Accessory subunits of complex I from *Yarrowia lipolytica*

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Albina Abdrakhmanova

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Gutachter : Prof. B. Ludwig
Prof. U. Brandt

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1 INTRODUCTION

1.1 Mitochondrial Respiratory Chain

The respiratory chain is a part of the process of oxidative phosphorylation. The components of the respiratory chain catalyse the transfer of electrons from reducing agents like NADH or $FADH_2$ onto molecular oxygen. The major part of the free energy released by electron transfer is used to generate a proton gradient and finally to produce ATP.

The respiratory chain includes three protein complexes embedded in the internal mitochondrial membrane:

- NADH:ubiquinone oxidoreductase (complex I)
- Ubiquinol:cytochrome *c*-oxidoreductase (complex III)
- Cytochrome *c*-oxidase (complex IV)

and two mobile electron carriers:

- Ubiquinone (UQ)
- Cytochrome *c*

Succinate:ubiquinone oxidoreductase, actually belonging to the citric acid cycle, can also be considered as complex II of the respiratory chain. ATP syntase is refered to as complex V though it does not participate in electron transfer.

The complexes of the respiratory chain are made from more than 80 polypeptides and contain different redox active cofactors. Among them are iron-sulfur centers (in I, II and III), cytochromes (in II, III and IV) and flavins (FMN or FAD in complexes I and II). Except for succinate:ubiquinone oxidoreductase all these complexes pump protons from the matrix space into the intermembrane space at the same time as they transfer reducing equivalents from one carrier to the next. Proton pumping creates substantial pH and electrical gradients across the inner mitochondrial membrane. The pumped protons re-enter the matrix space via the F_1F_O-ATPase, driving the synthesis of ATP as they return (Mitchell, 1961).

The knowledge of three-dimensional structures, catalytic and regulatory properties of the respiratory chain complexes are of medical significance, because they are involved in the development of human pathologies (Smeitink and Van den Heuvel, 1999). The detailed crystal structures for complex II (Lancaster *et al*., 1999; Yankovskaya *et al*., 2003), complex III (Iwata *et al.*, 1998; Hunte *et al*., 2000) and complex IV (Ostermeier *et al*., 1997) have already been obtained. While information about complex I is growing rapidly (Walker, 1992; Vinogradov, 1993; Brandt *et al*., 2003), no high resolution structure is available yet.

1.2 Mitochondrial Complex I

The key enzyme at the beginning of the mitochondrial electron transport chain, NADH:ubiquinone oxidoreductase (complex I), catalyses the transfer of two electrons from NADH to ubiquinone, coupled to the translocation of four protons across the membrane (Wikström, 1984). Almost all organisms possess complex I, however, in the yeasts *Saccharomyces* and *Kluyveromyces* complex I is absent (Balcavag and Mattoon, 1968; Büschges *et al*., 1994) and alternative mitochondrial NADH:Q oxidoreductases transfer the electrons from NADH to ubiquinone (Kerscher, 2000). It should be noted here that several organisms like plants and archaebacteria contain both complex I and alternative dehydrogenase (Rasmusson *et al*., 1999; Gomes *et al*., 2001).

As revealed by electron microscopy studies the enzyme has an L-shaped structure with two arms: a membrane-embedded part and a part protruding into the matrix called the peripheral arm (Grigorieff, 1998; Sazanov and Walker, 2000; Leonard *et al*., 1987; Djafarzadeh *et al*., 2000). The hydrophobic part possesses proton-pumping activity, whereas the hydrophilic part exhibit dehydrogenase activity. Presumably, the hydrophilic arm is also responsible for ubiqinone reduction (Zickermann *et al*., 2003).

Mitochondrial complex I has a higher complexity and molecular mass than the bacterial enzyme. Bacterial complex I is composed of only 14 subunits. They represent the minimal form of the enzyme required to ensure the coupling of electron transfer with proton pumping. All of them are conserved in eucaryotic complex I (Yagi *et al*., 1998). Seven of these 14 "central" subunits, ND1-6 and ND4L, are highly hydrophobic and encoded by mitochondrial DNA, the other seven (75-, 51-, 49-, 30-, 24-kDa, TYKY, PSST) are nuclear-encoded, hydrophilic and contain all know redox prostetic groups. Complex I from eucaryotes contains additional "accessory" subunits. The well-characterized enzyme from bovine heart with a molecular mass of over 980 kDa consists of 46 subunits (Hirst *et al*., 2003). In the green alga *Chlamydomonas reinhardtii*, 42 subunits were identified, which form a complex with a total molecular mass of about 970 kDa (Cardol *et al*., 2004). The *Neurospora crassa* complex I (1.1 MDa) contains 39 subunits (Marques *et al*., 2005). The possible role of several accessory subunits will be discussed below.

Complex I includes a number of redox active cofactors. FMN, non-covalently bound to the 51 kDa subunit, is the entry point for electron transfer from NADH to iron-sulfur clusters (Fecke *et al*., 1994; Sled *et al*., 1994). Results from different studies suggest the presence of eight to nine iron-sulfur clusters in complex I. Only six iron-sulphur clusters are visible in EPR

spectra of bovine heart complex I and are designated N1a, N1b, N2, N3, N4, N5 (Ohnishi, 1998), while in *Escherichia coli* nine clusters were identified (additional clusters N6a, N6b and N7, Rasmussen *et al*., 2001; Nakamaru-Ogiso *et al*., 2005), five clusters in the yeast *Yarrowia lipolytica* (N1-N5, Djafarzadeh *et al*., 2000; Kerscher *et al*., 2001) and only four in *Neurospora crassa* (N1-N4; Wang *et al*., 1991).

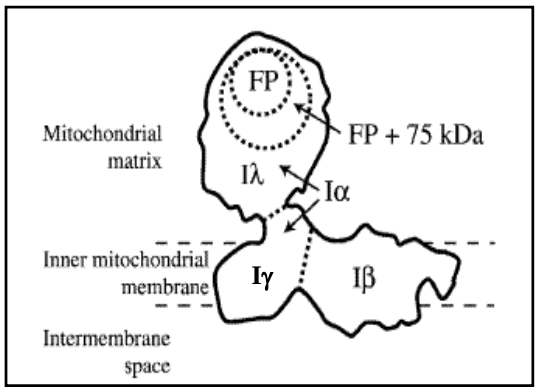

Figure 1.1 Structural relationships amongst the subcomplexes of complex I.

Complex I from bovine heart was fragmented into subcomplexes. Subcomplex Iα represents the extrinsic part (Iλ) of enzyme plus membrane part (Iγ) and Iβ is another part of the membrane arm. FP is the flavoprotein of bovine complex I. From Hirst *et al*., *Biochim. Biophys. Acta* (2003), **1604**, 135-150.

Purified complex I from bovine heart can be fragmented into various subcomplexes using chaotropic detergents like perchlorate (Fig. 1.1). The soluble flavoprotein subcomplex (FP) consists of the 51-, 24- and 10-kDa subunits and contains one molecule of FMN and six atoms of iron (Galante and Hatefi, 1979). The iron-protein fragment (IP) is also soluble and made of further "central" subunits (75-, 49-, 30-kDa, TYKY, PSST) (Masui *et al*., 1991; Walker, 1992). The precipitate is know as HP-fragment (hydrophobic protein, Walker, 1992). By other means, i.e. using sucrose gradient centrifugation (Finel *et al*., 1994) and ion exchange chromatography in the presence of non-denaturating detergents (Finel *et al*., 1992; Sazanov *et al*., 2000) complex I from bovine heart can be split into subcomplexes Iα and Iβ (Figure 1.1). Subcomplex Iα represents the extrinsic part (subcomplex Iλ) plus part of the membrane arm (Iγ) of the enzyme. Subcomplex Iβ is another part of the membrane domain. Subcomplex Iα contains all EPR-detectable iron-sulfur clusters and can transfer electrons from NADH to ferricyanide or ubiquinone. The subunit composition of the subcomplexes is summarized in Table 1.1.

B. taurus subunits	Molecular weight [kDa]	Modifications/ Cofactor	Coding	Subcomplex	*E. coli* subunits
75 kDa	77.0	Δ1-23/[2Fe-2S],[4Fe-4S]	Nuclear	Iλ	NuoG
51 kDa	48.5	Δ 1-20/[4Fe-4S], FMN	-//-	Iλ	NuoF
49 kDa	49.2	Δ 1-33/[4Fe-4S]	-//-	Iλ	NuoCD
30 kDa	26.4	Δ 1-38	-//-	Iλ	NuoCD
24 kDa	23.8	Δ 1-32/[2Fe-2S]	-//-	Iλ	NuoE
PSST	20.1	Δ 1-37/[4Fe-4S]	-//-	Iλ	NuoB
TYKY	20.2	Δ 1-36/2x[4Fe-4S]	-//-	Iλ	NuoI
ND1	36	-	mtDNA	Iγ	NuoH
ND2	39	-	-//-	Iγ	NuoN
ND3	13	-	-//-	Iγ	NuoA
ND4	52	-	-//-	Iβ	NuoM
ND5	67	-	-//-	Iβ	NuoL
ND6	19	-	-//-	Iγ	NuoJ
ND4L	11	-	-//-	Iγ	NuoK
42 kDa	36.7	Δ1-23	Nuclear	Iγ	
39 kDa	39.1	NADPH	-//-	Iγ	
18 kDa (AQDQ)	15.3	Phosphorylated?	-//-	Iλ	
15 kDa	12.5	-Met	-//-	Iγ	
13 kDa	10.5	Δ1-28	-//-	Iλ	
10 kDa	8.4	Δ1-34	-//-	Iλ	
AGGG	8.5	Δ1-36	-//-	Iβ	
ASHI	18.7	Δ1-28	-//-	Iβ	
ESSS	14.5	Phosphorylated?	-//-	Iβ	
KFYI	5.8	Δ1-27	-//-	Iγ	
MLRQ	9.3	-	-//-	Iγ and Iβ	
MNLL	7.0	-Met	-//-	Iβ	
MWFE	8.1	Phosphorylated?	-//-	Iγ	
PDSW	20.8	-Met	-//-	Iβ	
PGIV	20.0	-Met	-//-	Iγ	
SDAP	10.7	Pantetheine-4V-phosphat	-//-	Iγ and Iβ	
SGDH	16.7	Δ1-46	-//-	Iβ	
B22	21.7	-Met+Ac	-//-	Iβ	
B18	16.5	-Met+Myr	-//-	Iβ	
B17.2	17.1	+Ac	-//-	Iλ	
B17	15.4	-Met+Ac	-//-	Iβ	
B16.6	16.6	-Met+Ac	-//-	Iλ	
B15	15.1	-Met+Ac	-//-	Iγ	
B14.7	14.8	-Met+Ac	-//-	Iλ	
B14.5a	12.6	-Met+Ac	-//-	Iλ	
B14.5b	14.1	+Ac (partial)	-//-	Iβ	
B14	15.0	-Met+Ac	-//-	Iγ	
B13	13.2	-Met+Ac	-//-	Iλ	
B12	11.1	-Met+Ac (partial)	-//-	Iβ	
B9	9.3	-Met+Ac	-//-	Iα	
B8	11.0	-Met+Ac	-//-	Iλ	

Table 1.1 Central and accessory subunits of complexI from bovine heart (Bos taurus).

Abbreviations: - Met: N-terminal methionine is removed post-translationally; + Ac: N-terminal residue is acetylated; + Myr: N-terminal residue is myristoylated. Δa – b: known mitochondrial import sequence (residues a to b) are removed. From Hirst *et al*., *Biochim. Biophys. Acta* (2003), **1604**, 135-150.

Attempts have been made to assign the iron-sulfur clusters to subunits of complex I by determination of EPR-spectra of the subcomplexes. Binuclear cluster N1a and tetranuclear cluster N3 were found in the FP-fragment of bovine complex I (Ragan *et al*., 1982; Ohnishi *et al*., 1985). It was suggested that cluster N1a is associated with the 24 kDa subunit (Yano *et al*., 1994). However it was not possible to detect by EPR-spectroscopy this iron-sulfur cluster in *Y. lipolytica* and *N. crassa* (Brandt *et al*., 2003). Cluster N3 is located in the 51 kDa subunit (Fecke *et al*., 1994; Yano *et al*., 1996). As mentioned above this subunit also binds one molecule of FMN. Binuclear cluster N1b and tetranuclear cluster N7 could be bound in the 75 kDa subunit (Yano *et al*., 1995; Uhlmann and Friedrich, 2005), which also has a binding motif for tetranuclear clusters N4 (Yano *et al*., 1995) and probably N5 (Sled *et al*., 1993; Ohnishi, 1998). The binding site for cluster N2 was the subject of a long-lasting controversy. Two subunits were considered as possible candidates for cluster N2 binding, namely PSST (Rassmussen *et al*., 2001; Duarte *et al*., 2002) and TYKY (Albracht and Hedderich, 2000). Both subunits contain binding motifs for a tetranuclear cluster with highly conserved cysteins. However, site-directed mutagenesis in *Y. lipolytica*, *N. crassa* and *E. coli* provided evidence suggesting that cluster N2 is bound to the PSST subunit (Ahlers *et al*., 2000; Duarte *et al*., 2002; Flemming *et al*., 2003; Garofano *et al*., 2003) and resides at the interface between the PSST and the 49 kDa subunits (Kashani-Poor *et al*., 2001b; Kerscher *et al*., 2001c). It is supposed that cluster N2 is the immediate electron donor for ubiquinone, because of exhibits the most positive redox midpoint potential ($E_{m,7}$ = -150 mV) and interacts with semiquinone radicals detectable by EPR-spectroscopy (Ohnishi, 1998; Yano and Ohnishi, 2001).

It has been proposed that complex I was build from three different structural modules during evolution. The NADH dehydrogenase module (N-module) is part of the peripheral arm. The proton pump module (P-module) is probably located in the membrane arm (Zickermann *et al*., 1998) while the quinone reduction module (Q-module) is possibly located in the interface between the PSST and 49 kDa subunits (Dupuis *et al*., 1998; Kashani-Poor *et al.*, 2001b). Sequence alignment shows, that the electron input N-module of complex I, including the 75-, 51- and 24-kDa subunits is related to the NAD^+- reducing hydrogenase from *Alcaligenes eutrophus* (Pilkington *et al*., 1991). The 49 kDa and PSST subunits in the Q-module are homologues to the large and small subunits of water-soluble [NiFe] hydrogenases, respectively. Membrane bound type-3 hydrogenases encoded by the *hyc* operon in *E. coli* or the *ech* operon in *Methanosarcina barkeri* contain proteins that also show homology to the 49-kDa and PSST subunits as well as to the 30-kDa, TYKY, ND1 and ND5 subunits. The

ND2, ND4, and ND5 subunits of complex I show weak homology to each other and also to Na^+/H^+ antiporters of the type encoded by the *mrp* operon in *Bacillus subtilis* and the corresponding *mnh* operon in *Staphylococcus aureus* (Steuber, 2001; Mathiesen and Hägerhäll, 2002). In addition to the homologues of complex I subunits found in type-3 hydrogenases, type-4 hydrogenase from *E. coli* encoded by *hyf* operon (Andrews *et al.*, 1997) includes two more proteins of the Na^+/H^+ or K^+/H^+ antiporter type, that belong to the ND2/ND4/ND5 superfamily, and a hydrophobic protein, that shows homology to the NDL4 subunit of complex I (Finel, 1998).

The possible mechanisms of electron transport and proton translocation across the membrane were discussed in many reviews (Friedrich, 2001; Vinogradov, 2001; Brandt *et al.*, 2003). FMN oxidises NADH by uptake of two electrons that are then transferred one by one via iron-sulfur clusters to ubiquinone. Recent information about the structural organization of complex I and inhibitor binding support the hypothesis that the reduction of ubiquinone induces specific conformational changes that are then transmitted to the hydrophobic subunits in the membrane, acting as proton pumps (Brandt *et al.*, 2003).

1.2.1 Accessory subunits of complex I

In addition to the 14 “central” subunits, complex I from eucaryotic organisms contains a large number of “accessory” subunits: 32 subunits in bovine heart mitochondrial complex I (Hirst *et al.*, 2003), 21-23 subunits in the fungal enzyme (Videira *et al.*, 2002; Abdrakhmanova *et al.*, 2004) and about 16-28 subunits in photosynthetic organisms like *Vicia faba L* (Leterme and Boutry, 1993), *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* (Cardol *et al.*, 2004) were identified. Only some accessory subunits have been analysed in recent years, the function of most accessory subunits remains unknown.

The 39 kDa subunit has attracted special attention and will be described in more details below.

The subunit with the N-terminal sequence AQDQ, has been proposed to be the so-called “18 kDa subunit” that can become phosphorylated (Papa *et al.*, 1996; Papa, 2002). Phosphorylation was described to be cAMP-dependent and to lead to activation of complex I and mitochondrial respiration (Papa *et al.*, 2001). Several mutations in the nuclear gene encoding the AQDQ subunit were found in human patients. A 5bp-duplication at position 466-470 of

the coding sequence destroys a phosphorylation motif in this subunit. The mutation was lethal and the patient died of cardiorespiratory failure at the age of 16 month (Van den Heuvel *et al*., 1998). Another two mutations, a single nucleotide deletion in the middle part of the coding sequence leading to a truncated polypeptide and a nonsense mutation causing premature termination after only 14 amino acids of the putative mitochondrial targeting sequence were also lethal. All three mutations were associated with defective assembly of functional complex I (Petruzzella *et al*., 2001; Scacco *et al*., 2003).
However, Walker's group has shown that the phosphorylation site is in subunit ESSS with similar molecular weight and not in subunit AQDQ (Chen *et al*., 2004). This result seems to be convincing: by using Edman sequencing and mass spectrometric analysis of the radiolabeled band isolated by SDS-polyacrylamide gel electrophoresis, the same result was reproduced. In mammalian mitochondrial complex I the ESSS protein is essential for assembly of an active enzyme (Potluri *et al*., 2004). Thus, the question which one of both subunits becomes phosphorylated remains controversial.

Subunit B14.7 (Carroll *et al*., 2002) from bovine complex I exhibits homology to subunit 21.3b from *N. crassa*. It is related to the proteins, Tim22 of TIM22 complex, Tim17 and Tim23 of TIM23 complex, which mediate the transport of proteins from the cytosol across and into the inner mitochondral membrane (Rehling *et al*., 2004).

The B16.6 subunit is identical to the mammalian GRIM-19 protein (Fearnley *et al*., 2001) which is involved in the interferon- and retinoic acid-induced pathway of cell death (Angell *et al*., 2000). GRIM-19 may play also a crucial role in mitochondrial complex I assembly. By creating a GRIM-19 gene knockout in mice it was shown that lack of GRIM-19 destroys the assembly and electron transfer activity of complex I and also influences the other complexes in the mitochondrial respiratory chain (Huang *et al*., 2004). The dual role of GRIM-19/B16.6 may provide yet another paradigm that supports the notion, that mitochondria are directly involved in apoptotic cell death (Newmeyer and Ferguson-Miller, 2003).

The SDAP subunit of complex I belongs to the acyl carrier proteins (ACP) that contain a phosphopantethein group as cofactor that is covalently attached to a conserved serine residue (Sackmann *et al*., 1991; Runswick *et al*., 1991). It has been suggested that this subunit is part of a mitochondrial fatty acid synthase involved in the production of lipoic acid (Jordan and Cronan, 1997). The role of the SDAP protein as a subunit of complex I is unclear. In contrast

to other hydrophilic subunits, the absence of this matrix arm subunit in the *N. crassa* mutant does not only prevent the formation of the peripheral arm but also affects the assembly of the membrane arm (Schneider *et al*., 1995). Therefore, the SDAP subunit could participate in a spezialized synthetic pathway delivering a product which is needed for assembly of the membrane arm (Schneider *et al*., 1997).

Several mutants of the MWFE subunit in hamster cell lines were generated. It is a small (10 kDa) nuclear-encoded subunit, which can be a target for a cAMP-dependent kinase (Chen *et al.*, 2004). Yadava and coworkers (Yadava *et al*., 2004) have shown that the absence of this protein interferes with complex I assembly. However a number of subunits of the peripheral arm were attached to the inner membrane. The expression of a doxycycline-inducible HA-epitope-tagged MWFE protein could restore enzyme assembly, stability and activity after 48 hours, whereas the protein reached steady state levels after 24 hours. From these observations it was concluded that the MWFE subunit is required at an intermediate step for the complete assembly of complex I.

In the PGIV bovine protein that is homologous to the 20.8 kDa subunit from *N. crassa*, nine cysteine residues were suggested to be involved in disulfide bridges or to provide ligands for binding iron-sulfur cluster (Videira, 1998). In the *Chlamydomonas reinhardtii* homologues of bovine PDSW, PFFD and B18 subunits, two to four conserved cysteines were identified (Cardol *et al.*, 2004). The cysteine residues in the PFFD and B18 subunits can be aligned with four cysteines in complex IV chaperones COX17 and COX19 that are involved in copper import into mitochondria (Punter and Glerum, 2003). It was hypothesized that subunits PGIV, PDSW, PFFD and B18 play a role in iron-sulfur cluster binding or in metal transport into the mitochondria (Cardol *et al.*, 2004).

As was mentioned above, the function of most “accessory” subunits is still unknown. The major portion of these proteins are very likely not required for electron transport in complex I, but perform other essential functions needed for enzyme assembly and stability.

1.3 Biogenesis of complex I

Knowledge of the biogenesis of complex I could provide useful information about possible functions of accessory subunits. Complex I assembly has been the subject of several studies

carried out on eucaryotic organisms like mammals, the filamentous fungus *N. crassa* and the green alga *C. reinhardtii* (Duarte and Videira, 2000; Cardol *et al*., 2002; Ugalde *et al*., 2004).

In *N. crassa* it has been shown that the peripheral arm and the membrane arm are formed independently (Tuschen *et al*., 1990). Mutants lacking one of the nuclear encoded subunits of the peripheral arm were unable to assemble this arm but accumulated the membrane part of the enzyme. As mentioned above, the absence of only the acyl carrier protein of complex I affected assembly of the membrane arm (Schneider *et al.*, 1995). Similarly, the absence of mitochondrially encoded subunits of the membrane part did not prevent assembly of the hydrophilic arm. Both parts of the enzyme were shown to be assembly intermediates. The hydrophobic arm is formed by association of two assembly intermediates which contains also two extra proteins, called CIA30 and CIA84. They are no components of mature complex I, but are essential for assembly of the membrane arm in *N. crassa* (Küffner *et al*., 1998). In human cells only a homolog for CIA30 could be found (Janssen *et al*., 2002), but also another possible complex I chaperone, namely prohibitin, was identified (Bourges *et al*., 2004).

All proteins that in *N. crassa* are essential for association of the hydrophilic part have a counterpart in bacterial complex I and show homology to subunits of [NiFe] hydrogenases. They are the 49-, 30.4-, 21.3c- (TYKY) and 19.3-kDa (PSST) subunits (Videira and Duarte, 2001). For assembly of the membrane arm, subunits ND1-ND4, ND4L and ND6 are required and, interestingly, ND5 is not (Videira *et al.*, 2001). Mutations in the 51-, 40 -, 24-, 21.3a- and 21- kDa (AQDQ homologue) subunits lead to fully assembled, but inactive enzyme in *N. crassa* (Nehls *et al*., 1992; Ferreirinha *et al*., 1999; Schulte *et al*., 1999; Videira *et al.*, 2001).

Two different complex I assembly models were described in human cells which are in conflict to each other. According to the modular model proposed by Nijtmans' group (Ugalde *et al.*, 2004) the assembly of human complex I resembles the enzyme assembly in *N.crassa.* In contrast, the model proposed by the Shoubridge group suggests, that the peripheral and membrane arms are not assembled separately (Antonicka *et al*., 2003). These authors have observed in complex I-deficient patient cells several subcomplexes that are supposed to be intermediates of complex I assembly. One of the subcomplexes contained subunit ND1 and peripheral arm subunits, namely the 30-, 39- and 49-kDa subunits. Based on this finding they concluded that the peripheral and the membrane arms of human complex I are not assembled separately. Thus, these two models pose more questions than they give answers about complex I assembly in mammals.

Several findings however suggest distinct assembly pathways of complex I in different organisms. For example in *N. crassa,* the AQDQ homologue is not essential for complex I

assembly (Nehls *et al.*, 1992) and rotenone-sensitive activity (Ferreirinha *et al.*, 1999), whereas in human cells, mutations in the gene encoding this protein (*NDUFS4*) did affect complex I assembly (Scacco *et al.*, 2003). Site-directed mutagenesis of a highly conserved cysteine at position 85 in the 19.3-kDa (PSST) protein in *N. crassa*, proposed as a possible fourth ligand for N2 cluster, has resulted in a dramatic decrease of complex I activity (Duarte *et al.*, 2002). However, with similar mutations generated in *Y. lipolytica* no assembled complex I could be identified (Garofano, 2004, PhD thesis). It may be speculated that the biogenesis of complex I in different species occurs in different ways and may depend on its specific subunit composition.

1.4 The 39 kDa subunit of complex I

The nuclear-encoded 39 kDa subunit contains a nucleotide-binding motif near its N-terminus and can bind NADPH specifically (Yamaguchi *et al*., 2000). Localization of this subunit in subcomplex Iγ of complex I (Carroll *et al*., 2003) revealed that the 39 kDa subunit is likely to be located in the membrane part of the enzyme (Table 1.1).

Homology searches with the 39 kDa subunit sequence revealed that this protein is related to the SDR (short-chain dehydrogenases/reductases)-enzyme family. The members of this heterogeneous family participate in different redox and isomerization reactions using substrates ranging from alcohols, sugars, steroids and aromatic compounds to xenobiotics (Persson *et al*., 2003). Known members include hydroxysteroid dehydrogenases, cholesterol dehydrogenases, alcohol dehydrogenases, nucleotide sugar epimerases and isoflavone reductases (Kallberg *et al*., 2002a; Kallberg *et al*., 2002b).

Common to all enzymes is the use of NAD(H) or NADP(H) as cofactor. The NAD(P)H binding domain typically consists of a βαβ Rossman fold and contains three highly conserved glycines as part of the nucleotid-binding motif GX(X)GXXG or GXX(X)GXG. The pyrophosphate moiety of the cofactor makes close contact with the middle glycine, which is located at the turn following first the β-strand (Lesk, 1995). Determinant for selective interaction of the protein with NADH or NADPH is an amino acid at the end of the second β-strand. NADH-preferring enzymes have an acidic residue at this position that forms hydrogen bonds to the 2'- and 3'-hydroxyl groups of the adenine ribose moiety (Kallberg *et al.*, 2002a). NADPH-binding enzymes have either one or two basic residues: the first basic amino acid is located at the end of the second β-strand and the second basic residue is in the Gly-motif. It

was proposed that these basic residues bind the 2'-phosphate group of NADPH and assist in the neutralization of its negative charge (Kallberg *et al.*, 2002a).

