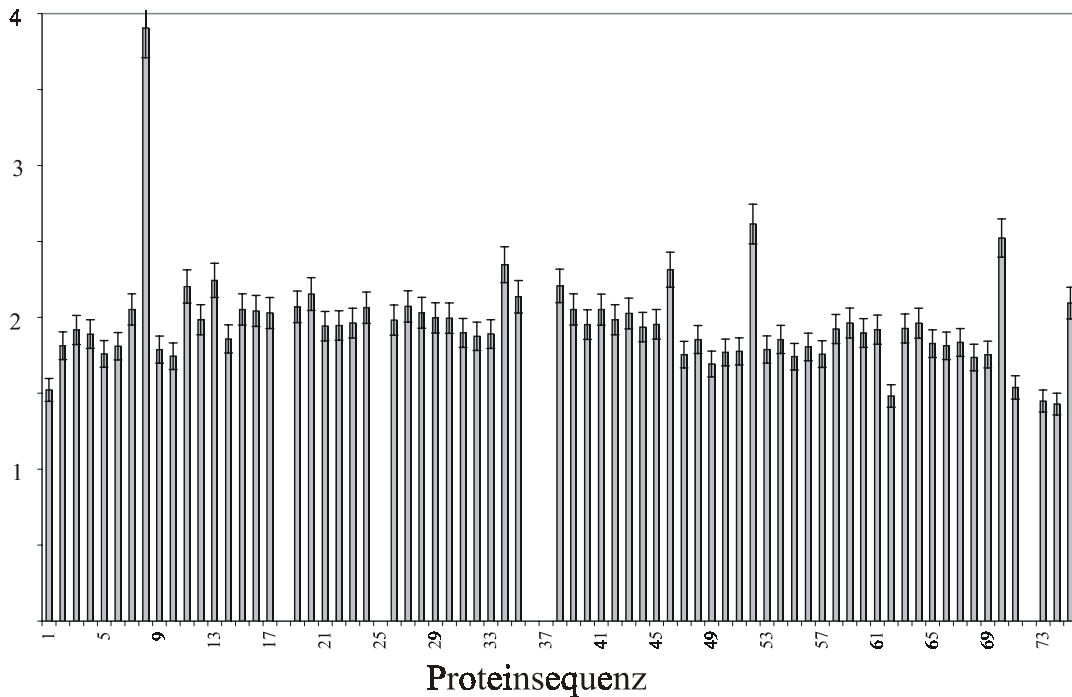


Abbildung 4.8: T_1/T_2 -Verhältnis im denaturierten Ubiquitin (pH 2, 8M Harnstoff, 303K, 10% D_2O , T_1 und T_2 Relaxationszeiten gemessen bei 600MHz Protonenfrequenz). Das Verhältnis ist für den mittleren Bereich des Proteins immer zwischen 1.8 und 2.1. Allerdings ist ein Abfall im N-terminalen (dieser jedoch nur sehr lokal) und im C-terminalen Bereich deutlich zu erkennen. Die Reste mit zu großem T_1/T_2 -Verhältnis sind Glycine, welche wir aus der Analyse der kreuzkorrelierten Raten ausgeschlossen haben.

4.2 Chemische Verschiebungsanalyse in denaturierten Proteinen

Die genaue Analyse dieser Vorzugskonformationen ist aber erst möglich durch die große Dispersion der ^{15}N chemischen Verschiebung. Diese ist im Gegensatz zu den $^1H^N$, $^{13}C_\alpha$ und $^{13}C_\beta$ Dimensionen ähnlich der Dispersion in nativ gefalteten Proteinen. Deshalb ist es möglich 1H - ^{15}N -HSQC Spektren zu messen und darauf aufbauend denaturierte Proteine vollständig zuzuordnen. Erst durch die Zuordnung aller chemischen Verschiebungen in denaturierten Proteinen kann man skalare Kopplungskonstanten, kreuzkorrelierte Raten, NOE und autokorrelierte Raten messen und so Information über die Struktur und Dynamik solcher Zustände gewinnen³².

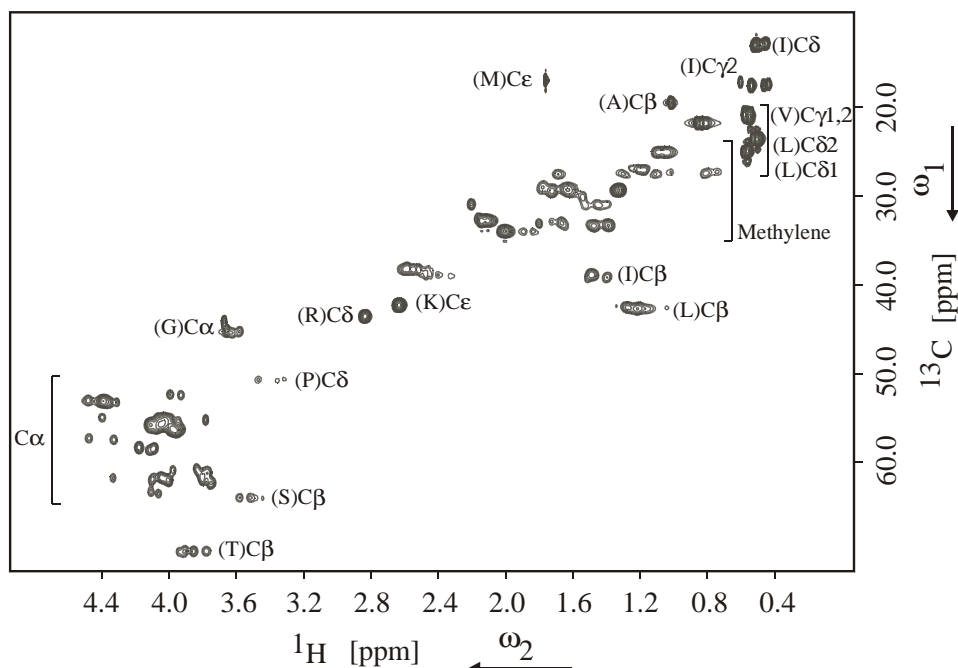


Abbildung 4.9: ^1H - ^{13}C HSQC Spektrum von denaturiertem Ubiquitin (pH 2, 8M Harnstoff, 303K, 10% D_2O , 600MHz Protonenfrequenz). Deutlich ist die geringe Dispersion der ^{13}C -chemischen Verschiebungen sichtbar. Die charakteristischen Verschiebungsbereiche sind eingezeichnet. Ein ^1H - ^{15}N HSQC Spektrum von denaturiertem Ubiquitin findet man in Peti et al.(Abbildung 1)¹⁴.

Die Differenz der nativen ^{13}C chemischen Verschiebungen von $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, ^{13}CO und $^1\text{H}_\alpha$ und von chemischen Verschiebungen aus entfaltenen Modellpeptiden³³ haben gezeigt, dass sie indikativ für bevorzugte Sekundärstrukturen³⁴ sind (CSI, chemical shift index^{35,36}). Durch die gleiche Analyse chemischer Verschiebungen kann auch der denaturierte Zustand charakterisiert werden^{37,38}. Sehr einfach kann man lokale Abweichungen vom denaturierten Zustand herausfinden. In Lysozym konnten so die aromatischen Aminosäuren als mögliche Nukleationskeime für die Faltung identifiziert werden^{8,9}. Wie in Abbildung 9 zu sehen, bilden sich in Ubiquitin keine Nukleationskeime um die aromatischen Aminosäuren (Phe 4, Phe 45, Tyr 59 und His 68). Jedoch kann man in den $^1\text{H}_\alpha$ chemischen Verschiebungen eine leicht positive systematische Erhöhung der Verschiebungsdifferenzwerte im N-terminalen Bereich in Ubiquitin finden. Das N-terminale antiparallele β -Faltblatt (Aminosäuren 2-17) ist auch autonom gefaltet³⁹. Auch autokorrelierte Relaxationsraten zeigen, dass denaturiertes Ubiquitin hier kein reines random coil Verhalten besitzt. Somit ist es möglich, dass der N-Terminus von Ubiquitin einen Faltungsstartpunkt darstellt.

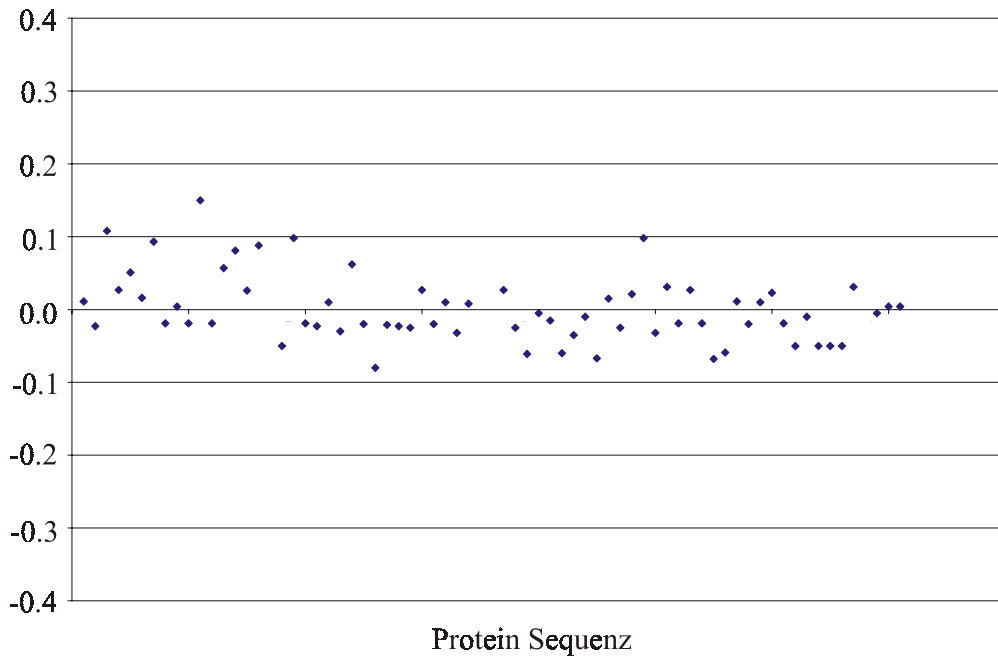
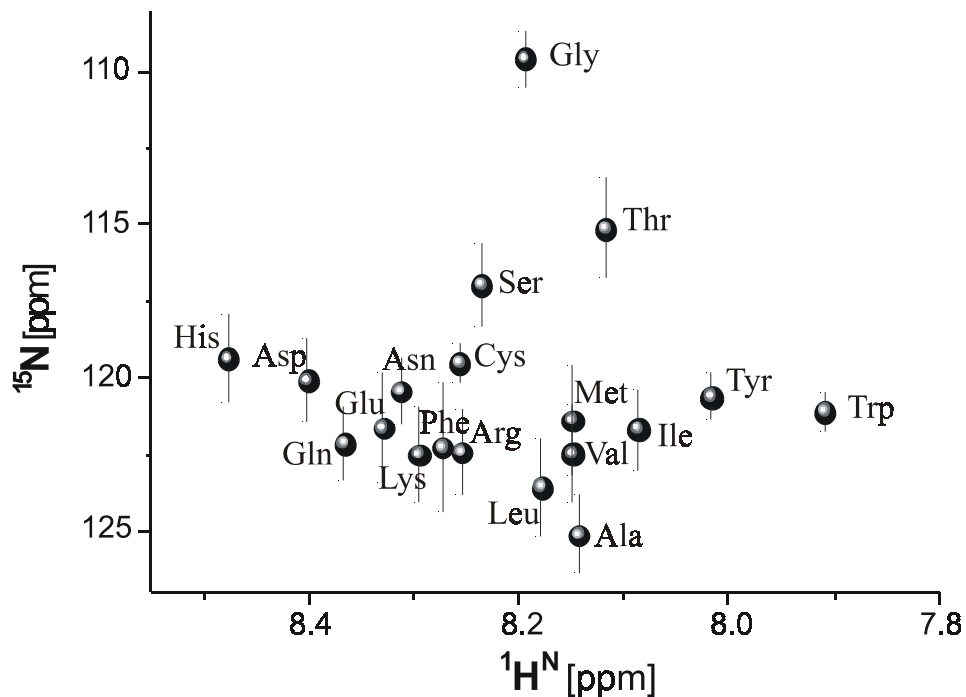


Abbildung 4.10: Abweichung der H_α chemischen Verschiebung vom Random Coil chemischen Verschiebungswert bestimmt an Modellpeptiden³³. Deutlich ist das Random Coil Verhalten entlang der Proteinsequenz erkennbar. Leichtere Abweichungen sind nur am N-terminalen Teil des Proteins erkennbar. Die hohe Stabilität des N-Terminus von Ubiquitin ist schon länger bekannt³⁹. Der N-Terminus stellt so möglicherweise einen Nukleationskeim für die Faltung dar. Allerdings sind keine Faltungsnukleationskeime an den aromatischen Aminosäuren, wie in Lysozym, erkennbar.



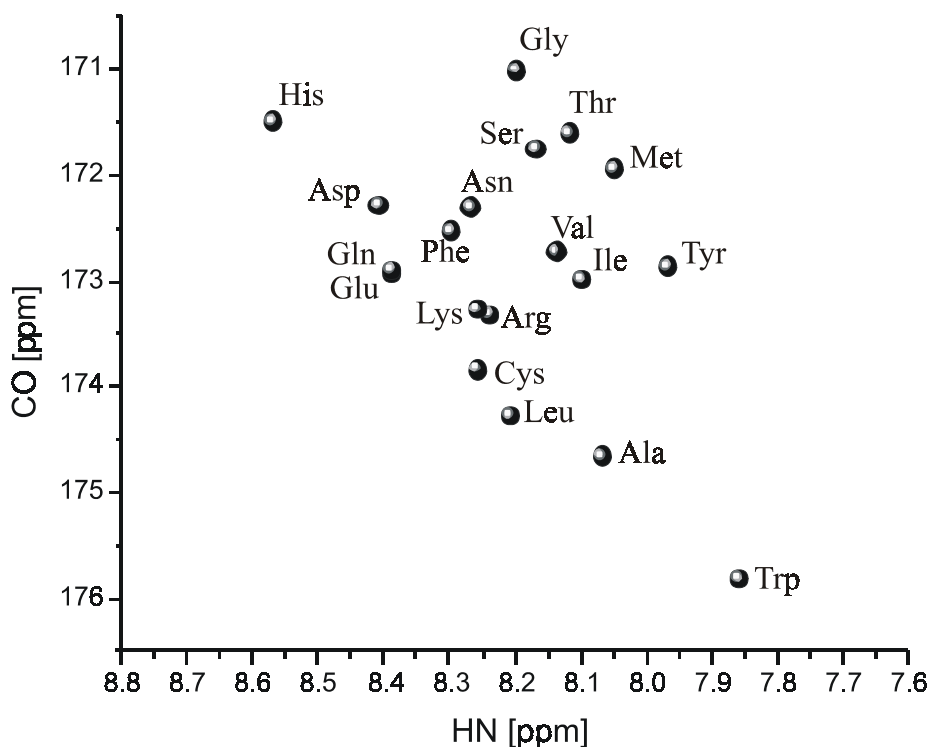


Abbildung 4.11: $^1\text{H}^N$ - ^{15}N HSQC (oben) und $^1\text{H}^N$ - ^{13}C HNCOC Spektrum (unten). Die ^{15}N und die ^{13}C CO Dispersion ist gewöhnlich auch in entfaltenen Proteinen ausreichend um mittels heteronuklearen multidimensionalen Spektren alle chemischen Verschiebungen eindeutig zuordnen zu können. Die Spektren wurden für die einzelnen Aminosäuren aus den mittleren chemischen Verschiebungen aufgebaut. Ähnliche Peakschemata findet man auch in nativen Proteinen, sodass diese Modell-Spektren bei der Zuordnung hilfreich sein können.

Natürlich interessiert die Frage warum die ^{15}N -chemischen Verschiebungen solch eine Dispersion zeigen und ob auch die chemischen Verschiebungen das vorgestellte Random Coil Modell erfüllen. Um die Datendichte der ^{15}N chemischen Verschiebungen zu erhöhen, habe ich chemische Verschiebungen aus drei Proteinen verwendet: Ubiquitin, reduziertes, carboxymethyliertes Lysozym und all-Ala- α -Lactalbumin (einer α -Lactalbumin Mutante, in welcher alle Cysteine gegen Alanin mutiert wurden). Somit hatte man 302 experimentelle ^{15}N chemische Verschiebungen.

Für die Kontrolle des Random Coil Modells haben wir als Datenbasis für native chemische Verschiebungen die BioMagResBank in Wisconsin benutzt (<http://www.bmrb.wisc.edu>). In der BioMagResBank Datenbank sind NMR-spektroskopisch spezifische Daten, wie eben chemische Verschiebungen, von 1781 Proteinen (Stand: 29.03.2001) abgelegt. Insgesamt findet man in der Datenbank 561116 chemische Verschiebungen, sodass man auch hier bei der Mittelung annehmen kann, dass die Kriterien des Random Coil Modells erfüllt werden. Vergleicht man die

aminosäurespezifisch gemittelten experimentellen Daten mit den aminosäurespezifisch gemittelten chemischen Verschiebungen aus der Datenbank, so kann man auch hier eine beachtliche Übereinstimmung ($R > 0.98$) finden¹⁴.

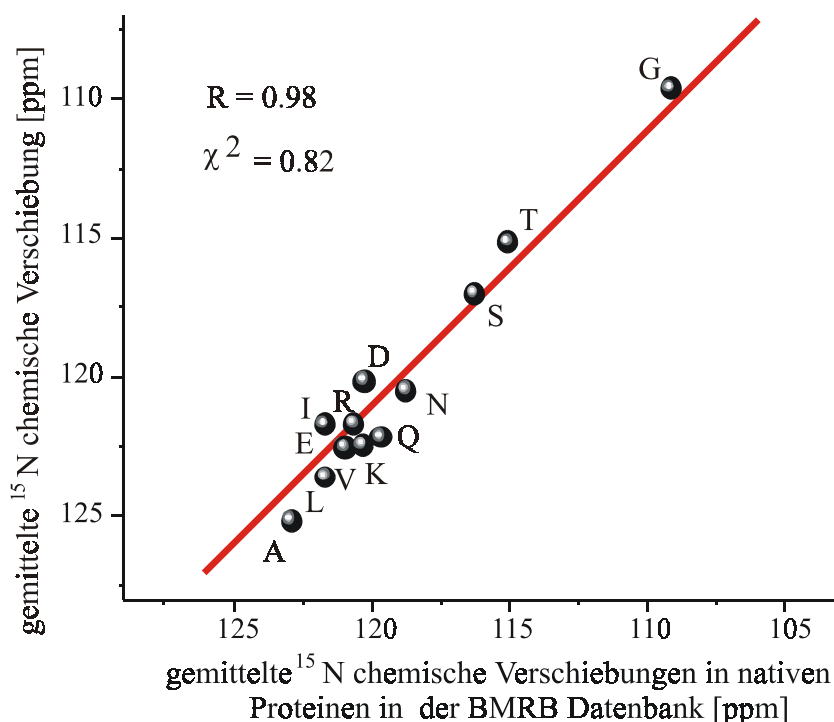


Abbildung 4.12: Vergleich der experimentellen gemittelten ¹⁵N chemischen Verschiebungen aus drei denaturierten Proteine, mit den gemittelten ¹⁵N chemischen Verschiebungen aus der BMRB-Datenbank. Die Korrelation ist beachtlich und erfüllt das Random Coil Modell.

Weiterhin bleibt die Frage bestehen, weshalb die ¹⁵N-chemischen Verschiebungen eine größere Dispersion zeigen. Wichtige Parameter für die ¹⁵N-chemische Verschiebung sind: Die Proteinrückgrat Winkel ϕ und ψ , die Seitenkettenorientierung (χ_1), Wasserstoffbrücken und Nachbareffekte, besonders von der vorhergehenden Aminosäure. Da man in denaturierten Proteinen langreichweitige Wechselwirkungen und Wasserstoffbrückenwechselwirkungen ausschließen kann, muss der Grund besonders in Nachbareffekten zu suchen sein. Dahingehend haben wir die chemischen Verschiebungen untersucht¹⁴. Interessant ist auch das aminosäurespezifisch auftretende starke Gruppieren bei bestimmten ¹⁵N-chemischen Verschiebungen. Besonders deutlich ist dies für Glycin, Serin und Threonin zu sehen. Das aminosäurespezifische Gruppieren der chemischen Verschiebung konnte auch schon früher gezeigt werden. Braun⁴⁰ in der Arbeitsgruppe von Kurt Wüthrich hat durch systematische Analyse (19 Gly-Gly-X-Ala Peptide und ein Gly-Gly-Lys-Ala-Ala Peptid wurden untersucht) eine empirische Formel für die ¹⁵N chemischen Verschiebungen entwickelt. Die vorhergesagten gemittelten ¹⁵N chemischen

Verschiebungen zeigen eine sehr gute Übereinstimmung mit den experimentellen chemischen Verschiebungen. Interessanterweise kann man die gemittelten Kopplungen sehr gut vorhersagen, die individuellen Unterschiede der chemischen Verschiebungen sind aber oft sehr groß. Für die denaturierten Proteine Ubiquitin, Lysozym und All-Ala-Lactalbumin haben wir versucht die chemischen Verschiebungen mit den ${}^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ Kopplungen, welche den Winkel ϕ beschreiben, zu korrelieren¹⁴. Für vereinzelte Aminosäuren konnte man die chemischen Verschiebungen und den Rückgratwinkel korrelieren. Leider ist im Moment die Datendichte zu gering, sodass zukünftig noch mehr Proteine analysiert werden sollten.

Allgemein bildet das Random Coil Modell eine Möglichkeit, den Anfangszustand der Proteinfaltung mit atomarer Auflösung zu beschreiben. Zukünftig wird es wichtig sein eine detaillierte Interpretation der dynamischen Effekte in diesen entfalten Zuständen zu charakterisieren. Dafür haben wir schon ${}^{15}\text{N}$ autokorrelierte Raten und heteronukleare NOE Daten gemessen. Allein die Interpretation dieser Raten ist nicht mit Modellen möglich^{30,41}, welche für native Proteine gelten (Lipari Szabo Modell), wie schon vorher angedeutet. Deshalb ist es hier wichtig alternative Ansätze zu entwickeln. Dies würde einerseits in einer Weiterentwicklung der Lipari Szabo Modells auf entfaltete Zustände möglich sein²⁸. In dieser nimmt man eine Verteilung der Korrelationszeit an. Andererseits ist es vielleicht möglich eine genaue dynamische Beschreibung zu erreichen, in dem man die Relaxationsraten mit einer Molekulardynamik Trajektorie vergleicht, wie dies für native Proteine möglich ist. Dies wurde vor kurzem auch für den A-Zustand⁴¹, einem vermutlichen Faltungsintermediat von Ubiquitin, gezeigt⁴². In Zusammenarbeit mit Rafael Brüschweiler und Jeanine J. Prompers haben wir eine 100ns Trajektorie von denaturiertem Ubiquitin berechnet. Die Daten sollen in den nächsten Wochen analysiert werden und ermöglichen uns hoffentlich einen weiteren Einblick in den Random Coil Zustand zu bekommen.

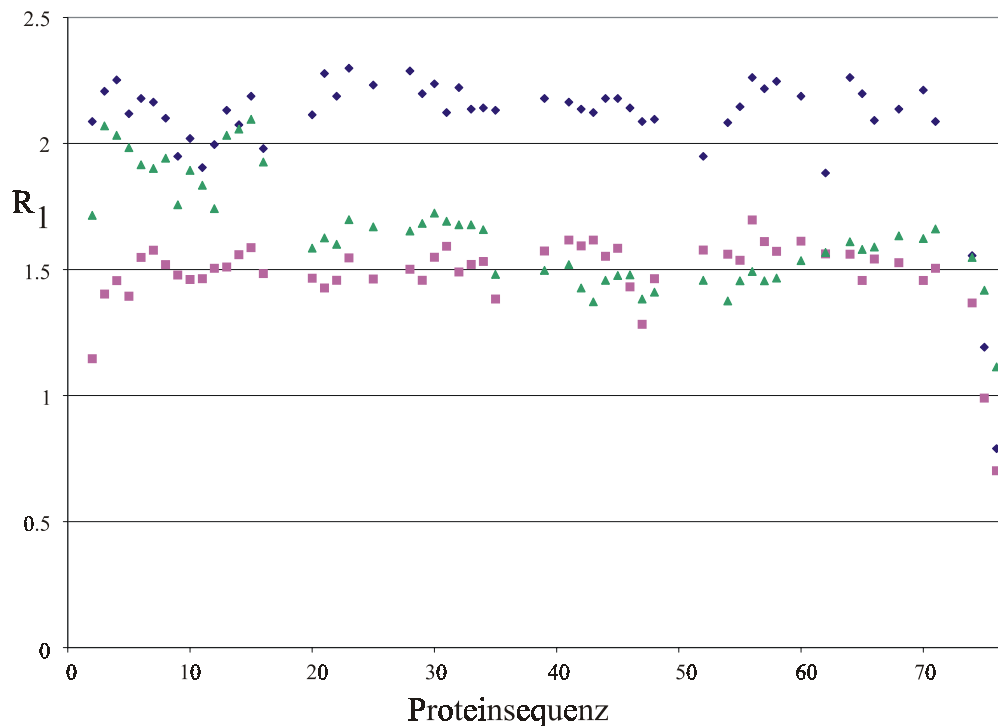


Abbildung 4.13: R_1 -autokorrelierte Raten gemessen bei 600 MHz Protonenfrequenz. Quadrate: denaturiertes Ubiquitin; Dreiecke: A-State Ubiquitin; Raute: natives Ubiquitin. Deutlich ist zu erkennen, dass der A-State zu Beginn natives Verhalten zeigt, der C-terminale Bereich jedoch vollständig denaturiert ist.

4.3 Literatur

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5 Zusammenfassung

In der vorliegenden Arbeit wurden neue Methoden für die hochauflösende NMR Spektroskopie entwickelt, um damit native und denaturierte Proteine charakterisieren zu können.

Neue Mess- und Anwendungsmethoden für dipolare Kopplungen bilden den Schwerpunkt der Doktorarbeit. Durch die Verwendung von flüssigkristallinen Medien ist es in NMR in Lösung möglich geworden dipolare Kopplungen zu bestimmen. Durch die Projektionen der einzelnen so bestimmten Vektoren auf andere Vektoren im Protein, welche beliebig weit entfernt sein können, kann weitreichende Orientierungsinformationen für die Strukturrechnung von Biomakromolekülen genutzt werden. Dies ist an den Beispielen Ubiquitin, Triggerfaktor und Raffinose in der Arbeit dargestellt. Durch diese Orientierungsinformation kann man auch Teile von Proteinen, wie zum Beispiel Domänen, gegeneinander ausrichten oder die relative Orientierung von Sekundärstrukturelementen nutzen um eine 3D Homologiesuche durchzuführen. Auch dies ist in der Arbeit beschrieben.

Die neuen Messmethoden erlauben erstmals die Bestimmung von ^1H - ^1H dipolaren Kopplungen mit Größe und Vorzeichen (J_{HH} -NOESY) und von allen drei dipolaren Kopplungen in einer Seitenkettenmethylgruppe (SPITZE-HSQC).

Ein weiterer wichtiger Punkt war die Extraktion dynamischer Parameter aus dipolaren Kopplungen. Der Vektor, der die wechselwirkenden Dipole verbindet, wird von der Dynamik des Proteins beeinflusst. Dadurch ist eine Analyse der Dynamik auf einer Zeitskala bis ca. 10ms möglich (bisher nur bis in den Bereich von ns). Besonders μs Dynamik, welche vorher nicht mittels NMR Methoden sichtbar, kann so dargestellt werden. Aus den dipolaren Kopplungen, welche in 11 verschiedenen Orientierungsmedien gemessen wurden, konnten modellfreie dipolare Ordnungsparameter extrahiert werden. Erstmals konnte zwischen axialsymmetrischen und anisotropen Bewegungen der NH Vektoren unterschieden werden. Unsere experimentellen Daten zeigen, dass Ubiquitin auf einer Zeitskala oberhalb der Korrelationszeit ähnlich viel Bewegung zeigt wie unterhalb der Korrelationszeit. Der mittlere Ordnungsparameter sinkt von 0.8 für Bewegungen bis zur Korrelationszeit auf 0.6 für alle Bewegungszeiten. Auch sieht man, dass viele Bewegungen bis zu 60% Anisotropie beinhalten.

TOCSY-Sequenzen sind wichtige Bausteine für Moleküle jeder Größenordnung. Hierfür wurden neue adiabatische TOCSY Sequenzen entwickelt, welche TOCSY Spektroskopie bei allen Magnetfeldern mit höherer Intensität erlauben. Wichtig ist auch noch die viel größere Robustheit gegen B_1 -Inhomogenitäten und Pulsmisskalibrierung. Dipolare TOCSY Sequenzen (MOCCA) erlauben besseren Transfer mittels den zuvor schon erwähnten dipolaren Kopplungen.

Durch die Analyse denaturierter Proteine wollte man ein besseres Verständnis dieses Zustandes erzielen. Hierfür wurden Kopplungskonstanten, kreuzkorrelierte und autokorrelierte Raten und chemische Verschiebungen gemessen und mit einem Modell, dem sogenannten Random Coil Modell verglichen. Mit Hilfe dieser experimentellen Daten sieht man erstmals direkt, dass Proteine im denaturiertem Zustand den Winkel ψ zwischen 60° (α -Helix) und -120° (β -Faltblatt) absuchen.

6 Schriftliche Veröffentlichungen

Die Veröffentlichungen sind alphabetisch nach dem Erstautor geordnet.

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7 Abkürzungen

BICELLEN: flache ovale Micellen aus mindestens 2 Bestandteilen, bilayered discoidal mixed micelles

BIRD: Zusammengesetzte Rotation, Bilinear Rotation Decoupling

BIR: B₁ Insensitive Rotation

CA: Konstante Adiabaticität, constant adiabaticity

CHAPSO: (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propansulfonat)

CONSTANT TIME: Klasse von Experimenten, bei denen die Modulation der Transferamplitude durch Evolution homo- oder heteronuklearer Kopplung nicht in einer Evolutionszeit, sondern in einer fixen Zeit stattfindet.

COSY: Korrelationsspektroskopie, Correlation spectroscopy

CSA: Chemische Verschiebungsanisotropie, chemical shift anisotropy

CTAB: Cetyltrimethylammoniumbromide

D: dipolare Kopplungskonstante, über den Raum vermittelt

DELAY: Wartezeit zur Evolution einer Kopplung

DFT: Dichte Funktionale Theorie Rechnungen (Density Functional Theory)

DHPC: L- α -Phosphatidylcholinedicaproyl (C6)(1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine)

DIODPC: 1,2-Di-O-dodecyl-*sn*-glycero-3-phosphocholine

DIPSI: Entkopplungs- und TOCSY-Sequenz, decoupling in the presence of scalar interactions

DLPC: L- α -Phosphatidylcholinedilauroyl (C12), (1,2-Didodecanoyl-*sn*-glycero-3-phosphocholine)

DMPC: L- α -Phosphatidylcholinedimyristoyl (C14), (1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine)

DQ/ZQ: Doppel- und Nullquanten Spektroskopie

HMQC: Heteronukleare Multiquantenkorrelation, Heteronuclear Multiple Quantum Correlation

HSQC: Heteronukleares Verschiebungskorrelations Spektrum, Heteronuclear Single Quantum Correlation

INEPT: Kohärenztransfer zwischen Spins unterschiedlicher Kerne, Insensitive Nuclei Enhanced by Polarization Transfer

J: skalare Kopplungskonstante, über die Bindungen vermittelt

K: Kelvin

NMR: Nuklear Magnetische Resonanz Spektroskopie; Kernspinresonanzspektroskopie

NOESY: Kern-Overhauser und Austausch Spektroskopie, nuclear Overhauser and exchange spectroscopy

PPM: millionste Teil, parts per million

RANDOM COIL: Grundzustand des entfalteten Proteins, eine große Anzahl von energieglichen, sehr schnell ineinander fluktuierenden Strukturen

RMSD: Mittlere quadratische Abweichung, Root Mean Square Deviation

SDS: Natrium-Dodecylsulfat

SPITZE-HSQC: Spinzustandsselektives HSQC Spektrum mit minimaler Überlappung der Peaks, SPIn sTate selective Zero overlap HSQC

S³E: Spinzustand selektive Anregung, Spin State Selective Excitation

τ_c : overall correlation time: Zeit die ein Molekül braucht um sich um seine eigene Achse zu reorientieren.

T₂*: effektive transversale Relaxationszeit

TROSY: Technik welche es erlaubt verschiedene Relaxationswege gegeneinander auszuspielen und so hohe Empfindlichkeit auch bei großen Molekülen ermöglicht; transverse relaxation optimized spectroscopy

TOCSY: totale Korrelationsspektroskopie, TOtal Correlated SpectroscopY

WURST: wideband, uniform rate, and smooth truncation

8 Anhang

8.1 *Praktische Aspekte bei der Herstellung von flüssigkristallinen Phasen*

Alle Angaben beziehen sich auf die Herstellung 1000 μ l einer flüssigkristallinen Phase. Ich beschreibe diese um eine wässrige 90% H₂O und 10% D₂O Lösung zu erhalten. Natürlich ist es auch möglich die meisten flüssigkristallinen Phasen in reinem D₂O herzustellen.

Flüssigkristalline Phasen basierend auf Phosphocholinen:

DLPC, DMPC und CHAPSO kann man bei Sigma-Aldrich kaufen. DHPC kann man aber nur bei Avanti Polar Lipids Inc. beziehen. Nur hier ist es möglich DHPC in reiner Pulverform zu kaufen und nur aus diesem DHPC lassen sich Bicellen formen. Achtung DHPC ist sehr hygroskopisch, sodass es sinnvoll ist unter Argon zu arbeiten.

DMPC: 677.9 g/mol, DHPC: 453.5 g/mol, DLPC: 621.8 g/mol, CHAPSO: 630.9 g/mol

Für eine 5% CHAPSO:DLPC Phase (als bestes molares Verhältnis hat sich bei uns 1:5 herauskristallisiert) braucht man: CHAPSO: 8.43 mg, DLPC: 41.56 mg.

Für CHAPSO/DMPC ist es besser ein etwas niedriges molares Verhältnis von nur 1:3 zu verwenden. SDS (Merck Chemikalien) und CTAB (Acros Chemikalien) können zur Modifikation eingesetzt werden. Bei CHAPSO/DLPC Systemen war die Endzusammensetzung 10:50:1, wobei der eine Anteil eben SDS oder CTAB war.

n-Alkyl-Polyethylenglykol/n-Alkylalkohol/H₂O Phasen:

Alle Substanzen können bei Sigma-Aldrich gekauft werden. Für eine 5% Stammlösung einer C12E5 (Polyoxyethylene-5-laurylether) / Hexanol Lösung muss man folgende Mengen verwenden:

C12E5 (406.18 g/mol; $\rho = 0.96 \text{ g/cm}^3$) wird im molaren Verhältnis von 0.96 zu Hexanol (102.18 g/mol; $\rho = 0.814 \text{ g/cm}^3$) eingesetzt. Die Gesamtmenge (C12E5/Hex) gilt als 5% zu H₂O. C12E5: 40.66 mg (42.35 μ l); Hexanol: 9.81 mg (12.05 μ l).

Helfrich Phasen Oberflächen Lösungen:

Auch hier ist es möglich alle Chemikalien von Sigma-Aldrich zu beziehen. Die folgenden Werte beziehen sich auf eine 3.5% Stammlösung. Es hat sich gezeigt, dass gerade in dieser

Lösung sehr starke elektrostatische Effekte auftreten und deshalb die Linienbreiten stark wachsen. Deshalb sollte eine 3.5% Lösung die obere Grenze der Konzentration der Oberflächenlipide darstellen.

CPBR (Cetylpyridiniumbromide Monohydrat): 402.47 g/mol.

Hexanol: 102.18 g/mol; $\rho = 0.814 \text{ g/cm}^3$

NaBr: 102.9 g/mol;

Als bestes Verhältnis von CPBR/ Hexanol hat sich 1:1.33 herausgestellt. Dies war wichtig, denn nur bei diesem Verhältnis braucht man eine NaBr Lösung welche nur 25-30 mM ist. Bei anderen Verhältnissen ist es notwendig die NaBr Konzentration bis auf 250 mM zu steigern, was die Messung der Kopplungskonstanten erschwert.

CPBR: 8.83 mg; Hexanol: 26.17 mg (32.14 μl)

25 mM NaBr Lösung: 25.72 mg / 10 ml

Purpur Membran Fragmente (Bacteriorhodopsin):

Hier gilt unser Dank der Arbeitsgruppe von Prof. Dr. D. Oesterhelt welche uns immer mit konzentrierten Purpur Membran Fragmenten versorgt haben. Wichtig war immer, dass die Proben kühl gelagert werden und die Ionenstärke in den Proben nicht zu hoch gewählt wird. Salzkonzentrationen über 100 mM lassen die Purpur Membran Fragmente schnell gelartig werden. Dann kann man mit der Phase nicht mehr orientieren. Vor der Vermischung mit dem Protein sollten die Purpur Membran Fragmente auch noch ca. 15 Minuten im Ultraschallbad gelassen werden um mögliche Klumpenbildung vorzubeugen.

Kristalline Zellulose Medien:

Hier wurden mir die meisten Tipps und Tricks von Steve Matthews zur Verfügung gestellt. Da ich seine Ausführungen übernommen habe möchte ich diese auch im Original hier wiedergeben:

“The grinding of the paper is critical (we use Whatman filter paper) if the grinding process is too harsh it will degrade the cellulose - this is evident from much shorter hydrolysis times. You must use a Wiley mill with a cutting action. During hydrolysis the sample should be stirred and kept at 45 degrees - check this regularly with a thermometer. The mixture does get

viscous and glassy, but this should stay this way for the course of the hydrolysis. A completely non viscous solution appears after the hydrolysis has gone too far (this should not happen before 1hr at 45°, also double check your acid concentration)

After dilution, dialysis, neutralisation and concentration the sample is left to separate into two phases (critical conc. ~4-5%). The phase is only observed through crossed polars. We observe the tube (long 8-10mm NMR tube) between two polarised sunglass lenses crossed at 90°. The liquid crystal layer is light and the isotropic is dark. We also check the anisotropic layer under the electron microscope - our published micrograph is optimal. The appearance of the cellulose is white and pearlescent similar to concentrated phage.

The anisotropic layer is taken and added directly to freeze dried protein or concentrated protein solution. High salt is not good so the extensive dialysis is critical. The HDO splitting is very broad (slow exchange) so we observe the alignment using the protein amide splittings.

Be careful of increasing ionic strength - the phase is very salt sensitive causing a gel - this process seems to be very protein dependant. You may wish to strip the sample of ions with +/-ve ion exchange resin and readjust pH before adding protein.”

8.2 *Pulsprogramme*

S³E Pulsprogramm:

```
;wp15nhsqcs3e
```

```
#include <Avance.incl>
```

```
#include <Grad.incl>
```

```
#include <Delay.incl>
```

```
#define GRADIENT3(ampl) p20:ngrad:c34
```

```
"p2=p1*2"
```

```
"p6=p5*2"
```

```
;"p14=256u" (q3 Inversionspuls zum Entkoppeln von 13Cα und 13CO)
```

```
"d11=30m"
```

```
"d4=2.4m"
```

```
"d12=20u"
```

```
"d5=d4+p6"
```

```
"d8=1.38m-p20-d16-50u"
```

```
"d29=d8+p2"
```

```
"d17=p1"
```

```
"d18=p5"
```

"d19=p16+d16+8u"
"d25=p16+d16+d0*2"
;"d25=p16+d16+d0*2+2*p14" (¹³C markierte Probe)

"I3=(td1/2)"

1 ze
d11 pl13:f3
2 30m do:f3
6m
3 6m
7 3m
8 d11
d11
4 6m
5 d11 do:f3
6 d12 pl1:f1
d12 pl3:f3
d1
d11
20u
(p1 ph1)
d4
(p6 ph1):f3
(p2 ph1)
d5
(p28 ph1):f1
(p1 ph2)
d12 pl9:f1
(p9 ph9):f1
50u
GRADIENT2(cnst21)
d16
(p5 ph3):f3
d8 pl1:f1
50u
GRADIENT3(cnst22)
d16 pl1:f1
(p2 ph1)
(p6 ph4):f3
d29
50u
GRADIENT3(cnst22)
d16
(p5 ph5):f3
(p5 ph6):f3
d0 ;pl0:f2
; (p14:sp13 ph0):f2 (q3 Inversionspuls zum Entkoppeln von ¹³Cα und ¹³CO)
; (p14:sp14 ph0):f2
d0
GRADIENT(cnst23)
d16
(p6 ph4):f3
d25
(p5 ph1):f3
d17
(p1 ph1)
d4
(p6 ph1):f3


```
(p2 ph1)
d4
(p5 ph11):f3
d18
(p1 ph11)
d4
(p6 ph1):f3
(p2 ph1)
d4
(p5 ph1):f3
d18
(p1 ph8)
d19
(p2 ph1)
4u
GRADIENT(cnst24)
d16 pl13:f3
4u BLKGRAD
go=2 ph31 cpd3:f3
30m do:f3 do:f2 wr #0 if #0 zd
3m ip8
3m ip8
lo to 3 times 2
3m ip5
3m ip6
lo to 7 times 2
3m rp5
lo to 8 times 2
d11 id0
d11
lo to 4 times l3
exit
```

```
ph0=0
ph1=0
ph2=1 3
ph3=(360)45
ph4=0
ph5=0
ph6=0
ph8=0
ph9=2
ph11=1
ph31=0 2
```

S3E_2D.c

C Programm zum Selektieren der FIDs in die zwei Unterspektren.
Geschrieben von Teresa Carlomagno

```
#include<stdio.h>
```

```
main()
{
FILE *fin1,*fout1,*fout2;
int v1[8192],v2[8192],v3[8192],v4[8192],vay[8192],vby[8192];
int v5[8192],v6[8192],v7[8192],v8[8192],vax[8192],vbx[8192];
```

```
int valfax[8192],vbetax[8192],valfay[8192],vbetay[8192];
int td1,td2,i,j,k;
char s1[80],s2[80],s3[80];
float scal_fac;

printf("path of input ser?");
scanf("%s",s1);
fin1=fopen(s1,"r");
printf("path of 1. output ser?");
scanf("%s",s2);
fout1=fopen(s2,"w");
printf("path of 2. output ser?");
scanf("%s",s3);
fout2=fopen(s3,"w");

printf("TD2? ");
scanf("%d",&td2);
printf("TD1? ");
scanf("%d",&td1);

td1=td1/2.0;

for(k=0;k<td1;k++){
fread(v1,4,td2,fin1);
fread(v2,4,td2,fin1);
fread(v3,4,td2,fin1);
fread(v4,4,td2,fin1);
fread(v5,4,td2,fin1);
fread(v6,4,td2,fin1);
fread(v7,4,td2,fin1);
fread(v8,4,td2,fin1);
for(j=0;j<td2;j++){
vax[j]=v1[j]-v3[j];
vay[j]=v2[j]-v4[j];
vbx[j]=v5[j]+v7[j];
vby[j]=v6[j]+v8[j];
valfax[j]=vax[j]+vbx[j];
valfay[j]=vay[j]+vby[j];
vbetax[j]=vax[j]-vbx[j];
vbetay[j]=vay[j]-vby[j];
}
fwrite(valfax,4,td2,fout1);
fwrite(valfay,4,td2,fout1);
fwrite(vbetax,4,td2,fout2);
fwrite(vbetay,4,td2,fout2);
}
fclose(fin1);fclose(fout1);fclose(fout2);
}
```

MOCCA Sequenz

verstärkter dipolarer Transfer; Spinlock longitudinal, daher bevorzugte Relaxationseigenschaften; MOCCA-Sequenz relaxiert nur mit T_1 und nicht mit einer Mittelung aus T_1 und T_2 wie DIPSI.

```
;wpbexy16z
;avance-version
;phase sensitive using States-TPPI
```

;water suppression using 3-9-19 pulse sequence with gradients
;allowing for presaturation during relaxation delay in cases
;of radiation damping

#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>

"d0=3u"
"d11=30m"
"d12=20u"
"d13=4u"

"p2=p1*2"

"DELTA=d0"

"l3=(td1/2)"

1 ze
2 d11
6m
3 d11
4 d12 pl9:f1
d1 cw:f1 ph29
d13 do:f1
d12 pl1:f1
p1 ph1
DELTA
p2 ph6
d0
p1 ph2
d20 pl10:f1
20u fq1:f1
5 43u
p6 ph20
86u
p6 ph21
86u
p6 ph20
86u
p6 ph21
86u
p6 ph21
86u
p6 ph20
86u
p6 ph21
86u
p6 ph20
86u
p6 ph23
86u
p6 ph24
86u
p6 ph23
86u
p6 ph24
86u

p6 ph24
86u
p6 ph23
86u
p6 ph24
86u
p6 ph23
43u

lo to 5 times l1

20u fq1:f1
d21 p11:f1
p1 ph3
50u p118:f1 UNBLKGRAD
p16:gp1
d16
p28*0.231 ph4
d19*2
p28*0.692 ph4
d19*2
p28*1.462 ph4
d19*2
p28*1.462 ph5
d19*2
p28*0.692 ph5
d19*2
p0*0.231 ph5
46u
p16:gp2
d16
4u BLKGRAD
go=2 ph31
d11 wr #0 if #0 ip1 zd
3m ip6
3m ip29
lo to 3 times 2
d11 id0
lo to 4 times l3
exit

ph1=0 2
ph2=0 0 0 0 2 2 2 2
ph3=0 0 2 2
ph4=0
ph5=2
ph6=1 3
ph20=0
ph21=1
ph23=2
ph24=3
ph29=0
ph31=0 2 2 0 2 0 0 2

Pulssequenz für das J_{HH} -NOESY

;wpjhhnoe

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
"p2=p1*2"
"p6=p5*2"
```

```
"d0=3u"
"d11=30m"
"d13=3u"
```

```
"d24=2.7m"
"d14=d24+p6"
"d25=d24-p16-d16"
```

```
"d8=200m"          (Noesy Mischzeit)
```

```
"d18=d8/2"
"d19=d8/2-p16-d16*2-50u"
```

```
"CEN_HN2=(p6-p2)/2"
"CEN_HN1=(p5-p1)/2"
```

```
"l3=(td1/2)"
```

```
1 ze
  d11 pl13:f3
2 d11
  d13 do:f3
  3m pl1:f1
3 d11
  3m
4 d12 pl3:f3
  d12 pl9:f1
  d1 cw:f1
  3u do:f1
  d12 pl1:f1
  (p1 ph1):f1
  d24
  (p6 ph6):f3
  (p2 ph1)
  d14
  (p28 ph1):f1
4u
  (p1 ph4)
50u UNBLKGRAD
GRADIENT(cnst21)
d16
(p5 ph3):f3
d0
(CEN_HN1 p1 ph1) (p5 ph4):f3
d25
GRADIENT(cnst22)
d16
(CEN_HN2 p2 ph1) (p6 ph6):f3
GRADIENT(cnst22)
d16
```

```
d25
(CEN_HN1 p1 ph4) (p5 ph1):f3
d25
GRADIENT(cnst23)
d16
(CEN_HN2 p2 ph1) (p6 ph6):f3
GRADIENT(cnst23)
d16
d25
(CEN_HN1 p1 ph4) (p5 ph4):f3
3u pl9:f1
d18 cw:f1
(p6 ph6):f3
d19 cw:f1
GRADIENT(cnst24)
d16 do:f1
d16 pl1:f1
(CEN_HN1 p1 ph4) (p5 ph1):f3
d25
GRADIENT(cnst25)
d16
(CEN_HN2 p2 ph1) (p6 ph6):f3
GRADIENT(cnst25)
d16
d25
(CEN_HN1 p1 ph4) (p5 ph4):f3
d25
GRADIENT(cnst26)
d16
(CEN_HN2 p2 ph1) (p6 ph6):f3
GRADIENT(cnst26)
d16 pl13:f3
d25 BLKGRAD
(p1 ph1):f1
go=2 ph31 cpd3:f3
d1 do:f3 wr #0 if #0 zd
3m ip3
lo to 3 times 2
d11 id0
lo to 4 times l3
exit
```

```
ph1=0
ph3=0 2
ph4=1
ph6=0
ph31=0 2
```

z-filter adiabatisches TOCSY

2 Versionen, implementiert in XWINNMR 3.1, kommerziell erhältlich November 2001

```
;wphcchatgp3d
;avance-version (00/12/01)
;HCCH-TOCSY
;3D sequence with
; inverse correlation using multiple inept transfer and
; C-C P9M16 spinlock sequence for adiabatic pulses
```

```
;  
; F1(H,t1) -> F2(C,t2) -> F2(C') -> F1(H',t3)  
;  
;off resonance C=O pulse using shaped pulse  
;phase sensitive using States-TPPI  
;L.E. Kay, G.Y. Xu, A.U. Singer, D.R. Muhandiram & J. D. Forman-Kay  
; J. Magn. Reson. B 101, 333 - 337 (1993)  
;W.Peti, C. Griesinger & W. Bermel, J. Biomol. NMR 18, 199 - 205 (2000)
```

```
#include <Avance.incl>  
#include <Grad.incl>  
#include <Delay.incl>
```

```
;;"d0=3u"  
;;"d10=3u"  
;;"d11=30m"  
;;"d12=20u"
```

```
"d4=1.6m"           ;tau a  
"d21=1.1m"          ;tau c  
"d23=475u"          ;tau b
```

```
"DELTA1=d4-p16-d16-4u"  
"DELTA2=d4-p16-d16-4u+d0*2+p4"  
"DELTA3=d23-p29-d16"  
"DELTA4=p22+p2+d10*2+4u"  
"DELTA5=d21-p16-d16-4u"  
"DELTA6=d4-p16-d16-p3*2-7u+p1"
```

```
"TAU=(p3*2+3u)-p1"
```

```
"CEN_HC1=(p3-p1)/2"  
"CEN_HC2=(p4-p2)/2"
```

```
"l3=(td1/2)"  
"l13=(td2/2)"
```

```
aqseq 312
```

```
1 d11 ze  
  d11 pl12:f2  
2 d11 do:f2  
  d11  
3 d11  
4 d11
```

5 d11

6 d1

50u UNBLKGRAD

d12 pl1:f1

(p1 ph3)

4u

p16:gp1

d16

DELTA1 pl2:f2

d0

(p4 ph1):f2

d0

(p2 ph1)

4u

p16:gp1

d16

DELTA2 pl3:f3

(p1 ph2)

p19:gp3

d16

(p3 ph4):f2

d10

(p22 ph1):f3

4u

p29:gp2

d16

DELTA3 pl0:f2

(p14:sp5 ph1):f2

4u

p29:gp2

d16

DELTA3 pl2:f2

p2 ph1

d10

(p4 ph1):f2

DELTA4

p29:gp2

d16

DELTA3 pl0:f2

(p14:sp5 ph1):f2

4u

p29:gp2

d16

DELTA3 pl2:f2

(p3 ph2):f2

4u

d12 pl0:f2

9 (p10:sp0 ph20):f2

3u

(p10:sp0 ph21):f2

3u

(p10:sp0 ph24):f2

3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph24):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph24):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph20):f2

3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph24):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph24):f2
3u
(p10:sp0 ph25):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph24):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph24):f2
3u
(p10:sp0 ph25):f2

3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph24):f2
3u

(p10:sp0 ph25):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

(p10:sp0 ph20):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
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(p10:sp0 ph23):f2
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(p10:sp0 ph24):f2
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(p10:sp0 ph21):f2
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(p10:sp0 ph20):f2
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(p10:sp0 ph20):f2
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(p10:sp0 ph21):f2
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(p10:sp0 ph23):f2
3u
(p10:sp0 ph26):f2
3u
(p10:sp0 ph23):f2
3u
(p10:sp0 ph24):f2
3u
(p10:sp0 ph21):f2
3u
(p10:sp0 ph20):f2
3u

(p10:sp0 ph24):f2
3u

(p10:sp0 ph25):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph22):f2
3u
(p10:sp0 ph27):f2
3u
(p10:sp0 ph20):f2
3u
(p10:sp0 ph25):f2
3u
(p10:sp0 ph24):f2
3u

lo to 9 times ll

d12
(p17 ph1)
(p17*2 ph2)
4u
p30:gp4
d16
(p1 ph1)
4u
p31:gp4
d16 pl2:f2

(p3 ph2):f2
4u
p16:gp1
d16
DELTA5
(CEN_HC2 p2 ph1) (p4 ph1):f2
4u
p16:gp1
d16
DELTA5
(CEN_HC1 p1 ph1) (p3 ph1):f2

4u
p16:gp1
d16
DELTA1
(CEN_HC2 p2 ph1) (p4 ph1):f2
4u
p16:gp1
d16
DELTA6
(TAU p1 ph1) (p3 ph1 3u p3 ph5):f2
4u pl12:f2
4u BLKGRAD
go=2 ph31 cpd2:f2
d11 do:f2 wr #0 if #0 zd

d11 ip3
lo to 3 times 2
d11 id0
lo to 4 times l3
d11 rd0 ip4
lo to 5 times 2
d11 id10
lo to 6 times l13

exit

ph1=0
ph2=1
ph3=0 2
ph4=0 0 2 2
ph5=0 0 0 2 2 2 2

ph20=(360) 0
ph21=(360) 15
ph22=(360) 90
ph23=(360) 165
ph24=(360) 180
ph25=(360) 195
ph26=(360) 270
ph27=(360) 345

ph31=0 2 2 0

;p10 : 120dB
;p11 : f1 channel - power level for pulse (default)
;p12 : f2 channel - power level for pulse (default)
;p13 : f3 channel - power level for pulse (default)
;p112: f2 channel - power level for CPD/BB decoupling
;sp0: f2 channel - shaped pulse 180 degree (adiabatic spinlock)
;sp5: f2 channel - shaped pulse 180 degree (C=O off resonance)
;p1 : f1 channel - 90 degree high power pulse
;p2 : f1 channel - 180 degree high power pulse
;p3 : f2 channel - 90 degree high power pulse
;p4 : f2 channel - 180 degree high power pulse
;p10: f2 channel - 180 degree shaped pulse for spinlock (adiabatic)
;p14: f2 channel - 180 degree shaped pulse
;p16: homospoil/gradient pulse [500 usec]
;p17: f1 channel - trim pulse [1 msec]
;p19: gradient pulse 2 [2 msec]
;p22: f3 channel - 180 degree high power pulse
;p27: f2 channel - trim pulse [2 msec]
;p29: gradient pulse 3 [300 usec]
;p30: gradient pulse 4 [5 msec]
;p31: gradient pulse 5 [4.4 msec]
;d0 : incremented delay (F1 in 3D) [3 usec]

```

;d4 : 1/(4J(CH)) - tau a          [1.6 msec]
;d10: incremented delay (F2 in 3D) [3 usec]
;d11: delay for disk I/O          [30 msec]
;d12: delay for power switching   [20 usec]
;d16: delay for homospoil/gradient recovery
;d21: 1/(6J(CH)) - tau c         [1.1 msec]
;d23: tau b                       [475 usec]
;l1: loop for P9M16 cycle:
;   mixing time = ((p9*9*16) * l1) [14 msec]
;l3: loop for phase sensitive 3D using States-TPPI : l3 = td1/2
;l13: loop for phase sensitive 3D using States-TPPI : l13 = td2/2
;in0: 1/(2 * SW(H)) = DW(H)
;nd0: 2
;in10: 1/(2 * SW(C)) = DW(C)
;nd10: 2
;NS: 16 * n
;DS: 32
;td1: number of experiments in F1
;td2: number of experiments in F2
;MC2: States-TPPI in F1
;MC2: States-TPPI in F2
;cpd2: decoupling according to sequence defined by cpdprg2
;pcpd2: f2 channel - 90 degree pulse for decoupling sequence

;use gradient ratio:  gp 1 : gp 2 : gp 3 : gp 4
;                    16 : 16 : 30 : 60

;for z-only gradients:
;gpz1: 16%
;gpz2: 16%
;gpz3: 30%
;gpz4: 60%

;use gradient files:
;gpnam1: SINE.100
;gpnam2: SINE.50
;gpnam3: SINE.100
;gpnam4: SINE.100

;wpatocsygpst19
;avance-version (00/12/01)
;homonuclear Hartman-Hahn transfer using X_M16 sequence
; for mixing
;MLEV16 cycle in pulseprogram, further cycling to be
; encoded in shape
;phase sensitive using States-TPPI
;water suppression using 3-9-19 pulse sequence with gradients
;allowing for presaturation during relaxation delay in cases
; of radiation damping
;M. Piotto, V. Saudek & V. Sklenar, J. Biomol. NMR 2, 661 - 666 (1992)
;V. Sklenar, M. Piotto, R. Leppik & V. Saudek, J. Magn. Reson.,
; Series A 102, 241 -245 (1993)
;E. Kupce, P. Schmidt, M. Rnace & G. Wagner, J. Magn. Reson. 135,
; 361-367 (1998)

```


;W.Peti, C. Griesinger & W. Bermel, J. Biomol. NMR 18, 199 - 205 (2000)

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
;;"d0=3u"
;;"d11=30m"
;;"d12=20u"
;;"d13=4u"
```

```
"t1=d9/(p29*16)+0.5"
```

```
"DELTA=d0"
```

```
"t3=(td1/2)"
```

```
1 ze
2 d11
  6m
3 d11
4 d12 p19:f1
  d1 cw:f1 ph29
  d13 do:f1
  d12 p11:f1
  p1 ph1
  DELTA
  p2 ph6
  d0
  p1 ph2
  d20 p10:f1
```

```
5 (p29:sp0 ph20):f1
  3u
  (p29:sp0 ph20):f1
  3u
  (p29:sp0 ph22):f1
  3u
  (p29:sp0 ph22):f1
  3u
```

```
(p29:sp0 ph20):f1
  3u
  (p29:sp0 ph22):f1
  3u
  (p29:sp0 ph22):f1
```

3u
(p29:sp0 ph20):f1
3u

(p29:sp0 ph22):f1
3u
(p29:sp0 ph22):f1
3u
(p29:sp0 ph20):f1
3u
(p29:sp0 ph20):f1
3u

(p29:sp0 ph22):f1
3u
(p29:sp0 ph20):f1
3u
(p29:sp0 ph20):f1
3u
(p29:sp0 ph22):f1
3u

lo to 5 times l1

d21 pl1:f1
p1 ph3
50u pl18:f1 UNBLKGRAD
p16:gp1
d16
p28*0.231 ph4
d19*2
p28*0.692 ph4
d19*2
p28*1.462 ph4
d19*2
p28*1.462 ph5
d19*2
p28*0.692 ph5
d19*2
p0*0.231 ph5
46u
p16:gp2
d16
4u BLKGRAD
go=2 ph31
d11 wr #0 if #0 ip1 zd
3m ip6
3m ip29
lo to 3 times 2
d11 id0
lo to 4 times l3
exit

ph1=0 2

```
ph2=0 0 0 0 2 2 2 2
ph3=0 0 2 2
ph4=0
ph5=2
ph6=1 3
```

```
ph20=0
ph22=2
```

```
ph29=0
ph31=0 2 2 0 2 0 0 2
```

```
;p11 : f1 channel - power level for pulse (default)
;p19 : f1 channel - power level for presaturation
;p118: f1 channel - power level for 3-9-19-pulse (watergate)
;sp0 : f1 channel - shaped pulse 180 degree (adiabatic spinlock)
;p0 : f1 channel - 90 degree pulse at p118
;
;      use for fine adjustment
;p1 : f1 channel - 90 degree high power pulse
;p16: homospoil/gradient pulse
;p28: f1 channel - 90 degree pulse at p118
;p29: f1 channel - 180 degree shaped pulse for spinlock (adiabatic)
;d0 : incremented delay (2D)           [3 usec]
;d1 : relaxation delay; 1-5 * T1
;d9 : TOCSY mixing time
;d11: delay for disk I/O                [30 msec]
;d12: delay for power switching         [20 usec]
;d13: short delay                       [4 usec]
;d16: delay for homospoil/gradient recovery
;d19: delay for binomial water suppression
;      d19 = (1/(2*d)), d = distance of next null (in Hz)
;d20: first z-filter delay              [10 usec]
;d21: second z-filter delay             [10 usec]
;l1: loop for MLEV16 cycle: ((p12*16) * l1) = mixing time
;l3: loop for phase sensitive 2D using States-TPPI method: l3 = td1/2
;in0: 1/(1 * SW) = 2 * DW
;nd0: 1
;NS: 8 * n
;DS: 16
;td1: number of experiments
;MC2: States-TPPI
```

```
;use gradient ratio:  gp 1 : gp 2
;                      30 : 30
```

```
;for z-only gradients:
;gpz1: 30%
;gpz2: 30%
```

```
;use gradient files:
;gpnam1: SINE.100
;gpnam2: SINE.100
```

;set p19 to 120dB when presaturation is not required
; use 75 - 80dB to reduce radiation damping

SPITZE-HSQC

:wpspitzehsqc-1

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
"p2=p1*2"
"p4=p3*2"
```

```
"d0=4u"
```

```
"d4=1.64m"
"d8=0.82m"
"d14=d4-p16-d16-50u"
"d25=d16+p19+8u" ;+p14"
"d7=d8-p19-d16-54u"
```

```
"d11=30m"
"d12=20u"
"d13=3u"
```

```
"d31=(p4-p2)/2"
"d30=(p3-p1)/2"
"l3=(td1/2)"
```

```
1 ze
  d11 pl12:f2
2 d11 do:f2
  6m
3 d11
5 d12 pl9:f1
  d1 cw:f1
  4u do:f1
  d12 pl1:f1
  d12 pl2:f2
  d12
  (p1 ph1)
  50u UNBLKGRAD
  GRADIENT(cnst20)
  d16
  d14
  (d31 p2 ph1):f1 (p4 ph0):f2
  50u
  GRADIENT(cnst20)
  d16
  d14
  (p9 ph1)
  (p1 ph2)
```

```

50u
GRADIENT(cnst21)
d16
(p3 ph14):f2
d0 pl20:f2
d0 pl2:f2
GRADIENT2(cnst22)
d16
(p4 ph0):f2
d25
(d30 p1 ph12):f1 (p3 ph11):f2
4u pl7:f1
4u pl8:f2

15 (p8*3.556 ph9):f1 (p8*3.556 ph9):f2
(p8*4.556 ph8):f1 (p8*4.556 ph8):f2
(p8*3.222 ph9):f1 (p8*3.222 ph9):f2
(p8*3.167 ph8):f1 (p8*3.167 ph8):f2
(p8*0.333 ph9):f1 (p8*0.333 ph9):f2
(p8*2.722 ph8):f1 (p8*2.722 ph8):f2
(p8*4.167 ph9):f1 (p8*4.167 ph9):f2
(p8*2.944 ph8):f1 (p8*2.944 ph8):f2
(p8*4.111 ph9):f1 (p8*4.111 ph9):f2

(p8*3.556 ph8):f1 (p8*3.556 ph8):f2
(p8*4.556 ph9):f1 (p8*4.556 ph9):f2
(p8*3.222 ph8):f1 (p8*3.222 ph8):f2
(p8*3.167 ph9):f1 (p8*3.167 ph9):f2
(p8*0.333 ph8):f1 (p8*0.333 ph8):f2
(p8*2.722 ph9):f1 (p8*2.722 ph9):f2
(p8*4.167 ph8):f1 (p8*4.167 ph8):f2
(p8*2.944 ph9):f1 (p8*2.944 ph9):f2
(p8*4.111 ph8):f1 (p8*4.111 ph8):f2

lo to 15 times l0          ; l0=20

4u pl1:f1
4u pl2:f2
(d30 p1 ph15):f1 (p3 ph13):f2
d8
(d31 p2 ph0):f1 (p4 ph0):f2
50u
GRADIENT2(cnst23)
d16
4u BLKGRAD
d7
(p1 ph21):f1
(p1 ph22):f1
(d30 p1 ph1):f1 (p3 ph1):f2
(d30 p1 ph20):f1 (p3 ph20):f2
go=2 ph31
d11 do:f2 wr #0 if #0 zd
3m ip11
3m ip11
lo to 3 times 2
d11 id0
lo to 5 times l3
exit

```

ph0=0
ph1=0
ph2=1 1 3 3
ph8=3
ph9=1
ph12=0
ph11=2
ph13=0
ph14=1 3
ph15=2
ph21=(360)45
ph22=(360)225
ph20=2
ph31=0 2 2 0

;wpspitzehsqc-2

#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>

"p2=p1*2"
"p4=p3*2"

"d0=4u"

"d4=1.64m"
"d8=0.82m"
"d14=d4-p16-d16-50u"
"d25=d16+p19+8u";+p14"
"d7=d8-p19-d16-54u"

"d11=30m"
"d12=20u"
"d13=3u"

"d31=(p4-p2)/2"
"d30=(p3-p1)/2"
"l3=(td1/2)"

1 ze
d11 pl12:f2
2 d11 do:f2
6m
3 d11
5 d12 pl9:f1
d1 cw:f1
4u do:f1
d12 pl1:f1
d12 pl2:f2
d12 ;pl3:f3
(p1 ph1)
50u UNBLKGRAD
GRADIENT(cnst20)
d16

d14
(d31 p2 ph1):f1 (p4 ph0):f2
50u
GRADIENT(cnst20)
d16
d14
(p9 ph1):f1
(p1 ph2)
50u
GRADIENT(cnst21)
d16
(p3 ph14):f2
d0 ;pl20:f2
d0 pl2:f2
GRADIENT2(cnst22)
d16
(p4 ph0):f2
d25
(d30 p1 ph12):f1 (p3 ph11):f2
4u pl7:f1
4u pl8:f2

15 (p8*3.556 ph9):f1 (p8*3.556 ph9):f2
(p8*4.556 ph8):f1 (p8*4.556 ph8):f2
(p8*3.222 ph9):f1 (p8*3.222 ph9):f2
(p8*3.167 ph8):f1 (p8*3.167 ph8):f2
(p8*0.333 ph9):f1 (p8*0.333 ph9):f2
(p8*2.722 ph8):f1 (p8*2.722 ph8):f2
(p8*4.167 ph9):f1 (p8*4.167 ph9):f2
(p8*2.944 ph8):f1 (p8*2.944 ph8):f2
(p8*4.111 ph9):f1 (p8*4.111 ph9):f2

(p8*3.556 ph8):f1 (p8*3.556 ph8):f2
(p8*4.556 ph9):f1 (p8*4.556 ph9):f2
(p8*3.222 ph8):f1 (p8*3.222 ph8):f2
(p8*3.167 ph9):f1 (p8*3.167 ph9):f2
(p8*0.333 ph8):f1 (p8*0.333 ph8):f2
(p8*2.722 ph9):f1 (p8*2.722 ph9):f2
(p8*4.167 ph8):f1 (p8*4.167 ph8):f2
(p8*2.944 ph9):f1 (p8*2.944 ph9):f2
(p8*4.111 ph8):f1 (p8*4.111 ph8):f2

lo to 15 times lo ; lo=20

4u pl1:f1
4u pl2:f2
(d30 p1 ph15):f1 (p3 ph13):f2
d8
(d31 p2 ph0):f1 (p4 ph0):f2
50u
GRADIENT2(cnst22)
d16
4u BLKGRAD
d7
(p1 ph21):f1
(p1 ph22):f1
(d30 p1 ph1):f1 (p3 ph1):f2
(d30 p1 ph20):f1 (p3 ph20):f2
go=2 ph31
d11 do:f2 wr #0 if #0 zd

```
3m ip11
3m ip11
lo to 3 times 2
d11 id0
lo to 5 times l3
exit
```

```
ph0=0
ph1=0
ph2=1 1 3 3
ph8=3
ph9=1
ph12=0
ph11=0
ph13=2
ph14=1 3
ph15=2
ph21=(360)45
ph22=(360)45
ph20=0
ph31=0 2 2 0
```

:wpspitzehsqc-3

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
"p2=p1*2"
"p4=p3*2"
```

```
"d0=4u"
```

```
"d4=1.64m"
"d8=0.82m"
"d14=d4-p16-d16-50u"
"d25=d16+p19+8u";+p14"
"d7=d8-p19-d16-54u"
```

```
"d11=30m"
"d12=20u"
"d13=3u"
```

```
"d31=(p4-p2)/2"
"d30=(p3-p1)/2"
"l3=(td1/2)"
```

```
1 ze
  d11 pl12:f2
2 d11 do:f2
  6m
3 d11
5 d12 pl9:f1
  d1 cw:f1
```


