A Central Functional Role for the 49-kDa Subunit within the Catalytic Core of Mitochondrial Complex I*

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We have analyzed a series of eleven mutations in the 49-kDa protein of mitochondrial complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica* to identify functionally important domains in this central subunit. The mutations were selected based on sequence homology with the large subunit of [NiFe] hydrogenases. None of the mutations affected assembly of complex I, all decreased or abolished ubiquinone reductase activity. Several mutants exhibited decreased sensitivities toward ubiquinone-analogous inhibitors. Unexpectedly, seven mutations affected the properties of iron-sulfur cluster N2, a prosthetic group not located in the 49-kDa subunit. In three of these mutants cluster N2 was not detectable by electron-paramagnetic resonance spectroscopy. The fact that the small subunit of hydrogenase is homologous to the PSST subunit of complex I proposed to host cluster N2 offers a straightforward explanation for the observed, unforeseen effects on this iron-sulfur cluster. We propose that the fold around the hydrogen reactive site of [NiFe] hydrogenase is conserved in the 49-kDa subunit of complex I and has become part of the inhibitor and ubiquinone binding region. We discuss that the fourth ligand of iron-sulfur cluster N2 missing in the PSST subunit may be provided by the 49-kDa subunit.

Oxidative phosphorylation is a universal process that converts most of the energy provided by foodstuffs into the general energy source ATP (1). During this process electrons pass through a series of membrane-bound multiprotein complexes that translocate protons across the membrane. The resulting proton motive force is used by ATP synthase to make ATP (2). Complex I (NADH:ubiquinone oxidoreductase) is the first of these electron transfer complexes and accounts for up to 40% of the pumped protons. In human mitochondria, complex I is regarded as a major source of deleterious reactive oxygen species. Hereditary and acquired defects affecting this multiprotein complex have been implicated in numerous degenerative diseases and seem to promote aging (3). The physiological and medical importance of complex I contrasts sharply with our still rather limited knowledge about the molecular structure and the catalytic mechanism of this multisubunit membrane protein with a total mass close to 1000 kDa (4).

The core of complex I is formed by 14 central subunits of which in animals and most fungi seven are encoded by the mitochondrial genome (5). The mitochondrial enzyme contains up to 29 additional "supernumerary" subunits. As bacterial enzymes comprise only the 14 central subunits, these must contain all functional modules required for electron and proton transfer. Except for the electron entry point that is formed by FMN and iron-sulfur center N3 in the 51-kDa subunit, the organization of these functional modules, the location and number of prosthetic groups, and the location of the ubiquinone substrate binding site are largely unknown. In particular, the function of nine of the 14 central subunits that carry none of the known prosthetic groups of complex I is obscure.

One of these proteins, the 49-kDa subunit, was found to be homologous to the large subunit of [NiFe] hydrogenases, a diverse family of enzymes found in archaebacteria and eubacteria (6). More recently, a "classical" mutation of *Rhodobacter capsulatus* selected for resistance to quinone analogous complex I inhibitors was found to affect this subunit (7). Prompted by these findings and guided by a more detailed analysis of the homology between the large [NiFe]-carrying subunit of hydrogenases and the 49-kDa subunit, we characterized site-directed mutations in the 49-kDa subunit using the yeast *Yarrowia lipolytica* as our newly developed model to study complex I (8).

We show that specific domains of the 49-kDa subunit are in fact of critical functional importance. Based on structural homology to water soluble, two-subunit [NiFe] hydrogenases, we propose that the protein fold around the [NiFe] active site of hydrogenases has been retained in the catalytic core of complex I (9).

EXPERIMENTAL PROCEDURES

Sequences were analyzed using the GCG-Husar server at the Deutsches Krebsforschungszentrum in Heidelberg. Structures were analyzed using Rasmol v2.7 and SwissProt PDB viewer v3.5 (10) software.