As evident from alignments, the amino acid composition of the 39 kDa subunit is highly conserved between different species (see appendix). The three glycines forming the nucleotide-binding motif GXXGXXG and the conserved arginine at the end of the second β-strand suggest that this protein is able to bind a molecule of NADPH (Figure 1.3).

The function of the 39 kDa protein is poorly understood, but most probably, it is not involved in respiratory electron transfer. The absence of this subunit in *N. crassa* leads to inactivation of the electron transfer of assembled complex I. The isolated enzyme showed wild type NADH:ferricyanide activity, but was unable to transfer electrons from NADH to quinones.

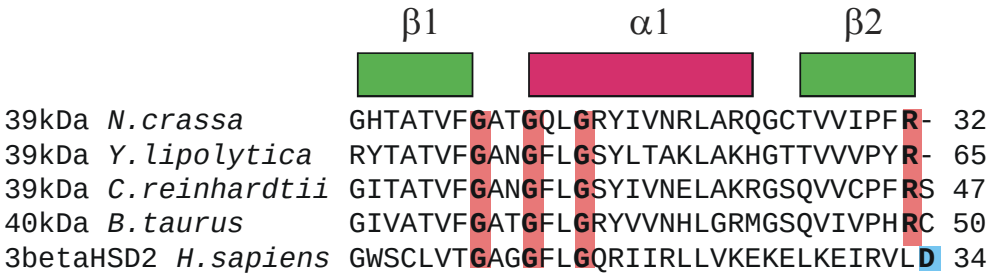

Figure 1.2 Partial alignment of sequences of a member of the SDRs enzyme family and several 39-kDa subunits of complex I.

39-kDa N.c = 39-kDa subunit, *Neurospora crassa* complex I; 39-kDa *Y.l.* = 39-kDa subunit, *Yarrowia lipolytica* complex I; 39-kDa C.r = 38 kDa subunit, *Chlamydomonas reihardtii* complex I*;* 40-kDa B.T. = 39 kDa, *Bos taurus*; 3betaHSD1 H.S = 3 beta-hydroxysteroid-dehydrogenase 2, *Homo sapiens* .

In addition, all iron-sulfur clusters that can be reduced by NADH were detected. Only the signal from N2 cluster showed some broadening in the deletion mutant. However, the signal assigne to a postulated new redox group named "X" (Schulte *et al.*, 1999; Friedrich *et al.*, 2000) was not found in the mutant. This new redox group was proposed on the basis of UV-visible spectroscopy. Its redox difference absorbance spectrum shows a negative peak at 430 nm, an isosbestic region at 360 nm and a positive peak at 300 nm with a difference absorption coefficient of ca 4 mM^{-1} at 430 nm. The midpoint redox potential is supposed to be more positive than -150 mV (Friedrich *et al.*, 1998; Schulte, 2001) and would fill the gap between cluster N2 and ubiquinone.

It was speculated that the 39 kDa subunit may be involved in the biosynthesis of this postulated redox group. The absence of this subunit would result in an inactive or instable

form of the redox group, thus causing a block in electron transfer activity (Schulte *et al.*, 1999).

However, at present, there is little experimental evidence to support this proposal. The existence of this redox group is hypothetical and no new convincing data were presented in recent years.

1.5 Yarrowia lipolytica as a Model Organism

To investigate complex I a model organism is needed. It should have a stable enzyme which is easy to purify and should be amenable to straightforward genetic manipulation. In this case *E. coli* is not an ideal model system, because its purified complex I is rather unstable and tends to dissociate during purification (Herter *et al*., 1997). Moreover, all structural genes for complex I subunits in *E.coli* are expressed and controlled by a single operon, what makes mutagenesis a non-trivial task. Stable complex I can be purified of mitochondria from bovine heart and the filamentous fungus *N. crassa*, but genetic manipulation in these organisms is either impossible or rather tedious. It should be noted here that the widely used lower eucaryotic model organism *Saccharomyces cerevisiae* does not contain complex I (Balcavag *et al*., 1968).

In contrast to the species mentioned before, the obligate aerobic yeast *Yarrowia lipolytica* provides all essential prerequisites for efficient investigation of complex I.

Yarrowia lipolytica strains are found in dairy products such as cheese and yoghurts. It is a non pathogenic organism with an optimum growth temperature of about 28°C. *Y. lipolytica* can use a variety of compounds as carbon sources: glucose (2 %), sodium acetate (up to 0.4 %), ethanol (up to 3 %) as well as n-, l-alkanes and fatty acids. This organism has a simple haplo/diplontic life cycle, with two naturally stable mating type alleles *MatA* and *MatB*. The genome of *Y. lipolytica* is small with few introns and several genetic markers are available for positive and negative selection. Sequencing of the genome of *Y. lipolytica* has been successfully accomplished under the framework of the Genolevures project recently. As a strictly aerobic organism *Y. lipolytica* has a constant high content of mitochondria with constitutive expression of stable complex I.

The respiratory chain of *Y. lipolytica* resembles its mammalian counterpart. In addition to the four main enzymes (complexes I-IV) it contains a hydrophilic external alternative non proton pumping NADH dehydrogenase (NDH 2) (de Vries and Marres, 1987; Rasmusson *et al*.,

1998) and one alternative terminal oxidase (Kerscher *et al*., 2002) that transfers electrons directly from ubiquinone to oxygen without proton pumping.

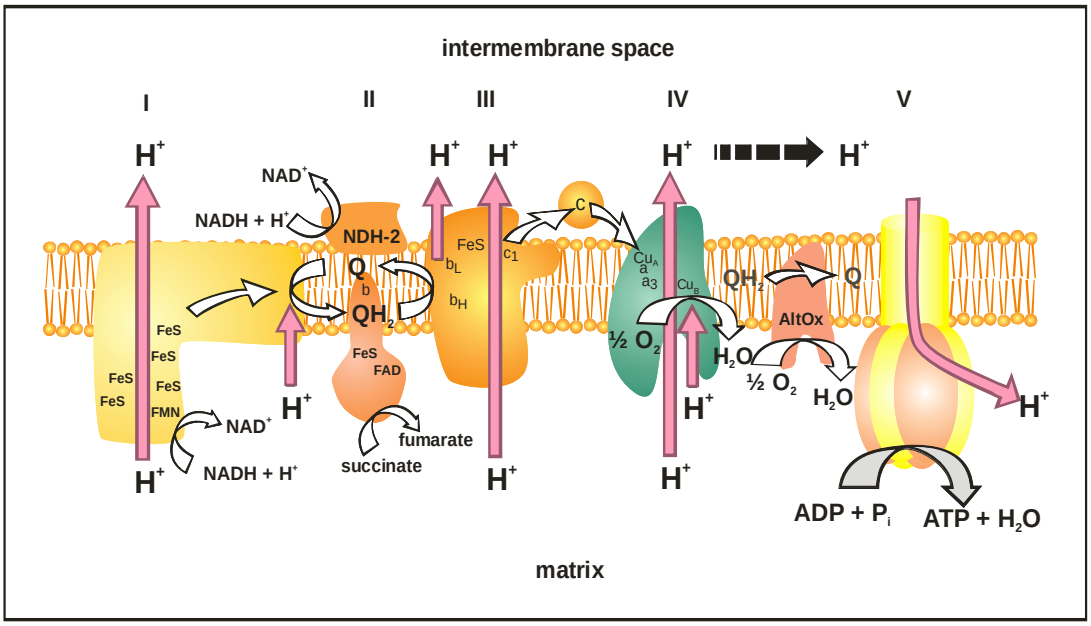

Figure 1.4 Mitochondrial respiratory chain of Yarrowia lipolytica.

Four electron transferring respiratory complexes are labelled as: I (NADH:ubiquinone oxidoreductase); II (succinate:ubiquinone oxidoreductase); III (cytochrome *c* reductase); IV (cytochrome *c* oxidase). Compared to the mammalian respiratory chain, *Y. lipolytica* has an extra external alternative NADH dehydrogenase labelled as (NDH2) and one alternative ubihydroquinone oxidase (AltOx).
The electron flow is shown with white arrows, whereas proton translocation is shown with pink arrows.
FeS: iron-sulphur centre; FMN: flavin mononucleotide; FAD: flavin - adenine dinucletide; NAD^+: nicotinamide-adenine dinucleotide; NADH: hydronicotineamide–adenine dinucleotide; Q: ubiquinone; QH_2: ubihydroquinone; *b*: heme *b*; b_H: high potential heme *b*; b_L: low potential heme *b*; a_3: heme a_3; *c*: cytochrome *c*; c_1: cytochrome c_1; Cu_A and Cu_B: copper centres.

NDH2 consists of a single water soluble subunit with non-covalently bound flavin adenine dinucleotide as cofactor (de Vries *et al*., 1987; Kerscher *et al*., 2001b) and transfers electrons from NADH to the ubiquinone pool without pumping protons. *Yarrowia lipolytica* has only one copy of the alternative NADH dehydrogenase (NDH2) gene. Because of its external orientation in *Y. lipolytica*, NDH 2 is not able to oxidise NADH generated in the matrix and in contrast to complex I it is not required for cell survival (Kerscher *et al*., 1999). To rescue lethal complex I mutants, the mitochondrial targeting sequence from the 75- kDa subunit of complex I and the NDH 2 gene were fused and an internal version of NDH-2i was generated (Kerscher *et al.*, 1999).

1.6 Complex I from Yarrowia lipolytica

The obligate aerobic yeast *Yarrowia lipolytica* was established in our laboratory as a powerful model organism for the investigation of mitochondrial complex I. Thanks to the completely sequenced genome of this organism and the available set of tools for genetic manipulation it is possible to efficiently create mutations in complex I subunit genes. The generation of an internal version of the alternative dehydrogenase (NDH2i) allowed for the survival of complex I deletion strains. One among other advantages of the yeast *Y. lipolytica* is the presence of a very stable complex I. A hexa-histidine sequence was attached to the C-terminus of the 30 kDa subunit (NUGM) of complex I. This allows to isolate very pure enzyme using Ni^{2+} affinity chromatography, followed by gel filtration (Kashani-Poor *et al*., 2001a). The original activity of purified complex I can be restored upon reconstitution into asolecin vesicles (Dröse *et al*., 2002). Electron microscopic analysis of single particles revealed that complex I from *Y. lipolytica*, like the enzyme from *N. crassa* and *B. taurus*, has an L-shaped form (Djafarzadeh *et al.*, 2000), exhibiting a peripheral and a membrane arm. The exact molecular weight of complex I from *Yarrowia lipolytica* and its subunit composition is still unknown.

1.7 Proteomic analysis of complex I

Identification strategies in the field of proteomic analysis often rely on peptide mapping by means of mass spectrometry. It is the analytical tool of choice to determine the molecular masses. In the case of proteins, the molecular mass of peptides, generated by proteolytic cleavage is usually measured. This method is widely accepted and applied, because it is highly sensitive, accurate, fast, and relatively easy to use.

Mass spectrometers consist of an ion source, a mass analyzer, an ion detector, and a data acquisition unit. First, molecules are ionized in the ion source. Then they are separated according to their mass-to-charge ratio (m/z) in the mass analyzer and the separate ions are detected. Many ionisation methods are avaible, which depend on the sample type, but two methods are most frequently used for biochemical analysis, namely Electrospray Ionisation (ESI) and Matrix Assisted Laser Desorption Ionisation (MALDI). Electrospray ionisation is suited for polar molecules ranging from 100 Da to 1 MDa in molecular mass. In electrospray ionization, the sample dissolved in polar, volatile solvent is pumped through a narrow capillary at a flow rate of between 1 µL/min and 1 mL/min. A high voltage is applied to the tip of the capillary and as a consequence of this strong electric field, the sample emerging

from the tip is dispersed into an aerosol of highly charged droplets. The ions generated by this ionisation method are multiply charged. MALDI (Hillenkamp *et al*., 1991) is used successfully for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. In MALDI, the sample that was pre-mixed with a matrix is bombarded with a laser light. The matrix molecules adsorb the laser energy and transform it into excitation energy for the sample. This leads to sputtering of singly charged analyte- and matrix-ions from the surface of the mixture. Commonly MALDI is combined with a time-of-flight (TOF) ion analyser and ESI with a quadrupole-TOF or ion trap mass. A tandem mass spectrometer (MS/MS) is a mass spectrometer that has more than one analyser. This instrumens can select specific ions, induce their fragmentation, and measure the m/z of the fragment ions. In proteomic analysis MS/MS is used for the sequencing of proteins or to determine their possible post-translational modifications.

In order to identify a protein the obtained ion-masses are analysed using genom or protein databases. The simplest search is done by a technique called peptide mass fingerprinting (PMF) which is usually carried out in combination with MALDI-TOF. The database search programs match the observed peptide masses to the theoretical masses derived from protein sequences. It should be noted here that the success rate of protein identification critically depends on the completeness of the database. Moreover sequences errors, and amino acid modifications, which may occur during sample preparation make the database searches a non-trivial task. In addition, post translational modifications of proteins are common that can shift both mass and charge.

Another critical point for protein identification is the sample preparation for MS measurements. The identification of protein from a mixture of different proteins is highly complex. Thus, it is advantageous to separate the proteins prior to mass analysis. The most commonly used method is gel-electrophoresis followed by in-gel digestion of individual protein spots with protease. However 1D SDS-PAGE provides a rather low-resolution separation of proteins and classical 2D-PAGE (IEF/SDS) is unable to detect membrane, highly basic or acidic proteins. In the case of membrane bound multiprotein complexes, doubled 2D SDS-PAGE discribed by Rais et al., (Rais *et al*., 2004) is a very simple and elegant method allowing separation not only of hydrophilic, but also hydrophobic proteins according their molecular mass.

At present, mammalian complex I is best characterized at the proteomic level. All but one subunit (46 subunits) were identified by MS in bovine heart mitochondria complex I. A lot of information about subunit topology within complex I subcomplexes has been obtained using

ESI-MS (Carroll *et al.*, 2003). Most recently, two new subunits, namely B14.7 and ESSS, were found in bovine complex I (Carroll *et al.*, 2002). Post-translation modifications of subunits are also best characterized in bovine and human enzyme (Papa *et al.*, 1996;Van den Heuvel *et al.*, 1998; Hirst *et al.*, 2003; Chen *et al.*, 2004). This was possible thanks to well established proteomic tools and the availability of the most accurate and complete databases. In contrast *Yarrowia lipolytica* sequence genome has been successfully sequenced just recently.

1.8 Goals of this Study

1.8.1 The determination of the subunit composition of complex I from *Y. lipolytica*.

Complex I from *Y. lipolytica* contains more than 20 "accessory" subunits in addition to the 14 "central" subunits. Previously, a limited number of the accessory subunits were identified by sequencing of the N-terminus by Edman degradation. Comparison of N-terminal amino acids with sequences from *N. crassa* and *B. taurus* resulted in the identification of the sequences for six *Y. lipolytica* homologues of accessory subunits (39-kDa subunit (NUEM), NUYM, NUZM, B13 (NUFM), ASHI (NIAM), B14 (NB4M)).

The first aim of this study was to determine the subunit composition of complex I from *Y. lipolytica*. The subunits of complex I have to be separated by 2D PAGE (doubled SDS and IEF/SDS) followed by the determination of the number and apparent masses of the subunits. Then, using MALDI-MS and the genome sequence databank, the sequences of proteins have to be identified and compared with other species to reveal the differences and similarities between complex I from different organisms.

It is important to establish the subunit composition for two reasons. Firstly, knowledge of the subunit composition is prerequisite for determining the atomic structure of complex I and for understanding its catalytic mechanism. Secondly, investigation of homologues of the subunits of complex I will provide useful information about the function and importance of the "accessory" subunits and should reveal possible interactions of the respiratory chain with biosynthetic and other metabolic pathways.

1.8.2 Mutagenesis of 39 kDa subunit of complex I

Construction and characterization of a 39 kDa deletion strain

Knock-out of the 39 kDa subunit of complex I in *N. crassa* led to complete loss of electron transport activity from NADH to ubiquinone (Schulte *et al.*, 1999). Based on this observation, it was proposed, that this subunit is involved in the biogenesis of a new redox group localised between cluster N2 and ubiquinone (see 1.2.4). To examine this hypothesis a mutant carrying a deletion of the 39 kDa subunit gene was generated and characterized in terms of assembly, catalytic activity of complex I and EPR-spectra of the redox groups.

Site-directed mutagenesis of the 39 kDa subunit

Based on sequence analysis and experimental data (Schulte *et al.*, 1999;Yamaguchi *et al.*, 2000) it had been proposed, that the 39 kDa subunit tightly binds one molecule of NADPH (see 1.2.4). Two parts of the nucleotid-binding domain are crucial for selective binding of NADPH, namely a motif which containes three glycines (GXXGXXG) and a basic residue at the end of the second β-strand. One of the aims of this study was to generate and characterize point mutants in this nucleotide-binding domain. Any changes in the glycine motif should interfere with nucleotide binding. The replacement of a basic residue that has shown to be responsible for the selective interaction with the 2'-phosphate group of NADPH was expected to change the affinity and/or selectivity of the cofactor to the protein. Nevertheless in comparison to a 39 kDa subunit deletion, it should affect complex I assembly and activity to a much lesser extent. To explore the role of tightly bound NADPH for complex I the point mutants had to be characterized in terms of complex I activity, stability and assembly.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Methanol, HPLC gradient grade (J.T. Baker, Deventer-Netherland); bovine serum albumin (BSA) (Biolabs, New England); n-Dodecyl-β-D-maltoside (Biomol Feinchemikalien GmbH, Hamburg-Germany); DEAE Bio-Gel A Agarose (Biorad Laboratories GmbH, München-Germany); Chelating Sepharose (Pharmacia Biotech AB, Uppsala-Sweden); Agar; bacto™ yeast extract, Trypton, selected peptone 140 (Gibco BRL Life Technologies, Paisley-United Kingdom); YNB (Difco Laboratories, Sparks, MD, USA); boric acid phenol developer, fixer and fixing buffer for X-ray films and X-ray films X-OMAT AR (BioMax MR (Kodak) Rochester-New York); acetone, ammonium peroxosulfate, chloroform acetic acid, Folin-Ciocalteus-Phenol reagent, isoamyl alcohol, isopropanol, $MgSO_4$, HCl, trichlorine acetic acid, chlorophorm, cholic acid, sodiumthiosulfate pentahydrate (Merck, Darmstadt-Germany); acetonitril, ammonium sulphate, EDTA, glas perls (0.25 – 0.5 mm), KCl, KOH, KH_2PO_4, sodium acetate, sodium citrate, NaCl, NaOH, $NiSO_4$, NaH_2PO_4, saccharose, X-Gal, NH_3HCO_3, (Carl Roth GmbH & Co, Karlsruhe-Germany); ATP, nucleotides, Ni-NTA Fast Flow Sepharose (Pharmacia); acrylamide, bisacrylamide, Coomassie-Blue G-250, urea, polyethylene glycol (PEG) 4000, dodecylsulphate sodiumsalt (SDS), Tricine, agarose, 6-amino caproic acid, amino acids, ampicilline, DMSO, ethidium bromide, glucose, glycerol (Pharmacia); hexaammine-ruthenuim(III)-chloride (HAR), Hepes, KCN, lithium acetate, mercaptoethanol, Mops, d-NADH, NADH, NADPH, FMN, NaN_3, PMSF, TEMED, Tris, asolectin, oligonucleotides, nitroblue-tetrazolium (NBT), bovine trypsin, 2,5-dihydroxy-benzoic acid (DHB), 4-hydroxy-a-cyanocinnamic acid (HCCA), trifluoracetic acid, luminol, coumaric acid, hydrogen peroxid (Sigma Chemie GmbH, Deisenhofen-Germany); asolectin, silver-nitrate (Fluka); oligonucleotides (MWG-Biotech Ebersberg-Germany).

2.1.2 Inhibitors

2-decyl-4-quinazolinyl amine (DQA) was a generous gift from Aventis CropScience, Biochemical Research, Frankfurt am Main, Germany; rotenone was purchased from Sigma Chemie GmbH, Deisenhofen, Germany.

2.1.3 Media and Solutions

Media for *Escherichia coli*:

LB-media: 1 % NaCl, 0.5 % yeast extract, 1 % bactotryptone, pH 7.5 (1.5 % agar for plates)

SOC-media: 0.5 % yeast extract, 2 % bactotryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

Media for *Yarrowia lipolytica*:

YPD-medium: 2 % Bacto™ peptone, 1 % yeast extract, 2 % glucose

Permanent culture medium: YPD-media + 40 % glycerol

Minimal synthetic medium (S): 1.7 % yeast nitrogen base without $(NH_4)_2SO_4$ and amino acids, 5 % $(NH_4)_2SO_4$, pH 5.0 were prepared as a 10 × stock solution and sterile filtrated. Carbon source (0.4 % acetate or 2 % glucose) were autoclaved and added to 10 × S-media. Depending of the type of selection one or several of the following components were added: 130 µM histidine, 200 µM lysine, 460 µM leucine, 180 µM uracil.

Buffers and solutions

10 × TAE-buffer: 400 mM Tris / acetate, 10 mM EDTA, pH 8.3

TE: 10 mM Tris / HCl, 1 mM EDTA, pH 8.0

20 × SSC-buffer: 3 M NaCl, 0.3 M sodium citrate, pH 7.0

One step buffer (freshly prepared): 45 % PEG4000, 0.1 M lithium acetate (pH 6.0), 100 mM DTE, 250 µg/ml salmon sperm DNA as carrier

Rehydratation buffer for IEF: 8 M Urea, 1.5 % Triton X-100, 10 mg/ml DTE, 0.5 % IPG buffer (ampholyte mixture), trace of bromphenol blue

SDS equilibration buffer for IEF: 50 mM Tris-HCl (pH 8.8), 6M Urea, 30 % Glycerol (v/v), 4 % SDS (w/v), 10 mg/ml DTE, trace of bromphenol blue

K-Cholate buffer: 1 % K-cholate, 0.15M KCl, 10 mM Tris-acetate, pH 7.5

Wash buffer: 1 mM EDTA, 0.1 mM PMSF, 10 mM Tris-acetate, pH 7.5

PBS-buffer: 80 mM $Na_2HPO_4 \times 2H_2O$, 20 mM $NaH_2PO_4 \times H_2O$, 100 mM NaCl, pH 7.5

ECL-1 buffer: 0.1 M Tris, 2.5 mM luminol, 450µM coumaric acid

ECL-2 buffer: 0.1 M Tris, 0.03 % H_2O_2, pH 8

2.1.4 Strains

Escherichia coli competent cells

strain	genotype
XL1-Blue	*recA*1 *endA1yrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F'*proAB lacl*^{q}*ZΔM*15 *Tn*10 *(Tet*^{r}*)*]
XL10-Gold	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F'*proAB lacI*^{q}*ZΔM*15 *Tn*10 (Tet^{r})]

Yarrowia lipolytica

strain	genotype
GB10	*30Htg2 Mat*B *ndh2i ura3*-302 *leu2*-270 *lys*-1
PIPO	30 Htg pop-in-pop-out *Mat*A, *lys*-1, *ura3*-302, *leu2*-270

2.1.5 Plasmids

name	property	source
pCR2.1	see product's description	Invitrogen, Groningen, The Netherlands
pBluescript SK(±)	see product's description	Stratagene, Heidelberg, Germany
pUB4	*Yarrowia lipolytica* "shuttle"-vector containing 1 kb fragment with *Hyg* B^{R} gene	Dr. Stefan Kerscher, Frankfurt/Main, Germany

2.1.6 Antibodies

First antibodies

antibody	specificity	provenience
30C10	NUBM (51 kDa)	Dr. Volker Zickermann Frankfurt am Main Germany
42A10	NUCM (49 kDa), internal epitop 31 PIPSGALGQKVPHV 45	
37G12	NUEM (39 kDa), internal epitop 263 VRHIELPKALYQAYTKATQAI 284	

Secondary antibody

Peroxidase conjugate anti-maus IgG developed in goat (Sigma, Germany)

2.1.7 Instruments

Centrifuges:

Heraeus Biofuge A (Osterode, Germany)

Heraeus Labofuge 400 (Osterode, Germany)

Heraeus Minifuge GL (Osterode, Germany)

Heraeus Cryofuge 8500i (Osterode, Germany)

Cool centrifuge J2-21, Beckman Instruments GmbH (München, Germany)

Ultracentrifuge L7-65 and L8-70M, Beckman Instruments GmbH (München, Germany)

Spectrophotometer:

UV 300 Shimadzu (Düsseldorf, Germany)

U-3210 Hitachi (Düsseldorf, Germany)

MultiSpec-1501, Shimadzu (Düsseldorf, Germany)

SPECTRAmax PLUS^{384}, Molecular Devices GmbH (Ismaning, Germany)

Thermocycler:

DNA Thermal Cycler 480, Perkin Elmer (Weiterstadt, Germany)

GeneAmp® PCR System 2400, Perkin Elmer (Weiterstadt, Germany)

cyclone® gradient, Peqlab, Biotechnologie GmbH (Erlangen, Germany)

Electroporation:

E. coli Pulser Bio-Rad (Hercules, USA)

DNA Sequencer:

ABI PRISM™ 310 Genetic Analyzer, Perkin-Elmer (Weiterstadt, Germany)

Sonifier:

B 15 Sonifier / Cell Disrupter, Branson (Danbury, UK)

EPR-Spectrometer:

ESP 300 E, Bruker (Rheinstetten, Germany) with continuous flow cryostat ESR 900, Tubney Woods Abingdon (Oxon, UK)

EPR-tubes:

Quartz glass Nr.: 707-SQ-250M (length: 250 mm, diameter: 4 mm), Rototec Spintec (Biebesheim, Germany)

Instruments for HPLC

UV/UV-Vis Detector, L-4000/L-4200 eguipped with Intellligent Inert Pump L-6210 and D-2500 Chromato-Integrator, Merck, Germany.

Columns

TSKgel G 4000 SW filtration column (210.5 mm × 600 mm), TosoHaas GmbH (Stuttgart, Germany)

TSKgel 3000 SW filtration column (75 mm x 600 mm), TosoHaas GmbH (Stuttgart, Germany)

RP-HPLC column: Hibar RT 250-4, LiChospher 100, RP 18 (5 µm), with pre-column LiChrospher 100 RP-18 (5µm), (Merck, Germany)

Other instruments:

10 l Fermenter, Biostat E; Braun (Melsungen, Germany)

Bead-Beater glass pearls mill, Biospec (Bartlesville, USA)

Cell-Desintegrator-C, Bernd Euler (Frankfurt/Main, Germany)

BioSys 2000 Workstation®, Beckman Instruments GmbH (München, Germany)

BioLogic HR Workstation, Bio-Rad Laboratories GmbH (München, Germany)

Photo camera MP4 land camera, Polaroid

GelSystem MINI, Biostep (Jahnsdorf, Germany)

Hybridisations oven HB-1D, Techne (Wertheim, Germany)

Microscope, Leitz (Wetzlar, Germany)

Ultrafree - 20 Centrifugal Filter Unit® with Biomax™ - 30 High Flux Polysulfone Membrane, Millipore GmbH (Eschborn, Germany)

Savant Speed Vac System SVC 100 H, (Fischer Laborbedarf, Germany)

Christ ALPHA I-6 Freeze-dryer (DAMON/IEC (U.K.) Ltd.)