4u do:f1
 d12 pl1:f1
 d12 pl2:f2
 d12 ;pl3:f3
 (p1 ph1)
 50u UNBLKGRAD
 GRADIENT(cnst20)
 d16
 d14
 (d31 p2 ph1):f1 (p4 ph0):f2
 50u
 GRADIENT(cnst20)
 d16
 d14
 (p9 ph1):f1
 (p1 ph2)
 50u
 GRADIENT(cnst21)
 d16
 (p3 ph14):f2
 d0
 d0 pl2:f2
 GRADIENT2(cnst22)
 d16
 (p4 ph0):f2
 d25
 (d30 p1 ph12):f1 (p3 ph11):f2
 4u pl7:f1
 4u pl8:f2

15 (p8*3.556 ph9):f1 (p8*3.556 ph9):f2
 (p8*4.556 ph8):f1 (p8*4.556 ph8):f2
 (p8*3.222 ph9):f1 (p8*3.222 ph9):f2
 (p8*3.167 ph8):f1 (p8*3.167 ph8):f2
 (p8*0.333 ph9):f1 (p8*0.333 ph9):f2
 (p8*2.722 ph8):f1 (p8*2.722 ph8):f2
 (p8*4.167 ph9):f1 (p8*4.167 ph9):f2
 (p8*2.944 ph8):f1 (p8*2.944 ph8):f2
 (p8*4.111 ph9):f1 (p8*4.111 ph9):f2

(p8*3.556 ph8):f1 (p8*3.556 ph8):f2
 (p8*4.556 ph9):f1 (p8*4.556 ph9):f2
 (p8*3.222 ph8):f1 (p8*3.222 ph8):f2
 (p8*3.167 ph9):f1 (p8*3.167 ph9):f2
 (p8*0.333 ph8):f1 (p8*0.333 ph8):f2
 (p8*2.722 ph9):f1 (p8*2.722 ph9):f2
 (p8*4.167 ph8):f1 (p8*4.167 ph8):f2
 (p8*2.944 ph9):f1 (p8*2.944 ph9):f2
 (p8*4.111 ph8):f1 (p8*4.111 ph8):f2

l0 to 15 times l0 ; l0=20

4u pl1:f1
 4u pl2:f2
 (d30 p1 ph15):f1 (p3 ph13):f2
 d8
 (d31 p2 ph0):f1 (p4 ph0):f2
 50u
 GRADIENT2(cnst22)
 d16

4u BLKGRAD
d7
(p1 ph21):f1
(p1 ph22):f1
(d30 p1 ph1):f1 (p3 ph1):f2
(d30 p1 ph1):f1 (p3 ph1):f2
go=2 ph31
d11 do:f2 wr #0 if #0 zd
3m ip11
3m ip11
lo to 3 times 2
d11 id0
lo to 5 times l3
exit

ph0=0
ph1=0
ph2=1 1 3 3
ph8=3
ph9=1
ph12=0
ph11=0
ph13=2
ph14=1 3
ph15=2
ph21=(360)45
ph22=(360)225
ph31=0 2 2 0

:wpspitzehsqc-4

#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>

"p2=p1*2"
"p4=p3*2"

"d0=4u"

"d4=1.64m"
"d8=0.82m"
"d14=d4-p16-d16-50u"
"d25=d16+p19+8u";+p14"
"d7=d8-p19-d16-54u"

"d11=30m"
"d12=20u"
"d13=3u"

"d31=(p4-p2)/2"
"d30=(p3-p1)/2"
"l3=(td1/2)"

l ze
d11 pl12:f2

2 d11 do:f2
 6m
 3 d11
 5 d12 pl9:f1
 d1 cw:f1
 4u do:f1
 d12 pl1:f1
 d12 pl2:f2
 d12 ;pl3:f3
 (p1 ph1)
 50u UNBLKGRAD
 GRADIENT(cnst20)
 d16
 d14
 (d31 p2 ph1):f1 (p4 ph0):f2
 50u
 GRADIENT(cnst20)
 d16
 d14
 (p9 ph1):f1
 (p1 ph2)
 50u
 GRADIENT(cnst21)
 d16
 (p3 ph14):f2
 d0; pl20:f2
 d0 pl2:f2
 GRADIENT2(cnst22)
 d16
 (p4 ph0):f2
 d25
 (d30 p1 ph12):f1 (p3 ph11):f2
 4u pl7:f1
 4u pl8:f2

 15 (p8*3.556 ph9):f1 (p8*3.556 ph9):f2
 (p8*4.556 ph8):f1 (p8*4.556 ph8):f2
 (p8*3.222 ph9):f1 (p8*3.222 ph9):f2
 (p8*3.167 ph8):f1 (p8*3.167 ph8):f2
 (p8*0.333 ph9):f1 (p8*0.333 ph9):f2
 (p8*2.722 ph8):f1 (p8*2.722 ph8):f2
 (p8*4.167 ph9):f1 (p8*4.167 ph9):f2
 (p8*2.944 ph8):f1 (p8*2.944 ph8):f2
 (p8*4.111 ph9):f1 (p8*4.111 ph9):f2

 (p8*3.556 ph8):f1 (p8*3.556 ph8):f2
 (p8*4.556 ph9):f1 (p8*4.556 ph9):f2
 (p8*3.222 ph8):f1 (p8*3.222 ph8):f2
 (p8*3.167 ph9):f1 (p8*3.167 ph9):f2
 (p8*0.333 ph8):f1 (p8*0.333 ph8):f2
 (p8*2.722 ph9):f1 (p8*2.722 ph9):f2
 (p8*4.167 ph8):f1 (p8*4.167 ph8):f2
 (p8*2.944 ph9):f1 (p8*2.944 ph9):f2
 (p8*4.111 ph8):f1 (p8*4.111 ph8):f2

 lo to 15 times lo ; lo=20

 4u pl1:f1
 4u pl2:f2
 (d30 p1 ph15):f1 (p3 ph13):f2

d8
(d31 p2 ph0):f1 (p4 ph0):f2
50u
GRADIENT2(cnst23)
d16
4u BLKGRAD
d7
(p1 ph21):f1
(p1 ph22):f1
(d30 p1 ph1):f1 (p3 ph1):f2
(d30 p1 ph20):f1 (p3 ph20):f2
go=2 ph31
d11 do:f2 wr #0 if #0 zd
3m ip11
3m ip11
lo to 3 times 2
d11 id0
lo to 5 times l3
exit

ph0=0
ph1=0
ph2=1 1 3 3
ph8=3
ph9=1
ph12=0
ph11=2
ph13=0
ph14=1 3
ph15=2
ph21=(360)45
ph22=(360)45
ph20=2
ph31=0 2 2 0

8.3 BMRB Zuordnungslisten

Tabelle 1 : DcuS resonance assignments; pH 6.5, 303 ° K (proton chemical shifts are given in parentheses).

residue	N	C	C ^α	C ^β	other
S12			57.184 (4.477)	62.219 (3.907,3.907)	
G13	109.987 (8.315)				
L14			55.966	40.613	C ^γ , 23.484(*)
V15	113.796 (8.587)				
P16			61.529 (4.327)	30.305 (2.218,1.831)	C ^γ , 25.839(*, *); C ^δ , 49.389(*, *)
R17	121.960 (8.385)		54.829	29.087	C ^γ , 25.869(*, *); C ^δ , 41.806(*, *)
G18	109.856 (8.433)		43.343		
H20	121.540 (7.970)		57.554	30.432	
S22		174.864	57.103 (4.400)	62.219 (3.973,3.973)	
D23	122.290 (8.392)	175.317	54.017 (4.492)	39.238 (2.668,2.668)	
M24	120.295 (8.303)	175.928	54.992 (4.449)	30.792 (2.561,2.057)	C ^γ , * (2.521,2.521); C ^ε , * (1.139)
T25	113.948 (8.019)	173.959	61.569 (4.377)	68.310 (3.941)	C ^γ 2, 25.809 (1.235)
R26	122.778 (8.497)	175.770	57.266 (3.902)	28.519 (1.842,1.842)	C ^γ , 25.758 (1.537,1.551); C ^δ , 41.836 (3.134,3.164)
D27	118.564 (8.392)	176.619	54.586 (4.422)	39.319 (2.638,2.638)	
G28	108.391 (8.154)	175.430	45.328 (3.827,3.966)		
L29	123.561 (8.210)	177.637	56.210 (4.041)	40.375 (1.746,1.746)	C ^γ , 24.377 (1.317); C ^δ 1, 21.616 (0.827); C ^δ 2, 21.907 (0.806)
A30	121.543 (8.568)	177.751	53.774 (3.966)	16.744 (1.434)	
N31	115.768 (8.195)	177.015	54.667 (4.438)	36.477 (2.893,2.722)	N ^δ 2, 113.083 (7.523,6.918)
K32	122.372 (7.791)	172.346	57.854 (4.309)	30.711 (1.531,1.531)	C ^γ , 23.240 (1.410,1.410); C ^δ , 25.352 (1.601,1.629); C ^ε , 40.700 (2.351,2.353)

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A33	122.091 (8.046)	177.977	53.692 (3.891)	16.581 (1.231)	
L34	120.498 (8.890)	176.987	56.291 (3.795)	40.375 (1.349,1.842)	C ^γ , 26.000 (1.367); C ^{δ1} , 22.272 (0.636); C ^{δ2} , 22.491 (0.826)
A35	121.628 (7.713)	179.590	53.936 (4.084)	15.932 (1.488)	
V36	119.517 (7.306)	176.675	64.412 (3.537)	29.737 (2.057)	C ^{γ1} , 19.860 (0.824); C ^{γ2} , 20.591 (0.661)
A37	123.745 (8.317)	177.241	53.900 (3.698)	15.932 (1.283)	
R38	116.978 (8.957)	177.524	57.996 (3.875)	29.412 (1.731,1.731)	C ^γ , * (1.501,1.501); C ^δ , 42.730 (4.278,4.278)
T39	115.909 (7.665)	176.053	65.061 (4.277)	67.497 (3.977)	C ^{γ2} , 19.933 (1.256)
L40	123.419 (7.863)	177.100	56.291 (4.061)	40.513 (1.821,1.335)	C ^γ , 24.551 (1.680); C ^{δ1} , 22.570 (0.800); C ^{δ2} , 22.716 (0.982)
A41	119.869 (8.408)	176.138	53.043 (3.787)	17.393 (1.466)	
D42	115.912 (7.237)	174.695	51.662 (4.925)	40.293 (2.813,2.594)	
S43	116.998 (7.579)		53.900 (4.891)	61.695 (4.171,4.203)	
P44		177.128	63.437 (4.245)	30.386 (1.972,2.366)	C ^γ , 26.245 (2.125,2.125); C ^δ , 49.632(*, *)
E45	116.682 (8.785)	178.628	58.484 (3.978)	27.707 (1.885,1.885)	C ^γ , 35.502 (2.329,2.329)
I46	122.163 (7.295)	175.006	60.514 (3.765)	33.797 (2.364)	C ^{γ1} , 26.651 (1.474,1.555); C ^{γ2} , 16.424 (0.709); C ^{δ1} , 9.192 (0.771)
R47	118.207 (7.523)	177.609	59.052 (3.666)	28.600 (1.897,1.814)	C ^γ , 26.732 (1.457,1.457); C ^δ , 42.648 (3.223,3.223);
Q48	115.465 (8.380)	178.458	56.453 (4.061)	26.731 (2.058,2.058)	C ^γ , 32.653 (2.400,2.400); N ^{e2} , 112.280 (7.440,6.800)
G49	109.112 (7.825)	173.789	45.978 (3.742,4.092)		
L50	119.388 (7.314)	175.826	54.829 (3.918)	40.700 (1.620,1.313)	C ^γ , 24.702 (0.445); C ^{δ1} , 19.505 (-0.146); C ^{δ2} , 19.716 (0.608)
Q51	115.514 (7.347)	174.780	54.180 (4.363)	27.097 (2.346,2.067)	C ^γ , 32.214 (1.962,1.962)
K52	119.961 (7.638)	174.044	52.474 (4.599)	33.229 (1.917,1.917)	C ^γ , 27.625 (1.281,1.281); C ^δ , 23.565 (1.709,1.709); C ^ε , 40.781 (3.027,3.027)
K53	121.004 (8.683)		53.169 (4.507)	28.970 (1.629,1.629)	C ^δ , * (2.293,2.293); C ^ε , * (3.788,3.788)
P54		177.807	64.736 (3.420)	30.062 (1.521,1.421)	C ^γ , 24.347 (2.128,2.128); C ^δ , 47.846 (3.197,3.197)
Q55	114.329 (8.972)	175.091	57.184 (4.050)	25.920 (2.123,2.003)	C ^γ , 32.335 (2.330,2.471)
E56	117.159 (7.681)	175.515	53.611 (4.641)	29.412 (2.287,1.937)	C ^γ , 34.609 (2.776,2.776)
S57	114.883 (7.426)	173.619	59.296 (4.203)	64.087 (3.656,3.656)	
G58	111.618 (8.793)	173.619	43.911 (4.084,4.084)		
I59	121.614 (8.298)	174.864	64.087 (3.574)	35.178 (2.062)	C ^{γ1} , 21.372 (0.806,0.806); C ^{γ2} , 15.363 (0.659); C ^{δ1} , 11.465 (0.944)
Q60	123.384 (8.550)	175.770	57.672 (4.466)	25.270 (1.860,2.386)	C ^γ , 28.925 (2.495,2.523)
A61	118.764 (7.803)	179.929	53.530 (4.106)	16.663 (1.402)	
I62	119.331 (7.104)	175.940	62.706 (3.776)	37.532 (1.795)	C ^{γ1} , 26.377 (0.860,0.860); C ^{γ2} , 15.171 (0.787); C ^{δ1} , 12.329 (1.688)
A63	121.805 (9.061)	178.260	54.261 (3.698)	16.663 (1.456)	
E64	115.732 (8.803)	177.128	56.535 (4.280)	28.843 (1.904,2.233)	C ^γ , 35.827(*, *)
A65	122.282 (7.408)	179.844	53.774 (4.138)	16.419 (1.552)	
V66	119.628 (8.060)	177.185	64.655 (3.442)	29.818 (1.917)	C ^{γ1} , 20.883 (0.750); C ^{γ2} , 21.176 (1.073)
R67	124.819 (9.024)	177.241	58.890 (3.612)	28.113 (2.057,2.057)	C ^γ , 26.540 (1.411,1.411); C ^δ , 40.375 (2.975,2.975)
K68	118.555 (8.246)	178.543	57.753 (4.083)	30.874 (1.893,1.893)	C ^γ , 23.890 (1.428,1.428); C ^δ , 27.707 (1.637,1.637); C ^ε , 40.375 (2.946,2.946)
R69	118.691 (7.708)	175.345	56.860 (4.137)	28.762 (1.992,1.828)	C ^γ , 25.595 (1.707,1.707); C ^δ , 41.918 (3.165,3.165)
N70	113.046 (7.243)	171.073	51.987 (4.728)	39.725 (2.700,2.260)	N ^{δ2} , * (6.859,6.636)
D71	120.295 (7.814)	172.657	53.530 (4.345)	37.939 (3.108,2.441)	
L72	115.835 (7.604)	174.695	51.825 (4.578)	41.755 (1.424,1.424)	C ^γ , 24.458 (1.452); C ^{δ1} , 20.723 (0.788); C ^{δ2} , 20.444 (0.738)
L73	124.031 (7.978)	175.600	55.723 (4.356)	41.268 (1.368,1.368)	C ^γ , 26.813 (1.601); C ^{δ1} , 20.885 (0.858); C ^{δ2} , 20.737 (0.805)
F74	104.778 (6.953)		54.874 (4.368)	38.715 (3.627,3.675)	C ^{δ1} , * (6.793); C ^{δ2} , * (6.780); C ^{ε1} , * (7.057); C ^{ε2} , * (7.042); C ^ζ , * (8.919)
I75		174.016	61.569 (4.647)	37.857 (1.753)	C ^{γ1} , 25.433 (0.568,0.568); C ^{γ2} , 14.308 (0.591); C ^{δ1} , 12.359 (0.807)
V76	135.590 (10.342)	171.893	59.458 (4.457)	34.122 (2.164)	C ^{γ1} , 20.079 (1.447); C ^{γ2} , 19.421 (1.377)
V77	130.343 (7.683)	174.242	58.559 (5.045)	31.193 (1.795)	C ^{γ1} , 19.421 (0.873); C ^{γ2} , 19.933 (0.775)
T78	119.761 (9.711)	172.516	57.996 (6.000)	69.609 (3.986)	C ^{γ2} , 19.069 (0.884)
D79	117.955 (7.661)	178.430	51.013 (5.050)	40.050 (2.829,3.548)	
M80	114.025 (7.835)	176.166	53.043 (4.663)	26.488 (1.982,2.121)	C ^γ , 30.143 (2.686,2.686)
Q81	119.730 (8.366)	174.638	53.692 (4.520)	27.138 (2.342,2.342)	C ^γ , 33.391 (2.501,2.501)
S82	111.425 (7.777)	170.818	58.565 (4.342)	61.326 (3.955,3.762)	
L83	122.844 (8.677)	176.477	53.774 (4.138)	39.481 (2.016,2.158)	C ^γ , 23.484 (0.606); C ^{δ1} , 20.398 (0.840); C ^{δ2} , 20.152 (0.877)
R84	117.763 (8.232)	176.675	56.042 (4.584)	29.569 (2.173,2.173)	C ^γ , 27.352 (0.964,0.964); C ^δ , 41.425 (2.526,2.619)
Y85	123.169 (10.629)	172.261	57.428 (4.302)	37.045 (2.966,2.769)	C ^{δ1} , * (7.396); C ^{δ2} , * (7.389); C ^{ε1} , * (6.313); C ^{ε2} , * (5.865)
S86	110.256 (7.836)	171.328	56.778 (4.978)	65.143 (3.865,3.865)	
H87	116.984 (9.029)		56.579 (4.443)	30.188 (3.154,3.162)	
P88		176.477	63.594 (4.313)	26.321 (1.672,2.407)	C ^γ , 30.625 (1.923,1.923); C ^δ , 48.084 (3.312,3.312)
E89	121.673 (11.724)	174.214	53.286 (4.457)	26.894 (1.731,2.355)	C ^γ , 34.823 (1.739,1.739)

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A90	127.361 (8.333)	177.977	53.003 (3.887)	16.222 (1.405)	
Q91	117.744 (9.176)	174.497	55.885 (4.149)	25.108 (1.949,1.949)	C ^γ , 31.361(*, *)
R92	117.754 (8.142)	175.855	54.423 (3.912)	26.245 (1.009,1.009)	C ^γ , 34.741 (1.380,1.380); C ^δ , 39.644 (2.181,2.137)
I93	122.609 (7.332)	175.402	62.457 (3.318)	35.659 (1.706)	C ^{γl} , 28.188 (1.736,1.736); C ^{γ2} , 15.033 (0.923); C ^{δ1} , 11.704 (0.643)
G94	115.578 (10.014)	170.847	43.217 (4.337,3.252)		
Q95	118.089 (7.591)		50.814 (4.783)	26.534 (1.692,1.692)	C ^γ , * (2.247,2.247); N ^{e2} , 113.083 (7.382,6.643)
P96		175.742	62.219 (4.352)	30.386 (2.307,2.307)	C ^γ , 26.563 (1.872,1.997); C ^δ , 49.226 (3.778,3.778)
F97	120.325 (8.545)	171.497	50.363 (4.708)	40.375 (2.815,2.815)	
K98	117.153 (8.441)	171.214	53.611 (4.916)	32.010 (1.740,1.601)	C ^γ , * (1.354,1.354); C ^δ , * (1.529,1.529); C ^e , 42.063 (3.140,3.140)
G99	115.309 (8.270)	173.195	43.054 (4.271,3.576)		
D100	128.201 (8.893)	175.317	53.443 (4.475)	38.096 (2.752,2.752)	
D101	119.730 (10.380)	174.214	52.474 (3.918)	35.096 (2.684,2.365)	
I102	115.532 (6.555)	175.091	60.270 (2.861)	36.396 (0.995)	C ^{γl} , 28.732 (0.702,0.702); C ^{γ2} , 13.658 (0.715); C ^{δ1} , 10.978 (0.035)
L103	122.341 (7.183)	178.458	57.028 (3.678)	38.574 (1.750,1.630)	C ^γ , 25.927 (1.335); C ^{δ1} , 23.149 (0.917); C ^{δ2} , 20.810 (0.787)
K104	117.382 (7.698)	176.789	57.672 (3.875)	31.523 (1.401,1.401)	C ^γ , 23.484 (1.211,1.211); C ^δ , 27.219 (1.530,1.530); C ^e , 40.293 (2.917,2.917)
A105	119.997 (6.489)	179.760	51.337 (5.178)	17.881 (1.080)	
L106	121.438 (7.784)	174.355	55.154 (3.842)	39.644 (1.795,1.379)	C ^γ , 24.864 (2.620); C ^{δ1} , 21.378 (0.736); C ^{δ2} , 20.992(*, N ^{δ2} , 113.619 (7.492,6.742)
N107	115.665 (7.166)	174.242	50.850 (4.882)	38.101 (3.065,2.550)	
G108	107.333 (7.622)	172.120	44.332 (4.159,3.677)		
E109	119.461 (7.511)	173.474	53.049 (5.054)	31.264 (1.735,2.252)	C ^γ , 34.914 (2.136,2.164)
E110	116.393 (8.174)	172.348	54.657 (4.638)	30.194 (2.028,2.028)	C ^γ , 36.089 (2.300,2.310)
N111	121.815 (8.052)	171.016	50.119 (5.296)	40.537 (2.389,2.228)	N ^{δ2} , * (6.801 *)
V112	123.949 (7.493)	173.195	59.783 (5.335)	33.635 (1.959)	C ^{γl} , 19.424 (0.992); C ^{γ2} , 19.202 (0.877)
A113	131.178 (9.909)	173.450	50.363 (4.717)	22.428 (1.638)	
I114	120.147 (8.371)	174.808	58.565 (5.279)	37.045 (1.706)	C ^{γl} , 26.651 (0.642,0.642); C ^{γ2} , 16.257 (0.778); C ^{δ1} , 11.628 (0.775)
N115	125.489 (8.822)	178.490	49.921 (4.974)	39.933 (2.848,2.821)	
R116	112.042 (7.252)	172.476	53.268 (4.909)	31.995 (1.716,1.716)	C ^γ , 22.561(*, *); C ^δ , 42.668(*, *)
G117	113.917 (7.055)	175.496	43.293 (3.728,3.934)		
F118	126.313 (9.240)	175.650	56.119 (4.322)	38.867 (3.169,3.169)	
L119	126.165 (10.206)		51.337 (4.567)	40.862 (1.467,1.467)	C ^γ , 28.813 (1.863); C ^{δ1} , 24.997 (0.869); C ^{δ2} , * (0.583)
A120	118.475 (7.275)	173.931	50.282 (4.106)	18.936 (1.467)	
Q121	117.855 (8.308)	174.978	56.210 (4.021)	26.732 (1.895,1.895)	C ^γ , 32.092 (2.170,2.182); N ^{e2} , 110.942 (6.771,7.162)
A122	129.299 (8.885)	173.648	49.434 (5.120)	20.931 (1.273)	
L123	123.243 (8.565)		52.925 (4.711)	41.836 (1.822,1.822)	C ^γ , 24.621 (1.300); C ^{δ1} , 22.915 (0.847); C ^{δ2} , 22.479 (0.949)
R124	129.631 (9.663)	172.686	53.205 (5.178)	31.523 (1.761,1.761)	C ^γ , 25.595 (1.736,1.781); C ^δ , 43.867 (3.755,3.635)
V125	114.039 (8.882)	172.403	56.046 (5.529)	31.776 (1.992)	C ^{γl} , 19.129 (0.950); C ^{γ2} , 17.886 (0.554)
F126	117.555 (8.524)	174.100	53.737 (5.618)	42.856 (2.518,2.518)	C ^{δ1} , * (6.973); C ^{δ2} , * (6.949); C ^{e1} , * (6.782); C ^{e2} , * (6.774); C ^ξ , * (8.047)
T127	114.869 (8.770)		56.661 (5.265)	69.409 (3.863)	C ^{γ2} , 19.813 (0.984)
P128		172.035	61.245 (4.620)	31.929 (1.784,1.302)	C ^γ , 25.676 (1.939,1.939); C ^δ , 51.013 (3.617,3.617)
I129	118.041 (7.518)	173.421	58.646 (4.159)	38.309 (0.982)	C ^{γl} , 25.758 (0.761,0.761); C ^{γ2} , 15.444 (0.652); C ^{δ1} , 14.308 (0.687)
Y130	127.530 (9.327)	174.157	55.235 (5.135)	40.781 (2.690,2.690)	C ^{δ1} , * (7.475); C ^{δ2} , * (7.475); C ^{e1} , * (6.570); C ^{e2} , * (6.558)
D131	119.629 (8.822)	176.873	50.363 (4.539)	39.400 (2.707,3.286)	
E132	117.922 (9.442)	175.147	57.103 (4.052)	27.138 (2.003,2.003)	C ^γ , 34.284 (2.380,2.380)
N133	118.557 (8.434)	173.223	51.094 (4.867)	37.532 (2.947,2.690)	N ^{δ2} , 115.760 (7.872,6.921)
H134	116.637 (8.339)	171.639	56.697 (3.752)	25.839 (3.494,3.001)	C ^{δ2} , * (6.580)
K135	121.591 (8.557)	175.232	53.368 (4.575)	31.036 (1.834,1.845)	C ^γ , 22.997 (1.300,1.300); C ^δ , 27.382 (1.623,1.623); C ^e , 40.781 (2.987,2.987)
Q136	128.671 (9.196)	175.204	55.885 (4.243)	24.910 (1.817,1.817)	C ^γ , 33.229 (0.981,0.863); N ^{e2} , 108.534 (6.477,7.734)
I137	118.392 (8.927)	173.704	59.783 (4.510)	38.669 (2.047)	C ^{γl} , 16.581 (0.790,0.790); C ^{γ2} , * (0.939); C ^{δ1} , 13.089 (0.872)
G138	109.115 (7.377)	170.733	45.085 (4.602,3.567)		
V139	124.410 (8.653)	172.516	60.270 (4.422)	35.827 (2.117)	C ^{γl} , 21.048 (0.795); C ^{γ2} , 20.883 (0.497)
V140	124.049 (8.912)	171.130	61.163 (4.345)	31.848 (2.003)	C ^{γl} , 20.398 (0.840); C ^{γ2} , 19.586 (0.795)
A141	131.818 (9.095)	174.497	47.440 (5.245)	21.697 (1.216)	
I142	123.134 (8.897)		57.716 (4.392)	38.471 (1.362)	C ^{γl} , * (0.909,0.909); C ^{γ2} , * (0.702); C ^{δ1} , 11.937 (0.619)
G143		169.828	42.562 (4.967,2.195)		
L144	123.780 (8.994)	173.931	51.825 (4.647)	42.892 (2.765,3.108)	C ^γ , 25.728 (1.856); C ^{δ1} , 24.215 (0.824); C ^{δ2} , 24.864 (0.989)
E145	126.183 (9.348)	175.883	56.697 (3.943)	28.843 (2.063,2.083)	C ^γ , 34.690 (3.173,3.173)
L146	130.308 (8.606)	177.185	54.312 (4.020)	40.095 (1.664,1.692)	C ^γ , * (1.273); C ^{δ1} , * (0.861)

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S147	114.285 (8.698)	174.214	58.808 (3.891)	60.839 (4.209,4.209)	
R148	120.568 (7.470)	176.025	55.723 (4.334)	29.006 (2.036,1.882)	C^{γ} , 25.971 (1.675,1.675); C^{δ} , 41.836 (3.174,3.174); N^{ϵ} , * (6.773)
V149	118.055 (7.736)	175.119	61.975 (3.988)	30.305 (2.282)	C^{γ^1} , 19.830 (0.945); C^{γ^2} , 19.640 (0.911)
T150	114.538 (8.087)	173.421	62.300 (4.170)	67.741 (4.355)	C^{γ^2} , 19.748 (1.207)
Q151	121.799 (7.984)	174.666	55.154 (4.234)	27.382 (2.078,2.078)	C^{γ} , 32.254 (2.398,2.398)
Q152	120.837 (8.206)	175.034	54.829 (4.267)	27.625 (2.024,2.024)	C^{γ} , 32.254 (2.349,2.387)
I153	121.137 (8.113)	175.345	60.229 (4.052)	36.883 (1.821)	C^{γ^1} , 25.595 (1.467,1.467); C^{γ^2} , 15.850 (1.158); C^{δ^1} , 11.628 (0.858)
N154	122.343 (8.479)	173.761	51.879 (4.326)	37.405 (2.833,2.833)	N^{δ^2} , * (7.375,7.731)
D155	121.578 (8.294)	174.666	52.718 (4.621)	39.725 (2.668,2.668)	
S156	116.415 (8.143)	172.176	56.697 (4.427)	62.300 (3.870,3.870)	
R157	128.704 (7.889)		55.443 (4.174)	29.214 (1.651,1.651)	C^{γ} , 29.476 (1.837,1.837); C^{δ} , 41.738 (3.143,2.885)

Tabelle 2 : Ubiquitin resonance assignments; pH 2.0, 303 ° K (proton chemical shifts are given in parentheses).

residue	N	C	C ^α	C ^β	other
M1		172.127	54.747	32.337	C ^γ , 30.345(*, *)
Q2	123.948 (8.908)	175.580	55.245 (4.371)	29.349 (1.872,1.908)	C ^γ , 33.333 (2.193,2.229)
I3	122.415 (8.360)	175.493	60.225 (4.157)	38.811 (1.765)	C ^γ , 26.361 (1.372,1.087); C ^{γ2} , 16.899 (0.837); C ^{δ1} , 11.919(*)
F4	124.494 (8.487)	174.976	56.241 (4.728)	39.309 (3.086,2.943)	C ^{ε1} , * (7.191)
V5	122.410 (8.334)	175.580	61.719 (4.157)	32.835 (1.979)	C ^{γ1} , 20.385(*); C ^{γ2} , 20.385 (0.873)
K6	125.102 (8.452)	176.788	55.743 (4.371)	32.835 (1.836,1.729)	C ^γ , 24.369 (1.444 *); C ^δ , 28.851 (1.622 *); C ^ε , 41.799 (3.015,2.979)
T7	115.893 (8.335)	174.889	61.221 (4.406)	69.687 (4.086)	C ^{γ2} , 20.883 (1.230)
L8	124.205 (8.509)	177.651	55.245 (4.443)	41.799 (1.658,1.658)	C ^γ , 26.361 (1.551); C ^{δ1} , 24.369(*); C ^{δ2} , 22.875 (0.908)
T9	112.630 (8.093)	175.148	61.221 (4.371)	69.687 (4.264)	C ^{γ2} , 20.883 (1.230)
G10	110.301 (8.316)	173.940	44.787 (3.943,4.014)		
K11	120.482 (8.100)	176.788	55.743 (4.086)	32.835 (1.836,1.729)	C ^γ , 24.369 (1.408,1.372); C ^δ , 28.353 (1.694 *); C ^ε , 41.799 (3.050,2.907)
T12	116.610 (8.377)	174.544	61.719 (4.371)	69.687 (4.157)	C ^{γ2} , 20.883 (1.194)
I13	123.520 (8.441)	176.011	60.225 (4.330)	38.811 (1.872)	C ^{γ1} , 26.361 (1.158,1.444); C ^{γ2} , 16.899 (0.908); C ^{δ1} , 12.417(*)
T14	118.645 (8.312)	174.199	61.221 (4.371)	69.687 (4.121)	C ^{γ2} , 20.883 (1.158)
L15	125.138 (8.390)	176.874	54.249 (4.407)	42.297 (1.587,1.551)	C ^γ , 26.361 (1.444); C ^{δ1} , 24.369(*); C ^{δ2} , 22.875 (0.908)
E16	121.515 (8.460)	175.839	55.245 (4.371)	28.353 (1.979,2.051)	C ^γ , 32.337 (2.443,2.443)
V17	121.457 (8.254)	175.925	61.719 (4.156)	32.337 (2.015)	C ^{γ1} , 20.385 (0.908); C ^{γ2} , 20.883 (0.908)
E18	124.448 (8.454)		55.245 (4.728)	28.851 (2.122,1.908)	C ^γ , * (2.122,2.515)
P19		176.961	62.715	31.839	C ^γ , 26.859(*, *); C ^δ , 50.265(*, *)
S20	115.309 (8.430)	174.717	58.233 (4.440)	63.711 (3.907,3.907)	
D21	120.466 (8.529)	175.321	52.755 (4.728)	37.815 (2.836,2.943)	
T22	114.056 (8.117)	174.544	61.719 (4.371)	69.687 (4.228)	C ^{γ2} , 20.883 (1.194)
I23	122.722 (8.139)	176.270	60.723 (4.157)	38.313 (1.872)	C ^{γ1} , 26.859 (1.158,1.479); C ^{γ2} , 16.899 (0.908); C ^{δ1} , 11.919(*)
E24	124.467 (8.541)	175.666	55.245 (4.300)	29.349 (1.979,2.086)	C ^γ , 33.333 (2.372 *)
N25	120.723 (8.560)		52.755 (4.730)	37.815 (2.872,2.943)	
V26	120.095 (8.097)	176.184	61.719 (4.192)	32.337 (2.301)	C ^{γ1} , 19.887 (0.873); C ^{γ2} , 20.883 (0.873)
K27	124.410 (8.346)	176.357	55.743 (4.300)	32.337 (1.836,1.836)	C ^γ , 24.369 (1.444,1.301); C ^δ , 28.851 (1.587,1.729); C ^ε , 41.799(*,2.872)
A28	124.955 (8.228)	177.651	52.257 (4.260)	18.891 (1.372)	
K29	121.002 (8.329)	176.702	56.241 (4.299)	32.835 (1.729,1.765)	C ^γ , 24.369 (1.479,1.444); C ^δ , 28.851(*, *); C ^ε , 41.799 (2.979,2.979)
I30	122.422 (8.255)	176.270	60.723 (4.157)	38.313 (1.836)	C ^{γ1} , 26.361 (1.194,1.479); C ^{γ2} , 16.899 (0.908); C ^{δ1} , 12.417(*)
Q31	123.618 (8.408)	175.666	55.245 (4.335)	28.353 (2.086,1.979)	C ^γ , 32.337 (2.443,2.372)
D32	120.303 (8.501)	175.235	52.755 (4.657)	37.815 (2.765,2.836)	
K33	121.987 (8.446)	176.443	56.241 (4.300)	32.337 (1.872,1.765)	C ^γ , 24.369 (1.408,1.444); C ^δ , 28.353(*,1.729); C ^ε , 41.799 (3.514,3.443)
E34	120.286 (8.334)	176.443	55.245 (4.300)	28.353 (2.015,2.158)	C ^γ , 32.337 (2.479,2.515)
G35	109.472 (8.352)	170.508	44.787 (3.907,3.978)		
I36	120.914 (8.046)		58.233 (4.371)	38.313 (1.836)	C ^{γ1} , * (1.158,1.479)
P38		177.047	63.213	31.839	C ^γ , 26.859(*, *); C ^δ , 50.265(*, *)
D39	117.154 (8.486)	175.407	52.755 (4.657)	37.317 (2.907,2.943)	
Q40	120.173 (8.321)	176.011	55.743 (4.335)	29.349 (1.944,2.122)	C ^γ , 33.333 (2.336,2.372)
Q41	120.938 (8.280)	175.925	55.743 (4.299)	28.851 (1.979,2.086)	C ^γ , 33.333 (2.336,2.372)
R42	122.016 (8.397)	176.011	55.743 (4.335)	30.345 (2.051,1.944)	C ^γ , 27.357 (1.587,1.765); C ^δ , 43.293 (3.193,3.157)
L43	123.797 (8.324)	176.788	54.747 (4.335)	42.297 (1.765,1.587)	C ^γ , 26.361 (1.408); C ^{δ1} , 24.369(*); C ^{δ2} , 22.875 (0.837)
I44	121.791 (8.181)	175.925	60.225 (4.120)	38.313 (1.729)	C ^{γ1} , 26.361 (1.087,1.372); C ^{γ2} , 16.899 (0.801); C ^{δ1} , 11.919(*)
F45	124.480 (8.421)	175.407	57.237 (4.585)	39.309 (2.979,3.122)	C ^{ε1} , * (7.226)
A46	125.963 (8.340)	177.738	51.759 (4.300)	18.891 (1.372)	
G47	107.253 (7.870)	174.026	44.787 (3.943,3.907)		
K48	120.443 (8.236)	176.702	56.241 (4.335)	32.835 (1.836,1.729)	C ^γ , 24.369 (1.408,1.444); C ^δ , 28.851(*, *); C ^ε , 41.799 (2.979,3.122)
Q49	121.372 (8.514)	176.011	55.743 (4.335)	28.851 (2.086,1.979)	C ^γ , 32.835 (2.336,2.372)
L50	123.626 (8.354)	177.392	54.747 (4.371)	42.297 (1.622,1.587)	C ^γ , 26.361 (1.551); C ^{δ1} , 24.369(*); C ^{δ2} , 22.875 (0.873)
E51	120.514 (8.457)	175.925	55.245 (4.371)	28.353 (2.122,1.979)	C ^γ , 32.337 (2.443,2.479)
D52	119.578 (8.531)	175.580	52.755 (4.728)	37.815 (2.907,2.979)	
G53	108.578 (8.376)	174.026	45.285 (3.943,3.978)		
R54	119.912 (8.151)	176.615	55.743 (4.371)	30.843 (1.872,1.765)	C ^γ , 26.361 (1.658,1.622); C ^δ , 42.795 (3.193,3.193)
T55	115.318 (8.272)	174.630	61.719 (4.371)	69.687 (4.228)	C ^{γ2} , 20.883 (1.230)
L56	124.446 (8.368)	177.392	55.245 (4.300)	42.297 (1.658,1.622)	C ^γ , 26.361 (1.587); C ^{δ1} , 24.369(*); C ^{δ2} , 22.875 (0.873)
S57	115.958 (8.356)	174.458	58.233 (4.407)	63.711 (3.800,3.836)	
D58	120.435 (8.436)	175.235	52.755 (4.657)	37.815 (2.836,2.800)	
Y59	120.095 (8.097)	175.407	57.735 (4.371)	38.313	C ^{δ1} , * (7.048)
N60	120.088 (8.360)	175.062	52.755 (4.692)	38.313 (2.800,2.658)	

I61	120.618 (8.000)	176.184	60.723 (4.121)	38.313 (1.836)	$C^{\gamma 1}$, 26.859 (1.158,1.444); $C^{\gamma 2}$, 16.899 (0.908); $C^{\delta 1}$, 12.417(*)
Q62	123.650 (8.377)	176.270	55.245 (4.371)	30.345 (1.944,2.086)	C^{γ} , * (2.479,2.443)
K63	123.890 (8.358)	176.702	56.241 (4.300)	32.835 (1.622,1.658)	C^{γ} , 24.369 (1.230,1.230); C^{δ} , 28.353 (1.515,1.587); C^{ϵ} , 41.799(*,2.907)
E64	120.949 (8.410)	176.270	55.245 (4.300)	28.353 (2.122,2.015)	C^{γ} , 32.337 (2.479,2.515)
S65	116.679 (8.442)	175.062	58.233 (4.513)	63.711 (3.907,3.907)	
T66	115.491 (8.241)	174.544	61.719 (4.371)	69.189 (4.050)	$C^{\gamma 2}$, 20.883 (1.194)
L67	123.757 (8.129)	177.047	54.747 (4.300)	42.297 (1.551,1.515)	C^{γ} , 26.361 (1.479); $C^{\delta 1}$, 23.871(*); $C^{\delta 2}$, 22.377 (0.837)
H68	119.824 (8.650)	174.113	54.249 (4.760)	28.353 (3.157,3.264)	
L69	124.029 (8.304)	176.874	54.747 (4.300)	42.297 (1.729,1.765)	C^{γ} , 26.361 (1.515); $C^{\delta 1}$, 23.871(*); $C^{\delta 2}$, * (0.873)
V70	122.524 (8.390)	175.925	61.719 (4.080)	32.337 (2.015)	$C^{\gamma 1}$, 20.385(*); $C^{\gamma 2}$, * (0.908)
L71	126.751 (8.383)	176.961	54.747 (4.300)	42.297 (1.551,1.515)	C^{γ} , 26.361 (1.587); $C^{\delta 1}$, 23.871(*); $C^{\delta 2}$, 22.875 (0.837)
R72	122.663 (8.489)		55.245 (4.371)	30.345 (1.765,1.622)	C^{γ} , * (1.587,1.515); C^{δ} , * (2.979,3.193)
L73		177.479	54.747	42.297	C^{γ} , 26.361(*); $C^{\delta 1}$, 24.369(*)
R74	121.985 (8.566)	176.874	56.241 (4.335)	30.345 (1.872,1.801)	C^{γ} , 26.361 (1.658,1.515); C^{δ} , 43.293 (3.229,3.193)
G75	109.911 (8.448)	174.285	44.787 (3.943,4.010)		
G76	108.828 (8.200)		43.791 (3.943,4.010)		



Adiabatic TOCSY for C,C and H,H J-transfer

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Abstract

Adiabatic pulses have been widely used for broadband decoupling and spin inversion at high magnetic fields. In this paper we propose adiabatic pulses and supercycles that can be used at high magnetic fields like 800 or 900 MHz to obtain broadband TOCSY sequences with C,C or H,H J-transfer. The new mixing sequences are equal or even superior to the well known DIPSI-2,3 experiments with respect to bandwidth. They prove robust against pulse miscalibration and B₁ inhomogeneity and are therefore attractive for fully automated spectrometer environments. These adiabatic mixing sequences have been incorporated in a novel z-filter HCCH-TOCSY experiment.

Introduction

The TOCSY building block (Braunschweiler and Ernst, 1983; Glaser and Quant, 1996) is one of the most important building blocks used in biomolecular NMR either for backbone or for side chain assignment experiments (Sattler et al., 1999). TOCSY achieves the fastest magnetization transfer between coupled spins and relies on the removal of chemical shifts from the Hamiltonian, which for a given bandwidth in ppm is getting more and more difficult as magnetic field strength increases. Therefore new TOCSY sequences need to be developed where the higher B₀ field is not compensated for by an increase in B₁ field, which would result in a quadratic increase of RF power. Here we emphasize on the design of building blocks and phase cycles, which require low power, have a broad bandwidth and show a square shaped coherence transfer efficiency diagram.

Adiabatic pulses (Tannus and Garwood, 1997) are used in high resolution liquid NMR for decoupling and spin inversion over large frequency bandwidths (Bendall, 1995; Fu and Bodenhausen, 1995; Kupce

and Freeman, 1995; Kupce et al., 1998; Starcuk et al., 1994) with a reasonable amount of RF-field strength. Additionally these pulses are also extremely robust against pulse miscalibration and B₁ field inhomogeneities. Therefore it is of obvious interest whether these adiabatic pulses are suitable to be used in TOCSY experiments for C,C and H,H J-transfer. Kupce et al. (1998) have shown that adiabatic pulses can be used in H,H-TOCSY experiments. However the published sequence, based on ca-WURST pulses (constant adiabaticity WURST-n, n = 2 or 8 (Kupce and Freeman, 1995)) expanded by the supercycle *P5M4* (Tycko et al., 1985), is far less broad banded than any of the traditional TOCSY sequences like MLEV-17, DIPSI-2,3 and FLOPSY-16. In this sequence an excellent transfer between resonances close to the diagonal is achieved, but the transfer efficiency diagram is far from being square shaped. Therefore we set out to find sequences with a more square shaped mixing profile that will also be suitable for C,C J-transfer. We will present pulses and supercycles that can be used in adiabatic TOCSYs for C,C and H,H J-transfer that perform better than the well known DIPSI-2,3 (for H,H and C,C J-transfer) (Shaka et al., 1988) and FLOPSY-16 (Kadkhodaie et al., 1991) (for H,H J-transfer) sequence.

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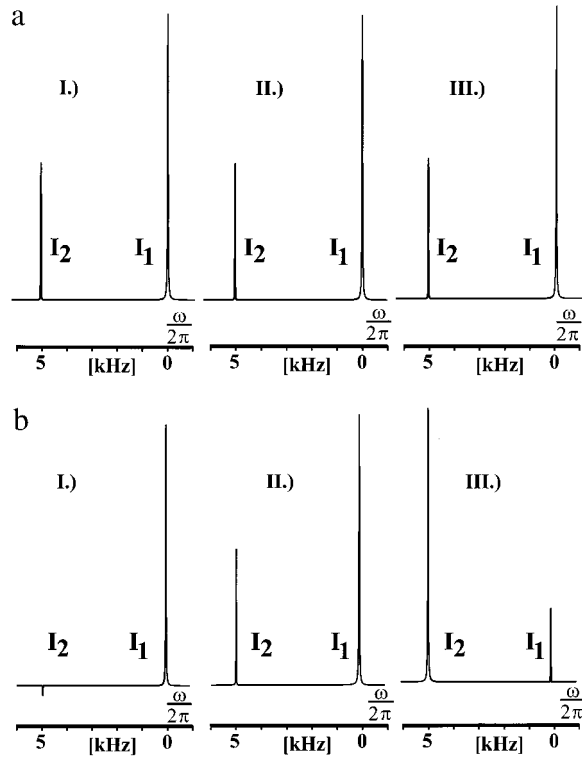


Figure 1. Efficiency of the adiabatic TOCSY transfer as a function of different parameters. All spectra were simulated as an ideal two spin system in which spin 1 was selectively excited and the magnetization was subsequently transferred to spin 2. In all simulations a ca-WURST-8 with a *P5M4* supercycle is used for magnetization transfer. In (a) can be seen that the time difference for the inversion of the two coupled spins Δt is not a critical parameter in TOCSY transfer. The following pulses were used to establish the magnetization transfer: (I) ca-WURST-8, total sweep width = 800 kHz, duration $T_p = 100 \mu\text{s}$, $\gamma B_1/2\pi = 62605 \text{ Hz}$, time difference between the inversion of the two spins $\Delta t = 0.4 \mu\text{s}$; (II) ca-WURST-8, total sweep width = 200 kHz, $T_p = 100 \mu\text{s}$, $\gamma B_1/2\pi = 31303 \text{ Hz}$, $\Delta t = 1.6 \mu\text{s}$; (III) ca-WURST-8, total sweep width = 50 kHz, $T_p = 100 \mu\text{s}$, $\gamma B_1/2\pi = 15651 \text{ Hz}$, $\Delta t = 6.5 \mu\text{s}$. As it can be seen in (b) the most important parameter for the transfer efficiency in adiabatic TOCSY schemes is the duration of the adiabatic pulses. The following pulses were used to establish the magnetization transfer: (I) ca-WURST-8, total sweep width = 400 kHz, $T_p = 200 \mu\text{s}$, $\gamma B_1/2\pi = 31303 \text{ Hz}$, $\Delta t = 1.6 \mu\text{s}$; (II) ca-WURST-8, total sweep width = 200 kHz, $T_p = 100 \mu\text{s}$, $\gamma B_1/2\pi = 31303 \text{ Hz}$, $\Delta t = 1.6 \mu\text{s}$; (III) ca-WURST-8, total sweep width = 100 kHz, $T_p = 50 \mu\text{s}$, $\gamma B_1/2\pi = 31303 \text{ Hz}$, $\Delta t = 1.6 \mu\text{s}$.

Materials and methods

Adiabatic pulses invert spins by a ‘slow’ passage of a chirped pulse through resonance. So spins having a different resonance frequency will be inverted at different times. This differs from the behavior during a square pulse, where all spins are inverted simultaneously. In order to assess the relevance of this feature

of adiabatic pulses for the efficiency of magnetization transfer, we carried out simulations on an ideal two spin system. The spins I_1 and I_2 are coupled to each other with a coupling constant J . After selectively exciting I_1 , magnetization is transferred by a TOCSY sequence to I_2 . The TOCSY sequence consists of a pulse with a WURST-8 shape subjected to a *P5M4* supercycle expansion (P5: $0^\circ, 150^\circ, 60^\circ, 150^\circ, 0^\circ$, expanded by MLEV4, details are given in the caption of Figures 1a and 1b) (Tycko et al., 1985). This is the same type of pulse and supercycle as has been used by Kupce et al. for establishing the H,H transfer. The frequency covered by the sweep nevertheless is significantly different in order to establish a truly adiabatic behavior. The resulting large RF field strength is of no concern in the context of the simulation. Figure 1a demonstrates that the time difference between the inversion of the two spins in an adiabatic passage is not a critical parameter for Hartmann-Hahn transfer. The most important parameter for the transfer, as is shown in Figure 1b, is the duration of the adiabatic pulse. So the ideal pulse should operate at a reasonable RF field strength (ca. 10 kHz) and be as short as possible, but still fulfilling the adiabatic condition. The key to the experiment is to find a pulse having these characteristics. Unfortunately this is not a trivial task, since the adiabatic behavior will suffer severely once the pulse length is getting too short. The pulse, which eventually worked best in our hands, is a tanh/tan pulse described in the context of the BIR pulse family (BIR - B_1 insensitive rotation) (Staewen et al., 1990; Garwood and Ke, 1991; Hwang et al., 1998), which performs a fast sweep at affordable RF power and an acceptable adiabaticity level. Even though short pulses can be constructed with other types of shapes as well, their performance with respect to the adiabatic behavior or the required peak power was not as good as for the tanh/tan pulse. This tanh/tan pulse can be constructed from the following adiabatic half passage and its time reversed half passage:

$$\begin{aligned}\gamma B_1(t) &= \gamma B_1^o f_B(t), \\ \Delta\omega(t) &= \Delta\omega^o f_\omega(t).\end{aligned}$$

$f_B(t)$ and $f_\omega(t)$ are dimensionless, time dependent modulation functions described by the hyperbolic tangent (tanh) and tangent (tan) function respectively, with $0 \leq t \leq T_p/2$ (Hwang et al., 1998):

$$\begin{aligned}f_B(t) &= \tanh[\xi 2t/T_p], \\ f_\omega(t) &= \tan[\kappa(1 - 2t/T_p)]/\tan[\kappa].\end{aligned}$$

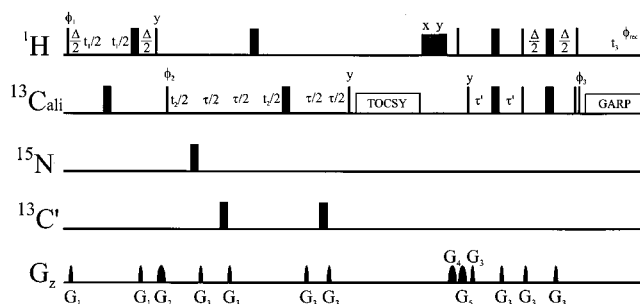


Figure 2. Pulse sequence of the z-filter HCCH-TOCSY experiment. Narrow and thick bars represent 90° and 180° pulses. Unless otherwise indicated the default phase for pulses is x . Phase cycling: $\phi_1 = x, -x, \phi_2 = 2(x), 2(-x), \phi_3 = 4(x), 4(-x), \phi_{\text{rec}} = x, 2(-x), x$. Quadrature detection in both dimensions is obtained by altering ϕ_1 and ϕ_2 respectively in the States-TPPI manner (Marion et al., 1989). Proton trim pulses of 1 ms were used. Delay durations: $\Delta = 3.2$ ms, $\tau = 475$ μs and $\tau' = 1.1$ ms. Carrier positions: $^1\text{H} = 4.65$ ppm, $^{13}\text{C} = 42$ ppm, $^{15}\text{N} = 114.5$ ppm. Proton pulses are applied using a 38.5 kHz rf field. $^{13}\text{C}_{\text{ali}}$ pulses are hard pulses with a field of 20.8 kHz. Off resonance Q3 shaped pulses with duration of 256 μs were used for $^{13}\text{C}'$ decoupling pulses. ^{15}N pulses are at a field of 2.6 kHz and ^{13}C GARP decoupling (Shaka et al., 1985) during acquisition is applied with a 2.5 kHz field. Gradients (sine bell shaped): $G_1 = (500$ $\mu\text{s}, 16$ G/cm), $G_2 = (1$ ms, 30 G/cm), $G_3 = (300$ $\mu\text{s}, 16$ G/cm), $G_4 = (5$ ms, 60 G/cm), $G_5 = (4.4$ ms, 40 G/cm). For the 2D version of the experiment 8 scans per t_2 (256 complex points, spectral width 12374.02 Hz) experiment were recorded with 2048 complex points in t_3 (spectral width 10000 Hz). A repetition delay of 2 s was used between scans, giving rise to a total measurement time of approximately 1.5 h for the 2D version of the experiment. Every TOCSY sequence explained in the text can be used with this z-filter HCCH-TOCSY sequence.

As seen in Figure 1b III nearly complete TOCSY transfer of magnetization from spin I_1 to spin I_2 can be achieved with a pulse duration of 50 μs . A tanh/tan pulse of 50 μs duration ($\tanh/\tan: \Delta\omega^0 = 150$ kHz, $\gamma B_1/2\pi^{\text{avr}} = 9750$ Hz, $\gamma B_1/2\pi^{\text{max}} = 10504$ Hz, $\zeta = 10$, $\tan \kappa = 20$, $Q = 2$ (Staewen et al., 1990; Garwood and Ke, 1991; Hwang et al., 1998)) has a comparable RF field strength to commonly used square pulses (duration of 90° pulse = 25 μs , $\gamma B_1/2\pi = 10000$ Hz). We have also used BIR-4 rotations (Staewen et al., 1990; Garwood and Ke, 1991), that are composed of two tanh/tan units, for C,C and H,H J-transfer (BIR-4: $\Delta\omega^0 = 150$ kHz, duration = 100 μs , $\gamma B_1/2\pi^{\text{avr}} = 9750$ Hz, $\gamma B_1/2\pi^{\text{max}} = 10504$ Hz, $\zeta = 10$, $\tan \kappa = 20$, $Q = 2$). The advantage of BIR-4 when compared to a tanh/tan rotation is its broader inversion profile that correlates with a higher adiabaticity. The double duration of BIR-4 compared to tanh/tan has no impact on the transfer performance. However, this will require a doubling of the mixing time in order to fully complete one expansion cycle. In the case of larger and more complicated expansions this makes the sequence less flexible for adaptation to an optimum mixing time dictated by the coupling topology of the spin system. The scheme of expanding the tanh/tan pulse to the BIR-4 pulse is generally applicable to other shapes as well.

To obtain a square shaped offset dependent mixing profile the basic building block of the TOCSY sequence needs to be expanded into supercycles. The supercycles most successfully used with adiabatic pulses

are composed of P5 (P5: $0^\circ, 150^\circ, 60^\circ, 150^\circ, 0^\circ$) and P9 (P9: $0^\circ, 15^\circ, 180^\circ, 165^\circ, 270^\circ, 165^\circ, 180^\circ, 15^\circ, 0^\circ$) (Cho et al., 1986; Skinner and Bendall, 1997; Tycko et al., 1985) expanded by MLEV-16 ($\bar{R}\bar{R}\bar{R}\bar{R}, \bar{R}\bar{R}\bar{R}\bar{R}, \bar{R}\bar{R}\bar{R}\bar{R}, \bar{R}\bar{R}\bar{R}\bar{R}$) (Jacobs et al., 1982; Levitt et al., 1982; Shaka et al., 1983). We found that multiple variants of these supercycles like *P5P9MI6* perform well in simulation and experiment. Long cycle times make these expansions ideally suited for H,H TOCSY experiments (Figure 6). All H,H TOCSY experiments can also be performed in a sensitivity enhanced manner yielding a gain of about 1.2.

In our study all C,C TOCSY experiments were performed as a z-filter version (Rance, 1987). Whereas in the original version (Kay et al., 1993) transverse magnetization is spin locked and then rotated to the z-axis before applying the ^1H -pulses/gradients for water suppression, in the version described here magnetization is brought to the z-axis immediately before the spinlock sequence. This way the TOCSY operates on z-magnetization. Simulation showed that TOCSY mixing sequences perform more robust (Glaser and Kramer, 2000) when applied in a z-filter. The novel HCCH-TOCSY version where the C,C TOCSY is performed in a z-filter is shown in Figure 2. Details of the experiment are given in the caption of Figure 2.

All simulations were done using NMRSIM 2.9.1.b software (Bruker Analytik GmbH, Rheinstetten, Germany), which is part of the Bruker NMR-SUITE. An ideal two spin system with a J-coupling of 7 Hz was

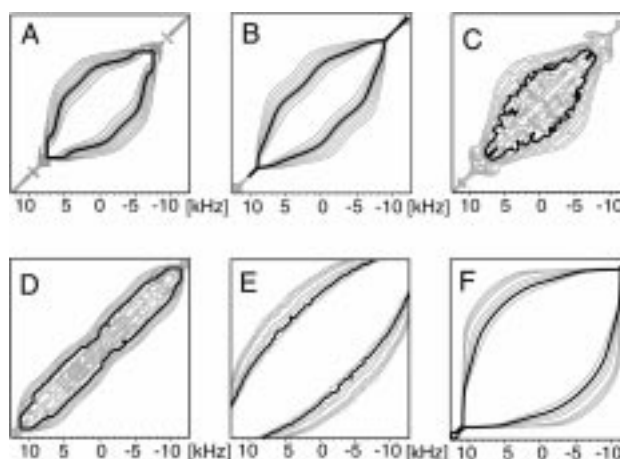


Figure 3. Comparison of different TOCSY schemes: The 80% transfer efficiency contour line is emphasized in black: (A) DIPSI-2 with $\gamma B_1/2\pi = 10$ kHz. (B) DIPSI-3 with $\gamma B_1/2\pi = 10$ kHz. (C) tanh/tan sequence: tanh/tan, *P9M16*: $T_p = 50 \mu\text{s}$, $\Delta\omega^0 = 150$ kHz, $\gamma B_1/2\pi^{\text{max}} = 11960$ Hz, $\zeta = 10$, $\tan \kappa = 20$. (D) ca-WURST-8, *P5M4*: $T_p = 150 \mu\text{s}$, total sweep width = 35 kHz, $\gamma B_1/2\pi^{\text{max}} = 10692$ Hz. (E) Using the *P9M16* supercycle with a BIR-4 rotation (BIR-4: $T_p = 100 \mu\text{s}$, $\Delta\omega^0 = 150$ kHz, $\gamma B_1/2\pi^{\text{max}} = 11960$ Hz, $\zeta = 10$, $\tan \kappa = 20$) provides again an increase of the diagonal and the antidiagonal bandwidth for the transfer. (F) FLOPSY-16 with $\gamma B_1/2\pi = 10$ kHz.

used for the simulations. The transfer time was always set to $1/2J$. Experimentally the C,C and H,H J-transfer based adiabatic TOCSY sequences were tested on a 10 mg $^{13}\text{C},^{15}\text{N}$ labeled sample of Ubiquitin in a $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 solution at pH 5.0 and 303 K (commercially available from VLI Research, Inc., Malvern, PA) in a 5 mm microcell Shigemitsu tube (320 μl). All experiments were performed on Bruker DRX 800 MHz and DRX 600 MHz spectrometers (Bruker Analytik GmbH, Rheinstetten, Germany) equipped with TXI HCN z-gradient probes. Spectra were processed using XWINNMR2.6 from Bruker. One z-filtered HCCH-TOCSY experiment was also carried out on a $^{13}\text{C},^{15}\text{N}$ labeled sample (1 mM) of the fumarate sensor histidine kinase *DcuS* (17.4 kDa). The protein sample was at pH 6.5, 50 mM sodium phosphate buffer, 200 mM NaCl, 0.8 mM CHAPS, 50 mM Glycine, 50 pM Pefabloc SC (Fluka AG, Buchs, Switzerland), 0.01% NaN_3 and $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10. Also this sample was measured at 303 K in a 5 mm microcell Shigemitsu tube.

Results and discussion

Figure 3 compares the offset dependent transfer efficiencies of two standard TOCSY variants, DIPSI-2 (A) and DIPSI-3 (B), with the adiabatic TOCSY variant published by Kupce et al. (D: ca-WURST-8, *P5M4* (Kupce et al., 1998)) and with our *P9M16*, tanh/tan based TOCSY experiments (C). The offset depen-

dence of the latter is similar to the DIPSI-3 sequence. DIPSI-2 is slightly more broadband along the antidiagonal but less broadband along the diagonal. The sequence shown in D, the original starting point of our work, is very broadband along the diagonal but deviates strongly from the desired square shaped offset dependence. Going to shorter pulses the broadband nature of the transfer improves dramatically both along the diagonal as well as along the antidiagonal. Using a BIR-4 pulse (Figure 3E) the bandwidth along the diagonal increases by a factor of two to three as compared to the DIPSI (A, B) or FLOPSY (F) sequence. The bandwidth along the antidiagonal increases about 50% when compared to the DIPSI-2 sequence.

To evaluate different TOCSY sequences we have used a novel z-filter HCCH-TOCSY experiment (Figure 2) that can accommodate different TOCSY schemes. TOCSY spectra of ubiquitin using the tanh/tan *P9M16* or the DIPSI-3 sequence are shown in Figure 4. Due to the fact that for a given average power the mixing schemes have discrete non identical durations we could not exactly match the mixing times for tanh/tan *P9M16* and DIPSI-3. To allow a fair comparison of the different mixing sequences we have taken 1D traces out of the 800 MHz 2D spectra (all shown in Figure 4C). Mixing times for DIPSI-3 were multiples of approx. 5.4 ms and multiples of approx. 7.2 ms for the tanh/tan *P9M16* sequence. This allows us to compare the intensities of the different mixing sequences. The adiabatic tanh/tan *P9M16*

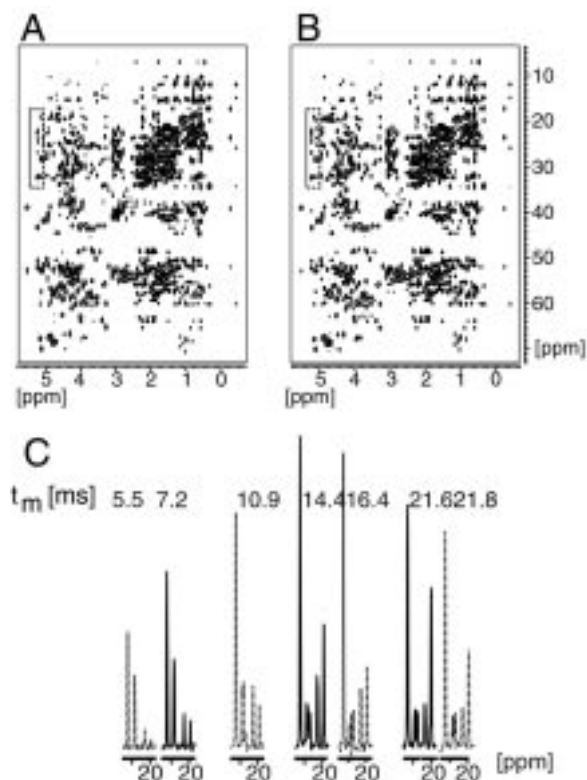


Figure 4. For ubiquitin 2D spectra of the HCCH-TOCSY sequence (Figure 2) are shown ($T_m \sim 12$ ms, 800 MHz spectrometer). (A) A DISPI-3 sequence was used for the C,C TOCSY (10 kHz). (B) A $P9M16$, tanh/tan (tanh/tan: $T_p = 50$ μ s, $\Delta\omega^0 = 150$ kHz, $\gamma B_1/2\pi^{\text{avr}} = 9750$ Hz, $\gamma B_1/2\pi^{\text{max}} = 10504$ Hz, $\zeta = 10$, $\tan \kappa = 20$, $Q = 2$) sequence was used. Due to different loop times of the mixing sequences (given by the different length of the supercycles) a direct comparison of the intensities of the spectra is difficult. Therefore slices out of the marked boxes are compared in (C). A build up curve against the mixing time is plotted (black lines are $P9M16$, tanh/tan and dashed lines are DISPI-3). For comparable mixing times (last two traces at $t_m = 21.6$ ms and $t_m = 21.8$ ms) a gain in sensitivity is observed (10–30%).

TOCSY is about 10–30% more sensitive than a DISPI-3 sequence. Comparing the last two traces in Figure 4, which are taken at 21.6 ms and 21.8 ms, respectively, this enhancement effect can be seen most easily. Even though this mixing time is not ideal, since magnetization has been lost due to relaxation, the adiabatic tanh/tan $P9M16$ TOCSY (full black line) has a higher intensity than the DISPI-3 sequence (dashed line).

The different mixing schemes proposed in this article are quite long and can therefore only accommodate a limited discrete set of mixing times. However, different pulses like tanh/tan and BIR-4 with different length and similar performance are available to provide more flexibility. For H,H J-transfer experiments we propose

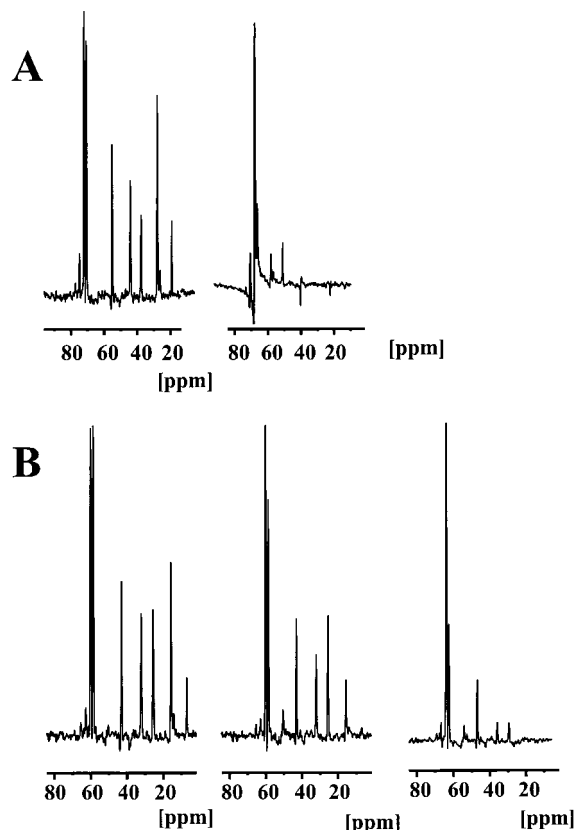


Figure 5. Traces out of 2D z-filter HCCH-TOCSY spectra of ubiquitin recorded with: (A) DIPS1-3 with a basic RF field strength of $\gamma B_1/2\pi = 10$ kHz (left) and one decreased by 2 dB (right); $T_m \sim 12$ ms. (B) BIR-4, $P9M16$ (BIR-4: $T_p = 100$ μ s, $\Delta\omega^0 = 150$ kHz, $\gamma B_1/2\pi^{\text{avr}} = 9750$ Hz, $\gamma B_1/2\pi^{\text{max}} = 10504$ Hz, $\zeta = 10$, $\tan \kappa = 20$, $Q = 2$) $T_m \sim 14$ ms. The actual RF field strength used was the nominal one ($\gamma B_1/2\pi^{\text{max}} = 10504$ Hz, left), the nominal one decreased by 2 dB (middle) and the nominal one decreased by 4 dB (right). The BIR-4 $P9M16$ sequence is much more robust towards pulse miscalibration and B_1 inhomogeneities.

a $P5P9M16$ ((P5: $0^\circ, 150^\circ, 60^\circ, 150^\circ, 0^\circ$) expanded first by P9 (P9: $0^\circ, 15^\circ, 180^\circ, 165^\circ, 270^\circ, 165^\circ, 180^\circ, 15^\circ, 0^\circ$) and then by MLEV16) supercycle with either tanh/tan or BIR-4 pulses. These supercycles provide excellent performance when compared with FLOPSY-16, which is known to be close to ideal considering the transfer efficiency along the antidiagonal (Glaser and Quant, 1996). $P5P9M16$ is even broader along the antidiagonal than FLOPSY-16 (Figure 6).