Y. lipolytica strains E129 (*Mat*A, *lys11*-23, *ura3*-302, *leu2*-270, *xpr2*-322) and E150 (*Mat*B, *his*-1, *ura3*-302, *leu2*-270, *xpr2*-322) were a kind gift from Prof. C. Gaillardin, Institut National de la Recherche Agronomique, Paris, France. The diploid strain GB1 was produced by mating E129 and E150. *Y. lipolytica* genetic techniques were carried out according to Barth and Gaillardin (11).

Heterozygous deletion strain NK2 was generated by double homologous recombination with a 5.6-kbp[1] *Sph*I fragment from the *NUCM* locus coding for the 49-kDa subunit in which codon positions 1–448 had been replaced with a 1.6-kbp polymerase chain reaction product containing the complete *URA3* gene in opposite orientation to the original *NUCM* open reading frame.

Random integration of a 4.7-kbp *Sal*I fragment containing a fusion of the *NUAM* mitochondrial targeting sequence and a truncated *NDH2* open reading frame to achieve expression of NDH2 on the matrix side of

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[1] The abbreviations used are: kbp, kilobase pair(s); DQA, 2-decyl-4-quinazolinyl amine; HAR, hexamineruthenium(III)-chloride; EPR, electron paramagnetic resonance; DBQ, 2-n-decylubiquinone.

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                          120                              222          456
Y. lipolytica complex I, 49 kDa   HRGTEKLIEYKTYMQALPYFDRLDYVSMMTN   GARLHAAYVRPGG   TMDLVFGEVDR
M. barkeri hydrogenase, EchE       HRGLETFINTKDFNQTTYVCERICGICSAHL   GNRVIHSISKVGG   TIDPCVSCTER
D. fructos. hydrogenase, large subunit FRGLEIILKGRDPRDAQHFTQRACGVCTYVH   GKNPHTQFTVVGG   AFDPCIACGVH
                          49                               224          539
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FIG. 1. **Partial alignment of sequences of the [NiFe] containing subunit of hydrogenase and the 49-kDa subunit of complex I.** The high sequence homology between the 49-kDa subunit of complex I and the *EchE* subunit of the membrane bound hydrogenase from *Methanosarcina barkeri* was used to identify the domains of the 49-kDa subunit of complex I that align with the nickel ligand carrying loops of hydrogenase and a third conserved region marked by a set of invariant glycines. The [NiFe]-ligating cysteins (marked in *yellow*) are in three cases replaced by residues that are highly conserved in all complex I 49-kDa sequences (marked in *red*). Residues found to be invariant in complex I and all [NiFe] hydrogenases are marked in *green*. Histidines conserved in only some subfamilies of [NiFe] hydrogenases and the 49-kDa subunit are marked in *blue*.

TABLE I
Characterization of 49 kDa mutants

Complex I content, activity, and inhibitor sensitivity in mitochondrial membranes of site-directed *Y. lipolytica* mutants in the 49-kDa subunit are compared to the plasmid complemented *nucm::URA3* (49 kDa) deletion strain (parental). Complex I content is given as specific NADH:HAR oxidoreductase activity in mitochondrial membranes ($100\% = 1\ \mu mol\ min^{-1}\ mg^{-1}$). NADH:DBQ oxidoreductase activity was normalized for complex I content ($100\% = 0.3\ \mu mol\ min^{-1}\ mg^{-1}$). EPR spectroscopy was performed using purified complex I (see Fig. 2). IC_{50}, concentration needed for 50% inhibition of the inhibitor-sensitive fraction of the NADH:DBQ oxidoreductase activity; *n.d.*, not determined.