IPGphor (Amersham Biosciense, Germany)

MALDI-TOF mass spectrometer Voyager De Pro (Applied Biosystems, USA).

2.1.8 Software

DNA and Protein Analysis software:

Mac Vector 3.5, IBI

VectorNTI Advance 9.0 (InfosMax, USA)

HIBIO DNASIS™ for Windows® Version 2, Hitachi Software Engineering Co., Ltd.

Husar (DKFZ, Heidelberg, Germany)

CLUSTALW (EMBL-EBI, Heidelberg, Germany)

Sequence Navigator (Applied Biosystems, USA)

Sequencing Analysis (Applied Biosystems, USA)

BCM Search Launcher (Baylor College of Medicine, USA)

Mascot (Matrix Science Ltd., London)

Protein Prospector (Mirrors at UCL-Ludwig, UK / Ludwig Institute Melbourne (Australia))

PROWL (ProteoMetrics, USA)

SOFTmax PRO, Molecular Devices GmbH (Ismaning, Germany)

Other software:

Microsoft Office Package

2.2 Methods of Molecular Biology / Gene Technology

2.2.1 Deletion strain of NUEM (Δ*nuem*)

The haploid deletion strain of the 39 kDa subunit of complex I from *Y. lipolytica* was generated using the one-step transformation method as described (Chen *et al*., 1997). The open reading frame (ORF) of the NUEM gene was replaced by a URA3 reporter gene. A fragment of NUEM gene in which the ORF had been replaced by URA3 gene was transformed into the haploid strain GB10 (*30Htg2 Mat*B, *ndh2i*, *ura3*-302, *leu2*-270, *lys*1). Subsequently a strain carrying the appropriate markers on minimal media plates was selected. Finally, the selected clons were checked by PCR and Southern Blotting.

2.2.2 DNA Gel Electrophoresis

DNA was separated according to standard procedures (Sambrook *et al*., 1989) in the presence of 0.5 µg/ml ethiduim bromide. Depending on the expected DNA fragment length agarose concentrations from 0.6 – 2.0 % in 1×TAE buffer were used. If the DNA fragments were extracted from the gel, TEA buffer with extra additive was used (UV-safe TAE, MWG-Biotech, Ebersberg). DNA molecular weight standards: 1 kb Ladder, 100 bp Ladder plus (MBI Fermentas, St. Leon-Rot).

2.2.3 Fill-in Reaction of 5`-Overhang

DNA blunt-ends were made with large fragments of *E. coli* DNA-polymerase I (Klenow-polymerase, New England Biolabs GmbH, Schwalbach/Taunus) as described by (Sambrook *et al.*, 1989).

2.2.4 DNA-Vector Dephosphorylation

To avoid self-ligation of empty vectors the DNA ends were dephosphorylated with SAP (Shrimp Alkaline Phosphatase, Boeringer Mannheim, Mannheim).

2.2.5 Phosphorylation of PCR-Products

To enable the ligation of PCR products it was necessary that both fragment ends were phosphorylated. Phosphorylation was done with T4 polynucleotide kinase (New England Biolabs) as described by Ausubel (2000). Alternatively, primers rather than DNA fragments, were phosphorylated before PCR.

2.2.6 DNA Extraction from Agarose Gels

DNA extractions from agarose gels were performed using the "Easy Pure Kit" (Biozym Diagnostic GmbH, Hess. Oldendorf) or the QIAprep® Gel Extraction Kit (Qiagen).

2.2.7 Ligation

T4 DNA-ligase (Gibco BRL Life Technologies) was used in the provided buffer to ligate DNA fragments. Usually ligation was carried out over night at 14°C.

2.2.8 Making of Electro-Competent *Escherichia coli* Cells

Electro-competent *E. coli* cells were made according the procedure described in "Current Protocols in Molecular Biology" (2000). Transformation efficiency was up to 2×10^9 colonies/µg DNA.

2.2.9 Transformation into *Escherichia coli* (electro-competent cells)

The transformation of plasmids (with Amp^R gene) into *E. coli* electro competent cells took place in an *E. coli* Pulser (Biorad) as described in "Current protocols in Molecular Biology" (2000). Transformants were then streaked out and grown over night on LB solid medium in the presence of ampicillin (50 µg/ml).

2.2.10 Preparation of Plasmid-DNA from *Escherichia coli*

Plasmid-DNA was prepared according to (Zhou *et al*., 1990) from a small volume of over night cultures (1.5-3 ml). Plasmid DNA for sequencing was prepared using the QIAprep® Spin Miniprep Kit (Qiagen).

2.2.11 DNA Sequencing

Double-strand DNA was used as template for sequencing. The sequencing reaction was made using the "ABI Prism dye terminator cycle sequencing kit" (Perkin Elmer, Weiterstadt). Sequencing was performed in an ABI Prism Automated Sequencer type ABI 310.

2.2.12 Polymerase Chain Reaction (PCR)

10 ng of plasmid-DNA resp. 100 ng of genomic DNA, 5 µl of both oligonucleotides (5 µM), 5 µl of provided 10X reaction buffer were combined in a total reaction volume of 50 µl. To avoid dimerisation of oligonucleotides as well as non-specific binding of oliogonucleotides to matrix DNA manual "hot-start" was applied. Used polymerases were: *Taq* DNA polymerase,

*Taq*2000™ DNA polymerase, *Pfu* DNA polymerase and *Pfu*Turbo™ DNA polymerase from Stratagene (Heidelberg) as well as *Taq* DNA polymerase from Sigma Chemie GmbH (Deisenhofen).

2.2.13 Generation of Point Mutations

The shuttle-vector pUB4 carrying a 4.91 kb insert coding for the NUEM gene was used as template for site directed mutagenesis. Point mutation was introduced by PCR with the "QuikChange™ site-directed mutagenesis kit" (Stratagene, Heidelberg). After amplification of the insert-containing plasmid using phosphorylated primers the reaction mixture was digested with *DpnI* to eliminate methylated template plasmid. Phosphorylated PCR products were ligated and transformed into electro-competent cells. To check the presense of the desired mutation and the absence of inadvertent sequence changes, the complete ORF of the mutagenised gene was sequenced and compared to the wild type ORF.

2.2.14 Southern Blot

Digested DNA (genomic DNA: 500 ng; plasmid DNA: 50 ng) was separated using agarose gel electrophoresis (1 %). The DNA was transferred over night onto Hybond N^+-membrane (Amersham, Braunschweig). Covalent crosslink of DNA to the membrane was achieved by UV-light irradiation (Stratalinker, Stratagene, Heidelberg).

2.2.15 ^{32}P DNA Labelling

DNA fragments were labelled with [α-^{32}P] dCTP (25 µCi for 25 ng DNA) using the "Random primer labelling – Prime-It®II" Kit (Stratagene, Heidelberg). The rate of radioactive labelling was checked by pipetting 3 µl of 1:100 diluted reaction mixtures onto two filter sheets (Whatman DE 81 ion exchange paper, Whatman International Ltd., Maidstone, England). One of the filters was washed two times for 5 minutes with 2 × SSC buffer and subsequently washed for 5 minutes in cold ethanol. To estimate incorporation of the radioactive label, count rates of both filters were controlled after drying using a Geiger counter.

2.2.16 Hybridisation of Radio Actively Labelled DNA Probes

Hybridisation took place in a rotating glass tube using a thermostatted hybridisation oven (HB-1D, Techne). The membranes were pre-hybridised for 15 minutes at 68°C followed by a main hybridisation period of 60 min at 68°C in "QuikHyb®" hybridisation solution (Stratagene, Heidelberg). For the main hybridisation, ^{32}P-labelled DNA fragment was added

together with 100 µl (10 mg/ml) salmon sperm DNA. Subsequently, blots were washed four times (2 × 15 min with 2 × SSC, 0.1 % SDS; 2 × 15 min with 0.1 × SSC, 0.1 % SDS) to remove non-specifically bound radioactive probe. Blots were exposed to Kodak X-Omat AR films with an amplifier-sheet over night at –80°C.

2.2.17 Transformation of *Yarrowia lipolytica*

Yarrowia lipolytica cells were transformed according to the method of (Chen *et al.*, 1997). A single colony was taken from a fresh plate. Alternatively, cells from 0.5 ml of an over night culture in complete medium were spun down. The cells were dispersed by vortexing for 1 min in 100 µl of freshly prepared one step buffer (45 % PEG4000, 0.1 M lithium acetate pH 6.0, 100 mM dithiothreitol, 250 µg/ml salmon sperm DNA as carrier). Subsequently the mixture was incubated for 1 h at 39°C and was spread on well dried selection plates. Transformants could be observed after 3 days incubation at 28°C.

2.2.18 Isolation of Total DNA of *Yarrowia lipolytica*

Total DNA isolation was performed according to the “rapid isolation of yeast chromosomal DNA” protocol described in “Current Protocols in Molecular Biology” (2000). Plasmid DNA was obtained by transformation of 150 ng of total DNA into *E. coli* competent cells.

2.3 Methods of Protein Chemistry

2.3.1 Growth of *Yarrowia lipolytica*

Yarrowia lipolytica parental strains were grown in YPD medium at 28°C in rotatory flasks. A clone of *Y. lipolytica* from an agarose YPD plate was taken for a 1 l pre-culture and shaken in a flask for 18 - 24 hours. Subsequently, the pre-culture was used to inoculate a 10 l fermenter (Biostat E; Braun, Melsungen). Fermentation lasted 14 - 18 hours. The yield was up to 90 g cells / l (wet weight).

Mutant strains were grown by fermentation in 10 l of YPD medium. The medium of the pre-culture depended on the plasmid. In the case of pUB4 YPD containing 100mg/l hygromycin B was used. The fermentation was inoculated in a 10 l fermenter with 1 l of pre-culture. Pre-cultures were shaken in flask for 24 hours and fermentation took futher 24 hours. Even without selective pressure during fermentation, no substantial loss of plasmid was observed.

2.3.2 Preparation of Mitochondrial Membranes

Mitochondrial membranes were prepared from freshly harvested cells or from cells that had been shock frozen in liquid nitrogen and kept at –80°C. To break the cell walls 0.5 mm glass beads (Bernd Euler Biotechnologie, Frankfurt) were used in a cell disintegrator. 300 – 500 g of cells were suspended in the same amount of buffer (600 mM sucrose, 20 mM Na/MOPS, 1 mM EDTA, pH 7.2). Cell breakage was carried out for at least 2 hours in the presence of 2 mM PMSF (protease inhibitor). Centrifugation for 25 min at 2000 × g was used to separate cell debris (pellet) from mitochondrial membranes (supernatant). To collect mitochondrial membranes this supernatant was ultracentrifuged for 1 hour at 100,000 × g. The homogenised membranes were resuspended in the same buffer as above but without EDTA, shock frozen and stored at –80°C.

Membrane quality was checked by recording cytochome absorption spectra (530-630 nm) of the reduced minus oxidised forms. Mitochondrial membranes were reduced by addition of dithionite and oxidised by addition of ferricyanide. Content of heme groups *b* and aa_3 (reduced minus oxidised form) was measured at 562 nm and at 605 nm, respectively. The concentration was usually 1-10 µM and the ratio heme *b*: heme aa_3 about 3:1.

2.3.3 Preparation of Mitochondrial Membranes in Small Amounts

Freshly harvested cells (4 - 8 g) were used at 1:1:1 cells to buffer to glass beads ratio (same as in 2.3.2). Cell breakage was achieved by vortexing in a Falcon tube for 10 × 1 min and intermittent cooling in ice for one minute. Centrifugations and further steps were the same as in 2.3.2.

2.3.4 Protein Quantitation

Protein determination was done according to the procedure of Lowry *et al*. (1951), as modified by Helenius and Simons (1972). Calibration was carried out with 0.1 – 2.0 mg/ml bovine serum albumin (BSA).

2.3.5 Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

Blue-native polyacrylamide gel electrophoresis was used to separate the components of the mitochondrial respiratory chain in membranes of *Y. lipolytica* (Schägger, 2003). 500 µg of total protein was solubilised with 1 g/g dodecyl maltoside and 500 mM amino caproic acid. The resulting solubilised mitochondrial membranes were put on 4-13% gradient gels.

2.3.6 Doubled SDS-Polyacrylamide Gel Electrophoresis (dSDS-PAGE)

Tricine dSDS-PAGE was used to separate the subunits of complex I from *Y. lipolytica* and was performed as described (Rais *et al.*, 2004). Briefly, lanes from 1D-gels (10% polyacrylamide, 6M urea) were incubated in acidic solution containing 100 mM Tris, 150 mM HCl, pH 2 for 30 min and analysed by SDS-PAGE as a second dimension using 16% polyacrylamide. The 2D-gels were stained with Coomassie blue G 250 or silver.

2.3.7 Isoelectric Focussing

IEF - separation as first dimension was done in a IPGphor apparatus (Amersham Pharmacia Biotech) using 13 cm immobilized pH gradient (IPG) strips (pH 3-10 or 6-11). The samples of complex I (~120µg) were rehydrated in a solution containing 8 M urea, 1.5% Triton X-100, 0.5% IPG Buffer and a trace of bromphenol blue for 12 h at 20 °C. Focusing was carried out by 50µA per IPG strip using the following steps: pH 3-10 gel, 200Vh (200V max), 500Vh (500V max), 1000 Vh (1000 V max) and 32000 Vh (8000V max) with a gradient step of 1125Vh (3500 V); pH 6-11 gel, 200 Vh (200V max), 500Vh (500V max), 1000Vh (1000V max) with a gradient step of 1125Vh (3500V max) and 64000Vh (8000V max). After focussing, strips were equilibrated using a buffer containing 50mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 4% SDS and a trace of bromphenol blue. The second dimension gel consisted of a Tricine-SDS 16% polyacrylamide gel.

2.3.8 Silver-staining of 2D-SDS gels

After electrophoresis the gel was incubated for 15 minutes in fixation solution, which consisted of 50% methanol and 10% acetic acid. Subsequently the gel was incubated in 0,005% sodiumthiosulfate-pentahydrate. The gel was washed in H_2O and incubated in 0.1% silver nitrate (w/v) for 30 minutes. Subsequently the gel was briefly washed in H_2O and developed in freshly prepared developer: 2% (w/v) sodium bicarbonate in 100 mL H_2O plus 100 µL 36.5% formaldehyde. The development reaction was stopped by 50mM EDTA solution.

2.3.9 Staining with nitro blue tetrazolium (NBT)

NBT-staining was used to detect the NADH activity of complex I in BN-PAGE. The non-fixed gel was incubated in a solution contaning 3mM NBT and 120 µM NADH for 5 minutes. In order to stop the reaction, the gel was incubated in 50 % methanol and 10 % acetic acid.

2.3.10 Western Blot

To investigate protein expression, a semidry immunoblotting procedure was followed using a polyvinylidendifluoride membrane (Immobilon ^{TM}P, Millipore). Membranes and gel-blotting paper were incubated in blotting-buffer (cathode buffer: 300mM 6-aminocaproic acid, 30 mM Tris, pH 9.2; anode buffer: 300mM Tris, 100mM Tricine, pH 8.7). Gels were blotted for 3 hours at 200mA and 20V. After blotting, membranes were incubated for 30 minutes in PBS buffer containing 0.4% (w/v) Triton-X100 to reduce the background caused by unspecific binding. After washing in PBS buffer once for 15 minutes and twice for 5 minutes, membranes were incubated over night with primary monolyclonal antibody against 51 kDa, 49 kDa and 39 kDa subunits of complex I diluted in PBS 1:1. After washing once for 15 minutes and twice for 5 minutes in PBS, incubation for 1 hour with anti-mouse IgG peroxidase conjugate (diluted 1:10000 in PBS) was performed. Proteins were detected by enhanced chemiluminescence (ECL). The washed membrane was incubated for 1 minute with a 1:1, (v/v) mixture of ECL-1 solution, containing 2.5 mM of luminol, 450 µM of coumaric acid (stock solution 90mM in DMSO), and ECL-2 solution, containing 0.03% hydrogen peroxide. The peroxidase oxidises luminol in the presence of hydrogen peroxide. Light emission occurring during luminal oxidation was detected by placing the blot in contact with an X-ray film.

2.3.11 Measurement of NADH:HAR activity

Detergent- and inhibitor-insensitive NADH:HAR [HAR: hexa-ammine-ruthenium(III) chloride] activity was measured using a Shimadzu MultiSpec-1501 or a Molecular Devices SPECTRAmax PLUS^{384} spectrophotometer by following NADH-oxidation at 340 minus 400 nm (ε=6.22 $mM^{-1}cm^{-1}$). Assays were performed in the presence of 200 µM NADH and 2 mM HAR, in 20 mM Na^+/Hepes, pH 8.0, 2 mM NaN_3 at 30°C (Sled and Vinogradov, 1993). This activity depends only on the presence of FMN and probably Fe-S cluster N3 (Gavrikova *et al.*, 1995). The reaction was started by the addition of 50 µg (total protein) of unsealed mitochondrial membranes.

2.3.12 Measurement of complex I catalytic activity

For measurement of complex I activity, dNADH was used as electron donor, and the ubiquinone analogue DBQ was used as electron acceptor. dNADH:DBQ activity at 60 µM DBQ and 100 µM dNADH was measured using a Shimadzu MultiSpec-1501 or a Molecular Devices SPECTRAmax PLUS^{384} spectrophotometer by following dNADH-oxidation at 340

minus 400 nm (ε=6.22 $mM^{-1}cm^{-1}$) at 30°C in 20 mM Na-MOPS pH 7.2 buffer containing 50 mM NaCl and 2 mM KCN. The reaction was started by adding mitochondrial membranes equivalent to a final concentration of 30 to 50 µg of protein/ml.

2.3.13 Purification of complex I

Complex I was purified from isolated mitochondrial membranes that were solubilised with n-dodecyl-β-D-maltoside as described (Kashani-Poor *et al.*, 2001a) with slight modifications. Purification was achieved by Ni^{2+}-affinity chromatography with a modest reduction of the imidazole concentration from 60 mM to 55 mM in the equilibration and washing buffer and subsequent gel filtration using a TSK4000 column.

2.3.14 Reactivation of purified complex I

In its natural environment, complex I is embedded in a lipid bilayer. Most of these lipids are lost during protein purification, resulting in significant loss of catalytic activity. To reactivate complex I dNADH:DBQ activity, asolectin was added (total soy bean extract with 20 % lecithin) in a 1:1 (w/w) protein-to-lipid ratio. The asolectin solution was 10 mg/ml solubilised by 1.6 % OG in 1 mM KP_i and 25 mM K_2SO_4 pH 7.2.

2.3.15 EPR-Spectra

Low temperature EPR spectra were obtained with a Bruker ESP 300E spectrometer equipped with a liquid helium continuous flow cryostat, ESR 900 from Oxford Instruments. Samples were mixed with NADH in the EPR tube and frozen in liquid nitrogen after 30 seconds reaction time. Spectra were recorded at 12 K or at 40 K with the following instrument settings: microwave frequency 9.475 GHz, microwave power 1 mW, modulation amplitude 0.64 mT. Under these conditions spectra show contributions from clusters N1, N2, N3 and N4. Spectra were recorded and analysed by Dr. Klaus Zwicker.

2.4 High Pressure Liquid Chromatography (HPLC) analysis of complex I bound NADPH and FMN

2.4.1 Extraction of NADPH and FMN from mitochondrial membranes and complex I

One volume of mitochondrial membranes was mixed with one volume of 1 % potassium cholate in Tris-acetate buffer (0.15 M KCl in 10 mM Tris-acetate buffer, pH 7.5) and incubated for 30 minutes on ice. After centrifugation at 35000 g for 1 hour the membranes were washed twice in washing buffer containing 1mM EDTA, 0.1 mM PMSF in 10 mM Tris-acetate buffer (pH 7.5). The pellet was resuspended in washing buffer. Extraction of nucleotides was performed as described (Schulte *et al.*, 1999) with some modifications. 3 mg of protein were lyophilized and then extracted for ten minutes with 500 µl of chloroform/ methanol (2:1, v:v) by vigorous shaking on ice. After addition of 600 µl of 0.1 M NaOH adjusted to pH 10 with glycine the sample was incubated for 15 minutes by vigorous shaking on ice. After centrifugation for ten minutes at 11000x g the upper phase was filtered through a cellulose minifilter (Minisart-RC/SRP, 0.20µm, Sartorius), lyophilized and then diluted in 50µl of filtrated water.

The same extraction procedure was carried out with complex I and standard solutions containing 1-5 nmol of nucleotide (NADH, NADPH, FAD or FMN) and 1mg/ml bovine serum albumin. 1 mg of complex I was extracted with 200 µl of chloroform/methanol and incubated with 300 µl of 0.1 M NaOH.

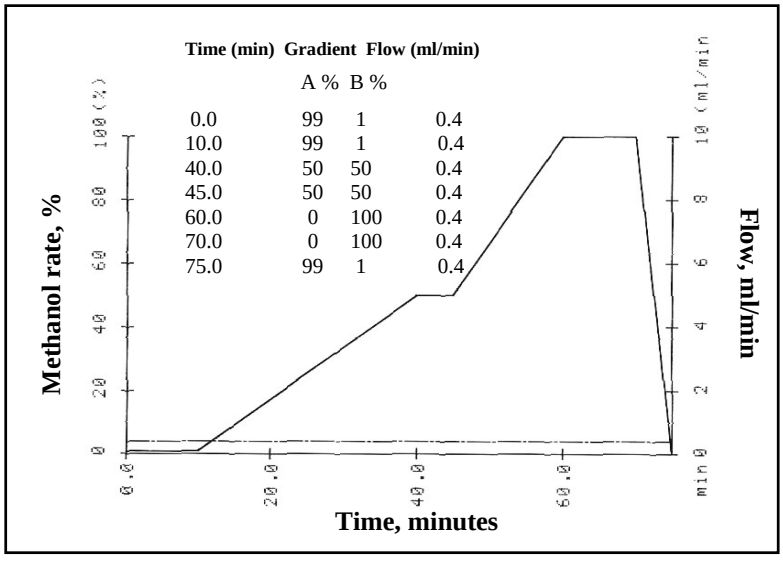

Figure 2.1 Profile of gradient programm for HPLC analysis of NADPH and FMN

A-0.05 M potassium dihydrogenphosphat buffer (pH 6); B-HPLC grade methanol.

2.4.2 Determination of NADPH and FMN

HPLC was performed with a MerckHitachi Intelligent Inert Pump L-6210 combined with UV-detector (L-400/L-4200 UV/UV-Vis, Merck Hitachi) set to 340 nm. A reversed-phase column (Hibar RT 250-4, LiChrospher 100, RP 18 (5 µm) with a pre-column LiChrospher 100 RP-18 (5µm), Merck, Germany) was used. Elution occured over 75 minutes at a flow rate of 0.4 ml/ minute with 1 % (v/v) methanol in 0.05 M potassium dihydrogenphosphate (pH 6), followed by a linear gradient from 1 % to 50 % (v/v) methanol in potassium dihydrogen-phosphate buffer over 40 minutes. Then a methanol/buffer (1:1 v/v) step of 5 minutes was followed by a linear gradient from 50 % to 100 % methanol over 15 minutes. Finally, the column was flushed with 100 % methanol for 10 minutes (Figure 2.1). Quantitative determination of nucleotides in complex I was carried out with standard solutions of the nucleotides, in a dilution series from 1–5 nmol (Figure 3.22).

2.5 Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS)

Stained protein spots were excised from dSDS polyacrylamide gels and treated following the protokol of van Monfort et al. (2002). The proteins were cleaved with trypsin (12.5 ng/µl) in digestion buffer containing 25 mM ammonium hydrogen cabonate, 5 mM $CaCl_2$ at 37°C over night.

The samples were analysed using MALDI-TOF mass spectrometry. Spectra were recorded in the positive ion mode with a Voyager De Pro MALDI-TOF mass spectrometer (Applied Biosystems, Germany). The samples were deposited on preparative plates by the fast evaporation method. DHB (2,5-dihydroxybenzoic acid) or HCCA (4-hydroxy-a-cyano-cinnamic acid) from Sigma were used as a matrix. Spectra were calibrated internally using bovine trypsin autolysis products (m/z 805.4167 and 2163.0567) or, if necessary, externally using a reference peptide mixture (bradykinin, angiotensin II, insulin, oxidized B chain, adreno corticotropic hormone (ProteoMass Peptide MALDI-MS Calibration Kit, Sigma) covering the m/z 757.3997-3494.6513 range. MALDI spectra were analysed by the Mascot software package (Matrix Science Ltd., London), Prowl software package (ProteoMetrics, LLC, New York, USA) or the Protein Prospector software package (Mirrors at UCL-Ludwig, UK / Ludwig Institute Melbourne (Australia)) using a proprietary *Y. lipolytica* genomic database.

2.6 Sequence analysis

DNA and protein sequences were analyzed using the DNASIS (Hitachi), HUSAR (http://genius.embnet.dkfz-heidelberg.de/) and PredictProtein (http://www.predictprotein.org/) program packages. Codon preference plots were generated using the *Y. lipolytica* codon usage table at http://www.kazusa.or.jp/codon/. Homology searches of mammalian and fungal databases were done using the BLAST server at http://www.ncbi.nlm.nih.gov/BLAST/. Alignments of fungal and mammalian proteins were generated using the program CLUSTALW at http://www.ebi.ac.uk/clustalw/index.html. Searches of the NCBI conserved domains database (Marchler-Bauer *et al*., 2003) were performed at the NCBI server (USA). (www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). Prediction of transmembrane helices using hidden Markov models (Tusnady, Simon, 1998; Krogh *et al*., 2001) was done using servers www.enzim.hu/hmmtop/ and www.cbs.dtu.dk/services/TMHMM/. All sequence analyses have been done in collaboration with Dr. Stefan Kerscher.

3 RESULTS

3.1 Subunit composition of complex I from Y. lipolytica

3.1.1 Isolation of mitochondrial complex from *Y. lipolytica* and identification of its subunits

The attachment of a hexa-histidine sequence to the C-terminus of the 30 kDa subunit (NUGM) allowed the isolation of very pure complex I from mitochondrial membranes (Kashani-Poor *et al.*, 2001a). Solubilized by n-dodecyl-β-D-maltoside, complex I was purified in two steps using Ni^{2+}-affinity and gel-filtration chromatography. After the first purification step the majority of other proteins were removed. Subsequent gel-filtration resulted in highly pure enzyme as judged by SDS-PAGE.