All adiabatic TOCSY sequences have in common that they are insensitive against pulse miscalibration and B_1 field inhomogeneities. In Figure 5 spectra of a DIPS1-3 sequence with $\gamma B_1/2\pi = 10$ kHz are compared with those of a BIR-4 $P9M16$ sequence (details are given in the caption of Figure 5). Reduction of

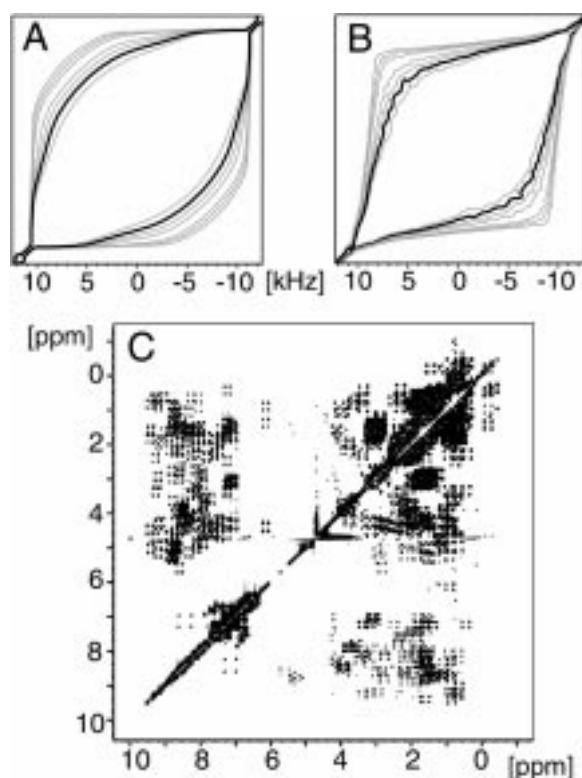


Figure 6. Simulation of the transfer efficiencies: (A) FLOPSY-16 with $\gamma B_1/2\pi = 10$ kHz, and (B) tanh/tan, P5P9M16 (tanh/tan: $T_p = 50$ μ s, $\Delta\omega^0 = 150$ kHz, $\gamma B_1/2\pi^{avr} = 9750$ Hz, $\gamma B_1/2\pi^{max} = 10504$ Hz, $\zeta = 10$, $\tan \kappa = 20$, $Q = 2$). (C) H,H TOCSY spectrum using (B) recorded on Ubiquitin.

the RF field strength in steps of two dB influences the transfer efficiency of DIPSI-3 much more than that of the BIR-4 P9M16 sequence. Even a RF field strength miscalibrated by +4 dB, which is equivalent to using a 50° pulse instead of a 90° pulse, still yields transfer with the BIR-4 P9M16 sequence. Adiabatic pulses are even more robust against an increase of RF power compared to the just discussed decrease since additional RF field strength will yield a higher Q factor and thus an even better adiabatic behavior. This is not the case for TOCSY sequences based on square pulses. Sequences like DIPSI and FLOPSY tolerate only small RF field strength miscalibrations in either direction. Especially FLOPSY-16 has shown to be very prone to miscalibration. The robustness of adiabatic TOCSY sequences therefore makes them highly attractive for automated biomolecular NMR spectroscopy where robustness of the sequence against miscalibration is beneficial.

Conclusions

We have presented novel and more efficient building blocks for adiabatic TOCSY J-transfer. Using tanh/tan or BIR-4 adiabatic pulses expanded by supercycles like P9M16 for C,C transfer or P5P9M16 for H,H transfer, we have achieved a gain in sensitivity of about 10–30% for the tanh/tan P9M16 C,C sequence when compared to a traditional DIPSI-3 C,C experiment. More important, these TOCSY sequences, especially when combined with BIR-4 pulses have shown to tolerate significant pulse miscalibration and B_1 inhomogeneity. These adiabatic TOCSY sequences have been tested using a novel z-filter version of a HCCH-TOCSY on ubiquitin and the histidine kinase fumarat sensor protein DcuS (17.4 kDa) yielding equally good spectra and therefore promise to be applicable to a wide range of systems of different sizes.

Acknowledgements

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Letter to the Editor: Assignment of ^1H , ^{13}C and ^{15}N resonances to the sensory domain of the membraneous two-component fumarate sensor (histidine protein kinase) DcuS of *Escherichia coli*

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Biological context

In bacteria various types of signal transduction proteins are used for the perception of environmental signals (Aizawa et al., 2000). Most of the sensors receive the signal at the periplasmic side of the membrane. By transferring the signal across the membrane, it is converted into a cellular signal, which is used for regulation of transcription, enzyme activity, or flagellar rotation. Two-component regulatory systems represent the most frequent systems of this type for transmembrane signalling in bacteria. They consist of a membraneous sensory histidine protein kinase and a cytoplasmic response regulator. Signal transfer between the sensor and the response regulator is effected by protein phosphorylation.

In the facultative anaerobic bacterium *Escherichia coli* the switch from aerobic to anaerobic metabolism is regulated at the transcriptional level in response to the electron acceptors O_2 , nitrate, and fumarate. The expression of the genes of anaerobic fumarate respiration, including fumarate reductase, an anaerobic C_4 -dicarboxylate (fumarate) carrier, and of fumarase B, is regulated by C_4 -dicarboxylates and the two-component regulatory system DcuSR (dicarboxylate uptake) (Zientz et al., 1998; Golby et al., 1999). The DcuSR system consists of the sensor DcuS located in the cytoplasmic membrane, and of the cytoplasmic response regulator DcuR. DcuS contains two transmembrane helices, one periplasmic domain, and the

C-terminal kinase and transmitter domain. The kinase domain extends into the cytoplasm of *E. coli* and catalyses autophosphorylation of a conserved histidine residue of the protein. Its activity is controlled by the external and possibly other signals. The periplasmic domain of about 140 amino acid residues is enclosed by the transmembrane helices. This domain binds fumarate and other C_4 -dicarboxylates (Zientz et al., 1998). Due to the periplasmic site for signal reception, the signal has to be transferred across the membrane from the sensory to the kinase and transmitter domain. From two-component sensors only the structure of the cytoplasmic CheA histidine kinase, which is involved in chemotaxis regulation (Bilwes et al., 1999), and of the cytoplasmic kinase domain of the osmosensor EnvZ (Tanaka et al., 1998) are known. Thus, for the process of transmembrane signalling no detailed structural information is available. To get an insight into the conformational changes involved in the signal transfer across the membrane, the structure of the periplasmic domain of the DcuSR fumarate sensory system was analysed by NMR spectroscopy. The ^1H , ^{15}N -HSQC with annotated assignments is shown in Figure 1.

Methods and experiments

The periplasmic domain (amino acid residues 45 to 180) of DcuS was overproduced in *E. coli* strain BL21DE3 carrying the DcuS_{45–180} expression plasmid pMW145. The plasmid was derived from pET28a with the codons for amino acid residues 45 to 180 of *dcuS* cloned in a frame behind the sequence for

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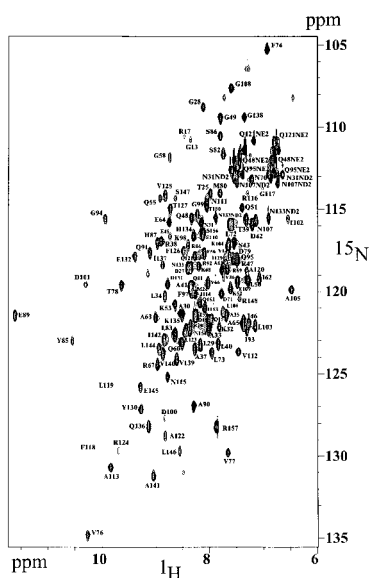


Figure 1. Annotated ^1H , ^{15}N -HSQC spectrum of DcuS recorded at 303 K and a ^1H frequency of 600 MHz. Sequential assignments are indicated with the one-letter amino acid code and the residue number.

the T7 promoter and an N-terminal His₆-tag. The induced strain BL21DE3(pMW145) was disrupted by a French Press, and the DcuS_{45–180} protein was isolated from the cell homogenate by chromatography on a Ni²⁺-NTA column. After elution, dialysis, and concentration by ultrafiltration, DcuS_{45–180} (20 mg protein/ml) was either stored at -80°C or directly used for NMR spectroscopy.

About 1 mM ^{15}N and ^{15}N , ^{13}C labeled DcuS were used for measurement in a 5 mm microcell Shigemi tube (300 μl). The protein sample was at pH 6.5, 50 mM sodium phosphate buffer, 200 mM NaCl, 0.8 mM CHAPS, 50 mM Glycin, 50 pM Pefabloc SC (Fluka AG, Buchs, Switzerland), 0.01% NaN₃ and H₂O/D₂O 90/10. The following experiments were carried out: ^{15}N labeled sample: 2D ^1H , ^{15}N -HSQC, 3D NOESY-HSQC (150 ms mixing time), 3D TOCSY-HSQC (60 ms mixing time), 3D HSQC-NOESY-HSQC (100 ms mixing time); ^{15}N , ^{13}C labeled sample: 3D HNC0, 3D CBCA(CO)NH, 3D HNCACB, 3D CC(CO)NH, 3D HBHACONH, 3D HCCH-COSY and 3D HCCH-adiabatic TOCSY (Peti et al., 2000). Pulsed field gradient versions of the experiments, with water flip-back pulses and sensitivity enhancement (Sattler et al. (1999) and references cited therein) were recorded on a Bruker DRX 600 and Bruker DRX 800 (TXI HCN z-grad) at 303 K (Bruker Analytic GmbH, Rheinstetten, Germany). Fourier transforma-

tion, mirror image linear prediction and data analysis were carried out using the programs XWINNMR 2.6 (Bruker) and XEASY (Bartels et al., 1995). Resonance positions were referenced using internal DSS to obtain ^1H , ^{13}C and ^{15}N chemical shifts as described in Wishart et al. (1995). The annotated ^1H , ^{15}N -HSQC is shown in Figure 1.

Extent of assignments and data deposition

^1H , ^{15}N , ^{13}C backbone resonances of 134 out of 151 non-proline residues could be assigned (the first 20 amino acids belong to the His₆-tag and a linker). The ^1H and ^{13}C resonances of all 6 prolines could be assigned. The side chain H _{α} , H _{β} , C _{α} , C _{β} resonances could be assigned for 136 of the 137 residues. Additional side-chain proton chemical shifts were identified in a TOCSY-HSQC, the NOESY-HSQC and the HCCH-COSY and HCCH-TOCSY spectra. The chemical shift values of proton, nitrogen and carbon resonances were deposited in the BioMagResBank in Madison, WI, U.S.A. (accession number BMRB-4821).

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Measurement of Magnitude and Sign of H,H-Dipolar Couplings in Proteins

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The determination of three-dimensional structures of proteins and nucleic acids by NMR relies on the measurement of a large number of NOE's and homonuclear and heteronuclear couplings.¹ Recently cross correlated relaxation rates² and dipolar couplings,^{3–5} were also introduced as new parameters for structure elucidation. Residual heteronuclear dipolar couplings proved to be a tool for orientation restraints which provide long-range structural information in proteins and have special impact on the structure determination of multidomain proteins.^{6,7} Size and sign of heteronuclear dipolar couplings can be measured from the difference of the splitting observed in partially aligned molecules, by comparison with the scalar couplings measured in isotropic solutions. Provided the amount of alignment is so weak that the absolute value of the dipolar coupling does not exceed the absolute value of the scalar coupling, the dipolar coupling can be directly extracted. This requirement is easily met for directly bound H–C and H–N pairs. For proton–proton dipolar couplings, however, this requirement cannot be met due to the much smaller size of proton–proton J -couplings. Therefore, experiments which allow measurement of size and sign of H,H dipolar couplings are of interest.

In this communication, we propose the measurement of H,H dipolar couplings based on the E.COSY principle^{8–10} in which the H,H dipolar coupling is related to an ¹⁵N–H splitting ($J + D$) whose sign is known. This method provides size and sign information for the H,H dipolar coupling which was only possible so far to measure for interresidual H^N,H^α dipolar couplings in ¹³C,¹⁵N labeled samples.¹¹ Transfer of coherence between the protons without decoupling the ¹⁵N and a planar mixing step before detection as achieved in a J_{HH} sequence¹² (Figure 1) provides an E.COSY type pattern in a ¹⁵N–H₁,H₂ moiety. The J_{HH} sequence is built from a ¹⁵N edited NOESY spectrum with planar mixing steps before and after the NOESY step. Such pulse sequences have been used for reliable extraction of J -coupling values.^{13–15} The relevant product operators are given under the pulse sequence in Figure 1. It turns out that in the first planar

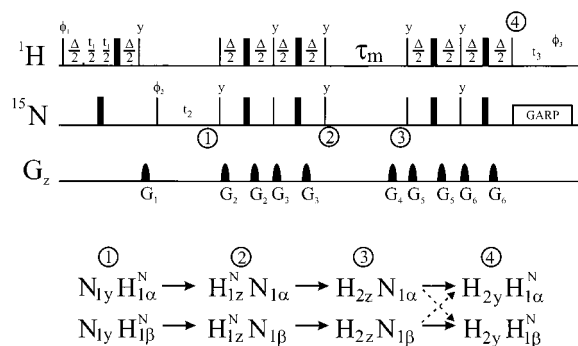


Figure 1. Pulse sequence of the 3D J_{HH} -NOESY. Narrow and thick bars represent 90° and 180° pulses. The default phase for pulses is x . Phase cycling: $\phi_1 = 2(x)$, $2(-x)$, $\phi_2 = x$, $-x$, $\phi_3 = x$, $2(-x)$, x . Quadrature detection in both dimensions is obtained by altering ϕ_1 and ϕ_2 , respectively, in the States-TPPI manner.²⁸ Water suppression was done with presaturation during the 2 s relaxation delay. Delay durations: $\Delta = 5.4$ ms and $\tau_m = 200$ ms. Carrier positions: ¹H = 4.65 ppm; ¹⁵N = 114.5 ppm. Proton pulses are applied using a 20.7 kHz rf field. ¹⁵N pulses are at a field of 8.2 kHz and GARP²⁹ decoupling during acquisition is applied with a 1 kHz field. Gradients (sine bell shaped): $G_1 = (1$ ms, 30G/cm), $G_2 = (1$ ms, 8G/cm), $G_3 = (1$ ms, 10G/cm), $G_4 = (1$ ms, 50G/cm), $G_5 = (1$ ms, 9G/cm), $G_6 = (1$ ms, 11G/cm). 24 scans per t_1 (32 complex points, spectral width 7183.9 Hz) and t_2 (32 complex points, spectral width 1666.6 Hz) experiment were recorded with 1024 complex points in t_3 (spectral width 7183.9 Hz). A repetition delay of 1.3 s was used between scans, giving rise to a total measurement time of 50 h for the 3D experiment. The measurement time for the 2D versions was 15 h. The relevant operator transfers are given around the two planar mixing periods and the NOE transfer step. Details are explained in the text. The pathway of operators is given under the pulse sequence for the crucial points in the sequence.

segment^{16–18} the operator transformations, $N_{1y}H_{1\alpha} \rightarrow H_{1z}N_{1\alpha}$ and similarly $N_{1y}H_{1\beta} \rightarrow H_{1z}N_{1\beta}$, are unique apart from other nondetectable coherences. Both transfers have an efficiency of $\sin[\pi(J + D)\Delta]$. However, for the second planar mixing, in addition to the desired transfer (full arrow), two undesired transfers are also possible (broken arrows) if Δ deviates from $(2(J + D))^{-1}$ (Figure 1). This complication will only occur in anisotropic (Bicelle, Phages) media where the values of $J + D$ are no longer uniform. The transfer amplitudes for the desired and undesired transfers are $(\sin[\pi(J_1 + D_1)\Delta](1 + \sin^2[\pi(J_1 + D_1)\Delta]))/2$ and $(\sin[\pi(J_1 + D_1)\Delta](\cos^2[\pi(J_1 + D_1)\Delta]))/2$, respectively, and $(J + D)(N_1, H_1)$ has been abbreviated by $(J_1 + D_1)$. The expected cross-peak pattern of the J_{HH} NOESY experiment is shown in Figure 2. In Figure 2 also sections out of the 2D spectra with displacement vectors are shown. To remove the undesired peak contributions the value of $(J + D)(N_1, H_1)$ needs to be determined. Then the amplitudes of the desired and undesired peak components can be derived. Taking into account that the biggest N,H^N dipolar couplings in our case are about ± 25 Hz, the biggest peak contribution is 8%. The undesired peak contributions can be completely removed as previously described.¹⁹ Undesired peak contributions also arise from spin flips during the NOESY mixing

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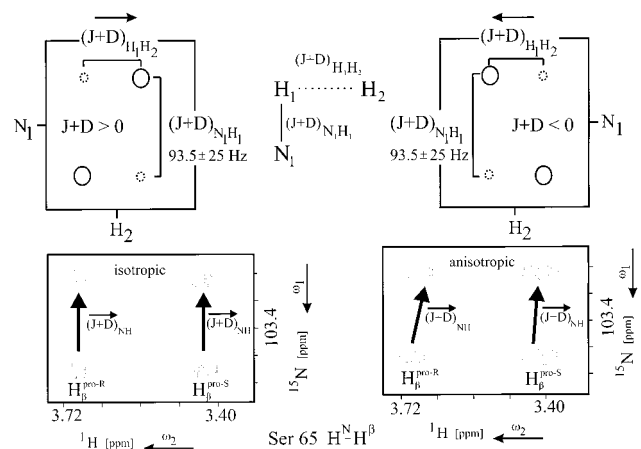


Figure 2. Schematic cross-peak pattern for the J_{HH} -NOESY. Due to the defined sign of $J_1 + D_1$ (93.5 ± 25 Hz provided sufficiently weak alignment is used), the sign of the H,H dipolar coupling can be extracted. The dotted cross-peak contributions arise from the normally weak undesired coherence transfers shown in Figure 1. They cannot be avoided since it is impossible to match Δ to all values of $J + D$. The dotted cross-peak contributions are also modulated by spinflips during the NOESY mixing time as indicated in the text. Also two peaks out of the spectra of Ubiquitin are shown. They display the peaks of Ser 65 H^N - H^B . In isotropic solution the 4J coupling is zero but in anisotropic solution a through space dipolar coupling can be extracted.

time. Therefore it is important that the ^{15}N T_1 time is longer than the NOESY mixing time. This condition is increasingly more easily accomplished going to larger proteins. The average error on the coupling constants was determined to be ± 0.8 Hz based on repeating the experiment.

The experiment was applied to ^{15}N labeled Ubiquitin (VLI Research, Inc., Malvern, PA). The alignment of the sample was achieved with the CHAPSO/DLPC/CTAB system (10:50:1) with 5% total lipid concentration. The reference sample was free of lipids. For both samples we used 3 mg of ^{15}N -labeled Ubiquitin (10 mM phosphate-buffer pH 6.5; $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10) in a 250 μL Shigemits microcell tube. This system allowed us to measure all experiments at 303 K. For the measurement of Ubiquitin, 2D versions of the J_{HH} sequence were also recorded. The digital resolution was put to 0.22 Hz/point in ω_2 . For small proteins, the couplings can be extracted from the 2D versions of the pulse sequence. The quality of the spectra is shown on representative traces in Figure S1. As expected the dipolar couplings show different signs. A total of 67 H,H dipolar coupling could be identified (Supporting Information).

We compared the measured dipolar couplings with the dipolar couplings calculated for structures of Ubiquitin derived from X-ray²⁰ crystallography and from NMR.^{21,22} The alignment tensor was calculated from the $^1D_{NH}$ dipolar coupling constants²³ in the frame of the pdb file using the software *DipoCoup*.²⁴ The Q -values of the H,N^H and the H,H dipolar couplings are listed in Table 1. It is observed that the dipolar H,H couplings fit the calculated NMR structure much better than the X-ray structure. Similar results on carbonyl chemical shifts were observed previously.²¹

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Table 1. Comparison of the Different Q Values²¹

	X-ray	$\langle\text{NMR}\rangle$	NMRopt	ΔQ	$\langle D^{\text{exp}}/D^{\langle\text{NMR}\rangle}$
$D(\text{N}-\text{H}^N)$	0.2312	0.2006	0.1991	14%	0.95
$D(\text{H}^N-\text{H}^X)$	1.0639	0.8324	0.8288	22%	0.70
$D(\text{H}^N-\text{H}^\alpha)$	0.9232	0.6843	0.6880	25%	0.78

^a Out of the 67 measured H^N, H^X dipolar couplings as indicated in Table S2 in the Supporting Information S3 are used for the calculation of the H^N, H^X Q -factor. 14 dipolar couplings mainly H^N, H^V and H^N, H^O were not used because of lack of stereospecific assignment. All 29 measured H^N, H^α couplings are used for the calculation of the H^N, H^α Q -factor. The percentages indicate the improvement of the Q -factor (ΔQ) referring to the X-ray structure versus the NMR structure. (NMR) is the average NMR structure of the 10 best structures and NMRopt is the best structure.²²

The percentage of decrease of the Q -factors for the H,H couplings from the X-ray structure to the NMR structure is even higher compared with that of the N, H^N couplings (Table 1). The larger discrepancies (Q -factors) of experimental and structure derived dipolar couplings for the side chain have also been observed for CH dipolar couplings²⁵ and have been attributed to larger dynamics of the side chains compared to the protein backbone. This is substantiated by our finding that the slope of $D_{HH}^{\text{exp}}/D_{HH}^{\langle\text{NMR}\rangle} = 0.7$ instead of 1 indicating that $\langle S_{HH} \rangle = 0.7 \langle S_{HN} \rangle$. The larger Q -factors observed for the H,H dipolar couplings underlines the potential of these couplings to improve the description of protein structures and dynamics.²⁶

In conclusion, we have presented a method for measuring the sign and value of H,H dipolar couplings. These dipolar couplings are easily extractable and can be used as long-range distance restraints in biomacromolecules. Due to the knowledge of the sign of the proton–proton dipolar couplings also angular restraints can be evaluated. This method allows one to extract all possible H,H dipolar couplings out of two spectra. In principle, all methods for the measurement of H,H scalar couplings based on E.COSY or DQ/ZQ methodology can be used for sign and size determination of H,H dipolar couplings. The E.COSY method proposed here is among the most sensitive methods even for large proteins. It requires minimal isotopic labeling and can be combined with S^3E ²⁷ elements for further resolution enhancement.

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Supporting Information Available: A listing of the eigenvalues of the alignment tensor and the orientation in the bicelle medium used for the measurement and formula for calculation of the dipolar couplings; all measured H,H and N, H^N dipolar couplings in Ubiquitin, graphs for the correlation of the experimental and the calculated theoretical values, and some representative traces are shown; transfer amplitudes for the J_{HH} NOESY experiment are discussed (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>. See any current masthead page for ordering information and Web access instructions.

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A new method for the simultaneous measurement of magnitude and sign of $^1D_{CH}$ and $^1D_{HH}$ dipolar couplings in methylene groups*

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Key words: dipolar couplings, methylene groups, spin state selective transfer, structure determination, SPITZE-HSQC, ubiquitin

Abstract

Heteronuclear dipolar couplings of the protein backbone have proven to have a big impact on the accuracy of protein NMR structures. H,H dipolar couplings might have the same impact on side chains. Here we present a method that combines both heteronuclear and homonuclear dipolar couplings to investigate the local conformation of methylene groups. A new pulse sequence (SPITZE-HSQC) is presented, that allows to measure the two C,H and the H,H dipolar couplings at the same time, using spin state selective transfers. The new method has been applied to the methylene groups of glycines in the protein ubiquitin. The C,H and the H,H dipolar couplings might have a key role in fast stereospecific assignment of protons in CH₂ groups.

Abbreviations: S₃E, spin state selection; HSQC, heteronuclear single quantum correlation; TOCSY, total correlated spectroscopy.

Introduction

Dipolar couplings (Tolman et al., 1995; Tjandra and Bax, 1997) constitute a powerful tool to obtain long-range structural restraints. Residual heteronuclear dipolar couplings have a special impact on structural studies of multidomain proteins (Fischer et al., 1999) where long range restraints are needed to define the orientation of one domain with respect to the other. The size and the sign of residual dipolar couplings can be measured from the difference of the splitting observed in partially aligned and isotropic solutions of the molecule, provided that the amount of alignment is so weak that the absolute value of the dipolar coupling is smaller than the absolute value of the scalar coupling. Dipolar couplings can be used to obtain

local information as well. Here we introduce a new method to measure D_{CH_1} , D_{CH_2} and $D_{H_1H_2}$ dipolar couplings in methylene groups through an S³E-type HSQC (Meissner et al., 1997) in aligned solutions. The sign of the $D_{H_1H_2}$ coupling is obtained as well.

Local structural information on methylene groups is particularly important for hydrophobic side chains located in protein cores. The D_{CH} and D_{HH} couplings can be used to refine the local environment and to obtain the stereospecific assignment of the protons, provided that additional structural information is known. Recently, a method for the measurement of methylene and methyl dipolar couplings has been reported (Ottiger et al., 1998). In this work only the sum of the two CH dipolar couplings in methylene groups could be measured and several spectra needed to be recorded to obtain an accurate fit of the experimental data. With the present approach dipolar couplings for each individual CH vector and also for the HH vector can be easily measured from only four experiments. The H,H dipolar coupling provides additional infor-

*Dedicated to Prof. H. Kessler on the occasion of his 60th birthday.

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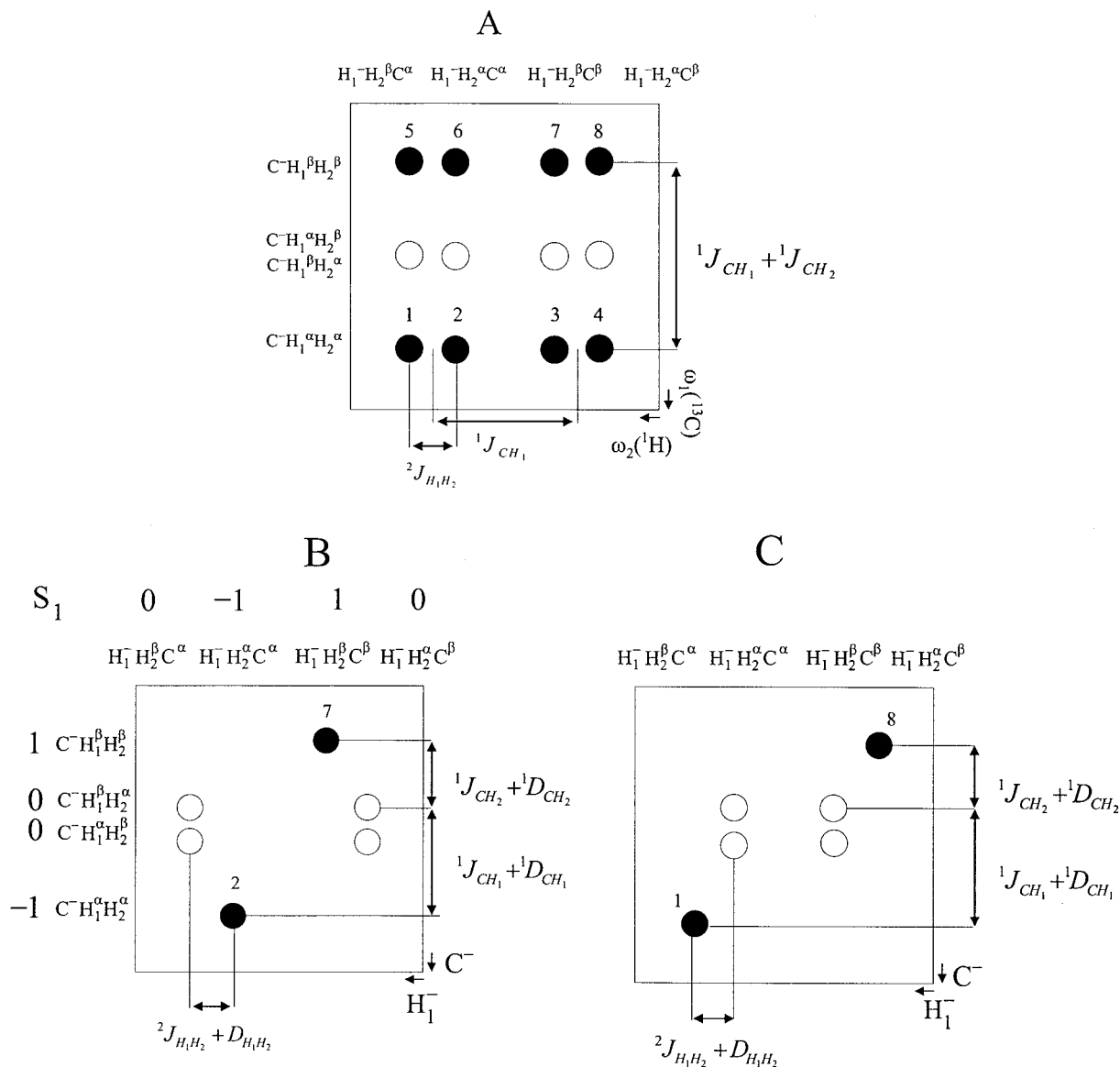


Figure 1. (A) Schematic representation of the peak deriving from a CH_1H_2 group at the frequency of the carbon in ω_1 and of proton H_1 in ω_2 . The central lines (empty circles) are missing provided that J_{CH_1} and J_{CH_2} are equal. The splitting in ω_1 equals the sum of the two coupling constants $J_{CH_1} + J_{CH_2}$. Peaks 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are separated by the $J_{H_1H_2}$ couplings, and they are generally not resolved in proteins due to the large linewidth. (B and C) Schematic representation of the selection rules for the mixing operators $\mathbf{H}_1 = \pi(J_{CH_1} + D_{CH_1})(C_x H_{1x} + C_y H_{1y}) + \pi(J_{CH_2} + D_{CH_2})(C_x H_{2x} + C_y H_{2y})$ in case $J_{CH_1} + D_{CH_1} \neq J_{CH_2} + D_{CH_2}$. The selection rules were derived classifying the coherences according to the symmetry operators $S_1 = C_z H_{1z} + C_z H_{2z}$ and $S_2 = H_{1z} + H_{2z} + C_z$, which commutes with both \mathbf{H}_1 and with the free evolution Hamiltonian. The eigenvalues of all coherences ρ for S_2 are: $[S_2, \rho] = -\rho$. Panel B gives the transfer pattern for the sequence of Figure 3 and panel C for the sequence of Figure 4.

mation with respect to the two C,H dipolar couplings, as shown in simulations, and the combination of the three dipolar couplings restricts the methylene group to four symmetry related orientations.

Theory

A schematic representation of a fully coupled HSQC (Müller, 1979; Bodenhausen and Ruben, 1980) for a CH_1H_2 group at the frequency of the carbon in ω_1 and of proton H_1 in ω_2 is reported in Figure 1A. The central lines (empty circles) are missing since the two contributions from $C^-H_1^\alpha H_2^\beta$ and $C^-H_1^\beta H_2^\alpha$ cancel out, provided that $J_{CH_1} + D_{CH_1}$ and $J_{CH_2} + D_{CH_2}$ are equal. The splitting in ω_1 equals the sum of the two coupling constants $J_{CH_1} + D_{CH_1} + J_{CH_2} + D_{CH_2}$. The peaks that appear in a coupled HSQC are the dark gray ones, which have been numbered in the figure from 1 to 8. Peaks 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are separated by the $J_{H_1H_2} + D_{H_1H_2}$ couplings, and they are generally not resolved in proteins due to the large linewidth. The experiment we are going to describe achieves selective transfer between the following operators through a S³E scheme:

$$\begin{aligned} C^-H_1^\alpha H_2^\alpha &\rightarrow H_1^-H_2^\beta C^\alpha && \text{peak 1} \\ C^-H_1^\beta H_2^\beta &\rightarrow H_1^-H_2^\alpha C^\beta && \text{peak 8} \end{aligned} \quad (1)$$

or

$$\begin{aligned} C^-H_1^\alpha H_2^\alpha &\rightarrow H_1^-H_2^\alpha C^\alpha && \text{peak 2} \\ C^-H_1^\beta H_2^\beta &\rightarrow H_1^-H_2^\beta C^\beta && \text{peak 7} \end{aligned} \quad (2)$$

This provides high reduction of spectral overlap and the possibility to measure all three involved couplings. Four spectra are obtained, each of which contains only one peak per CH_2 group. For partially aligned systems, the selectivity is independent of the identity of the couplings $J_{CH_1} + D_{CH_1}$ and $J_{CH_2} + D_{CH_2}$. The selective transfer presented here has several advantages in comparison to a coupled HSQC. First, for large molecules, where peaks 1 (8) and 2 (7) are in general not at all or only partially resolved, this approach allows the measurement of $J_{H_1H_2} + D_{H_1H_2}$ coupling constants, as peaks 1 (8) and 2 (7) now appear in two different spectra. Second, whereas the $J_{CH_1} + D_{CH_1}$ and $J_{CH_2} + D_{CH_2}$ couplings are measurable also in a coupled HSQC, the new approach allows the separation of the corresponding lines in two spectra, with greatly reduced spectral overlap. Third, due to the selectivity of the transfer described in Equations 1 and

2, the magnetization corresponding to the $C^-H_1^\alpha H_2^\alpha$ and the $C^-H_1^\beta H_2^\beta$ terms is not distributed on four lines, as in a coupled HSQC, but concentrated on one single line with maximum possible transfer efficiency (Glaser et al., 1998). This leads to a gain of a factor 4 in signal-to-noise with respect to a coupled HSQC for molecules for which the $J_{H_1H_2}$ coupling is resolved, and of a factor 2 for those molecules for which the $J_{H_1H_2} + D_{H_1H_2}$ coupling is not resolved, as has been found in spectra of diacetonglucose (data not shown).

The design of the new pulse sequence, which we call SPITZE-HSQC (SPIn sTate selective ZERO overlap HSQC), can be understood most easily in terms of selection of desired transitions in an energy level diagram (Figure 2). We will describe the design of the pulse sequence which achieves the operator transfers of Equation 1. The following explanation is rigorously valid only assuming $J_{CH_1} + D_{CH_1} = J_{CH_2} + D_{CH_2}$. The energy level diagram is that of two protons which sum up to a spin 1 and a spin 0 particle coupled to ^{13}C , which is the spin 1/2. The spin 0 of the two protons is not excited assuming equal $J_{CH} + D_{CH}$ couplings and is thus ignored in the level diagram. The energy level diagram consists of the following six levels: $|\alpha, 1\rangle$, $|\alpha, 0\rangle$, $|\alpha, -1\rangle$, $|\beta, 1\rangle$, $|\beta, 0\rangle$ and $|\beta, -1\rangle$, where for example $|\alpha, 0\rangle$ means that the carbon is in the α spin state and the spin 1 particle formed by the two protons has $F_z = H_{1z} + H_{2z} = 0$. These levels can be expressed as product functions with $|\alpha_C/\beta_C\rangle$ referring to carbon in the α or β state:

$$\begin{aligned} |\alpha/\beta, 1\rangle &= |\alpha_C/\beta_C\rangle |\alpha_{H_1}\rangle |\alpha_{H_2}\rangle \\ |\alpha/\beta, 0\rangle &= \frac{1}{\sqrt{2}} |\alpha_C/\beta_C\rangle (|\alpha_{H_1}\rangle |\beta_{H_2}\rangle \\ &\quad + |\beta_{H_1}\rangle |\alpha_{H_2}\rangle) \\ |\alpha/\beta, -1\rangle &= |\alpha_C/\beta_C\rangle |\beta_{H_1}\rangle |\beta_{H_2}\rangle \end{aligned} \quad (3)$$

The operators $C^-H_1^\alpha H_2^\alpha = |\beta, 1\rangle \langle \alpha, 1|$ and $C^-H_1^\beta H_2^\beta = |\beta, -1\rangle \langle \alpha, -1|$ describe the two transitions indicated respectively with continuous and dashed black arrows in Figure 2A. These operators must be transferred selectively to the terms $(H_1^-H_2^\alpha C^\alpha + H_2^-H_1^\alpha C^\alpha) = \sqrt{2} |\alpha, 0\rangle \langle \alpha, 1|$ and $(H_1^-H_2^\beta C^\beta + H_2^-H_1^\beta C^\beta) = \sqrt{2} |\beta, -1\rangle \langle \beta, 0|$, respectively, which correspond to the two transitions indicated with continuous and dashed black arrows in Figure 2B. These selective transfers are obtained interchanging the energy levels that are connected by the wavy lines in Figure 2A. Both transfers are brought about by a zero quantum rotation $C_x F_x + C_y F_y$ by an

1 and 8

2 and 7

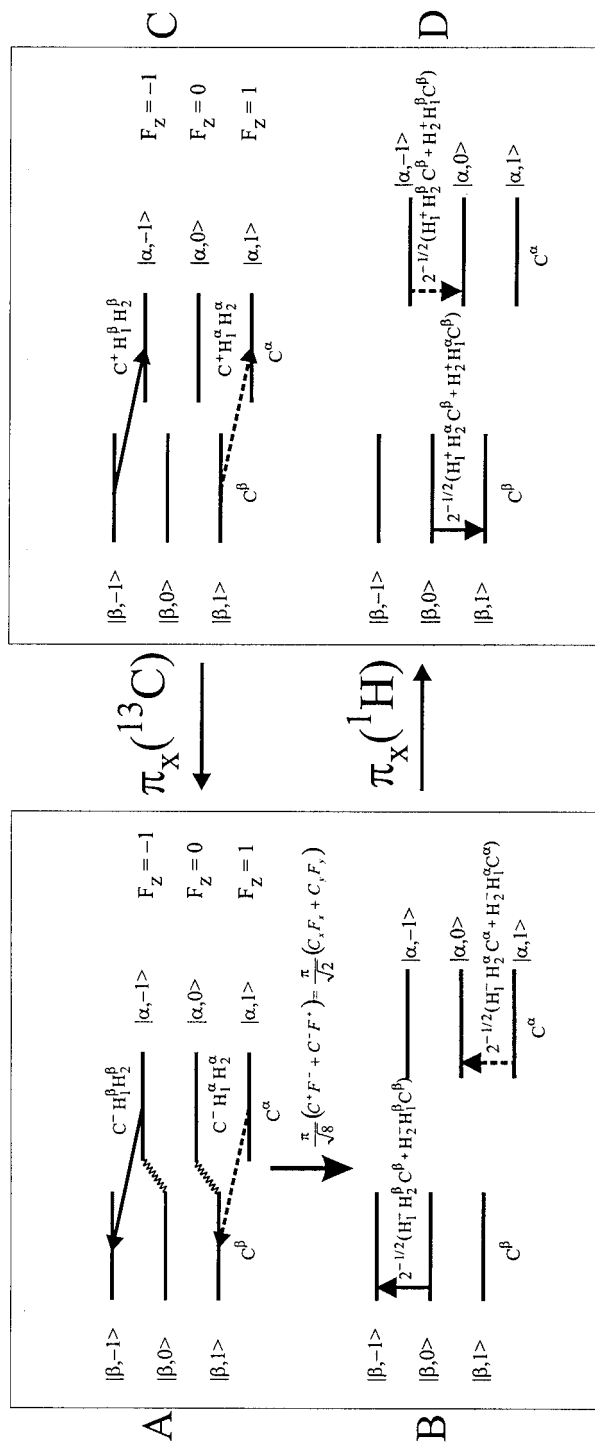


Figure 2. Symmetry adapted energy level diagram for a CH_1H_2 group. The arrows represent the desired transitions of Equations 1 and 2 in ω_1 (panel A) and ω_2 (panel B). The two levels connected by wavy lines must be interchanged to obtain the desired selective transfers, as explained in the text. For the selection of peaks 1 and 8, a $\pi_X(^{13}C)$ is introduced before the mixing, which transforms the coherences of C into A. Then the mixing from A to B is applied and finally the coherences are transformed to those in D by a $\pi_X(^1H)$ pulse. Altogether the transfer $C^+ \rightarrow H^+$ is shown. The desired transfer $C^- \rightarrow H^-$ is obtained with equal efficiency due to the hermiticity of all Hamiltonians in NMR.

angle $\pi/\sqrt{2}$:

$$\begin{aligned}
 & e^{i\frac{\pi}{\sqrt{8}}(F^+C^-+F^-C^+)} |\beta, 1\rangle \langle\alpha, 1| e^{-i\frac{\pi}{\sqrt{8}}(F^+C^-+F^-C^+)} = \\
 & e^{i\frac{\pi}{2}(|\alpha, -1\rangle\langle\beta, 0|+|\alpha, 0\rangle\langle\beta, 1|+|\beta, 1\rangle\langle\alpha, 0|+|\beta, 0\rangle\langle\alpha, -1|)} \\
 & |\beta, 1\rangle \langle\alpha, 1| \quad (4) \\
 & e^{-i\frac{\pi}{2}(|\alpha, -1\rangle\langle\beta, 0|+|\alpha, 0\rangle\langle\beta, 1|+|\beta, 1\rangle\langle\alpha, 0|+|\beta, 0\rangle\langle\alpha, -1|)} = \\
 & i |\alpha, 0\rangle \langle\alpha, 1|
 \end{aligned}$$

The rotation operator $(\pi/\sqrt{2})(C_xF_x + C_yF_y)$ is implemented in the following way: $\pi J(C_xF_x + C_zF_z)$ is implemented by a heteronuclear DIPSI (Shaka et al., 1988; Rucker and Shaka, 1989), leading to a rotation by $\pi/\sqrt{2}$ after $\tau = 1/\sqrt{2}(J_{CH} + D_{CH})$; application of $90_x^\circ(^1\text{H}, ^{13}\text{C})$ and $90_{-x}^\circ(^1\text{H}, ^{13}\text{C})$ pulses before and after the DIPSI sequence, respectively transforms this Hamiltonian into the desired zero quantum rotation operator. The pulse sequence is shown in Figure 3A and delivers peaks 2 and 7 of Figure 1; the quadrature detection in t_1 is obtained by echo-antiecho selection. Evolution of homonuclear scalar couplings and dipolar couplings has no influence on the selectivity of the transfer since the functions of Equation 3 are eigenfunctions for the J-coupling Hamiltonian $2\pi J_{12}\hat{H}_1 \cdot \hat{H}_2$ and for the dipolar coupling Hamiltonian, which for the DIPSI mixing sequence in Figure 3A is $-\pi D_{12}(H_{1z}H_{2z} - \frac{1}{2}(H_{1x}H_{2x} + H_{1y}H_{2y}))$ on resonance.

Selection of peaks 1 and 8 is achieved by introduction of a $180_x^\circ(^{13}\text{C})$ pulse before and $180_x^\circ(^1\text{H})$ pulse after the zero quantum rotation, as indicated in Figures 2C and D. The transfer is depicted there for $C^+ \rightarrow H^+$. Due to the hermicity of all operators, the transfer $C^- \rightarrow H^-$ occurs with equal efficiency. The pulse sequence for this transfer (Figure 4A) is derived from the pulse sequence in Figure 3A by appending a $180_x^\circ(^{13}\text{C})$ pulse before and a $180_x^\circ(^1\text{H})$ pulse after the mixing performed in Figure 3A.

The peaks 2 and 7 as well as 1 and 8 of the schematic pulse sequence of Figures 3A and 4A, respectively, can be separated in two different spectra by an S^3E -type selection after the TOCSY transfer (Figures 3B,C and 4B,C). The two final spectra are obtained by adding and subtracting the two experiments of Figures 3 B and C and of Figures 4B and C, respectively. In the sequence of Figure 3B, the magnetization terms present at point a and corresponding to peaks 2 and 7 can be represented by the two arrows of Figure 5A. These terms defocus with respect to the $J_{CH} + D_{CH}$ couplings for a total delay $1/(4(J_{CH} + D_{CH}))$, during which they acquire a phase difference of 90° ($\pm 45^\circ$). In the experiment of Figure 3B, no

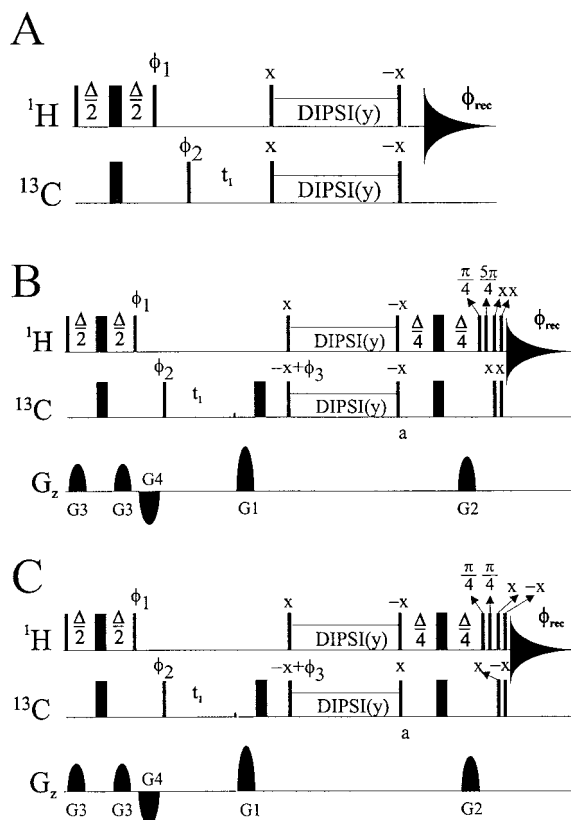


Figure 3. (A) Schematic sequence which implements the zero quantum Hamiltonian for the selection of peaks 2 and 7. The DIPSI sequence is along y and is irradiated with a field of 6250 Hz. (B and C) Pulse sequences for the selection of peaks 2 and 7 and their separation in two spectra. All pulses are hard. Phases not explicitly given are equal to x ; $\phi_1 = y, -y$; $\phi_2 = x, x, -x, -x$; $\phi_{\text{rec.}} = x, -x, x, -x$. Gradients are $G1 = 4G2$. Quadrature detection in t_1 is achieved by adding and subtracting two FIDS acquired with $\phi_3 = 0$, $G1 = 4G2$ and $\phi_3 = \pi$, $G1 = -4G2$ in the experiment of panels B and C. The experiments resulting from the pulse sequences of panels B and C must be added and subtracted to obtain the desired separation. The two $180_x^\circ(^{13}\text{C}, ^1\text{H})$ pulses before the acquisition time can be shifted after the TOCSY mixing and before the defocusing delay $\Delta/2$ and therefore translated into a phase shift of the 90° pulse flanking the TOCSY mixing on the corresponding nucleus. This achieves a reduction of the number of pulses needed for the desired selection.

effective pulse is applied at the end of this delay. The experiment of Figure 3C differs from the experiment of Figure 3B until point a by a $180_x^\circ(^{13}\text{C})$ pulse; therefore peaks 4 and 5 are selected instead of peaks 2 and 7 from the TOCSY sequence. The two $180_x^\circ(^{13}\text{C}, ^1\text{H})$ pulses during the defocusing delay $1/(4(J_{CH} + D_{CH}))$ transform them in peaks 1 and 8. At the end of this delay a 180° pulse with 45° phase is applied to the proton. This pulse turns the magnetization corresponding to peaks 1 and 8 in 2 and 7 (with negative amplitude),

Table 1. $J_{H_1H_2}$, J_{CH_1} and J_{CH_2} scalar couplings for glycines in non-oriented ubiquitin (columns 1–3); $D_{H_1H_2}$, D_{CH_1} and D_{CH_2} experimental (columns 4–6) and calculated (columns 7–9 (X-RAY) and 10–12 (NMR)) dipolar couplings for glycines in oriented ubiquitin (details are given in the text). The error of measurement of the couplings is ± 0.4 Hz.

	Non-oriented ubiquitin scalar couplings			Oriented ubiquitin: dipolar couplings			Theoretical dipolar couplings (X-RAY)			Theoretical dipolar couplings (NMR)		
	$J_{H_1H_2}$ (Hz)	J_{CH_1} (Hz)	J_{CH_2} (Hz)	$D_{H_1H_2}$ (Hz)	D_{CH_1} (Hz)	D_{CH_2} (Hz)	$D_{H_1H_2}$ (Hz)	D_{CH_1} (Hz)	D_{CH_2} (Hz)	$D_{H_1H_2}$ (Hz)	D_{CH_1} (Hz)	D_{CH_2} (Hz)
	Gly10	-17.6	143.7	142.4	-12	52.3	-16.6	-19.9	43.4	-33.6	-14.1	64.5
Gly35	-16.5	145.2	142.8	18.7			21.6	-33.9	94.2	36.1	-31.4	118.0
Gly47	-17.6	143.4	139.5	-34.2	45.5	-29.4	-12.8	58.6	-44.1	-25.8	53.3	-39.7
Gly75	-16.6	142.3	142.3	-3.0	8.3	8.3	4.9	32.7	-44.8	-25.0	0.0	-78.4
Gly76	-17.3	140.4	140.6	-1.9	4.8	4.4	-37.6	-39.5	-31.9	-19.5	-9.0	-62.8

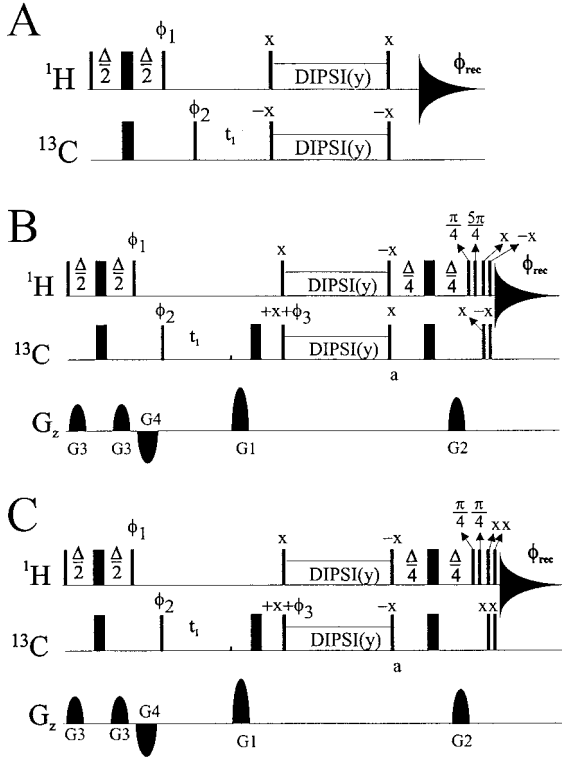


Figure 4. (A) Schematic sequence for the selection of peaks 1 and 8, obtained adding a $180_x^{(13C)}$ pulse before and a $180_x^{(1H)}$ pulse after the mixing time of Figure 3A. The DIPSI sequence is along y and is irradiated with a field of 6250 Hz. (B and C) Pulse sequences for the selection of peaks 1 and 8 and their separation in two spectra. All pulses are hard. Phases not explicitly given are equal to x ; $\phi_1 = y, -y$; $\phi_2 = x, x, -x, -x$; $\phi_{rec.} = x, -x, x, -x$. Gradients are $G1 = 4G2$. Quadrature detection in t_1 is achieved by adding and subtracting two FIDS acquired with $\phi_3 = 0, G1 = 4G2$ and $\phi_3 = \pi, G1 = -4G2$ in the experiment of panels B and C. The experiments resulting from the pulse sequences of panels B and C must be added and subtracted to obtain the desired separation. The two $180_x^{(13C, 1H)}$ pulses before the acquisition time can be shifted after the TOCSY mixing and before the defocusing delay $\Delta/2$ and therefore translated into a phase shift of the 90° pulse flanking the TOCSY mixing on the corresponding nucleus. This achieves a reduction of the number of pulses needed for the desired selection.

respectively. Adding and subtracting the two experiments 3B and 3C, separation of the peaks 2 and 7 in two different spectra is obtained. Analogously, the two sequences of Figures 4B,C achieve separation of the peaks 1 and 8 in two spectra. Since the $J_{CH} + D_{CH}$ couplings are different for each CH vector in each methylene group, no optimal value for the defocusing delay Δ can be used for every peak. We set Δ equal to $1/(4J_{CH})$, which introduces a phase difference between lines 1, 2 and 7, 8 equal to $2\pi D_{ch}\Delta$. This phase difference must be corrected for interactively

with coupling constants extraction, as described by Carlomagno et al. (1998), to avoid systematic errors.

If $J_{CH_1} + D_{CH_1} \neq J_{CH_2} + D_{CH_2}$ the transfers described in Equations 1 and 2 are still selective, as will be demonstrated in the following. In addition, $C^-H_1^\alpha H_2^\beta$ and $C^-H_1^\beta H_2^\alpha$, corresponding to the central lines (empty circles) of Figure 1A, are now excited and can be observed. These transitions are selectively transformed into the transitions $H_1^- C_\alpha H_2^\beta$, $H_1^- C_\beta H_2^\alpha$, $H_2^- C_\alpha H_1^\beta$ and $H_2^- C_\beta H_1^\alpha$ by the sequence of Figure 3A. The selective transfers for the case $J_{CH_1} + D_{CH_1} \neq J_{CH_2} + D_{CH_2}$ can be understood in the following way. The ZQ mixing Hamiltonian $\mathbf{H}_1 = \pi(J_{CH_1} + D_{CH_1})(C_x H_{1x} + C_y H_{1y}) + \pi(J_{CH_2} + D_{CH_2})(C_x H_{2x} + C_y H_{2y})$, implemented by the DIPSI sequences of Figure 3B, commutes with the two symmetry operators $\mathbf{S}_1 = C_z H_{1z} + C_z H_{2z}$ and $\mathbf{S}_2 = H_{1z} + H_{2z} + C_z$, which is the operator measuring the coherence order. The free evolution Hamiltonian before and after the mixing also commutes with \mathbf{S}_1 and \mathbf{S}_2 . Therefore coherences before and after the heteronuclear TOCSY mixing, brought about by \mathbf{H}_1 , can be classified according to both symmetry operators \mathbf{S}_1 and \mathbf{S}_2 (Levitt et al., 1985). One finds the following eigenvalues for the commutators $[\mathbf{S}_1, \rho] = s_1\rho$ and $[\mathbf{S}_2, \rho] = s_2\rho$:

	\mathbf{S}_1	\mathbf{S}_2
$H_1^- C_\alpha H_2^\alpha, H_2^- C_\alpha H_1^\alpha \longleftrightarrow C^- H_1^\alpha H_2^\alpha$	\longleftrightarrow	$-1 \quad -1$
$H_1^- C_\alpha H_2^\beta, H_2^- C_\alpha H_1^\beta \longleftrightarrow C^- H_1^\alpha H_2^\beta$	\longleftrightarrow	$0 \quad -1$
\otimes		
$H_1^- C^\beta H_2^\alpha, H_2^- C^\beta H_1^\alpha \longleftrightarrow C^- H_1^\beta H_2^\alpha$	\longleftrightarrow	$0 \quad -1$
$H_1^- C^\beta H_2^\beta, H_2^- C^\beta H_1^\beta \longleftrightarrow C^- H_1^\beta H_2^\beta$	\longleftrightarrow	$1 \quad -1$

The heteronuclear TOCSY achieves transfer only between coherences which have the same eigenvalue for both the symmetry operators \mathbf{S}_1 and \mathbf{S}_2 . Therefore, only the peaks shown in Figure 1B are selected by the experiment of Figure 3A. The selection rule for the experiment of Figure 4 is derived by adding a $\pi_x^{(13C)}$ pulse before and a $\pi_x^{(1H)}$ after the DIPSI mixing scheme, as explained above. This inverts the coherence orders before and after the mixing and in addition the spin state of the coupled proton in ω_2 , yielding peaks 1 and 8 (Figure 1C). The presence of the additional peaks deriving from the $C^-H_1^\alpha H_2^\beta$ and $C^-H_1^\beta H_2^\alpha$ coherences in ω_1 increases spectral overlap but does not hinder the reliable extraction of the three $J + D$ couplings, since they appear at a different frequency in ω_1 compared to the interesting

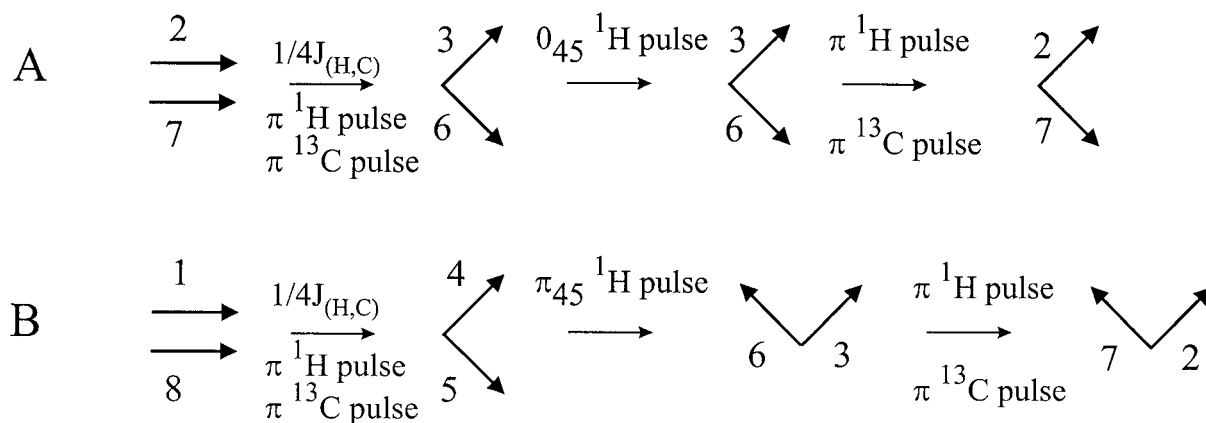


Figure 5. Pictorial explanation of the peak selection which takes place in sequences 3B and 3C after point *a*. In panel A the magnetization terms corresponding to peaks 2 and 7 are defocused with respect to the $J_{CH} + D_{CH}$ coupling for a total delay $1/(4(J_{CH} + D_{CH}))$; as a consequence they acquire a 90° phase difference in the proton dimension. In panel B inter-conversion between various lines occurs due to the presence of $\pi(^{13}\text{C})$ and $\pi(^1\text{H})$ pulses, as reported in the figure. The final terms are the same as those in panel A, but peak 7 has undergone a sign inversion, as desired for the S^3E selection.

traces. Moreover, in large macromolecules these peaks have a broader linewidth in ω_1 than the desired ones, due to cross-correlated relaxation Γ_{CH_1,CH_2} , and may partially cancel out depending on the relative size of the $J_{CH_1} + D_{CH_1}$ and $J_{CH_2} + D_{CH_2}$ couplings. In the oriented sample measured for the present study these peaks could not be observed, although some of the D_{CH} couplings were up to 50 Hz large.

The S^3E element does no longer work perfectly when $J_{CH} + D_{CH}$ deviates from $1/(2\Delta)$. In this case, the initial estimation of the $J_{CH} + D_{CH}$ coupling constant can be obtained from the SPITZE-HSQC spectrum. This can be used for phase correction in ω_2 for the two spectra to be added and subtracted for the S^3E selection. In this way a clean suppression of the undesired peaks is achieved.

Experimental and results

The new SPITZE-HSQC sequences have been applied to a 10 mg ^{13}C , ^{15}N labeled sample of ubiquitin in a $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 solution at pH 5.0 and 303 K (commercially available from VLI Research, Inc., Malvern, PA) in 5 mm NMR tubes (500 μl). All experiments were performed at 300 K on Bruker DRX 600 MHz spectrometers (Bruker AG, Rheinstetten, Germany) equipped with TXI HCN z-grad probes. Spectra were processed using FELIX 98.0 (MSI, San Diego, CA).

The spectra resulting from the pulse sequences of Figures 3 and 4 after the post-acquisition data treatment explained in the previous section are shown in

Figure 6 for the region containing the CH_2 correlations of glycines. Separation of peaks 1 and 8 has been achieved in the two spectra shown in the two upper panels, while peaks 2 and 7 are in the spectra shown in the two lower panels. The difference in the resonance frequencies of peaks 1 and 8 corresponds to $J_{CH} + D_{CH} + J_{HH} + D_{HH}$, that of peaks 2 and 7 to $J_{CH} + D_{CH} - J_{HH} - D_{HH}$ and that of peaks 1 and 2 and 7 and 8, respectively, to $J_{HH} + D_{HH}$. From the SPITZE-HSQC spectra the three coupling constants $J_{H_1H_2} + D_{H_1H_2}$, $J_{CH_1} + D_{CH_1}$ and $J_{CH_2} + D_{CH_2}$ can be measured for all six glycines (Table 1). The aforementioned advantages of the SPITZE-HSQC with respect to a coupled HSQC are illustrated in Figure 7. For peak 1 of the H_1 of Gly10 a gain in signal-to-noise of a factor 3 is found in the SPITZE-HSQC experiment with respect to the coupled HSQC. Moreover, in the coupled HSQC peak 8 of Gly10 overlaps with peak 1 of Gly47, hindering the extraction of the J_{CH_1} . In the novel approach peaks 1 and 8 are separated in two spectra and all J_{CH} couplings can be measured without ambiguities.

The same experiments have been repeated for 1.5 mg of ^{13}C , ^{15}N labeled ubiquitin (VLI Research) dissolved in 5% CHAPSO/DLPC/CTAB (1:5:0.1) (pH 5, 10 mM phosphate buffer, 90/10 $\text{H}_2\text{O}/\text{D}_2\text{O}$) bicelles in Shigemitsu microcell tubes (250 μl) (Losonczi and Prestegard, 1998; Wang et al., 1998). Traces for residue Gly 10 are shown in Figure 8. The alignment tensor was calculated based on the X-ray and NMR structure and has been calculated with the program *Dipocoup* (Meiler et al., 2000) using $^1\text{D}_{\text{NH}}$ dipolar

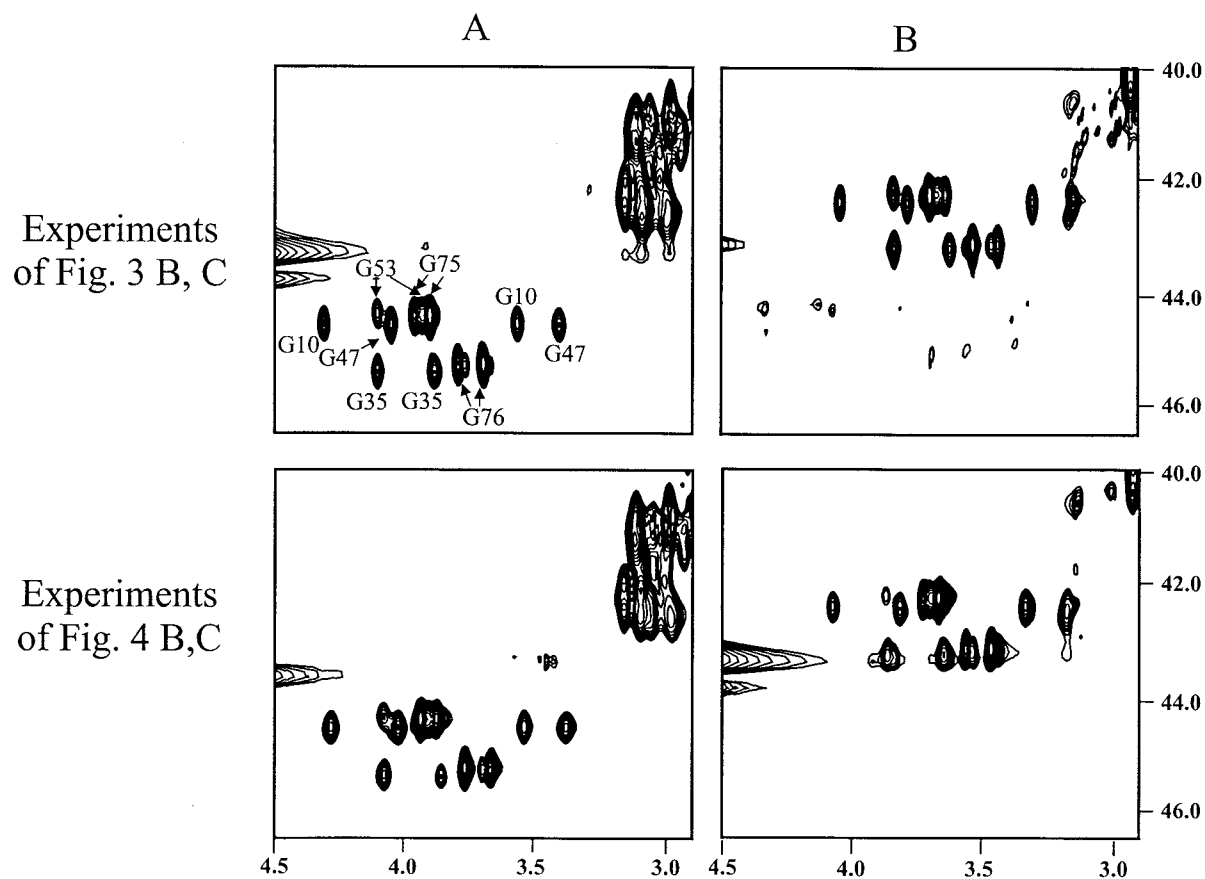


Figure 6. Spectra deriving from adding (panel A) and subtracting (panel B) the experiment of Figures 3B,C and 4B,C for a 1.5 mM sample of $^{13}\text{C}/^{15}\text{N}$ labeled ubiquitin. ^{15}N and ^{13}C were decoupled in t_1 with 180° pulses. The region corresponding to the glycine CH_2 groups is shown. Each experiment has run for 8 h for a total of 2048×512 real points. Data were linear predicted in t_1 to 512 complex points and then zero-filled to 1024 points.

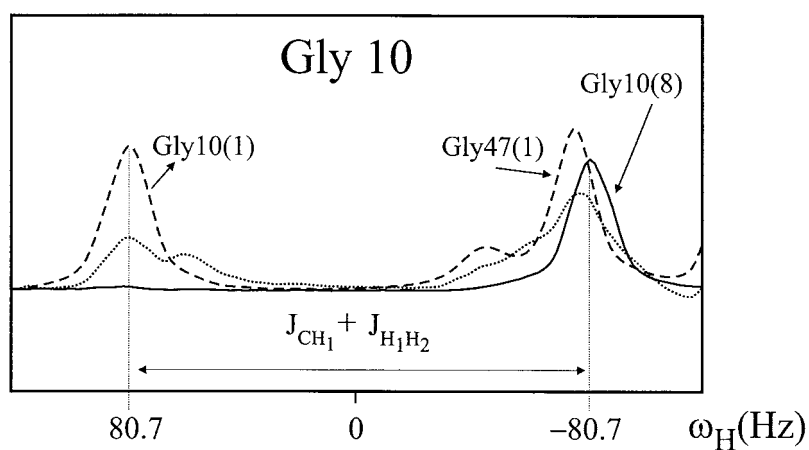


Figure 7. Traces extracted from the spectra in the upper panels of Figure 6 (dashed line: panel A; continuous line: panel B) at the C_α chemical shift of Gly10. The dotted line represents the corresponding trace in a coupled HSQC, which has run for as long as one SPITZE-HSQC sequence. The trace has been multiplied by 2 to account for the fact that the two SPITZE-HSQC experiments of Figure 4, which give rise to the corresponding dashed and continuous traces, have run together for twice as long as the HSQC experiment. In the HSQC trace peak 1 of Gly47 overlays with peak 8 of Gly10, hindering the extraction of J_{CH_1} . In the SPITZE-HSQC experiments peaks 1 and 8 are separated in two spectra and all J_{CH} couplings can be measured without ambiguities.

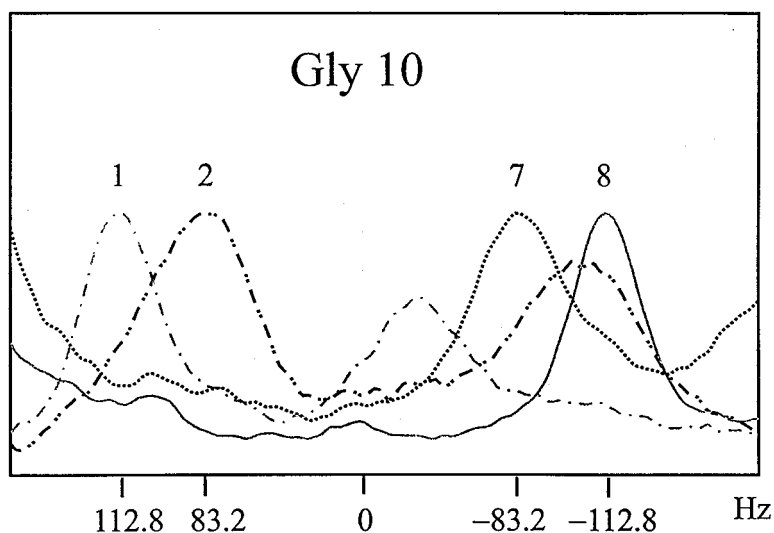


Figure 8. Traces corresponding to peaks 1 (dashed line), 2 (dashed dotted line), 7 (dotted line) and 8 (continuous line) for residue Gly 10 of ubiquitin dissolved in 5% CHAPSO/DLPC/CTAB (1:5:0.1) (pH 5 10 mM phosphate buffer, 90/10 H₂O/D₂O) bicelles at the chemical shift of proton H₁. $J_{CH} + D_{CH}$ couplings can be extracted from the chemical shift difference of lines 1 and 7 or 2 and 8, while $J_{HH} + D_{HH}$ is obtained from the chemical shift difference of lines 1 and 2 or 7 and 8.

couplings measured with the S³E (Meissner et al., 1997) method. A total of 56 ¹D_{NH} dipolar couplings could be measured. The Q-factor (Cornilescu et al., 1999) of the experimental dipolar couplings compared with the one calculated from the crystal structure of ubiquitin (Vijay-Kumar et al., 1987) is 0.28, which equals a correlation coefficient of $R = 0.96$. The eigenvalues of the alignment tensor are defined: $D_{xx} = 11.6$ Hz, $D_{yy} = 49.3$ Hz, $D_{zz} = -60.9$ Hz ($D_{ax} = -30.5$ Hz, $R = 0.41$). Q is equal to 0.199 ($R = 0.98$) by comparison with a refined NMR structure obtained taking dipolar couplings into account (Cornilescu et al., 1999a,b) (alignment tensor: $D_{xx} = 13.8$ Hz, $D_{yy} = 47.8$ Hz, $D_{zz} = -61.6$ Hz, $D_{ax} = -30.8$ Hz, $R = 0.37$).