Strain	Complex I content	Complex I activity	IC_{50}		N2 by EPR
			DQA	Rotenone	
	%		*nM*		
Parental	100	100	17	610	normal
Residues corresponding to nickel ligands					
D143E	170	23	38	630	unstable
D143C	110	30	30	580	normal
V460A	90	<5	n.d.	n.d.	not detectable
V460M	100	28	53	760	decreased
E463Q	60	20	34	780	decreased
Surrounding conserved residues					
R141A	130	17	21	570	not detectable
Y144H	70	<5	n.d.	n.d.	altered
D458A	93	28	520	5200	normal
Conserved histidines					
H120A	93	39	24	580	normal
H226A	100	25	18	770	not detectable
H384A	70	47	12	550	normal

the mitochondrial membrane[2] resulted in DQA resistance and permitted isolation of haploid *nucm::URA3* deletion strain NK2.1. Point mutants were generated by the QuikChange™ method (Stratagene). The polymerase chain reaction template was a 2780-kbp *NUCM* fragment subcloned into the *Nhe*I site of pINA240, which carries the *LEU2* marker.

Protein was determined according to a modified Lowry protocol (12). Unsealed mitochondrial membranes were prepared from mutant strains as described previously (13). All mutant strains expressed fully assembled complex I in essentially normal amounts as seen by blue native electrophoresis (14) of mitochondrial membranes (not shown) and specific HAR oxidoreductase activity (cf. Table I) that only depends on a functional 51-kDa subunit of complex I (15) and can be used to quantitate complex I. The observed variations of complex I content were within the range commonly observed between batches of the parental strain. Purification of complex I from mitochondrial membranes and measurements of steady state dNADH:DBQ activity and detergent- and inhibitor-insensitive NADH:HAR activity were performed as described previously (16).

RESULTS

Design of Site-directed Mutations in the 49-kDa Subunit— The sequence homology between the large subunit of water soluble [NiFe] hydrogenases (17) and the 49-kDa subunit of complex I is too poor to unambiguously align the sequences around the cysteines ligating the [NiFe] prosthetic group. However, the corresponding subunit from membrane-bound [NiFe] hydrogenases like the archeal *Ech* hydrogenase (18) exhibits significantly higher homology to the 49-kDa subunit of complex I than to the large subunit of its water soluble counterpart.

This allowed for a reliable alignment of the regions around the [NiFe]-ligating cysteines that are arranged in two pairs located near the N terminus and the C terminus of the sequences (Fig. 1). The four [NiFe] ligating cysteines in the hydrogenase sequence are substituted by two acidic residues, a small residue (serine, glycine, or alanine), and a valine in all known 49-kDa subunit sequences. Remarkably, the valine is the residue that was found to be mutated to methionine in an inhibitor-resistant mutant in *R. capsulatus* (7). An arginine two positions upstream of the N-terminal domain cysteine pair and an aspartate two positions upstream of the C-terminal cysteine pair were found to be invariant in all known [NiFe] hydrogenase *and* 49-kDa subunit sequences. This arginine is part of a RGXEX_{16}RXCGXCXXXH motif reported by Albracht (17) to be present in most sequences of the large [NiFe] hydrogenase subunit.

Based on this sequence analysis, we reasoned that the domains around the [NiFe] active site of hydrogenase, despite the loss of the prosthetic group, may have retained an important catalytic function in the 49-kDa subunit of complex I. To test this hypothesis we constructed and characterized a series of site-directed mutations in the homologous domains of the 49-kDa subunit of *Y. lipolytica* complex I.

The inhibitor-resistant mutant found in *R. capsulatus* (7) suggested a possible involvement of the 49-kDa subunit in forming the ubiquinone binding site of complex I. As histidine is known to be in a critical position in all ubiquinone binding sites that have so far been characterized in detail based on molecular structures (19, 20), we included the mutagenesis of three histidines that are strictly conserved in all complex I

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[2] S. Kerscher, A. Eschemann, P. Ahlers, and U. Brandt, manuscript in preparation.