Using ESI-MS it has been shown that the molecular mass of isolated complex I from bovine heart mitochondria is 980 kDa (Hirst *et al.*, 2003). According to BN-acrylamide gel electrophoresis the band corresponding to *Y. lipolytica* complex I migrates slightly faster than that of complex I from bovine heart. The position of the band of complex I from *Y. lipolytica* corresponds to a molecular mass of approximately 900 kDa. NADH dehydrogenase activity of this band can be demonstrated by incubating BN-gels with NADH as electron donor and nitro blue tetrazolium as electron acceptor (Figure 3.1).

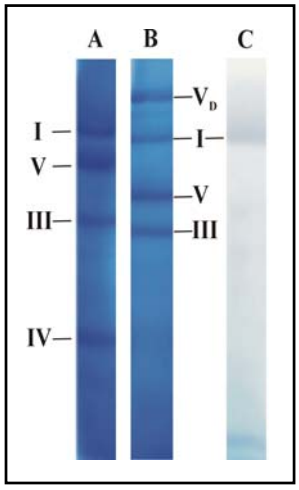

Figure 3.1 The respiratory chain complexes from B. taurus (A) and Y. lipolytica (B,C) separated by BN-PAGE

Coomassie-blue stained respiratory chain complexes from *B. taurus* (A) and *Y. lipolytica* (B), respectively; C-detection of NADH dehydrogenase activity in mitochondrial membranes from *Y. lipolytica*. The BN-gel was stained with nitro blue tetrazolium, according to the procedures described in 2.3.9. Roman numbers show individual complexes of the respiratory chain from bovine heart mitochondria.

The subunits of *Y. lipolytica* complex I were separated by doubled SDS-PAGE (dSDS-PAGE) or isoelectric focusing followed by SDS-PAGE in the second dimension. Some 39 protein spots were counted in the Coomassie- and silver-stained dSDS gels (Figure 3.2).

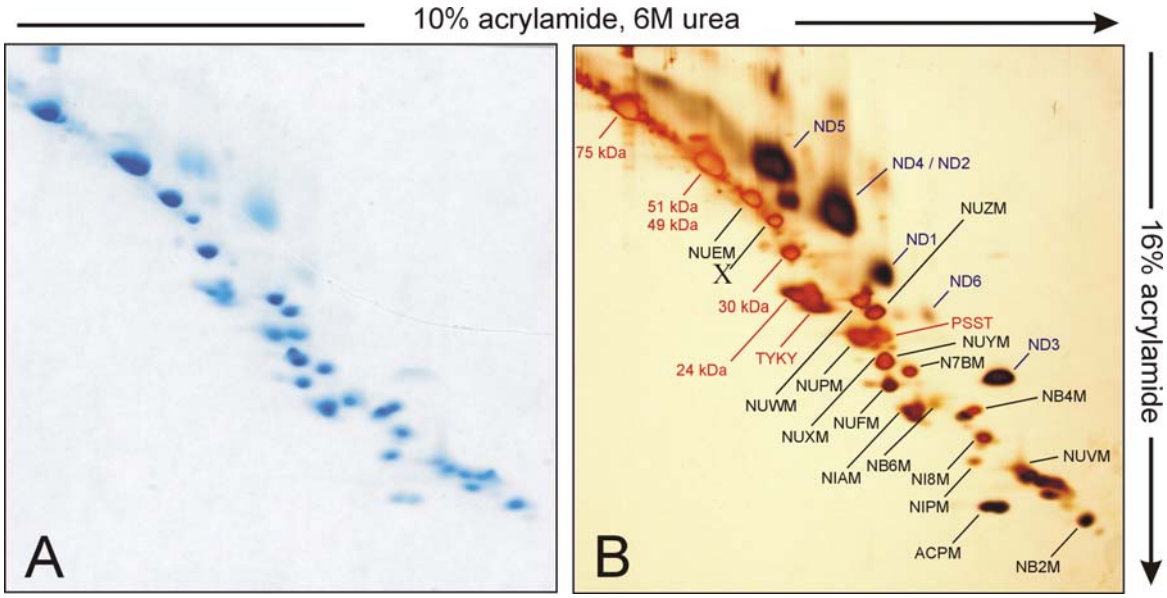

Figure 3.2 dSDS-PAGE of purified complex I from Y. lipolytica.

A-coomassie blue stained dSDS gel with 80 µg of complex I; B-silver stained dSDS gel with 60 µg of complex I. The seven central, nuclear coded and six of the seven central, mitochondrially coded subunits are labeled in red and blue, respectively, using bovine nomenclature. Spots that could be assigned to individual accessory subunits by mass spectrometry are labeled in black using the *Y. lipolytica* nomenclature. The protein labeled "X" is a complex I associated protein which shows homology to the thiosulfate sulfurtransferase enzyme family.

As shown in Figure 3.2 the well separated complex I subunits were dispersed around the diagonal, with the hydrophobic proteins found above this diagonal. It is apparent from the picture that the level of the protein labeled "X" may be substoichiometric.

By combination of isoelectic focusing as the first dimension and Tricine-SDS PAGE in the second dimension 32 protein spots were identified. However, this method did not allow the separation of highly hydrophobic gene products and therefore the mitochondrially encoded "ND" proteins were not detectable (Figure 3.3).

In order to systematically identify the proteins, all 39 protein spots were excised from dSDS gels and digested with trypsin.The peptide mixture was analysed by MALDI-MS using peptide mass fingerprint (PMF).

Thirty seven subunits of complex I representing a total molecular mass of about 950 kDa were identified (Tables 3.1 and 3.2). The sequence of the 14 central subunits had been determined earlier from the cloned genes (Djafarzadeh *et al.*, 2000) or in the case of the ND-subunits from the mitochondrial genome of *Y. lipolytica* (Kerscher *et al.*, 2001a). The identity of the seven central hydrophilic proteins in dSDS gels was defined using MALDI-MS. The seven

mitochondrial encoded proteins were assigned based on their predicted molecular mass and their strong hydrophobicity that places them clearly above the electrophoretic diagonal.

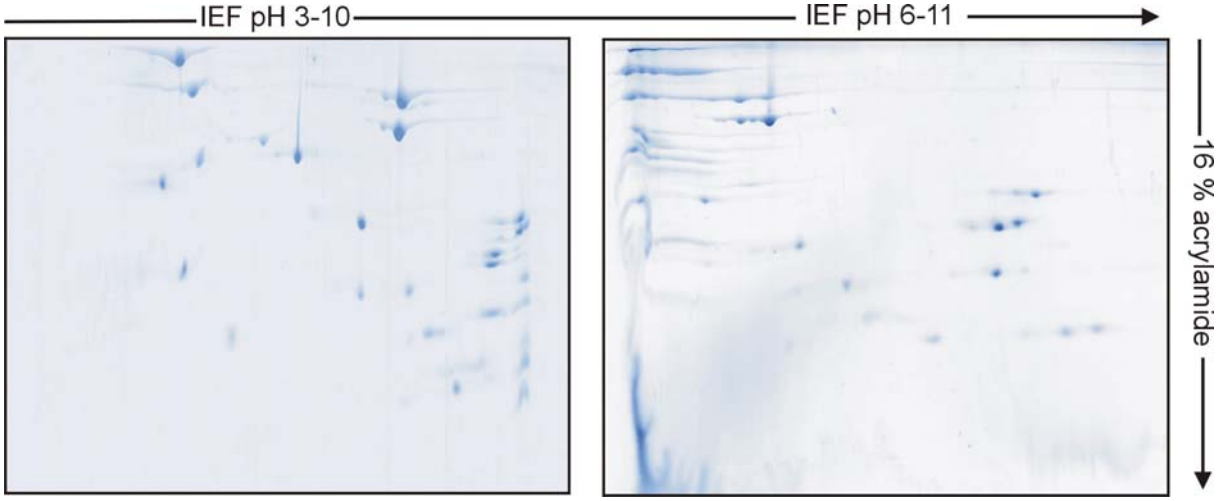

Figure 3.3 Coomassie stained 2D PAGE (IEF / SDS-PAGE) of purified complex I from Y. lipolytica.

The first-dimension separation of purified complex I subunits (100µg) was carried out by isoelectic focusing (IEF) in the pH ranges of 3-10 and 6-11. As second dimension 16 % Tricine-SDS acrylamide gel was used. Some 32 protein spots were counted. The seven central mitochondrially encoded subunits were not detectable.

By searching the *Y. lipolytica* genome database, the sequences of 23 accessory subunits were found, based on their homology to *B. taurus* (Table 3.2) and *N. crassa* subunits. Sixteen of these subunits were identified in the purified protein using MALDI-MS (Table 3.2, appendix). The N-termini of nine of the accessory subunits had previously been sequenced by Edman degradation. One extra protein named "X" exhibited homology to the thiosulfate sulfurtransferase enzyme family and could be regarded as the 24th accessory subunit of *Y. lipolytica* complex I with yet unknown function.

Gene name	*Bos taurus* homologue	Protein mass		Amino-terminal sequence	TMDs[4]	MALDI-TOF[5]	
		precursor	Mature			matching peptides	sequence coverage
		Da	Da			found/total	%
NUAM[1,]	75 kDa	78,701	75,195	AEIELT[1]	0/0	7/72[6]	11[6]
NUBM[1]	51 kDa	53,753	51,657	ATTQDA[1]	0/0	8/56[7]	21
NUCM[1]	49 kDa	52,427	49,942	ATTALP[1]	0/0	3/43	18
NUGM[1]	30 kDa	32,344	29,225	QAAPSS[1]	0/0	5/31	15
NUHM[1]	24 kDa	27,215	24,067	IVSVHR[3]	0/0	4/20	12
NUIM[1]	TYKY	25,650	22,320	APATDS[3]	1/0	6/23	54
NUKM[1]	PSST	23,429	20,425	SAPAGT[1]	0/0	5/19[7]	26

gene name	*Bos taurus* homologue	predicted mass	
NU1M[2]	ND1	38,345	10/10
NU2M[2]	ND2	53,328	14/13
NU3M[2]	ND3	14,469	3/3
NU4M[2]	ND4	54,477	13/12
NU5M[2]	ND5	73,701	18/16
NU6M[2]	ND6	20,757	5/5
NULM[2]	ND4L	9,810	2/2

Table 3.1: Central subunits of Yarrowia lipolytica complex I

[1] Djafarzadeh et al., 2000; [2] Kerscher et al., 2001; [3] Kerscher et al., submitted; [4] transmembrane domains predicted using servers www.enzim.hu/hmmtop/ and www.cbs.dtu/services/TMHMM/; [5] data for tryptic peptides, using 50 ppm error; [6] data for V8 (DE) digested peptides, using 50 ppm.

gene name	*Bos taurus* homologue	protein mass		Amino-terminal sequence	TMDs[4]	MALDI-TOF[5]	
		precursor	mature			matching peptides	sequence coverage
		Da	Da			found/total	%
NUEM	39 kDa	42,704[2]	40,432	MNSFEN	0/0	4/43	14
"X"protein		34,600			0/0	10/33	42
NUWM	ESSS	28,434[2]	23,436	FALRAY	1/0	13/33	55
NUZM	-	19,748[3]	19,748[3]	MLPGGP	0/0	8/21	38
NUYM	18 kDa	24,995[2]	19,219	QKKDVP	0/0	4/22	59
NUPM	PGIV	17,375 [2]			0/0	4/20	25
NUXM	-	18,564[3]			3/2	4/19	26
N7BM	B17.2	18,504[3]			0/0	4/19	34
NUJM[1]	B14.7	17,307[2]			2/2		
NUFM	B13	16,634[3]	15,571[3]	NVSKGV	0/0	4/16	41
NIAM	ASHI	17,305[3]		IRASFD	1/1	6/17	57
NI2M	B22	15,266[2]			0/0		
NB6M	B16.6	14,091[3]			1/1	6/16	30
NB4M	B14	11,965	11.965	AIIATA	0/0	4/24	58
NB8M	B18	15,896[3]	11,067	AEFPPL	0/0		
NUMM	13	14,256[2]			0/0		
NIDM	PDSW	12,859[3]			0/0		
ACPM	SDAP	12,039[3]			0/0	5/20	44
NUVM	B15	10,478[3]	10,347	VELKPS	1/1	4/13	42
NIPM	15	10,018[3]			0/0	5/14	33
NIMM	MWFE	9,792[3]			1/1	4/10	35
NB2M	B12	9.423[2]			1/1	4/14	28
NI8M	B8	8,700[2]			0/0	5/19	38
NI9M	B9	7,835[3]			1/1		

Table 3.2: Accessory subunits of Yarrowia lipolytica complex I

[1] TREMBL entry for *B. taurus*: Q8HXG6; [2] single exon predicted; [3] two exons predicted; [4] transmembrane domains predicted using servers http://www.enzim.hu/hmmtop/ and www.cbs.dtu/services/TMHMM/; [5] data for tryptic peptides, using 50ppm error.

3.1.2 Database search for homologues of complex I subunits from *Y. lipolytica*

Homologous subunits of *Y. lipolytica* complex I were identified by searching the *Y. lipolytica* genome with sequences of human, *B. taurus, N. crassa, A. thaliana* and *C. reinhardtii* complex I proteins. The results of database searching are summarized in Tables 3.3 and 3.4. The subunits from the different organisms were named according to their molecular mass or in some cases according to the first four amino acids of the mature subunit. To avoid possible confusion the SwissProt nomenclature and accession number of the complex I subunits was used in this study.

The 14 central subunits representing the minimal form of complex I are highly conserved between different species. In most eukaryotic organisms, the seven hydrophilic subunits (NUAM, NUBM, NUCM, NUGM, NUHM, NUIM (TYKY), NUKM (PSST)) forming the NADH-dehydrogenase part of the enzyme are nuclear coded. However, in higher plants the NUCM and NUGM proteins are encoded by the mitochondrial *nad7* and *nad9* genes, respectively (Heazlewood *et al*., 2003). All central nuclear encoded subunits were found in *Y. lipolytica* at the genomic (Djafarzadeh *et al.*, 2000) and proteomic level. On average, the sequences of the nuclear encoded proteins from *Y. lipolytica* have about 50 % identity (Table 3.3) to their orthologues in other species. The mitochondrially encoded proteins (NU1M-NU6M, NULM) of *Y. lipolytica* complex I exhibited highest sequence similarity to fungal and higher plant proteins (Table 3.3).

By detailed database searching the orthologues for 23 accessory subunits of *Y. lipolytica* complex I were identified (Table 3.4). Seventeen of them are conserved among the eukaryotes analyzed here.

Two ACPM (YALI0D24629g and YALI0D24643g) orthologs were found in the *Y. lipolytica* genome. The proteins sequences have an average identity of 43 % and both could be aligned with the ACPM subunits from bovine, *N. crassa, A. thaliana* and *C. reinhardtii* complex I. The difference between sequence similarities of both subunits and their orthologs in other species were insignificant (Table 3.3). However, the ACPM1 (YALI0D24629g) subunit was directly identified in *Y. lipolytica* complex I by MALDI-MS analysis (Table 3.2).

The molecular mass of the NB4M subunits from various species is about 15 kDa. The predicted mass of the *Y. lipolytica* NB4M subunit, derived from a single large open reading frame corresponded to a molecular mass of 21.3 kDa. However, only the first half of the predicted *Y. lipolytica* translation product could be aligned with the *N. crassa* homologue, while the second half was absent in the *N. crassa* protein. Searching for splicing donor and acceptor sites (CTGAG and CTAACTCAG in *Y. lipolytica*, respectively) revealed that the

NB4M protein in *Y. lipolytica* in fact consists of two exons (see appendix). The second exon is very short and encodes only three amino acids. The molecular mass of the NB4M subunit predicted from these two exons (11.9 kDa) corresponded to its localisation in 2D gels (Figure 3.2).

For eleven subunits of bovine complex I no homologue could be detected in *Y. lipolytica*, *N. crassa* and in photosynthetic eukaryotes like *A. thaliana* and *C. reinhardtii* (Cardol *et al.*, 2004). They are NIKM, NINM, NIGM, NI9M, NUML, NUOM, N4AM, N4BM, NISM, BN7M, NUDM. All of them seem to be specific to mammalian complex I.

The NUZM subunit that was detected in the genomes of *Y. lipolytica* and *N. crassa* is conserved among fungi and exhibits no significant homology to any of the subunits of the other species. The 9.5 kDa protein from fungal complex I was proposed to be homologous to bovine subunit B9. However, Cardol and coworkers (Cardol *et al.*, 2004) showed that these subunits are probably not homologues to each other. Thus, NI9M and NUZM present a set of fungus-specific subunits. In addition to these two proteins, *N. crassa* has another subunit (NURM), which was not found in the genome of other eukaryotes. Previously reported as being fungus specific, the *Y. lipolytica* NUVM subunit (Abdrakhmanova *et al.*, 2004) was also detected in mammalian complex I. The sequence of this subunit shares 58 % similarity and 37 % identity with the 7 kDa XP_322246 protein from *N. crassa*. On the other hand the sequences of the bovine B15 and the *N. crassa* 7 kDa polypeptides exhibit 36 % similarity and 24 % identity. Their hydrophobicity profiles match closely in the region aligned with the fungal protein sequence (Cardol *et al.*, 2004). Based on these similarities it could be proposed that the NUVM subunit of *Y. lipolytica* complex I is the orthologue of the bovine B15 (NB5M) protein.

No homologues could be identified for the *Y. lipolytica* 23 kDa subunit (NUWM) in standard BLAST searches of the bovine and *N. crassa* genomes. Detailed sequence analysis of NUWM provided evidence that this protein could be an orthologue of the NESM subunit. This will be described in more detail below.

Finally, a novel protein named “X” with a molecular mass of 34.6 kDa was identified from the 2D gel of purified *Y. lipolytica* complex I using MALDI-TOF-MS. The analysis of fingerprint data revealed that this protein shows close sequence similarity to the thiosulfate sulfurtransferase enzyme family and exhibits no homology to any other known subunit of complex I.

Table 3.3

Y. lipolytica (37subunits)	*H. sapiens* S/I	Mammals (*B. taurus*) S/I	Fungi (*N. crassa)* S/I	Higher plants (*A. thaliana*) S/I	Green algae (*C. reinhardtii*) S/I
Central subunits					
NUKM	74/65	72/65	80/75	67/63	78/72
NUIM	67/61	66/60	77/72	62/57	57/52
NUHM	62/54	63/54	67/60	53/44	58/49
NUGM	57/48	60/52	70/59	56/47	56/46
NUCM	73/64	75/66	77/72	71/63	68/60
NUBM	75/68	75/69	80/75	76/69	73/67
NUAM	63/53	63/53	75/69	59/51	59/51
NU1M	53/42	56/44	65/53	53/42	52/43
NU2M	39/23	40/24	53/38	39/27	42/25
NU3M	42/32	43/33	46/38	46/36	46/32
NU4M	46/32	46/32	56/42[1]	49/37	49/35
NULM	45/27	46/29	64/52	47/39	43730
NU5M	47/35	48/34	60/47	50/39	47/36
NU6M	35/20	35/20	50/35[1]	47/31	45/29
Accessory subunits (Eukaryote-specific subunits (17 subunits))					
NIMM	42/31	41/31	53/38	48/36	35/28
ACPM1/ACPM2	46/35 47/37	55/42 54/38	62/57 56/53	51/45 51/39	51/44 44/36
NI8M	40/34	41/35	46/34	41/32	41/34
NB2M	38/33	39/32	51/49	45/38[2]	47/36
NUFM	47/35	46/35	43/34	49/35	37/26
NUMM	43/31	44/32	50/44	36/29	34/25
NB4M	38/26	41/28	52/43	33/22	35/19
NESM*	47/32	50/34	44/38	34/24	67/38
NIPM	37/32	33/30	45/34	40/32	40/32
NB6M	50/42	49/40	60/48	48/37	41/35
N7BM	44/38	43/37	65/53	47/35	43/34
NB8M	42/27	43/27	43/27	46/39	49/38
NUYM	43/33	44/33	62/50	43/35	47/34
NUPM	44/32	46/36	65/58	37/28	29/21
NI2M	36/26	37/27	47/36[1]	41/32	41/30
NIDM	32/20	33/18	48/38	42/33	22/19
NUEM	44/33	47/34	63/50	47/32	45/34
Subunits identified in at least two lineages (4 subunits)					
NUJM	41/38	45/36	39/31	N.I.	36/28
NB5M	41/29	30/17	58/37	N.I.	N.I.
NIAM	32/22	35/23	39/30	N.I.	N.I.
NUXM		N.I.	52/44	36/27	34/27
Fungus-specific subunits (2 subunits)					
NI9M		N.I.	45/36	N.I.	N.I.
NUZM		N.I.	46/42	N.I.	N.I.

Table 3.3 Sequence similarity and identity of complex I subunits

The subunit sequences were alingned with their orthologues from Y. lipolytica using the GAP progroamm from the HUSAR (genius.embnet.dkfz-heidelberg.de) program package in standard mode. S-sequence similarity in percent; I-identity in percent. *using “gap extention penalty” 1 and allowing gap extensions longer than 20 amino acids. [1] -*Podospora anserina* sequences [2] - *Oryza sativa* gene product identified in complex I.

Table 3.4

SwissProt Nomenclature (for mammals and fungi)	Bacteria (*E. coli*) 14 subunits	*H. sapiens*	Mammals (*B.taurus*) 46 subunits[0]	Fungi (*N. crassa*) 39 subunits	Higher plants (*A. thaliana*) 41 subunits	Green algae (*C. reinhardtii*) 42 subunit	Yeasts (*Y. lipolytica*) 37 subunits
Bacterial NADH dehydrogenase orthologues (14 subunits)							
NUKM	NUOB	NDUFS7	PSST (20) P42026	19.3 O47950	24 At5g11770	18 AAQ6369	YALI0F06050g CAB65525
NUIM	NUOI	NDUFS8	TYKY (23) P42028	21.3c Q12644	25.5 At1g16700	23 AAQ63697	YALI0F00924g CAB65524
NUHM	NUOE	NDUFV2	24 M22539	24 X78083	28.3 At4g02580	27 AAQ63695	YALI0D00737g CAB65523
NUGM	NUOC	NDUFS3	30 P23709	31 P23710	ND9 (22.6) Q95748	25 AAQ55457	YALI0F02123g CAB65522
NUCM	NUOD	NDUFS2	49 S04104	49 X54508	ND7 (44.6) P93306	43 AAQ63700	YALI0F17248g CAB65521
NUBM	NUOF	NDUFV1	51 P25708	51 P24917	53.5 At5g08530	50 AAQ63696	YALI0B20372g CAB65520
NUAM	NUOG	NDUFS1	75 J02877	78 X57602	81.5 At5g37510	75 AAQ73136	YALI0D05467g CAB65519
NU1M	NUOH	ND1	ND1 (36) P03887	ND1 (42) P08774	ND1 (36) NP_085565	ND1 (31.6) AAB93446	ND1 CAC28089 [7]
NU2M	NUON	ND2	ND2 (39) P03892	ND2 (66) A25096	ND2 (55) NP_085584	ND2 (42.4) AAB93444	ND2 CAC28115 [7]
NU3M	NUOA	ND3	ND3 (13) P03898	ND3 Q35141	ND3 (14) NP_085553	14 AAQ55461	ND3 CAC28116 [7]
NU4M	NUOM	ND4	ND4 (52) P03910	ND4 S02153[1]	ND4 (55) NP_085518	ND4 (48.7) AAB93441	ND4 CAC28103 [7]
NULM	NUOK	ND4L	ND4L (11) P03902	ND4L (10) P05509	ND4L (11) NP_051111	24.2[3] AAO61142	ND4L CAC28106 [7]
NU5M	NUOL	ND5	ND5 (67) P03920	ND5 (80) P05510	ND5 (74) NP_085478	ND5 (59) AAB93442	ND5 CAC28107 [7]
NU6M	NUOJ	ND6	ND6 (19) P03924	ND6 S02156[1]	ND6 (23.5) NP_085495	ND6 (17.7) AAB934	ND6 CAC28088 [7]
Eukaryote-specific subunits (17 subunits)							
NIMM		NDUFA1	MWFE (7.5) Q02377	9.8 CAE85571	7.5 At3g08610	7.5[3] AAS48198	Not assigned yet
ACPM		NDUFAB1	SDAP (8) P52505	9.6 S17647	14 At1g65290	14[3] AAQ73138	YALI0D24629g and YALI0D24643g
NI8M		NDUFA2	B8 X63219	10.5 Q07842	10.8 At5g47890 41/32	11 AAQ63699	YALI0C03201g
NB2M		NDUFB3	B12 Q02365	10.6[3] XP_331394	7 AK059007[2]	6.5[3] AAS48194	Not assigned yet
NUFM		NDUFA5	B13 P23935	29.9 P24919	19.2 At5g52840 49/35	18 AAQ73139	YALI0E23089g
NUMM		NDUFS6	13 S28238	18.4[3] EAA26933	12.2 At3g03070 36/29	13 AAQ64639	YALI0D19030g
NB4M		NDUFA6	B14 X63211	14.8 CAA53963	15 At3g12260 33/22	14 AAQ84469	YALI0A01419g
NESM		NDUFB11	ESSS (14.5) Q8HXG5	11.7[3] XP_324110	13[3] At3g57785	17 AAS48192	YALI0E29095g
NIPM		NDUFS5	PFFD (15) Q02379	11.5[3] EAA31476	14 At3g62790	11[3] AAQ98888	Not assigned yet
NB6M		NDUFA12	B16.6 Q95KV7	13.5[3] EAA29209	16.1 At1g04630	16 AAQ64637	Not assigned yet
N7BM		NB7M	B17.2 O97725	13.4[3] EAA31813	18 At3g03100	18 AAQ64638	YALI0B00792g
NB8M		NDUFB7	B18 Q02368	89.7[3] EAA28195	12 At2g02050	12 AAQ73135	Not assigned yet
NUYM		NDUFS4	AQDQ (18) X63215	21 P25711	17.1 At5g67590	19 AAQ64640	YALI0B14861g

Table 3.4 (continued)

NUPM		NDUFA8	PGIV (19) P42029	20.8 EAA35830	12 At5g18800	12.9[3] AAQ5546	YALI0A20680 9g
NI2M		NDUFB9	B22 S28256	18[3] CAD60692[1]	13.6 At4g34700	13.9[3] AAQ73134	YALI0D07216g
NIDM		NDUFB10	PDSW (22) Q9DCS9	12.3 X68965	12.5 At1g49140,At3 g18410	17 AAQ55459	Not assigned yet
NUEM		NDUFA9	39 X59418	40 P25284	44 At2g20360	38 AAQ55458	YALI0D24585g
Subunits identified in at least two lineages (4 subunits)							
NUJM		NDUFA11	B14.7 NP_783649	21.3b S14277	N.I.[4]	23 AAS58499	YALI0E11891g
NB5M		NDUFB4	B15 P48305	7[3] XP_322246	N.I.	N.I.	Not assigned yet
NIAM		NDUFB8	ASHI (19) S28242	20.1[3] XP_332152	N.I.[4]	N.I.[4]	YALI0D04939g
NUXM			N.I.	20.9 Q02854	9 At4g16450	13 AAQ64641	YALI0E28424g
Mammal-specific subunits (11 subunits)							
NIKM		NDUFC1	KFYI (6)[6] Q02376	N.I.	N.I.	N.I.	N.I.
NINM		NDUFB1	MNLL (7) Q02378	N.I.	N.I.	N.I.	N.I.
NIGM		NDUFB2	AGGG (8) Q02374	N.I.	N.I.	N.I.[4]	N.I.
NI9M		NDUFA3	B9[5,6] Q02371	N.I.[5]	N.I.	N.I.	N.I.[5]
NUML		NDUFA4	MLRQ (9) Q01321	N.I.	N.I.	N.I.	N.I.
NUOM		NDUFV3	10[6] P25712	N.I.	N.I.	N.I.	N.I.
N4AM		NDUFA7	B14.5a Q05752	N.I.	N.I.	N.I.	N.I.
N4BM		NDUFC2	B14.5b Q02827	N.I.	N.I.	N.I.	N.I.
NISM		NDUFB5	SGDH (16) Q02380	N.I.	N.I.	N.I.	N.I.
NB7M		NDUFB6	B17 Q02367	N.I.	N.I.	N.I.	N.I.
NUDM		NDUFA10	42 P34942	N.I.	N.I.	N.I.	N.I.
Fungus-specific subunits (4 subunits)							
NI9M			N.I.[5]	9.5[5] A44210	N.I.	N.I.	Not assigned yet
NURM			N.I.	17.8 X71414	N.I.	N.I.	N.I.
NUZM			N.I.	21.3a P19968	N.I.	N.I.	YALI0A02651g
10.4[9]			N.I	10.4 NCU01467.1	N.I	N.I	N.I
Plant-specific subunits (12 subunits)							
			N.I.	N.I.	6 At4g20150	10 AAS58501	N.I.
			N.I.	N.I.	8 At2g31490	13 AAS48193	N.I.
			N.I.	N.I.	17[3] At3g07480	19 AAS58502	N.I.
			N.I.	N.I.	25 (γCA-like)[8] At5g63510	27 AAS48195	N.I.
			N.I.	N.I.	27 (γCA-like)[8] At3g48680	N.I.	N.I.
			N.I.	N.I.	30 (γCA-like)[8] At1g47260	32 AAS48197	N.I.
			N.I.	N.I.	32 (γCA-like)[8] At5g66510	29 AAS48196	N.I.
			N.I.	N.I.	11 (NDH11) At1g67350	N.I.	N.I.
			N.I.	N.I.	16 (NDH16) At2g27730	N.I.	N.I.
			N.I.[4]	N.I.	N.I.	9 AAS58498	N.I.