For this partially aligned sample the resonances of Gly53 are too broad to be detectable and the resonances of the two H_α protons of Gly35 are shifted under the water resonance for two of the four SPITZ-ESQC spectra. The values of the $D_{H_1H_2}$, D_{CH_1} and D_{CH_2} for the remaining four glycines are reported in Table 1, together with the theoretical values calculated from the NMR and X-ray structure. The sign of the $J_{H_1H_2} + D_{H_1H_2}$ coupling is the same as for the $J_{H_1H_2}$ coupling if the relative position of lines 1 and 2 is not inverted with respect to that of the non-oriented sample (peak 1 downfield, peak 2 upfield). All calculations of dipolar couplings have been performed with the program *DipoCoup* (Meiler et al., 2000).

The $D_{H_1H_2}$, D_{CH_1} and D_{CH_2} dipolar couplings for Gly10 and Gly47 and the $D_{H_1H_2}$ coupling for Gly35 do not agree well with the X-ray structure but better with the NMR structure. The numerical agreement is not excellent, as for other H,H dipolar coupling measurement (Cai et al., 1999; Peti and Griesinger, 2000), which reflects the presence of motion as well as deviations from the final structure. The agreement for the mentioned glycine residues can be increased assuming an order parameter of 0.85, which is in line with the order parameter measured for the NH vectors from relaxation measurements. Thus most of the deviation between measured and predicted dipolar couplings can be attributed to motion.

The measured dipolar couplings for Gly75 and Gly76 do not agree with the calculated ones due to the high degree of disorder at the C-terminal part of the molecule. The experimental value of D_{CH_1} is equal to that of D_{CH_2} for both residues, as is expected in the presence of extensive conformational averaging.

For side-chain methylene groups a lower sensitivity is expected due to the presence of $J_{CC} + D_{CC}$ couplings. While the quality of the selectivity is not affected by the presence of other carbon coupling partners, as shown in theoretical simulations and in experiments on diacetone-glucose (unpublished results), the sensitivity of the transfer will be. The mixing time $1/(\sqrt{2}J_{CH})$ is almost three times shorter than that required for a complete transfer through J_{CC} couplings.

Nevertheless, a signal-to-noise loss of up to a factor 2 can take place for non-oriented samples, and perhaps more in oriented systems, depending on the D_{CC} . In special cases selective carbon mixing sequences could be used to avoid the magnetization transfer to neighboring carbons. Moreover, resolution in the carbon dimension can be enhanced by a constant time version of the experiment or a 3D correlation.

Conclusions

We have presented a method to measure the $D_{H_1H_2}$, D_{CH_1} and D_{CH_2} dipolar couplings in methylene moieties based on an S^3E -type correlation. The proposed experiments allow the extraction of magnitude and sign of all three dipolar couplings with minimal spectral overlap and increased signal-to-noise with respect to a coupled HSQC. The method has been demonstrated on the glycine residues of partially oriented ubiquitin. The measured dipolar couplings can be used in local structural refinements and constitute a great help in the stereospecific assignment of protons.

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DipoCoup: A versatile program for 3D-structure homology comparison based on residual dipolar couplings and pseudocontact shifts

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Abstract

A program, *DipoCoup*, is presented that allows to search the protein data bank for proteins which have a three dimensional fold that is at least partially homologous to a protein under investigation. The three dimensional homology search uses secondary structure alignment based on chemical shifts and dipolar couplings or pseudocontact shifts for the three dimensional orientation of secondary structure elements. Moreover, the program offers additional tools for handling and analyzing dipolar couplings.

Introduction

One goal of post genomic research is to determine all protein folds. The number of folds is expected to be limited (Sali, 1998; Fischer and Eisenberg, 1999). Sequence profile methods nowadays have a big impact in fold recognition. *Ab initio* structure prediction works up to 40–60 amino acids and may emerge as a powerful tool for structure prediction in the future (Moult, 1999). To obtain a complete coverage of folds most effectively, it is important to focus on the elucidation of structures with novel folds rather than rediscovering known folds on new proteins. Blast threading and *ab initio* approaches rely on the analysis of primary and secondary structure in the context of a three dimensional structure database. We will present experimental tools that allow to compare the 3D fold of a new protein to all known folds in an early stage of NMR based structure determination. This approach has the potential to predict folds of a new protein with little homology to proteins with known folds. By the same token, structure elucidation of a new protein with

a structure homologous to a known fold will be accelerated. There is so far only one example of using experimental NMR parameters in an early stage for 3D homology searches (Annala et al., 1999). Recently the possibility for using protein fragments generated from PDB and chosen by aligning similar dipolar couplings and chemical shifts for structure determination was shown (Delaglio et al., 2000). The availability of orientation information from NMR experiments in terms of residual dipolar couplings (Tolman et al., 1995; Tjandra and Bax, 1997; Bax and Tjandra, 1997; Clore et al., 1998a; Fischer et al., 1999; Peti and Griesinger, 2000; Meiler et al., 2000) offers new possibilities in this field. In this paper, we present a versatile program, *DipoCoup*, that uses chemical shifts for the alignment of secondary structure elements and tertiary structure alignment from dipolar couplings and pseudocontact shifts for the homology search in the PDB. We will show, using examples, that the program is fast enough to search through a large number of pdb files.

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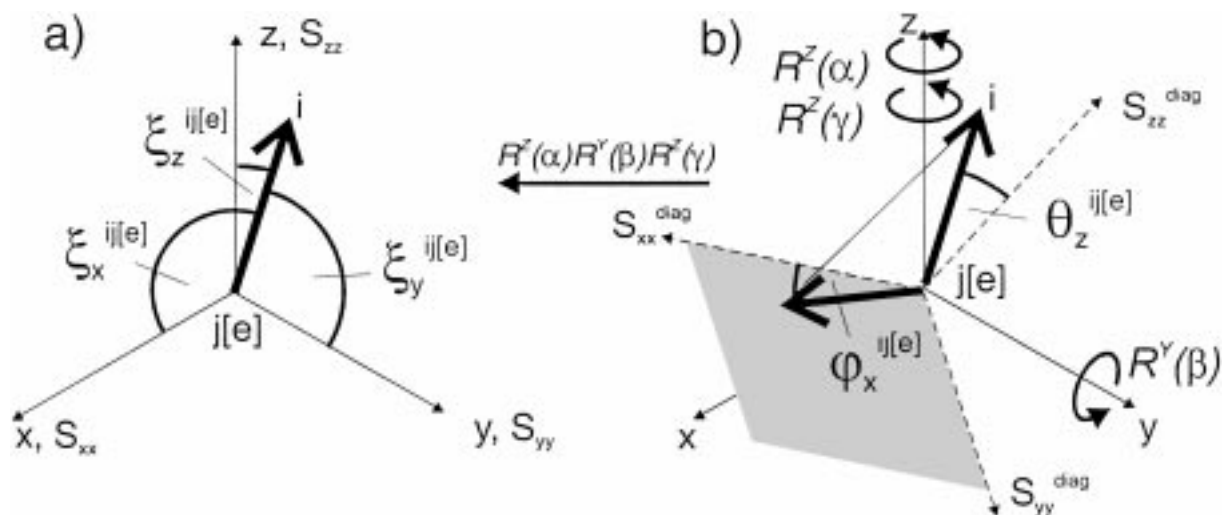


Figure 1. (a) Coordinate system of the molecule (x, y, z) with a bond vector between the two nuclei i and j or a nucleus i and an electron e . The projection angles of the vector onto the x, y, z axes are $\xi_x^{ij[e]}$, $\xi_y^{ij[e]}$ and $\xi_z^{ij[e]}$, respectively. (b) Representation of the vector in the frame of the tensor S_{xx}^{diag} , S_{yy}^{diag} , S_{zz}^{diag} . The Euler rotation transforms the tensor into the coordinate system of the molecule. The orientation of the bond vector \vec{r}_{ij} is defined by the angles θ_z^{ij} and ϕ_x^{ij} .

Theory

Experimental dipolar couplings between nuclei i and j (D^{ij}) and pseudocontact shifts between nucleus i and electron e (δ_{PC}^{ie}) are related to the alignment tensor (principal components: A_{xx} , A_{yy} , A_{zz}) or to the magnetic susceptibility tensor (principal components: χ_{xx} , χ_{yy} , χ_{zz}) and to the orientation of a specific vector with respect to the alignment tensor expressed by the projection angles θ_z^{ij} and ϕ_x^{ij} according to Equation 1. The vector is either the vector \vec{r}_{ij} between the two coupled atoms i and j in case of dipolar coupling (Equation 1a) or the vector \vec{r}_{ie} between the electron spin e (paramagnetic center) and the active nucleus i (Equation 1b).

$$D^{ij}(\theta_z^{ij}, \phi_x^{ij}) = \frac{-\mu_0 h S \gamma_i \gamma_j}{8\pi^3 r_{ij}^3} \left[\frac{1}{6}(2A_{zz} - A_{xx} - A_{yy})(3 \cos^2 \theta_z^{ij} - 1) + \frac{1}{2}(A_{xx} - A_{yy}) \cos 2\phi_x^{ij} \sin^2 \theta_z^{ij} \right], \quad (1a)$$

$$\delta_{PC}^{ie}(\theta_z^{ie}, \phi_x^{ie}) = \frac{10^6}{6\pi r_{ie}^3} \left[\frac{1}{6}(2\chi_{zz} - \chi_{xx} - \chi_{yy})(3 \cos^2 \theta_z^{ie} - 1) + \frac{1}{2}(\chi_{xx} - \chi_{yy}) \cos 2\phi_x^{ie} \sin^2 \theta_z^{ie} \right] \quad (1b)$$

In case of paramagnetic alignment the susceptibility tensor $\hat{\chi}$ is related to the alignment tensor \hat{A} by $\hat{\chi} = \hat{A}(15\mu_0 k T / 4B_0 \pi)$. Equation 1 uses the alignment tensor as the frame of reference (Figure 1). The formal dependence of dipolar couplings and pseudo contact shifts is the same while the prefactors differ. The prefactor is constant for dipolar couplings (Equation 1a) if the distance r_{ij} is constant. For pseudocontact shifts and also for dipolar couplings between nuclei whose distance is not fixed a priori in the bonding network they vary because the distance r_{ie} or r_{ij} cannot be regarded as constant in this case (Ghose and Prestegard, 1997; Clore and Garrett, 1999).

However, the measured orientation value cannot be translated directly in a combination of θ_z^{ij} and ϕ_x^{ij} . An infinite number of combinations of θ_z^{ij} and ϕ_x^{ij} exist, that fulfill an experimental value. Still if one pair of angles θ_z^{ij} and ϕ_x^{ij} can be found to be correct due to the alignment of a whole molecule, four orientations of the molecule fulfill all experimental values, since the signs of angles θ_z^{ij} and ϕ_x^{ij} can be reversed independently in Equation 1 without the change of either dipolar couplings or pseudo contact shifts.

In the context of a 3D homology search, the coordinate system of a protein in the 3D structure data file (e.g. PDB) is the natural frame of reference. Therefore we express Equation 1 in this coordinate system which is rotated by three Euler angles α, β, γ with respect to the alignment tensor (Figure 1). Equation 2 expresses

the dipolar couplings in the molecular frame (an identical equation is obtained for pseudocontact shifts δ_{PC}^{ie} by replacing j with e , all following equations are only given for dipolar couplings):

$$\begin{aligned}
 D^{ij}(\xi_x^{ij}, \xi_y^{ij}, \xi_z^{ij}) &= \\
 &= F_{ij} \begin{pmatrix} \cos \xi_x^{ij} \\ \cos \xi_y^{ij} \\ \cos \xi_z^{ij} \end{pmatrix}^T \begin{pmatrix} -S_{yy} - S_{zz} & S_{xy} & S_{xz} \\ S_{xy} & S_{yy} & S_{yz} \\ S_{xz} & S_{yz} & S_{zz} \end{pmatrix} \begin{pmatrix} \cos \xi_x^{ij} \\ \cos \xi_y^{ij} \\ \cos \xi_z^{ij} \end{pmatrix} \\
 &= F_{ij} \begin{pmatrix} (\cos^2 \xi_y^{ij} - \cos^2 \xi_x^{ij})S_{yy} + (\cos^2 \xi_z^{ij} - \cos^2 \xi_x^{ij})S_{zz} \\ + (2 \cos \xi_x^{ij} \cos \xi_y^{ij})S_{xy} + (2 \cos \xi_x^{ij} \cos \xi_z^{ij})S_{xz} \\ + (2 \cos \xi_y^{ij} \cos \xi_z^{ij})S_{yz} \end{pmatrix} \quad (2)
 \end{aligned}$$

with

$$F_{ij} = \frac{-\mu_0 h S \gamma_i \gamma_j}{8\pi^3 r_{ij}^3}$$

In this molecular frame the alignment tensor is no longer diagonal and can be expressed by a symmetric three by three traceless matrix holding five independent elements S_{xx} , S_{zz} , S_{xy} , S_{xz} and S_{yz} , the elements of the Saupe matrix (Saupe, 1968). The eigenvalues of this matrix S_{xx}^{diag} , S_{yy}^{diag} , S_{zz}^{diag} are identical to the principal components of the alignment tensor A_{xx} , A_{yy} , A_{zz} . The angles ξ_x^{ij} , ξ_y^{ij} , ξ_z^{ij} define the projection angles of the bond vector \vec{r}_{ij} or the vector between the nucleus and the electron \vec{r}_{ie} using pseudocontact shifts onto the molecular frame. For a given structure and experimental dipolar couplings D_{exp}^{ij} , the five independent tensor contributions can be determined directly by solving the linear system of equations given from Equation 3 for a set of experimental dipolar couplings for n pairs of nuclei i and j requiring $D_{\text{exp}}^{ij} = D_{\text{theor}}^{ij}$ (Losonczi et al., 1999).

$$\begin{aligned}
 \begin{pmatrix} D_{\text{exp}}^{ij1}/F_{ij} \\ \vdots \\ D_{\text{exp}}^{ijn}/F_{ij} \end{pmatrix} &\stackrel{!}{=} \begin{pmatrix} D_{\text{theor}}^{ij1}/F_{ij} \\ \vdots \\ D_{\text{theor}}^{ijn}/F_{ij} \end{pmatrix} = \\
 \begin{pmatrix} \cos^2 \xi_y^{ij1} - \cos^2 \xi_x^{ij1} & \cos^2 \xi_y^{ij1} - \cos^2 \xi_x^{ij1} & 2 \cos \xi_x^{ij1} \cos \xi_y^{ij1} & \dots \\ \vdots & \vdots & \vdots & \vdots \\ \cos^2 \xi_y^{ijn} - \cos^2 \xi_x^{ijn} & \cos^2 \xi_y^{ijn} - \cos^2 \xi_x^{ijn} & 2 \cos \xi_x^{ijn} \cos \xi_y^{ijn} & \dots \end{pmatrix} \\
 \begin{pmatrix} 2 \cos \xi_x^{ij1} \cos \xi_y^{ij1} & 2 \cos \xi_x^{ij1} \cos \xi_z^{ij1} \\ \vdots & \vdots \\ 2 \cos \xi_x^{ijn} \cos \xi_y^{ijn} & 2 \cos \xi_x^{ijn} \cos \xi_z^{ijn} \end{pmatrix} \begin{pmatrix} S_{yy} \\ S_{zz} \\ S_{xy} \\ S_{xz} \\ S_{yz} \end{pmatrix} &= \vec{C} \vec{S} \quad (3)
 \end{aligned}$$

This system of equations can be solved by multiplication of the pseudo inverse of the rectangular matrix \vec{C} , i.e., by calculating the Moore-Penrose-Inverse of the matrix yielding the vector \vec{S} . Rebuilding the Saupe matrix from these values and analyzing its eigensystem yields the eigenvalues of the tensor S_{xx}^{diag} , S_{yy}^{diag} , S_{zz}^{diag} as well as its orientation given by the eigenvectors. It can be expressed in terms of three Euler angles in α , β , and γ .

$$\begin{aligned}
 \begin{pmatrix} -S_{yy} - S_{zz} & S_{xy} & S_{xz} \\ S_{xy} & S_{yy} & S_{yz} \\ S_{xz} & S_{yz} & S_{zz} \end{pmatrix} &= \left(R^Z(\alpha) R^Y(\beta) R^Z(\gamma) \right)^T \\
 &\begin{pmatrix} S_{xx}^{\text{diag}} & 0 & 0 \\ 0 & S_{yy}^{\text{diag}} & 0 \\ 0 & 0 & S_{zz}^{\text{diag}} \end{pmatrix} \quad (4) \\
 &R^Z(\alpha) R^Y(\beta) R^Z(\gamma)
 \end{aligned}$$

The solution of the Moore Penrose inversion problem is equivalent to finding a solution D_{theor}^{ij} with the least square deviation for a given experimental set of D_{exp}^{ij} . Experimental errors cannot be directly taken into consideration during this approach. Therefore a careful analysis afterwards is necessary according to Losonczi et al. (1999).

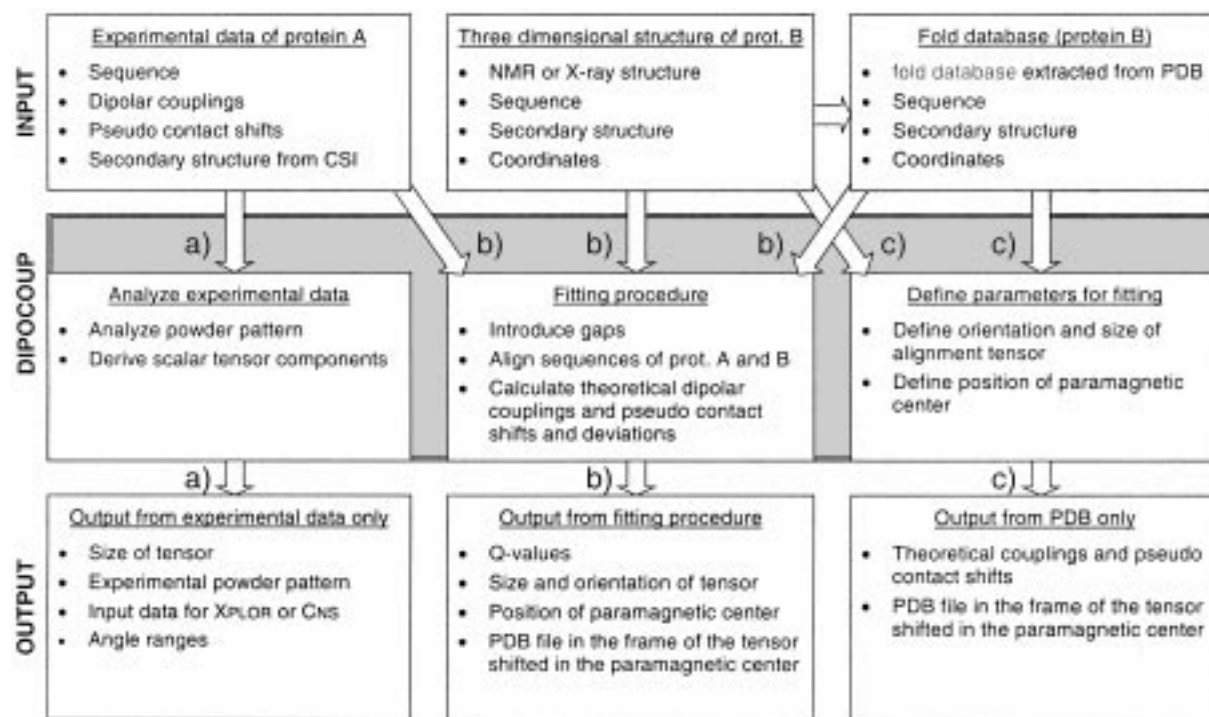


Figure 2. Schematic features of the program *DipoCoup*. Arrows (a) describe the analysis of experimental dipolar couplings and/or pseudocontact shifts from protein **A** without the knowledge of its three dimensional structure and without the use of the database. Arrows (c) describe the analysis of the three dimensional structure of protein **B** for calculating theoretical dipolar couplings or pseudocontact shifts. Arrows (b) indicate the fitting procedure of protein **A** to the known three dimensional structure of protein **B**. This is used to obtain the orientation of the alignment tensor derived from the experimental data for **A** in the molecular frame of protein **B**. The quality of the fit is measured by the Q -value. Alternatively to a single molecule **B** a whole database can be searched finding homologous structures or structure fragments.

Materials and methods

The 3D homology search program *DipoCoup* was written in C++ and can be run on every standard PC working with either Windows95/98 or WindowsNT. The program offers three general means of analyzing dipolar couplings and pseudocontact shifts (Figure 2) of the protein **A** under investigation by comparing it to one or several selected proteins **B** from the database. In procedure (a) one can analyze the experimental dipolar couplings and/or pseudocontact shifts of protein **A** as well as obtain secondary structure information from ^{13}C chemical shift index data, CSI (Spera and Bax, 1991; Wishart et al., 1992, 1995; Wishart and Sykes, 1994). The program is able to handle different sets of dipolar couplings in combination with pseudocontact shifts for one alignment tensor. Dipolar couplings for atom pairs with defined distances (e.g., N-H^N, C α -H α , C α -CO) in protein **A** scaled with F_{ij}^{-1} can be visualized in a histogram yielding a powder pattern. The eigenvalues S_{xx}^{diag} , S_{yy}^{diag} ,

S_{zz}^{diag} of the tensor can then be determined from the histogram (Clare et al., 1998b). With this information the program can generate input files for XPLOR- or CNS-annealing protocols which use residual dipolar couplings as restraints. It also calculates the angle projection ranges that allow to use dipolar couplings in XPLOR- or CNS-calculations without the necessity to define the orientation of the alignment tensor (Meiler et al., 2000).

3D homology searching is prepared in (c) by calculating NMR properties of a potentially homologous protein **B** which is extracted from a structure data base. From the given three dimensional structure of protein **B** a set of dipolar couplings and pseudocontact shifts can be generated. To do this, the program first adds hydrogen atoms that may be missing in the structure and corrects the bond lengths between all heavy atoms and their bound hydrogen atoms according to Bax and Ottiger (1998). For a given alignment tensor and paramagnetic center theoretical dipolar couplings and pseudocontact shifts can be calculated, visualized

and exported to disk, respectively. Also the three dimensional structure can be exported to disk oriented in the frame of reference of the alignment tensor and shifted to the appropriate position with respect to the paramagnetic center.

Finally in procedure (b), both the experimental data from the protein **A** under investigation and the three dimensional structure from the protein **B** can be checked for matching 3D folds. One or several proteins **B** from the protein data bank (PDB) can be used, allowing one to compose the experimental data to a database of proteins. Hydrogen atoms are added or corrected for proteins **B** as already described in (c). The secondary structure elements of proteins **B** are calculated from the coordinates by analyzing hydrogen bonds and φ - and ψ -angles. Then the alignment of residues *a* of **A** and *b* of **B** is done such that $D_{\text{exp}}(a)$ is assigned to the respective atoms of residue *b* of protein **B**. This set of 'experimental' dipolar couplings is used to calculate the alignment tensor and its orientation according to Equation 3. In this case no analysis of the histogram needs to be performed. As a quality measure the *Q*-value of the dipolar couplings (analogous for pseudocontact shifts) is used: $Q = \sqrt{\sum_{ij}(D_{\text{exp}}^{ij} - D_{\text{theor}}^{ij})^2 / \sum_{ij}(D_{\text{exp}}^{ij})^2}$ (Cornilescu et al., 1998). *Q* is a normalized square deviation and is equivalent to $\sqrt{2}$ times the *R*-factor (Clare and Garrett, 1999). Moreover, the program calculates the correlation coefficient *R* (not to be mixed up with the *R*-factor) and offers therefore a second quality value.

The alignment of the residues *a* of protein **A** and *b* of protein **B** is not based on primary sequence homology. Rather, the sequences will be aligned to have a minimum *Q*-value. The program aligns first all amino acids of protein **A** over the amino acids of protein **B** starting with the first for both proteins, respectively. After calculation of the *Q* factor for this alignment the sequence of protein **A** is shifted by one residue and the procedure is repeated until the last amino acid of **A** is aligned with the last amino acid of **B**. This ensures that terminal secondary structure elements of protein **A** are fully used in the alignment process. This procedure avoids to find false positive hits due to a changing number of dipolar couplings and CSI data used. A check for matches of the secondary structure elements is performed. Secondary structure elements are derived from CSI for protein **A** and by analysis of H-bonds and ϕ and ψ (procedure (c) in Figure 2) for protein **B**. To achieve optimal alignment, the secondary structure elements of **A** can be

disconnected and aligned individually with matching secondary structure elements of **B**. By default, disconnection of secondary structure elements in **A** occurs at boundaries of secondary structure elements, e.g. from β -sheet to random coil. However, the user may also suggest other positions for disconnecting the sequence, if additional information has to be used or other ideas have to be tested. If no secondary structure alignment is possible the alignment with minimal *Q* without the use of secondary structural information is presented. The program allows for a search over the whole or part of the PDB database, as will be described subsequently.

If pseudocontact shifts are given, the position of the paramagnetic center either can be explicitly defined in the three dimensional structure or can be optimized by an interactive grid search protocol. For optimization to proceed, a starting position, a starting step, and the size of the cube to be searched has to be supplied. The program searches this given cube using the starting step size and restarts this search with the best point of the previous search and a decreased step size and size of the cube, until the step size is smaller than a predefined target value (e.g., 0.1 Å).

The program can be downloaded together with an example and two databases of 125 (Rost and Sander, 1994) and 500 representative folds out of the PDB from: <http://krypton.org.chemie.uni-frankfurt.de/~mj/software.html>

$^1J_{\text{NH}}$ and $^1D_{\text{NH}}$ couplings were measured for the protein *HgiCIC* (C46 → S) using the direct measurement of the $^1J_{\text{NH}}$ splitting in the ^{15}N dimension of 2D ^1H - ^{15}N HSQC spectra and $^1J_{\text{NH}}$ modulated spectra (Tjandra et al., 1996). To measure the dipolar couplings, two ^{15}N labeled samples of *HgiCIC* (C46 → S) were prepared: One for measuring isotropic $^1J_{\text{NH}}$ couplings and one sample where the weak alignment to the magnetic field is induced using CHAPSO/DLPC lipid bicelles (Wang et al., 1998). Both samples contained 2.5 mM protein, 10 mM phosphate buffer at pH 6.5, 0.03% NaN_3 , 0.1 mM Pefabloc SC, 600 mM NaCl, and 500 μl of 95% H_2O /5% D_2O in an 5 mm NMR tube.

The cyclophilin A sample was approximately 0.7 mM in 100 mM potassium phosphate buffer at pH 6.5 and 0.03% NaN_3 . Solutions of 250 μl (95% H_2O /5% D_2O) were measured in Shigemitsu microcell tubes. Alignment was achieved by CHAPSO/DLPC/CTAB bicelles (5% total lipid conc.: 1: 5: 0.1; Losonczy and Prestegard, 1998).

All measurement were carried out on Bruker DRX-600 or Bruker DRX-800 (Bruker, Rheinstetten, Germany) spectrometers equipped with standard 5 mm triple-resonance, z-gradient probes. The temperature for all measurements was 303 K. The measurements of the $^1J_{\text{NH}}$ splitting in the ^{15}N dimension of 2D ^1H - ^{15}N HSQC spectra were collected with $512 (t_1) \times 2048 (t_2)$ complex data points. $^1J_{\text{NH}}$ modulated spectra were collected with $128 (t_1) \times 2048 (t_2)$ complex data points. Data processing and analysis were performed using either XWinNMR 2.6 (Bruker, Karlsruhe, Germany) or Felix98.0 (MSI, San Diego, CA, USA).

Results and discussion

We have applied the program to three different protein structures: For rhodniin (Friedrich et al., 1993; van de Locht et al., 1995) we calculated a theoretical set of dipolar couplings and pseudocontact shifts using an NMR structure (Maurer and Griesinger, personal communication). A 3D homology search is performed on a restricted database of proteins according to procedure (b) of Figure 2. For cyclophilin A we recorded experimental dipolar couplings and procedure (a) of Figure 2 is used to analyze the experimental data. The dipolar couplings are fitted against the known NMR and X-ray structures, and the orientation of the alignment tensor is determined. The third example is the protein *HgiCIC* which is currently under investigation in our laboratory. This protein contains a helix-turn-helix motif. Using experimentally derived dipolar couplings a 3D homology search on a restricted set of the PDB was performed.

Rhodniin consists of 103 amino acid and contains two similarly folded domains of 45 amino acids connected by a flexible linker of 10 amino acids. A set of $^1D_{\text{NH}}$ dipolar couplings and pseudocontact shifts for amide hydrogens was calculated from the known NMR structure of the protein for the N-terminal domain assuming a specific size and orientation of the alignment tensor and a specific position of a paramagnetic center. Only 36 couplings in rigid parts of the domain were used for the following calculations. The eigenvalues were set to be $S_{zz}^{\text{diag}} = 4.58 \times 10^{-4}$, $S_{yy}^{\text{diag}} = -2.96 \times 10^{-4}$ and $S_{xx}^{\text{diag}} = -1.62 \times 10^{-4}$, amounting to a rhombicity of 0.2. This set of dipolar couplings and pseudocontact shifts is used as an 'experimental' test set.

Measured dipolar couplings were fitted to the NMR structure of rhodniin, by omitting and including pseudocontact shifts. As expected, the dipolar couplings are reproduced in the first case (Figure 3, Table 1) when pseudocontact shifts were omitted. With a normalized square deviation of $Q = 0.00$ the tensor size and orientation exactly reproduce the predefined values. The Q -value is found to be 0.08 in the second case when the tensor and the position of the paramagnetic center were recalculated. The paramagnetic center is found with a deviation of 0.786 \AA to its original position. This deviation is caused by the grid search step size of 0.5 \AA yielding a maximum deviation of $\frac{1}{2}\sqrt{3} \text{ \AA} \approx 0.866 \text{ \AA}$. This deviation is also the reason for $Q > 0.00$. Additionally, deletion of one, two or three amino acids after residues 15 and 39, as well as the addition of amino acids at the same positions do not influence the result of the calculation. Sequence alignment is always found correctly, irrespective of the usage of pseudocontact shifts.

The 'experimental' set of dipolar couplings was fitted to the X-ray structure of ovomucoid (a homologous protein to the N-terminal domain of rhodniin). The 'experimental' values as well as the values calculated for the best fit are given in Figure 3, together with the visualization of both structures in the frame of the resulting alignment tensor. The program finds an eight amino acid shift in the sequence alignment (Table 1) which agrees with the primary sequence alignment for rhodniin and ovomucoid. In this case, the normalized square deviation was found to be $Q = 0.30$.

To speed up the process of three dimensional homology search, a subset of 125 folds was extracted from the PDB with a diverse set of folds according to Rost and Sander (1994). Loading the data and calculating secondary structure elements for all proteins in the fold database takes about 5 min on a 450 MHz Pentium II processor. The search itself takes only below 1 s for the whole database, if no gaps are introduced. This time increases to be 48 s if disconnecting of protein parts as explained above with a gap size of up to 5 amino acids is allowed.

The search over this database using the earlier mentioned theoretical set of dipolar couplings for the N terminal domain of rhodniin (a typical Kazal inhibitor) yields ovomucoid (1ovo_a) as 2nd best hit with a Q -value of 0.45 and porcine pancreatic secretory trypsin inhibitor (1tgs_i) as 16th best hit with a Q -value of 0.53. Both proteins are known as Kazal inhibitors and are homologous to rhodniin. In 9 out of these best 16 examples the α -helix of the rhodniin

Table 1. Results of fitting the experimental set of dipolar couplings of the N-terminal domain of rhodniin to ovomucoid. Identical amino acids in both sequences are labeled by | and similar amino acids are labeled by *

rhodniin	:	<u>12</u>	L	H	R	V	C	G	S	D	G	E	T	Y	S	N	P	C	T	L	N	C	A	K	F	N	G	K	P	E	L	V	L	V	H	D	G	C	<u>47</u>
					*	*																																	
ovomucoid	:	<u>20</u>	T	R	P	L	C	G	S	D	N	K	T	Y	G	N	P	C	N	F	C	N	A	V	V	E	S	N	P	T	L	T	L	S	H	F	G	C	<u>55</u>

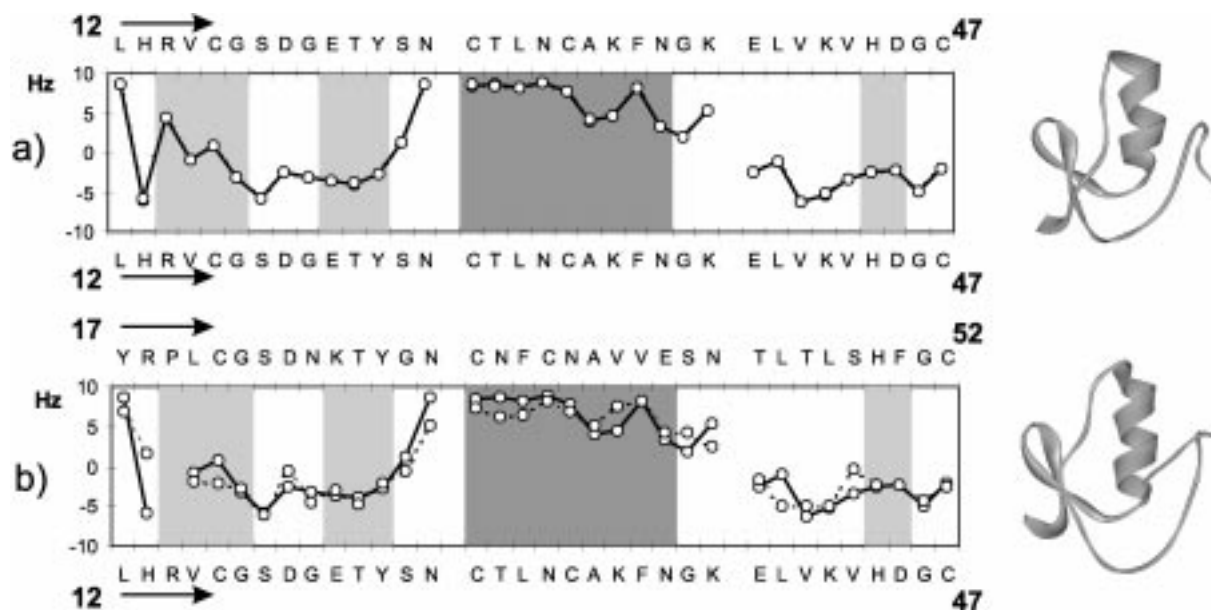


Figure 3. Results of fitting a theoretical set of dipolar couplings for the N-terminal domain of rhodniin to the rhodniin structure (protein A) itself (a) and to ovomucoid (protein B, a Kazal inhibitor), which is homologous in sequence and structure (b). The black lines indicate the theoretical calculated coupling values, the dotted lines indicate dipolar couplings calculated for the final fit. On the upper x-axis the amino acid number of protein B, on the lower x-axis the amino acid number of protein A is found. Secondary structure elements are shown by light gray areas (β -sheet) and dark gray areas (α -helix). The three dimensional structures are given in the coordinate system of the tensor (y - and z -axis are in the paper plane, the x -axis is perpendicular to the paper plane).

Table 2. Results of fitting the experimental set of dipolar couplings of the N-terminal domain of rhodniin to rhodniin itself and to an ensemble of eight Kazal inhibitors, some of which are in complex with serine proteases. For 1tbq the data of the N-terminal domain are fitted to the homologous C-terminal domain of rhodniin

Protein name	pdb code	Fit range	Q
Rhodniin	–	12–47	0.00
Rhodniin in complex with thrombin (Res.: 2.6 Å)	1tbr	12–47	0.27
Rhodniin in complex with thrombin (Res.: 3.1 Å)	1tbq	65–101	0.27
Ovomucoid	1ovo	20–55	0.30
Human pancreatic secretory inhibitor in complex with trypsin	1cgi	20–55	0.30
Procine pancreatic secretory inhibitor in complex with trypsin	1tgs	19–54	0.32
Human pancreatic secretory trypsin inhibitor	1hpt	20–55	0.36
Pig proteinase inhibitor (Kazal type)	1pce	24–59	0.38
Leech-derived inhibitor with procine in complex with trypsin	1ldt	10–45	0.49

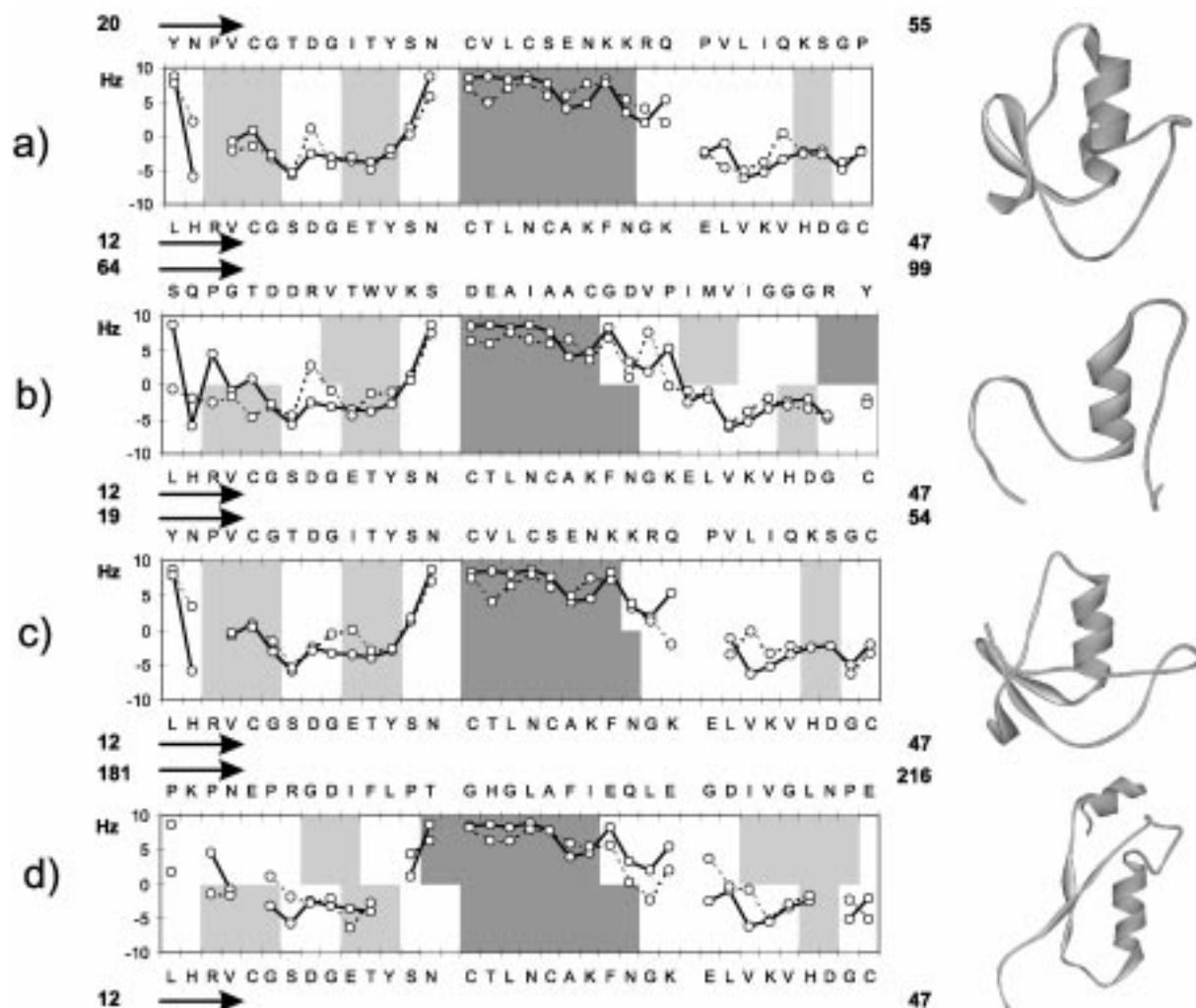


Figure 4. Results for a search in a database of 125 folds extracted from PDB for the theoretical set of dipolar couplings for the N-terminal domain of rhodniin. The black lines indicate the experimental coupling values (protein **A**, rhodniin), dotted lines indicate dipolar couplings calculated for protein **B** from the database. The upper and lower x -axes show the amino acid number of protein **B** and protein **A**, respectively. Secondary structure elements are represented similar to Figure 3. The results are ordered by increasing normalized square deviations (Q -values). (a) ovomucoid (1ovo_a) with a Q -value of 0.30, (b) fragment of an oxidoreductase (6dfr residues 64–99) with a Q -value of 0.31, (c) is again a proteinase inhibitor (1tgs_i) with a Q -value of 0.32 and (d) is a part of an intramolecular oxidoreductase (4xia_a residues 181–216) with a Q -value of 0.35. Subsequent hits have considerably worse matches with Q -values above 0.40.

domain is fitted over a β -strand of the protein from the PDB. This observation can be explained by the parallel orientation of $N-H^N$ bond vectors in both secondary structure elements. Dipolar couplings are therefore of the same size in both secondary structure elements which makes a distinction difficult.

Much more significant results with less false positive answers and lower Q -values are obtained when secondary structure information from CSI is utilized by two simple rules: first, the alignment of β -strands over α -helices is excluded and second, only residues

in well defined secondary structure regions are used for the calculation of Q -values. Using these rules, the two Kazal inhibitors of our database are ranked 1st (ovomucoid, 1ovo_a, $Q = 0.30$) and 3rd (porcine pancreatic secretory trypsin inhibitor, 1tgs_i, $Q = 0.32$). Figure 4 presents the first four hits of this search for which structures are displayed in the coordinate system of the tensor. The 2nd result is part of dihydrofolate reductase (6dfr) with a Q -value of 0.31 and the fourth result is part of D-xylose isomerase (4xia_a) with a Q -value of 0.35. Results (b) and (d) have a

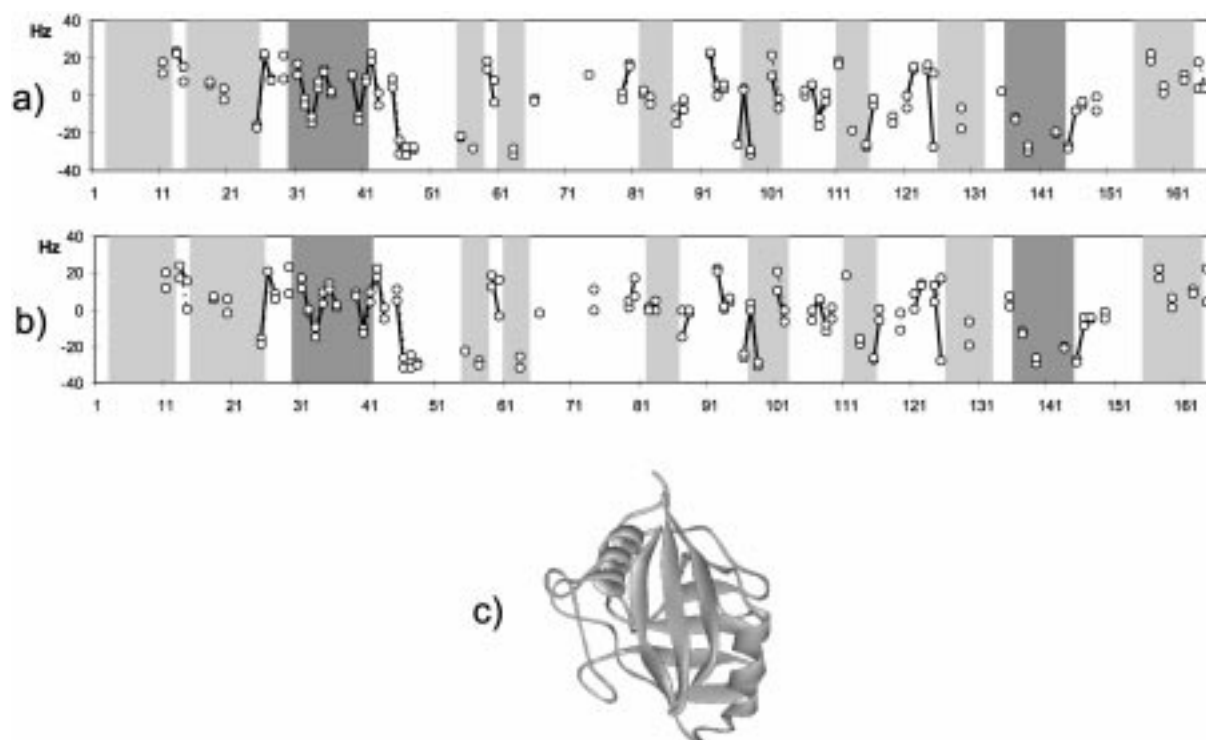


Figure 5. Results of fitting an experimental set of dipolar couplings for cyclophilin A (protein **A**) to the NMR structure (a) and to the X-ray structure (b) (protein **B**). Definition of lines and shaded areas is like in Figure 3. Q -values are 0.28 and 0.21 for (a) and (b) respectively. The three dimensional structure is given in the coordinate frame of the tensor extracted from the fitting procedure (c).

similarly oriented α -helix and at least one of the three β -strands present in rhodniin with a similar orientation with respect to each other. Since the three β -strands are very short (three residues per strand) and nearly parallel, all dipolar couplings within them are of the same size. Therefore this matches very well with one larger β -strand or an extended region when all N-H^N bonds are parallel (d). Matches (b) and (d) have a primary sequence homology of only 11% and 5%. Thus the program finds 3D homology irrespective of sequence homology.

The result of this first homology search suggests rhodniin to be homologous to other Kazal inhibitors. Therefore a more thorough search for Kazal type inhibitors was performed in the PDB and a subset of such inhibitors was extracted. The Q -values of all eight structures range from 0.27 to 0.49 (Table 2).

The second example is cyclophilin for which only 69 fast and easily determinable dipolar couplings were extracted and fitted to the NMR structure (Ottiger et al., 1997) and X-ray (Weber et al., 1982) structures. Results are given in Figure 5 together with the three dimensional structures in the alignment tensor frame

of reference. Q -values are 0.28 and 0.21 for NMR- and X-ray-structure, respectively. The good agreement of both structures with the experimental data proves that it is not necessary to determine all couplings for fitting. Moreover, the possibility of calculating dipolar couplings for other residues allows to accelerate further interpretation of spectra. While we detect 3D homology to other known cyclophilins, searching in a data bank of 125 folds only finds small parts of the whole sequence, in particular helix-strand-strand motives. It appears that cyclophilin has a rather unique 3D fold.

In the soilbacteria *Herpetosiphon giganteus* many restriction modification systems could be characterized. One of these systems is the *HgiCI* system of which the C-protein (Control protein) *HgiCIC* (expressed with a His₆ tag and a C46 \rightarrow S mutation) of 10 kDa molecular mass is currently under investigation in our laboratory and was used as a test system for *DipoCoup*. A total of 62 ¹D_{NH} dipolar couplings could be extracted for the 88 residue protein *HgiCIC*. The dipolar couplings range from -7.5 to 7.1 Hz. To establish weak alignment we used CHAPSO/DLPC (1:5) bicelles with a total lipid concentration of 5%.

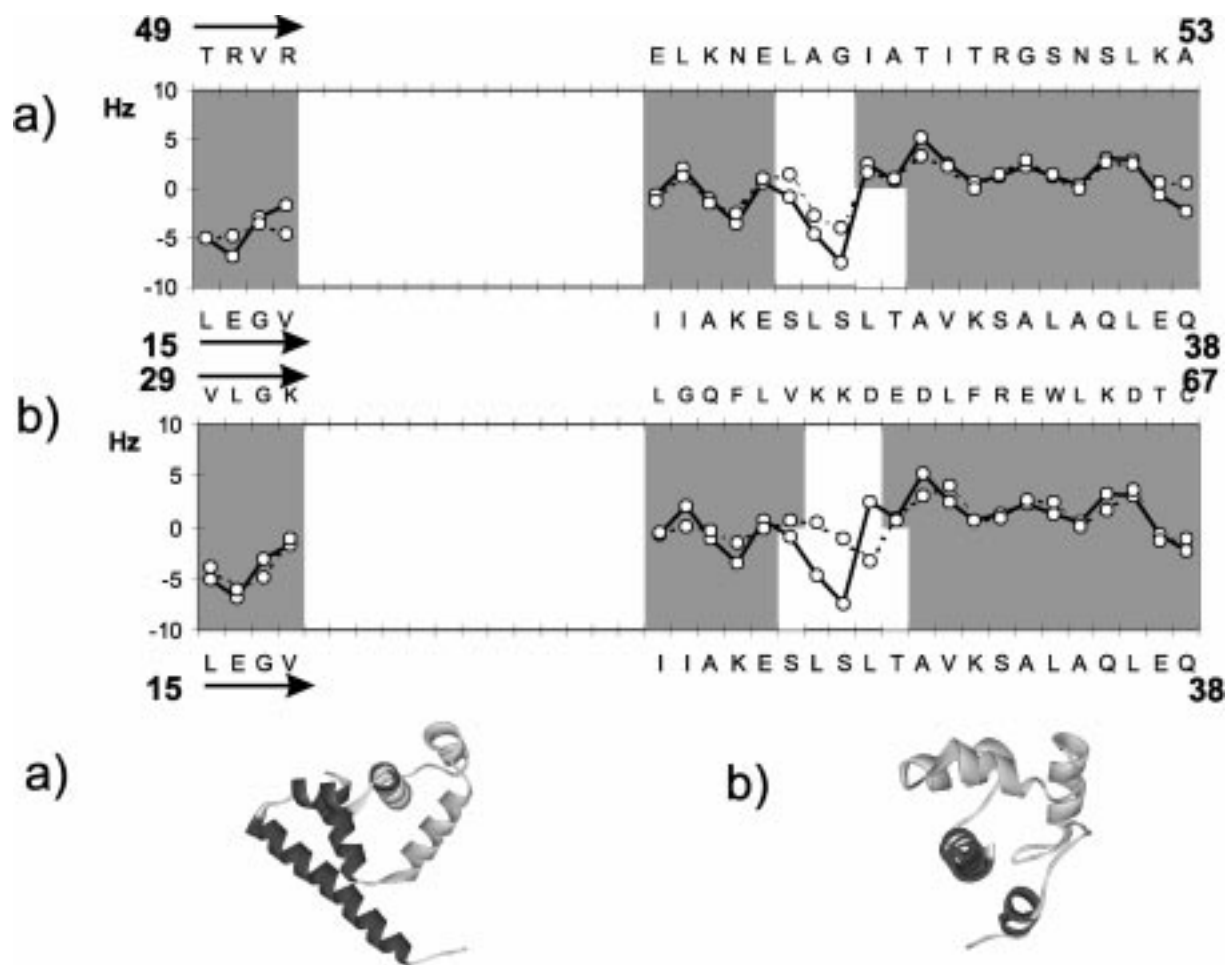


Figure 6. Result of the alignment of residues 15 to 53 of the protein *HgiCIC* (C46S) to a database especially designed for helix-turn-helix proteins. The results with lowest Q -value are trp Repressor (a, $Q = 0.42$) and BAF (b, $Q = 0.43$). Definition of lines and shaded areas is as in Figure 3. The upper and lower x-axes show the amino acid number of protein **B** and protein **A**, respectively. A comparison of the dipolar couplings and corresponding structures in the alignment tensor coordinate system is shown for the two best fits. Light gray parts represent the fitted parts and dark gray parts are not fitted.

Secondary structure alignment indicated the protein might be a typical representative of the helix-turn-helix (HTH) fold family (Brennan and Matthews, 1989; Patto and Sauer, 1992; Harrison, 1999). Therefore we searched for known representatives of the HTH family in the DPInteract (<http://arep.med.harvard.edu/dpinteract>) database. There are two groups of known HTH proteins. One comprises all α -helical proteins and the other $\alpha+\beta$ proteins with a HTH motive. With this information we built a database with 19 helix-turn-helix proteins (also at <http://krypton.org.chemie.uni-frankfurt.de/~mj/software.html>). From the experimental $^1D_{NH}$ dipolar couplings of *HgiCIC* the alignment tensor was calculated and the alignment search

according to Figure 2b was performed with *DipoCoup*, including CSI data. The whole *HgiCIC* (C46 \rightarrow S) protein proved to be too large for an alignment with the structures of the database. We therefore partitioned the protein into two overlapping parts. The first part contained the residues 15 to 53 and the second one residues 32 to 71. Both regions can be aligned with parts of proteins in the HTH database. Alignment of the first part (residues 15 to 53) shows good match with the trp repressor (Q -value: 0.42) and the cellular factor BAF (Q -value of 0.43, Figure 6). The second part (residues 32–71), which includes also the HTH motif, does not match as well as the first stretch of amino acids. We find a best match with the structures of LexA (Q -value: 0.64) and with the struc-

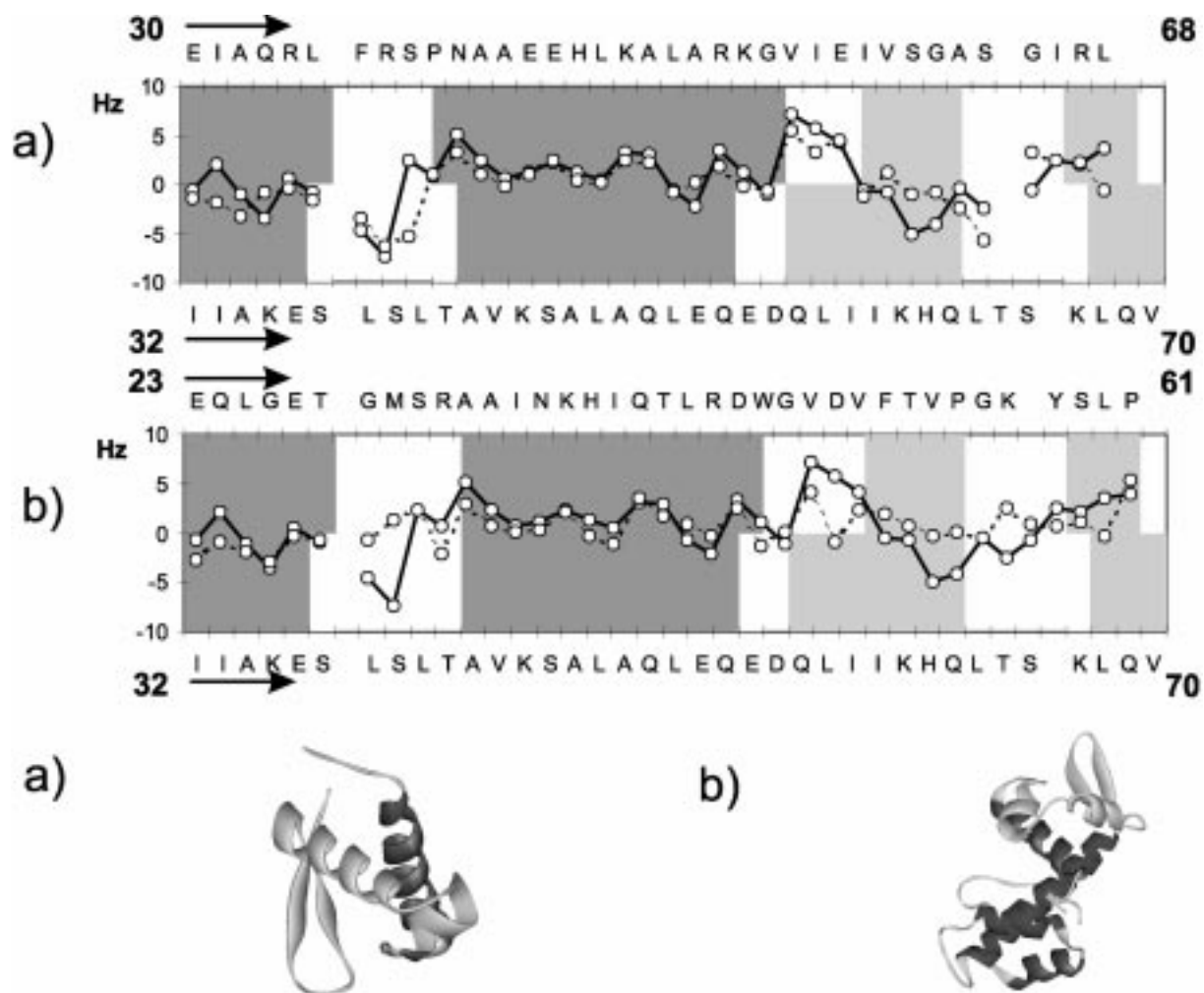


Figure 7. The best match of residues 32 to 71 of the protein *HgiCIC* (C46S) with HTH protein database is shown. Measured dipolar couplings of the protein are plotted against the calculated dipolar couplings of the two best fitting proteins LexA, and diphtheria toxin repressor (b, $Q = 0.71$). The upper and lower x -axes show the amino acid number of protein **B** and protein **A**, respectively. The residue by residue match of the dipolar couplings is much better than the rather high Q value would suggest. Light gray parts represent the fitted parts and dark gray parts are not fitted.

ture of the diphtheria toxin repressor (Q -value: 0.71). Even though the Q -values for the alignment are quite high, the experimental and the calculated dipolar couplings match rather well on a residue by residue basis (Figure 7). A few large deviations can cause large Q -values, since Q depends quadratically on the deviation of dipolar couplings. Although *HgiCIC* is not very similar to any of the already known HTH-proteins in total, two parts of its structure match known protein folds from which a 3D model of the protein can be derived.

C proteins are also known to bind DNA. The HTH motif in *HgiCIC* is consistent with the finding that

HgiCIC binds DNA as observed by band shift assays. The observed shifts upon DNA titration are most prominent for the amino acids in the helix-turn-helix motif.

Conclusions

We have demonstrated the possibility to use residual dipolar couplings and pseudocontact shifts together with secondary structure information to perform 3D structure homology searches in representative sub-databases of the PDB. We present the program *DipoCoup* which performs this homol-

ogy search in a fast, accurate and user friendly way. Moreover, *DipoCoup* can be used to perform additional analysis of experimentally determined orientation data or 3D structures of proteins. The program is free for academic use, and can be downloaded from <http://krypton.org.chemie.uni-frankfurt.de/~mj/software.html>.

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NMR Structure Determination of Saccharose and Raffinose by Means of Homo- and Heteronuclear Dipolar Couplings

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Residual dipolar couplings have dramatically improved the accuracy and precision of high-resolution NMR structures during the last years. This was first demonstrated for proteins. In this article, we describe, with raffinose and saccharose as examples, that dipolar couplings improve the precision of structures of carbohydrates for which usually very few structural parameters are available. The relative orientation as well as the dynamics of the monosaccharide moieties with respect to each other can be determined with the help of ^{13}C , ^1H and ^1H , ^1H dipolar couplings, which can easily be measured. Significant differences between the solution and the X-ray crystal structure exist. These results indicate that residual dipolar-coupling data may provide a more complete and dynamic model of carbohydrates in particular, and small molecules in general.

1. Introduction. – The use of residual dipolar couplings [1][2] observed when proteins are subjected to an orienting environment has already had considerable impact on the precision of protein-structure determination by means of high-resolution NMR spectroscopy of liquids [3–5]. In uniformly ^{13}C , ^{15}N -labeled proteins, a large number of dipolar couplings is experimentally accessible. These dipolar couplings can be measured for internuclear vectors such as NH , NC_α , $\text{C}_\alpha\text{C}'$, NC' , and $\text{H}_\alpha\text{C}_\alpha$, which are isotropically distributed for many globular proteins [6–8]. Heteronuclear dipolar couplings have also been utilized to derive structures of small ligands bound to proteins [9–11]. For small molecules, few NOEs and scalar coupling constants can be measured that define, for example, the conformation across the glycosidic bonds in oligosaccharides [12]. However, there are several major difficulties that need to be considered in oligosaccharides. First, in contrast to proteins, fewer C,H dipolar couplings across a single bond can be measured. Second, the distribution of C,H bond vectors is not isotropic due to the geometry of the pyranose rings [13–17]. As a consequence, an alignment tensor cannot easily be derived for oligosaccharides oriented in bicelles [18]. Third, it is known that oligosaccharides are often flexible around the glycosidic bonds that connect the different sugar moieties. Therefore, different alignment tensors can be observed for the monosaccharides constituting the oligosaccharide. However, investigations of oligosaccharides published so far rely on C,H dipolar couplings from which individual alignment tensors for the monosaccharides cannot be derived. Therefore, in these studies, it is assumed without proof that the alignment tensor is the same for all monosaccharide moieties [13][15–17].

To overcome the potential problems, we increased the number of experimental dipolar couplings by measuring C,H dipolar couplings but also including H,H dipolar couplings. For this study, residual dipolar couplings were recorded for saccharose (= β -D-fructofuranosyl- α -D-glucopyranoside) and raffinose (= β -D-fructofuranosyl O- α -D-

galactopyranosyl-(1 → 6)- α -D-glucopyranoside). With the additional homonuclear residual dipolar couplings, it was possible to obtain an alignment tensor for each monosaccharide. This enables the evaluation of the mobility of each of the monosaccharides.

2. Results and Discussion. – 2.1 *Measurement of Homo- and Heteronuclear Dipolar Couplings.* Since heteronuclear and homonuclear dipolar couplings are observed on top of scalar couplings, the general strategy is to record couplings on a sample of the oligosaccharide with and without alignment. The methods used are the HSQC [19] without decoupling in the ^{13}C dimension for measurement of the C,H couplings, the SPITZE-HSQC [20] for the measurement of the C,H and H,H dipolar couplings of CH_2 groups, and E.COSY [21–23], for the first time, for the measurement of the H,H dipolar couplings in carbohydrates. All these experiments provide residual dipolar coupling constants with the sign and size [24][25] required to translate them in the least ambiguous way into orientational information. As for C,H-HSQC and the SPITZE HSQC residual dipolar-coupling measurement have been described in [19][20], we subsequently focus on the extraction of H,H dipolar couplings from E.COSY spectra. In the discussion below, referring to a coupling constant corresponds to the superposition of the scalar (J) and the dipolar coupling (D). Consider a system consisting of three mutually coupled spins A, B, and C. From an E.COSY cross-peak between spins A and B we can extract the C,B coupling constant from the ω_2 component of the displacement vector due to C, provided the sum of the A,C coupling is resolved in ω_1 . The size and the sign of the C,B coupling constant can be determined provided the sign of the associated coupling constant between A and C is known [23]. Since the sign of the coupling constant is known only if the scalar couplings are larger than the dipolar couplings, the alignment should be rather weak.

Three types of E.COSY cross-peaks can be observed in anisotropic solutions. First, we see cross-peaks between scalar coupled protons. An example for this case is the extraction of dipolar couplings from the cross-peaks between the vicinal protons H3,H2 and H3,H4 of glucose, which is part of raffinose, in the isotropic (*Fig. 1, a*) and the anisotropic medium (*Fig. 1, b*). The passive spins are H1 and H4 in the H2/H3 cross-peak, and H2 and H5 in the H4/H3 cross-peak. While in the isotropic phase the $^4J(\text{H,H})$ couplings are usually close to zero, the H3,H2 cross-peak in the oriented phase shows a sizeable $(D + J)(\text{H2,H4}) = -1.74$ Hz coupling, as does the H3/H4 cross-peak. The sign of the coupling is derived from the fact that the vector lies in the second or fourth quadrant of the ω_2, ω_1 coordinate system, which means that the sign is opposite to the positive sign of the associated $(D + J)(\text{H3,H4})$ or $(D + J)(\text{H3,H2})$ coupling. This procedure provides dipolar couplings between protons separated by four bonds from the cross-peaks between vicinal protons in the oligosaccharide.

The second case are cross-peaks when the active coupling is due to residual dipolar couplings, and which, thus, are not observed in isotropic media. For example, the fructose H6'/H4 cross-peak (*Fig. 2*) originates from the $D(\text{H6}',\text{H4})$ coupling alone. This cross-peak contains three displacement vectors due to couplings of H6, H5, and H3, with H6' and H4. They can be assigned due to the fact that H6 shows the largest displacement in ω_1 , H3 shows no displacement in ω_2 , and H5 causes a displacement in both ω_1 and ω_2 . Again, sign and size of the coupling constants can be derived.

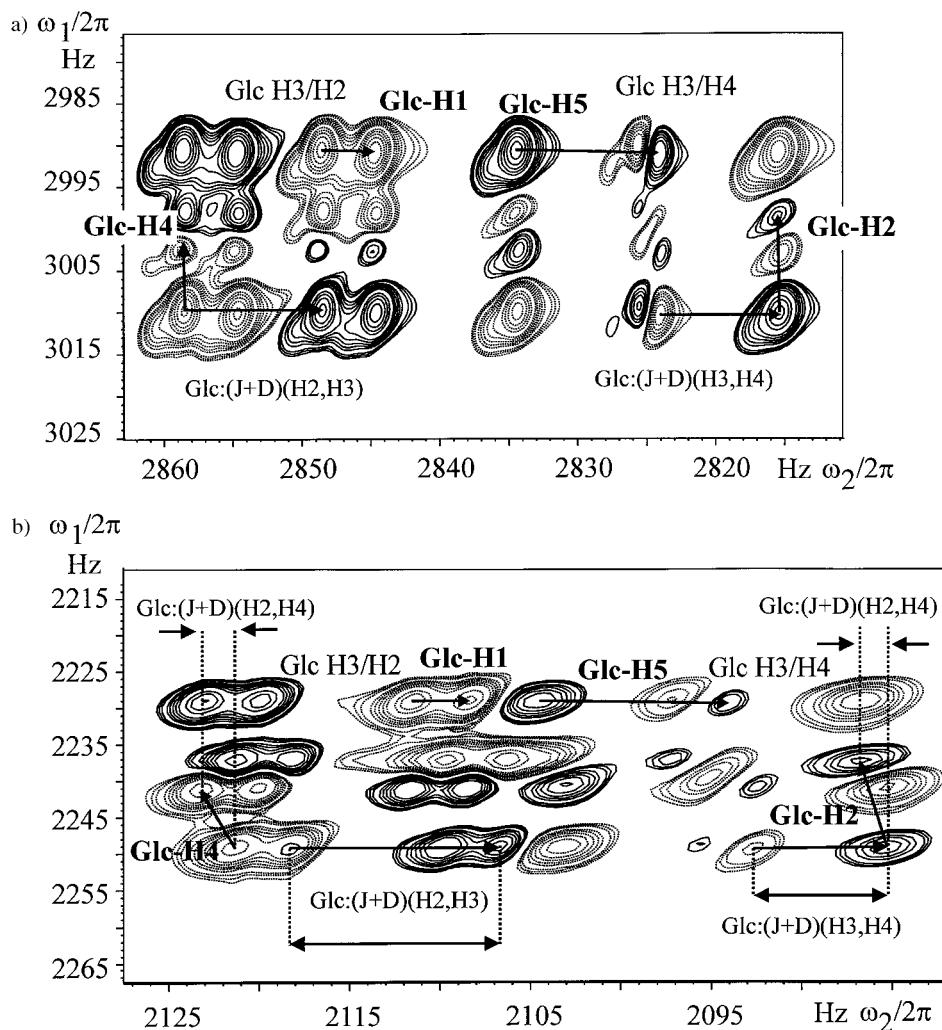


Fig. 1. The H3/H2 and H3/H4 cross-peaks of glucose in raffinose recorded in a) isotropic solution and in b) CHAPSO/DMPC (1/3.5; 7.5%). Positive contours are full, negative contours are plotted in dashed lines. The passive spins and the respective displacement vectors are given in bold. Measuring the displacement preferably in ω_2 yields size and sign of the coupling constants of interest. The displacement vectors for Glc-H2 and Glc-H4 lack an ω_2 component in isotropic phase, whereas they reflect the pure dipolar Glc (H2,H4) coupling in the anisotropic medium.