FIG. 2. **EPR spectra of purified complex I from 49-kDa mutants**. The EPR spectra of complex I from *Y. lipolytica* reduced with NADH were recorded at a temperature of 12 K, a microwave frequency of 9.48 GHz, a microwave power of 2 mW, and a modulation amplitude of 0,8 millitesla. Under these conditions the spectra show contributions from clusters N1, N2, N3, and N4 (16). The *dashed line* indicates the position of the g_{xy} and the g_z signal of the iron-sulfur cluster N2. Some spectra show changes in both regions, but strong overlap by other clusters makes interpretation of changes in the g_{xy} region of the spectra difficult. *A*, nickel ligands (cf. Table I); *B*, surrounding conserved residues and conserved histidines (cf. Table I). In complex I from mutant Y144H the spectrum of cluster N2 is shifted and decreased. The g_z − signal was found at 2.054.
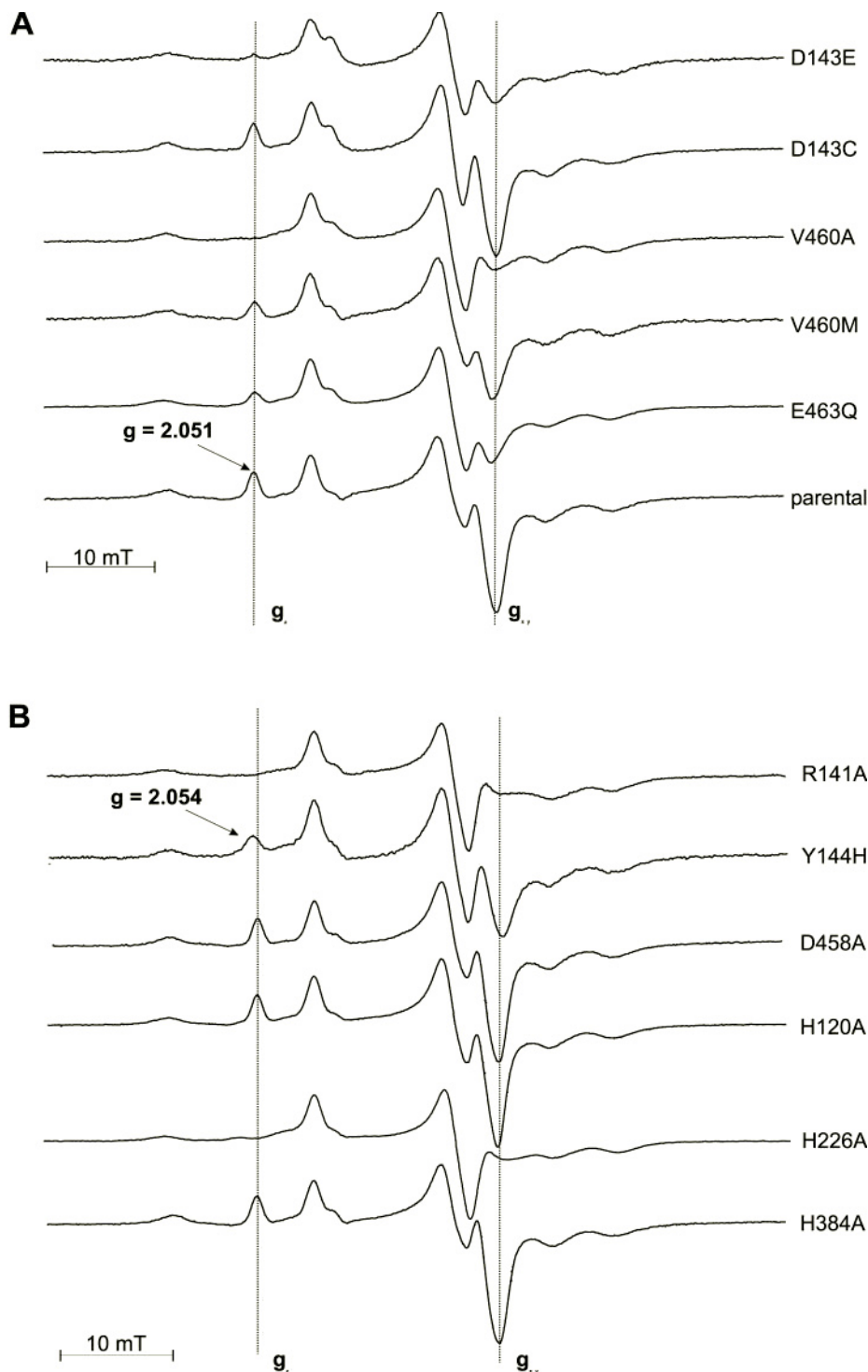
sequences. The first of these histidines (His-120) is located immediately before the RG*X*E motif. The second histidine (His-226) is in the middle of a pattern of glycines (G X_3HX_6GG) found invariably in complex I and all [NiFe] hydrogenases (Fig. 1). The third histidine (His-384) lies in a 30-amino acid-long insertion of the 49-kDa subunit that is missing in the sequences of the large subunit of [NiFe] hydrogenases.