			N.I.	N.I.	N.I.	16 AAS58503	N.I.
			N.I.	N.I.	N.I.	19 AAS58500	N.I.

Table 3.4 Orthology of complex I subunits from prokaryotic and eukaryotic organisms.

ND proteins are mitochondria-encoded subunits. [0] Sequence data available for 45 subunits only. Unknown subunit has a molecular mass of about 10.6 kDa. [1] *Podospora anserina* sequences. [2] *Oryza sativa* gene product identified in complex I. [3] Molecular masses predicted from sequence analyses only. (If not indicated otherwise, molecular masses of nuclear coded subunits are based on data from proteomic analyses). [4] No clear homologous sequence was identified. [5] Previously described as orthologues. SwissProt continues to use the same name for them, but this should be revised. [6] No related sequences in non-mammalian species have been identified for these three proteins. [7] EMBL entries with the annotation "Method: conceptual translation supplied by author" use the correct *Y. lipolytica* mitochondrial code (S.Kerscher et al., *Comp. Funct. Genom.* 2, 80-90). GenBank entries which have the annotation "REVIEWED REFSEQ: This record has been curated by NCBI staff" erroneously use the *S. cerevisiae* mitochondrial code. [8] similar to bacterial γ-type carbonic anhydrases, [9] not annotated yet.

3.1.3 The NUWM subunit of *Y. lipolytica* complex I

As reported previosely (Abdrakhmanova *et al.*, 2004) no homologue of the complex I subunit NUWM from *Y. lipolytica* was revealed by standard BLAST searching in fungal and mammalian genomes. Similarly, no homologous sequence for the NURM subunit of *N. crassa* complex I was found in the genomes of other organisms. It was proposed, that these two subunits are specific to complex I from *Y. lipolytica* and *N. crassa*, respectively.

The NUWM and NURM proteins have N-terminal targeting sequences which are cleaved off during mitochondrial import (45 and 26 amino acids in the NUWM and NURM, respectively. See appendix). Although both subunits show no sequence similarity they are comparable in size (23.4 kDa for NUWM and 17.8 kDa for NURM) and for both proteins a single transmembrane domain was predicted. Remarkably, the extramembranous domains of the two proteins share common characteristics. While one domain is small and fairly acidic, the other has a high content of both acidic and basic residues. Three aspartates and three glutamates, but no basic residues are found within the 31 N-terminal amino acids of the mature *N. crassa* NURM subunit, resulting in a theoretical pI of 5.4. Similarly, seven aspartates, six glutamates, but also four arginines and seven lysines are found in the 64 C-terminal amino acids of the mature *Y. lipolytica* NUWM subunit, resulting in a theoretical pI of 5.2. These domains are likely to reside in the mitochondrial matrix. In contrast, the 111 amino acid C-terminal domain of *N. crassa* NURM has five arginines, eleven lysines, six aspartates and ten glutamates and a theoretical pI of 7.8. The 122 amino acid N-terminal domain of *Y. lipolytica* NUWM has nine arginines, thirteen lysines, seven aspartates and thirteen glutamates and a theoretical pI of 9.3. These domains are likely to reside in the intermembrane space.

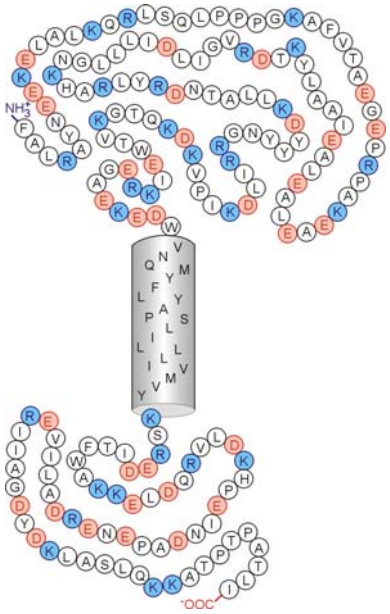

Figure 3.5 Predicted structural fold of the NUWM subunit.

The predicted membrane helix is shaded in gray; basic and acidic residues in the extramembraneous domains are shown in blue and red, respectively. The N-terminal domain of the subunit is predicted to point towards the intermembrane space.

In bovine heart complex I several subunits are present that are designed in a similar fashion as *Y. lipolytica* NUWM. These include subunits which have no counterpart in the *Y. lipolytica* enzyme (NESM, NB7M, NISM, NUML, NIGM, N4BM, NINM, NIKM; Table 3.4) and subunits for which homologues could be detected in *Y. lipolytica* (NIAM, NB6M, NB2M, B9M, NIMM; Table 3.4). The NB7M, NISM, NUML, NIGM, N4BM, NINM, NIKM are also missing in *N. crassa*, *A. thaliana* and *C. reinhardtii* complex I. Remarkably, the NESM subunit was identified in fungal and higher plant genomes recently (Cardol *et al.*, 2004). The NESM protein from *Chlamydomonas* complex I displays significant similarity to complex I proteins from rice and to an 11.7 kDa unknown protein from *N. crassa*. By position-specific iterative BLAST analysis (PSI-BLAST) it was revealed that these four proteins are distant relatives to the bovine NESM (ESSS) subunit of complex I. Interestingly, the PSI-BLAST search showed that the NUWM subunit from the *Y. lipolytica* and the NESM subunit from *N. crassa* are putative homologues. The NESM subunits from *N. crassa* (11.7 kDa) is much smaller than NUWM (23.4 kDa), but seems to have a similar design with a single transmembrane domain and two highly charged extramembraneous domains (Figure 3.5). By sequence alignment allowing longer gaps than in standard analysis it was found that both proteins share 44% similarity and 38 % identity (Table 3.3).

```
Y.l_NUWM      MLKLHYRNFITAQHSTTNTTPTMIASVCKRAGLRAGPRAYPGVRQFALRAYNEEKELALK 60
N.c_NESM      ----------------------MPAPTILRAGALASRRAFSTSRAVRSGGAPHYD----- 33
                                    * *..  ***  *. **:.  * .   .  . .

Y.l_NUWM      QRLSQLPPPGKAFVTAEGEPRPAKEAELAELAEIAALYKTDRVGILDILLLGNKHARLYR 120
N.c_NESM      ------PPSGWLFGVRPGE---------------------------------------- 46
                    **.*  * .  **

Y.l_NUWM      DNTALLKDYYYNGRRILDKIPVKDKQTGKVTWEIKREGAEKEDWVNQMYFLYAPSLILLL 180
N.c_NESM      --------------------------------EYKREG-----WEIPFFYGFCGSFAVAT 69
                                             * ****     *   ::: :. *: :

Y.l_NUWM      IVMVYKSREDITFWAKKELDQRVLDKHPEINDAPENERDALIVERIIAGDYDKLASLQKK 240
N.c_NESM      IAYAFKPDTSIQTWALEEARRRLEAEG--ILEDPHPEK---------------------- 105
              *. .:*.  .*  ** :*  :*:  :   * : *. *:

Y.l_NUWM      ATPTPATLI 249
N.c_NESM      --------- 
```

Figure 3.6 Alignment of NUWM from Y. lipolytica and NESM from N. crassa

The sequences were aligned using the CLUSTALW program (www.ebi.ac.uk/clustalw/index.html). * - conserved amino acids; **:** - conserved substitutions; . - semi-conserved substitutions. Small and hydrophobic amino acids are red, acidic are blue, basic are magenta; hydroxyl + amine + basic are green. The transmembrane damain is shown in grey background.

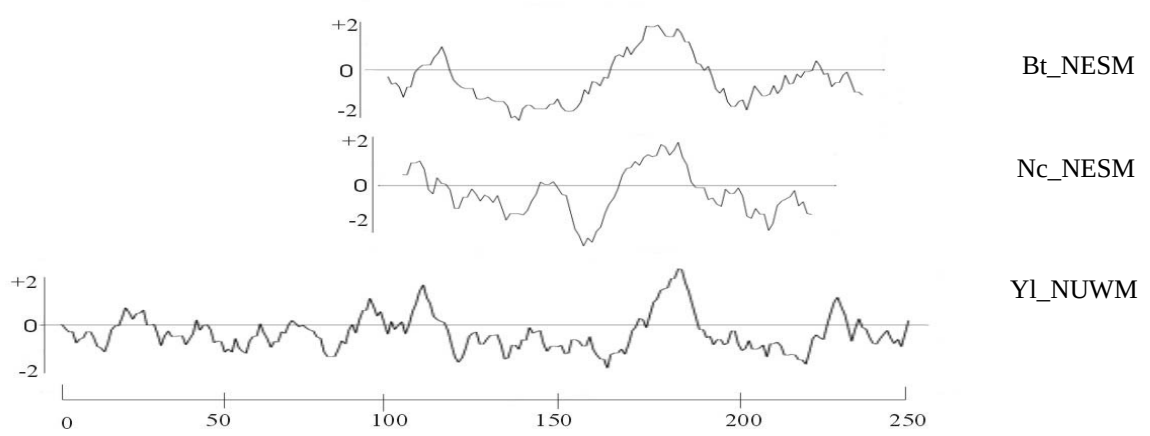

Figure 3.7 Comparison of the hydrophathy profiles of the NUWM and NESM subunits

The hydrophathy profiles were done using the HUSAR (htt://genius.embnet.dkfz-heidelberg.de/) program package. The NUWM protein from *Y. lipolytica* (Yl) is not compatible in size with the NESM subunits from bovine (Bt) and *N. crassa* (Nc), but shows a similar design. A single transmembrane domain is predicted for all three proteins.

3.2 Exploring the accessory 39 kDa subunit of complex I from Y. lipolytica

3.2.1 Generation of 39 kDa subunit (NUEM) mutants

3.2.1.1 Δnuem strain

The *NUEM* gene encoding the accessory 39-kDa subunit of complex I from *Y. lipolytica* was deleted by homologous recombination with a *URA*3-marked deletion allele. First, the genomic 4.91 kb *Not*I/*Sal*I fragment was subcloned into the pBluescript SK(-) vector. A region of about 1.2 kb including the complete *NUEM* open reading frame (ORF) was replaced by a *BamH*I/*Eco*RI restricted PCR product carring the *URA*3 gene from *Y. lipolytica* in opposite orientation of the original *NUEM* gene. The 5.2 kb *Not*I/*Sal*I *URA*3 containing fragment was used for transformation of haploid *Y. lipolytica* GB10 cells (3*0Htg2 Mat*B *ndh2i ura3*-302 *leu2*-270 *lys*-1).

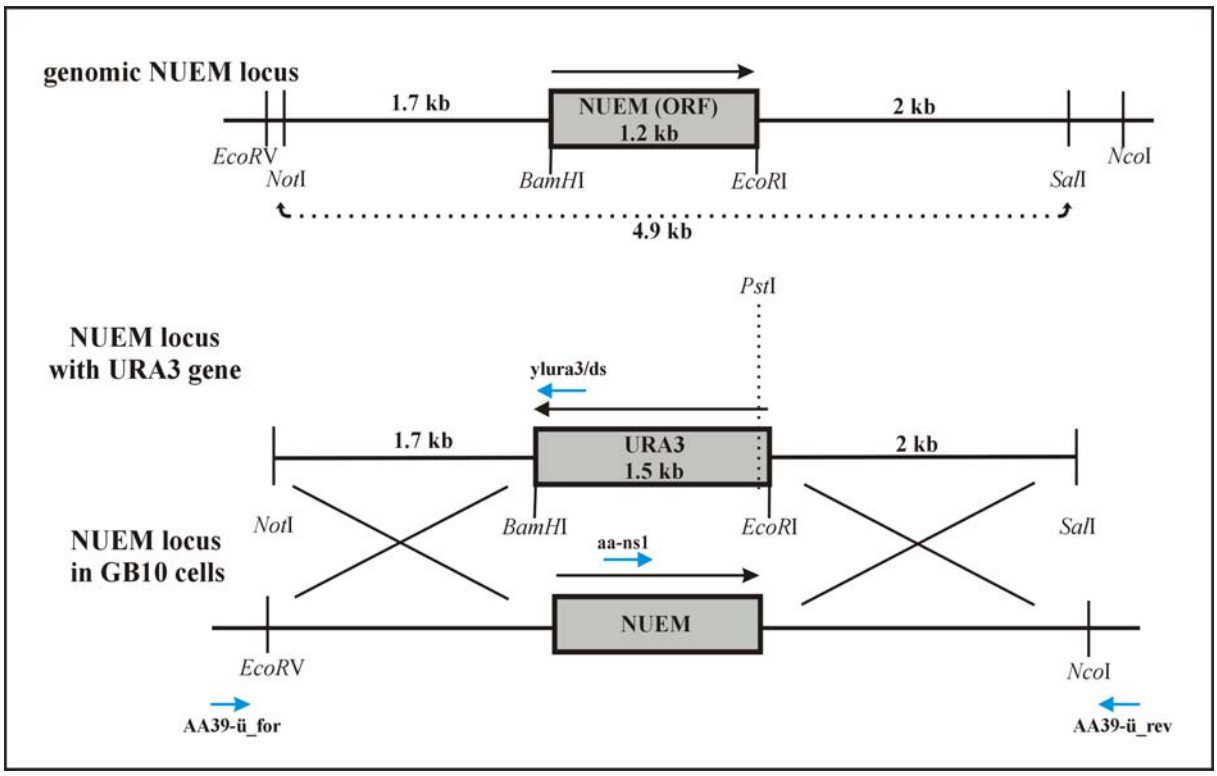

Figure 3.11 Strategy for generation of Δnuem strain

The ORF in the genomic *NUEM* locus was replaced with the *URA*3 marker gene by homologous recombination. A fragment of a construct carrying a *URA*3 gene flanked with genomic sequences was used for transformation of *Y. lipolytica* GB10 cells. The transformants were selected and screened for homologous recombination. PCR products from primer pairs (AA39-ü_for, ylura3/ds) and (AA39-ü_rev, aa-ns1) and Southern Blot were used for verification (see Figure 3.12).

The transformants were selected on minimal media without uracil and screened for homolougous recombination at the chromosomal *NUEM* locus by PCR (Figure 3.12) and Southern blot analysis (Figure 3.13). For verification, a primer pair (AA39-ü_for/ylura3/ds) was used wich resulted in a PCR-product spanning the upstream region of *NUEM* including the sequence of the inserted *URA*3 gene (Figure 3.11). Only clones with inserted *URA*3 gene resulted in a PCR-product of 1.7kb (Figure 3.12A). Additionally, the absence of the wild type NUEM gene was checked in transformants by PCR with primer pair aa-ns1 and AA39ü_rev (Figure 3.11 and 3.12B). Six correct deletion mutants were identified, but for subsequent analysis of the Δ*nuem* strain and creation of point mutants strain AA39/25.1 was used.

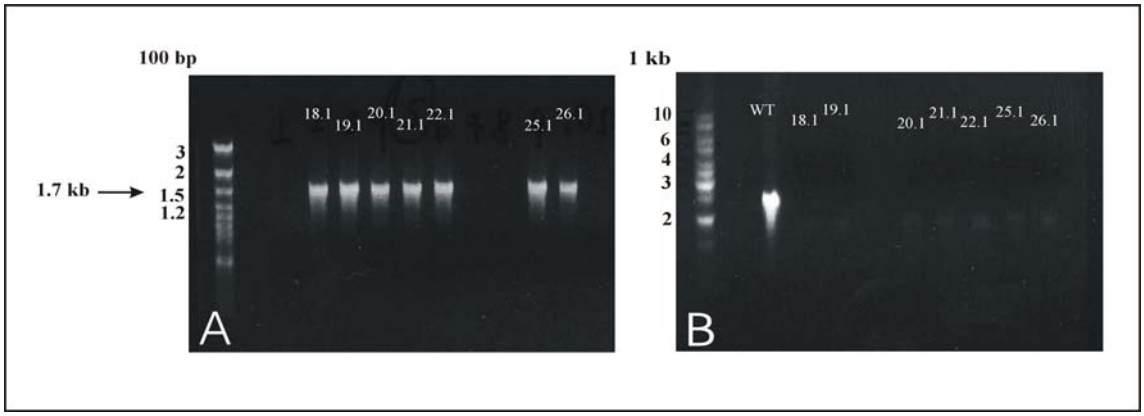

Figure 3.12 PCR test for deletion of the NUEM gene.

A) PCR product was generated with primer pair AA39-ü_for, ylura3/ds. B) PCR product was generated with primer pair AA39-ü_rev, aa-ns1. The wild type product (2.5 kb) was missing in the selected transformants.

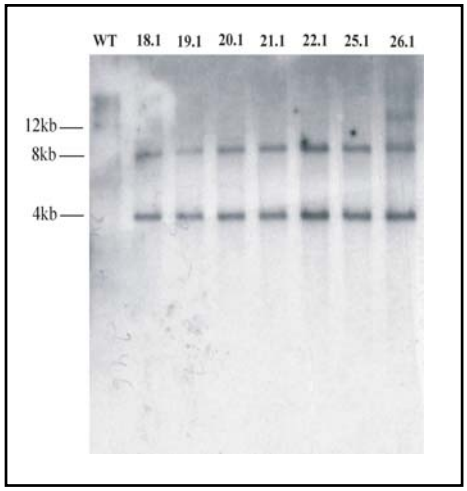

Figure 3.13 Southern Blot test to verify deletion of the NUEM gene

Genomic DNA from transformants was extracted and digested with *Pst*I. The transformants that contain the *URA3* gene have a *Pst*I site in the ORF of the marker gene (Figure 3.12) and therefore 2 bands should be detectable by a *Not*I/*Sal*I probe. Transformant 26.1 has NUEM (12 kb band) and *URA3* (8 and 4 kb band) indicating non-homologous recombination.

3.2.1.2 Site-directed mutagenesis of subunit NUEM

To create point mutations in the NUEM gene, the deletion strain was complemented with plasmid pUB4 with an insert carrying a mutated 39 kDa subunit gene. The *NUEM* gene was amplified and subcloned into pPCR 2.1. The resulting vector was cut with *Eco*RI to obtain a *NUEM* containing fragment that was cloned into "shuttle"-vector pUB4 harboring the *Hyg* B^R gene. Transformants carrying plasmid pUB4/*NUEM* were able to grow on YPD complete medium with hygromycin as selection marker instead of SD minimal medium with *URA*3 as selection marker. In the following, the Δ*nuem* strain complemented with plasmid pUB4/*NUEM* is named 'parental'. The plasmid pUB4/*NUEM* was used as a template for site directed mutagenesis in *NUEM* gene. Several point mutations in the nucleotide binding motif and of the basic residue at the end of the second β-strand were generated (Figure 3.14). Since the pyrophosphate moiety of NADPH makes close contact with the middle glycine (Lesk, 1995) in the nucleotide binding motif attention was focussed on glycine-43.

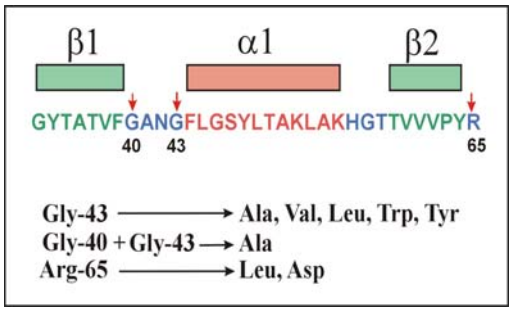

Figure 3.14 Point mutations in the NUEM gene

Mutations were generated in the nucleotide binding motif GXXGXXG and in arginine-65 supposed to be responsible for selective interaction with the 2'-phosphate group of NADPH.

3.2.2 Characterisation of 39-kDa subunit (NUEM) mutants

3.2.2.1 Δ*nuem* mutant

Mitochondrial membranes from strain Δ*nuem* of *Y. lipolytica* were isolated and analysed using BN-PAGE in the first dimension followed by Tricine-SDS-PAGE as the second dimension. First, the complexes of OXPHOS were solubilized from the membranes with 1g n-dodecyl-β-D-maltoside (LM) per g of total protein and separated under native conditions using a 4 to 13 % polyacrylamide gradient gel. As critical test to identify the assembly of complex I, the respiratory chain complexes were divided into subunits on denaturating 16% Tricine SDS-PAGE in the second dimension. As shown in Figure 3.14A, complex I of the

parental strain can be identified between complex V dimer and complex V monomer. In contrast, complex I was not observed in the deletion mutant (Figure 3.14B). Even if a lower concentation (0.7 g of LM per g protein) of detergent was used, no assembled complex I could be identified in strain Δ*nuem* (data not shown).

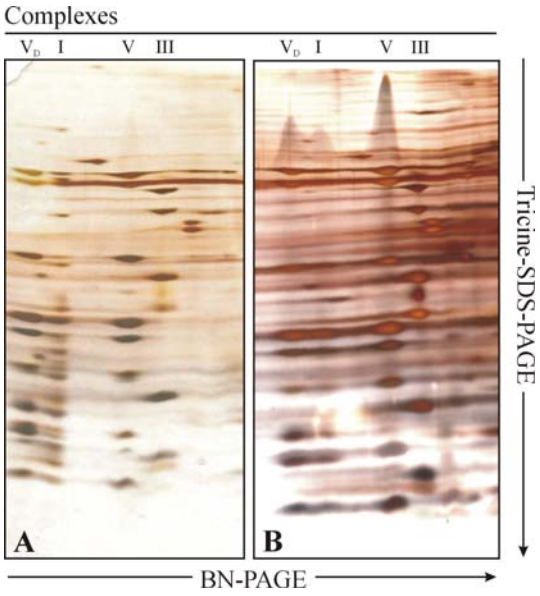

Figure 3.15 2D-PAGE of mitochondrial membranes from parental (A) and Δnuem (B) strains

The complexes of the respiratory chain were solubilized with 1g n-dodecyl-β-D-maltoside (LM) per g of total protein and loaded on a blue native gel (4-13% acrylamide gradient). In the second dimension, the complexes were separated by 10 % Tricine-SDS-PAGE. Roman numbers indicate individual complexes of the respiratory chain of *Y. lipolytica*. V_D – complex V dimeric form.

The NADH:HAR activities of isolated mitochondrial membranes from parental and deletion strains were measured. This activity is based on the ability of complex I to oxidize NADH in the presence of the electron acceptor hexammineruthenium-III (HAR). Because this process is dependent on the presence of FMN in the NUBM (51 kDa) subunit, but independent of ubiquinone oxidoreductase activity and proton pumping, the NADH:HAR activity can reflect the amount of assembled complex I in membrane preparations. Since all transformants contained the internal version of alternative dehydrogenase (NDH2i) (Kerscher *et al.*, 2001b), which is able to oxidize NADH, but not dNADH (deamino-NADH), for measurements of complex I activity dNADH was used to discriminate between complex I and the alternative enzyme.

Mitochondrial membranes from strain Δ*nuem* exhibited lower dNADH:HAR activity than wild type membranes (16 % of the parental strain activity). However, no specific ubiquinone reductase activity was detected in the deletion strain (Table 3.5). The absence of assembled complex I in BN-gels and the loss of complex I activity in membrane preparations provided evidence that complex I was not assembled in the deletion strain. The residual dNADH:HAR activity could have been due to subcomplexes containing the NUBM subunit.

3.2.2.2 Point mutations

As judged by BN-PAGE (Figure 3.16), complex I was fully assembled to near wild type expression levels only in mutants G43A, R65L, R65D. Although after replacement of glycine-43 to valine dNADH:HAR activity in membranes still corresponded to 29 % of the parental strain activity (0.4 and 1.4 µmol min^{-1} mg^{-1} for G43V and the parental strain, respectively), no assembled complex I was found in this mutant by BN-PAGE.