Finally in the last case, the E.COSY spectrum can also contain cross-peaks that do not show an E.COSY pattern, because one of the two possible couplings between spins A and C, or B and C is zero. This situation is encountered for protons that belong to different monosaccharides in the trisaccharide. Then, only the extraction of the active coupling is possible, for which the sign cannot be determined. The extraction is done according to the DISCO [26–28] method by comparison with other cross-peaks. This is

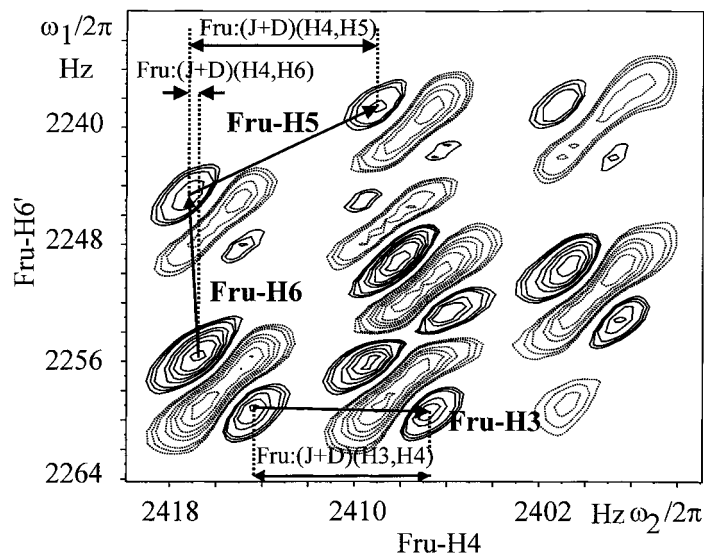


Fig. 2. $H6/H4$ Cross-peak of fructose in raffinose recorded in CHAPSO/DMPC (1/3.5; 7.5%). Positive and negative contours marked as in Fig. 1. The passive spins are H6, H5, and H3. They can be easily distinguished due to the different vector components in ω_1 and ω_2 . Size and sign of the coupling constants of interest are measured from the ω_2 component of the displacement vectors and reported in Table 2.

shown using the Gal-H1/Glc-H6 cross-peak that is compared with the Glc-H6/H6 cross-peak in Fig. 3. The dipolar as well as the scalar coupling constants that could be extracted from raffinose and saccharose are compiled in Tables 1 and 2 and 6 and 7, respectively.

2.2. Materials and Methods. Raffinose and saccharose were purchased from Fluka (Buchs, Switzerland) and used without further purification. Samples of 20 mM raffinose or saccharose in 500 μ l D_2O in regular thin-wall NMR tubes were used. For the alignment, bicelles were formed from CHAPSO/DMPC (1/3.5, 7.5%) (CHAPSO/DMPC purchased from Sigma (St. Louis, MO, USA); SDS purchased from Merck (Darmstadt, Germany), and used without further purification) [29–31]. For saccharose, dipolar couplings were measured with CHAPSO/DMPC (1/3.5, 7.5%). For raffinose, two sets of dipolar couplings in CHAPSO/DMPC (1/3.5, 7.5%) and CHAPSO/DMPC/SDS (1/3.5/0.2, 7.5%) were measured. Since small carbohydrates have no charge, the difference between the two data sets is small. Therefore, all calculations have been performed with the CHAPSO/DMPC data set. Interestingly, the CHAPSO/DLPC bicelles did not align the carbohydrates very well. This is different compared with proteins [20][30][32]. All experiments were performed either on a Bruker DRX-600 or Bruker DRX-800 spectrometer (Bruker AG, Rheinstetten, Germany) equipped with TXI HCN z -grad probes. The temperature for all measurements was set to 308 K, except for the NOESY spectra (280 K). All spectra were processed with XWINNMR 2.6 (Bruker AG, Karlsruhe, Germany).

For all experiments, the proton carrier was set to 4.7 ppm (HDO resonance) and for carbons to 82 ppm. The recycle delay was set between 2 and 5 s according to inversion

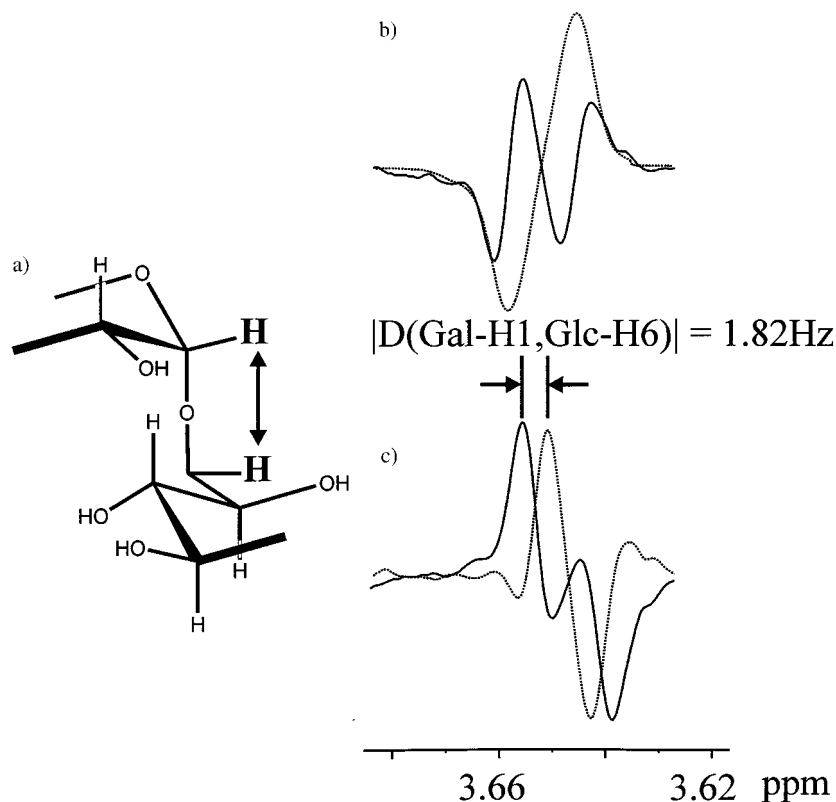


Fig. 3. a) Schematic picture of the galactose and glucose linkage in raffinose. b) Solid line: trace obtained by summation along ω_1 over the Gal-H1, Glc-H6 cross-peak that is purely caused by the dipolar coupling. Broken line: trace obtained in the same way from the Glc-H6', H6 cross-peak. c) DISCO Procedure applied to the two traces of b, taking the sum (solid line) and difference (broken line). The difference in the line positions of the two peaks provides the absolute value of the desired (Gal-H1, Glc-H6) dipolar coupling constant, but not its sign.

recovery T_1 experiments. Coupled HSQC spectra were recorded with $2\text{k} \times 4\text{k}$ and zero-filled to establish 0.4-Hz resolution for the exact measurement of the $^1J(\text{C}, \text{H})$ couplings (measurement time 6 h). The SPITZE HSQC was recorded as described by *Carlomagno et al.* (measurement time 8 h for each experiment, zero-filled to obtain a resolution of 0.3 Hz in ω_2 .) [20]. H,H Dipolar couplings were recorded according to the P.E.COSY [22][33] method. $4\text{k} \times 1\text{k}$ Data points (measurement time 10 h) were recorded in the experiment and then zero-filled to $8\text{k} \times 4\text{k}$ to obtain the desired resolution of 0.2 Hz. 2D NOESY Spectra were recorded at different mixing times to obtain distance restraints (measurement temperature 280 K to increase τ_c). Build-up curves [34] were measured at 800 MHz from 200 to 1100 ms (measurement time 11 h). For raffinose, the optimal mixing time is 500 ms ($4\text{k} \times 1\text{k}$ and zero-filled to $16\text{k} \times 4\text{k}$). To suppress zero-quantum peaks, the NOESY spectra were recorded with an incremented mixing time and the 500-ms NOESY with a random variation of the mixing time [34][35].

Structure Refinement. The two carbohydrate structures were refined in order to determine the relative orientation of the monosaccharides using the X-Plor program [36] together with an optimized force field for saccharides [37], and NOEs and dipolar-coupling restraints. A standard simulated annealing protocol was used together with dipolar coupling restraints implemented as angle restraints [38]. NOE Distances were translated into a standard X-Plor potential. For calculating the angle restraints from the dipolar couplings, as well as for fitting the structures to the experimental data and for determining the alignment tensor size and orientation, the program *DipoCoup* [39] was used. In every experiment, 100 structures were generated and those 10 with the lowest energies were further analyzed.

Determination of the Alignment Tensor for Each Monosaccharide. The determination of the tensor size according to *Clore et al.* [18] cannot be performed due to the relatively low number of dipolar couplings available per monosaccharide. For an individual structural unit, for instance, one of the monosaccharides, one needs at least five linearly independent equations to obtain the alignment tensor [25][39][40]. However, for glucose and galactose there are only two linearly independent C,H orientations, which lead to only two linearly independent equations. Thus, the tensor determination for each monosaccharide unit from C,H couplings alone is impossible. Nevertheless, including H,H dipolar couplings, this number can be increased well over five independent equations. To obtain insight into the error propagation of dipolar couplings with respect to the alignment tensor, we did the following test: the lowest-energy structure of raffinose (see below) and the firm experimental dipolar couplings ('firm' dipolar couplings are those that are used for structure calculation) were used, and the alignment tensor was individually fitted to the monosaccharides. For galactose, we obtained: $D_{ax} = -3.09 \pm 0.26$ Hz, $R = 0.45 \pm 0.19$, for glucose: $D_{ax} = -5.60 \pm 0.73$ Hz, $R = 0.56 \pm 0.09$, and for fructose: $D_{ax} = 7.20 \pm 0.93$ Hz, $R = 0.60 \pm 0.08$ (D_{ax} is the value for the axial component and R for the rhombicity of the alignment tensor [2][39]). The errors have been derived by randomly adding or subtracting an experimental uncertainty of ± 0.2 Hz to or from the experimental H,H dipolar couplings and ± 0.4 Hz to or from the experimental C,H dipolar couplings. For the structure calculations, an error of *ca.* $\pm 20\%$ for the axial component and the rhombicity of the tensor was derived. Due to the uncertainty of the alignment tensor, a grid search over this region was performed. The starting size of the tensor was taken from the experimental dipolar couplings and the neutron diffraction or X-ray structure as a model for the solution structure. The step size of the grid search was chosen to contain all possible and structural meaningful values for the axial component and the rhombicity of the alignment tensor. After selection of ten structures with the lowest Q values (Q -value filtration), the alignment tensor was back-calculated from those structures by *Moore-Penrose* inversion [39] to check for self-consistency of the alignment tensor. Cross validation on the basis of unused dipolar-coupling data was carried out for those structures that had a self-consistent alignment tensor.

2.3. Structure Calculation of Raffinose. Measured scalar and dipolar couplings are given in *Tables 1* and *2*. Not all of them were used for the structure calculations. Since the protocol does not allow ensemble calculations, only one conformation could be calculated in each run. Therefore, couplings that are subject to conformational averaging are excluded from the fitting for the determination of the alignment tensor as

well as from the structure calculation. The same holds for protons with overlapping chemical shifts. The dipolar couplings of the glucose C6 H₂ group could be used for the structure calculation, because the population of the C5,C6 torsion angle is mainly g^- (g^- :75%, g^+ :25%, t :0%) as derived from J -coupling analysis [42]. The stereochemical assignment of the diastereotopic protons could be accomplished on the basis of the Glu-H6,H4 NOE ($\delta(\text{C6 H}^{pro-R}) = 4.08$ ppm, $\delta(\text{C6 H}^{pro-S}) = 3.74$ ppm). In contrast, the fructose C6 H₂ group adopts two conformations, 66% of g^+ and 32% of g^- ($\delta(\text{C6 H}^{pro-R}) = 3.82$ ppm, $\delta(\text{C6 H}^{pro-S}) = 3.88$ ppm) and could, therefore, not be used in the structure-calculation protocol. The two protons at the galactose C6 are degenerate as are the protons at fructose C1. All the excluded couplings are marked with a star in *Table 1* and are used for cross validation. We call the other dipolar couplings firm experimental couplings and use them for the derivation of the monosaccharide alignment tensors as well as for the structure determination.

Table 1. *Dipolar Couplings Determined for Raffinose in CHAPSO/DMPC (1/3.5; 7.5%)*. The three dipolar couplings with undefined sign were extracted according to the described DISCO procedure. The couplings not marked by stars are the ‘firm’ couplings as introduced in the text.

	$D(\text{C,H})$ [Hz]		$D(\text{H,H})$ [Hz]
Gal-C1/H1	2.60	Gal-H1/H2	-1.10
Gal-C2/H2	-3.64	Gal-H2/H3	0.23
Gal-C3/H3	-3.42	Gal-H3/H4	0.20
Gal-C4/H4	3.38	Gal-H4/H5	-0.99
Gal-C5/H5	-3.94	Gal-H3/H5	± 1.78
*Gal-C6/H6;H6'	-2.34	Glc-H1/H2	-0.61
Glc-C1/H1	5.28	Glc-H2/H3	1.70
Glc-C2/H2	2.73	Glc-H3/H4	-1.00
Glc-C3/H3	2.34	Glc-H4/H5	-0.30
Glc-C4/H4	1.52	*Glc-H5/H6	-2.50
Glc-C5/H5	3.29	Glc-H6/H6'	2.90
Glc-C6/H6	3.90	Glc-H2/H4	-1.74
Glc-C6/H6'	1.90	Glc-H3/H5	0.00
*Fru-C1/H1;H1'	4.55	Fru-H3/H4	-2.48
Fru-C3/H3	-6.54	Fru-H4/H5	0.69
Fru-C4/H4	-4.76	*Fru-H5/H6	-1.20
Fru-C5/H5	-2.60	*Fru-H5/H6'	-1.24
*Fru-C6/H6	1.80	*Fru-H6/H6'	1.70
*Fru-C6/H6'	-2.00	Fru-H3/H5	1.01
*Gal-H1/Glc-H6'	± 1.82	*Fru-H4/H6	0.50
*Gal-H5/Glc-H4	± 1.56	*Fru-H4/H6'	0.70
*Gal-H1/Glc-H6	± 1.45		

Alignment tensors for each monosaccharide from firm heteronuclear $D(\text{C,H})$ dipolar couplings and the combination of firm heteronuclear and homonuclear dipolar $D(\text{C,H}) + D(\text{H,H})$ dipolar couplings were extracted by fitting these firm dipolar-coupling constants against the X-ray structure of raffinose [42] individually for each monosaccharide and for the whole structure [39]. Using the firm dipolar couplings, we find the following tensor sizes for the monosaccharides from the CHAPSO/DMPC data: galactose: $D_{\text{ax}} = -3.11$ Hz, $R = 0.35$, glucose: $D_{\text{ax}} = -5.60$ Hz, $R = 0.57$, fructose: $D_{\text{ax}} = 7.01$ Hz, $R = 0.56$ (*Table 3*). The tensor sizes for glucose and fructose are similar

Table 2. *Scalar Couplings Determined for Raffinose in Isotropic Solution.* Also NOE distances measured as described in the text of the paper are listed. The Glc-H1/H2 cross-peak was used as reference peak. The star indicates the sum of two couplings.

	$^1J(\text{C,H})$ [Hz]		$J(\text{H,H})$ [Hz]		NOE [\AA]
Gal-C1/H1	170.6	Gal-H1/H2	3.93	Gal-H1/Glc-H6'	2.52
Gal-C2/H2	146.3	Gal-H2/H3	10.30	Gal-H1/Glc-H6	2.78
Gal-C3/H3	145.5	Gal-H3/H4	3.45	Glc-H1/H2*	2.41
Gal-C4/H4	146.8	Gal-H4/H5	1.20	Glc-H4/H6	2.62
Gal-C5/H5	142.9	Glc-H1/H2	3.96	Gal-H5/Glc-H4	3.05
Gal-C6/H6;H6'	287.9*	Glc-H2/H3	9.89	Glc-H4/H6	3.33
Glc-C1/H1	169.7	Glc-H3/H4	8.76	Glc-H1/Frau-H1	2.44
Glc-C2/H2	144.6	Glc-H4/H5	10.40	Fru-H4/H6'	2.71
Glc-C3/H3	145.0	Glc-H5/H6	3.96	Fru-H4/H6	2.99
Glc-C4/H4	145.9	Glc-H6/H6'	-10.90	Gal-H1/H2	2.37
Glc-C5/H5	145.5	Fru-H3/H4	8.79	Gal-H3/H5	2.73
Glc-C6/H6	145.5	Fru-H4/H5	8.45	Glc-H3/H5	2.60
Glc-C6/H6'	144.3	Fru-H5/H6	3.00		
Fru-C1/H1;H1'	290.1*	Fru-H5/H6'	7.32		
Fru-C3/H3	149.8	Fru-H6/H6'	-12.21		
Fru-C4/H4	144.2	Glc-H5/H6'	2.26		
Fru-C5/H5	144.2				
Fru-C6/H6	143.6				
Fru-C6/H6'	142.6				

Table 3. *Tensor Sizes for Raffinose in CHAPSO/DMPC (1/3.5; 7.5%).* The average alignment tensor of the ten structures with lowest energy from 100 XPLOR calculations is given. The values for the two calculated data sets with the lowest Q values, and the most self-consistent D_{ax} and R values are shown. The galactose, glucose, and fructose structures are derived from the two best data sets of the solution structure of raffinose. The items indicated with § refer to the neutron-diffraction structure of saccharose, the item indicated with § refers to the X-ray structure of raffinose. The tensor size and rhombicity using only heteronuclear dipolar couplings ($D(\text{C,H})$ in Table 3) are not sufficient to determine the alignment tensor for each of the monosaccharides due to too few linearly independent orientations.

	D_{ax} [Hz]	R	Q
§Gal: $D(\text{C,H}) + D(\text{H,H})$	-3.11	0.35	0.26
Gal: $D(\text{C,H}) + D(\text{H,H})$	$-3.41 \pm 0.03 / -3.45 \pm 0.01$	$0.24 \pm 0.03 / 0.25 \pm 0.02$	$0.15 \pm 0.00 / 0.13 \pm 0.00$
§Glc: $D(\text{C,H}) + D(\text{H,H})$	-5.60	0.57	0.27
Glc: $D(\text{C,H}) + D(\text{H,H})$	$-5.59 \pm 0.01 / -5.64 \pm 0.01$	$0.52 \pm 0.00 / 0.49 \pm 0.00$	$0.24 \pm 0.00 / 0.23 \pm 0.00$
§Fru: $D(\text{C,H}) + D(\text{H,H})$	7.01	0.56	0.09
Fru: $D(\text{C,H}) + D(\text{H,H})$	$-6.26 \pm 0.01 / -6.29 \pm 0.03$	$0.39 \pm 0.01 / 0.46 \pm 0.02$	$0.05 \pm 0.00 / 0.04 \pm 0.00$
§Sacc: $D(\text{C,H})$	5.04	0.22	0.09
§Sacc: $D(\text{C,H})$	4.00	0.46	0.34
Sacc: $D(\text{C,H})$	$3.48 \pm 0.06 / 3.66 \pm 0.09$	$0.28 \pm 0.02 / 0.26 \pm 0.03$	$0.35 \pm 0.00 / 0.34 \pm 0.00$
§Sacc: $D(\text{C,H}) + D(\text{H,H})$	-5.49	0.56	0.26
§Sacc: $D(\text{C,H}) + D(\text{H,H})$	-4.99	0.47	0.48
Sacc: $D(\text{C,H}) + D(\text{H,H})$	$-4.92 \pm 0.03 / -5.08 \pm 0.01$	$0.52 \pm 0.00 / 0.52 \pm 0.01$	$0.29 \pm 0.00 / 0.25 \pm 0.00$
§Raff: $D(\text{C,H})$	3.87	0.57	0.42
Raff: $D(\text{C,H})$	$5.63 \pm 0.16 / 5.76 \pm 0.11$	$0.32 \pm 0.02 / 0.28 \pm 0.00$	$0.25 \pm 0.01 / 0.26 \pm 0.01$
§Raff: $D(\text{C,H}) + D(\text{H,H})$	-3.87	0.40	0.54
Raff: $D(\text{C,H}) + D(\text{H,H})$	$-4.17 \pm 0.03 / -4.24 \pm 0.02$	$0.58 \pm 0.01 / 0.58 \pm 0.02$	$0.36 \pm 0.00 / 0.34 \pm 0.00$

Table 4. ^{13}C Relaxation Rates for Raffinose. All measurements were performed twice to estimate the error of the relaxation rates.

	T_1 [ms]	r.m.s.d. T_1 [ms]	T_2 [ms]	r.m.s.d. T_2 [ms]	hetNOE
Gal C1	576	30	282	7	0.37
Gal C2	509	15	300	37	0.38
Gal C3	519	11	198	9	0.38
Gal C4	594	18	225	27	0.40
Gal C5	516	41	208	16	0.38
Avr. Gal	543		243		0.38
Glc C1	466	53	310	23	0.36
Glc C2	460	34	323	10	0.37
Glc C3	494	13	253	36	0.37
Glc C4	459	7	226	15	0.37
Glc C5	481	6	223	15	0.37
Avr. Glc	472		267		0.37
Fru C3	518	43	330	32	0.36
Fru C4	518	40	369	20	0.36
Fru C5	487	19	246	18	0.38
Avr. Fru	508		315		0.37
Gal C6	519	42	244	11	0.38
Glc C61	284	9	174	9	0.38
Glc C62	265	6	161	14	0.38
Fru C1	359	17	243	31	0.38
Fru C61	455	26	217	25	0.39
Fru C62	391	1	225	46	0.39

within 20%. However, for galactose we observe a reduction of the tensor size to *ca.* 60% compared to the other two tensors. We attribute this reduction to higher amplitude dynamics of the galactose moiety. This motion is reflected in T_1 , T_2 , and in heteronuclear NOE data for the C-atoms (Table 4). An overall correlation time τ_c of *ca.* 150 ps was calculated for raffinose. The average T_1 values of galactose are larger than in glucose and fructose, indicating a larger amplitude for the mobility. The mobility is anisotropic since the two axial CH vectors at C1 and C4 in galactose show a higher mobility than the equatorial vectors. This finding would be in agreement with a rotation about the φ_1 angle of the glycosidic linkage between galactose and glucose in raffinose (Fig. 4).

The structure calculations were performed on the basis of NOE distance information in addition to the dipolar couplings as given in Tables 1 and 2. For the calculation of the structure, the axial tensor component was changed from ± 4.5 to ± 8.0 Hz with a step size of 0.5 Hz, and the rhombicity was changed from 0.417 to 0.666 with a step size of 0.083 for the CHAPSO/DMPC dipolar couplings as described above (Materials and Methods) and based on the monosaccharide tensors. For $D_{\text{ax}} = -4.50$ Hz, $R = 0.667$ and $D_{\text{ax}} = 4.50$ Hz, $R = 0.583$, the back-calculated values from the ten lowest-energy structures were $D_{\text{ax}} = -4.17 \pm 0.02$ Hz, $R = 0.58 \pm 0.01$ and $D_{\text{ax}} = -4.24 \pm 0.01$ Hz, $R = 0.58 \pm 0.02$, respectively. For the second data set (written in brackets below), the change of sign of the axial component does not indicate strong differences of the tensor, which is obvious when comparing the x , y , and z components of the initial tensor: $D_{xx} = -0.57$ Hz, $D_{yy} = -8.44$ Hz, $D_{zz} = 9.00$ Hz to the final tensor:

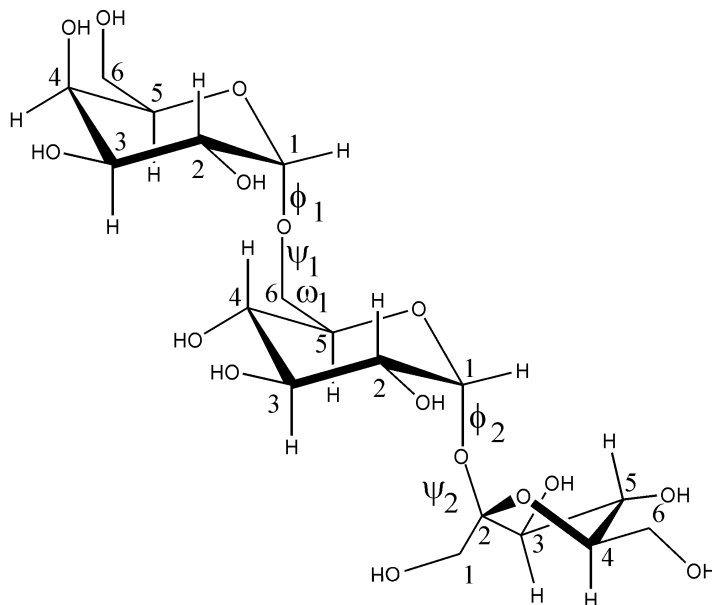


Fig. 4. Conformation of raffinose with the five rotatable dihedral angles as defined by $\varphi_1 = \text{Gal}(O5, C1, O1)\text{Glc}(C6)$, $\psi_1 = \text{Gal}(C1, O1)\text{Glc}(C6, C5)$, $\omega_1 = \text{Glc}(O6, C6, C5, O5)$, $\varphi_2 = \text{Glc}(O5, C1, O1)\text{Fru}(C2)$, $\psi_2 = \text{Glc}(C1, O1)\text{Fru}(C2, O5)$

$D_{xx} = 0.54$ Hz, $D_{yy} = 7.90$ Hz, $D_{zz} = -8.47$ Hz (with $D_{ax} = \frac{2D_{zz} - D_{yy} - D_{xx}}{6}$ and $R = \frac{2(D_{xx} - D_{yy})}{3D_{zz}}$ and keeping in mind that the axis labels can be interchanged without changing the physics). The back-calculated monosaccharide alignment tensors for glucose and fructose agree quite well with the overall alignment tensor. The back-calculated galactose tensor remains smaller by *ca.* 60% than the two other ones.

Fig. 5 shows a stereoplot of the ten lowest-energy structures of raffinose calculated with the experimental data with $D_{ax} = 4.50$ Hz and $R = 0.583$ ($Q = 0.34$). For the ten structures with lowest energies and lowest Q values, the glycosidic linkage between glucose and fructose is described by $\varphi_2 = 98.4 \pm 1.6^\circ$ ($\varphi_2 = 95.9 \pm 2.7^\circ$) and $\psi_2 = -151.9 \pm 1.1^\circ$ ($\psi_2 = -153.0 \pm 1.5^\circ$) (angles in braces describe the second tensor mentioned above). The glycosidic linkage between galactose and glucose is described by $\varphi_1 = 57.3 \pm 1.5^\circ$ ($\varphi_1 = 55.5 \pm 0.6^\circ$), $\psi_1 = -178.3 \pm 0.7^\circ$ ($\psi_1 = -176.8 \pm 0.6^\circ$) and $\omega_1 = -62.8 \pm 0.5^\circ$ ($\omega_1 = -62.4 \pm 0.5^\circ$) with a mean Q value of 0.36 (0.34). A recalculation of the glucose–fructose linkage ignoring the galactose experimental restraints did not change the result.

The result of the structure calculation did not change when further experimental restraints were taken into account. This is true for the interglycosidic NOE cross-peak (GlcH1/FruH1) that translates into a distance of 2.38 Å based on the GlcH1/H2 distance (2.41 Å in the neutron-diffraction structure of saccharose[43][44]) as a reference, as well as for the $^3J(\text{H},\text{H})$ coupling constants between H3,H4 and H4,H5 protons in the fructose ring. These coupling constants were parameterized by means of

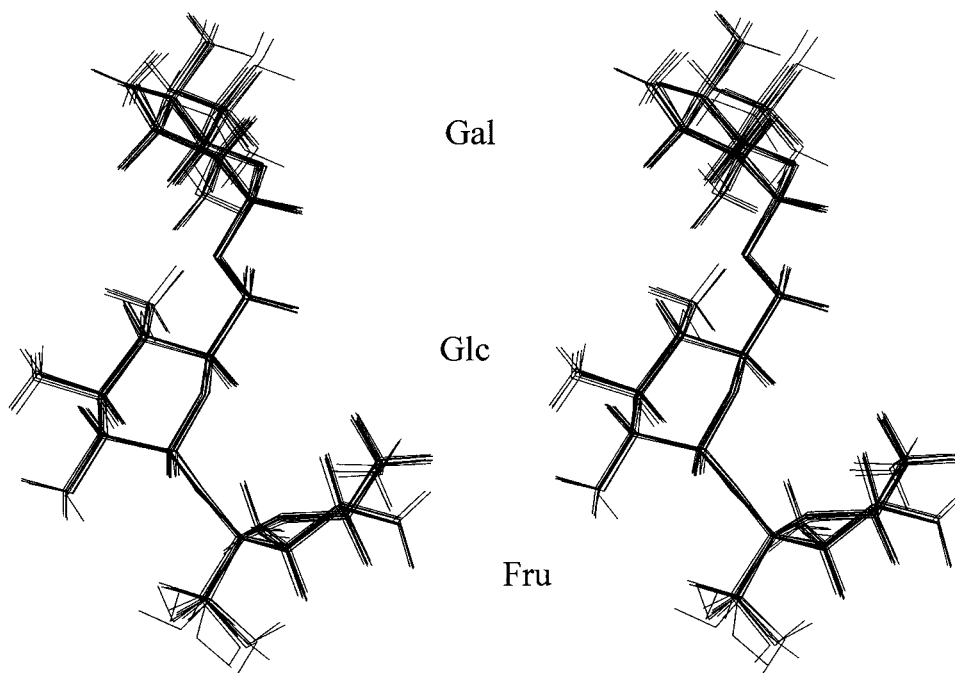


Fig. 5. Stereoplot of the 10 structures of raffinose with lowest energy as obtained from the dipolar, NOE, and J-coupling restraints. The glucose and galactose are in the chair conformation, the fructose in the 4T_3 conformation. The r.m.s.d. of the structures is 0.28 Å (heavy atoms).

the Karplus curve of Haasnoot *et al.* [45][46]. Calculations with and without these coupling constants showed no difference and yielded the 4T_3 conformation of the fructose ring.

The cross validation of the dipolar couplings is summarized in Table 5. The experimentally measured but so far unused dipolar couplings are reproduced quite well. Of special interest is the Fru-C6 H₂ group that has a distribution of conformations of 66% of g^+ and 32% of g^- based on scalar coupling constants. Provided the correct structure of the trisaccharide was calculated, it should be possible to determine the

Table 5. Cross Validation of Raffinose Structure with Measured D(H,H) Dipolar Couplings, Which Were Not Used in the Structure Calculations. The two theoretical couplings are derived from the two optimal alignment tensors as described in the text. The errors are the standard deviations derived from the ten best NMR-derived structures of raffinose.

	Exper. [Hz]	Theory [Hz]
Fru-C6/H6	1.80	2.10/2.07
Fru-C6/H6'	-2.00	-1.78/ -1.66
Fru-H6/H6'	1.70	0.85/0.93
Gal-H1/Glc-H6'	± 1.82	-1.62 ± 0.06/ -1.60 ± 0.06
Gal-H5/Glc-H4	± 1.56	-1.66 ± 0.06/ -1.72 ± 0.07
Gal-H1/Glc-H6	± 1.45	0.81 ± 0.02/0.82 ± 0.03

distribution of populations based on the experimental dipolar couplings alone. Indeed, we calculate 62.5% of g^+ , 32% of g^- , and 7.5% of t from the dipolar couplings (C6,H6', C6,H6, and H6',H6). Thus, it should be possible to predict the conformation also for those CH₂ groups where an analysis based on scalar coupling constants and NOEs was not possible. This is the case for the Fru-C1 H₂ and the Gal-C6 H₂ groups, for which the protons have identical chemical shifts and only the C,H dipolar couplings could be measured. From this sum of the dipolar couplings, we can calculate a range of populations for the staggered conformations. For Gal-C6 H₂, the population-distributing parameterization is $p(g^-) = 0.22 + 0.2x$; $p(g^+) = 0.78(1 - x)$ and $p(t) = 0.58x$ with x varying between 0 and 1. This prediction is in quite good agreement with the population distribution found for the C6 H₂ of galactose of $p(g^-) = 21\%$, $p(g^+) = 45\%$, $p(t) = 25\%$ [47], in a study using selective deuteration in the C6 position of galactopyranose, if we set $x = 0.406$. According to the same analysis with dipolar couplings for the Fru-C1 H₂, we find a population parameterization: $p(g^-) = 0.29$; $p(g^+) = 0.71(1 - x)$ and $p(t) = 0.71x$ with x varying between 0 and 1.

Fitting of known substructures to the experimental dipolar couplings provides a measure for conformational similarity. Fitting the neutron-diffraction structure of saccharose [43][44] to the firm dipolar couplings for the saccharose part of raffinose yields a Q factor of 0.26, which is of the same size as Q factors obtained when fitting the dipolar couplings of the individual monosaccharides to an alignment tensor. Thus, it can be expected that the solution structure of the saccharose part in raffinose agrees well with the neutron-diffraction structure of saccharose. Indeed, the dihedral angles for the Glc-Fru linkage of $\varphi_2 = 98.4 \pm 1.6^\circ$ ($\varphi_2 = 95.9 \pm 2.7^\circ$) and $\psi_2 = -151.9 \pm 1.1^\circ$ ($\psi_2 = -153.0 \pm 1.5^\circ$) agree very well with the neutron-diffraction data ($\varphi_2 = 107.8^\circ$ and $\psi_2 = -159.8^\circ$). However, fitting the X-ray structure [42] of raffinose to the firm dipolar couplings yields a Q value of 0.54. This indicates that the solution structure of raffinose deviates from its X-ray structure. Indeed, the angles describing the Glc-Fru linkage $\varphi_2 = 81.6^\circ$ and $\psi_2 = -105.5^\circ$ deviate from the previously mentioned angles for the solution structure of raffinose.

2.4. Structure Calculation of Saccharose. The procedure used for raffinose was also used for the structure calculation of saccharose. Measured scalar and dipolar couplings are given in *Tables 6* and *7*. Dipolar couplings marked with a star in *Table 6* are again subject to conformational averaging or proton chemical-shift overlap and therefore not used for the refinement. The firm dipolar-coupling constants were fitted against the neutron-diffraction structure [43][44] of saccharose individually for each monosaccharide and for the whole structure [39]. The tensor sizes and rhombicities as well as the Q factors [40] are given in *Table 8* for hetero- and homonuclear dipolar couplings in combination ($D(\text{C,H}) + D(\text{H,H})$). Fitting of the dipolar couplings of glucose and fructose to the monosaccharide structure from the neutron-diffraction structure of saccharose yielded almost identical tensors ($^{\text{G}}\text{Glc}$: $D(\text{C,H}) + D(\text{H,H})$ and $^{\text{F}}\text{Fru}$: $D(\text{C,H}) + D(\text{H,H})$ in *Table 8*). Eight linearly independent vector orientations could be measured for glucose. Only five are available for fructose, which is the reason for $Q = 0.0$. Based on this result, for the structure calculation of saccharose, one tensor was assumed for the whole disaccharide. The axial tensor component (D_{ax}) was varied from ± 2.25 to ± 3.25 Hz with a step size of 0.25 Hz and the rhombicity (R) of the alignment tensor was changed from 0.325 to 0.625 with a step size of 0.05. For $D_{\text{ax}} =$

Table 6. *Dipolar Couplings Determined for Saccharose in CHAPSO/DMPC (1/3.5; 7.5%)*. The couplings not marked by stars are the firm couplings as described in the text.

$D(\text{C,H})$ [Hz]		$D(\text{H,H})$ [Hz]	
Glc-C1/H1	1.95	Glc-H1/H2	-2.00
Glc-C2/H2	1.88	Glc-H2/H3	0.81
Glc-C3/H3	1.99	Glc-H3/H4	0.34
Glc-C4/H4	1.11	Glc-H4/H5	-0.45
Glc-C5/H5	1.80	Glc-H2/H4	0.00
*Glc-C6/H6	-0.88	Glc-H3/H5	0.00
*Fru-C1/H1	-1.25	Fru-H3/H4	-0.11
Fru-C3/H3	-3.31	Fru-H4/H5	-0.30
Fru-C4/H4	-2.73		
Fru-C5/H5	-4.49		
*Fru-C6-H6	-1.63		

Table 7. *Scalar Couplings Determined for Saccharose in Isotropic Solution*.

$^1J(\text{C,H})$ [Hz]		$^3J(\text{H,H})$ [Hz]	
Glc-C1/H1	169.6	Glc-H1/H2	4.02
Glc-C2/H2	144.3	Glc-H2/H3	10.07
Glc-C3/H3	145.4	Glc-H3/H4	9.13
Glc-C4/H4	144.8	Glc-H4/H5	9.94
Fru-C1/H1	289.4	Fru-H3/H4	8.77
Fru-C3/H3	144.8	Fru-H4/H5	8.45
Fru-C4/H4	144.6		
Fru-C5/H5	148.5		
Fru-C6/H6	287.6		

Table 8. *Tensor Sizes for Saccharose in CHAPSO/DMPC (1/3.5; 7.5%)*. The average alignment tensor and the standard deviation derived from the ten structures with lowest energy from 100 XPLOR calculations are given. The glucose and fructose structures are derived from the best solution structure of saccharose. The items indicated with \$ refer to the neutron diffraction structure of saccharose.

	D_{ax} [Hz]	R	Q
$^{\text{\$}}\text{Glc: } D(\text{C,H}) + D(\text{H,H})$	-2.68	0.44	0.05
Glc: $D(\text{C,H}) + D(\text{H,H})$	-2.58 ± 0.46	0.46 ± 0.01	0.07 ± 0.01
$^{\text{\$}}\text{Fru: } D(\text{C,H}) + D(\text{H,H})$	-2.32	0.61	0.00
Fru: $D(\text{C,H}) + D(\text{H,H})$	-2.26 ± 0.02	0.63 ± 0.08	0.00 ± 0.00
$^{\text{\$}}\text{Sacc: } D(\text{C,H})$	3.19	0.65	0.06
Sacc: $D(\text{C,H})$	-2.28 ± 0.02	0.66 ± 0.08	0.04 ± 0.02
$^{\text{\$}}\text{Sacc: } D(\text{C,H}) + D(\text{H,H})$	-2.74	0.37	0.16
Sacc: $D(\text{C,H}) + D(\text{H,H})$	-2.51 ± 0.02	0.43 ± 0.02	0.09 ± 0.01

-2.50 Hz and $R=0.425$, the ten structures with the lowest energy have a Q value of 0.09. These structures have a self-consistent tensor with $D_{\text{ax}} = -2.51$ Hz and $R = 0.43$ (Sacc: $D(\text{C,H}) + D(\text{H,H})$ in Table 8). All other initial D_{ax} and R values were of lower self consistency. The back-calculated monosaccharide tensors agree well with the overall alignment tensor (Table 8). The ten structures with lowest energy not only have a low Q value of 0.09 but also a very small heavy-atoms r.m.s.d. value of 0.39 Å. The glycosidic linkage between glucose and fructose is described by $\varphi = 99.7 \pm 3.8^\circ$ and $\psi = -159.3 \pm 3.5^\circ$. This is in excellent agreement with the neutron-diffraction structure of

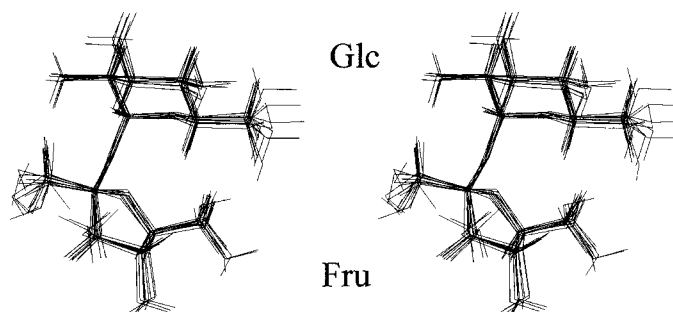


Fig. 6. Stereoview of the 10 structures of saccharose with lowest energy as obtained from the dipolar, NOE, and J-coupling restraints. The glucose is in the chair conformation, the fructose in the 4T_3 conformation. The r.m.s.d. of the structures is 0.39 Å (heavy atoms).

saccharose: $\varphi = 107.8^\circ$ and $\psi = -159.8^\circ$. The structure of saccharose is shown in Fig. 6 as a stereoplot.

The excellent agreement of the solution structure and neutron-diffraction structure of saccharose could be expected, because fitting of the firm experimentally determined dipolar couplings to the neutron-diffraction structure of saccharose yields a Q value of 0.16 (${}^8\text{Sacc}$: $D(\text{C,H}) + D(\text{H,H})$ in Table 8). This is almost as small as the Q values obtained for the individual monosaccharides in saccharose. Therefore, the solution structure of saccharose is expected to be highly similar to its neutron-diffraction structure.

The CH_2 groups were left out from the analysis because of overlap or conformational averaging. However, with the known tensor size the distribution of conformations for the CH_2 groups can be obtained like in raffinose. We find for the glucose C6H_2 group: $p(g^-) = 0.61x$; $p(g^+) = 0.30 + 0.09x$ and $p(t) = 0.78(1 - x)$ with x varying between 0 and 1. Again, this parameterization corresponds well with the exact values determined from selectively deuterated carbohydrates [48] ($p(g^-) = 57\%$, $p(g^+) = 38\%$, $p(t) = 5\%$), resulting in an x value of 0.935. The following conformational population was found for fructose: C1H_2 : $p(g^-) = 0.31$; $p(g^+) = 0.69(1 - x)$ and $p(t) = 0.69x$ with x varying between 0 and 1; C6H_2 : $p(g^-) = 0.37x$; $p(g^+) = 0.2 + 0.16x$ and $p(t) = 0.80(1 - x)$.

3. Conclusion. – We have shown that it is possible to measure $D(\text{H,H})$ in addition to $D(\text{C,H})$ dipolar coupling constants in sugars with the E.COSY method. From both types of dipolar-couplings, it is possible to determine a well-defined alignment tensor for the individual monosaccharides and check for differential monosaccharide mobility. Monosaccharide Q values are a good measure to check whether the solution structure agrees with structural models of oligosaccharides derived from modeling or crystal structures. Inclusion of the H,H dipolar couplings quite dramatically increases the precision of carbohydrate structures compared to those derived from NOE and J coupling constants alone. This is reflected in the sharp decrease of the r.m.s.d. values of the heavy atoms of the raffinose structures based on only NOE and J coupling data: r.m.s.d. = 1.02 Å, and when using a combination of NOE, J couplings, and hetero- and homonuclear dipolar couplings: r.m.s.d. = 0.28 Å. In principle, four symmetry-related

orientations of the monosaccharides are possible. However, due to the short linkers between the monosaccharides only one of these orientations was found in this work [9]. Therefore, one alignment was sufficient to fully define the entire conformation. Differential dynamic behavior was clearly observed for the galactose moiety, which had a significantly reduced alignment tensor when compared to those of the other monosaccharides. Therefore, further refinement of the model is expected when the full dynamic information of the monosaccharide moieties are taken into account.

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Model-Free Approach to the Dynamic Interpretation of Residual Dipolar Couplings in Globular Proteins

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Abstract: The effects of internal motions on residual dipolar NMR couplings of proteins partially aligned in a liquid-crystalline environment are analyzed using a 10 ns molecular dynamics (MD) computer simulation of ubiquitin. For a set of alignment tensors with different orientations and rhombicities, MD-averaged dipolar couplings are determined and subsequently interpreted for different scenarios in terms of effective alignment tensors, average orientations of dipolar vectors, and intramolecular reorientational vector distributions. Analytical relationships are derived that reflect similarities and differences between motional scaling of dipolar couplings and scaling of dipolar relaxation data (NMR order parameters). Application of the self-consistent procedure presented here to dipolar coupling measurements of biomolecules aligned in different liquid-crystalline media should allow one to extract in a “model-free” way average orientations of dipolar vectors and specific aspects of their motions.

1. Introduction

Since the first measurements of nuclear dipolar spin–spin couplings in proteins caused by the partial alignment of the proteins with respect to the external magnetic field,^{1–5} these parameters have become widely used for the determination and refinement of structures of biomolecules in solution.^{6–11} While in most applications residual dipolar couplings (rdc) are interpreted in the context of a static structure, it has been suggested from early on that these couplings also probe protein dynamics.¹² In multimodular systems, such as multidomain

proteins and complex sugars, differences in alignment tensors determined for individual domains were attributed to differential motions between the domains.^{13–17}

In the context of biomolecular structure determination, dipolar couplings are used to refine structures by optimizing agreement between experimental couplings, D_j^{exp} , and dipolar couplings predicted from the structural model, D_j^{calc} . A commonly used measure for the agreement is the Q value, defined by¹⁸

$$Q = \frac{\sum_j (D_j^{\text{exp}} - D_j^{\text{calc}})^2}{\sum_j (D_j^{\text{exp}})^2} \quad (1)$$

The smaller Q , the better is the agreement between the structural model and the experimental data. In case of perfect agreement ($Q = 0$), $D_j^{\text{exp}} = D_j^{\text{calc}}$ for all j (values of Q larger than 1 are of little interest, since $Q = 1$ can always be achieved by setting $D_j^{\text{calc}} = 0$). In our experience, Q values for dipolar couplings determined directly from X-ray structures and NMR structures determined without the use of dipolar couplings typically lie between 0.2 and 0.5.^{18,19} Possible reasons for Q values deviating from zero are experimental uncertainties, dynamic and exchange

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effects, and errors in the 3D structures, for example due to crystal packing in X-ray structures. NMR structures that are refined using dipolar couplings typically exhibit Q values between 0.05 and 0.3, depending also on the quality of the experimental data.^{19,20}

In this work we investigate the effects of motions on Q values of backbone dipolar couplings using a 10 ns molecular dynamics (MD) simulation of ubiquitin and discuss several different scenarios for the structural and dynamic interpretation of dipolar couplings that explicitly take dynamics contributions into account. A practical procedure for including certain aspects of dynamics is the division of each dipolar coupling value by the corresponding Lipari–Szabo order parameter, S_{LS} , obtained from NMR relaxation experiments.²¹ This procedure is valid only to a first-order approximation.²² The quality of this approximation is quantitatively assessed here using the MD simulation as a reference from which averaged dipolar couplings as well as S_{LS} order parameters are computed and compared with each other. The inverse problem is then addressed to directly extract information on biomolecular structure and motions from dipolar couplings measured in multiple liquid-crystalline environments that give rise to different alignments. The proposed treatment is based on the assumptions that the structure and intramolecular motion are not significantly altered by the liquid-crystalline environment and that the alignment process is not affected by intramolecular motions. The treatment allows the determination of residual dipolar coupling order parameters, S_{rdc} , that probe motion up to the millisecond range and thus are complementary to the relaxation-derived Lipari–Szabo order parameters S_{LS} . We do not discuss here larger scale dynamics of (partially) unfolded proteins or interdomain dynamics of multidomain proteins. In the following section, the theoretical background of motional averaging effects on dipolar couplings is developed. In subsequent sections the theory is applied to the MD trajectory.

2. Motional Averaging of Dipolar Couplings

The residual dipolar couplings, which give rise to resonance splittings, result from the secular part of the magnetic dipole–dipole interactions between nuclear spins of molecules that are partially aligned in an anisotropic liquid. The dipolar splitting $\langle D \rangle$ (in units of hertz) between directly bonded heteronuclei X and H can be expressed in the laboratory frame as

$$\langle D \rangle = -\frac{\mu_0}{4\pi^2} \gamma_X \gamma_H \frac{h}{2\pi} (4\pi/5)^{1/2} \langle r_{\text{XH}}^{-3} \rangle \langle P_2(\cos \chi) \rangle \quad (2)$$

where $P_2(\cos \chi) = (3 \cos^2 \chi - 1)/2$, χ is the angle of the internuclear vector to the external B_0 field, $\mu_0/4\pi = 10^{-7} \text{ V}\cdot\text{s}/\text{A}\cdot\text{m}$, γ_X , γ_H are the gyromagnetic ratios, and r_{XH} is the distance between the two spins. The angular brackets denote an ensemble average over orientations χ and distances r_{XH} or, assuming that the system is ergodic, a time average over a single molecule. In eq 2, it is assumed that radial and angular averaging are statistically separable, as is the case for directly bonded N–H and C–H atom pairs. Furthermore, the radial part $\langle r_{\text{XH}}^{-3} \rangle$ can often be considered to be identical for nuclear X–H pairs of the same kind.

For an internally static molecule, the dipolar couplings can alternatively be expressed in a molecular fixed frame in terms of a traceless reduced alignment tensor \mathbf{D} (in units of hertz),

with eigenvalues D_{xx} , D_{yy} , and D_{zz} , where $|D_{zz}| \geq |D_{yy}| \geq |D_{xx}|$.²² In the eigenframe of this tensor, the dipolar coupling between two nuclei connected by an internuclear vector with orientation $\Omega = (\theta, \varphi)$, where θ, φ denote the polar angles in the eigenframe of \mathbf{D} , is given by D_{stat} .²²

$$D_{\text{stat}} = D_a \left\{ 3 \cos^2 \theta - 1 + \frac{3}{2} R \sin^2 \theta \cos 2\varphi \right\} \quad (3)$$

where $D_a = D_{zz}/2$ is the axial component and $R = {}^{2/3}(D_{xx} - D_{yy})/D_{zz}$ is the rhombicity of \mathbf{D} with $0 \leq R \leq {}^{2/3}$. If \mathbf{D} is symmetric, D_{zz} corresponds to the principal axis value along the symmetry axis of \mathbf{D} . For a given alignment tensor \mathbf{D} , D_{zz} is the largest coupling possible for the considered type of X–H spin pairs.

In the presence of intramolecular molecular dynamics, the experimental dipolar coupling corresponds to a conformational average, denoted by angular brackets, relative to the alignment tensor frame:

$$\langle D \rangle = D_a \left\{ \langle 3 \cos^2 \theta - 1 \rangle + \frac{3}{2} R \langle \sin^2 \theta \cos 2\varphi \rangle \right\} \quad (4)$$

Equation 4 assumes that intramolecular motion does not interfere with the alignment process, i.e., that the alignment process is not significantly affected by internal motions. In the case of an alignment process due to steric effects,²³ this condition is fulfilled for motions that do not much alter the shape of the molecule. For small-amplitude, short-range motions, which can have a local or a concerted character,²⁴ eq 4 is expected to be more accurate than for larger amplitude motions of loops and termini, for example.

It is useful to express eq 4 in terms of normalized second-order spherical harmonic functions $Y_{2M}(\theta, \varphi)$:¹⁵

$$\frac{\langle D \rangle}{D_{zz}} = \sqrt{\frac{4\pi}{5}} \left(\langle Y_{20}(\theta, \varphi) \rangle + \sqrt{\frac{3}{8}} R \langle (Y_{22}(\theta, \varphi) + Y_{22}^*(\theta, \varphi)) \rangle \right) \quad (5)$$

where $Y_{20}(\theta, \varphi) = \sqrt{5/(16\pi)}(3 \cos^2 \theta - 1)$, $Y_{2\pm 2}(\theta, \varphi) = \sqrt{15/(32\pi)} e^{\pm 2i\varphi} \sin^2 \theta$.²⁵ In what follows, $Y_{2\pm 1}(\theta, \varphi) = \mp \sqrt{15/(8\pi)} e^{\pm i\varphi} \cos \theta \sin \theta$ will also be used.

In analogy to eq 3, D_{stat} can be defined for reference purposes as the dipolar coupling expected from a static internuclear vector pointing along the average orientation $(\theta_{\text{av}}, \varphi_{\text{av}}) = (\langle \theta \rangle, \langle \varphi \rangle)$:

$$\frac{D_{\text{stat}}}{D_{zz}} = \sqrt{\frac{4\pi}{5}} \left(Y_{20}(\theta_{\text{av}}, \varphi_{\text{av}}) + \sqrt{\frac{3}{8}} R (Y_{22}(\theta_{\text{av}}, \varphi_{\text{av}}) + Y_{22}^*(\theta_{\text{av}}, \varphi_{\text{av}})) \right) \quad (6)$$

The effect of intramolecular reorientational motion on the dipolar coupling can be expressed by the *dipolar scaling factor*, λ_{rdc} :

$$\lambda_{\text{rdc}} = \langle D \rangle / D_{\text{stat}} \quad (7)$$

In the absence of motion, $\lambda_{\text{rdc}} = 1$, and in the presence of motion, $-\infty < \lambda_{\text{rdc}} < \infty$.

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A benefit of eq 5 is that it can be easily transformed into a new reference frame related to the old reference frame by a three-dimensional rotation $\mathbf{R}(\alpha, \beta, \gamma)$ using the well-known transformation properties of the spherical harmonics $Y_{2M}(\theta, \varphi)$ under a three-dimensional rotation specified by the Euler angles α , β , and γ :²⁵

$$\mathbf{R}(\alpha, \beta, \gamma) Y_{2M}(\theta, \varphi) = \sum_{M'=-2}^2 e^{-i\alpha M'} d_{M'M}^{(2)}(\beta) e^{-i\gamma M} Y_{2M'}(\theta, \varphi) \quad (8)$$

Provided that the average protein structure and the dynamics do not vary with different alignment media, it follows that the dipolar coupling $\langle D \rangle$ measured in a new alignment frame i with axial component $D_{zz}^{(i)}$ and rhombicity $R^{(i)}$ that is related to the old frame by the rotation $\mathbf{R}(\alpha^{(i)}, \beta^{(i)}, \gamma^{(i)})$ can be expressed as

$$\frac{\langle D^{(i)} \rangle}{D_{zz}^{(i)}} = \sqrt{\frac{4\pi}{5}} \left(\sum_{M'=-2}^2 e^{-iM'\alpha^{(i)}} d_{M'0}^{(2)}(\beta^{(i)}) \langle Y_{2M'} \rangle \right) + \sqrt{\frac{4\pi}{5}} \sqrt{\frac{3}{8}} R^{(i)} \left(\sum_{M'=-2}^2 e^{-iM'\alpha^{(i)}} d_{M'2}^{(2)}(\beta^{(i)}) e^{-2i\gamma^{(i)}} \langle Y_{2M'} \rangle + e^{-iM'\alpha^{(i)}} d_{M'-2}^{(2)}(\beta^{(i)}) e^{2i\gamma^{(i)}} \langle Y_{2M'} \rangle \right) \quad (9)$$

Note that eq 9 is linear in the five motionally averaged spherical harmonics $\langle Y_{2M}(\theta, \varphi) \rangle$. If the couplings $\langle D^{(i)} \rangle$ belonging to a certain dipolar interaction are measured for five (or more) known alignments tensors $\{D_{zz}^{(i)}, R^{(i)}, \alpha^{(i)}, \beta^{(i)}, \gamma^{(i)}\}$, the five quantities $\langle Y_{2M} \rangle$, $M' = -2, -1, 0, 1, 2$ belonging to this dipolar interaction can be determined by solving the linear system of equations of eq 9 using, for example, singular-value decomposition or Moore–Penrose inversion.²⁶ The average vector orientation $(\theta_{\text{av}}, \varphi_{\text{av}})$ can be approximated by the effective orientation $(\theta_{\text{eff}}, \varphi_{\text{eff}})$ that is found by minimizing the sum

$$\sum_{M=-2}^2 \left(\langle Y_{2M}(\theta, \varphi) \rangle - Y_{2M}(\theta_{\text{eff}}, \varphi_{\text{eff}}) \right)^2 \quad (10)$$

To discuss the effects of symmetry in the motional distributions of an internuclear vector, it is useful to describe the distribution in a frame with the z axis pointing along the average orientation of the vector. In this new frame the instantaneous orientation of a vector is denoted by (θ', φ') . If $\langle e^{\pm i\varphi'} \rangle = \langle e^{\pm 2i\varphi'} \rangle = 0$, as is the case for axially symmetric reorientational motion, it follows $\langle Y_{2M} \rangle = 0$ except for $\langle Y_{20} \rangle$. To calculate the dipolar coupling, a coordinate transformation into the alignment frame is necessary, which is achieved by the rotation $\mathbf{R}'(\alpha' = 0, \beta' = -\theta_{\text{av}}, \gamma' = -\varphi_{\text{av}})$.

The extent of *nonaxial symmetry* of the motion can be quantified by the *motional asymmetry parameter* η fulfilling $0 \leq \eta \leq 1$:

$$\eta = \left(\frac{\sum_{M=\pm 1, \pm 2} \langle Y_{2M}(\theta', \varphi') \rangle \langle Y_{2M}^*(\theta', \varphi') \rangle}{\sum_{M=0, \pm 1, \pm 2} \langle Y_{2M}(\theta', \varphi') \rangle \langle Y_{2M}^*(\theta', \varphi') \rangle} \right)^{1/2}$$

$$= \frac{(S^2 - \langle P_2(\cos \theta') \rangle^2)^{1/2}}{S} \quad (11)$$

where the generalized S^2 order parameter has been introduced, which plays a key role in the “model-free” interpretation of heteronuclear NMR spin relaxation data by Lipari and Szabo,²¹

$$S^2 = \frac{4\pi}{5} \sum_{M=-2}^2 \langle Y_{2M}(\theta, \varphi) \rangle \langle Y_{2M}^*(\theta, \varphi) \rangle \quad (12)$$

The S^2 order parameter extracted from spin relaxation data is sensitive to motions faster than the overall tumbling correlation time and is denoted here as S_{LS}^2 . In contrast, an S^2 order parameter can be determined from residual dipolar couplings using eqs 9 and 12, which probes the much wider submillisecond time scale range and which is denoted as S_{rdc}^2 . Therefore, S_{LS}^2 is an upper limit for S_{rdc}^2 , $S_{\text{rdc}}^2 \leq S_{\text{LS}}^2$. Note that when using the 10 ns MD trajectory for calculating S_{LS}^2 and S_{rdc}^2 , the two parameters probe the same time scales and are therefore identical.

For *axially symmetric motion* with respect to the average orientation $(\theta_{\text{av}}, \varphi_{\text{av}})$, for which $\langle e^{\pm i\varphi'} \rangle$ and $\langle e^{\pm 2i\varphi'} \rangle$ vanish, the average dipolar coupling $\langle D \rangle$ can be expressed in a more compact way. Using eq 9 with $\langle Y_{2M}(\theta', \varphi') \rangle = 0$ for $M' = \pm 1, \pm 2$ it follows

$$\begin{aligned} \frac{\langle D \rangle_{\text{sym}}}{D_{zz}} &= \sqrt{\frac{4\pi}{5}} \langle Y_{20}(\theta', \varphi') \rangle \left(d_{00}^{(2)}(\beta') + \sqrt{\frac{3}{8}} R (d_{02}^{(2)}(\beta') e^{-2i\gamma'} + d_{0-2}^{(2)}(\beta') e^{2i\gamma'}) \right) \\ &= \left(\frac{1}{2} (3 \cos^2 \theta' - 1) \right) \left(\frac{1}{2} (3 \cos^2 \beta' - 1) + \frac{3}{4} R \sin^2 \beta' \cos 2\gamma' \right) \quad (13) \end{aligned}$$

where $\beta' = -\theta_{\text{av}}$, $\gamma' = -\varphi_{\text{av}}$. Consequently, the dipolar coupling of an internuclear vector is scaled under axially symmetric motion as compared to a static vector pointing along the average direction by

$$\lambda_{\text{rdc, sym}} = \frac{\langle D \rangle_{\text{sym}}}{D_{\text{stat}}} = \langle P_2(\cos \theta') \rangle \quad (14)$$

where θ' is the angle between an instantaneous internuclear vector and the average vector orientation $(\theta_{\text{av}}, \varphi_{\text{av}})$. Note that $\lambda_{\text{rdc, sym}}$ does not depend on the relative orientation $(\theta_{\text{av}}, \varphi_{\text{av}})$ with respect to the alignment frame. In the case of axially symmetric motion, S_{rdc}^2 simplifies to $S_{\text{rdc, sym}}^2 = (4\pi/5) \langle Y_{20}(\theta', \varphi') \rangle^2 = \langle P_2(\cos \theta') \rangle^2$ and thus

$$\lambda_{\text{rdc, sym}} = S_{\text{rdc, sym}} \quad (15)$$

From eq 14 follows that $-0.5 \leq \lambda_{\text{rdc, sym}}, S_{\text{rdc, sym}} \leq 1$.

Knowledge of λ_{rdc} is useful for the determination of an average 3D protein structure using residual dipolar couplings. D_{stat} values, which are directly related to $(\theta_{\text{av}}, \varphi_{\text{av}})$ by eq 6, could then be obtained by dividing experimental couplings $\langle D \rangle$ by λ_{rdc} according to eq 7. Since in practice λ_{rdc} values are not readily available, they sometimes are approximated by their respective S_{LS} values extracted from spin relaxation experiments.¹⁸ $\lambda_{\text{rdc}} = S_{\text{LS}}$ holds if (i) internal reorientational motion is axially symmetric and (ii) all relevant motions take place on nanosecond and subnanosecond time scales. Condition (i) can be tested by

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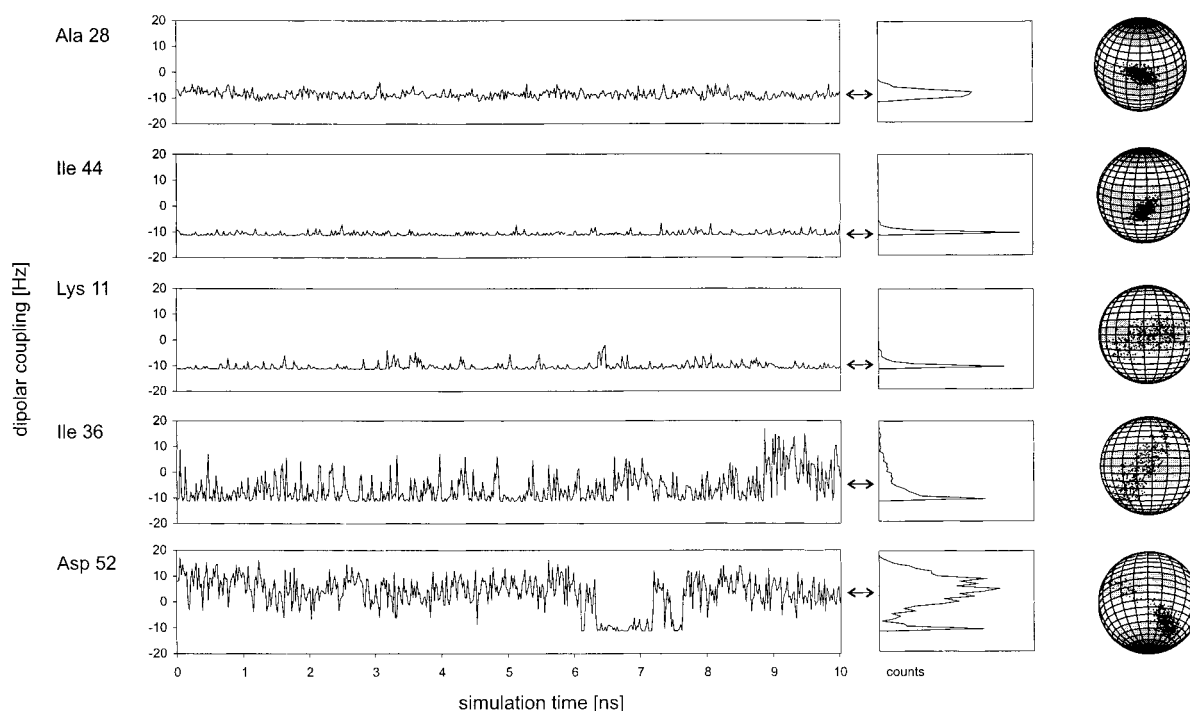


Figure 1. Time dependence of ^{15}N – ^1H residual dipolar coupling values for selected amino acids of ubiquitin extracted from a 10 ns molecular dynamics (MD) simulation. Ala 28 belongs to the α helix, Ile 44 to a β strand, and Lys 11, Ile 36, Asp 52 to loop regions. In the middle, the distributions of the couplings are plotted vertically, with the horizontal arrows indicating average dipolar coupling values that would be observed experimentally. The dots plotted on the surface of the spheres (right) correspond to the N– ^1H orientations sampled during the MD trajectory (500 snapshots).

using a molecular dynamics (MD) simulation, which is done in the following section. Presently, condition (ii) can be assessed only by comparison with experimental data. If condition (i) is fulfilled but (ii) is not fulfilled, $S_{\text{LS, sym}}$ represents an upper limit, $\lambda_{\text{rdc, sym}} = S_{\text{rdc, sym}} \leq S_{\text{LS, sym}}$.

In addition, it was assumed here that the alignment tensor \mathbf{D} is a priori known. In practice, however, \mathbf{D} is iteratively adjusted during structure refinement based on residual dipolar couplings. Thus, the best fitting alignment tensor \mathbf{D} implicitly includes certain motional contributions. In the following, a 10 ns MD simulation of ubiquitin is used to elucidate the influence of molecular motion on the interpretation of residual dipolar couplings.

3. Dipolar Couplings Calculated from MD Trajectory

A MD simulation of native ubiquitin was carried out under periodic boundary conditions using the program CHARMM 24.^{27,28} An energy-minimized all-atom representation of the X-ray structure of ubiquitin²⁹ was embedded in a cubic box with a side length of 46.65 Å, containing a total of 2909 explicit water molecules. The simulation was performed at a temperature of 300 K with an integration time step of 1 fs. Details of this simulation have been reported elsewhere.³⁰ During a simulation

time of 11 ns, snapshots were stored every 500 fs. From a 10 ns section of the trajectory, covering the range between 1 and 11 ns, 500 snapshots were selected with an increment of 20 ps for calculating dipolar couplings.

The 500 snapshots were reoriented and translated with respect to the snapshot at 6 ns by a least-squares superposition of their backbone atoms belonging to regular secondary structures. An average structure was constructed from the 500 reoriented snapshots by averaging over the Cartesian coordinates of all heavy atoms. The average positions of hydrogen atoms were determined by adding averaged X–H vectors (X = N or C atoms), which were rescaled to their standard lengths (1.02 Å for N–H and 1.09 Å for C–H), to the position of the corresponding X atom.

The shape of ubiquitin undergoes only small changes during the trajectory, as was assessed by computing inertia tensors for the 500 snapshots. The standard deviations of the moments of inertia tensor lie between 1% and 2%, which supports the validity of the assumptions underlying eq 4. It is assumed in the following that the MD trajectory represents a realistic description of the internal dynamics of ubiquitin, and thus slower time scale motions, which are not represented by the 10 ns simulation, are ignored.

To characterize the effect of dynamics, dipolar couplings were calculated from the 500 snapshots for a fixed alignment tensor \mathbf{D} with $D_{zz} = 20$ Hz (with respect to ^{15}N – ^1H couplings), $R = 0$, $\alpha = \beta = \gamma = 0$. The time dependence of backbone ^{15}N – ^1H couplings is depicted in Figure 1 for a selection of five amino acids that experience variable amounts of motion: Ala 28 (α helix), Ile 44 (β sheet), Lys 11 (loop), Ile 36 (loop), and Asp 52 (loop). Also given in Figure 1 are the distributions of the dipolar couplings over the trajectory. Most of the displayed distributions, which also depend on the size and orientation of the alignment tensor, show quasi-singularities and are unimodal

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Table 1. Back-Calculated Alignment Tensors and Q Values for Ubiquitin According to Scenario I (No Scaling)

nuclei ^a	$(\tilde{D}_{zz}/D_{zz})^b$	R^c	α^d (deg)	β^e (deg)	γ^f (deg)	Q_{all}^g	Q_{sec}^h	Q_{loop}^i
true	1.00	0.00	0	0	0	0.00	0.00	0.00
N H ^N	1.00	0.00	0	0	0	0.15	0.08	0.24
C ^{α} H ^{α}	1.00	0.00	0	0	0	0.10	0.08	0.12
H ^N H ^{α}	1.00	0.00	0	0	0	0.07	0.04	0.09
true	1.00	0.33	0	0	0	0.00	0.00	0.00
N H ^N	1.00	0.33	0	0	0	0.16	0.08	0.22
C ^{α} H ^{α}	1.00	0.33	0	0	0	0.09	0.07	0.11
H ^N H ^{α}	1.00	0.33	0	0	0	0.07	0.04	0.09
true	1.00	0.67	0	0	0	0.00	0.00	0.00
N H ^N	1.00	0.67	0	0	0	0.16	0.09	0.23
C ^{α} H ^{α}	1.00	0.67	0	0	0	0.10	0.08	0.11
H ^N H ^{α}	1.00	0.67	0	0	0	0.07	0.04	0.09
N H ^N	1.00	0.00	0	0	0	0.15	0.08	0.24
	1.00	0.00	0	45	0	0.14	0.07	0.19
	1.00	0.00	0	45	90	0.12	0.09	0.15
	1.00	0.00	0	45	180	0.10	0.07	0.13
	1.00	0.00	0	45	270	0.14	0.08	0.18
	1.00	0.00	0	90	0	0.12	0.06	0.24
	1.00	0.00	0	90	45	0.09	0.07	0.14
	1.00	0.00	0	90	90	0.14	0.06	0.20
	1.00	0.00	0	90	135	0.16	0.08	0.26

^a Pairs of nuclei for which dipolar couplings are computed. ^b Ratio of the best fitting and the predefined tensor size ($D_{zz} = 20$ Hz for N–H^N couplings). ^c Rhombicities and ^{d,e,f} orientations of the predefined and back-calculated tensors. ^{g,h,i} Q_{all} , Q_{sec} , and Q_{loop} are the Q values (eq 1) for the whole protein, the secondary structural elements, and the loops, respectively.

except for Asp 52, where larger scale backbone modulations lead to a bimodal distribution.