Mutagenesis of Conserved Residues in the 49-kDa Subunit Corresponding to Hydrogenase [NiFe] Ligands—Mutation V460M, a reconstruction of the inhibitor-resistant mutant in *R. capsulatus*, resulted in a 70–80% loss of complex I activity and a 3-fold increase in the IC$_{50}$-value for the ubiquinone-analogous inhibitor DQA (Table I). EPR-spectra of the isolated mu-

tant enzyme revealed that the signal of iron-sulfur cluster N2 was significantly decreased. The signals of all other EPR-detectable iron-sulfur clusters (16) were unchanged (Fig. 2*A*). The more drastic mutation V460A led to complete loss of activity. With all other iron-sulfur clusters unaffected, cluster N2 was not detectable in the EPR spectra of isolated complex I from this mutant. Mutations D143C, D143E, and E463Q also led to a 70–80% decrease in complex I activity and a 2-fold resistance to the ubiquinone-analogous inhibitor DQA with normal or slightly increased IC$_{50}$ values for rotenone. Mutation D143E resulted in destabilized iron-sulfur cluster N2 that was lost from the complex upon purification if high concentrations of detergent were used. If in complex I preparations from this
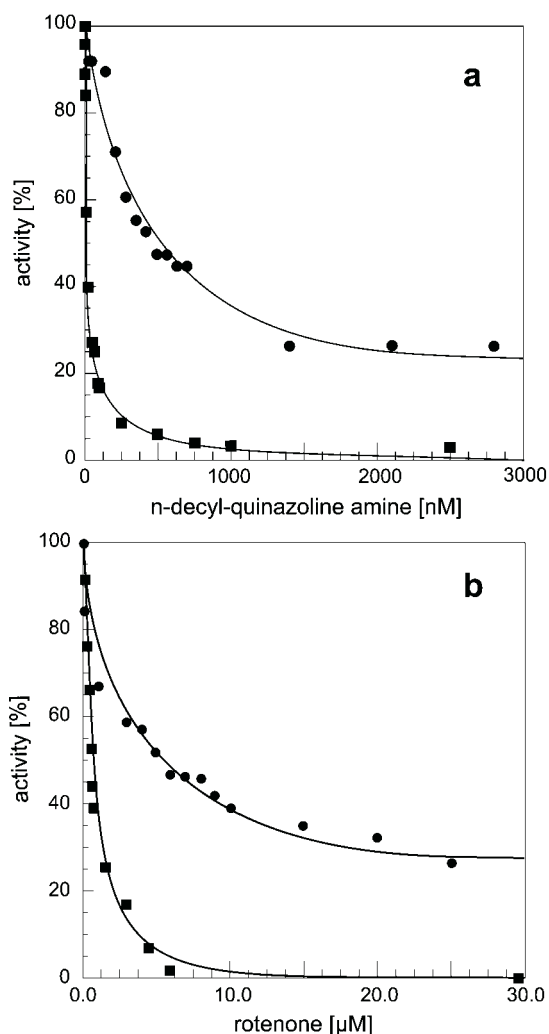
FIG. 3. **Inhibitor titrations of mitochondrial membranes from mutant D458A.** The diagrams show the inhibition of the dNADH:nonylubiquinone oxidoreductase activity of wild type (■) and mutant D458A (●) mitochondrial membranes by increasing concentrations of n-decyl-quinazoline-amine (*a*) and rotenone (*b*). Compared with wild type membranes a 30-fold higher concentration of n-decyl-quinazoline-amine and a 10-fold higher concentration of rotenone were required for 50% inhibition of inhibitor-sensitive complex I activity in D458A membranes (see Table I).