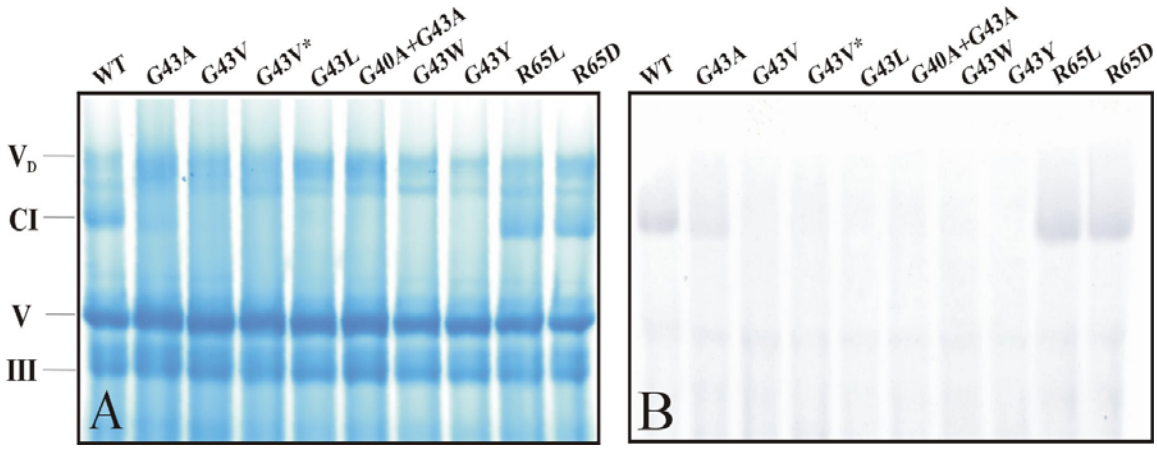

Figure 3.16 BN-PAGE of point mutants of the NUEM subunit

The respiratory chain complexes were solubilised from membranes with 1g n-dodecyl-β-D-maltoside (LM) per g of total protein. A – Coomassie stained BN-gel. B – Nitro blue tetrazolium stained BN-gel to show in-gel complex I activity. WT – parental strain, G43V*- cells were grown at 20°C, others at 27°C.

Based on the assumption that the change of glycine-43 to valine may have led to formation of a more labile enzyme, the G43V mutant was also grown at lower temparature (20°C). The respiratory chain complexes of strain G43V grown at 20°C were separated using the milder detergent digitonin described by Schägger (2003). Still, no assembled complex I was observed in a BN-gel under these conditions, but dNADH:HAR activity increased to 43 % of the parental strain activity. A very weak signal of complex I NADH dehydrogenase activity in the G43V mutant could be identified by an in-gel complex I activity assay using nitro blue tetrazolium under native condition (Figure 3.17B). Western blotting with antibodies against NUBM (51-kDa), NUCM (49-kDa) and NUEM (39-kDa) subunits revealed the presence of fully assembled complex I in small amounts in the G43V cells grown at 20°C. Remarkably, NUBM (51-kDa) and NUCM (49-kDa) subunits were found in fully assembled complex I as well as in the subcomplexes, whereas NUEM (39-kDa) subunit was detected at the front of the gel, probably as the monomeric subunit (Figure 3.18). These data show that the

replacement of glycine-43 to a valine resulted in the formation of less stable enzyme, which probably contains loosely bound subunit NUEM.

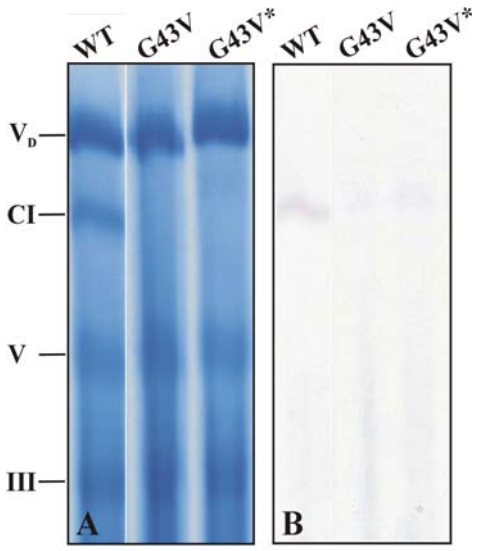

Figure 3.17 Coomassie (A) and nitro blue tetrazolium (B) stained BN gel of G43V mutant

Mitochondrial membranes from mutant G43V grown at normal (27°C) and G43V* lower (20°C) temperature were solubilised from membranes with digitonin (3g per gram of protein) and OXPHOS complexes were separated by BN-PAGE.

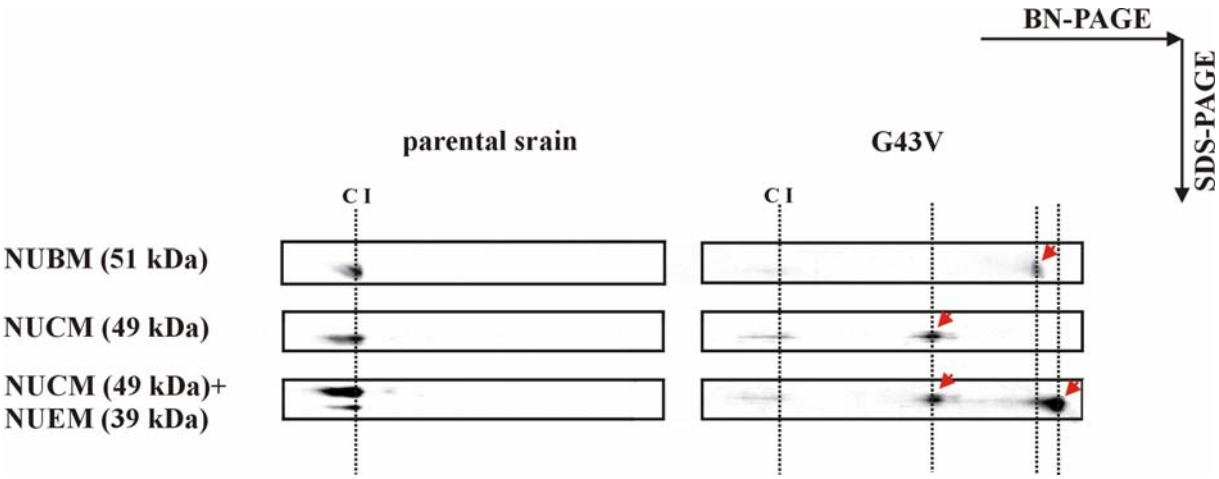

Figure 3.18 Western blot analysis of mutant G43V grown at 20°C

First dimension BN-PAGE with mitochondrial membranes was followed by Tricine SDS-PAGE as second dimension. The proteins were blotted on PVDF membrane and tested using antibodies against NUBM, NUCM, NUEM subunits. The subcomplexes are marked with red arrows. The 39-kDa subunit was found at the front of the gel, probaly as the monomeric subunit.

3.2.3 Mutants G43A, R65L, R65D

The mutation of glycine-43 to alanine (mutant G43A) took place at a position that is common to all nucleotide binding motifs whereas the replacement of argine-65 to a leucine or aspartate (mutants R65L and R65D) affected a position in the cofactor binding site that is responsible for selective binding of NADPH. As mentioned above all three mutants contained fully assembled complex I, which exibited in-gel NADH dehydrogenase activity (Figure 3.16B). Although the enzyme content in mutant mitochondria decreased to 50-60 % of the parental strain (Table 3.5), no significant changes in cells growth rates were observed (not shown). All mutants retained specific ubiquinone reductase activity of complex I (dNADH:DBQ) at the level of 80-90 % of the wild type (Table 3.5).

Assembled complex I could be purified from all three mutants according to procedures described in section 2.3.13. The use of n-dodecyl-β-D-maltoside (1:1 w/w) did not affect the subunit pattern of complex I from the mutants. All subunits were still present in the mutant enzyme in amounts comparable with parental complex I (Figure 3.19). Interestingly, the "X" protein which was identified by mass spectrometry and exhibits close similarity to the thiosulfate sulfurtransferase family was present in lower amounts in mutants G43A and R65L than in wild type. In mutant R65D, only traces of this protein could be identified.

Strain	Complex I content(%)	Vmax µmol min^{-1} mg^{-1}	Vmax (%)
Parental	100	0,41 ± 0,06	100
ΔNUEM	16	n.d	-
G43A	50	0,37 ± 0,03	90
R65D	64	0,33 ± 0,001	80
R65L	50	0,39 ± 0,04	95

Table 3.5 Complex I activity measured on mitochondrial membranes from wild type and mutants

Complex I content and activity in mitochondrial membranes of *Y. lipolytica* mutants in the NUEM subunit are compared to parental cells. Complex I content is given as specific dNADH:HAR oxidoreductase activity in mitochondrial membranes (100% = 1.4 μmol $min^{-1}mg^{-1}$), Vmax - dNADH:DBQ oxidoreductase activity normalized for complex I content (100% = 0.41 μmol $min^{-1}mg^{-1}$).

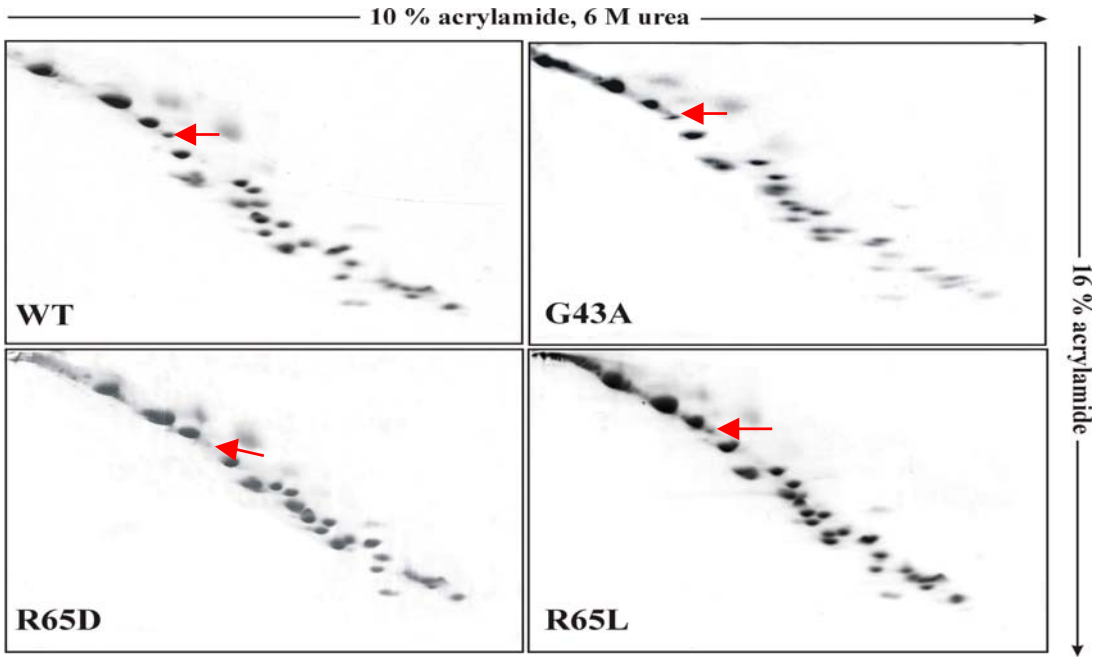

Figure 3.19 Doubled SDS-PAGE of complex I isolated from parental and mutant strains.

Coomassie stained dSDS-gels with 70-80µg of isolated complex I. All subunits could be identified in purified enzyme of the mutants, except protein "X"(noted with red arrows). This protein was present in lower amount than in parental strain or was missing (mutant R65D).

Isolated complex I was reactivated by incubation with asolectin (mixture of soybean phospholipids). All three mutants were able to oxidise NADH, but showed a decrease in specific ubiquinone reductase activity of complex I. The G43A mutant had retained about 80 % of dNADH:DBQ activity, whereas R65D and R65L had 13% and 32 % activity respectively (Table 3.6).

Strain	Complex I content (%)	Vmax µmol min^{-1} mg^{-1}	Vmax (%)
Parental	100	3,1 ± 0,2	100
G43A	60	2,4 ± 0,2	78
R65D	74	0,4 ± 0,07	13
R65L	81	1,0 ± 0,08	32

Table 3.6 Activity tests on purified complex I from wild type and mutants.

Isolated enzyme was reactivated with asolectin at a 1:1 (w/w) protein-to-lipid ratio. Complex I content is given as specific dNADH:HAR oxidoreductase activity (100% = 48 μmol $min^{-1}mg^{-1}$), Vmax - dNADH:DBQ oxidoreductase activity was normalized for complex I content measured as dNADH:HAR activity (100% = 3.1 μmol $min^{-1}mg^{-1}$).

The EPR spectra of iron sulfur clusters N1, N2, N3 and N4 of mutant complex I preparations showed no significant changes compared to the parental strain. Only in the spectrum of mutant R65D a slightly diminished intensity of cluster N2 signal was observed (Figure 3.20). Thus only those mutants that exhinited a marked decrease in catalytic activity showed rather minor changes in their EPR spectra. However, while a dramatic decrease of dNADH:DBQ activity was observed for mutant R65D, in membrane preparations of this mutant specific complex I was 80 % of the parental strain. Probably, the observed loss of activity that was observed for two of the mutants was rather unspecific and reflected instability of the enzyme during the purification procedure.

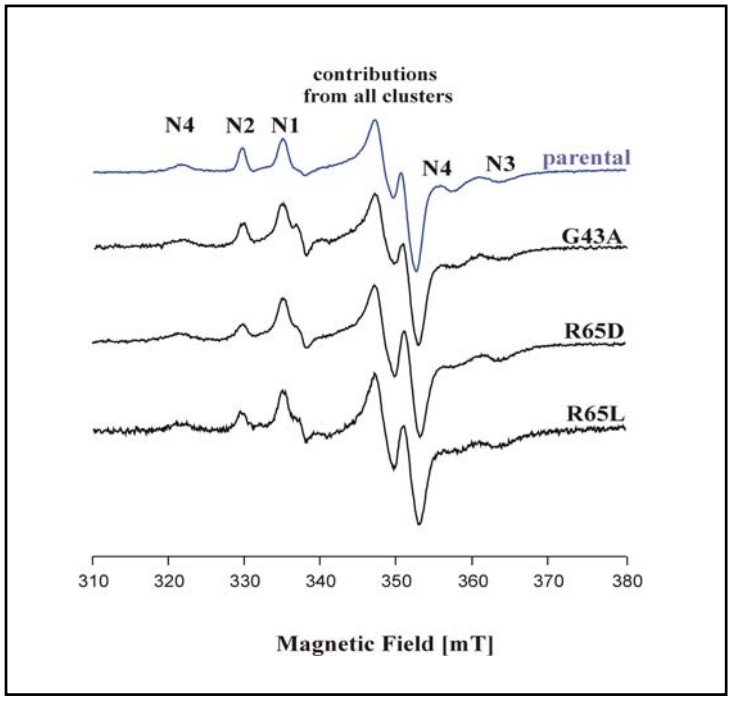

Figure 3.20 EPR spectra of complex I purified from parental strain and mutants.

EPR spectra of purified complex I from parental and mutant strains. EPR spectra of complex I 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 mT. Under these conditions spectra show contributions from clusters N1, N2, N3 and N4 (Djafarzadeh *et al.*, 2000). The N2 signal in R65D shows a slightly decrease. The spectra were obtained by Dr. Klaus Zwicker.

3.2.3.1 Determination of NADPH and FMN content in complex I

In order to determine the content of NADPH in complex I, purified enzyme was treated with an alkaline chloroform/methanol mixture. Then the extract was analysed by reversed-phase HPLC (RP-HPLC). In RP-HPLC, compounds are separated based on their hydrophobic character. Unlike traditional HPLC, RP-HPLC uses a stationary phase that is unpolar while the mobile phase is polar. Therefore the more hydrophobic the analyte the better it binds to the column and the later it will be eluted from the column.

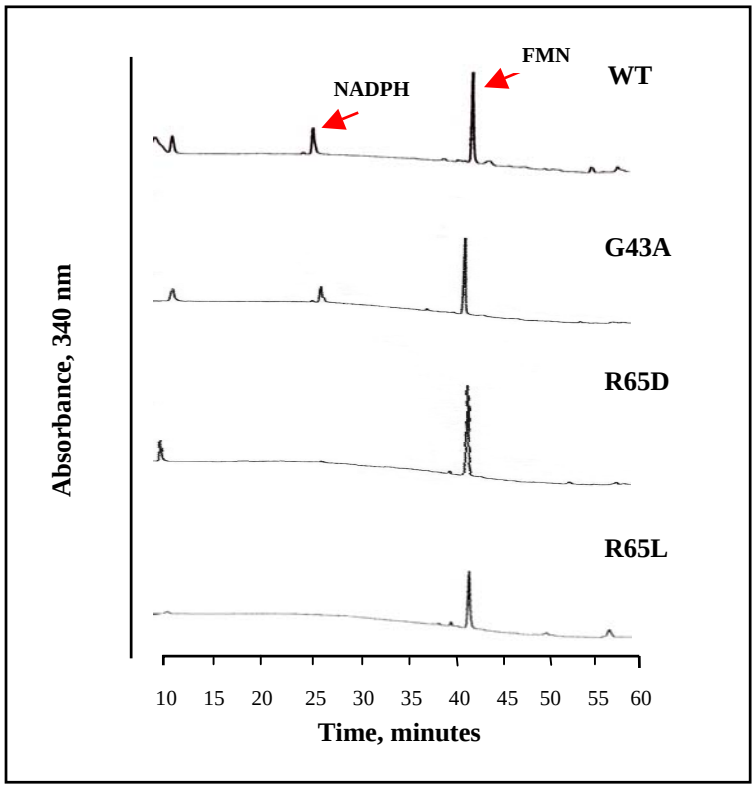

Figure 3.21 RP-HPLC elution profile of NADPH and FMN extracted from complex I of parental and mutants strains.

NADPH and FMN were extracted from purified enzyme with an alkaline chloroform/ methanol mixture. The extracts were analysed using RP-HPLC detecting at 340 nm. The peak obtained at 27 and 42-43 minutes showed the characteristic absorption spectrum of NADPH and FMN, respectively (not shown).

Analysis of wild type complex I yielded FMN and NADPH, but no NADH (Figure 3.21). As revealed by RP-HPLC with standard solutions containing 0.5-5 nmol of NADH, NADPH and FMN, NADH appeared at a retention time of 31 minutes (not shown), whereas NADPH due to its higher polarity eluted at 27 minutes retention time. This experiment provided additional evidence that the 39 kDa subunit can bind NADPH and not NADH. In the extract from G43A complex I both peaks were observed, but in lower amounts than in the parental strain. Mutants R65D and R65L did not contain NADPH, but FMN was still present in both mutants.

Compared to NADPH in standard solutions (Figure 3.22), the absorption intensity of NADPH bound to complex I from the wild type corresponded to 0.56 nmol of NADPH per mg of complex I (Table 3.7). It should be emphasized that for the calibration curve NADPH and FMN were extracted from a mixture with BSA and treated like the complex I samples. The FMN content in parental complex I corresponded to 1 nmol of FMN per nmol of enzyme, consistent with data obtained for *Y. lipolytica* complex I (Djafarzadeh, Dissertation Universität Frankfurt 2000). The mutant G43A enzyme was still able to bind NADPH, but with lower affinity than the wild type enzyme. The FMN amount in this mutant correspond to almost 90 % of the wild type value. This finding could be taken as evidence that the impairment of NADPH binding in mutant G43A was caused by changes in the nucleotide binding domain and not by global disturbance of the enzyme structure. Intactness of complex

I was also suggested by the near normal specific activity measured in mitochondrial membranes (Table 3.5).

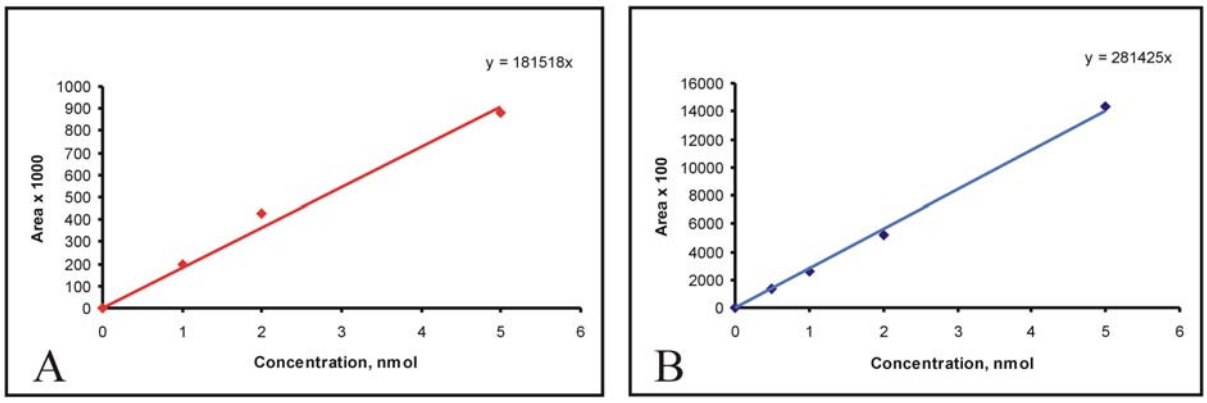

Figure 3.22 Calibration plots of NADPH (A) and FMN (B)

Aliquots of standard solutions containing 1-5 nmol of NADPH or FMN and 1mg/ml of bovine serum albumin were treated with an alkaline chloroform/methanol mixture. The extracts were analysed using RP-HPLC at 340 nm. The Y axis corresponds to the area of NADPH or FMN peaks observed at 27 and 42-43 minutes, respectively; the X axis correspond to the concentration of the nucleotides in standard solutions.

The FMN yield of R65D mutant was comparable to wild type amounts, whereas in mutant R65L its content decreased to 66 % of the parental strain. As mentioned above NADPH was missing in both mutants.

Strain	NADPH	FMN
	nmol / mg of complex I	
parental	0.56	0.93
G43A	0.35	0.81
R65D	n.d	0.99
R65L	n.d	0.61

Table 3.7 Content of NADPH and FMN in complex I from parental strain and mutants

Determination of nucleotide content in complex I was carried out using calibration solutions of NADPH and FMN in a dilution series from 0.5-5 nmol (see Fig. 3.22). The standard solutions were treated following the same procedure as for purified complex I and analysed by RP-HPLC. n.d - not detectable.

3.2.3.2 Determination of NADPH and FMN in mitochondrial membranes

It was supposed that arginine-65 is crucial for selective binding of NADPH to the 39-kDa subunit. The absence of NADPH in isolated complex I of R65D and R65L mutants and the dramatic decrease of enzyme activity could have been caused by changes of complex I structure during purification. It was also unclear wheather the mutation had abolished the protein's ability to bind the cofactor or wheather the cofactor was simply lost during the purification procedure. To answer these questions, cholate-treated mitochondrial membranes were analysed by RP-HPLC. Washing of the membrane with cholate was done to reduce the amounts soluble and of other weakly membrane-associated proteins.

Y. lipolytica membranes were prepared as described in part 2.3.3 and washed with buffer containing 1% of potassium cholate. The ratio of cholate to protein was 1:1 (w/w). The cholate-treated membranes from the parental strain and mutant R65L retained about 90% of complex I specific ubiquinone reductase activity compared to untreated membranes (Table 3.8). In contrast, washing of membranes from strain R65D with cholate containing buffer reduced complex I activity to about 50 %.

Strain	Complex I activity (%)	
	before treatment with K-Cholate	after
Parental	100	91
R65D	70	54
R65L	97	86

Table 3.8 Complex I activity tests measured in cholate-treated mitochondrial membranes prepared from wild type and mutants

The membranes were treated with potassium cholate at 1:1 (w/w) protein-to-cholate ratio. Complex I activity is given as dNADH:DBQ oxidoreductase activity (100% = 0.4 μmol $min^{-1}mg^{-1}$) normalized for complex I content in mitochondrial membranes measured as specific dNADH:HAR oxidoreductase activity (100% = 1.1 μmol $min^{-1}mg^{-1}$).

Peaks corresponding to NADPH and FMN in the R65D and R65L mutants were analysed following RP-HPLC analysis of washed mitochondrial membranes (Figure 3.23).

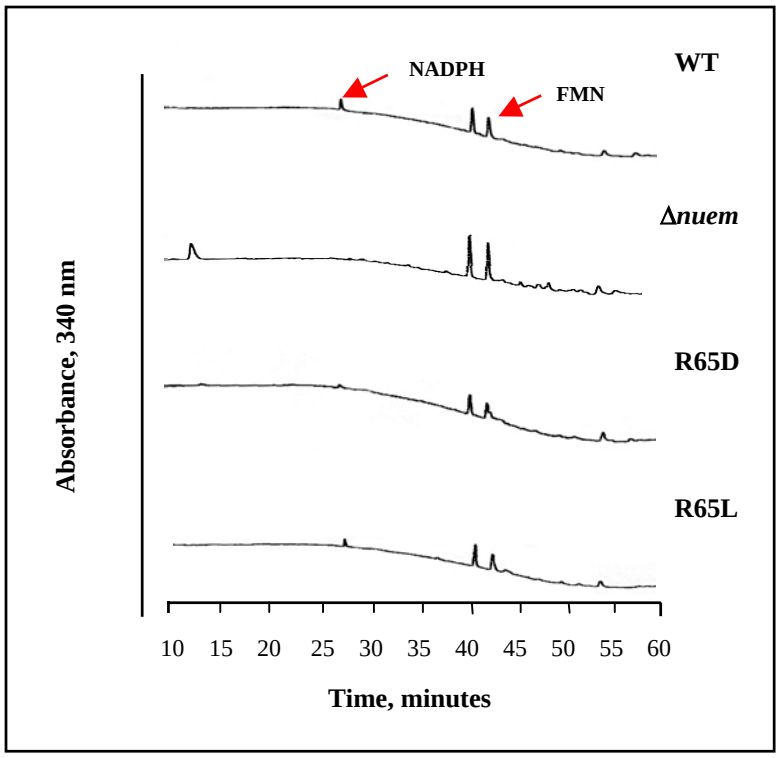

Figure 3.23 HPLC analysis of washed mitochondrial membranes from parental and mutants strains

The fragments of mitochondrial membranes were treated with 1 gram of potassium cholate per 1 gram of protein. The peaks obtained at 27 and 42-43 minutes showed the characteristic absorption spectrum of NADPH and FMN, respectively.

In both mutants FMN was present in wild type amounts, whereas NADPH content was reduced. The replacement of arginine-65 to aspartate led to a more dramatic decrease in affinity for the cofactor. Only traces of NADPH were found in this mutant. In all membranes the content of NADPH was too low for quantitative analysis. The additional peak observed at a retention time of 41-41.5 minutes may correspond to FAD, a cofactor of complex II (succinat dehydrogenase) and NDH2 (alternative dehydrogenase) and several other quinone reductases of the *Y. lipolytica* respiratory chain.