4. Influence of Motion on Q Values

In practice, experimental dipolar couplings are commonly refined toward a single static structure. It is investigated here what level of agreement can be expected between experimental couplings and couplings calculated from the average structure in the presence of molecular motion occurring during the 10 ns MD trajectory of ubiquitin.

For the following analyses, sets of dipolar couplings belonging to 11 alignment tensors with different orientations and rhombicities were constructed from the 500 snapshots taken from the trajectory (see Table 1). Here and in the following, it is assumed that changes in the alignment tensor leave intramolecular motions unaffected. For three alignment tensors with $D_{zz} = 20$ Hz (for N–H^N dipolar couplings) and R values set to 0, $1/3$, and $2/3$, respectively, dipolar couplings were computed for N–H^N, C ^{α} –H ^{α} , and H^N–H ^{α} spin pairs. In addition, eight more alignment tensors with $R = 0$ and $D_{zz} = 20$ Hz were defined by reorienting the original tensor using rotation matrixes $\mathbf{R}(\alpha, \beta, \gamma)$ with the following Euler angles to sample a representative distribution of tensor orientations:

$$(\alpha, \beta, \gamma) = \{(0^\circ, 45^\circ, 0^\circ), (0^\circ, 45^\circ, 90^\circ), (0^\circ, 45^\circ, 180^\circ), \\ (0^\circ, 45^\circ, 270^\circ), (0^\circ, 90^\circ, 0^\circ), (0^\circ, 90^\circ, 45^\circ), (0^\circ, 90^\circ, 90^\circ), \\ (0^\circ, 90^\circ, 135^\circ)\}$$

For these eight alignment tensors, only N–H^N couplings were computed.

On the basis of the MD simulation of ubiquitin, the effect of motion on Q was analyzed for three scenarios, I, II, and III, that involve different treatments of the data:

I. In this scenario, for a given alignment tensor, dipolar couplings were averaged over the 500 MD snapshots and compared with the dipolar couplings calculated from the average

structure described in the previous section using the same alignment tensor.

II. In this scenario, for a given alignment tensor, dipolar couplings were averaged over the 500 MD snapshots and compared with the dipolar couplings calculated from the average structure using an optimized alignment tensor that was varied in size and orientation to minimize Q .

III. In this scenario, for a given alignment tensor, dipolar couplings were averaged over the 500 MD snapshots and subsequently divided by their respective S_{LS} order parameters calculated from the same snapshots. These rescaled dipolar couplings were then compared with the dipolar couplings calculated from the average structure using an optimized alignment tensor that was varied in size and orientation to minimize Q .

Scenario I corresponds to a situation where the “true” alignment tensor is known from external sources, for example theoretical calculations^{23,31} or paramagnetic alignment. In this case, Q values become largest and motional effects are strongest, since they are not included in the form of a scaled alignment tensor. For scenario II, which is equivalent to overall scaling of all dipolar couplings combined with reorientation of the alignment tensor, readjustment of the alignment tensor can partially absorb internal motional effects. For example, if intramolecular motion reduces all dipolar couplings by 10% (compared to the couplings of the average structure), a new alignment tensor for the average structure that is 10% smaller would still yield $Q = 0$. This approach is equivalent to scaling of all couplings by a uniform λ_{rdc} value. Since the amplitudes of intramolecular motion generally vary between different protein sites, there will be no uniform scaling of dipolar couplings. Instead, individual motional scaling of dipolar couplings must be explicitly taken into account, which is the approach followed in scenario III. In the absence of any other information, a commonly used guess for the scaling factors are the S_{LS} order parameters of eq 12 obtained from spin relaxation measurements. As was shown in section 2 (eq 15), the scaling by S_{LS} values is adequate if all intramolecular motions are axially symmetric and take place on nanosecond and subnanosecond time scales that are accessed by spin relaxation experiments.

All three scenarios were analyzed for all 11 alignment tensors, and the results are compiled in Tables 1–3. For scenarios II and III, which involve fitting of the alignment tensors, the program DipoCoup¹⁹ was used, performing a Moore–Penrose inversion, also known as singular-value decomposition,^{26,32} previously used for the analysis of NMR relaxation data for anisotropic tumbling.³³ Since the results significantly differ between protein backbone parts with a well-defined secondary structure and loop regions, Q values were calculated for these different parts as well as for the whole protein backbone. Amino acids that belong to either a helix or a β sheet have residue numbers 2–7, 12–17, 23–34, 41–45, 49–50, and 65–72.

For scenario I, the Q values vary between 0.04 and 0.26 (see Table 1). They clearly depend on the type of vectors: N–H^N vectors show Q values that are larger than those of C ^{α} –H ^{α} vectors, which in turn have Q values that are larger than those of H^N–H ^{α} vectors. This is not surprising since the H^N–H ^{α} distances are longer than the one-bond distances, and thus a displacement of the H^N or H ^{α} atom causes only a minor change

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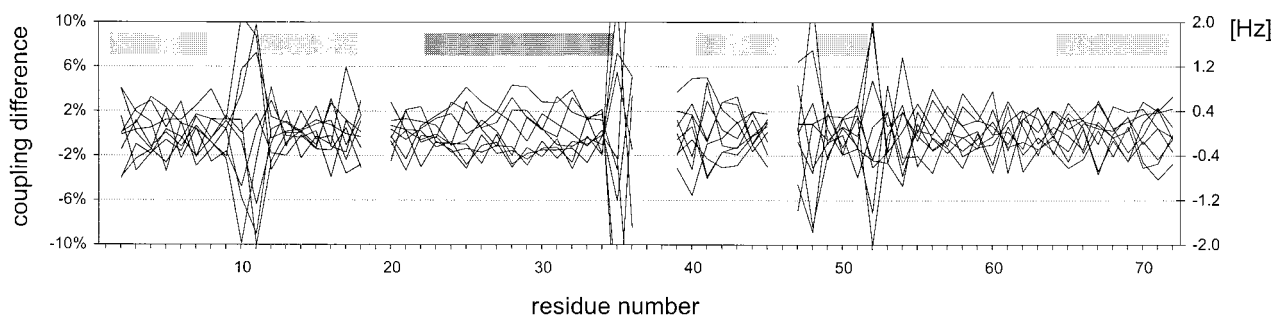


Figure 2. Differences between dipolar N–H^N couplings averaged over the 500 MD snapshots of ubiquitin and the back-calculated couplings determined for the (static) N–H^N vectors of the average structure as a function of residue number. The alignment tensor was optimized according to scenario II (see text). The calculation was done for each of the nine axially symmetric alignment tensors ($R = 0$) given in Table 2. The light gray bars on top of this figure (and also of Figures 3 and 6) indicates the β strands and the dark bar the α helix.

Table 2. Back-Calculated Alignment Tensors and Q Values for Ubiquitin According to Scenario II (Uniform Scaling)

nuclei ^a	$(\tilde{D}_{zz}/D_{zz})^b$	R^c	α^d (deg)	β^e (deg)	γ^f (deg)	Q_{all}^g	Q_{sec}^h	Q_{loop}^i	S_{LS}^j
true	1.00	0.00	0	0	0	0.00	0.00	0.00	
N H ^N	0.90	0.04	0	0	0	0.10	0.05	0.17	0.91
C ^{α} H ^{α}	0.92	0.01	0	1	0	0.06	0.04	0.07	0.94
H ^N H ^{α}	0.95	0.01	0	-1	0	0.04	0.03	0.05	0.95
true	1.00	0.33	0	0	0	0.00	0.00	0.00	
N H ^N	0.89	0.33	0	0	-2	0.11	0.06	0.15	0.91
C ^{α} H ^{α}	0.92	0.35	0	1	0	0.05	0.04	0.06	0.94
H ^N H ^{α}	0.95	0.33	0	-1	0	0.04	0.03	0.05	0.95
true	1.00	0.67	0	0	0	0.00	0.00	0.00	
N H ^N	0.91	0.64	1	0	1	0.08	0.04	0.14	0.91
C ^{α} H ^{α}	0.95	0.65	0	89	0	0.06	0.03	0.07	0.94
H ^N H ^{α}	0.97	0.65	0	0	0	0.04	0.02	0.07	0.95
N H ^N	0.90	0.04	0	0	0	0.10	0.05	0.17	0.91
	0.91	0.03	0	45	0	0.09	0.05	0.13	0.91
	0.90	0.02	0	45	91	0.06	0.05	0.07	0.91
	0.92	0.01	0	45	180	0.06	0.03	0.08	0.91
	0.91	0.01	0	45	270	0.09	0.06	0.11	0.91
	0.91	0.02	0	90	1	0.08	0.04	0.16	0.91
	0.93	0.01	0	90	45	0.05	0.03	0.09	0.91
	0.90	0.02	0	90	90	0.10	0.06	0.13	0.91
	0.89	0.02	0	90	135	0.11	0.05	0.18	0.91

^a Pairs of nuclei for which dipolar couplings are computed. ^b Ratio of the best fitting and the predefined tensor size ($D_{zz} = 20$ Hz for N–H^N couplings). ^c Rhombicities and ^{d,e,f} orientations of the predefined and back-calculated tensors. ^{g,h,i} Q_{all} , Q_{sec} , and Q_{loop} are the Q values (eq 1) for the whole protein, the secondary structural elements, and the loops, respectively. ^j S_{LS} is the average order parameter for these vectors calculated from the MD trajectory according to eq 12.

in the vector orientation. The Q values depend on the orientation of the alignment tensor but they are nearly independent of R . Significant differences in Q are observed between regular secondary structures and loop regions.

The results of scenario II, which are summarized in Table 2, demonstrate the effect on Q values if the alignment tensor is allowed to vary. As compared to Table 1, the Q values drop by about 30%. The motional effects are contained in modified alignment tensors $\tilde{\mathbf{D}}$. The directions of the principal axes change typically by less than 1° , and the rhombicity changes by 0.04 or less. The largest effects are seen in the new \tilde{D}_{zz} values, which are scaled relative to the original D_{zz} values by factors between 0.89 and 0.95. Table 2 contains also average S_{LS} and S_{rdc} order parameters calculated from the 500 snapshots according to eq 12. The S_{LS} and S_{rdc} values vary between 0.91 and 0.95, which is comparable to the scaling factor variations \tilde{D}_{zz}/D_{zz} . The Q values depend on the details of the motional distributions and average orientations of the internuclear vectors relative to the alignment tensors. The Q values vary for the chosen alignment

tensors by as much as a factor of 2. In Figure 2, the differences in back-calculated and “true” N–H^N dipolar couplings are plotted as a function of the residue number for the nine alignment tensors with different orientations defined in the lower part of Table 2. For individual N–H^N couplings, the motional influences characteristically depend on the directions sampled by the N–H^N vector relative to the alignment tensor. In the absence of rhombicity, $R = 0$, motion has the strongest influence for the average directions $\theta_{\text{av}} = 0^\circ, \pm 90^\circ, 180^\circ$, for which $P_2(\cos \theta)$ has maximal curvature. The differences between back-calculated and “true” couplings, which are distributed around zero, are largest for the loop regions that do not belong to regular secondary structure. N–H^N vectors of these regions have calculated S^2 order parameters lower than 0.8 (see Supporting Information). For some but not all of these vectors, the orientational distributions have not converged during the 10 ns MD trajectory, which can also be seen for some of the examples shown in Figure 1. Figure 2 illustrates that the effect of dynamics on the observable dipolar coupling value depends on the orientation of the alignment tensor. The alignment tensor defines the projection along which motion is observable. The possibility to reconstruct characteristic motional features from dipolar couplings collected for different alignment tensors is discussed below.

For scenario III (Table 3), where the average dipolar couplings are individually divided by their S_{LS} , S_{rdc} values, the fitted alignment tensors almost identically reproduce the “true” alignment tensors with changes in R smaller than 0.03 and \tilde{D}_{zz} values lying within 1% of D_{zz} . All Q values are further decreased as compared to the values in scenario II, with the largest reductions found for the mobile loop regions, where Q drops between 0.02 and 0.10. Figure 3 demonstrates the improved agreement for the individual N–H^N pairs as compared to the case in Figure 2. However, the Q values can still significantly differ from zero (see Table 3): for N–H^N dipolar couplings they vary between 0.04 and 0.07. This behavior is indicative of non-axially symmetric reorientational local motions of these internuclear vectors. Thus, the order parameter S_{LS} , S_{rdc} does not always accurately represent the motional scaling λ_{rdc} of dipolar couplings during the MD simulation. The residual discrepancies shown in Figure 3 are smallest for N–H^N vectors belonging to regular secondary structures, where dynamics is smaller and more closely matches axial symmetry than in the loop regions, where more complicated motion occurs that is generally more asymmetric. Analogous analyses carried out for alignment tensors with increasing rhombicities R indicate that changes in R can also have non-negligible effects on dipolar couplings.

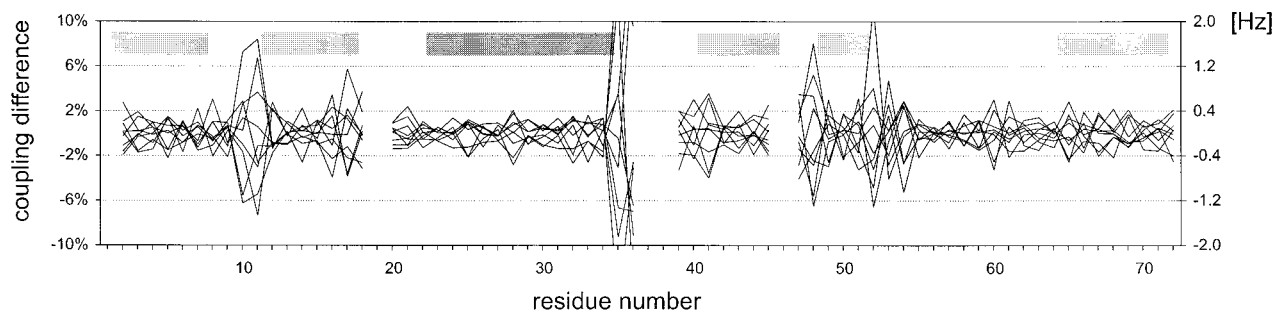


Figure 3. Differences between scaled dipolar N–H^N couplings averaged over the 500 MD snapshots of ubiquitin and the back-calculated couplings determined for the (static) N–H^N vectors of the average structure as a function of residue number. The dipolar couplings determined by averaging over the MD snapshots were scaled with their Lipari–Szabo order parameter S_{LS} according to scenario III. The calculation was done for each of the nine axially symmetric alignment tensors ($R = 0$) given in Table 3.

Table 3. Back-Calculated Alignment Tensors and Q Values for Ubiquitin According to Scenario III (Individual Scaling)

nuclei ^a	$(\bar{D}_{zz}/D_{zz})^b$	R^c	α^d (deg)	β^e (deg)	γ^f (deg)	Q_{all}^g	Q_{sec}^h	Q_{loop}^i
true	1.00	0.00	0	0	0	0.00	0.00	0.00
N H ^N	0.99	0.03	0	0	0	0.07	0.04	0.11
C ^α H ^α	1.00	0.00	0	0	0	0.02	0.02	0.02
H ^N H ^α	1.00	0.01	0	−1	0	0.03	0.02	0.04
true	1.00	0.33	0	0	0	0.00	0.00	0.00
N H ^N	1.00	0.35	0	1	2	0.08	0.05	0.11
C ^α H ^α	1.00	0.34	0	0	0	0.03	0.03	0.03
H ^N H ^α	1.00	0.34	0	1	0	0.03	0.02	0.04
true	1.00	0.67	0	0	0	0.00	0.00	0.00
N H ^N	1.01	0.65	0	0	0	0.04	0.02	0.07
C ^α H ^α	1.01	0.66	0	0	0	0.04	0.02	0.04
H ^N H ^α	1.01	0.66	−1	0	0	0.04	0.02	0.07
N H ^N	0.99	0.03	0	0	0	0.07	0.04	0.11
	1.00	0.00	0	45	1	0.07	0.03	0.10
	1.00	0.01	0	46	90	0.05	0.03	0.07
	1.00	0.03	0	45	180	0.06	0.03	0.08
	1.00	0.01	0	45	270	0.06	0.04	0.07
	0.99	0.01	0	90	1	0.04	0.02	0.08
	1.01	0.01	0	90	45	0.04	0.02	0.06
	1.01	0.01	0	90	90	0.06	0.04	0.08
	0.99	0.01	0	90	135	0.05	0.03	0.08

^a Pairs of nuclei for which dipolar couplings are computed. ^b Ratio of the best fitting and the predefined tensor size ($D_{zz} = 20$ Hz for N–H^N couplings). ^c Rhombicities and ^{d,e,f} orientations of the predefined and back-calculated tensors. ^{g,h,i} Q_{all} , Q_{sec} , and Q_{loop} are the Q values (eq 1) for the whole protein, the secondary structural elements, and the loops, respectively.

5. Reconstructing Motional Distributions from Multiple Alignment Data

From the previous analysis, it becomes clear that static and motional contributions to a dipolar coupling measured for a single alignment cannot readily be separated. The questions are addressed here of how this task can be accomplished by combining dipolar couplings measured for different alignment tensors and what aspects of the motional distributions of the internuclear vectors can be reconstructed.

For this purpose, five N–H^N pairs were selected in ubiquitin that show differential motional properties: Ala 28 (α helix) and Ile 44 (β sheet), which are part of regular secondary structures, and Lys 11, Ile 36, and Asp 52, which belong to the more mobile loop regions. The distinct motional behavior of these residues in the MD trajectory is reflected in their φ, ψ dihedral angle fluctuations. The right column in Figure 4 shows the φ, ψ distributions for the 500 MD snapshots: Ala 28 and Ile 44 show quite narrow φ, ψ distributions characteristic of α helix and β sheet structures, while Lys 11 and Ile 36 exhibit significantly wider distributions, in particular in their φ dihedral angle. Asp

52 exhibits a less regular behavior, indicative of a multimodal distribution. Dipolar couplings are sensitive to reorientations related to fluctuations of nearby dihedral angles as well as to longer range motions related to fluctuations of dihedral angles that are farther away.

In the left and middle panels of Figure 4, the orientations of the above-mentioned N–H^N vectors are displayed for the 500 MD snapshots indicated as dots as a function of the polar angles (θ, φ). The orientational distributions of the 500 snapshots are in all cases elongated (approximately elliptical for Ala 28 and Ile 44); i.e., they do not exhibit axial symmetry. The large filled circle in the center of each panel represents the orientation of the N–H^N vector in the average structure. The superimposed solid lines represent N–H^N vector orientations that are consistent with the dipolar couplings averaged over the 500 snapshots for the nine different alignment tensors with $R = 0$ described in the previous section (see Tables 2 and 3). Thus, any static N–H^N vector that points along a (θ, φ) direction belonging to a certain line could accurately reproduce the (scaled) dipolar coupling averaged over the trajectory for the alignment tensor associated with this line.

The panels in the left column correspond to scenario II, with the fitted alignment tensors given in the lower part of Table 2, while the panels in the middle column correspond to scenario III, where the couplings were divided by their individual S_{LS} order parameter. If all nine lines intersect at a single point, then a static N–H^N vector pointing along the intersection can simultaneously reproduce all MD-averaged couplings for the nine alignments. For the regular secondary structural residues Ala 28 and Ile 44, this behavior is approximately found for scenario II (left column of Figure 4), while it is not fulfilled for the three other residues, Lys 11, Ile 36, and Asp 52. For the latter residues, MD-averaged dipolar couplings measured for multiple alignments cannot be quantitatively reproduced by a static structural model.

Individual S_{LS} scaling of dipolar couplings (scenario III, middle column of Figure 4) improves the situation, in particular for Ala 28 and Ile 44, which belong to regular secondary structures although the reorientational distributions of these vectors are not axially symmetric. For these vectors, the intersections coincide with the dipolar coupling predicted from the average structure (filled circle). Thus, the MD-averaged dipolar couplings obtained in multiple alignment media scaled by their respective S_{LS} values allow for these residues the reconstruction of highly accurate average orientations. For a set of dipolar coupling measurements performed for a sufficiently large number of different alignments (five or more), it is conceivable to use an effective dipolar scaling factor $\lambda_{rdc,eff}$ as a fitting parameter. Since $\lambda_{rdc,eff}$ covers besides the relaxation-

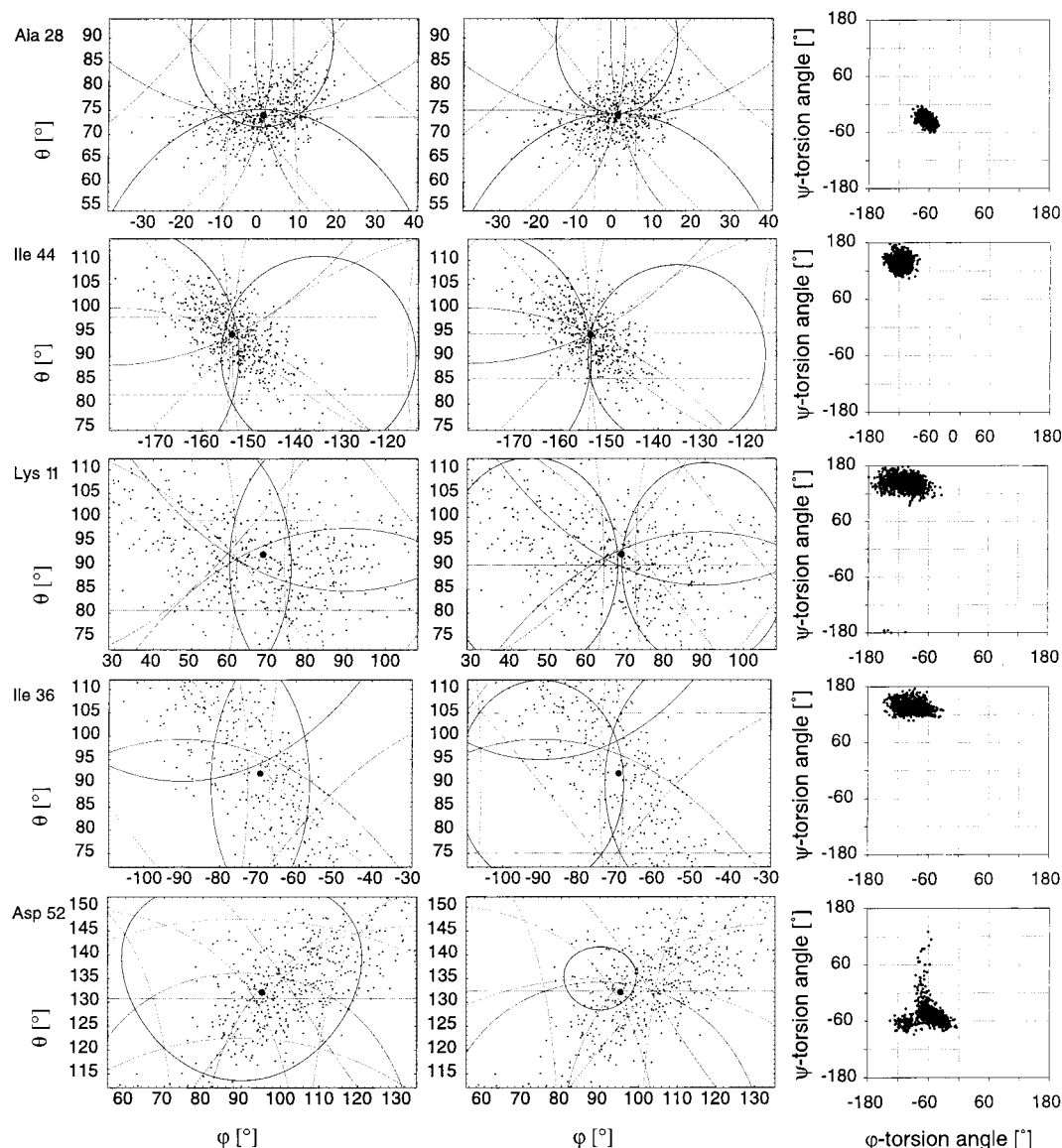


Figure 4. Determination of average N–H^N directions from dipolar couplings measured for nine different alignment tensors exemplified for residues Ala 28, Ile 44, Lys 11, Ile 36, and Asp 52. The dots in the panels in the left and the middle columns correspond to the orientations of the N–H^N vectors of 500 snapshots in the selected angular ranges. The full distributions are displayed on the spheres of Figure 1. The solid lines represent all static orientations that reproduce the MD-averaged couplings for the corresponding alignment tensors. The left panel corresponds to scenario II (no individual scaling of couplings), while the middle panel corresponds to scenario III (each coupling is scaled by its Lipari–Szabo order parameter S_{LS}). The panels in the right column show the φ, ψ dihedral angle distributions for these residues.

active motional time scales also slower time scales, comparison of $\lambda_{rdc,eff}$ with experimentally determined S_{LS} parameters should allow one to gain important insight into intramolecular motions occurring between nanosecond and millisecond time scales. It is expected that generally $\lambda_{rdc,eff} \leq S_{LS}$.

In contrast, for the mobile residues Lys 11, Ile 36, and Asp 52, none of the scenarios yields satisfactory results for the average orientations (left and middle columns of Figure 4). For scenario III (middle column), scaling by S_{LS} somewhat narrows down the range of possible average orientations, but obviously simple scaling remains insufficient for a quantitative determination of the average orientations because of the mathematical inequivalence of S_{LS} and λ_{rdc} for non-axially symmetric orientational distributions (cf. eqs 7, 9, and 12). Since according to eqs 7 and 9 λ_{rdc} directly depends on the average orientation of the dipolar vector, extraction of the average orientation and of the motional averaging effects becomes more complicated.

Model-Free Extraction of $\langle Y_{2M} \rangle$ and $(\theta_{eff}, \varphi_{eff})$ Quantities.

The following two-step procedure is proposed using experimental dipolar couplings and S_{LS} order parameters:

1. Absolute alignment tensors \mathbf{D} for multiple liquid-crystalline media are determined from experimental dipolar couplings using S_{LS} order parameters obtained from relaxation experiments for residues belonging to well-defined secondary structures.

2. The average orientation of a dipolar vector belonging to a more mobile region is extracted by fitting the averaged spherical harmonics $\langle Y_{2M} \rangle$, $M = -2, -1, 0, 1, 2$, to the dipolar couplings collected in all available alignment media using eq 9 with \mathbf{D} of 1 and by determining the orientations $(\theta_{eff}, \varphi_{eff})$ by a least-squares fit according to eq 10.

The averaged spherical harmonics $\langle Y_{2M} \rangle$ quantities provide a “model-free” representation of motional effects on dipolar couplings in analogy to S_{LS}^2 order parameters in spin relaxation studies.²¹ In fact, the $\langle Y_{2M} \rangle$ quantities contain information about

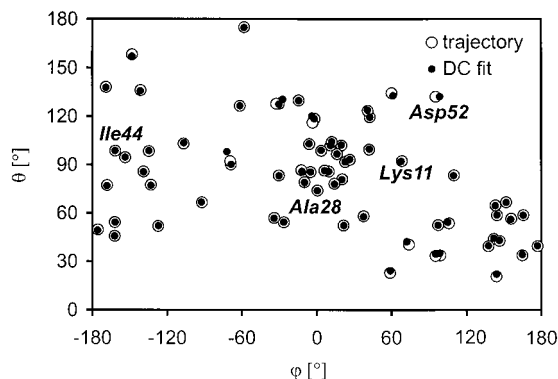


Figure 5. Comparison between average N–H^N directions ($\theta_{av}, \varphi_{av}$) determined from the MD trajectory and estimates ($\theta_{eff}, \varphi_{eff}$) determined by solving the linear system of equations (eq 9) followed by the minimization of the sum of eq 10. The figure shows that the estimate is generally within 2° of the exact average.

motional asymmetry, which has become lost in S_{LS}^2 . The above procedure was applied to the MD-averaged dipolar couplings, with results shown in Figures 5 and 6. Comparison between average N–H^N directions ($\theta_{av}, \varphi_{av}$) computed from the MD trajectory and estimates ($\theta_{eff}, \varphi_{eff}$) determined by solving the overdetermined linear system of equations (eq 9), including the residual dipolar couplings determined for all nine alignment media, followed by the minimization of the sum of eq 10, yields differences that are less than 2° for residues in secondary structural elements (Figure 5). Although the deviation can be larger for loop regions (up to 5°), the ($\theta_{eff}, \varphi_{eff}$) values provide, on average, a much better and more reliable estimate for ($\theta_{av}, \varphi_{av}$) than the (θ, φ) values that are consistent with a single dipolar coupling value. From the extracted $\langle Y_{2M} \rangle$ quantities S_{rdc}^2 values were determined according to eq 12. As expected, they turn out to be identical with the S_{LS}^2 values determined directly from the trajectory.

Furthermore, asymmetry parameters η , which reflect the amount of asymmetry in reorientational motion (eq 11), were determined from the extracted $\langle Y_{2M} \rangle$ quantities, and they are shown in Figure 6 as a function of the amino acid number. The largest asymmetry is found in ubiquitin for residues in mobile loop regions with η values exceeding 10% (see Figure 6), while in secondary structural elements the asymmetry is typically well below 5%.

6. Conclusion

Intramolecular motions affect residual dipolar couplings in the form of a scaling by a factor λ_{rdc} , which generally also depends on the average orientation of the internuclear vector with respect to the alignment frame. Using a MD simulation as a reference, motional averaging effects of dipolar couplings have been described in detail, and a solution to the inverse problem has been presented that used theoretical dipolar couplings,

assuming an optimal set of different alignment tensors. The proposed self-consistent analysis of dipolar couplings should allow the extraction of accurate structural information in terms of average orientations also when applied to experimental data.

Alignment tensors that are fitted to dipolar couplings tend to absorb a significant amount of intramolecular motional effects. If no information on S_{LS} order parameters is available, refinement of a static structural model should be “stopped” at Q values of about 0.05 for secondary structural parts and of about 0.1 for more mobile loop regions. If S_{LS} values are available, refinement to smaller Q values is conceivable, provided that no slower time scale motions are present.

Information on such slower time scale motions that are not reflected in spin relaxation data can be obtained from dipolar couplings measured in different liquid-crystalline media. The results presented here suggest that the combined use of dipolar coupling data sets measured in five or more different environments allows the accurate reconstruction of average positions and the retrieval of unique information on motional averaging of spherical harmonic functions of rank 2, $\langle Y_{2M} \rangle$, that is not readily accessible by S_{LS}^2 order parameters obtained from spin relaxation measurements. Besides the longer time scales probed by dipolar couplings, also direct information about motional asymmetry of individual internuclear vectors, expressed by the parameter η , is available. For rapid axially symmetric reorientational motion of an internuclear vector, λ_{rdc} becomes equal to S_{rdc} . The $\langle Y_{2M} \rangle$ quantities have a “model-free” character similar to the model-free order parameters S_{LS}^2 extracted from NMR spin relaxation experiments.²¹ In analogy to the NMR relaxation field, interpretation of the $\langle Y_{2M} \rangle$ quantities in terms of concrete motional models, such as the 3D GAF model,³⁰ is possible as a subsequent step of data interpretation.

The basic assumption made here is that the liquid-crystalline environment does not affect biomolecular structure and dynamics. This assumption can be experimentally tested to some extent by verifying that chemical shifts, line widths, and homo- and heteronuclear relaxation parameters do not significantly change with the liquid-crystalline environment. In the case that the average protein structure varies for different alignment media, such variations would be reflected also in the $\langle Y_{2M} \rangle$ quantities.

At present, the requirement of five different liquid-crystalline environments may seem demanding. Moreover, the different alignment tensors should significantly differ with respect to each other in order to minimize the influence of experimental uncertainties in the residual dipolar couplings. Rapid progress in the development and understanding of aligning tools, however, makes it likely that soon a sufficient number of different alignment media will become available that lead to different alignment tensors.^{4,5,34–52} Application of the presented protocol to experimental data is currently under way.

After submission of this work, a paper by Tolman et al.⁵³ appeared, in which the effects of protein motions on dipolar

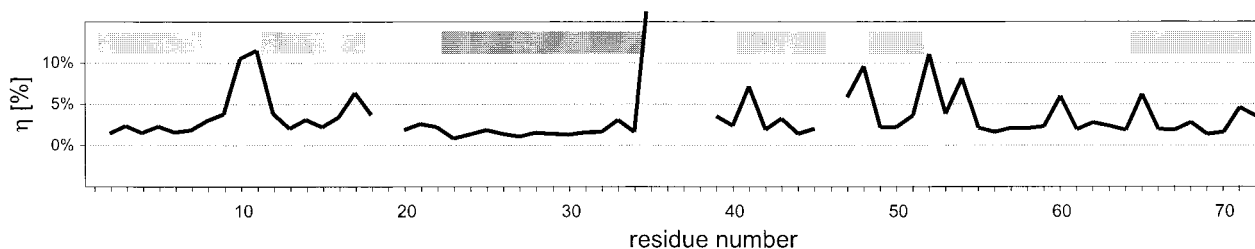


Figure 6. Motional asymmetry parameter η defined in eq 11 for N–H^N vectors as a function of the residue number. In regular secondary structure, η varies between 1% and 6%, while in more mobile loop regions the asymmetry can exceed 10%.

couplings measured for a single alignment tensor in ubiquitin were discussed. It differs from the one presented here in the following way. In the paper by Tolman et al., experimental dipolar couplings of different vectors in the peptide plane measured for one alignment medium were interpreted using analytical motional models, whereas in the present work MD-generated dipolar couplings of a single vector measured in multiple alignments were interpreted in a “model-free” way.

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Supporting Information Available: Two tables with structural and dipolar coupling averaging information determined from the 10 ns trajectory of ubiquitin; figure exemplifying the effect of errors in the dipolar couplings on the extraction of motional and structural information (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>. JA010002Z

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Model-Free Analysis of Protein Backbone Motion from Residual Dipolar Couplings

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Introduction:

Over the last few years, residual dipolar couplings (RDC's) (Tjandra & Bax, 1997; Tolman et al., 1995) have emerged as highly useful NMR parameters for the elucidation and refinement of biomolecular structures in solution. RDC's complement the traditional NMR parameters such as NOE and scalar couplings as well as other new types of constraints such as cross-correlated relaxation parameters (Reif et al., 1997) and trans-hydrogen bond J couplings (Dingley & Grzesiek, 1998) due to their long-range nature.

A main application of RDC's aims at the reduction of the number of required NOE's for determining the 3D structure of a protein (Bewley et al., 1998; Cai et al., 1998; Clore et al., 1999; Drohat et al., 1999). In another use of RDC's the PDB database is searched for protein structures that are consistent with a given set of RDC's (Aitio et al., 1999; Andrec et al., 2001; Annala et al., 1999; Meiler et al., 2000b). In yet another approach, structures of protein fragments are identified from the database and the 3D backbone structure of the protein is then reconstructed by properly assembling these fragments (Delaglio et al., 2000). Going even a step further, it was demonstrated that the backbone structure of ubiquitin can be determined by aligning sequential peptide planes solely based on RDC's, i.e. without using database information (Hus et al., 2001).

It was recognized from early on that dipolar couplings do not only reflect the average structure but also structural fluctuations allowing the characterization of conformational flexibility in proteins (Tolman et al., 1997). For cyanometmyoglobin, which contains eight helices, Tolman et al. (Prestegard et al., 1999; Tolman et al., 1997) presented two dynamical models to reproduce the experimental dipolar couplings (Tolman et al., 1995; Tolman & Prestegard, 1996a; Tolman & Prestegard, 1996b). The dynamical models were based on X-

ray (Kuriyan et al., 1986), neutron diffraction (Cheng & Schoenborn, 1991) and NMR (Ösapay et al., 1994) derived structures. The first model describes motions of the helices as a cooperative wobbling in a cone with symmetry axis along the helix axes. In the second model the helix was treated as a rigid unit that undergoes rotations about an axis perpendicular to the helix axis. In both models scaling factors S for the dipolar couplings were introduced that decreased dipolar couplings predicted from a static structure. The extracted scaling factors varied between 0.45 to 0.7 for individual helices, corresponding to sizeable cone opening angles (e.g. $\alpha=42^\circ$ for $S = 0.65$) if axially symmetric motions are assumed.

More recently, Tolman et al. presented an approach to extract dynamics from a set of RDC's measured along the backbone of ubiquitin (Tolman et al., 2001). They used a single alignment medium and measured 7 different heteronuclear dipolar couplings including the spin pairs N-HN, C^i -HN $^{(i+1)}$, C^i -N $^{(i+1)}$, C α -C', C'-H α , C α -H α , C α -C β within the backbone of each amino acid. Assuming a fixed geometry of these vectors for each amino acid moiety in the protein, they derived a general degree of order value ϑ (GDO) that reflects the motional scaling of the dipolar couplings for each peptide moiety. The success of the approach however critically depends on the accurate knowledge of the local bonding geometry of all atoms involved (bond lengths and bond angles) and the ϕ torsion angle.

Recently, we introduced a "model-free" approach to the dynamic interpretation of RDC's of a single dipolar vector measured in multiple alignments (Meiler et al., 2000a). The approach used a 10 ns molecular dynamics (MD) simulation of ubiquitin from which averaged RDC's were computed for backbone NH dipolar vectors using 9 different alignments. A mathematical framework was developed that describes the extraction of averages of spherical harmonics of rank 2, $\langle Y_{2M}(\theta, \phi) \rangle$, and of effective vector orientations $(\theta_{eff}, \phi_{eff})$ that correspond in good approximation to the average orientations (θ_{av}, ϕ_{av}) as extracted from the

trajectory. The $\langle Y_{2M}(\theta, \phi) \rangle$ quantities reflect motional averaging without necessitating a concrete motional model, in analogy to the model-free approach of Lipari & Szabo (Lipari & Szabo, 1982a; Lipari & Szabo, 1982b) used for the interpretation of NMR spin relaxation data. The $\langle Y_{2M}(\theta, \phi) \rangle$ can be used to calculate an S_{rdc}^2 order parameter that is similar to the Lipari & Szabo S_{LS}^2 order parameter with the important difference that S_{rdc}^2 reflects motions on a time-scale range between femtoseconds and milliseconds while S_{LS}^2 reflects motions on (sub-) nanosecond time scales.

In the past motions on time scales of μs to ms were primarily accessed using $T_{1\rho}$ measurements. Off-resonance $T_{1\rho}$ experiments further extend the accessible time-scale range (Akke et al., 1998; Zinn-Justin et al., 1997). The translation of $T_{1\rho}$ data into dynamic models is hampered by the fact that the chemical shift information of the different conformers does not allow the derivation of structural models of the interconverting conformers. Therefore additional dynamical information is highly desirable to characterize structural processes on these slow time scales. In addition scalar J couplings also provide detailed information on slow time scale motion (Case et al., 2000).

Here we apply the theoretical approach of Meiler et al. (Meiler et al., 2001) to experimental NH dipolar couplings of ubiquitin. Ubiquitin is perhaps the protein that is best studied by NMR. Numerous ^{15}N backbone relaxation studies have been reported (Carlomagno et al., 2000; Lienin et al., 1998; Schneider et al., 1992; Tjandra et al., 1995). Using different approaches it is well established that in ubiquitin only the residues I23 and N25 exhibit conformational exchange (de Alba et al., 1999; Tjandra et al., 1995; Tjandra et al., 1996). Also H-D exchange data are available measured for ubiquitin (Johnson et al., 1999; Sivaraman et al., 2001). Residual dipolar coupling data were published for bicelle media (Cornilescu et al., 1999b; Ottiger & Bax, 1998b; Tolman et al., 2001). In this work RDC's of

ubiquitin were measured for 11 different alignment media in order to probe intramolecular motions in a model-free way (see Table 1). The data are interpreted using the framework described in the theory paper (Meiler et al., 2001) yielding effective NH vector orientations, fluctuation amplitudes expressed in terms of the RDC derived order parameter S_{rdc}^2 and information about the asymmetry of intramolecular motions. Adaptations of the introduced mathematical analysis that proved to be useful in context of experimental data are summarized in the following section and in the flow-chart diagram given in Figure 1.

Theoretical results

The dipolar coupling observed in a certain anisotropic medium (denoted by index i) are given by:

$$D_i^{\text{exp}} = D_{i,zz} \left\{ \langle 3 \cos^2 \theta_i^{at} - 1 \rangle + \frac{3}{2} R_i \langle \sin^2 \theta_i^{at} \cos 2\phi_i^{at} \rangle \right\} \quad \text{Eq.1}$$

The angular brackets denote conformational averaging. $D_{i,zz}$ is the main component and R_i is the rhombicity of the alignment tensor. θ^{at} and ϕ^{at} are the orientations of the vectors in the frame of the alignment tensor (at). To analyze dipolar couplings in the context of dynamics it is useful to express equation 1 using normalized second-order spherical harmonic functions as given in equation 2 (a more detailed description is given in the Appendix):

$$\frac{D_i^{\text{exp}}}{D_{i,zz}} = \sum_{M=-2}^2 F_{i,M} \langle Y_{2,M}(\theta^{mol}, \phi^{mol}) \rangle \quad \text{Eq. 2}$$

where $\langle Y_{2M}(\theta, \phi) \rangle$ are the averaged spherical harmonics for a given NH vector. The superscript mol in equation 2 describes the change of the coordinate system from the alignment tensor frame into the molecular frame. The $F_{i,M}$ are functions of the three Euler

angles α_i , β_i , and γ_i that relate the molecular frame to the dipolar frame (see Appendix). In the following we will write θ and ϕ where we refer to the molecular frame of the protein.

We have shown in the theory paper in scenario II that fitting the dipolar couplings to NH vectors contained in secondary structure elements of one rigid NMR structure (Cornilescu et al., 1999a) will yield a motion averaged alignment tensor characterized by $\tilde{D}_{i,zz}$ and \tilde{R}_i as well as the angles $\tilde{\alpha}_i$, $\tilde{\beta}_i$, and $\tilde{\gamma}_i$. The orientation $(\tilde{\alpha}_i, \tilde{\beta}_i, \tilde{\gamma}_i)$ and the rhombicity (\tilde{R}_i) of the scaled tensor are virtually indistinguishable from those of the true tensor and the motion is reflected only in a scaling of the principal value according to: $\tilde{D}_{i,zz} = S_{overall} \cdot D_{i,zz}$ ($S_{overall} = \lambda_{overall}$ in scenario II in the theoretical paper). The rhombicity and the orientation of the scaled versus the true tensor were independent of the exact value of the overall scaling as well as the structure used for fitting the tensor provided that the column vectors \vec{F}_i derived from the \hat{F} matrix are sufficiently linearly independent from each other. We can thus rewrite equation 2 based on the experimentally scaled alignment tensor using scaled spherical harmonics $\langle \tilde{Y}_{2M}(\theta, \phi) \rangle$:

$$\frac{D_i^{\text{exp}}}{\tilde{D}_{i,zz}} = \sum_{M=-2}^2 F_{i,M} \langle \tilde{Y}_{2,M}(\theta^{mol}, \phi^{mol}) \rangle = \sum_{M=-2}^2 F_{i,M} \left(\langle Y_{2,M}(\theta^{mol}, \phi^{mol}) \rangle / S_{overall} \right) \quad \text{Eq. 3}$$

Model Free Approach

The inversion of the \hat{F} matrix in equation 3 yields the averages of the spherical harmonics. Since we enforce $\langle Y_{22}(\theta, \phi) \rangle^* = \langle Y_{2-2}(\theta, \phi) \rangle$ and $\langle Y_{21}(\theta, \phi) \rangle^* = -\langle Y_{2-1}(\theta, \phi) \rangle$ in equation 3, 5 independent variables remain for the calculation. Therefore we need at least five alignments that need to be realized experimentally to calculate the $\langle Y_{2M}(\theta, \phi) \rangle$ values. However, from

equation 3 we only obtain $\langle Y_{2M}(\theta, \phi) \rangle / S_{overall} = \langle \tilde{Y}_{2M}(\theta, \phi) \rangle$ and not the desired $\langle Y_{2M}(\theta, \phi) \rangle$ values. We therefore need to determine $S_{overall}$ as will be described in the course of the paper. The $\langle Y_{2M}(\theta, \phi) \rangle$ values yield a model free analysis of the motion and make available a much more detailed picture of the motion than it is provided by S_{LS}^2 the Lipari-Szabo order parameter.

In order to achieve this more detailed picture we rotate each individual NH vector into a frame with primed axes x', y', z' such that $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ is maximized:

$$\max \langle \tilde{Y}_{20}(\theta', \phi') \rangle = \sum_{M=-2}^2 D_{M,0}(\phi_{eff}^{(1)}, \theta_{eff}^{(1)}, 0) \langle \tilde{Y}_{2M}(\theta, \phi) \rangle = \sqrt{\frac{4\pi}{5}} \sum_{M=-2}^2 Y_{2,-M}(\theta_{eff}^{(1)}, \phi_{eff}^{(1)}) \langle \tilde{Y}_{2M}(\theta, \phi) \rangle$$

Eq. 4

Maximizing $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ puts the new z' axis into the center of the distribution for the given NH vector and thus defines $\theta_{eff}^{(1)}$ and $\phi_{eff}^{(1)}$. As shown in the appendix, the $\langle \tilde{Y}_{21}(\theta', \phi') \rangle$ and $\langle \tilde{Y}_{2-1}(\theta', \phi') \rangle$ values vanish. Then the $\langle \tilde{Y}_{22}(\theta', \phi') \rangle$ and $\langle \tilde{Y}_{2-2}(\theta', \phi') \rangle$ averages reflect the asymmetry of the motion. In order to derive parameters that are easier to grasp we define a relative amplitude η of anisotropy of the motion defined in equation 5 that is equivalent to the definition of η in the theoretical paper (equation 11). This η parameter is independent of the overall scaling $S_{overall}$:

$$\eta = \sqrt{\frac{\sum_{M=-2,2} \langle \tilde{Y}_{2M}(\theta', \phi') \rangle \langle \tilde{Y}_{2-M}(\theta', \phi') \rangle}{\sum_{M=-2}^2 \langle \tilde{Y}_{20}(\theta', \phi') \rangle \langle \tilde{Y}_{20}(\theta', \phi') \rangle}}$$

Eq. 5

The ratio of the imaginary and the real part of $\langle \tilde{Y}_{22}(\theta', \phi') \rangle$ and $\langle \tilde{Y}_{2-2}(\theta', \phi') \rangle$ define the orientation of the anisotropic motion.

$$\bar{\phi}' = \frac{1}{2} \arctan \frac{\langle \tilde{Y}_{22}(\theta', \phi') \rangle - \langle \tilde{Y}_{2-2}(\theta', \phi') \rangle}{i(\langle \tilde{Y}_{22}(\theta', \phi') \rangle + \langle \tilde{Y}_{2-2}(\theta', \phi') \rangle)} \quad \text{Eq. 6}$$

It should be noted that due to the ϕ dependence of the $Y_{22}(\theta, \phi)$ and $Y_{2-2}(\theta, \phi)$ functions the $\bar{\phi}'$ angle has a π periodicity. It is obvious from the definition of $\bar{\phi}'$ that it is different from the average ϕ' of a distribution of vector orientations $\langle \phi' \rangle$. The translation of the five averaged spherical harmonics into the primed coordinate system amounts to the definition of five new parameters, namely: $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ which reflects the axial order, $\theta_{eff}^{(1)}$ and $\phi_{eff}^{(1)}$ which reflect the average orientation of the vector and η and $\bar{\phi}'$ which reflect the amount of anisotropic disorder and the direction of this anisotropic motion in the x', y' plane.

In addition to dissecting the orientational disorder into its axially symmetric and asymmetric distribution we can also calculate a traditional order parameter S_{rdc}^2 based exclusively on dipolar couplings and that is formulated in analogy to the well known Lipari-Szabo order parameter:

$$S_{rdc}^2 = \frac{5}{4\pi} \sum_{M=-2}^2 \langle Y_{2M}(\theta, \phi) \rangle \langle Y_{2M}^*(\theta, \phi) \rangle \quad \text{Eq. 7}$$

By contrast to S_{LS}^2 , S_{rdc}^2 covers motion up to the NMR time scale that is defined by the inverse of the differences of chemical shifts or dipolar couplings of exchanging conformations. It should be noted that the S_{rdc}^2 values form a distribution with an average of $S_{overall}^2$ and that they are always smaller than 1. Consequently, the \tilde{S}_{rdc}^2 derived from the $\langle \tilde{Y}_{2M}(\theta, \phi) \rangle$ values form a distribution with an average of 1 and therefore certain \tilde{S}_{rdc}^2 will exceed 1 for some of the NH vectors.

Derivation of average orientations

In the theoretical paper we were interested in the calculation of the averaged orientations of the vectors (θ_{av}, ϕ_{av}) defined as the polar angles of an averaged NH vector over the trajectory.

We found those can be obtained very accurately from the averages of the spherical harmonics $\langle Y_{2M}(\theta, \phi) \rangle$ and even better from the experimentally directly accessible $\langle Y_{2M}(\theta, \phi) \rangle / S_{overall} = \langle \tilde{Y}_{2M}(\theta, \phi) \rangle$. There are several options to derive these effective angles θ_{eff} and ϕ_{eff} that we call effective instead of average since they are not identical with the averages.

The first approach for the optimization of $(\theta_{eff}, \phi_{eff})$ uses the average spherical harmonics $\langle \tilde{Y}_{2M}(\theta, \phi) \rangle$ obtained by equation 3 and rotates them in a primed reference frame that the new average $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ is maximized according to equation 4. This approach was already explained in the previous paragraph. The superscript 1 on θ_{eff}, ϕ_{eff} indicates that this is just the first way to derive the information about the effective θ_{eff}, ϕ_{eff} . It is obvious that $\langle \tilde{Y}_{2,M}(\theta', \phi') \rangle$ is at maximum when the average vector is parallel to the z' -axis. This approach is used to derive the motional models from the averaged spherical harmonics (equation 5 and 6).

The second approach is to find two angles θ_{eff} and ϕ_{eff} that minimize the following expression:

$$\sum_{M=-2}^2 \left(\langle Y_{2,M}(\theta, \phi) \rangle - Y_{2M}(\theta_{eff}^{(2)}, \phi_{eff}^{(2)}) \right)^2 \quad \text{Eq. 8}$$

It has the drawback that individual order parameters for each NH vector are not taken into account.

Therefore a third approach is used, in which the directly accessible $\langle \tilde{Y}_{2,M}(\theta, \phi) \rangle$ values are scaled assuming axially symmetric motion. The scaling factor $\tilde{S}_{axial} = \frac{S_{axial}}{S_{overall}}$ is individually optimized for each NH vector but uniform for all alignment media. The superscript “axial” is used for this parameter since the value enforces a uniform scaling of all spherical harmonic $\langle \tilde{Y}_{2,M}(\theta, \phi) \rangle$ values and therefore a uniform scaling of all experimental dipolar couplings. This is true for axially symmetric motion as proven in the theoretical paper.

$$\sum_{M=-2}^2 \left(\left(\langle \tilde{Y}_{2M}(\theta, \phi) \rangle / \tilde{S}_{axial} \right) - Y_{2M}(\theta_{eff}^{(3)}, \phi_{eff}^{(3)}) \right)^2 \quad \text{Eq. 9}$$

Comparison with GDO approach

The scaling by S_{axial} is very similar to the internal generalized degree of order (GDO) analyzed by Tolman et al. (Tolman et al., 2001) based on the measurement of several dipolar couplings in one alignment medium. The residue specific internal GDO is defined as the ratio between the local and the overall alignment tensor (index o)(see equation 2 in (Tolman et al., 2001)):

$$\vartheta(\text{int}) = \sqrt{\frac{\sum_{ij} S_{ij}^2}{\sum_{ij} S_{ij,o}^2}} \quad \text{Eq. 10}$$

where S_{ij} are the indices of the Saupe matrix. This leads to:

$$\vartheta(\text{int}) = \frac{\left| \frac{1}{2} \sqrt{(4 + 3R^2)} \cdot S_{zz} \right|}{\left| \frac{1}{2} \sqrt{(4 + 3R_o^2)} \cdot S_{zz,o} \right|} \quad \text{Eq. 11}$$

where R is the rhombicity ($R = \frac{S_{xx} - S_{yy}}{S_{zz}}$) and S_{zz} the axial component of the alignment tensor. If the rhombicity R is identical to R_o the GDO is reduced to $\frac{S_{zz}}{S_{zz,o}}$. Even if the rhombicity is different for the overall and the fragment specific Saupe matrices, the influence of the rhombicity is rather minor. The maximum change from $R_o = \frac{2}{3}$ to $R = 0$ for the individual fragment or the reverse would change the GDO only by 15% while a change of R by 0.1 invariably leads to a change of the GDO by less than 1%. Thus the GDO is quite insensitive to the motional anisotropy. Therefore the GDO measures axially symmetric motion similar to the axial order parameter S_{axial} introduced here. Anisotropic motion is difficult to assess using the GDO. This is quite distinct from our approach as elaborated in the previous paragraph.

Error analysis

In the following we investigate the errors of our analysis. Due to the inversion of the \hat{F} matrix in equation 3 the errors on the dipolar couplings will translate into errors on the average spherical harmonics from which all further results are derived. The error propagation critically depends on the sampling of the five dimensional vector space spanned by the \vec{F}_i vectors. In the optimal situation, five alignment media would provide five orthogonal vectors. However, as will be found this ideal situation cannot readily be realized for experimental conditions. We measure the degree of non-singularity of the \hat{F} matrix by calculating its singular values and the ratio between the largest and the smallest singular value (condition number). Ideally, the condition number is close to 1, in reality however, the condition number is larger than 1.

The error on the experimental dipolar couplings was found to be $\pm 0.5\text{Hz}$ (the errors in different media slightly depend on the signal to noise ratio). The isotropic couplings were measured using the HSQC-J technique that allows measuring the couplings with higher accuracy. Using all 11 experimental dipolar couplings an average statistical error of 2.2% on the spherical harmonics $\langle \tilde{Y}_{2M}(\theta, \phi) \rangle$ is found with the largest error on one of the spherical harmonics being 3.7%. This yields a statistical error of the dipolar order parameter \tilde{S}_{rdc} that is smaller than 2%, the largest statistical error on a single spherical harmonic being 3.2%. It should be noted that this analysis explicitly addresses only the statistical errors while potential systematic errors are ignored.

Similar small errors as found for the 11 alignment media can be obtained with only five or six alignment media. The optimal combination of 6 or 5 alignment media (table 1) yields an average statistical error on the dipolar order parameter of 2.2% and 3.1% with the largest error on the spherical harmonics being 4.2% and 6%, respectively. The largest possible error on the dipolar order parameter is 3.5% and 4.4%. Thus the optimal selection of six alignment media would have provided similar results as the combination of all 11 alignment media. However, due to the unpredictability of the alignment tensors in the molecular frame we had to test a larger set.

Using exclusively the information provided by the bicelle media the error propagation is less favorable. The largest error on the spherical harmonics is 56.6% (average statistical error 26.7%) and the statistical average error of the dipolar order parameter is 22.7%. Thus with the 6 optimal or 11 used alignment media the errors are low enough to obtain statistically significant conclusions.

Results and Discussion:

Measurement of different alignment tensors

As mentioned in the introduction, the alignment tensors need to be sufficiently different so that the error propagation of the experimental dipolar couplings does not transform into an exceedingly large error on the averaged spherical harmonics $\langle Y_{2M}(\theta, \varphi) \rangle$ and the derivation of the θ_{eff} and ϕ_{eff} angles are faithful. Therefore a major objective of the experimental work was to find alignment media that provide a sufficiently small condition number. Since there is no theory to predict the orientation of the main axes of the alignment tensor with respect to the molecular frame for nearly all presently available alignment media we had to determine these orientations experimentally for ubiquitin. For 11 alignment media we determined the alignment tensors in the molecule fixed coordinate system (Fig. 2). First we used dilute liquid crystal media made from Phosphocholines commonly referred to as bicelles (Bax & Tjandra, 1997b; Sanders & Schwonek, 1992; Sanders II et al., 1994; Tjandra & Bax, 1997). The bicelles can be charged by additives that introduce electrostatic interactions between the biomacromolecule and the bicelles and thus rotate the orientation of the tensor. Using CTAB (Cetyltrimethylammoniumbromide) the bicelles are charged positively while SDS (Sodium dodecyl sulfonate) introduces a negative charge. However, unfortunately, the tensor did not change considerably. There is also no difference as expected when changing the mixture of the bicelles from CHAPSO/DLPC to DHPC/DMPC. This supports the notion that the shape rather than the charge distribution of the protein determines the alignment. This can also be derived from the small effect induced by the charged bicelles. Therefore, it was more important to find other experimental approaches to obtain sufficiently different orientations of the alignments. This was achieved using Pf-1 phage (Hansen et al., 1998), purple membrane (Bacteriorhodopsin) (Koenig et al., 1999; Sass et al., 1999), Helfrich phase surfactant lipids (Barrientos et al., 2000; Prosser et al., 1998), uncharged, nonionic *n*-dodecyl-penta(ethylene

glycol)/*n*-Hexanol phase (Rückert & Otting, 2000) and polyacrylamide gel (Sass et al., 2000; Tycko et al., 2000) for alignment. Some of these methods introduce much more charge onto the liquid crystals and therefore lead to bigger changes of the alignment tensors. By comparing the different phases, we also found rather interesting equivalences of media for ubiquitin alignment. For example, the uncharged CHPASO/DLPC bicelles and the nonionic *n*-dodecyl-penta(ethylene glycol)/*n*-Hexanol phase yielded almost the same orientation of the alignment tensor in the molecular frame. Thus for these two alignment media, the mechanism of alignment appears to be based on shape and not on the charge of the protein. This observation is also supported by the sharp lines of the spectra in these liquid crystal media and additionally confirmed by unchanged transverse relaxation times (T_2) as compared to water solution.

In all charged liquid crystal media the line widths are broader due to decreasing T_2 times and larger interaction with the orienting media. Both positive and negative charge on the alignment media induces a T_2 effect. For ubiquitin we found larger variation of the orientation of the alignment tensor when we measured in completely different alignment media rather than trying to modify one alignment method for example by addition of charges (CTAB or SDS in bicelle media). For example, using bicelle media alone provided the following scaled singular values 3.889, 1.248, 0.479, 0.103 and 0.026 yielding a condition number of 152.1. All 11 alignment media, however, yielded the scaled singular values: 4.909, 2.260, 0.844, 0.675 and 0.594 and a condition number of 8.2. Table 1 contains the set of media that had the smallest fifth singular value and the largest condition number for 5 to 11 alignment media used. It is interesting to note that the condition number for the optimal set of 6 alignment media is the smallest condition number, also smaller than that found for 11 alignment media. Due to the fact that the absolute values of the singular values are responsible for the error propagation the 11 media are still better than the 6. For ubiquitin, DMPC/DHPC,

CHAPSO/DLPC/SDS, Bacteriorhodopsin, Pf-1 phages, the *n*-dodecyl-penta(ethylene glycol)/*n*-hexanol phase and the Helfrich phases turned out to yield the optimum information. Whether these alignment media will prove equally useful for other proteins remains to be seen. It should be noted, however, that although ubiquitin is quite stable and therefore compatible with many alignment media it also has major drawbacks for alignment since it has an almost spherical shape and little charge due to its pI of 7.6.

In the following analysis we used only those residues for that dipolar couplings could be obtained in enough different media that the condition number was smaller than 10 in order to obtain statistically relevant data. To decrease the condition number even further one would have to measure in even more alignment media. New media are found on a regular basis (Desvaux et al., 2001) and therefore it is not unlikely that five or more alignment media for many biomolecules can be found.

An alternative would be the measurement of several dipolar couplings in the peptide plane (Fushman et al., 2000). However, there are drawbacks to this method as well. Other dipolar couplings than the NH dipolar couplings are often less accurately measured due to smaller gyromagnetic ratios and longer distances and therefore smaller dipolar coupling constants and their associated internuclear distances need to be accurately calibrated in order to exclude overestimation of motional anisotropies. NH^N internuclear distances are well calibrated and understood (Ottiger & Bax, 1998b). The estimation of internuclear distances is critical. This was for example found when using chemical shielding anisotropies and dipolar couplings for structure calculation (Cornilescu & Bax, 2000).

Extraction of θ_{eff} and ϕ_{eff} and comparison with different structures of ubiquitin and the MD trajectory

As described in the introduction, θ_{eff} , ϕ_{eff} can be calculated by using equations 4, 8, and 9. We see the best agreement between the effective orientations (θ_{eff} , ϕ_{eff}) and the average vector orientation (θ_{av} , ϕ_{av}) using the NMR structure that was already refined against two sets of dipolar couplings (Cornilescu et al., 1999a; Cornilescu et al., 1999b). The NMR structure was also used for the back calculation of the alignment tensors. The orientations of the NH vectors derived using equation 4, differ slightly from those obtained in the three structures (trajectory, X-ray (Vijay-Kumar et al., 1987) and NMR) especially in the very mobile regions; the C-terminal part, the first loop (Leu8-Lys11) between the two β -strands, around glycine 47 and glycine 53 (figure 3a,b, figure 4a,b). These are also the residues with the lowest GDO in the analysis of Tolman et al. (Tolman et al., 2001).

Absolute overall scaling

For all residues for which a sufficient number of dipolar couplings were available and thus the condition number was smaller than 10 we were able to calculate the averaged spherical harmonics from equation 3. The order parameter derived from the $\langle \tilde{Y}_{2M}(\theta, \phi) \rangle$ values according to equation 7 will deliver \tilde{S}_{rdc}^2 that can be larger than 1 for residues whose mobility is smaller than the averaged mobility. This behavior of \tilde{S}_{rdc}^2 is different from the desired S_{rdc}^2 . For 43 out of 76 residues the condition number was below 10 and the order parameters \tilde{S}_{rdc}^2 range between 0.108 (Gly 76) and 1.62 (Glu18) using the sizes of the alignment tensors as obtained by direct fitting of the experimental dipolar couplings to the static NMR structure.

To obtain the non-scaled dipolar order parameters S_{rdc}^2 from \tilde{S}_{rdc}^2 the value of $S_{overall}$ that reflects the overall dynamics absorbed by the alignment tensors needs to be known. The sequence averaged $\sqrt{\langle S_{rdc}^2 \rangle}$ order parameter will be the $S_{overall}$ scaling parameter. This overall scaling $S_{overall}$ cannot be mathematically derived from the experimental dipolar couplings in a unique way. Due to the fact that dipolar couplings are able to display motions over a very large time range even the expected value is unclear. We will present three approaches to derive $S_{overall}$ values for ubiquitin.

The distribution of the non-scaled dipolar order parameters \tilde{S}_{rdc}^2 yields an average of 1 and a maximum of 1.62 corresponding to the largest found dipolar order parameter. By scaling the largest experimental order parameter to 1 the average of the dipolar order parameter is $S_{overall} = 0.78$.

The second approach relies on the assumption that S_{rdc}^2 is smaller than the relaxation derived Lipari-Szabo order parameter S_{LS}^2 , since S_{rdc}^2 is also sensitive to motions slower than the overall tumbling correlation time. The largest experimental \tilde{S}_{rdc}^2 values are found for the helix indicating that this is the most rigid secondary structural element in ubiquitin. Since residues 23 and 25 show conformational exchange, only residues 24, 26, 28, 29 and 32 were used for the following analysis. The average S_{LS}^2 value was found to be 0.89 ± 0.02 for the helix residues from relaxation measurements (Lienin et al., 1998; Schneider et al., 1992; Tjandra et al., 1995). A fixed τ_c value of 4.05 ns, a ^{15}N CSA of -160 ppm and a r^{NH} distance of 1.02 \AA were used. Since the largest \tilde{S}_{rdc}^2 are found for the NH vectors of the helix and $S_{rdc}^2 \leq S_{LS}^2$ should be fulfilled, the most conservative assumption corresponds to the limiting case of

$S_{rdc}^2 = S_{LS}^2$ where $S_{overall}$ is set such that $\frac{\tilde{S}_{rdc}^2}{S_{overall}} = S_{rdc} = S_{LS}$ for the NH vector with the largest

$\frac{\tilde{S}_{rdc}^2}{S_{LS}^2}$ ratio (Ile 30). Fulfilling the equation for the residue with the largest ratio in the helix

yields an overall scaling factor of $S_{overall} = 0.78$, which reproduces the value from the first approach.

The third approach relies on the calculation of alignment tensors based on dipolar couplings that are less sensitive to dynamics. H,H coupling that depend on larger distances are less scaled by motion than NH dipolar couplings. This was also shown by analyzing the MD trajectory where $\bar{S}_{LS_{NH}} = 0.9$, $\bar{S}_{LS_{HNH\beta}} = 0.93$, $\bar{S}_{LS_{HNH\alpha}} = 0.95$ was found. H,H dipolar couplings can be measured by the J_{HH} -NOESY method (Peti & Griesinger, 2000). Although we showed that the measurement of the HH dipolar couplings is rather accurate the difference between the experimental dipolar couplings D_{HH}^{exp} and the dipolar couplings calculated from the ubiquitin structure D_{HH}^{theo} using the D_{NH} derived alignment tensor was rather large even when using the ensemble of NMR derived structures of ubiquitin (Cornilescu et al., 1999a; Cornilescu et al., 1999b). However, the D_{HH}^{exp} and D_{HH}^{theo} could be correlated. The slope of the correlation was 0.75 indicating that the NH derived alignment tensor used for the prediction of the H,H dipolar couplings was too small and that the H,H vectors experience a larger alignment tensor (figure 6). This result depends on the selection of the dipolar couplings. This factor is 0.6 taking only $H^N H^\beta$ couplings into account and 0.78 when only $H^N H^\alpha$ dipolar couplings are used. The $H^N H^\alpha$ dipolar couplings are expected to be most reliable since their distance variation only depends on the backbone φ torsion angle fluctuations. The $H^N H^\beta$ dipolar couplings depend in addition on the side chain χ_1 torsion angle.

There is further evidence that the alignment tensor derived from NH dipolar couplings experience motion-induced scaling beyond what is expected from S_{LS} values. Tolman et al.

compared the alignment tensor derived from RDC's with the one derived from pseudocontact shifts and found that they differ by about 20% in Cyanometmyoglobin (Tolman et al., 1997).

A different explanation (Bax & Tjandra, 1997a) was checked by a second more detailed analysis with the same results (Prestegard et al., 1999). The scaling factor of 0.8 is similar to the scaling factor found in our analysis ($S_{overall} = 0.78$).

Comparable results as the ones reported by Prestegard et al. were obtained on the protein calbindin D_{9k} (Allegrozzi et al., 2000; Bertini et al., 2000; Bertini et al., 2001) complexed with different paramagnetic lanthanides including Ce(III), Yb(III) and Dy(III). The overall scaling of the RDC derived alignment tensor as compared to the pseudocontact derived alignment tensor was found to be between 5% and 50% (Banci, 2001). Simple analytical models indicate a distance dependent increase of the pseudocontact shift derived alignment tensor. Thus, if short-range H^N protons are also included the pseudo contact shifts derived alignment tensor is systematically reduced. The overall scaling factor of 0.78 found in the present study fits well to these findings.

As a consequence a substantial part of motion of ubiquitin occurs on time scales slower than the overall tumbling correlation time τ_c .