mutant iron-sulfur cluster N2 was detectable by EPR spectroscopy (Fig. 2), it exhibited an altered g_{xy} region. Although this finding is difficult to interpret as several clusters contribute to this part of the EPR spectrum, it was probably due to heterogeneity of the sample. Complex I from mutant E463Q consistently exhibited a significantly decreased electron paramagnetic resonance signal of cluster N2 (Fig. 2). Again, no effects on other EPR-detectable iron-sulfur clusters were observed.

Mutagenesis of Conserved Residues in the 49-kDa Subunit Corresponding to Residues in the Vicinity of the Cysteine Pairs of [NiFe] Hydrogenase— Mutation R141A resulted in more than 80% loss of complex I activity, but sensitivities toward DQA and rotenone were unaffected. In complex I from this mutant iron-sulfur cluster N2 was not detectable, and all other EPR-detectable iron-sulfur clusters were unchanged (Fig. 2). Mutation D458A also resulted in about 70% loss of activity, and EPR spectra of complex I from D458A were normal. With IC_{50} value increases of 30-fold for DQA and 10-fold for rotenone, this mutant exhibited by far the most pronounced inhibitor resistance (Table I; Fig. 3). Exchanging the fully conserved Tyr-144 with histidine resulted in complete loss of activity. With all

other iron-sulfur clusters unchanged, this mutation changed the EPR spectrum of iron-sulfur cluster N2 as characterized by a general broadening of the signal and significant increase of the g_z value (Fig. 2).

Mutagenesis of Conserved Histidines in the 49-kDa Subunit—Mutations H120A and H384A resulted in moderately decreased complex I activities (Table I) but did not affect the spectra of the EPR-detectable iron-sulfur clusters. Except for a slightly elevated IC_{50} value for DQA in H120A, no changes in inhibitor sensitivity were observed in these two mutants (Table I). Membranes from mutant H226A exhibited a 75% loss of activity and normal sensitivity toward DQA and rotenone. However, in the otherwise unchanged EPR spectra of complex I from this mutant, the signal of iron-sulfur cluster N2 was not detectable (Table I, Fig. 2).

DISCUSSION

The Pattern of Functional Changes Suggests Conservation of Functional Domains—Guided by sequence homology to the hydrogen reactive subunit of [NiFe] hydrogenases, we have characterized a series of 11 point mutations in the 49-kDa subunit of *Y. lipolytica* complex I. Complete loss of activity was caused by two mutations, V460A and Y144H. One mutation, D458A, increased the IC_{50} values for DQA and rotenone dramatically, while five other exchanges, D143E, D143C, V460M, E463Q, and H120A, caused moderate resistance to DQA. Although no effects on any other EPR-detectable iron-sulfur clusters were observed, isolated complex I from seven mutants exhibited changes in the EPR spectrum of iron-sulfur cluster N2; in V460M and E463Q cluster N2 was decreased, and in V460A, R141A, and H226A it was not detectable. In D143E this iron sulfur cluster was destabilized and in Y144H a broadening of the N2 EPR spectrum and a specific shift of the g_z value was observed. The fact that some of these mutations had pronounced, but specific effects on ubiquinone reductase activity, inhibitor sensitivity, or the EPR spectrum of iron-sulfur cluster N2 demonstrated that the 49-kDa subunit plays a central functional role in complex I. Some amino acid exchanges near or at the residues corresponding to the cysteine pairs ligating the [NiFe] center in hydrogenase had rather severe consequences on complex I function, whereas two of the histidine to alanine exchanges further away (H120A, H384A) had only very minor effects. This strongly supports the notion that domains around the hydrogen reactive [NiFe] site of hydrogenase have been retained as a functionally critical module in the 49-kDa subunit of complex I.