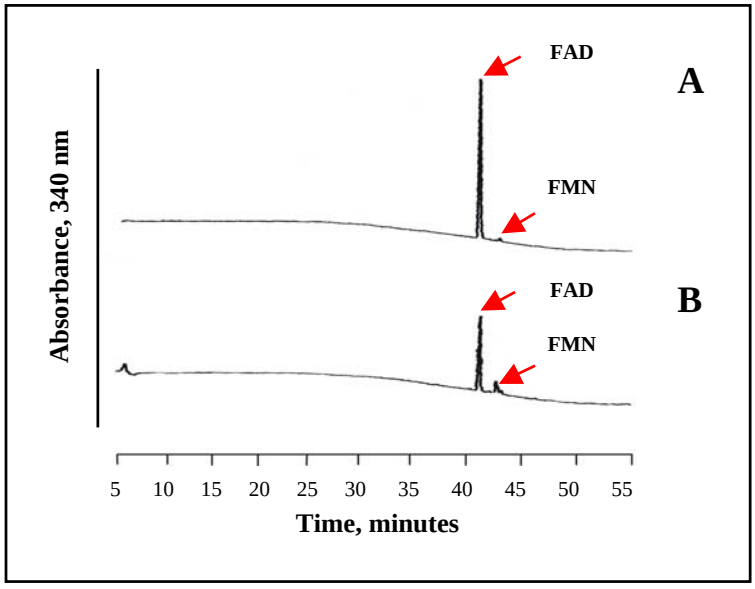

Figure 3.24 HPLC analysis of the pure FAD dissolved in water (A) and treated with alkaline chloroform/methanol mixture (B)

The FAD peak was observed at the retention time of 41.5 minutes. Only traces of FMN (43.6 minutes) were identified in a sample dissolved in water while its content was higher after treatment with alkaline chloroform/methanol mixture. Presumably, the FAD is partially turned into FMN under alkaline conditions.

In order to verify this assumption, pure FAD was treated with an alkaline chloroform/ methanol mixture. Then the extract was analysed by RP-HPLC. As shown in Figure 3.24B, two peaks were identified in the extract of pure FAD. The higher peak at the retention time of 41- 41.5 minutes corresponds to FAD and concides with the peak observed at the same retention time in the membrane fraction. The second peak visible at the retention time of 43.6 minutes seems to coorespond to FMN that was derived from FAD due to alkali treatment. The very low size of this peak in a control sample (pure FAD dissolved in water) could confirm this assumption (Figure 3.24A).

Surprisingly, in strain Δ*nuem* that was analyzed as a negative control a FMN peak could be observed that was higher than in wild type. Similarly, the FAD peak was higher in the deletion strain, but no fully assembled complex I was present in the deletion mutant (see above). On the other hand, the deletion mutant exhibited 16% of parental strain dNADH:HAR activity. As this residual activity and the FMN found by HPLC analysis may have resulted from subcomplexes containing the NUBM (51kDa) subunit, Δ*nuem* membranes were analysed by Western blotting using antibodies against the NUBM (51-kDa) and NUCM (49-kDa) subunits. As clearly seen in Figure 3.25 the two subunits were detected at near wild-type levels, demonstrating the presence of subcomplexes in the deletion strain. As expected, no signal was detected using antibodies against the NUEM subunit.

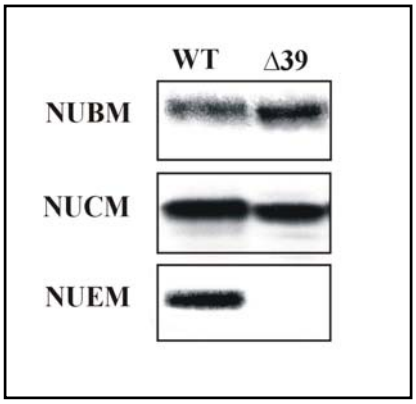

Figure 3.25 Western blot analysis of mitochondrial membranes isolated from parental (WT) and Δnuem strains

Mitochondrial membrane proteins were separated on a SDS-Tricine gel. Then, the proteins were transferred on PVDF membrane and tested with antibodies against NUBM (51-kDa), NUCM (49-kDa) and NUEM (39-kDa) subunits of complex I. No signal from the 39-kDa subunit was observed in the deletion strain.

4 DISCUSSION

4.1 Complex I from Y. lipolytica

Mitochondial NADH:ubiquinone oxidoreductase (complex I) is a very large multiprotein complex. In addition to the 14 strictly conserved central subunits it contains a variable number of accessory subunits. At present, the best characterized enzyme is complex I from bovine heart with a molecular mass of about 980 kDa and 32 accessory proteins (Carroll *et al.*, 2002; Hirst *et al.*, 2003). In this study, the subunit composition of mitochondrial complex I from the aerobic yeast *Y. lipolytica* has been analysed by a combination of proteomic and genomic approaches. The purified enzyme was resolved into 39 spots by doubled SDS-PAGE. This method which has been developed especially for the mass spectrometric analysis of multimeric membrane proteins (Rais *et al.*, 2004) allowed to separate well not only the hydrophilic but also the hydrophobic constituents of complex I. The hydrophobic subunits were found above the electrophoretic diagonal which is defined by the hydrophilic subunits. Remarkably, the hydrophilic proteins are not found exactly on the diagonal. In the second dimension, some proteins migrated somewhat faster than expected (Figure 3.2). For example, the mature NB4M and NI8M subunits have almost similar molecular masses of 11.9- and 11-kDa, respectively. However, NI8M migrated much faster when compared to the NB4M subunit. The position of the ACPM1 subunit in the acrylamide gel was also anomalous. It could be speculated that the subunits that migrated anomalously in the second dimension have in vivo modifications.

Using MALDI-TOF mass spectrometry, 23 proteins were identified, including the seven central subunits of complex I. The seven central hydrophobic subunits were not unambiguously detected by MS. The positions of the ND-subunits in 2D acrylamide gels were predicted based on their molecular weight and hydrophobicity that places them above the electrophoretic diagonal. Identification of the hydrophobic proteins by peptide mass fingerprinting (PMF) is generally difficult. Since charged residues, that in many cases are the target site for proteases, are rare in membrane-spanning regions, enzymatic cleavage tends to lead to the generation of large hydrophobic fragments. These are mostly poorly soluble leading to poor MS detection and low sequence coverage. Moreover, proteins that contain only few trypsin cleavage sites, generate only a limited number of peptides in the mass range suitable for PMF (1-3.5 kDa).

By genome database searches, 37 open reading frames could be assigned to complex I. These are 14 central and 23 accessory subunits. Assuming that complex I contains only one copy of

each subunit, its molecular mass in *Y. lipolytica* is approximately, 930 kDa, consistent with its apparent native molecular weight in BN- gels (about 900 kDa). It should be noted that the actual molecular mass of complex I could differ from the calculated mass. The protein masses were predicted from sequence analysis of the predicted gene translation products, which were compatible with and/or displayed significant sequence similarities to complex I sequences present in the genomes of *N. crassa* and other fungi. The mature protein masses of the central nuclear coded subunits were calculated from single exons and some N-terminal sequences obtained by Edman degradation (Kerscher *et al.*, 2001a). For thirteen accessory subunits two exons were predicted, and in most cases splice site predictions were well supported. However, only for a few accessory subunits the N-terminal ends have been sequenced. For many other proteins information about mitochondrial import sequences was missing and only precursor molecular masses could be calculated. In addition, post-translation modifications like phosphorylation, myristoylation, acetylation, are common and could change the actual protein masses.

In summary, 37 coding sequences of complex I subunits from *Y. lipolytica* were identified, which compose the complex with a predicted total molecular mass of approximately 930 kDa. It is still possible that *Y. lipolytica* complex I contains more subunits than were counted in dSDS acrylamide gels.

4.2 Conserved subunits of complex I

The successful completion of several genomic sequencing projects allowed or the determination of the subunit composition of complex I from different eucaryotic organisms. The number of subunits that were identified as being conserved between eucaryotic kingdoms increased as new genome databases became available. Comparison of the complex I constituents from bovine heart, the filamentous fungus *N. crassa*, the higher plant *A. thaliana* and the green alga *C. reinhardtii* revealed 31 conserved subunits (Cardol *et al.*, 2004). In this study, it has been attempted to compare all identified *Y. lipolytica* complex I subunits with enzyme components from the species mentioned above. Apart from the 14 highly conserved central subunits which also have counterparts in bacterial NADH dehydrogenase, all examined organisms contain 18 additional conserved proteins (Tables 3.3 and 3.4). The average sequence identity score for the *Y. lipolytica* accessory subunits was 35 % that is significantly lower than the value of 47 % for the nuclear coded core subunits.

The 18th conserved subunit NUJM (B14.7) was identified recently in bovine complex I (Carroll *et al.*, 2003) and found to be conserved among mammals, fungi (Abdrakhmanova *et al.*, 2004) and green algae (Cardol *et al.*, 2004). This subunit also shows homology to TIM22 and TIM23 complex proteins, which are involved in the transport of proteins from the cytosol across and into the inner mitochondral membrane (Rehling *et al*., 2004). For the *Y. lipolytica* NUJM homologue, two transmembrane helices were predicted. The molecular mass of the NUJM subunit calculated from a single exon corresponds to 17.3 kDa and no cleavable mitochondrial import sequence can be predicted. The C-teminal extension which brings the *N. crassa* B14.7 homologue up to 21.3-kDa is not present in *Y. lipolytica* NUJM subunit. The B14.7 bovine homologue was also identified in the genome of the green alga *C. reinhardtii* (Cardol *et al*., 2004). However, no clear homologues of the B14.7 subunit could be found in the worm *C. elegans* and in the genomes of the higher plants *A. thaliana* and *O. sativa* (Cardol *et al*., 2004; Heazlewood *et al*., 2003).

The function of most of the 18 conserved accessory subunits in eucaryotic complex I is still largely unknown. Since they do not have counterparts in the bacterial enzyme it can be assumed that they are not involved in electron transfer and in proton translocation across the membrane. It has been suggested that accessory subunits may play a role in improving the structural stability of the enzyme and in protecting the complex against oxidative stress. Because the majority of the supernumerary subunits were found to be localized in subcomplex Iα (Iγ and Iλ subcomplexes) of bovine complex I (Carroll *et al*., 2003; Table 1.1 see Introduction), it was speculated that they could form stabilizers to keep the redox groups in the right position. Thus the accessory subunits could prevent the escape of electrons and the production of superoxide radicals (Friedrich and Weiss, 1996). Moreover, some of them show some homology to enzymes or proteins with alternative function and so they may also have a specific role essential not only for complex I activity or assembly. The influence of several accessory subunits on the activity or assembly of complex I has been studied (see 1.2.1). However, a potential involvment of these subunits in other synthetic pathways was not supported in most cases by experimental evidence.

As mentioned above (see 1.2.1), two complex I subunits with similar molecular mass are supposed to be phosphorylated by a cAMP dependent protein kinase, namely NUYM (AQDQ in bovine; Papa *et al.*, 1996; Papa, 2002) and NESM (ESSS in bovine; Chen *et al.*, 2004). The phosphorylation site in the NUYM protein is conserved among mammals, but has been deduced only from sequence motifs and not determined experimentally. This putative kinase motif is absent from the homologous proteins in all other examined organisms. Apparently,

this control device is not implemented in the enzyme of other eucaryotic species. In contrast, the phosphorylation of serine-20 in the bovine NESM subunit was shown experimentally by Edman sequencing and mass spectrometric analysis of the radiolabeled band isolated by SDS-polyacrylamide gel electrophoresis. However, this residue is not conserved even among mammals, despite good conservation of the protein. The homologue of the bovine NESM subunit was also identified in *Y. lipolytica*. Previosely reported as an organism specific complex I protein, the NUWM subunit shows homology to the XP_324110 protein from *N. crassa*, which has been reported to be a distant relative of the bovine NESM subunit (Cardol *et al.*, 2004). The NUWM protein of *Y. lipolytica* is not compatible in size with its homologues in bovine and *N. crassa*, but they seem to be similar in design, with a single transmembrane domain and two small, but highly charged extramembraneous domains. Similarly designed are several other subunits conserved among eukaryotes (NIAM, NB6M, NB2M, B9M, NIMM; Table 3.4) and subunits, which have no counterpart in the *Y. lipolytica* enzyme (NB7M, NISM, NUML, NIGM, N4BM, NINM, NIKM; Table 3.4). It is tempting to speculate that the common functional requirements for this group of subunits are structure- rather than sequence-specific. By their ability to readily insert into the mitochondrial inner membrane these single transmembrane helix subunits could support assembly of the membrane arm, function as chaperones by interacting with transmembrane helices of other subunits. Some support for this proposal comes from the fact that a similar situation was reported for one of the accessory subunits of mitochondrial cytochrome bc_1 complex (Brandt *et al*., 1994): The 6.4 kDa subunit from bovine and the 8.0 kDa subunit from *Saccharomyces cerevisiae* bc_1 complex exhibit very low sequence similarity and consist of a single transmembrane helix bounded on both ends by highly charged domains.

The NB6M subunit (bovine B16.6 homologue) was identified only recently as a complex I component in mammals (Fearnley *et al.*, 2001; Carroll *et al.*, 2002). This subunit is identical to the mammalian GRIM-19 protein, which is involved in apoptotic cell death induced by interferon-β and retinoic acid (Lufei *et al*., 2003). The observation that GRIM-19 and NB6M are the same protein involved in two independent cellular processes has been disscussed (Hirst *et al.*, 2003). The identification of a *Y. lipolytica* homologue supports the view that this subunit is a genuine component of complex I. It should be noted however that sequence similarity between the fungal and mammalian proteins is low around the 40 C-terminal amino acids representing the part of GRIM-19 that has been demonstrated to be essential for its pro-apoptotic function (Angell *et al.*, 2000).

Two genes coding for ACPM proteins were identified in *Y. lipolytica*. It is known that most eukaryotes contain two mitochondrial ACPs (Schulte, 2001). One ACPM, most likely located in the mitochondrial matrix is needed for fatty acid synthesis (Jordan *et al.*, 1997). Another ACPM is part of complex I and essential for its assembly. Disruption of the complex I associated ACP gene in *N. crassa* resulted not only in a deficiency of complex I assembly, but also in an increase of the lysophospholipid content of the mitochondrial membranes (Schneider *et al.*, 1995; Schneider *et al.*, 1997). It has been speculated that the ACP subunit of complex I is involved in recycling of lysophospholipids formed from lipid hydroperoxides by a phospholipase. Both ACPM proteins discovered in *Y. lipolytica* show close homology to the complex I ACP subunit from bovine heart and *N. crassa*. However, sequence identity of ACPM2 was 46%, i.e somewhat lower than the identity score of 50% found for ACPM1. Moreover, using MALDI-MS analysis of the purified complex I revealed that *Y. lipolytica* ACPM1 is the isoform incorporated into complex I.

4.3 Nonconserved components of complex I

Besides the subunits conserved among all eukaryotic species there are several proteins, which represent a set of phylum specific complex I subunits. It has been shown that eleven subunits are specific to mammalian species and have no related sequences in non-mamalian species. Three proteins are specific for fungi and six are typical for photosyntetic organisms like *A. thaliana* and *C. reinhardtii* (Heazlewood *et al.*, 2003; Cardol *et al.*, 2004). The subunits NI9M, NURM and NUZM were found in *N. crassa* complex I and were described as fungus specific proteins. However, the NURM subunit was not found in the *Y. lipolytica* genome and is likely to be specific for *N. crassa* complex I. The NUJM, NB5M and NIAM subunits are conserved in mammals and *N. crassa* (Cardol *et al.*, 2004). As it was reported previousely no homologue was found for the NUVM subunits of *Y. lipolytica* (Abdrakhmanova *et al.*, 2004). Detailed analysis of the NUVM sequence revealed that this subunit is similar to the NB5M protein from *N. crassa*. Thus, these three subunits represent a set of proteins specific for mammalian and fungal complex I. And finally, there is one subunit (NUXM) common to fungi and plants (Abdrakhmanova *et al.*, 2004; Cardol *et al.*, 2004).

In addition to possible functions such as improving enzyme stability and/or protecting complex I against reactive oxygen species, it was proposed that the non conserved components play important roles in enzyme assembly. As was discussed above (see 1.3), biogenesis of complex I in various species could occur in different ways depending on its

subunit composition. Thus, deletion of the NUXM subunit in *N. crassa* prevented the formation of the membrane arm but did not affect the assembly of the peripheral arm (Schulte and Weiss, 1995). Remarkably, this subunit is the only one that does not have a counterpart in the animal enzyme and is common for fungal and plant complex I. This may point to a specific role of this non-conserved "accessory" subunit in the assembly of the membrane arm in non-mammalian enzymes (Cardol *et al.*, 2004).

A novel protein named "X" with a molecular mass of 34.6 kDa was identified in *Y. lipolytica* complex I by MALDI-MS. It is apparent from the gel electrophoresis pattern that the level of this protein may be substoichiometric (Figure 3.2). The are two possible explanations for this observation: either protein "X" is a novel subunit that is loosely bound to complex I or it is an independent protein the attachment of which to complex I is strong enough to be stable during purification. This protein exhibits no homology to any other known subunit of complex I. However, database searching revealed that protein "X" is related to the thiosulfate:cyanide sulfurtransferase enzyme family, often referred to as rhodaneses (TSTs, EC 2.8.1.1). Rhodaneses are ubiquitous enzymes present in all living organisms, from bacteria to man. In mammals, these enzymes are predominantely located in mitochondria (Matthies *et al*., 2004), where they form stable complexes through disulfide bonds with membrane-bound enzymes (Hatzfeld and Saito, 2000). In vitro, the members of this enzyme family catalyse the transfer of a sulfane sulfur atom from thiosulfate to cyanide, yielding sulfite and thiocyanate as final reaction products. The reaction occurs via a double displacement mechanism involving the transient formation of a persulfite-containing intermediate (Rhod-S), in which the transferring sulfur is covalently bound to the invariant catalytic cysteine residue. The biological role of rhodanese is largely speculative, because their in vivo substrates remain unknown. Proposed functions include cyanide detoxification (Sorbo, 1957), maintenance of the sulfane pool (Westley, 1988), selenium metabolism (Osagawara *et al*., 2001; Bordo and Bork, 2002) and thiamine biosynthesis (Palenchar *et al*., 2000). Due to their ability to transfer sulfur atoms and their mitochondrial location it was suggested that rhodaneses could catalyze the formation of iron-sulfur centers (Ogata and Volini, 1990). There have been reports which show that the sulfur transferred by rhodanese activates NADH dehydrogenase activity (Pagani and Galante, 1983). In bovine heart, phosphorylation of rhodanese leads to loss of its activity and possibly converts the enzyme into a protein sulfurase, which would extract "labile" sulfur from iron-sulfur centers of the respiratory chain. It was speculated that the rhodanese may regulate the respiration rate by controlling the status of iron-sulfur centers of enzymes of the respiratory chain (Ogata *et al*., 1990).

In the context of previous work on sulfotransferases it appears very likely that the "X" protein of *Y. lipolytica* is an independent enzyme which is stronly bound to complex I and stable during the purification. However, the "X" protein was found in a dissolved crystal of complex I (Zickermann, personal communication) which could be a valid reason to believe that this protein may be the 38th "*bona fide*" subunit of *Y. lipolytica* complex I. The substoichiometric levels of the "X" protein in 2D gel patterns and its absence in some mutants (see 3.2.3) of *Y. lipolytica* complex I could reflect a loose attachment to complex I. Similarly, the NUDM (42 kDa) subunit of bovine complex I was also found in substoichiometric amounts in purified enzyme. This subunit was lost gradually during chromatography, suggesting that it is only loosely bound to the complex (Hirst *et al*., 2003).

The identification of complex I-associated proteins with a putative function for iron-sulfur clusters modification supports the idea that the activity of *Y. lipolytica* respiratory chain enzymes could be regulated via control of the status their iron-sulfur clusters.

4.4 NADPH binding to the accessory 39 kDa subunits of Y. lipolytica complex I

An attempt had been made previously to determine the function of the 39 kDa (NUEM) subunit of *N. crassa* complex I. Schulte and coworkers (1999) had shown that in *N. crassa* this subunit is not required for assembly of complex I, but is crucial for catalytic activity. However, the postulated function of the 39 kDa subunit in the biosynthesis of the hypothetical but yet unidentified redox group "X" is not supported by the experimental evidence presented here.

4.4.1 Deletion mutant

Unlike in *N. crassa,* complex I was not assembled in the Δ39 kDa mutant of *Y. lipolytica*. The absence of the enzyme in BN-gels and the loss of specific complex I activity demonstrated the absence of assembled complex I. This result might indicate different pathways or control of complex I assembly in both organisms. In this context, the additional subunit NURM present in *N. crassa* and absent in *Y. lipolytica* complex I may be involved by playing a specific role for enzyme assembly and stability.

The residual dNADH:HAR activity and the FMN detected by HPLC analysis in the mitochondrial membranes from the deletion strain could arise from membrane associated

subcomplexes including the NUBM (51 kDa) and NUCM (49 kDa) subunits. These subunits were identified in the membrane by Western-Blot analysis. The higher amount of FAD in mitochondrial membranes of Δ*nuem* may reflect an increased content of FAD-binding enzymes. There are at least two enzymes of the *Y. lipolytica* respiratory chain containing FAD as a cofactor, namely succinate:dehydrogenase (complex II) and alternative dehydrogenase (NDH2 and NDH2i). Apparently, the higher expression level of both enzymes is a compensatory mechanism in the cells as a response to the lack of assembled complex I.

4.4.2 Site-directed mutagenesis in the nucleotide binding domain

The typical nucleotide binding site consists of three glycines (GXXGXXG). The middle one makes close contact to the pyrophosphate moiety of the cofactor and is crucial for nucleotide binding (Lesk, 1995). It should be noted here that it is not clear whether NADPH is exchangeable or tightly bound to the protein and required for structural stability.

Replacement of glycine-43 with alanine in the 39 kDa subunit seems to result in damage of the cofactor binding site within the protein, accompanied by minimal overall changes in protein structure. Complex I from mutant G43A retained almost 90% of wild type activity, but enzyme content in mitochondrial membranes was reduced to 50%. Interestingly, the NADPH amount in isolated complex I was also reduced to 40 % in comparison to the parental strain, pointing to a decrease in affinity for the cofactor. As the decrease in NADPH binding correlated with a corresponding defect in the assembly of complex I one might speculate that the 39 kDa subunit may participate in a biosynthetic pathway, delivering a product which is needed for complex I assembly.

After replacement of glycine-43 with valine no assembled complex I was found under standard conditions. Only small amounts of assembled enzyme were identified in cells grown at lower temperature, indicating the formation of an unstable enzyme complex that did not contain the 39 kDa subunit. This protein was found at the front of the gel as a free pool. It remains unclear whether in this mutant the 39 kDa protein was not at all associated with the assembled enzyme or whether it was lost during solubilisation. A number of reports show that at earlier stages of complex I assembly, the 39 kDa subunit forms a subcomplex with other proteins including the 49 kDa and 30 kDa subunit (Antonicka *et al.*, 2003; Ugalde *et al.*, 2004). However, no signal was observed in the subcomplex containing the 49 kDa subunit using antibodies against the 39 kDa protein. Considering that NADPH is tightly bound to the protein seems likely that exchanging glycine-43 to valine led to complete displacement of the cofactor from the nucleotide-binding pocket due to the much bulkier side chain of valine in

comparison to glycine. The loss of the ability to bind the nucleotide could cause steric alteration of the protein, which may prevent incorporation of the 39 kDa subunit into the subcomplex. This assumption is however speculative and needs futher investigation.
Overall, the results presented here indicate a key role for the 39 kDa subunit for complex I assembly in *Y. lipolytica*.

4.4.3 Mutants R65D and R65L

The presence one or two basic residues determines the ability of SDRs to bind NADPH: the first basic amino acid is located at the end of the second β-strand (Figure 4.1) and the second basic residue is in the Gly-motif. In the 39 kDa subunit of *Y. lipolytica* complex I, the highly conserved arginine at position 65 is predicted to reside at the end of the second β-strand (Figure 4.2).

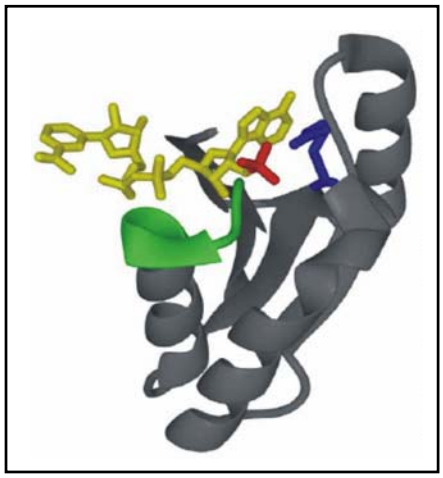

Figure 4.1 Cofactor binding region of human 17β-hydrosteroid dehydrogenase (17β-HSD1), an SDR enzyme (pdb ID 1QYV)

The presence of arginine at the end of the second β-strand in 17β-HSD1 determines the selective binding of NADPH. The arginine residue (blue) forms a salt bridge with the 2'-phosphate (red) of NADPH (yellow). The GXXXGXG motif of 17β-HSD1 is shown in green. From Agarwal and Auchus, Endocrinology, (2005) Mar 17; [Epub ahead of print 2005].

The positively charged arginine-65 could interact with the 2'-phosphate of NADPH. It stabilizes the binding of the cofactor and neutralizes its intrinsic negative charge through two hydrogen bonds (Persson *et al.*, 2003). NADH-prefering enzymes have an acidic amino acid at the same position. Computer simulations of the NADPH binding poket in 17β–hydrosteroid dehydrogenase type 3 (17β–HSD3), a member of the SDR enzyme family, have shown that the trunk of the side chain of arginine-80 corresponding to arginine-65 in the 39 kDa subunit of *Y. lipolytica* forms part of the hydrophobic pocket for the purine ring of adenosine while its guanidinium moiety is in contact with the 2'-phosphate of NADPH (McKeever *et al.*, 2002). The position of this basic residue is probably quite conserved so that removal of arginine-80 would appear to be prerequisite to reduce the enzyme's affinity for NADPH (Scrutton *et al.*, 1990; Perham *et al.*, 1991)

Arginine-65 in the 39 kDa subunit was changed to a neutral (leucine) and a negatively charged (aspartate) amino acid. In both mutants, fully assembled complex I was found. Despite a reduced content of enzyme in mitochondrial membranes, specific activity of complex I from mutants R65L and R65D was 80-90% of wild type. After replacement of arginine-65 in the 39 kDa subunit a peak corresponding to the NADPH was still observed after HPLC analysis of washed mitochondrial membranes. However, it was much smaller for mutant R65L and hardly detectable for R65D indicating significantly reduced binding of NADPH to the protein. Leucine is neutral and can not make a hydrogen bond with 2'-phosphate of NADPH and neutralize its negative charge, but it has a short side chain and can fulfill the role of the hydrophobic portion of arginine. Therefore it is very likely that leucine did not prevent the cofactor binding, but weakened its binding to the protein. In contrast, the change of arginine-65 to aspartate resulted in a dramatic reduction in the affinity for NADPH and only traces of the cofactor were found in the mitochondrial membrane fraction. As expected, the negatively charged side chain of aspartate severely interferred with binding of NADPH to the protein. Interestingly, no NADH could be extracted from complex I of the R65D mutant. As revealed by HPLC analysis of pure NADH, it has a longer retention time (30-31 minutes) than NADPH (27 minutes). This suggested that replacement of a basic residue with an acidic one did not change the cofactor preference from NADPH to NADH. A similar result was obtained for 17β–HSD3 when changing arginine-80 to aspartate (McKeever *et al.*, 2002), this mutant was inactive when NADPH was used as cosubstrate but was also not able to bind NADH. Modelling of aspartic acid at position 80 in this enzyme showed that aspartate is not able to effectively interact with the 2'- and 3'-hydroxyls of NADH due to its short side chain.