As displayed in figure 7 the dipolar order parameter S_{rdc} is smaller than the axial dipolar order parameter S_{axial} but both are smaller than the order parameters from relaxation studies S_{LS} (sequence average: $\langle S_{LS} \rangle = 0.89$; $\langle S_{axial} \rangle = 0.85$; $\langle S_{rdc} \rangle = 0.78$). There are also a few residues that have larger S_{rdc}^2 than S_{LS}^2 values. Val 5, Val 17 and Ile 23 have S_{rdc}^2 values that are larger than their S_{LS}^2 values but are still in the error range of the S_{rdc}^2 values. Nevertheless Glu 18, Ile 36 and Gln 49 have S_{rdc}^2 values are larger than the corresponding S_{LS}^2 values even including the error. Interesting enough both Glu 18 and Ile 36 precede a Proline residue (Pro 19 and Pro 37). This cannot be explained presently. It might be due to an even smaller $S_{overall}$.

We also correlate the axial order parameter S_{axial} with the internal GDO values calculated by J. R. Tolman (Tolman et al., 2001). The GDOs and the S_{axial} order parameters on average have comparable values (figure 8).

S_{axial} only reports on the axial part of the motion, thus the inequality $S_{axial} \geq S_{rdc}$ should hold. Furthermore, for a rigid vector $S_{axial} = S_{rdc} = 1$ hold (see Appendix). Indeed we find that S_{rdc} is smaller than S_{axial} ($S_{axial} \geq S_{rdc}$) (figure 7). Then it is not surprising that the anisotropy of the motion is rather large in some regions of ubiquitin including the loops and especially the very flexible C-terminus. On average, the η value is 0.16, where amino acids in the non-secondary elements show rather large η values of up to 0.66 (Gly76) (figure 9). The average η value in the secondary structure elements is 0.12. This is in quite good agreement with the finding that S_{axial}^2 is always larger than the S_{rdc}^2 values. If we correlate S_{axial}^2 and S_{rdc}^2 we find a slope of the correlation function of 0.8 which indicates again an average asymmetry of the motion of 20% ($S_{axial}^2 = 0.79 \cdot S_{rdc}^2 + 0.20$) (figure 10). This is in big contrast to the results from the 10 ns trajectory of ubiquitin where the asymmetry was on average 3.8%. However this might be due to the different time ranges of motions that are sampled by the experimental data and the trajectory.

To obtain a more specific picture of the nature of the anisotropic motions, we performed the model free analysis based on the $\langle \tilde{Y}_{2M}(\theta, \phi) \rangle$ values extracted from equation 3 where the average orientations $\theta_{eff}^{(1)}, \phi_{eff}^{(1)}$ and the amplitude of the axial motions $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ (figure 11) are derived.

The information on the anisotropy of the motion is then reflected in the η and $\bar{\phi}'$ values. $\bar{\phi}'$ is the direction of the anisotropic motion in the x', y' plane for every residue. Figure 12a shows a $\bar{\phi}'$ plot for the experimental data and respective data derived from the trajectory. It is

quite interesting that there is a rather good correlation of these values (figure 12b) despite the considerable error on the experimental $\bar{\phi}'$ values.

The helix is rather immobile and has the highest experimental $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ values and rather small η values. This is in good agreement with the $\langle Y_{20}(\theta'^{raj}, \phi'^{raj}) \rangle$ extracted from the trajectory. Nevertheless there is some anisotropy of the motion detectable. The average angle $\bar{\phi}'$ over the helix NH vectors is -20° with a distribution ranging from $+20^\circ$ to -55° . This indicates preferential mobility of all NH vectors in this direction. Whether this motion is correlated or uncorrelated cannot be derived from the dipolar couplings.

The β -sheets have considerable smaller $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ and S_{rdc} than S_{LS} values indicating large motions probably in the ns to μ s time scale because large $T_{1\rho}$ effects are absent in ubiquitin. The β -sheets exhibit a fair amount of anisotropic motion according to the η values. Similar to the helix we find a clustering of the $\bar{\phi}'$ values around -130° in β -sheet 2 (residues 12-17). This clustering of $\bar{\phi}'$ values can be found throughout the protein in other β -strands and also in loop regions.

It is quite interesting to note that the $\bar{\phi}'$ values for the secondary structure elements that have been determined experimentally and from the trajectory fit rather well with the exception of Ile 30, Thr 66 and His 68 (figure 12b). It is interesting to note that the His 68 NH is involved in a weak hydrogen bridge across an anti-parallel β -sheet, the NH of Ile 30 and Thr 66 are not at all involved in an internal hydrogen bridge. Thus, it seems that the force field potentials used for internal hydrogen bridges reflect the direction of the motion well whereas this is not the case for NH's that are not involved in internal hydrogen bridges. However, the amplitudes of the motions are too small in the trajectory that could be due to the limited duration of the trajectory.

In the following we discuss some specific residues that are known for interesting dynamical features: The first loop consisting of the amino acid sequence Thr⁷-Gly⁸-Thr⁹-Gly¹⁰-Lys¹¹-Thr¹² connecting two β -strands of ubiquitin exhibits enhanced internal mobility as seen in all dynamical studies so far (Lienin et al., 1998; Schneider et al., 1992; Tjandra et al., 1995). The dipolar order parameters are also smaller for this loop (on average $S_{rdc} = 0.7$) than the average dipolar order parameter of 0.78 found in this analysis.

For those two residues that show slow conformational exchange based on relaxation experiments (de Alba et al., 1999) we find rather large dipolar order parameters. These are Ile 23 ($S_{rdc}^2 = 0.95$) and Asn 25 ($S_{rdc}^2 = 0.83$). Val 70 that also shows slow conformational exchange as observed in $T_{1\rho}$ measurements in super cooled water has no conspicuous RDC derived order parameter of $S_{rdc}^2 = 0.77$ (Skalicky et al., 2000).

Possible different explanations

The amount of motion reflected in the order parameter in our analysis is approximately twice that previously derived from relaxation measurements. We assume in our analysis that the average structure of ubiquitin is not changed in the different alignment media. However, the alignment media might not only orient the protein but also upon interactions influence its conformation. In order to check on the integrity of the ubiquitin structure we measured ¹⁵N-T₂ times and H,H-NOESY spectra of ubiquitin in a Helfrich phase, CHAPSO/DLPC/CTAB 5% phase and in isotropic solution. Helfrich phases were chosen because they induced the largest line broadening in the ubiquitin spectra. The CHAPSO/DLPC/CTAB 5% phase was chosen to represent the behavior of the bicelles as one of the most commonly used and most stable Phosphocholine mixture. The NOESY spectra were recorded with mixing times that were adapted to the effective correlation time of ubiquitin in water and in the liquid crystal media.

After the ^{15}N - T_2 time measurement 2D NOESY spectra with ^{15}N - T_2 time adapted mixing times have been quantitatively analyzed. The NOESY mixing times were set according to

$$\left(\left(\frac{\tau_{m,\text{isotrop}}}{T_{2,\text{isotrop}}} \right) = \left(\frac{\tau_{m,\text{aligned}}}{T_{2,\text{aligned}}} \right) \right)$$

since all rates relevant in the NOESY scale with the correlation time of the molecule, which in turn is proportional to the reciprocal ^{15}N - T_2 times. The fact that the NOE's recorded in the two phases differ at maximum by less than $\pm 6\%$ indicates that the structure of ubiquitin does not change upon binding in the liquid crystal medium (Helfrich phase $\pm 6\%$, CHAPSO/DLPC/CTAB phase $\pm 4.5\%$). The errors on the NOESY cross peaks in the liquid crystal media is about 5%. The background noise is rather strong due to the liquid crystal media when compared with spectra recorded in isotropic solution. Thus, the NOESY cross peak integrals between the aligned and the isotropic structure are not significant.

The ^{15}N - T_2 times are smaller in all regularly secondary elements in the aligned phases. This indicates homogeneous interactions of the protein with the alignment media. For the Helfrich phase the ^{15}N - T_2 times of the C-terminal residues become larger than the isotropic values, which is presently not understood. It could indicate unfolding of the terminal β -sheet, which in turn could increase the time scale of mobility of the C-terminus. However, we do not see any indication of this from the NOE's in this region or from the ^{15}N - T_2 times.

Another alternative explanation that the alignment tensor is averaged due to the side chain mobility of the protein can be excluded since this would affect all dipolar couplings in the same way, which is in disagreement with the proton-proton dipolar couplings.

In order to study the impact of the static reference structure on the results we took the NH vector orientations $\theta_{\text{eff}}^{(1)}, \phi_{\text{eff}}^{(1)}$ and used them in our analysis protocol. Although most of the changes in orientations were within a range of $\pm 10^\circ$ the back calculated alignment tensors were less favorable for our analysis since the smallest singular value decreases by 20% pushes

the average condition number from 7.6 to 9.9. Still the main conclusions hold for the changed alignment tensors: The \tilde{S}_{rdc}^2 values for the initial and the new tensors correlate very well (R=0.97) meaning that the differences in motion for the different parts of the molecule are reproduced. However, the $S_{overall}$ value derived from the new orientations would be smaller than 0.78 clearly indicating that this parameter requires support from other experimental input. It should also be noted that the new tensors exhibit a smaller condition number thus increasing the error of the analysis. Thus a condition number of a maximum 10 is required to perform this part of the analysis.

Summary

We have shown that a model free analysis of motion derived from NH dipolar couplings when measured in at least 5 to 6 media is possible (table 1) due to the differences of the alignment processes. We have quantified the differences of the alignment tensors by the condition number and the singular values of the \hat{F} matrix. We have found that the average vector orientations derived from the measurement in the different alignment media deviate slightly from the previously known experimental and theoretical structures. In addition we could derive average spherical harmonics for the NH vectors that describe the amplitude as well as the anisotropy of the motion. Using the spherical harmonics we have derived a residual dipolar coupling order parameter that is found to deviate strongly from the relaxation derived Lipari-Szabo order parameter especially in the β -sheets and loops. This indicates that on the time scale slower than the correlation time but faster than the detection limit of $T_{1\rho}$ considerable motion is occurring. In accordance with this finding the rather small sequence averaged $\langle S_{rdc} \rangle = S_{overall} = 0.78$ is smaller than the average Lipari-Szabo order parameter of

$$\langle S_{LS} \rangle = 0.9. \text{ If we assume axial wobbling in a cone according to } \left(\frac{1}{2} \cos \alpha (1 + \cos \alpha) = S_{axial} \right)$$

the amplitude for the fast motion of $S_{LS} = 0.9$ would imply an opening half-angle of about 22° . The additional disorder $\langle S_{rdc} \rangle$ derived from the residual dipolar couplings call for an additional wobbling in the cone half-angle of about 24° . The motions are uncorrelated.

From the analysis of the individual spherical harmonics we observe a considerable amount of anisotropy in the motion. This effect is predominant in β -sheets and loops but less expressed in helices. We have observed similarities of the directions of the anisotropic motions in secondary structure elements and agreement with the trajectory in this respect.

Material and Methods:

^{15}N and ^{13}C labeled ubiquitin was bought from VLI Research, Inc. (Malvern, PA) and used without further purification. For all samples between 1.5 and 3 mg of ubiquitin were dissolved in 10mM phosphate-buffer pH 6.5 ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10) in a 300 μl Shigemi microcell tube. All experiments were recorded on either Bruker-DRX-600 MHz or Bruker-DRX-800 MHz spectrometers (Bruker AG, Rheinstetten, Germany) equipped with a TXI HCN z-grad probe (measurement temperature was set to 303K for all experiments). All spectra were processed using XWINNMR 2.6 (Bruker AG, Karlsruhe, Germany) and FELIX 98.0 or FELIX 2000 (MSI, San Diego, USA).

Liquid crystal media:

Alignment of the protein was achieved using the following bicelles (Sanders & Schwonek, 1992; Sanders II et al., 1994): CHAPSO/DLPC (1:5; 5%), CHAPSO/DLPC/CTAB (10:50:1; 5%), CHAPSO/DLPC/CTAB (10:50:1; 4%), CHAPSO/DLPC/SDS (10:50:1; 5%) (Losoncz & Prestegard, 1998; Ottiger & Bax, 1998a; Wang et al., 1998). CHAPSO/DLPC was purchased from Sigma (St. Louis, MO, USA); SDS was purchased from Merck, (Darmstadt, Germany); and CTAB was purchased from ACROS (New Jersey, USA) and used without further purification. Dipolar couplings of ubiquitin in DHPC/DMPC and DHPC/DMPC/SDS were taken from the literature (Ottiger & Bax, 1998b).

In addition to bicelle media we used other liquid crystalline media to obtain NH dipolar couplings in ubiquitin. For the measurement using purple membrane fragments (Bacteriorhodopsin) (Koenig et al., 1999; Sass et al., 1999) (2mg/ml) we had to increase the salt concentration up to 100mM to decrease the electrostatic interaction between the highly charged Bacteriorhodopsin and ubiquitin. For the same reason 50 mM NaCl was used with Pf-1 phages (5mg/ml, 50mM NaCl, ASLA Ltd., Riga, Latvia) (Clare et al., 1998; Hansen et al., 1998). We also achieved alignment using surfactant lipids

((Cetylpyridiniumbromide/Hexanol = 1:1.33), 25mM NaBr, 5%) commonly named Helfrich phases (Barrientos et al., 2000; Prosser et al., 1998). NH dipolar couplings in polyacrylamid gels (7% paa) were taken from the literature (Sass et al., 2000; Tycko et al., 2000).

Using the nonionic liquid crystal medium composed of *n*-dodecyl-penta(ethylene glycol) and *n*-hexanol (Rückert & Otting, 2000) dipolar couplings could be obtained. The very small line widths comparable to those of the proton resonances in isotropic solution rendering this medium ideal for measuring other heteronuclear backbone dipolar couplings in proteins.

The determination of the alignment tensor and all other calculation were done using the home written software *DipoCoup* (Meiler et al., 2000b) and Mathematica 4.0 (Wolfram Res., Inc., Oxfordshire, U.K.) on PC or SGI computer systems.

All NH dipolar couplings were measured using the S³E-¹H-¹⁵N HSQC (Meissner et al., 1997) pulse sequence. In addition a ¹H-¹⁵N HSQC spectrum without decoupling during t_1 was recorded. $t_{1\max}$ was set to the average ¹⁵N T₂ relaxation time of 160ms that was estimated by constant time ¹H-¹⁵N HSQC experiments and T₂ time measurements.

MD simulation:

A MD simulation of native ubiquitin was carried out under periodic boundary conditions using the program CHARMM 24 (MacKerell et al., 1998). An energy-minimized all-atom representation of the X-ray structure of ubiquitin (Vijay-Kumar et al., 1987) was embedded in a cubic box with a side length of 46.65 Å containing a total of 2909 explicit water molecules. The simulation was performed at a temperature of 300 K with an integration time step of 1 fs. Details of this simulation have been reported elsewhere (Lienin et al., 1998; Meiler et al., 2001).

We also used the experimental dipolar coupling to test the MD run. Comparison of the experimental results with the MD trajectory were done by using the eleven different alignment tensors for calculating theoretical dipolar couplings from the MD trajectory and

comparing them with the experimental dipolar couplings. The RMSD-values between the experimental and the theoretical dipolar couplings show large differences occurring in the less structured regions such as the β -turn (Leu8-Lys11) and around the prolines (Pro19, Pro37, Pro38). Also the dynamical features of the very flexible C-terminal part have a rather poor description in the calculated trajectory. The agreement between trajectory and experiment is however rather good for the secondary structure elements. Another way of visualization of the differences between the experiment and the trajectory can be derived from the comparison of the experimental θ_{eff} , ϕ_{eff} values and the trajectory-derived θ_{av} , ϕ_{av} values. In the highly dynamic loop regions the average angle values derived from the trajectory do not agree with the experimental values. These differences are much larger than those using the NMR or the X-Ray structure to calculate the θ_{av} , ϕ_{av} values. Interestingly, the trajectory described the N-terminal part of ubiquitin much better than the C-terminal part. This is due to the fact that ubiquitin has secondary structure elements in the N terminus right from the start. In fact, the secondary structure elements are so stable that they are even retained in media that normally completely unfold proteins.

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Appendix:

The transformation from equation 1 to equation 2 transforms the orientations in the individual alignment frames given by $\theta_i^{at}, \phi_i^{at}$ into orientations in the molecular frame given by θ^{mol}, ϕ^{mol} by virtue of Wigner rotation matrices.

$$\frac{D_i^{exp}}{D_{i,zz}} = \sqrt{\frac{4\pi}{5}} \left(\langle Y_{20}(\theta_i^{at}, \phi_i^{at}) \rangle + \sqrt{\frac{3}{8}} R_i \left(\langle Y_{22}(\theta_i^{at}, \phi_i^{at}) \rangle + \langle Y_{2-2}(\theta_i^{at}, \phi_i^{at}) \rangle \right) \right) \quad \text{Eq. A1}$$

$$= \sqrt{\frac{4\pi}{5}} \left(\sum_{M=-2}^2 e^{-iM\alpha^i} d_{M0}^2(\beta^i) \langle Y_{2,M}(\theta^{mol}, \phi^{mol}) \rangle \right) \quad \text{Eq. A2}$$

$$+ \sqrt{\frac{4\pi}{5}} \sqrt{\frac{3}{8}} R \left(\sum_{M=-2}^2 e^{-iM\alpha^i} d_{M2}^2(\beta^i) e^{-2i\gamma^i} \langle Y_{2,M}(\theta^{mol}, \phi^{mol}) \rangle + e^{-iM\alpha^i} d_{M-2}^2(\beta^i) e^{2i\gamma^i} \langle Y_{2,M}(\theta^{mol}, \phi^{mol}) \rangle \right)$$

where $D_{i,zz}$ is the axial value of the dipolar couplings, R_i is the rhombicity of the alignment and $\langle Y_{2M}(\theta, \phi) \rangle$ are the averaged spherical harmonics for a given NH vector

$$\left(\left\langle Y_{20}(\theta, \phi) \right\rangle = \sqrt{\frac{5}{16\pi}} (3\cos^2\theta - 1); \quad \left\langle Y_{2\pm 1}(\theta, \phi) \right\rangle = \sqrt{\frac{15}{8\pi}} e^{\mp i\phi} \cos\theta \sin\theta; \right.$$

$$\left. \left\langle Y_{2\pm 2}(\theta, \phi) \right\rangle = \sqrt{\frac{15}{32\pi}} e^{\pm 2i\phi} (\sin^2\theta) \right). \text{ The superscript } at \text{ describes the spherical harmonics in}$$

each of the alignment tensor frames. As already mentioned in the text the superscript *mol* describes the change of the coordinate system from the alignment tensor frame into the molecular frame brought about by the three Euler angles α_i , β_i , and γ_i . This transformation defines the matrix $F_{i,M}$:

$$F_{i,M} = \sqrt{\frac{4\pi}{5}} \left(e^{-iM\alpha^i} d_{M0}^2(\beta^i) + \sqrt{\frac{3}{8}} R \left(e^{-iM\alpha^i} d_{M2}^2(\beta^i) e^{-2i\gamma^i} + e^{-iM\alpha^i} d_{M-2}^2(\beta^i) e^{2i\gamma^i} \right) \right) \quad \text{Eq. A3}$$

In the text we are using θ and ϕ referring always to the reference frame of the molecule.

For the coordinate frame x', y', z' in which the $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ are maximized according to equation 4, the anisotropy of the motion is only reflected in the $\langle \tilde{Y}_{22}(\theta', \phi') \rangle$ and

$\langle \tilde{Y}_{2-2}(\theta', \phi') \rangle$ averages. The averaged $\langle \tilde{Y}_{21}(\theta', \phi') \rangle$ and $\langle \tilde{Y}_{2-1}(\theta', \phi') \rangle$ values are zero, because for the maximum condition for $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ the derivatives with respect to any rotation about the primed axes $\frac{\partial \langle \tilde{Y}_{20}(\theta', \phi') \rangle}{\partial \delta_{x', y', z'}}$ must vanish. Since an infinitesimal rotation about the primed

axes $(\delta_{x'}, \delta_{y'})$ is identical to the application of the angular momentum operators we find:

$$0 = \frac{\partial}{\partial \delta_{x'}} \langle \tilde{Y}_{20}(\theta', \phi') \rangle = \frac{L_{x'} \langle \tilde{Y}_{20}(\theta', \phi') \rangle}{i\hbar} = \frac{\langle \tilde{Y}_{21}(\theta', \phi') \rangle + \langle \tilde{Y}_{2-1}(\theta', \phi') \rangle}{i\hbar} \quad \text{Eq. A4}$$

$$0 = \frac{\partial}{\partial \delta_{y'}} \langle \tilde{Y}_{20}(\theta', \phi') \rangle = \frac{L_{y'} \langle \tilde{Y}_{20}(\theta', \phi') \rangle}{i\hbar} = \frac{(\langle \tilde{Y}_{21}(\theta', \phi') \rangle - \langle \tilde{Y}_{2-1}(\theta', \phi') \rangle)}{\hbar} \quad \text{Eq. A5}$$

we find that the $\langle \tilde{Y}_{21}(\theta', \phi') \rangle$ and $\langle \tilde{Y}_{2-1}(\theta', \phi') \rangle$ averages are zero.

There is no analytical expression for S_{axial} as a function of S_{rdc} and η . However the empirical correlation: $\left(\sqrt{\frac{4\pi}{5}} \langle Y_{20} \rangle \right) \cdot (S_{axial}) \approx S_{rdc}^2$ holds with a correlation coefficient of

R=0.89 for our experimental data.

Figure Captions:

Figure 1:

Overview of the most important steps to extract structural and model free dynamical information from dipolar couplings. D_i^{exp} experimental dipolar couplings measured in i different alignment media; $\tilde{D}_{i,zz}$ largest principal component of the alignment tensor extracted from the experimental data using a rigid model (NMR, X-Ray or trajectory); $F_{i,M}$ elements of \hat{F} matrix including all the alignment tensor information and the Wigner rotation elements for the translation from the individual alignment frames to the molecular frame; $\langle \tilde{Y}_{2M} \rangle$ spherical harmonic functions derived from the experimental dipolar couplings; $\theta_{\text{eff}}^{(1,2,3)} \phi_{\text{eff}}^{(1,2,3)}$ effective orientations of the NH vectors derived using three different approaches. The first approach is used for the model free analysis. $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ isotropic part of the motion; η and $\bar{\phi}'$ the amplitude and the orientation of the anisotropic motion, respectively. S_{overall} overall scaling factor that reflects the overall scaling of the back calculated alignment tensors due to motion; S_{axial} residue specific order parameter describing the axially symmetric motion; S_{rdc} dipolar order parameter. Details can additionally be found in the text of the manuscript.

Figure 2:

The alignment tensors of the 11 different alignment media used for the dynamical analysis are shown. Six tensors are from bicelle media. Here the difference of orientations of the tensor is rather minor. The DHPC/DMPC, CHAPSO/DLPC bicelles and the n -dodecyl-penta(ethylene glycol) and n -hexanol phases are uncharged and have nearly the same alignment tensor.

Figure 3:

Comparison of the effective orientations of the NH vectors θ (a) and ϕ (b). The black spheres show the experimental model free derived orientations calculated using equation 4. The

triangles show the orientation of the NH vectors in the NMR structure, the square the orientations in the X-ray structure.

Figure 4:

Experimental effective orientations of the NH vectors in polar coordinates θ (a) and ϕ (b) calculated using equation 4 as compared to the orientations derived from the 10ns MD trajectory (stars).

Figure 5:

Histogram of the distribution of the order parameter \tilde{S}_{rdc}^2 (left). The distribution varies between 0.18 and 1.62. Scaling of the distribution by division by $S_{overall} = 0.78$ such that the maximal S_{rdc}^2 is 1 leads to the distribution of S_{rdc}^2 (right).

Figure 6:

Correlation of experimental D_{HH}^{exp} and calculated D_{HH}^{calc} proton, proton dipolar couplings. The experimental couplings were measured using the J_{HH} -NOESY experiment. The calculated couplings were derived from the ensemble of NMR structures using an alignment tensor $(\tilde{D}_{zz}, \tilde{D}_{yy}, \tilde{D}_{xx})$ back-calculated from the same structure and the NH dipolar couplings. The slope of the correlation is 0.75 ± 0.05 indicating that the NH dipolar coupling derived alignment tensor is smaller by this factor than the effective alignment tensor for the HH dipolar couplings $(D_{zz}^{HH}, D_{yy}^{HH}, D_{xx}^{HH})$.

Figure 7:

Comparison of the three order parameters discussed in the text. S_{rdc}^2 and S_{axial}^2 are order parameters derived from the dipolar couplings. They reflect structural variation up to the milliseconds range. S_{LS}^2 is the relaxation derived order parameter that describes motions faster than the reciprocal correlation time (4ns for ubiquitin at 30°C).

Figure 8:

Plot of the GDO of Tolman et. al and the S_{axial}^2 order parameters along the protein sequence. Both order parameters reflect axially symmetric motion.

Figure 9:

Representation of the parameter η along the ubiquitin sequence. The anisotropy of the orientational distribution derived from the experimental dipolar couplings and the trajectory is shown. The anisotropy found in the experiment is much larger than in the trajectory. This is especially true for the loops, bends and β -sheet regions.

Figure 10:

Correlation between S_{rdc}^2 and S_{axial}^2 . The slope is 0.79 indicating about 20% anisotropic motion in ubiquitin. This is in agreement with η displayed in figure 9 averaged over the protein sequence.

Figure 11:

Experimental $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ values and the values derived from the trajectory along the ubiquitin sequence. The $\langle \tilde{Y}_{20}(\theta', \phi') \rangle$ values of the central helix in ubiquitin show the largest values. This is in accordance with the trajectory.

Figure 12:

(a) Comparison of $\bar{\phi}'$ angles derived from the experimental dipolar couplings and from the trajectory. The values correlate quite well in the secondary structure elements except for the residues Ile 30, Thr 66 and His 68. The correlation plot shown in (b) has a correlation coefficient of $R=0.84$ excluding the three outliers mentioned before.

Table 1:

Number of used alignment conditions	5	6	7	8	9	10	11
DMPC/DHPC	0	1	1	1	1	1	1
DMPC/DHPC/SDS	0	0	0	0	0	0	1
Purple Membrane Fragments	1	1	1	1	1	1	1
CHAPSO/DLPC/SDS	0	1	1	1	1	1	1
CHAPSO/DLPC	1	0	0	0	1	1	1
CHAPSO/DPLC/CTAB 4%	0	0	1	1	1	1	1
CHAPSO/DPLC/CTAB 5%	0	0	0	0	0	1	1
Polyacrylamide Gel	0	0	0	1	1	1	1
Helfrich Phase	1	1	1	1	1	1	1
Pf-1 Phages	1	1	1	1	1	1	1
<i>n</i> -dodecyl-penta(ethylene glycol)/ <i>n</i> -hexanol	1	1	1	1	1	1	1
Condition Number	6.362	6.184	6.548	7.039	7.367	7.817	8.267
Singular value 1	3.045	3.373	3.759	4.094	4.333	4.617	4.909
Singular value 2	1.804	1.898	2.013	2.071	2.203	2.220	2.260
Singular value 3	0.618	0.723	0.725	0.775	0.796	0.833	0.844
Singular value 4	0.532	0.600	0.600	0.646	0.646	0.675	0.675
Singular value 5	0.479	0.546	0.574	0.582	0.588	0.591	0.594

Table 1 compares the singular values and the condition number for the five to 11 best alignment media. In all cases with less than 11 alignments the combination of the alignment media is given that provides the largest fifth singular value. The smallest singular value of the \hat{F} matrix is most significant for the propagation of errors into the model free analysis. Thus six experimental alignment tensors are almost as good as 11 for the purposes of this paper.

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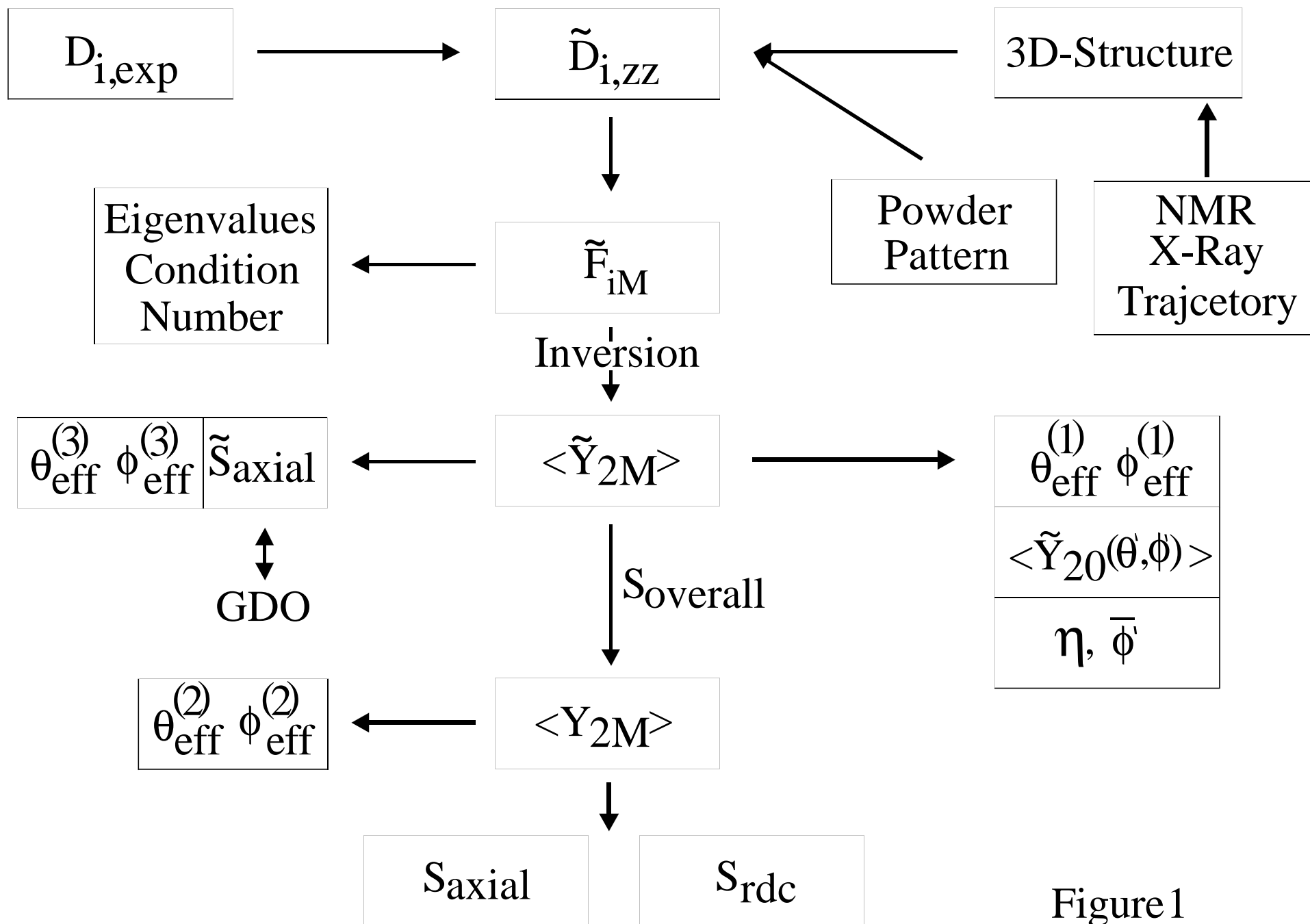
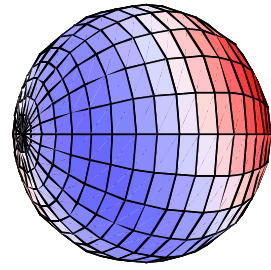
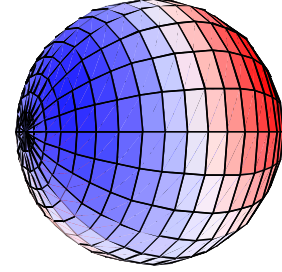


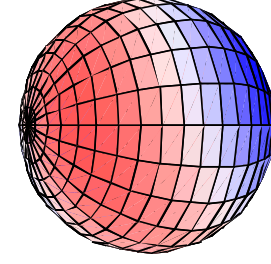
Figure 1



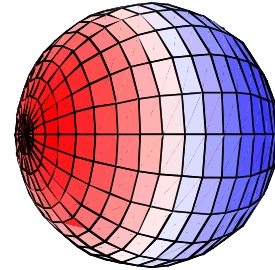
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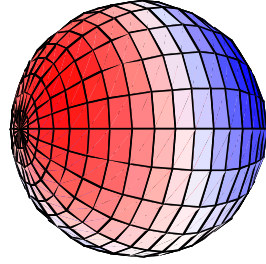
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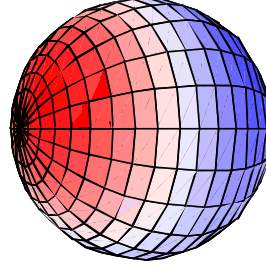
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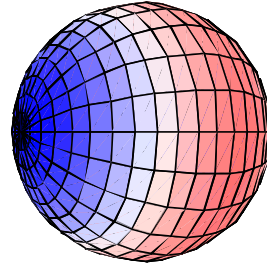
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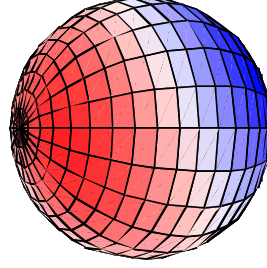
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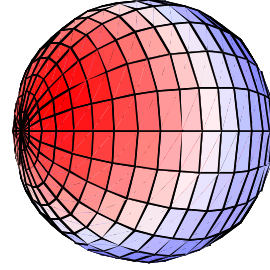
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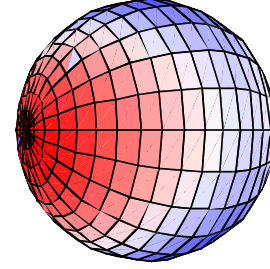
Bacterio Rhodopsin



Polyacrylamid gel



**nonionic *n*-alkyl-polyethylen-
glycole/*n*-alkylalcohol**



Helfrich phases

Pf-1 phases

Figure 2

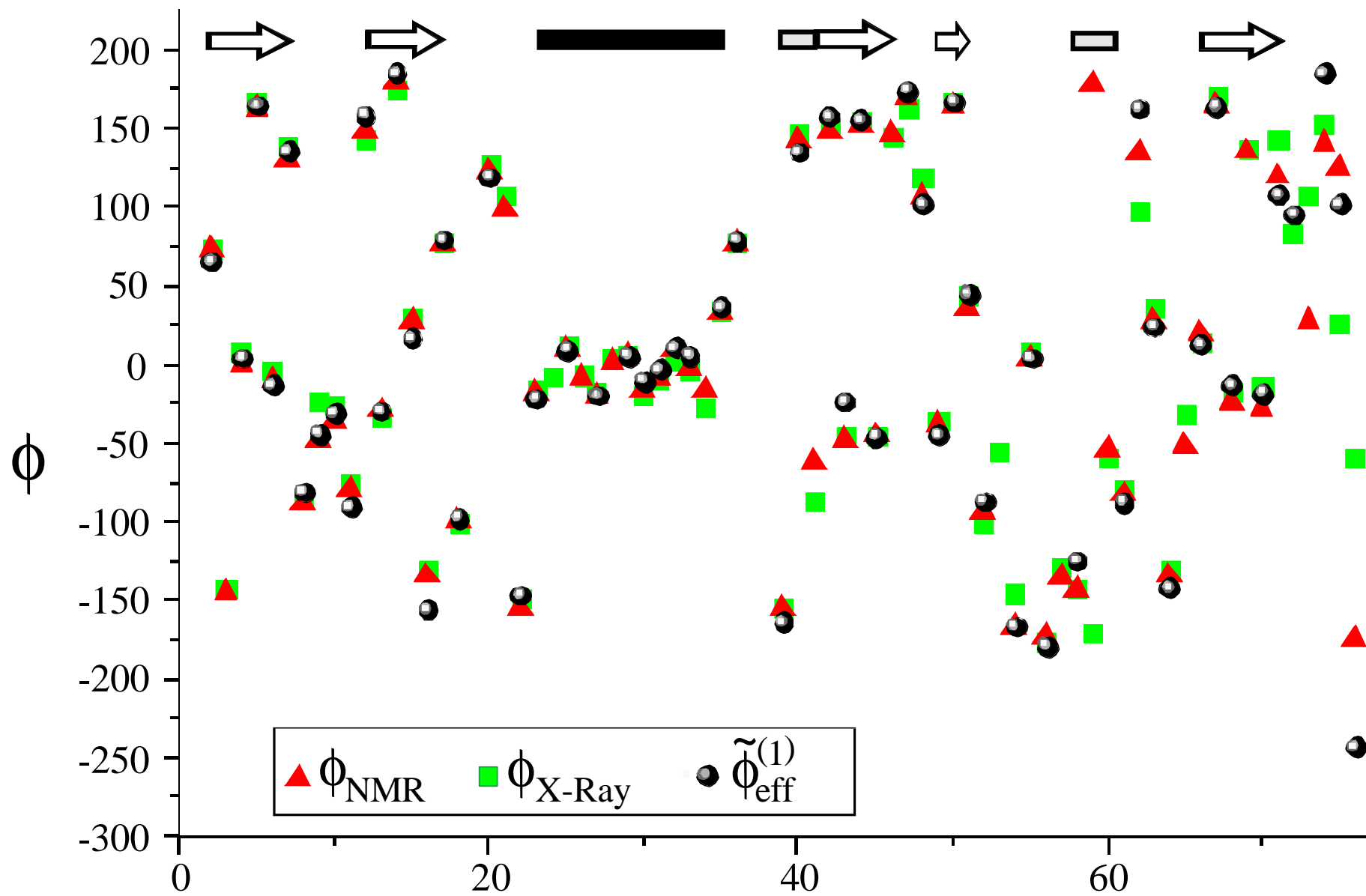


Figure 3a

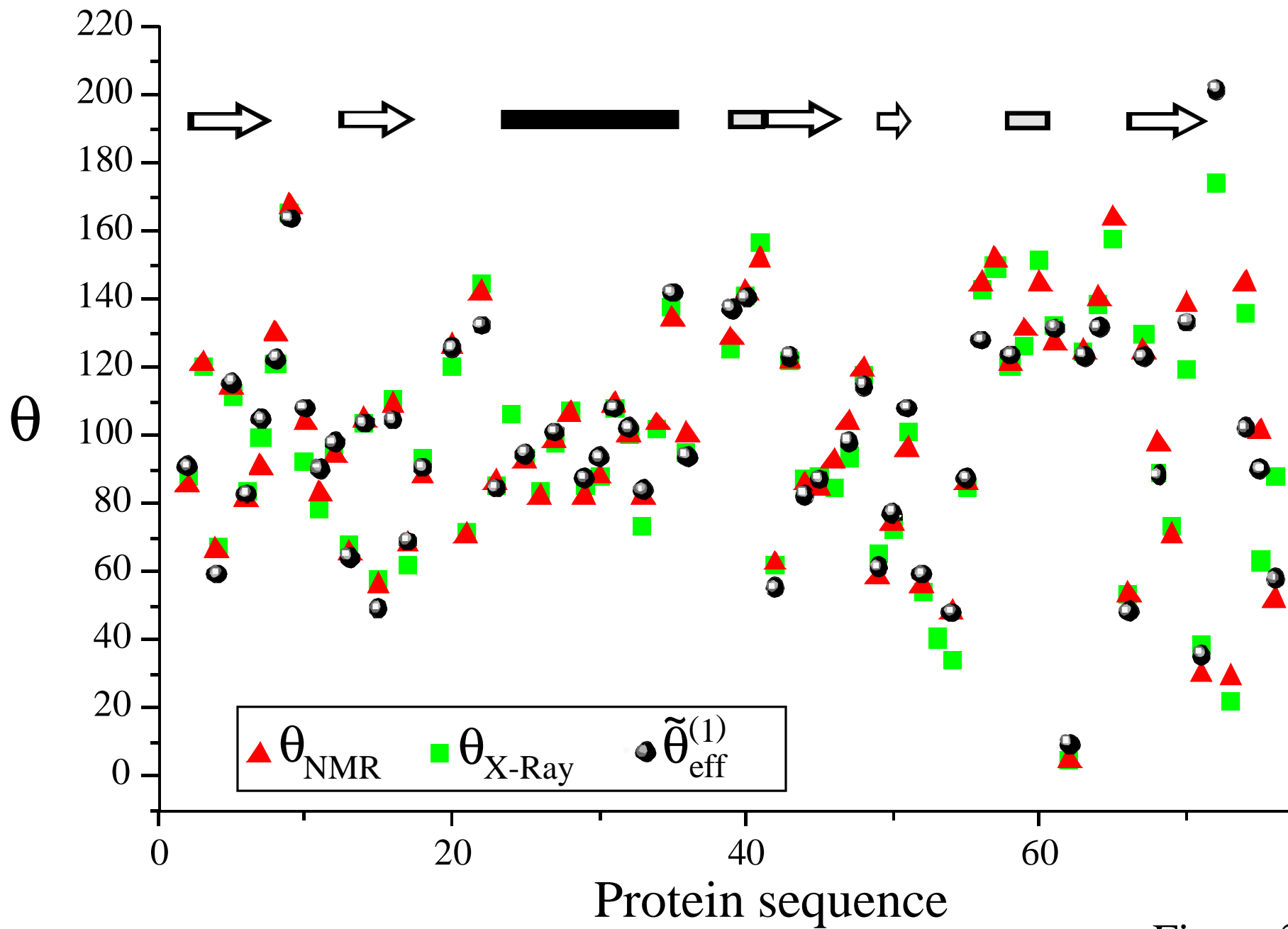


Figure 3b

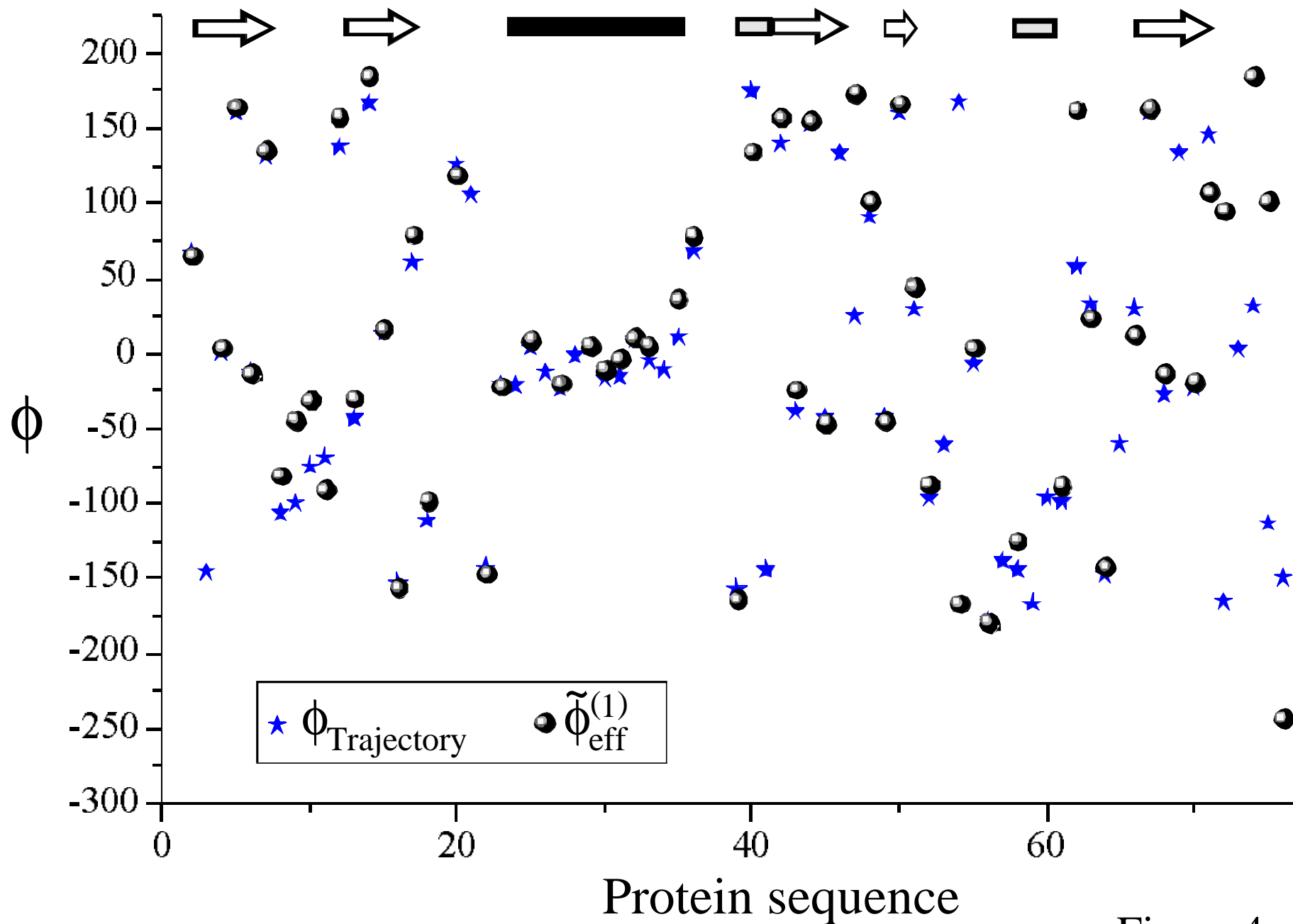


Figure 4a

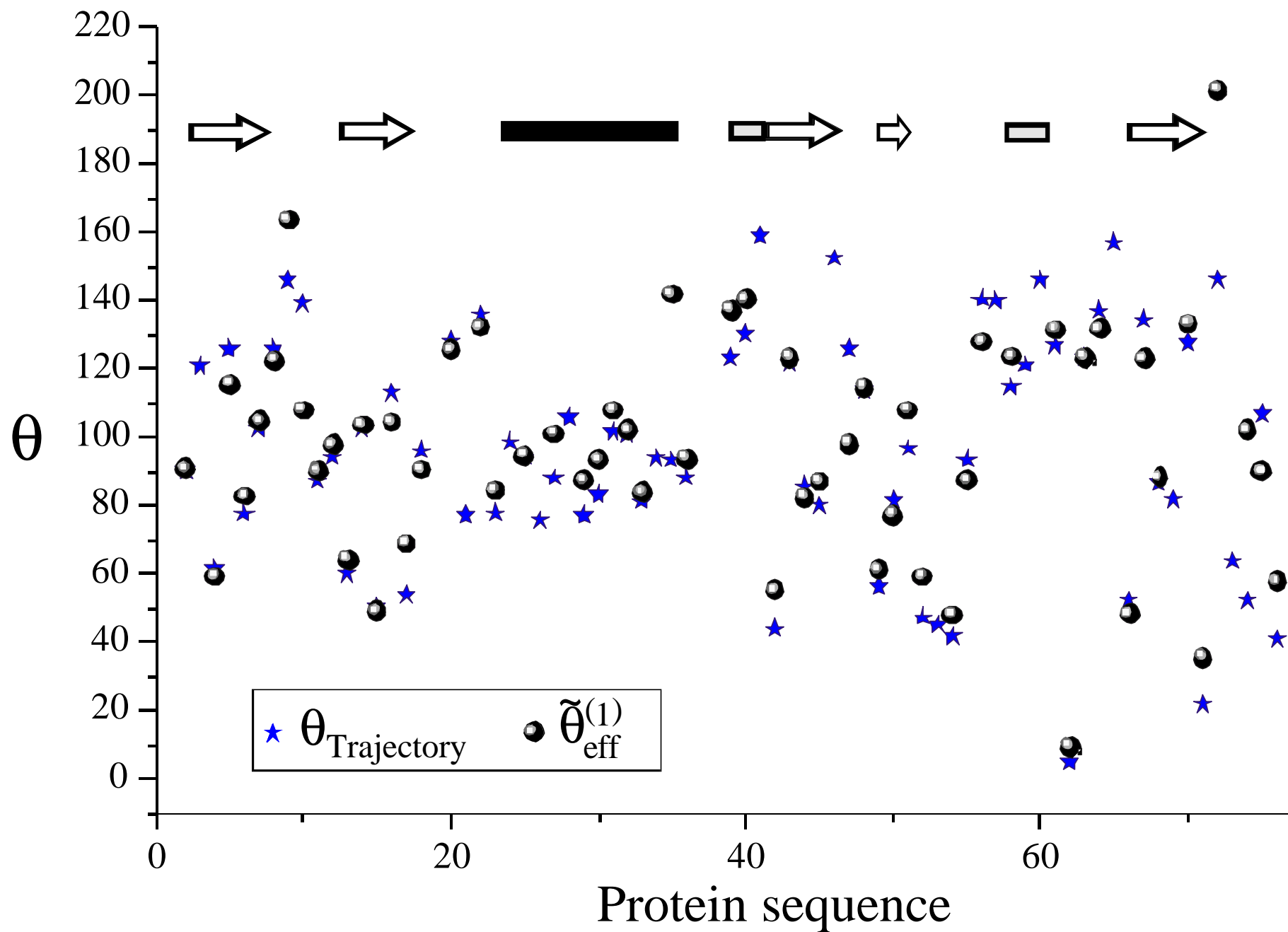
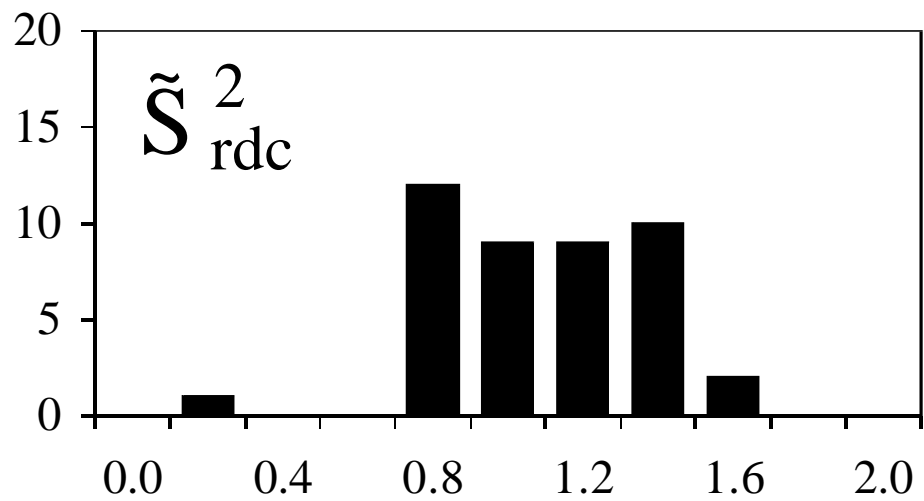


Figure 4b



$(1/S_{\text{overall}})^2$

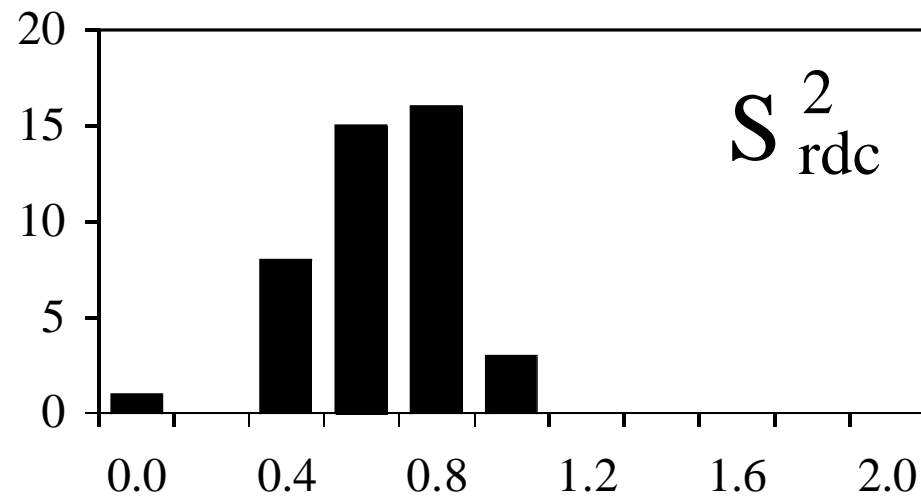


Figure 5

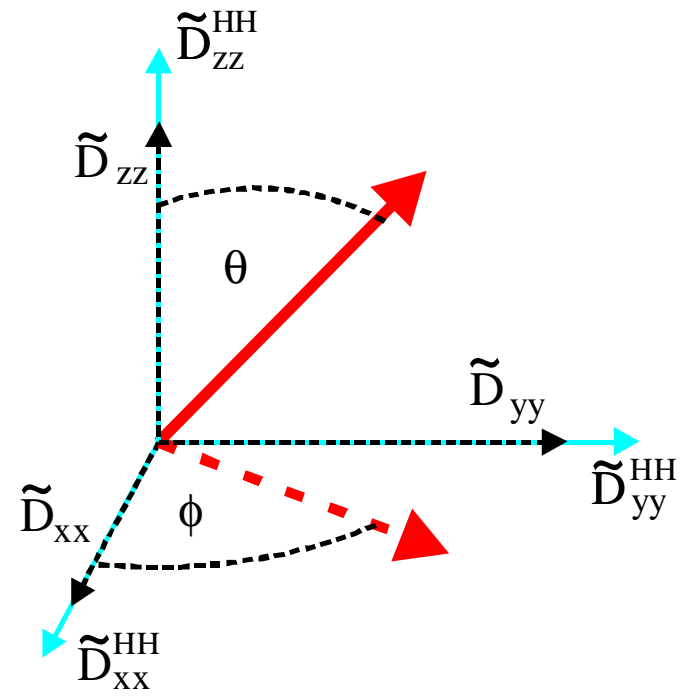
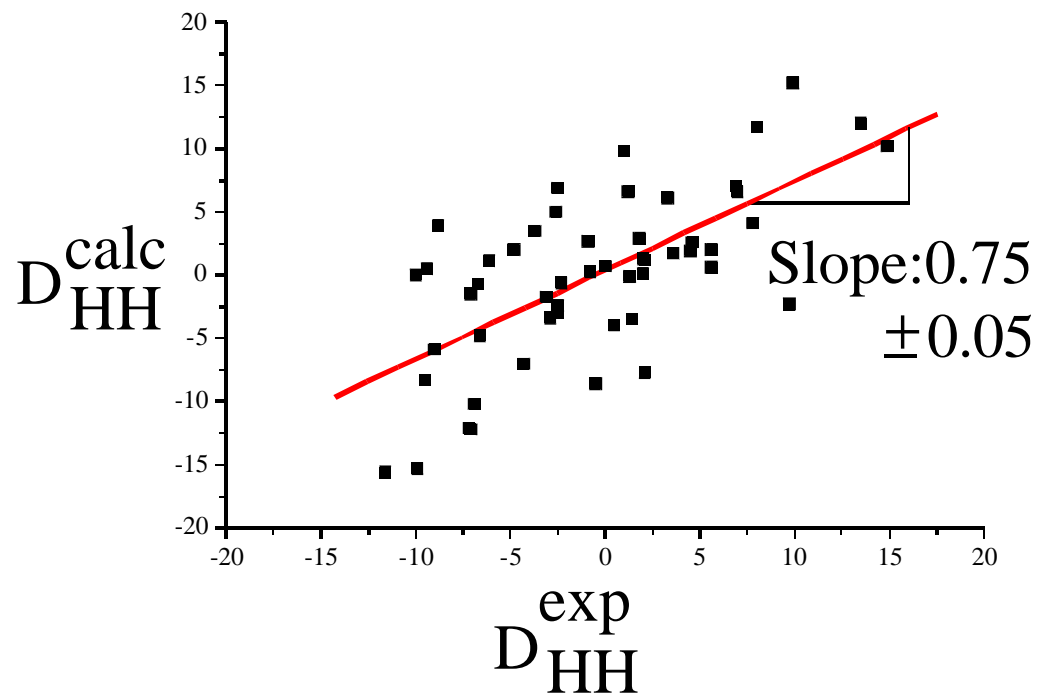