Structural Homology Explains Effects on Iron-Sulfur Cluster N2—The Fe_4S_4 cluster N2 is assumed to be the immediate electron donor to ubiquinone in complex I. By virtue of its pH-dependent redox potential (21) it has been suggested to be an important component of the proton-translocating machinery (22, 23). There has been conflicting evidence whether this redox group resides in the TYKY (24) or in the PSST subunit (25). In either case one would not have expected mutations in the 49-kDa subunit to have pronounced and specific effects on iron-sulfur cluster N2, as was observed in this study.

A straightforward explanation became obvious when we combined information from the molecular structure of the water soluble [NiFe] hydrogenase from *Desulfovibrio fructosovorans* (26) and the sequence homology to model the arrangement of the mutated residues in the 49-kDa subunit of *Y. lipolytica* complex I (Fig. 4, 9): In hydrogenase, the [NiFe] site is located at the interface between the large and small subunit adjacent to the proximal iron-sulfur cluster of the small subunit. The small subunit of hydrogenase is homologous to the PSST subunit of complex I (17). Three of the cysteines ligating the proximal iron-sulfur cluster are conserved in the PSST

FIG. 4. **Structural model for the interface between the 49-kDa and PSST subunits of complex I from *Y. lipolytica* based on the structure of water soluble [NiFe] hydrogenases.** *A,* side chains of critical residues in the large (*L*) and small (*S*) subunit of the water soluble [NiFe] hydrogenase from *D. fructosovorans* (26) (coordinates Protein Data Bank entry 1FRF). The [NiFe] center and the proximal iron-sulfur cluster are also shown; *B,* residues of the 49-kDa (no index) and PSST (index PSST) subunits of *Y. lipolytica* complex I lining up with the residues highlighted in *panel A* (see also Fig. 1) were placed into the structure of the *D. fructosovorans* [NiFe] hydrogenase.
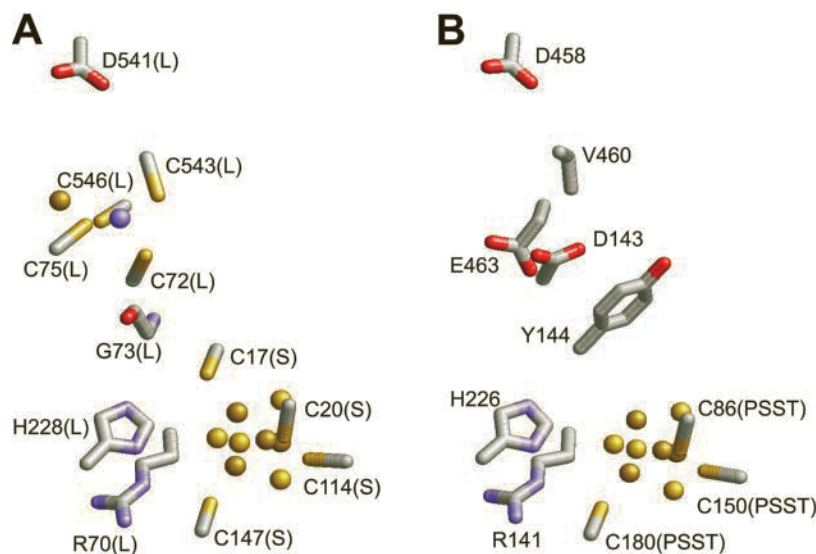
subunit (Cys-86, Cys-150, and Cys-180) and are therefore expected to ligate iron-sulfur cluster N2 of complex I (if this subunit in fact binds this redox group). It is immediately obvious from Fig. 4 that the effects on iron-sulfur cluster N2 by the mutations analyzed here correlate well with the proximity of the corresponding residues in water soluble hydrogenase to the proximal iron-sulfur cluster. It is most remarkable, that H226A, the only mutation studied here with a sequence position distant from the residues corresponding to the [NiFe]-ligating cysteines, exhibited such a pronounced effect on complex I abolishing the EPR signal of iron-sulfur cluster N2. In the hydrogenase structure the corresponding residue His-228 resides at the tip of a loop flanked by three invariant glycines (cf. Fig. 1). Thus in the light of the molecular structure of [NiFe] hydrogenase, analysis of our data strongly suggests that the proximal iron-sulfur cluster has in fact become cluster N2 in the PSST subunit of complex I and resides at the interface to the 49-kDa subunit. However, as one of the cysteines ligating the proximal iron-sulfur cluster is missing in PSST, it remains an unsolved question which residue may serve as the fourth ligand of cluster N2. Glu-89 had been proposed as a possible candidate (22) but could be excluded recently by site-directed mutagenesis (8). Based on the structural homology outlined above one is tempted to speculate that the fourth ligand of N2 actually resides on the 49-kDa subunit. Possible candidates are the invariant His-226, which already in hydrogenase makes a hydrogen bond to the proximal iron-sulfur cluster (26), and fully conserved Tyr-144, which corresponds to a conserved glycine in hydrogenase (cf. Fig. 1). Observations were made that may lend support to either option; mutant H226A in the 49-kDa subunit did not exhibit an EPR signal for iron-sulfur cluster N2 and Y144H led to a total loss of complex I activity along with a shift in the g_z value of iron-sulfur cluster N2.

It should be noted that although no iron-sulfur cluster N2 was detectable in complex I from mutants R141A and H226A, complex I in these two mutants exhibited significant residual ubiquinone reductase activity of around 20–30%. This seems difficult to explain if iron-sulfur cluster N2 is in fact an essential component of the electron pathway of complex I. However, as both residues are predicted to be in close vicinity to iron-sulfur cluster N2, the mutations may have shifted the midpoint potential to very negative values. This may prevent reduction of the iron-sulfur cluster by NADH necessary for detection by EPR spectroscopy. Although this is one possible explanation, further studies will be needed.

Inhibitor Resistance Suggests Involvement of the 49-kDa

Subunit in Ubiquinone Binding—Several of the mutations analyzed here exhibited resistance to ubiquinone-analogous inhibitors of complex I that was most pronounced for the residue most distant from the predicted position of iron-sulfur cluster N2. We conclude that this part of the 49-kDa subunit contributes to the inhibitor (and ubiquinone) binding pocket of complex I and has evolved from the [NiFe]-binding fold of hydrogenase. The very recent identification of two additional inhibitor resistant point mutations G409A and D412E (Gly-462 and Asp-465 in *Y. lipolytica*) in the 49-kDa subunit in *R. capsulatus* (27) by Dupuis and co-workers corroborates this view. Also the finding that the PSST subunit is labeled specifically by a derivative of pyridaben, another ubiquinone-analogous inhibitor of complex I (28) fits well with the structural model of Fig. 4. A recent report from the Nijmegen Center for Mitochondrial Disorders that two independent point mutations on the loop carrying the possible fourth ligand of cluster N2 cause hypertrophic cardiomyopathy and encephalomyopathy in humans (29), provides further support for this paradigm. It seems inevitable to conclude that the 49-kDa and the PSST subunit form a central part of the catalytic core of complex I and contribute to the ubiquinone binding domain.

Our results demonstrate a remarkable degree of structural conservation between a homologous subunit from two classes of quite functionally different enzymes on a molecular level. We conclude that not only the overall fold of the active site is the same in [NiFe] hydrogenase and complex I but also that both enzyme families share a number of functionally important residues that are invariant in all known sequences. This is of particular significance as a central part of the catalytic core of complex I itself seems to have evolved from a hydrogen reactive [NiFe] center to a site reducing the much larger ubiquinone that may comprise the proton pumping module as well. We suggest that the residues invariant between the large subunit of hydrogenase and the 49-kDa subunit of complex I may be involved in electron and proton pathways serving the active site as these functions have to be present in both enzymes.

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