Figure 6

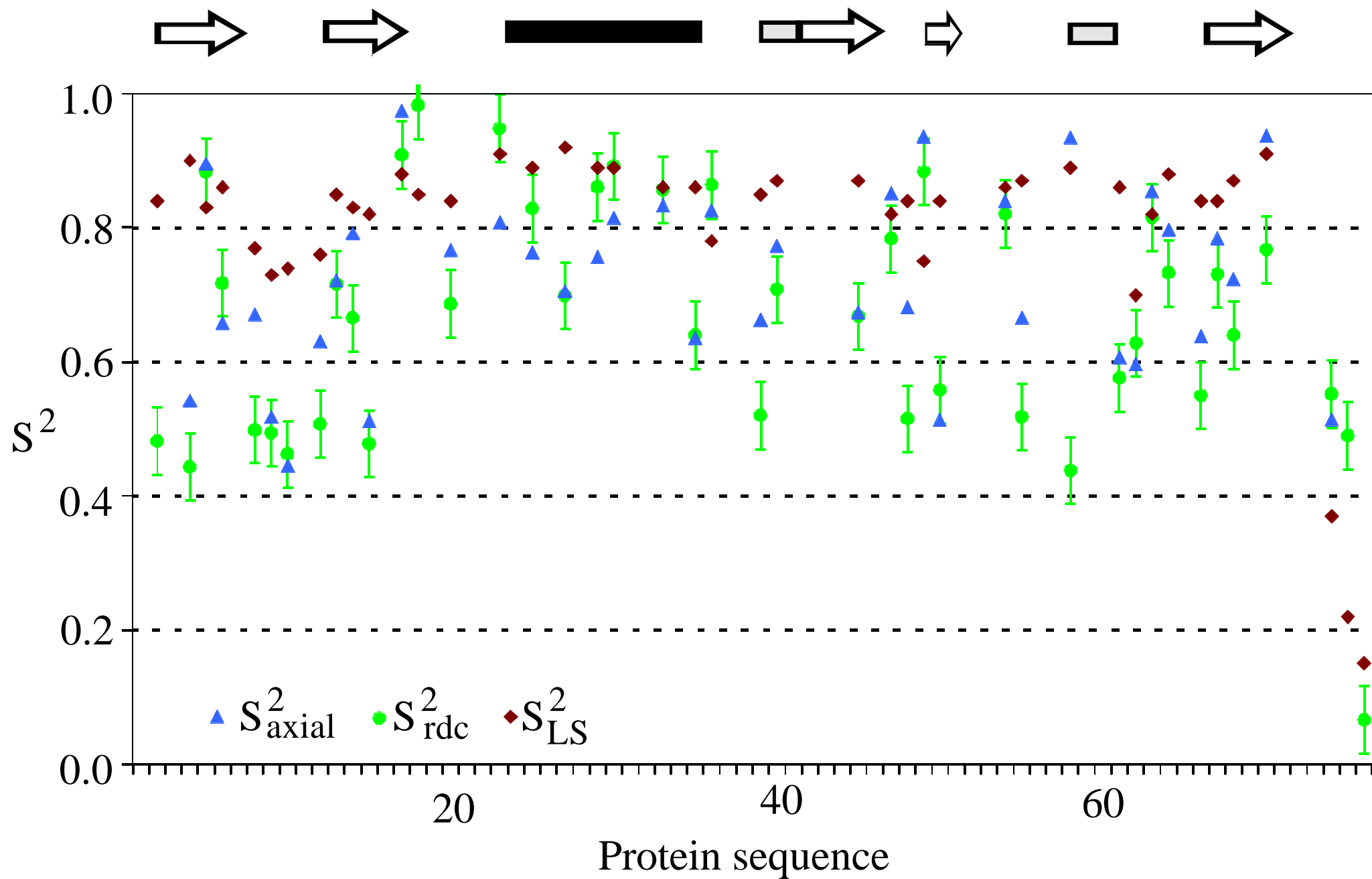


Figure 7

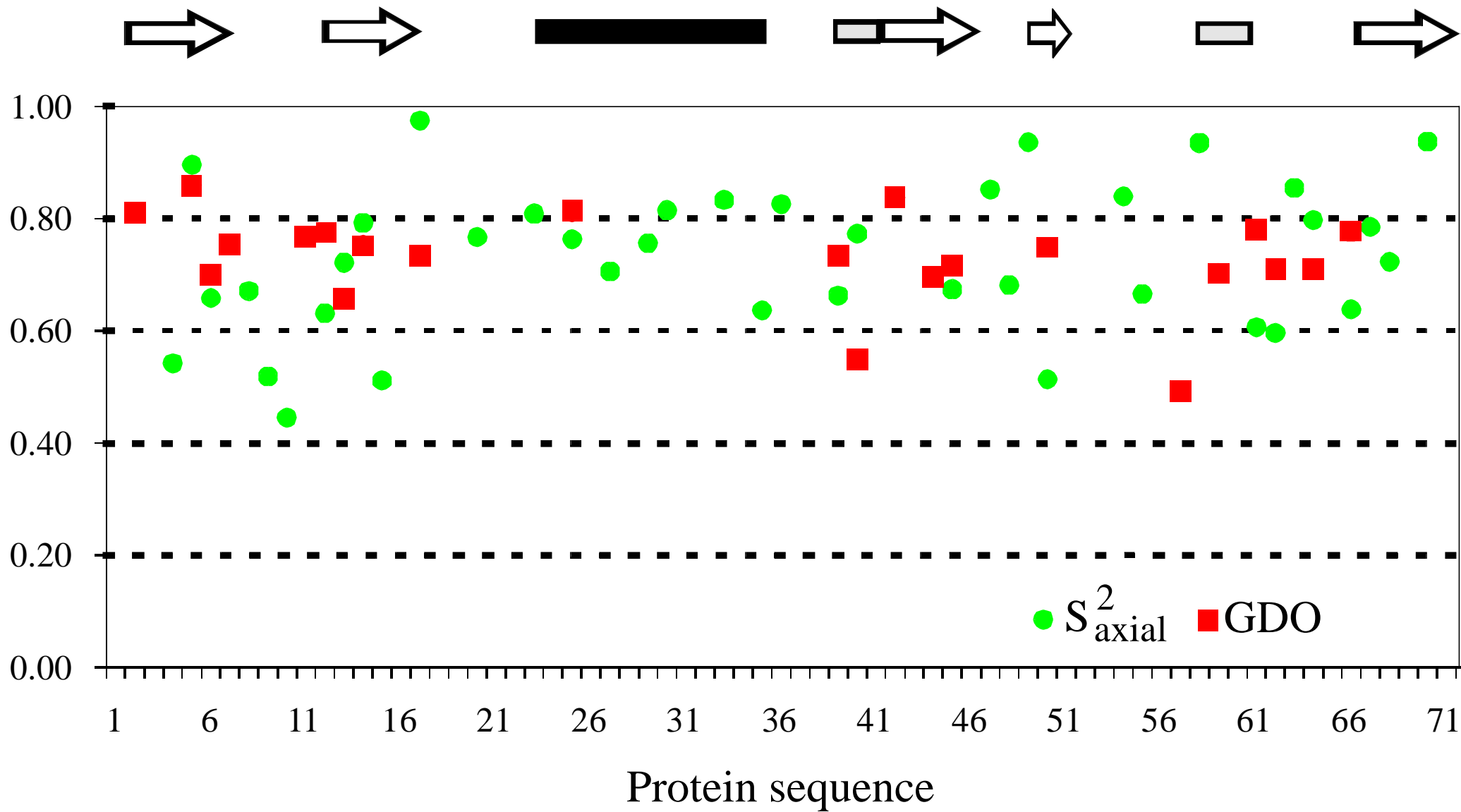


Figure 8

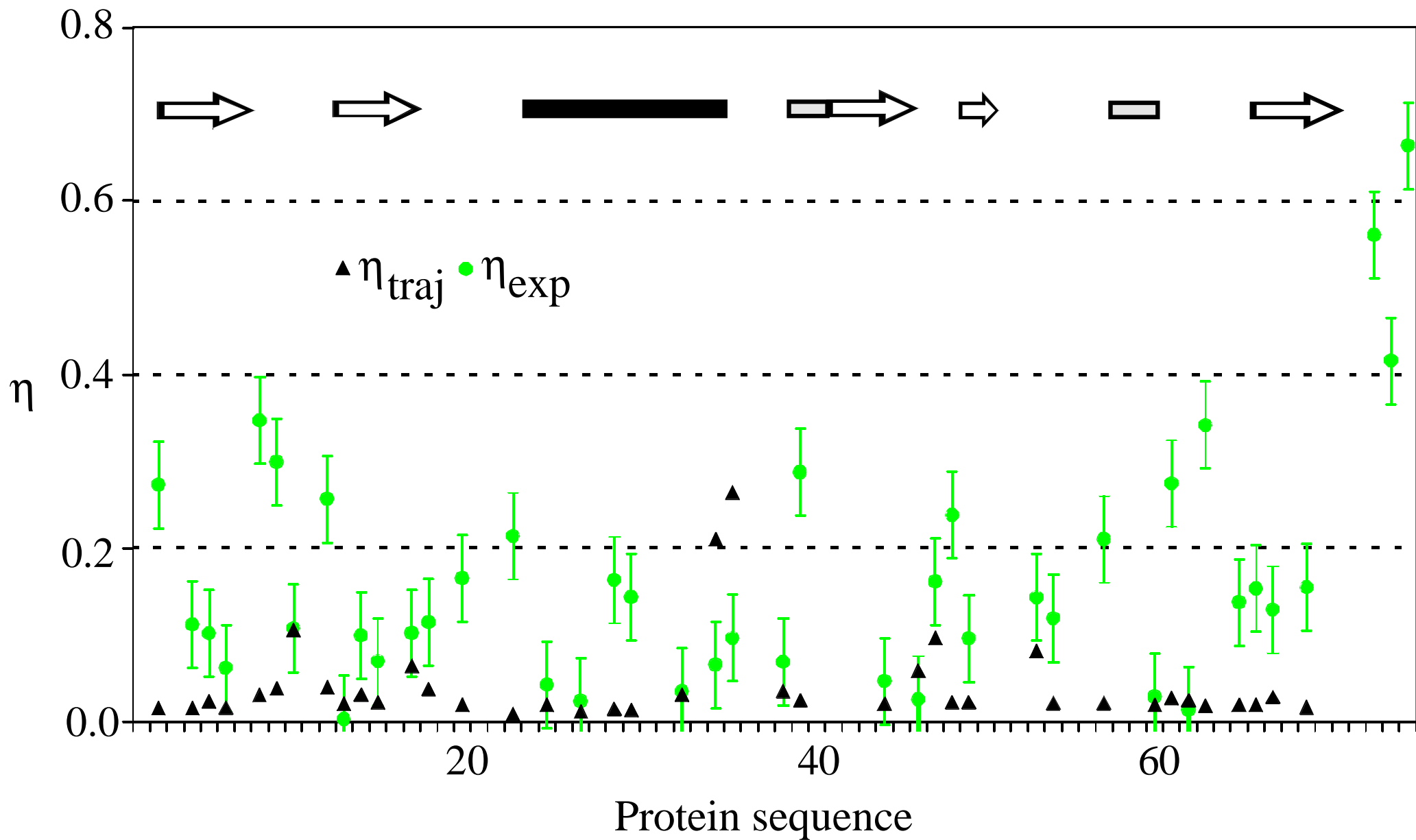


Figure 9

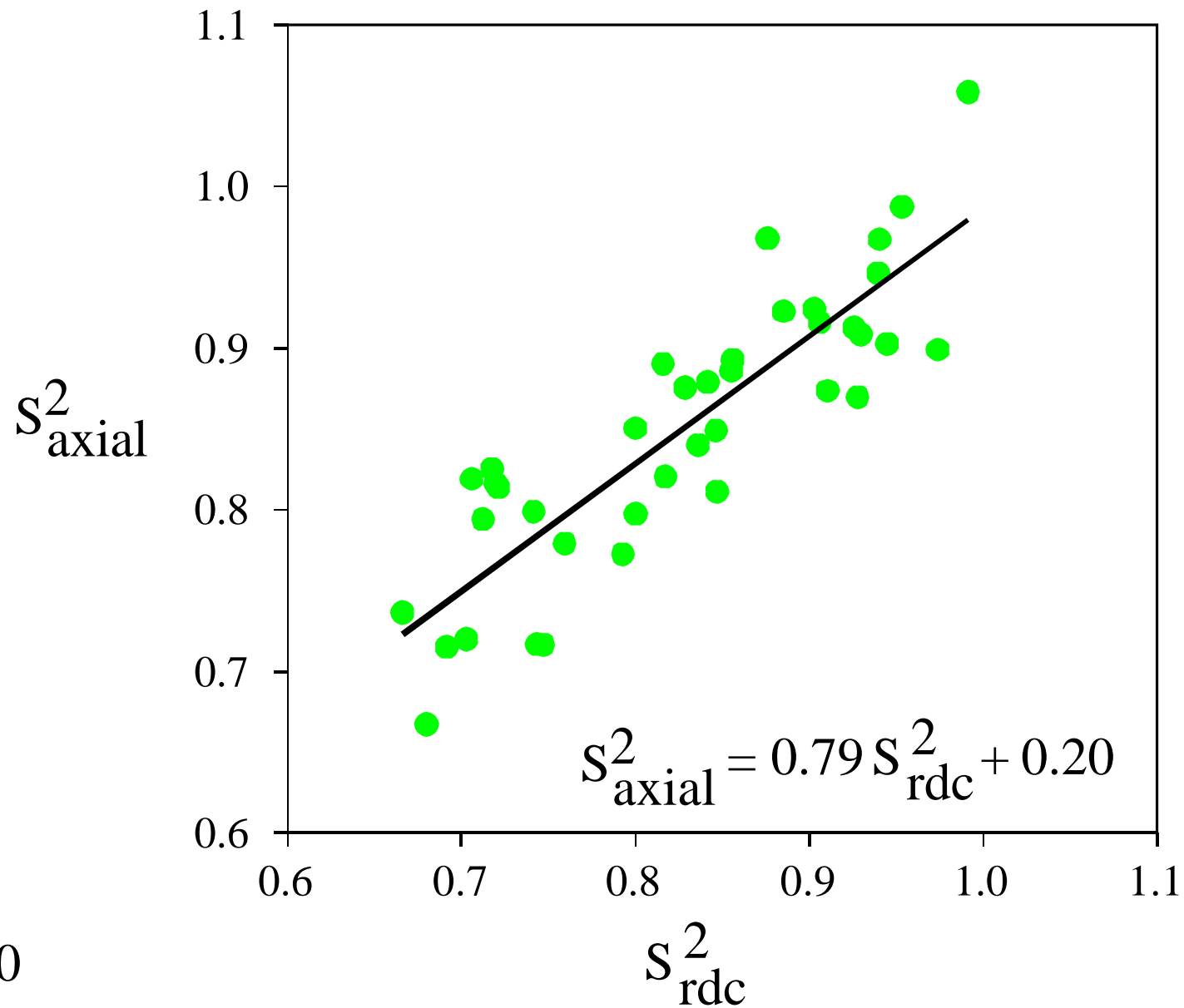


Figure 10

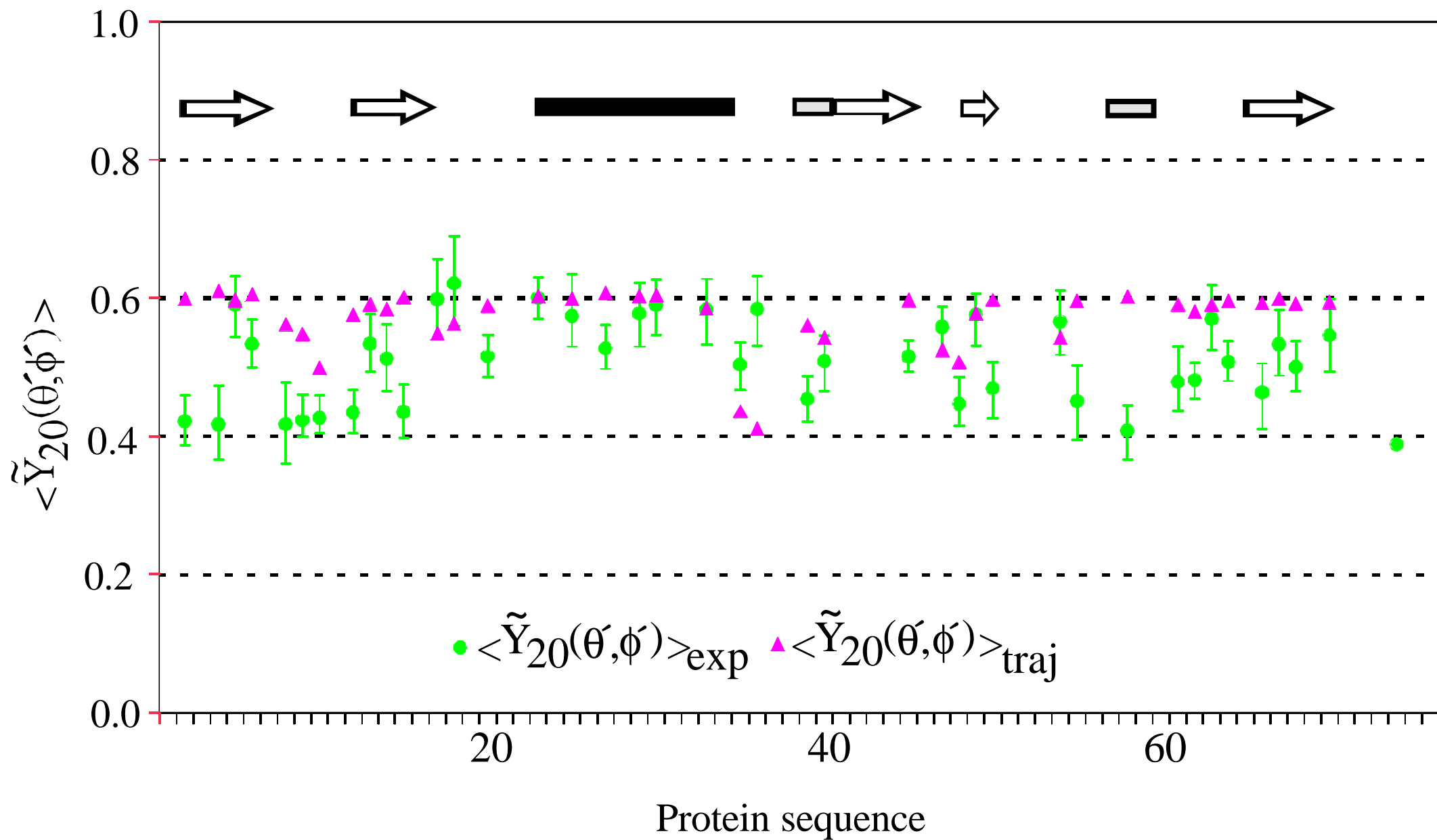


Figure 11

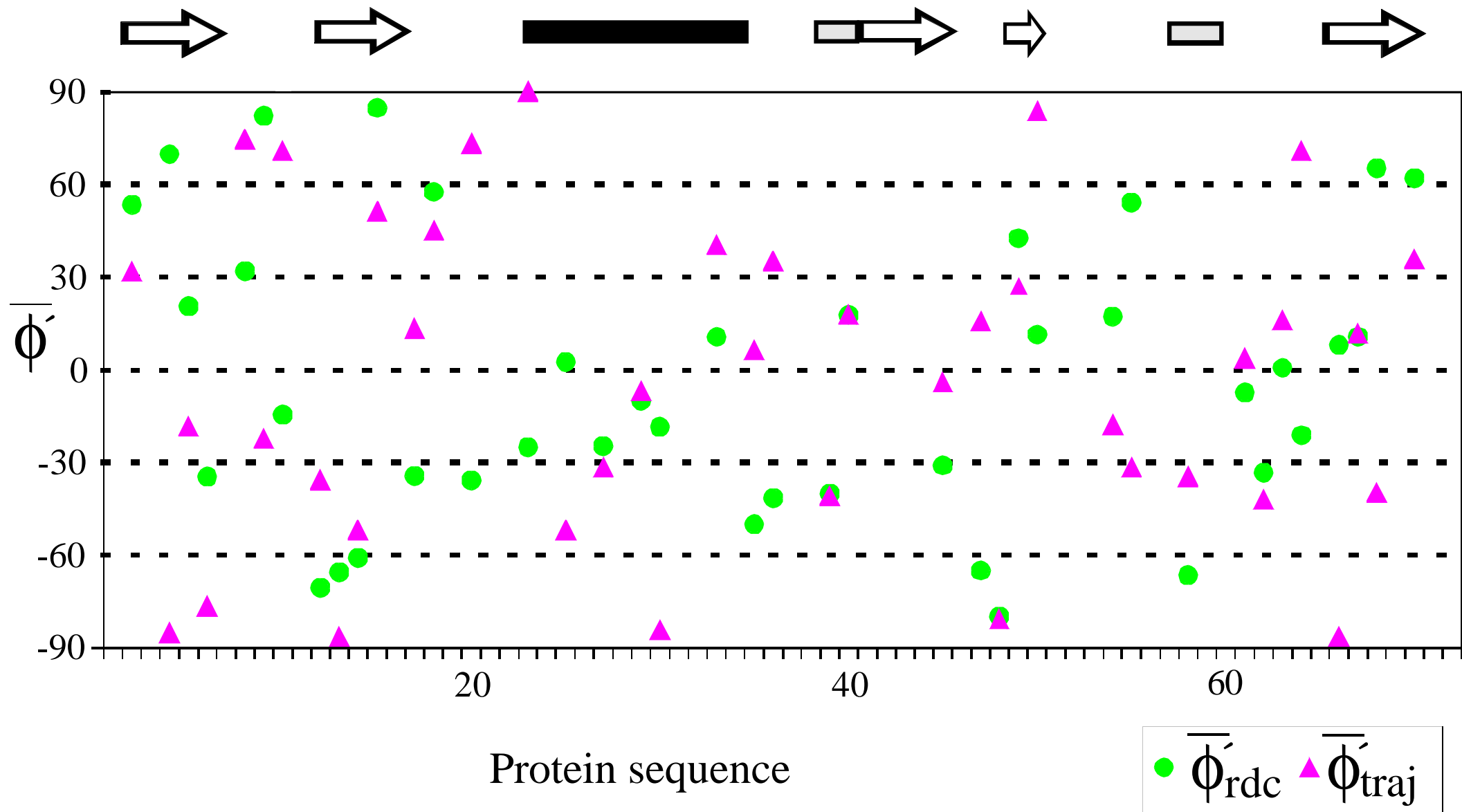


Figure 12a

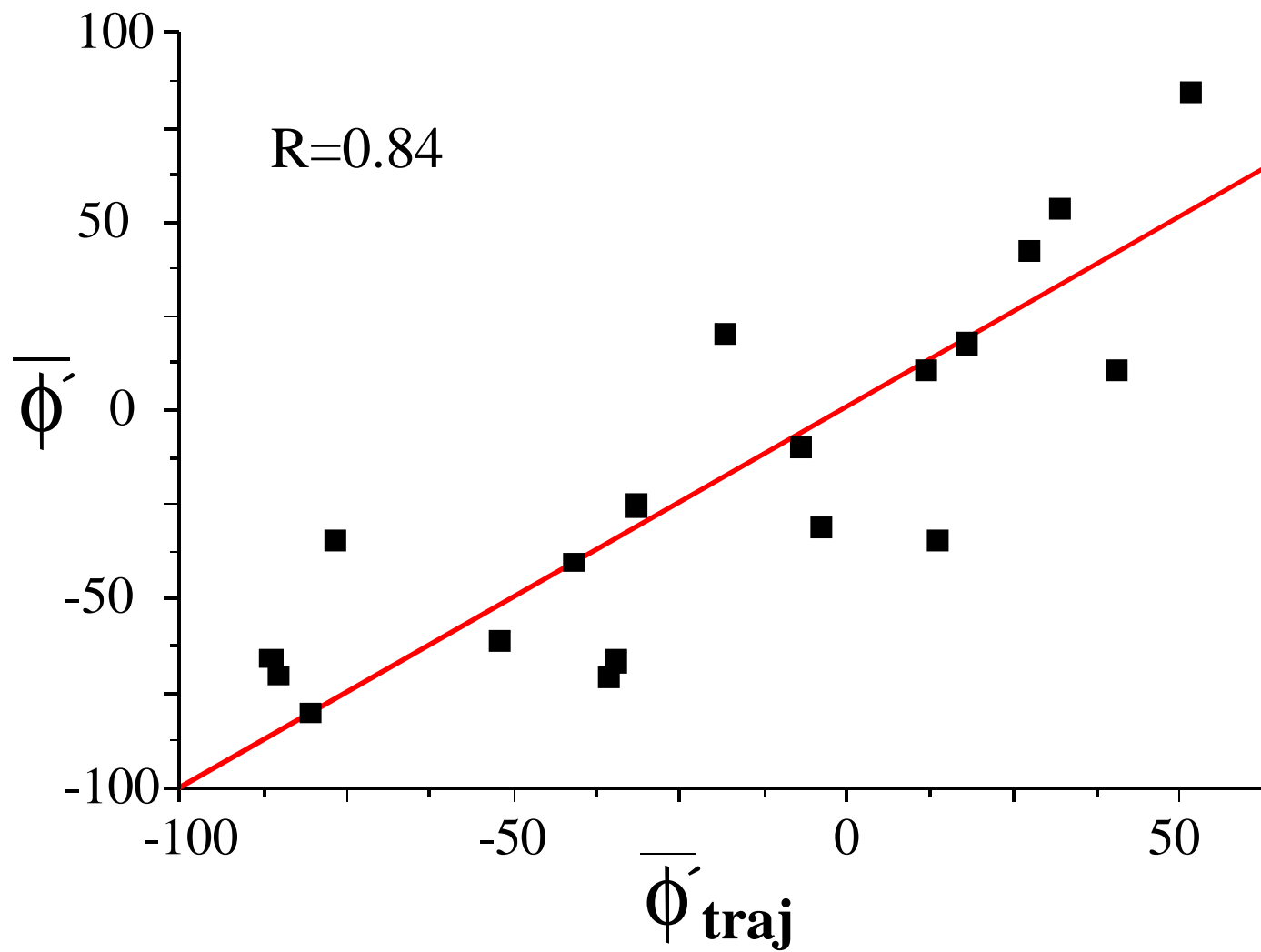


Figure 12b

NMR Spectroscopic Investigation of ψ Torsion Angle Distribution in Unfolded Ubiquitin from Analysis of $^3J(C_{\alpha},C_{\alpha})$ Coupling Constants and Cross-Correlated $\Gamma_{H^{N},C_{\alpha}H_{\alpha}}^C$ Relaxation Rates

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The denatured state of a protein consists of an ensemble of conformers. Statistical models have been developed to describe the conformational averaging process for this dynamic state of peptides¹ and proteins.^{2,3} Our model^{2,3} assumes that all the interactions in a polypeptide chain studied at high concentrations of denaturant such as 8 M urea are local. Moreover, the distribution of conformers can be described by a statistical analysis of the distribution of torsion angles of residues in a database of native folded proteins not located in secondary structure elements. The Ramachandran diagram for all amino acids (Figure 1) shows that ϕ sampling is mainly restricted to values $-60^\circ > \phi > -180^\circ$, while ψ sampling covers $\psi \approx -30^\circ$ and $100^\circ < \psi < 180^\circ$ of the diagram.

Heteronuclear NMR spectroscopy has been key to validate predictions based on our model. NMR studies revealed a remarkable correlation between predictions taken from the protein database and amino acid specific variations of the rotamer distribution around the angles ϕ^6 and χ_1^4 for the protein lysozyme denatured in 8 M urea at pH 2 as well as in studies of small unstructured peptides.⁷

For denatured ubiquitin a good correlation is observed between the predicted and the measured $^3J(H^N,H_{\alpha})$ coupling constants averaged for a given residue type, for example, all alanines (Figure 2A and B). A set of 70 out of 73 possible $^3J(H^N,H_{\alpha})$ coupling constants could be determined. This observation supports our

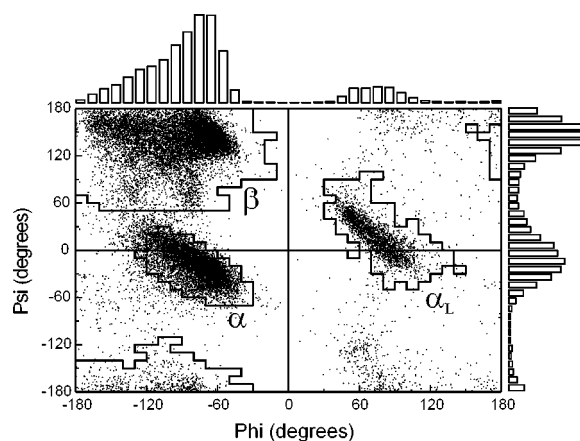


Figure 1. The distribution of ϕ, ψ torsion angles for all amino acids not located in secondary structure elements in a database of 402 high-resolution crystal structures of native folded proteins.⁴ The α, β and α_L (combined α_{left} and γ_{left}) regions of ϕ, ψ space, as defined by Swindells et al. are labeled.⁵

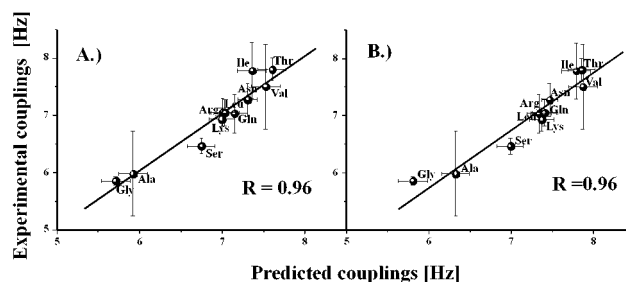


Figure 2. Correlation between experimental $^3J(H^N,H_{\alpha})$ averaged for a given residue type, e.g., all alanines in ubiquitin denatured in 8 M urea, pH 2, and $^3J(H^N,H_{\alpha})$ coupling constants predicted for amino acids excluding aromatic amino acids and Asp and Glu residues. Mean values for a given residue type are depicted as balls, error bars represent standard deviations. Three different Karplus parametrizations were used for the calculated couplings, error bars for predicted couplings represent standard deviations. (Complete information on experimental conditions and predictions is given in Table S3 in Supporting Information) (A) Comparison with predictions for residues that are not located in secondary structure elements in the database of native folded protein structures with ϕ, ψ torsion angles covering all regions of the Ramachandran diagram. (B) Comparison with predictions for residues that are not located in secondary structure elements and that have positive ψ torsion angles only.

model that predicts that conformational preferences in the denatured polypeptide chain are local and provides further evidence that the model does not depend on a specific peptide sequence.

However, since $^3J(H^N,H_{\alpha})$ depend only on the angle ϕ , it is difficult to differentiate between two possible models of conformational averaging: a model in which ϕ, ψ sampling is restricted to positive ψ torsion angle space as opposed to a model in which the polypeptide chain is sampling both positive and negative ψ torsion angles. $^3J(H^N,H_{\alpha})$ are indeed predicted to be similar if residues not located in secondary structure elements are taken into account that have positive ψ torsion angles only (Figure 2B) or that have both positive and negative ψ torsion angles (Figure 2A). The presence of experimental NOE contacts H^N_i, H^N_{i+1} and of $H_{\alpha i}, H^N_{i+1}$ of similar intensity in denatured proteins indicate that averaging involves sampling of positive and negative ψ torsion angles, which is compatible with predictions of our model.⁸

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However, a quantitative analysis of populated rotamers from NOE intensities is difficult due to the r^{-6} averaging and complex dynamical properties that are potentially nonuniform along the denatured peptide chain.

In the present study, two NMR experiments were carried out to probe the conformational averaging around the protein backbone angle ψ . In particular, $^3J(C_\alpha, C_\alpha)$ coupling constants⁹ and dipole, dipole cross-correlated relaxation rates^{10–13} have been measured. The dependence of the cross-correlated relaxation rate $\Gamma_{H^N, C_\alpha H_\alpha}^c$ between the N, H^N and H_α, C_α dipole tensors on their projection angle θ ^{10,11} is given in eq 1.1.

$$\Gamma_{H^N, C_\alpha H_\alpha}^c = \frac{\gamma_H \gamma_N}{(r_{N, H^N})^3} \frac{\gamma_H \gamma_C}{(r_{C_\alpha, H_\alpha})^3} \left(\frac{\hbar \mu_0}{4\pi} \right)^2 \times \frac{1}{5} (3 \cos^2 \theta - 1) S^2 \tau_c \quad (1.1)$$

For the native state of ubiquitin (10 mM phosphate, pH = 4.7, $T = 303$ K), in agreement with Pelupessy et al.,¹³ large negative relaxation rates of -11.8 ± 1.8 Hz are observed for the β -sheet regions of the protein (Figure 3a, triangles). More positive relaxation rates are observed in α -helical and loop regions of the protein. For denatured ubiquitin, uniform cross-correlated relaxation rates with an average of -4.3 ± 1.8 Hz are observed (Figure 3a, balls). The structural interpretation of the cross-correlated relaxation rates for a dynamic system is difficult, since neither the overall correlation time τ_c nor the order parameter S^2 can be unambiguously determined for a denatured polypeptide chain. The ^{15}N T_1/T_2 ratios for residues 4–64 are 2.0 ± 0.3 indicating a relative uniform effective correlation time for the center part of denatured ubiquitin. However, the experimental cross-correlated relaxation rates in denatured ubiquitin are small and negative. The rates for residues 23–34 that are in the α -helix in the native state are more negative in the denatured state but do not reach the values found for the β -sheet region of the native state of ubiquitin. We interpret the more negative relaxation rates as a conformational averaging, in which residues sample both positive and negative ψ torsion angles in the denatured state.

To further gain insight into the conformational averaging in denatured ubiquitin, $^3J(C_\alpha, C_\alpha)$ coupling constants shown in Figure 3B were measured and compared with data for native ubiquitin. $^3J(C_\alpha, C_\alpha)$ depend⁹ on the angle ψ . This unexpected relation, which is in contradiction to a Karplus-type dependence on the intervening torsion angle ω , was found for data obtained for native ubiquitin (Figure 3B, triangles). The mean experimental coupling constants $^3J(C_\alpha, C_\alpha)$ for residues in β -sheet regions of native ubiquitin are 1.69 ± 0.1 Hz, while coupling constants are too small to be observed in α -helical regions. In denatured ubiquitin, $^3J(C_\alpha, C_\alpha)$ are larger than 0.7 Hz throughout the sequence including residues 23–34 that are located in the α -helical region in the native state (Figure 3B, balls). The mean $^3J(C_\alpha, C_\alpha)$ coupling constant is 0.85 ± 0.2 Hz with a pair wise rmsd of 0.1 Hz (Table S2, Supporting Information) for the 41 resonances that are sufficiently resolved to allow coupling constants to be determined. As an exception,

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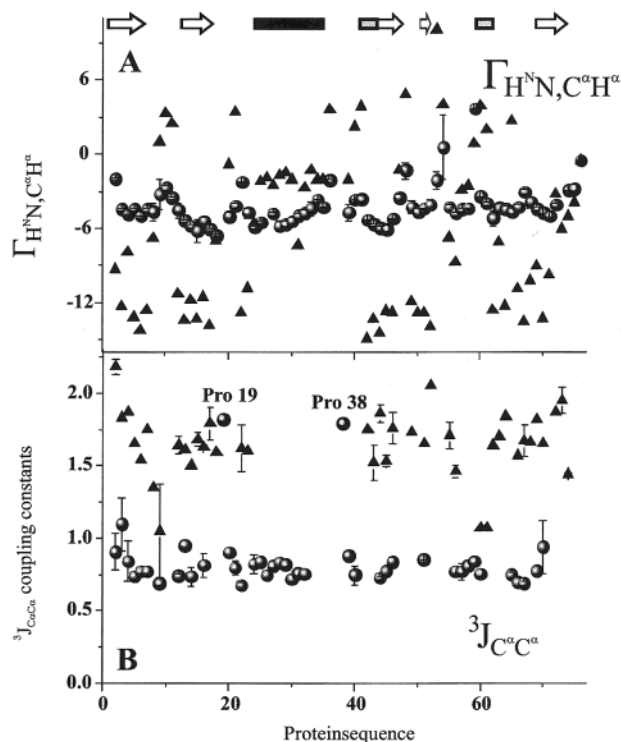


Figure 3. A. Cross-correlated relaxation rates $\Gamma_{H^N, C_\alpha H_\alpha}^c$ and B. coupling constants $^3J(C_\alpha, C_\alpha)$ in Hz measured for native (triangles) and for denatured (balls) ubiquitin. Pairwise root-mean-square differences of coupling constant values, which could be measured twice for successive residues, are given as error bars. Error bars for relaxation rates represent standard deviations from repeated experiments. Secondary structure elements in the native state of ubiquitin are indicated. All experiments were performed at 600 MHz. Experiments were carried out as described by Hennig et al.⁹ and Pelupessy et al.,¹³ respectively.

large $^3J(C_\alpha, C_\alpha)$ are observed for Pro19 and Pro38. This might reflect a strong preference of prolines for ϕ, ψ torsion angles in the polyprolyl region of ϕ, ψ space ($\phi \approx -60^\circ, \psi \approx +150^\circ$) and potentially restricted ψ sampling.

In conclusion, the two NMR parameters show that in a denatured polypeptide chain, individual amino acids sample both positive and negative ψ torsion angles. This observation has consequences for our understanding of the overall shape and tumbling of a polypeptide chain denatured in high concentrations of denaturant. The experiments reported here provide complementary tools to study the nature of non-native states of proteins and deviations found in intermediate states by heteronuclear NMR spectroscopy.

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Supporting Information Available: A listing of measured coupling constants, cross-correlated relaxation rates of denatured ubiquitin, predictions of averaged coupling constants for different Karplus parametrizations and error analysis (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.



Chemical shifts in denatured proteins: Resonance assignments for denatured ubiquitin and comparisons with other denatured proteins

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Key words: chemical shifts, denatured proteins, α -lactalbumin, lysozyme, random coil, resonance assignment, triple resonance NMR, ubiquitin

Abstract

Chemical shift assignment is reported for the protein ubiquitin denatured in 8M urea at pH 2. The variations in ¹⁵N chemical shifts of three different proteins (ubiquitin, disulfide reduced, carboxymethylated lysozyme, all-Ala- α -lactalbumin), all without disulfides and denatured in 8M urea at pH 2 are compared to 'random coil shifts' of small model peptides (Braun et al., 1994) and to the averaged native chemical shifts taken from the BMRB database. Both parameterizations show a remarkable agreement with the averaged measured ¹⁵N chemical shifts in the three denatured proteins. Detailed analysis of these experimental ¹⁵N chemical shifts provides an estimate of the influence of nearest neighbors and conformational preferences on the chemical shift and provides a direct means to identify non-random structural preferences in denatured proteins.

Introduction

A large number of different techniques can be used to derive structural information for native states of proteins. This is different for the denatured state of a protein, for which only a few methods exist to obtain information at atomic resolution. It is now clear that NMR spectroscopy is the major technique to investigate the structural and dynamical characteristics of denatured proteins. ¹H, ¹⁵N, ¹³C resonance assignments for the denatured state have been reported for a number of proteins (Neri et al., 1992a, b; Arcus et al., 1994, 1995; Logan et al., 1993, 1994; Buck et al., 1995; Frank et al., 1995; Shortle, 1996; Wong et al., 1996; Yang and Kay, 1996; Schwalbe et al., 1997; Blanco et al., 1998; Dyson and Wright, 1998; Eliezer et al., 1998; Penkett et al., 1998; Hennig et al., 1999). The availability of methods to obtain site-specific res-

onance assignments forms the basis for a detailed analysis of NMR parameters such as NOE effects, J-couplings, and heteronuclear relaxation rates. The analysis of the NMR parameters of denatured proteins is challenging, since they reflect an average over the ensemble of conformers populated in the random coil state of a protein and methods have to be developed to describe the conformational averaging in denatured proteins. We have developed a statistical model for the random coil state of proteins (Fiebig et al., 1996; Smith et al., 1996a, b; Schwalbe et al., 1997; Hennig et al., 1999). Our random coil model assumes that all interactions in the unfolded polypeptide chain of a protein are local and that the conformational distribution can be described from the distribution of structure in native proteins. On this basis, NMR parameters such as J-couplings and chemical shifts should be predictable from analysis of their distribution observed in the native state of proteins.

The purpose of this study is to analyze the residual chemical shift dispersion observed in ¹H, ¹⁵N corre-

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lation experiments for denatured states of proteins. Here, we report the ^1H , ^{15}N , ^{13}C chemical shifts of ubiquitin denatured in 8M urea at pH 2. The chemical shift assignment of ubiquitin in its denatured state may serve as a reference for NMR studies in its native state. It contains 76 amino acids (8.6 kDa) without prosthetic groups or disulfide bridges. Ubiquitin is found in all eukaryotes and plays an important role as a control component of the ATP-dependent eukaryotic system for regulation of protein turnover (Rechsteiner, 1988; Hershko and Ciechanover, 1998). The structure of ubiquitin has been studied under a variety of different conditions. The native state of ubiquitin is well characterized by high-resolution X-ray crystallography (Vijay-Kumar et al., 1987) and NMR spectroscopy. NMR investigations of native ubiquitin have involved measurement of homonuclear NOEs, homo- and heteronuclear coupling constants (Di Stefano and Wand, 1987; Weber et al., 1987; Tjandra et al., 1995; Cavanagh et al., 1996) as well as residual dipolar couplings (Cornilescu et al., 1999). Auto- and cross-correlated relaxation rates have been measured to provide an insight into the backbone dynamics of ubiquitin (Schneider et al., 1992; Tjandra et al., 1995, 1996; Fushman et al., 1998; Fushman and Cowburn, 1998; Carlomagno et al., 2000).

Ubiquitin remains folded even at temperatures of 80 °C and for pH values ranging from 1.2 to 13 (Lenkinski et al., 1977; Jenson et al., 1980; Nash and Jonas, 1997). In 60% aqueous methanol at pH 2, ubiquitin forms a so-called A-state. In this state, the antiparallel β -sheet and the central α -helix are conserved in the N-terminal half of the protein (Brutscher et al. (1997) and references cited therein). The C-terminal half, which is rich in β -sheet character in the native state, undergoes a methanol-induced transition to a dynamic state that is purely α -helical. A wide range of dynamic properties were measured to confirm this model of a non-native state of ubiquitin. In 8M urea, ubiquitin is essentially completely denatured (Lenkinski et al., 1977). Under these conditions ubiquitin provides a good model for examining the ^{15}N chemical shift dispersion in the denatured state of a polypeptide chain without disulfides.

In order to analyze nearest neighbor effects on the $^1\text{H}^{\text{N}}$, ^{15}N chemical shifts in denatured proteins, we compare $^1\text{H}^{\text{N}}$, ^{15}N resonance assignments in human ubiquitin with chemical shift data for disulfide reduced, carboxymethylated hen egg white lysozyme (Schwalbe et al., 1997; Hennig et al., 1999), and a mutant form of human α -lactalbumin in which all cys-

teines have been replaced by alanines (Redfield et al., 1999). In addition, trends observed for chemical shift deviations are correlated with a large set of $^3\text{J}(\text{H}^{\text{N}}, \text{H}^{\alpha})$ coupling constants measured for the three different proteins. All proteins were studied in their unfolded state obtained by denaturation in 8M urea at pH 2.

Material and methods

^{15}N labeled ubiquitin and ^{15}N , ^{13}C labeled ubiquitin (both 5 mg; gifts from VLI Research Inc., Malvern, PA) were denatured in 8M urea at pH 2 in 95% $\text{H}_2\text{O}/5\%$ D_2O . 10 mg of ^{15}N , ^{13}C labeled ubiquitin was dissolved at pH 4.7 in 95% $\text{H}_2\text{O}/5\%$ D_2O for the native state reference sample. Experiments for the assignment of backbone resonances are summarized in Table 1. Pulsed field gradient versions of the experiments, with water flip-back pulses (Grzesiek and Bax, 1993) and sensitivity enhancement (Sattler et al. (1999) and references cited therein) were recorded on a Bruker DRX 600 (TXI HCN z-grad) at 303 K. A recycle delay of 1.5 s was used for all experiments. The carrier positions were: 117.5 ppm for ^{15}N , 174 ppm for $^{13}\text{C}'$, 38.2 ppm for aliphatic ^{13}C , and 4.8 ppm for ^1H , respectively. All ^1H pulses were centered around the water resonance. All carbon pulses were implemented as Gaussian cascades (Emsley and Bodenhausen, 1990); Q3 (Emsley and Bodenhausen, 1992) pulses for inversion and G4 and time reversed G4 pulses for excitation were used. Fourier transformation, mirror image linear prediction and data analysis were performed using the program Felix 98.0 (Molecular Simulation Inc., San Diego, CA). Resonance positions were referenced using internal DSS and TSP to obtain ^1H chemical shift referencing and for the ^{13}C and ^{15}N dimensions by calculation as described in Wishart et al. (1995). No difference was observed between DSS and TSP referencing. For ubiquitin, a total of 71 out of 73 possible $^1\text{H}^{\text{N}}$, ^{15}N correlation peaks can be resolved at a proton resonance frequency of 600 MHz.

Coupling constant determination

The measurement of $^3\text{J}(\text{H}^{\text{N}}, \text{H}^{\alpha})$ coupling constants was performed as described for denatured human ubiquitin (Peti et al., 2000). $^3\text{J}(\text{H}^{\text{N}}, \text{H}^{\alpha})$ coupling constants for all-Ala α -lactalbumin were measured using a 0.4 mM sample of ^{15}N labeled protein dissolved in 8M urea (95% $\text{H}_2\text{O}/5\%$ D_2O) at pH 2 and 293 K (Redfield et al., 1999). A 2D HMQC-J (Kay and Bax,

Table 1. Summary of experiments carried out for the assignment of ubiquitin unfolded in 8M urea at pH 2

Experiment	No. of data points/sweep width			References
	t ₃	t ₂	t ₁	
2D ¹ H, ¹⁵ N-HSQC		4096(¹ H); 5482.45 Hz	512(¹⁵ N); 1149.425 Hz	
3D NOESY-HSQC (200 ms)	1024(¹ H); 5482.45 Hz	64(¹⁵ N); 1149.425 Hz	256(¹ H); 5482.45 Hz	(Driscoll et al., 1990; Marion et al., 1989a)
3D TOCSY-HSQC (60 ms, 80 ms)	1024(¹ H); 5482.45 Hz	64(¹⁵ N); 1149.425 Hz	256(¹ H); 5482.45 Hz	(Marion et al., 1989b)
3D HSQC-NOESY-HSQC (150 ms)	1024(¹ H); 5482.45 Hz	48(¹⁵ N); 1149.425 Hz	64(¹⁵ N); 1149.425 Hz	(Frenkiel et al., 1990)
3D HNCO	1024(¹ H); 5482.45 Hz	64(¹³ C); 1666.67 Hz	64(¹⁵ N); 1149.425 Hz	(Kay et al., 1990)
3D CBCA(CO)NH	1024(¹ H); 5482.45 Hz	64(¹⁵ N); 1149.425 Hz	128(¹³ C); 9615.385 Hz	(Girzesiek and Bax, 1992)
3D HNCACB	1024(¹ H); 5482.45 Hz	128(¹³ C); 9615.385 Hz	128(¹⁵ N); 1149.425 Hz	(Wittekind and Mueller, 1993)
3D CC(CO)NH	1024(¹ H); 5482.45 Hz	64(¹⁵ N); 1149.425 Hz	128(¹³ C); 9615.385 Hz	(Girzesiek et al., 1993)

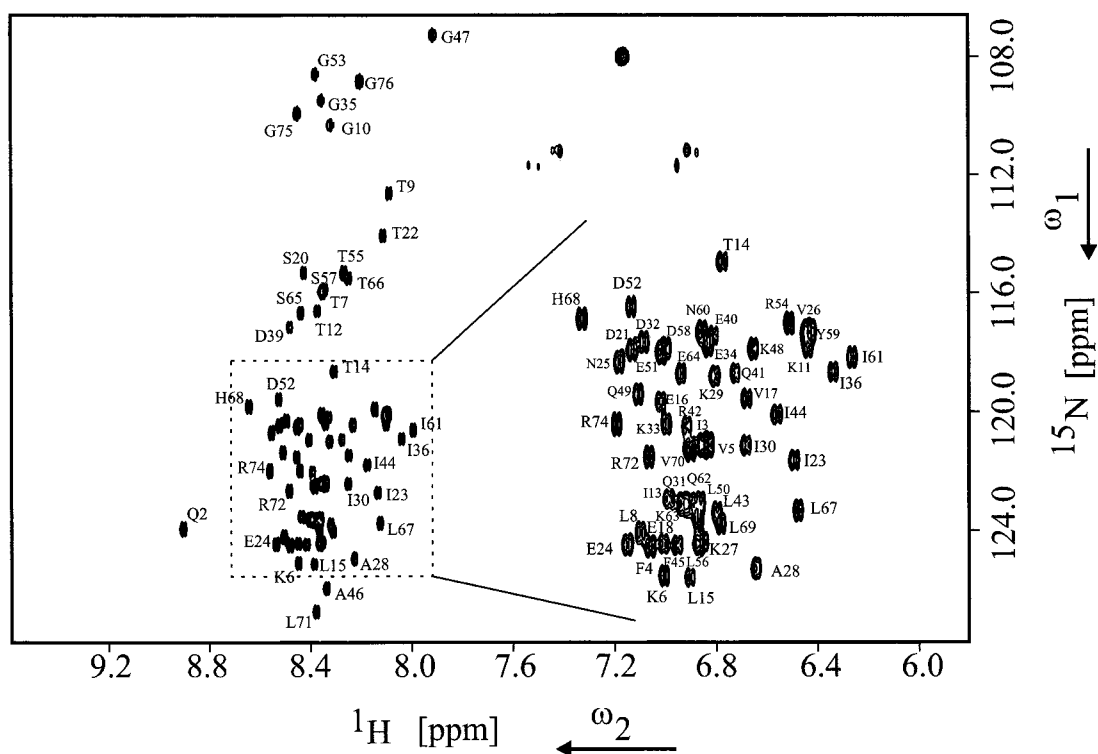


Figure 1. Annotated $^1\text{H},^{15}\text{N}$ -HSQC spectrum of ubiquitin unfolded in 8M urea, pH 2, recorded at 303 K and a ^1H frequency of 600 MHz. Sequential assignments are indicated with the one-letter amino acid code and the residue number.

1990) dataset was collected at 600 MHz. The sweep widths were 6514.66 and 1152.07 Hz in ω_2 and ω_1 , and 2048 and 300 complex points were collected in t_2 and t_1 , respectively. After zero-filling, the digital resolution in ω_1 (^{15}N) was 0.56 Hz/point. The coupling constants were extracted from ω_1 columns using an in-house fitting program. Lysozyme chemical shift and coupling constant data were analyzed taking previously published data (Schwalbe et al., 1997; Hennig et al., 1999).

Results and discussion

Chemical shift assignments

The $^1\text{H},^{15}\text{N}$ -HSQC spectrum, with annotated assignments, of ubiquitin denatured in 8M urea at pH 2 is shown in Figure 1. The proton chemical shift dispersion in denatured ubiquitin is only 0.6 ppm (excluding the second amino acid at the N-terminus of the protein). The ^{15}N chemical shifts in ubiquitin show an amino acid type specific clustering: this is clearly visible for glycine, threonine and serine residues. The other amino acids cluster together with ^{15}N chemical

shifts in the range 117–125 ppm (Table 2). For a given amino acid, the residual chemical shift dispersion of ^{15}N is large compared to the narrow chemical shift dispersion of $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$ and $^{13}\text{C}'$.

A second characteristic feature observed in experiments on denatured proteins is the narrow line width. The long ^1H and ^{15}N transverse relaxation properties ($T_2(^{15}\text{N}) \approx 350$ ms for ubiquitin at 60 MHz ^{15}N frequency) are very uniform for denatured states of proteins lacking disulfide bridges. This enables experiments to be performed on denatured proteins that are only feasible in native proteins after deuterium incorporation. Increased mobility, as evidenced by a negative heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE, might provide anNOE, might provide an additional hint to identify terminal amino acids in unfolded proteins.

In ubiquitin, sequential NOE cross peak patterns are uniform throughout the sequence and $\text{H}^{\text{N}}, \text{H}^{\text{N}}$ ($i, i+1$) and $\text{H}^\alpha, \text{H}^{\text{N}}$ ($i, i+1$) cross peaks are observed with similar intensity. This reflects the fact that in a random coil both the conformational regions corresponding to the α and β main chain ϕ, ψ conformations are populated (Smith et al., 1996a, b). Provided that the primary sequence of the polypeptide chain

Table 2. Averaged chemical shifts for three different proteins denatured in 8M urea at pH 2. ^{13}C chemical shifts are reported for human ubiquitin and cysteine reduced, carboxymethylated hen eggwhite lysozyme. Std. is the standard deviation of the chemical shifts

	^{15}N (ppm)	Std. (ppm)	$^1\text{H}^{\text{N}}$ (ppm)	Std. (ppm)	$^{13}\text{C}^{\alpha}$ (ppm)	Std. (ppm)	^{13}CO (ppm)	Std. (ppm)	Number of residues
Ala	125.11	1.27	8.14	0.15	52.26	0.25	177.10	0.37	25
Arg	122.41	1.42	8.25	0.19	55.96	0.38	175.80	0.46	15
Asn	120.43	1.05	8.31	0.14	52.79	0.19	174.71	0.40	17
Asp	120.09	1.36	8.40	0.09	52.66	0.16	174.91	0.49	23
Cys	119.49	0.64	8.26	0.19	55.05	0.02	173.82	0.22	3
Gln	122.13	1.17	8.37	0.18	55.46	0.25	175.67	0.42	16
Glu	121.63	1.79	8.33	0.13	55.26	0.12	175.74	0.56	17
Gly	109.56	0.91	8.19	0.20	44.93	0.34	173.58	0.37	23
His	119.36	1.46	8.48	0.16	54.64	0.56	174.15	0.06	4
Ile	121.66	1.31	8.09	0.17	60.47	0.26	175.68	0.33	23
Leu	123.55	1.61	8.18	0.20	54.79	0.32	177.01	0.34	29
Lys	122.49	1.57	8.30	0.16	55.87	0.49	175.97	1.35	23
Met	121.38	1.82	8.15	0.13	55.12	0.37	174.50	2.05	4
Phe	122.27	2.08	8.27	0.19	57.39	0.11	175.13	0.32	9
Pro					63.47	0.36	176.27	0.46	3
Ser	116.95	1.34	8.24	0.16	58.11	0.36	174.25	0.53	20
Thr	115.11	1.64	8.12	0.14	61.37	0.63	174.27	0.54	21
Trp	121.08	0.62	7.91	0.13	57.19	0.44	175.81	0.30	8
Tyr	120.61	0.74	8.02	0.09	57.87	0.12	175.38	0.25	8
Val	122.47	1.59	8.15	0.22	61.91	0.26	175.36	0.73	11

is not repetitive, ^{13}C labeling is not always required for the ^1H and ^{15}N resonance assignment (Schwalbe et al., 1997; Redfield et al., 1999). For the sequential ($i\pm 1$) assignment, the NOESY-HSQC and HSQC-NOESY-HSQC spectra were found to provide the best resolution. Figure 2 shows strips taken from $^1\text{H},^{15}\text{N}$ -NOESY-HSQC and $^1\text{H},^{15}\text{N}$ -TOCSY-HSQC spectra of denatured ubiquitin with annotated ($i,i+1$) connectivities. In contrast to folded proteins, for which $\text{H}^{\text{N}},\text{H}^{\text{N}}(i,i+1)$ cross peaks in $^1\text{H},^{15}\text{N}$ -HSQC-NOESY-HSQC are observed only in α -helical secondary structure elements, these cross peaks are observed throughout the polypeptide chain in denatured proteins and therefore provide useful correlations for the sequential assignment in cases for which the $\text{N}^{\text{H}},\text{N}^{\text{H}}(i,i+1)$ cross peaks are overlapped in the $^1\text{H},^{15}\text{N}$ -NOESY-HSQC spectrum. Residues with neighboring glycine, threonine or serine residues serve as starting points for assignment: the ^{15}N chemical shift for these three amino acids differ from those of other amino acid type (see Figure 1) and differentiation between serine and threonine can readily be obtained from the $^1\text{H},^{15}\text{N}$ -TOCSY-HSQC spectrum. While a majority of residues can be assigned in denatured proteins labeled

with ^{15}N , incorporation of both ^{15}N and ^{13}C has often been reported to be vital for complete resonance assignment in denatured proteins. Standard 3D assignment pulse sequences like HNCACB, CBCA(CO)NH or HNCOC resolving ^{13}C chemical shifts in the relatively well resolved $^1\text{H},^{15}\text{N}$ plane can be applied as they are for native states of proteins. However, due to the observation that the variation in the carbon chemical shift (e.g. C^{α} and C^{β}) is very small, analysis of $^1\text{H},^{15}\text{N}$ NOESY-HSQC spectra is often vital for the assignment of denatured proteins.

$^1\text{H},^{15}\text{N},^{13}\text{C}$ backbone resonances for 71 out of the 73 non-proline residues of ubiquitin in 8M urea, pH 2, could be assigned. The ^1H and ^{13}C resonances of two prolines were also assigned. Residues around Pro19 showed a doubling of the $^1\text{H}^{\text{N}},^{15}\text{N}$ correlation peaks. The side chain $\text{H}^{\alpha},\text{H}^{\beta},\text{C}^{\alpha},\text{C}^{\beta}$ resonances could be assigned for 75 of the 76 residues (no assignment was possible for Pro37 because there are two neighboring prolines in the sequence). Additional side chain proton chemical shifts were identified in the ^{15}N edited TOCSY-HSQC and the NOESY-HSQC spectra. The chemical shift values of proton, nitrogen and carbon resonances have been deposited in the

Table 3. Comparison of averaged experimental chemical shifts for each amino acid compared with the averaged chemical shifts predicted using the empirical values by Braun et al. (column a) and the BMRB values (column b). Column c reports the correlation coefficient of the average ^{15}N chemical shift for each amino acid compared with the chemical shifts predicted by Braun et al. (1994), column d reports the χ^2 value between experiment and predictions and column e gives the number of residues in the three protein datasets

	^{15}N averaged measured values		(c) Correlation coefficient	(d) χ^2	(e) Number of residues
	(a) averaged Braun et al.	(b) averaged BMRB			
Ala	-0.59	2.14	0.88	0.32	25
Arg	-0.29	2.04	0.88	0.44	15
Asn	-0.07	1.63	0.79	0.41	17
Asp	-0.71	-0.22	0.83	0.58	23
Cys	-0.61	0.19	0.65	1.06	3
Gln	0.23	2.42	0.64	0.78	16
Glu	-0.17	0.95	0.93	0.65	17
Gly	-1.14	0.36	0.73	0.39	23
His	-0.74	0.01	0.96	0.16	4
Ile	-0.14	-0.09	0.88	0.41	23
Leu	-0.25	1.84	0.81	0.92	29
Lys	-0.61	1.48	0.92	0.36	23
Met	-0.52	1.34	0.94	0.4	4
Phe	0.37	1.54	0.87	1.08	9
Ser	-0.65	0.64	0.85	0.72	20
Thr	-0.79	0.01	0.89	0.56	21
Trp	-1.62	-0.18	0.61	1.02	8
Tyr	-1.39	-0.09	0.50	0.68	8
Val	1.37	1.42	0.92	0.42	11

BioMagResBank in Madison, WI, U.S.A. (accession number 4375, <http://www.bmrwisc.edu>).

An important step in the refolding of ubiquitin is thought to involve a cis-trans proline isomerization as described by Briggs and Roder (1992) using hydrogen-deuterium exchange labeling in conjunction with rapid mixing methods and two-dimensional NMR analysis. Ubiquitin has three proline residues. In our studies, a doubling of resonances with a ratio of 12.5% to 87.5% for the cis and trans conformations of Pro19, respectively, was observed for resonances of residue Ser20, indicating slow cis-trans proline isomerization in denatured ubiquitin (cis-Pro19: C^α : 61.396 ppm, C^β : 33.507 ppm; trans-Pro19: C^α : 61.98 ppm, C^β : 31.13 ppm). These findings are in good agreement with the earlier study using pulsed hydrogen exchange experiments in which cis/trans proportions of 9% to 91%, 14% to 86% and 19% to 81% were found for Pro19, Pro37 and Pro38, respectively.

Analysis of chemical shifts

Residual dispersion of chemical shifts in denatured proteins is mainly observed for backbone amide nitrogen and carbonyl atoms, while deviation of individual ^{13}C and other ^1H chemical shifts from their mean is small and decreases for side chain atoms (Table 2). Interestingly, the standard deviation of the carbonyl shifts is small and comparable to the other carbon chemical shift deviations. The mean chemical shifts found here and in other proteins are close to the averaged values commonly referred to as random coil chemical shifts that have been measured in a large number of small peptide constructs and for which solvent effects have been characterized (Wishart et al., 1991a, b; Thanabal et al., 1994; Plaxco et al., 1997). For example, the peptide constructs GGXGG are assumed to adopt no preferred conformation and therefore to represent the random coil in solution (Merutka et al., 1995). An extensive characterization of random coil chemical shifts was carried out by Wishart et al. (1995) using the peptides GGXAGG and GGXPGG to quantify the influence of a proline residue on the

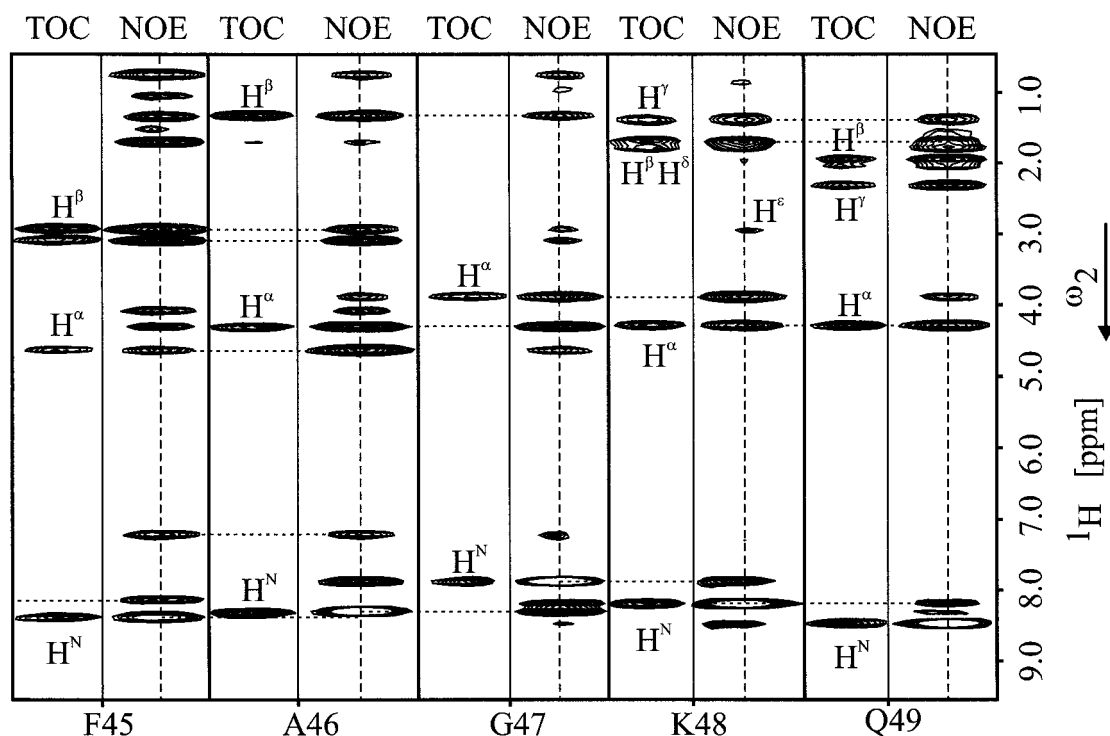


Figure 2. Strips taken from the $^1\text{H},^{15}\text{N}$ -NOESY-HSQC and the $^1\text{H},^{15}\text{N}$ -TOCSY-HSQC spectra of unfolded ubiquitin. The strips of residues F45 to Q49 show features typical of NOESY spectra for unfolded proteins. A uniform pattern of sequential (i,i+1) NOE cross peaks for $\text{H}^{\text{N}}, \text{H}^{\text{N}} (i,i+1)$ and $\text{H}^{\alpha}, \text{H}^{\text{N}} (i,i+1)$ is observed with similar intensity throughout the entire sequence, in contrast to native folded sequences. The intraresidual correlation peaks are annotated.

chemical shifts of the preceding residue. The reported chemical shift values are routinely used to interpret deviations as being indicative for specific ranges of values of the backbone angles ϕ and ψ . Interestingly, C' , C^{α} and C^{β} , but not ^{15}N chemical shifts are used to predict the secondary structure of proteins (Glushka et al., 1989; Spera and Bax, 1991; Wishart et al., 1992, 1995, 1997; Braun et al., 1994; Wishart and Sykes, 1994a, b; Wishart and Nip, 1998). Recently, Cornilescu et al. (1999) proposed an elegant way to extract the angle information from these chemical shifts to restrain ϕ , ψ torsion angles in the structure calculation of native proteins using a database approach.

Here, we concentrated on the ^{15}N chemical shifts since these have a significant dispersion in the denatured state, and we analyzed data for three proteins denatured in 8M urea at pH 2: human ubiquitin, reduced carboxymethylated hen egg white lysozyme and all-Ala α -lactalbumin. We compared the residue-specific averaged chemical shift of these denatured proteins with averaged chemical shifts of native proteins as deposited in the BMRB chemical shift database. The

average chemical shift statistics of all native folded diamagnetic proteins was used as its last update in February 1999. Aromatic amino acids, cysteine and methionine residues were excluded from the statistical analysis because of the lack of experimental data in our dataset of denatured proteins (less than 10 chemical shifts) and because aromatic residues have been found to be involved in non-random structure at least in lysozyme (Schwalbe et al., 1997; Hennig et al., 1999). The analysis reveals that the mean observed chemical shifts in native proteins are very close to the mean observed values in denatured proteins (shown in Figure 3); the correlation coefficient is 0.98. In native states of proteins a large spread of chemical shifts around the mean is observed. For the unfolded proteins studied here, the distribution is narrow.

In 1994, Braun et al. investigated the ^{15}N chemical shifts in random coil peptides. The chemical shift for a given amino acid averaged over all 20 possible dipeptide pairs as given by Braun et al. correlates well ($R = 0.99$, shown in Figure 4) with the averaged ^{15}N chemical shift for a given amino acid derived from the proteins studied here (Figure 4). Braun et al. (1994) re-

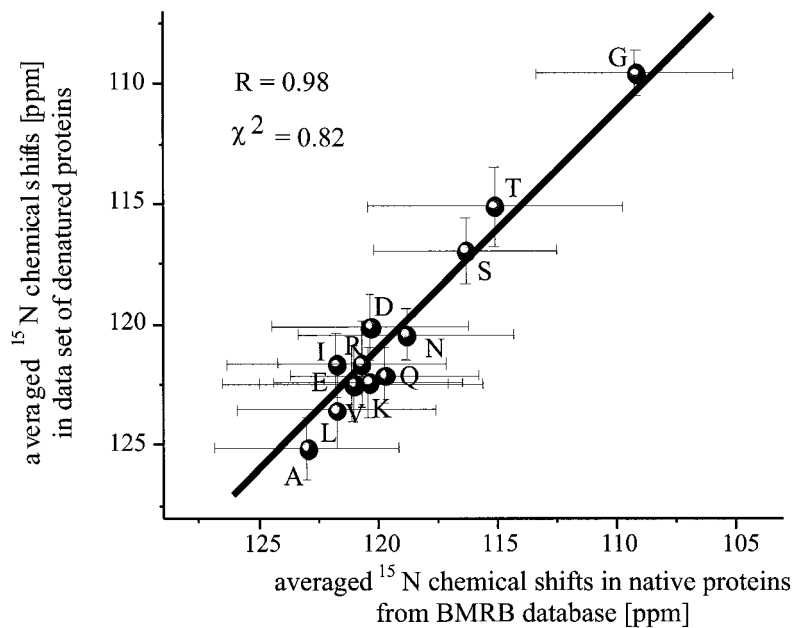


Figure 3. Comparison of the averaged ^{15}N chemical shifts of three denatured proteins with the averaged ^{15}N chemical shifts of folded proteins in the BMRB database. The average was taken for all amino acids of a given kind. The balls represent the mean values and the error bars are standard deviations. Aromatic amino acids, cysteine and methionine were excluded from this comparison as discussed in the text. Large variations in ^{15}N chemical shifts are observed for native proteins. A much smaller variation can be seen for the ^{15}N chemical shift of the three denatured proteins studied.

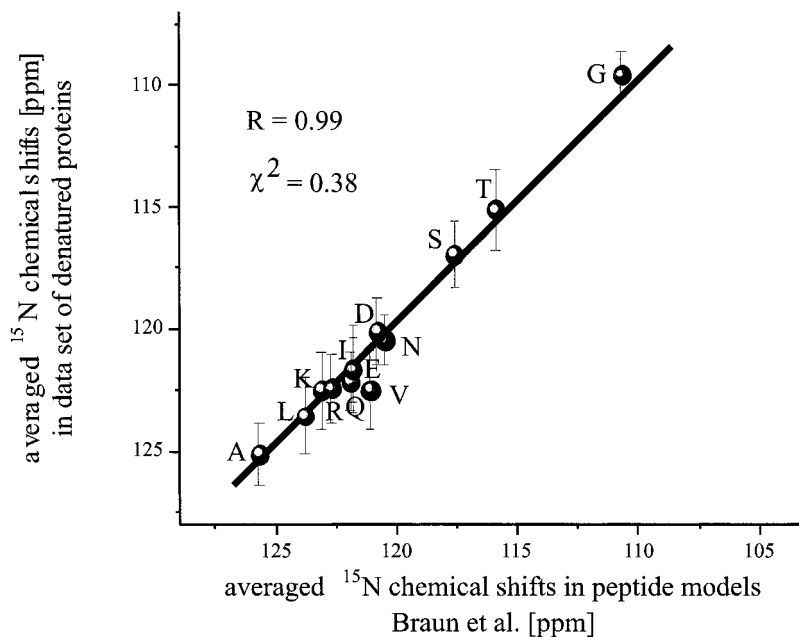


Figure 4. Comparison of the averaged ^{15}N chemical shifts of three denatured proteins with the ^{15}N chemical shift calculated by Braun et al. (1994).

ported a correction factor to account for the influence of the $i-1$ amino acid on the ^{15}N chemical shift of amino acid i . A comparison with the averaged experimental data provided here is shown for all residues in Figure 4 and is summarized in Table 3. The data show that the chemical shift for a specific amino acid corrected for the $i-1$ amino acid and averaged over the three datasets from ubiquitin, lysozyme and all-Ala α -lactalbumin correlates well with the random coil data from the peptide work.

Figure 5 shows the correlation of experimental $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constants in the three proteins individually and averaged over the three different proteins and predictions from the random coil model. The coupling constants for a random coil were predicted (Smith et al., 1996) using an enlarged database of 402 high resolution protein crystal structures (Henig et al., 1999). Predictions were made using only those residues that are not in regions of recognized secondary structure (COIL parameter set) using three different Karplus parameterizations (Pardi et al., 1984; Ludvigsen et al., 1991; Vuister and Bax, 1993).

The correlations reveal the following: using all amino acids, the averaged correlation coefficient R is found to be 0.85. Excluding Asp and Glu as residues that are protonated at low pH, the averaged correlation coefficient increases to 0.89. This can also be seen for the individual proteins. Aromatic residues are often involved in non-random conformations in the denatured states of proteins. This is also reflected here. Reduction of the dataset ($-$ aromatic residues and $-D$, $-E$) increases the correlation coefficient (ubiquitin: $R = 0.96$; lysozyme: $R = 0.94$; all-Ala- α -lactalbumin: $R = 0.90$; and $R = 0.95$ for all three proteins). The increase in correlation between prediction and experiment is independent of the protein and seems therefore independent of both the primary sequence and the native structure of the protein. Lysozyme and α -lactalbumin contain a significant portion of α -helices in their native state, while ubiquitin is mainly a β -sheet protein. This result is in agreement with the database approach to describe the denatured state of proteins; no bias of the experimental coupling constants depending on the secondary structure in the native protein is observed. This comparison is also in agreement with the observation that aromatic residues are involved in non-random conformations in the denatured states of proteins. Analysis of coupling constants and chemical shifts shows that aromatic residues are found to deviate from the model predictions. Therefore, measurement of ^{15}N chemical shifts appears to

be a very sensitive method to measure this deviation from random conformational sampling.

Figure 6 shows the correlation of the experimental chemical shifts of the 29 assigned leucine residues in the three datasets with predictions using the correction factors of Braun et al. (1994). It is apparent that the size of the neighboring amino acid side chain influences both the chemical shifts as well as the coupling constants (summarized in Table 4). In principle, both electronic and steric factors exerted by amino acid $i-1$ will influence the ^{15}N chemical shift of amino acid i and will therefore account for the variation between *different* dipeptides. For a given dipeptide, the variations are likely to reflect differences in the conformational sampling of the specific subunit. Therefore, it is interesting to investigate the relationship between the variation of chemical shifts and experimental parameters like $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constants that depend on the angle ϕ . An extensive investigation is beyond the scope of this paper, mainly because of the limited dataset of only 230 measured coupling constants, which are summarized in Table 4. A previous analysis of $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constant data for unfolded fibronectin binding protein (Penkett et al., 1997) showed that there is an increase in coupling constant values when residue ($i-1$) has a β branched or aromatic side chain. The increased coupling constants reflect a greater population of β ϕ,ψ conformers in the ensemble of rotamers. Overall, a similar trend is seen here. For example, for asparagine the average $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constant is 7.3 Hz when residue ($i-1$) has a β branched or aromatic side chain compared to 6.7 Hz for other residue types at position ($i-1$), while for isoleucine the average $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constant is 7.6 Hz when residue ($i-1$) has a β branched or aromatic side chain compared to 6.7 Hz when another residue is in the preceding position. Additional factors to these are also important, however. It is interesting to see that there is a considerable variation in both the chemical shift and the $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constant *within* the dipeptide pair Thr/Leu (Thr7/Leu8: $\delta^{15}\text{N} = 124.21$ ppm, $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha}) = 6.9$ Hz; Thr14/Leu15: $\delta^{15}\text{N} = 125.14$ ppm, $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha}) = 7.4$ Hz; Thr55/Leu56: $\delta^{15}\text{N} = 124.45$ ppm, $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha}) = 7.0$ Hz; Thr66/Leu67: $\delta^{15}\text{N} = 123.76$ ppm, $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha}) = 7.6$ Hz). The most low-field ^{15}N chemical shift of Leu15 corresponds with the largest $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constant. For Thr14/Leu15, Thr55/Leu56, and Thr66/Leu67, there is a correlation between downfield $^1\text{H}^{\text{N}}$ and ^{15}N chemical shift and increase in $^3\text{J}(\text{H}^{\text{N}},\text{H}^{\alpha})$ coupling constant,

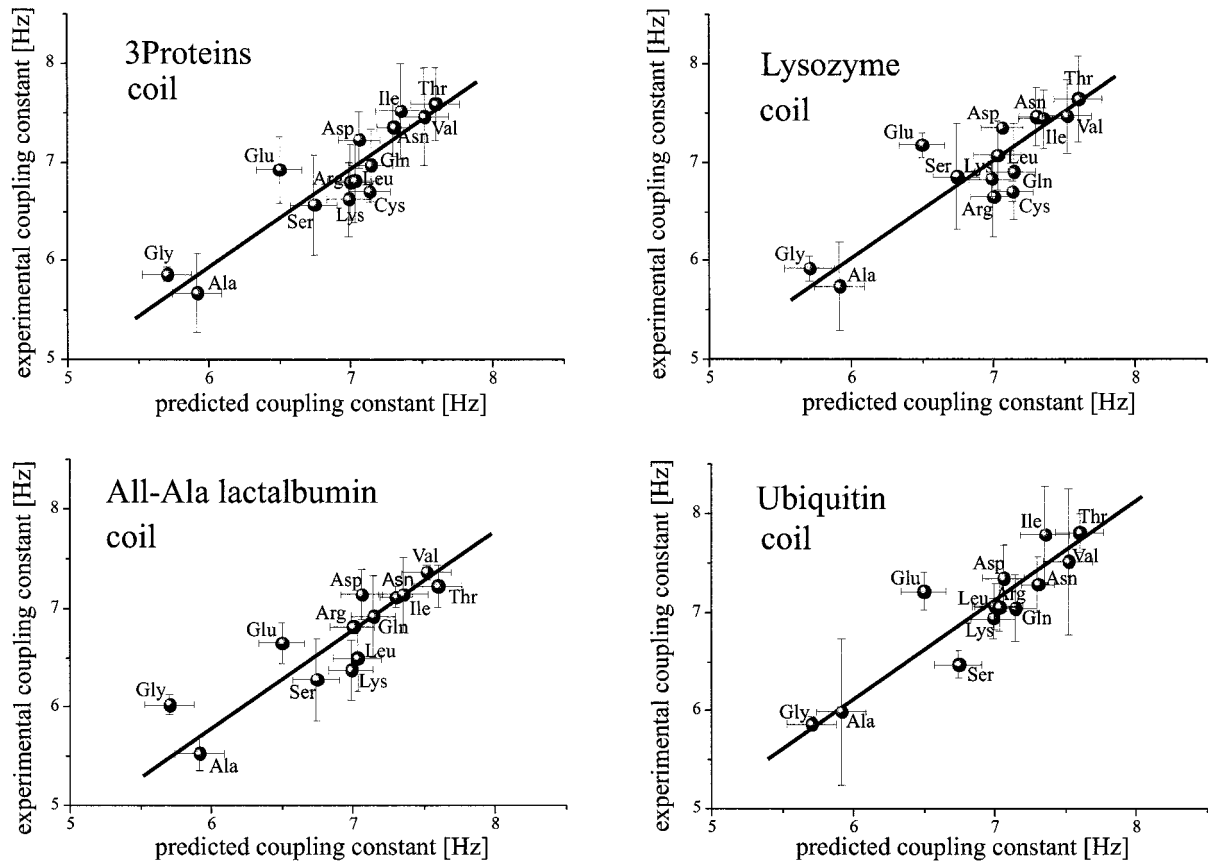


Figure 5. Comparison of experimental $^3J(\text{H}^{\text{N}}, \text{H}^{\alpha})$ coupling constants (aromatic residues are excluded) with predicted values from the statistical model for a random coil. The values were predicted as described by Smith et al. (1996a, b) using an enlarged database of 402 native protein structures (Hennig et al., 1999). Values for correlation including aromatic residues are given in Table 4.

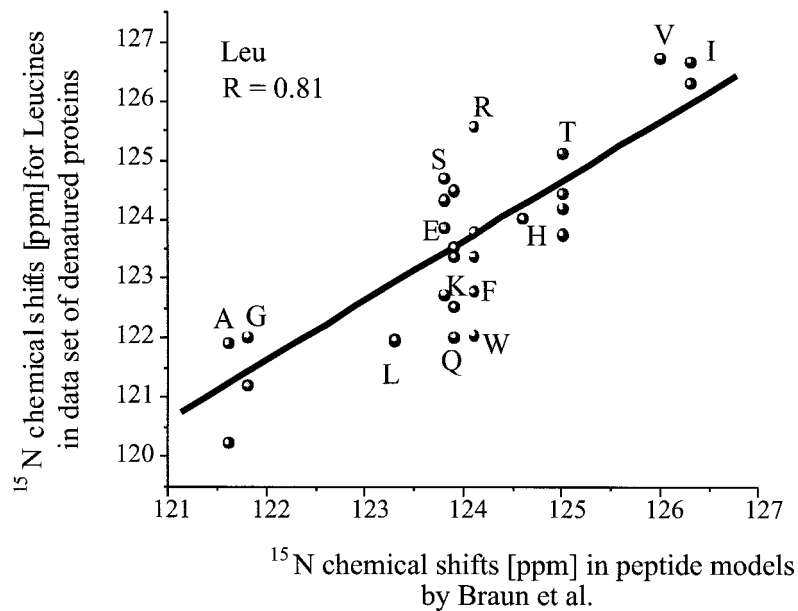


Figure 6. Comparison of experimental ^{15}N chemical shifts of 29 leucine residues with predicted values of Braun et al. (1994).

suggesting that both chemical shifts and coupling constants reflect on the tendency towards more extended structure. For Thr7/Leu8, for which this correlation is not observed, we propose a non-random structure in ubiquitin as evidenced by the differences in chemical shifts and by increased heteronuclear relaxation rates (Peti and Schwalbe, in preparation).

Conclusions

The chemical shift assignments for denatured ubiquitin have been reported. A good correlation between the mean chemical shifts in the database of native folded proteins and the random coil values of the three proteins investigated here has been found. Taking nearest neighbor effects into account, the averaged experimental chemical shifts can be correlated with the prediction given by Braun et al. (1994). Further analysis, however, also reveals pronounced effects that cannot be predicted only by analysis of dipeptide pairs in the unfolded states of proteins. This implies that, in order to describe with accuracy the averaged conformation in a denatured protein, additional nearest neighbor effects need to be taken into account.

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