Enzyme purification from both mutants resulted in a significant decrease of complex I activity and complete loss of NADPH. The negative effect of purification was more pronounced in mutant R65D, since specific complex I activity was reduced from 80 % of wild type activity in mitochondrial membranes to 13 % in purified enzyme. This was accompanied with slightly diminished intensity of the cluster N2 signal in this mutant. It is very likely that the changes observed in the mutants were due to some instability of the enzyme during the purification procedure.

As shown from secondary structure analysis (Figure 4.2) of the 39 kDa subunit, arginine-65 is located at the end of the second β-strand. From this observation and from general considerations on structural dynamics of membrane proteins (Ash *et al.*, 2004; Roux and Schulten, 2004) one can conclude that a single mutation at position 65 may lead to structural

changes of the protein. This is especially important for mutant R65D, because a positively charged arginine is replaced by a negatively charged aspartate. This mutation might change electrostatic interactions in the region close to the NADPH pocket and may also explain loss of activity in the mutant. In comparison, mutation R65L does not introduce additional charge and may not change electrostatic interactions in it surroundings.

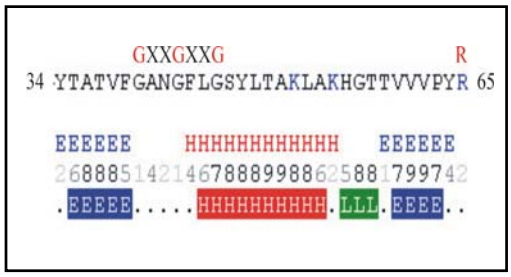

Figure 4.2 Secondary structure prediction of the nucleotide-binding domain of the 39-kDa complex I subunit from Y. lipolytica

The prediction of secondary structure was done using the PredictProtein programm package from Internet (www.predictprotein.org). E – β-strand, H- α-helix, L- loop.

Remarkably, the amount of protein "X" also correlated with complex I activity. It may be speculated that this protein could also influence enzyme stability.

These results confirm the conclusions made from experiments with the deletion strain and mutant G43A, namely that the 39 kDa subunit is not essential for complex I activity but is involved in assembly and stability of the enzyme complex. The basic residue located at the end of the second β-sheet in the cofactor binding domain of the 39 kDa subunit of complex I is required for binding of NADPH to the protein. Replacement of this amino acid not only led to decreased affinity of the protein for NADPH but also caused sterical changes in the 39 kDa subunit.

5 SUMMARY

5.1 Subunit composition of Y. lipolytica complex I

Complex I, the largest multiprotein enzyme of the respiratory chain, consists of 23-32 accessory subunits in addition to the 14 central subunits that are highly conserved among procaryotes and eucaryotes. The first aim of this work was the identification and sequence determination of accessory subunits of complex I from the aerobic yeast *Yarrowia lipolytica*. 39 protein spots were counted on doubled SDS gels, the identity of 23 complex I subunits including the seven central nuclear coded subunits was defined using MALDI-MS. By a combination of proteomic and genomic approaches, the sequences of 37 complex I subunits were identified. The sum of their individual molecular masses (about 930 kDa) was consistent with the native molecular mass of approximately 900 kDa for *Y. lipolytica* complex I obtained by BN-PAGE. A genomic analysis with *Y. lipolytica* and other eukaryotic databases to search for homologues of complex I subunits revealed 31 conserved proteins among the examined species. Detailed sequence analysis of the NUWM complex I subunit, previously postulated to be a *Y. lipolytica* specific protein showed that this subunit is a putative homologue of the NESM subunit from *N. crassa.*

A novel protein named “X” was found in purified *Y. lipolytica* complex I by MALDI-MS. This protein exhibits homology to the thiosulfate sulfurtransferase enzyme referred to as rhodanese. Human rhodaneses are mostly located in mitochondria where they form stable complexes through disulfide bonds with membrane associated enzymes. It has been proposed that rhodaneses could regulate the respiration rate by reversible sulfuration of iron-sulfur clusters of respiratory chain complexes. The finding of a rhodanese-like protein in isolated complex I of *Y. lipolytica* suggests a specific regulatory mechanism of complex I activity through control of the status of its iron-sulfur clusters.

The function of most accessory subunits is still largely unknown. In contrast to the 14 central subunits which present the minimal form of the enzyme, accessory subunits do not have a counterpart in the bacterial enzyme. Most likely, they are not involved in electron transfer and proton translocation across the membrane. It has been suggested that they could play a role in improving enzyme stability and in protecting complex I against oxidative stress. Some of the accessory subunits show homology to enzymes or proteins with alternative functions and thus are probably essential for complex I assembly and/or activity.

5.2 The 39 kDa subunit of complex I

The second part of this study was aimed at investigating the possible role of the accessory 39 kDa subunit of *Y. lipolytica* complex I. This subunit shows homology to the short-chain dehydrogenase/reductase enzyme family and is able to bind NADPH as cofactor. However, it is not clear whether NADPH is exchangeable or tightly bound to the protein and whether it is required for structural stability.

The postulated function of the 39 kDa subunit in the biosynthesis of a yet unidentified redox group for *N. crassa* complex I, is not supported by any new experimental evidence at present.

In contrast to the situation in *N. crassa*, deletion of the 39 kDa encoding gene in *Y. lipolytica* led to the absence of fully assembled complex I. This result might indicate a different pathway of complex I assembly in both organisms.

The replacement of the middle glycine-43 with alanine in the NADPH-binding site (GXXGXXG) did not prevent assembly of active complex I. The G43A mutant retained 90% of wild type activity. However, the reduced amount of enzyme in mitochondrial membranes correlated with reduced cofactor content in the mutant complex I. Based on these findings and on the assumption that NADPH is exchangeable, it could be speculated that NADPH bound to the 39 kDa subunit is needed for a biosynthetic pathway delivering a product which is needed for complex I assembly. The conservation of wild type activity in the G43A mutant complex I after partitial loss of the cofactor indicated that the 39 kDa subunit is probably not essential for complex I activity. Surprisingly, after changing glycine-43 to valine, no assembled complex I was detected by BN-PAGE. Only traces of assembled complex I were found by Western blot analysis in cells grown at lower temperature. While the mutant complex I did not contain the 39 kDa subunit, the subunit was observed at the front of the gel as a monomeric protein. If NADPH is tightly bound to the protein, the displacement of the cofactor from the nucleotide-binding domain due to the longer side chain of valine could lead to the conformational change in the protein. This could prevent the incorporation of the 39 kDa subunit into complex I.

Mutations of arginine-65 that is located at the end of the second β-strand and responsible for selective interaction with the 2'-phosphate group of NADPH retained complex I activity in mitochondrial membranes but the affinity for the cofactor was markedly decreased. Complex I from mutant R65L was able to bind NADPH while only traces of cofactor were found in cholate-washed mitochondrial membranes of mutant R65D. Purification of complex I from mutants R65L and R65D resulted in decrease or loss of ubiquinone reductase activity,

respectively. It is very likely that replacement of arginine-65 not only led to a decrease in affinity for NADPH but also caused instability of the enzyme due to steric changes in the 39 kDa subunit.

6 OUTLOOK

Further investigation will be needed to identify all proteins present in a preparation of complex I from *Y. lipolytica* and to confirm which of them are true subunits. Sequencing of cDNAs for the accessory subunits will be required to confirm some splicing events experimentally. ESI-MS measurements of the masses of intact mature subunits and comparison of these value with masses calculated from sequences of cDNAs will allow to identify potential post-translation modifications of complex I subunits.

The finding of a sulfurtransferase-like protein in purified *Y. lipolytica* complex I is of special interest. The generation of a deletion mutant of the gene encoding this protein will allow to investigate its possible role for complex I activity and biogenesis.

The investigation of subcomplexes in the Δ39 and G43V mutants could shed light on the assembly pathway of complex I in *Y. lipolytica* and allow to determine the role of the 39 kDa subunit in this process.

Mutant R65D shows the characteristics needed to study the function of the 39 kDa subunit for complex I activity: the enzyme retains wild type activity while it is almost devoid of NADPH. But unfortunately the complex in the R65D mutant is unstable during purification procedure. Therefore, it should be interesting to explore the R65D mutant complex I in terms of enzyme activity in more detail in submitochondrial particles while preserving its native structure.

7 ZUSAMMENFASSUNG

Die mitochondriale Atmungskette ist Teil der oxidativen Phosphorylierung. Die Komponenten der Atmungskette katalysieren die Übertragung von Elektronen aus den Reduktions-Äquivalenten NADH und $FADH_2$ auf molekularen Sauerstoff. Die freie Energie der Redoxreaktionen wird für das Pumpen von Protonen verwendet. Die Protonen werden aus der Matrix in den Intermembranraum zwischen der inneren und äußeren Mitochondrien-membran transportiert. Dabei einsteht ein elektrochemisches Protonenpotential, das schließlich zur ATP-Synthese genutzt wird. Insgesamt sind vier Enzymkomplexe und zwei mobile Elektronüberträger an der Atmungskette beteiligt. Ein Enzymkomplex, die ATP-Synthase, sorgt für die ATP-Synthese. Die Enzyme der Atmungskette sind: NADH:Ubichinon Oxidoreduktase (Komplex I), Succinat:Ubichinon Dehydrogenase (Komplex II), Ubihydrochinon:Cytochrom c-Oxidoreduktase (Komplex III), Cytochrome c-Oxidase (Komplex IV). Ubichinon (UQ) und Cytochrome *c* fungieren als mobile Elektronencarrier.

Die NADH:Ubichinon Oxidoreduktase (Komplex I) ist das größte und komplizierteste Enzym der Atmungskette, es katalysiert den Transfer von zwei Elektronen von NADH auf Ubichinon. Daran gekoppelt ist die Translokation von vier Protonen über die innere Membran. Elektronenmikroskopische Untersuchungen haben gezeigt, dass Komplex I eine L-förmige Gestalt aufweist, wobei der sogenannte Membranarm in die innere mitochondriale Membran eingebettet ist und der periphere Arm in die mitochondriale Matrix hineinragt. Im Vergleich zum bakteriellen Enzym ist der mitochondriale Komplex I komplizierter aufgebaut und hat eine größere molekulare Masse. Die 14 sogenannten zentralen Untereinheiten, die auch im bakteriellen Komplex I vorkommen, bilden die „minimale“ Form des Enzyms. Sieben davon befinden sich im peripheren Arm und sind kernkodiert (75, 51, 49, 30, 24 kDa, TYKY, PSST). Diese enthalten alle bekannte Redoxgruppen des Komplex I: FMN und die Eisen-Schwefel Zentren N1a, N1b, N2-N5, N6a,b. Der Membranarm besteht aus weiteren sieben Untereinheiten (ND1-ND6, ND4L), die hydrophob sind und von mitochondrialer DNA kodiert werden. Der eukaryotische Komplex I besitzt zusätzlich zu den 14 zentralen Untereinheiten noch 23-32 „akzessorische“ Untereinheiten. Komplex I der Säugetiere hat ein Molekulargewicht von etwa 1 MDa und besteht aus 46 Untereinheiten (Skehel *et al.*, 1998; Carroll *et al.*, 2003), Komplex I der Grünalge *Chlamydomonas reinhardtii* besteht aus 42 (970 kDa) (Cardol *et al.*, 2004) Untereinheiten und der vom Fadenpilz *Neurospora crassa* aus 39 Untereinheiten (1.1 kDa) (Marques *et al.*, 2005) Die Funktion der meisten akzessorischen

Untereinheiten ist unbekannt. Da sie keine Homologe im bakteriellen Enzym haben, ist es sehr wahrscheinlich, dass die akzessorischen Untereinheiten nicht an der Elektronenübertragung und dem Protonenpumpen beteiligt sind. Einige von ihnen zeigen eindeutige Homologie zu Enzymen oder Proteinen mit alternativen Funktionen, und könnten weitere Aufgaben übernehmen, die für die Aktivität und/oder Assemblierung des Komplex I erforderlich ist. Derzeit sind nur wenige der akzessorischen Untereinheiten detailliert untersucht worden.

In den letzten Jahren wurde *Yarrowia lipolytica,* eine obligat aerobe Hefe, als Modellsystem in unserem Labor etabliert. Im Vergleich zur Atmungskette der Hefe *S. cerevisiae,* die keinen Komplex I beinhaltet, besitzt *Y. lipolytica* einen stabilen Komplex I. Das Enzym lässt sich gut reinigen und ist dem Komplex I aus *N. crassa* sehr ähnlich. Die Sequenzierung des Genoms von *Y. lipolytica* wurde vor kurzem abgeschlossen. All dies macht diese Hefe zu einem hervorragenden Modellsystem für strukturelle und funktionelle Untersuchungen an Komplex I (Kerscher *et al.*, 2002).

Das erste Ziel dieser Arbeit war die Bestimmung der Untereinheiten, aus denen sich Komplex I von *Y. lipolytica* zusammensetzt. Kenntnisse der Untereinheitenzusammensetzung können für die Bestimmung der Enzymstruktur und des Reaktionsmechanismus wichtig sein. Außerdem liefert die Aufdeckung von Homologien einzelner Untereinheiten nützliche Informationen über mögliche Aufgaben der akzessorischen Untereinheiten und mögliche Wechselwirkungen der Atmungskette mit weiteren biosynthetischen Prozessen.
Die His-tag Markierung der 30 kDa Untereinheit des Komplex I ermöglicht die Isolierung des hochreinen Enzyms mittels Ni^{2+}-Affinitätschromatographie und nachfolgender Gelchromatographie (Kashani-Poor *et al.*, 2001a). Der isolierte Komplex I wurde mithilfe von dSDS-PAGE (Rais *et al.*, 2004) in einzelne Untereinheiten zerlegt. Die meisten Proteine liegen auf einer Diagonalen, wobei die hydrophoben Untereinheiten sich oberhalb derselben befinden. 39 Proteinspots wurden im Gelmuster separiert, davon wurden 23 Untereinheiten mittels Massenspektrometrie (MALDI-MS) identifiziert, inklusive der sieben zentralen kernkodierten Untereinheiten. Die sieben zentralen hydrophoben Untereinheiten lassen sich mittels MALDI-MS bisher nicht eindeutig identifizieren. Aufgrund ihrer molekularen Masse und ihrer Hydrophobizität wurde ihre Position in 2D-Polyacrylamidgelen vorausgesagt. Durch die Kombination von proteinbiochemischen und genomanalytischen Methoden wurden insgesamt 37 Untereinheiten von Komplex I aus *Y. lypolytica* identifiziert. Die Summe ihrer individuellen

molekularen Massen (zirka 930 kDa) stimmt gut überein mit der molekularen Masse von ungefähr 900 kDa für *Y. lipolytica* Komplex I, die mithilfe der BN-PAGE bestimmt wurde. Die Datenbanksuche nach Untereinheithomologien für *Y. lipolytica* Komplex I zeigte, dass 31 Untereinheiten zwischen Säugetieren, Pilzen und photosynthetisch aktiven Organismen wie *Chlamydomonas reinhardtii* und *Arabidopsis thaliana* konserviert sind.

Die NUWM Untereinheit aus *Y. lipolytica* Komplex I wurde zunächst als organismusspezifisches Protein betrachtet (Abdrakhmanova *et al.*, 2004). Eine detaillierte Analyse der Proteinsequenzen zeigte jedoch, dass diese Untereinheit Homologie zum XP_324110 Protein von *N. crassa* aufweist, das als entfernter Verwandter der NESM Untereinheit aus Rind beschrieben wurde (Cardol *et al.*, 2004). Obwohl das NUWM Protein von *Y. lipolytica* in Bezug auf die Größe nicht mit seinen Homologen in Rind und *N. crassa* kompatibel ist, scheinen diese drei Proteine im Aufbau ähnlich zu sein: sie haben eine einzelne transmembranäre Domäne und zwei kleine, aber stark geladene extramembranäre Domänen.

Weitere Untersuchungen sind erforderlich, um alle Untereinheiten von Komplex I zu identifizieren und zu verifizieren, welche von ihnen authentische Komplex I Untereinheiten sind. Sequenzierung von cDNAs für die akzessorischen Untereinheiten ist nötig, um das Spleißen die Exon-Intron-Strukturen der zugrundeliegenden Gene experimentell zu bestätigen. Potenzielle posttranslationale Modifikationen der Untereinheiten können identifiziert werden, indem die aus den cDNA-Sequenzen berechneten molekularen Massen mit den Ergebnissen von ESI-MS-Messungen an intakten reifen Untereinheiten vergleichen werden.

Ein neues Protein "X" wurde in gereinigtem *Y. lipolytica* Komplex I mithilfe von MALDI-MS identifiziert. Kein Homolog dieses Proteins konnte in Komplex I von anderen eukaryotischen Organismen gefunden werden. Es zeigt aber eindeutige Homologie zu Mitgliedern der Thiosulfat Sulfurtransferase Enzymfamilie, welche auch als Rhodanesen bezeichnet werden (TSTs, EC 2.8.1.1). Menschliche Rhodanese wird größtenteils in Mitochondrien gefunden (Matthies *et al.*, 2004), wo sie sich durch Disulfidbindungen an Membranproteinkomplexe binden können (Hatzfeld *et al.*, 2000). Die Mitglieder dieser Enzymfamilie katalysieren *in vitro* die Übertragung eines Sulfan Schwefel-Atoms von Thiosulfat auf Zyanid, dabei entstehen Sulfit und Thiocyanat als Endprodukte. Es ist spekuliert worden, dass die Rhodanesen die Atmungsrate durch reversible Sulfurierung von Eisen-Schwefel-Clustern von

Atmungskettenkomplexen regulieren könnten (Ogata *et al*., 1989). Die Identifizierung eines möglichen Sulfotransferase-Proteins im gereinigten *Y. lipolytica* Komplex I könnte ein erster experimenteller Hinweis darauf sein, dass *Y. lipolytica* einen regulatorischen Mechanismus für Atmungsketten Komplexe besitzt, der über den Status ihrer Eisen-Schwefel Cluster wirkt. Weitere Untersuchungen sind nötig um die mögliche Rolle des Proteins „X“ für *Y. lipolytica* Komplex I abzuklären.

Im zweiten Teil dieser Arbeit wurde die Funktion der NUEM (39 kDa) Untereinheit untersucht. Die akzessorische 39 kDa Untereinheit gehört zu der short-chain Reduktase/ Dehydrogenase Enzymfamilie. Diese Enzyme enthalten in der Nähe des N-Terminus eine typische Nukleotid-Bindungsstelle für NADPH oder NADH. Diese bestehen üblicherweise aus einer βαβ Rossmann-Faltung und drei Glycinen in der Anordnung GXXGXXG, wobei das mittlere Glycin für die Nukleotidbindung besonders wichtig ist (Kallberg *et al.*, 2002b; Persson *et al.*, 2003). Da die 39 kDa Untereinheit einen basischen Rest am Ende des zweiten β-Strangs besitzt, wird NADPH als Cofaktor gebunden. Es wird angenommen, dass dieser basische Rest mit der 2’-Phosphatgruppe des NADPH eine Wasserstoffbrückenbindung eingehen kann, wodurch wird die negative Ladung des Phosphats neutralisiert und die ganze Struktur stabilisiert wird (Lesk, 1995;Kallberg *et al.*, 2002b; Persson *et al.*, 2003). Jedoch ist nicht klar, ob das NADPH ein austauschbares Substrat darstellt oder ob es fest an das Protein gebunden ist und für die Strukturstabilität des Proteins erforderlich ist.
Deletion der NUEM (39 kDa) Untereinheit in *N. crassa* lieferte einen assemblierten, aber inaktiven Komplex I. UV/Vis-spektroskopische Signale, die als Hinweis auf eine neuartige, bisher nicht identifizierte Redoxgruppe „X“, die sich möglicherweise zwischen Cluster N2 und Ubichinon befindet interpretiert worden waren, wurden in der Deletionsmutante nicht identifiziert. Es ist daher postuliert worden, dass die 39 kDa Untereinheit an der Biosynthese der Redoxgruppe „X“ beteiligt ist (Schulte *et al.*, 1999). Jedoch wurden seither keine neuen Nachweise erbracht, die diese Hypothese bestätigen könnten. Außerdem ist die Existenz der Redoxgruppe „X“, deren chemische Natur nach wie vor unklar ist, insbesondere durch die sorgfältige chromatographische und massenspektroskopische Untersuchung von Komplex I aus Rinderherz, in Frage gestellt.

Im Gegensatz zu *N. crassa*, lieferte die Deletion der 39 kDa Untereinheit in *Y. lipolytica* keinen assemblierten Komplex I. Assembliertes Enzym war nicht im BN-Gel nachweisbar, selbst bei niedriger Detergenzkonzentration. Jedoch zeigten mitochondriale Membranen des

Deletionsstammes 16 % dNADH:HAR Aktivität. Da die dNADH:HAR Aktivität nur von der NUBM (51 kDa) Untereinheit abhängt, kommt diese restliche Aktivität vermutlich von Subkomplexen, die die NUBM (51 kDa) Untereinheit besitzen. Eindeutige Signale der NUBM (51 kDa) und NUCM (49 kDa) Untereinheiten wurden in Membranen des Δ*nuem* Stamms mittels Western-Blot Analyse nachgewiesen.
Dieses Ergebnis weist darauf hin, dass *N. crassa* und *Y. lipolytica* möglicherweise verschiedene Assemblierungswege des Komplex I haben.

Der Deletionsstamm konnte erfolgreich komplementiert werden. Es wurde eine Reihe von Punktmutationen in die 39 kDa Untereinheit eingeführt. Die Punkmutationen wurden direkt in der Nukleotidbindunsstelle (mittleres Glycin-43) und an der Position des basischen Restes am Ende des zweiten β-Strangs (Arginin-65) generiert.
Anhand eines Blau-Nativ Gels konnte gezeigt werden, dass der Austausch von Glycin-43 gegen Alanin in der 39 kDa Untereinheit eine vollständige Assemblierung von Komplex I erlaubt. Komplex I der G43A Mutante zeigte fast 90 % der wildtypischen DBQ-Aktivität, jedoch ist der Enzymgehalt in der mitochondrialen Membran auf 50 % reduziert. Auch das molare Verhältnis von NADPH zu Komplex I beträgt nur 40 %, was auf eine niedrigere Affinität des mutierten Proteins für NADPH hinweisen könnte. Ausgehend von diesen Ergebnissen und der Annahme eines austauschbaren NADPH kann spekuliert werden, dass das NADPH der 39 kDa Untereinheit an einem biosynthetischen Prozess beteiligt ist, welcher ein Produkt liefert, das für die Komplex I Assemblierung erforderlich ist. Die Tatsache, dass die G43A, bei partiellem Verlust des Kofaktors wildtypische DBQ-Aktivität besaß, zeigte an, dass die 39 kDa Untereinheit wahrscheinlich nicht für die Komplex I Aktivität notwendig ist.
Überraschenderweise konnte nach dem Austausch von Glycin-43 gegen Valin kein assemblierter Komplex I im Blau-Nativen Gel nachgewiesen werden. Nur Spuren des Enzyms wurden mithilfe der Western-Blot Analyse in Zellen identifiziert, die bei einer niedrigen Temperatur angezogen wurden. Dabei enthielt der Komplex I keine 39 kDa Untereinheit. Diese wurde an der Front des Gels als monomere Untereinheit gefunden. Es kann spekuliert werden, dass wegen der längeren Seitenkette von Valin der Kofaktor aus der Bindingdomäne verdrängt wird. Ausgehend von der Annahme, dass das NADPH fest an das Protein gebunden und nicht austauschbar ist, könnte dies zu strukturellen Änderungen des Proteins führen. Dies könnte die Integration der 39 kDa Untereinheit in den Komplex I verhindern.

Mutationen des Arginin-65 am Ende des zweiten β-Stranges, das für die selektive Bindung von NADPH verantwortlich ist, lieferte in den mitochondrialen Membranen einen assemblierten und aktiven Komplex I. Jedoch war die Menge des Kofaktors in den Mutanten im Vergleich zum Elternstamm geringer. Die Mutante R65L war noch in der Lage NADPH zu binden, jedoch wurden nur noch Spuren des Kofaktors in Cholat-gewaschenen mitochondrialen Membran der R65D Mutante gefunden. Die Reinigung des Komplexes I aus den Mutanten R65L und R65D führte zu einer starken Reduzierung (Mutante R65L) bzw. zum Verlust (Mutante R65D) der Ubichinon-Reduktase Aktivität. Vermutlich führte der Austausch von Arginin-65 nicht nur zur Abnahme der Affinität des Proteins für NADPH, sondern auch zu einer verminderten Enzyminstabilität aufgrund von strukturellen Änderungen des Proteins. Es wäre sehr interessant, die Mutante R65D detaillierter zu untersuchen, da diese viele Eigenschaften besitzt, die für die Untersuchungen der 39 kDa Funktion nötig sind: Sie enthält einen aktiven Komplex I, der NADPH sehr schwach binden kann. Leider war das Enzym dieser Mutante jedoch zu instabil. Trotzdem wäre es sehr interessant, den Komplex I der R65D Mutante z.B. in submitochondrialen Partikeln ausführlicher zu erforschen.

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9 APPENDIX

9.1 NB4M gene, ACC. No YALI0A01419g

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TGTAGTTCTGGAACTCGGGCTCGGTCATATGGACAGAGGAGTCTGAGCCGCCCCACCCCTCGCCGCCAAAAACTT
GTCGCAGAAACGGCTCACTGCCCTCTCGGGTAGCAAAGTCCTTGGAAGCATCTCCGATAAGATATGCCTGGTAGG
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TGCGCAGTATGGGAATCTGTTTTCGCCATCCATACCAGCTATCGGGGAAGCCGTGGCACAGCATCAGGGTCTTTC
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CATTTTTAGCAGCAAGCTGTTACTTCGTGTTCTGCTGTTGGGCCCTAAGACAAAGGGTTGTGCTGGAGTAGTTGG
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CACCTTTCTTACTTCTTTCTTACTTCGTCTACGCTTTTCCAAACCAGCAGCAACTGTCCCAATTCTCACAGATGA
GACTCCTGACATTCCAACATCTTCCACCACCTCTTTCGTATACTAACTCAGAACCGTGCTTAAACACATTATATA
TTGAAATGATACACTA

Figure 9.1 Y. lipolytica NB4M subunit encoding gene.

Gene has two exons (marked with blue) and one intron (marked with red). The second exon is very short and encoded only three amino acids. The predicted from two exons molecular mass of NB4M subunit is 11.9 kDa.

9.2 Mass spectra

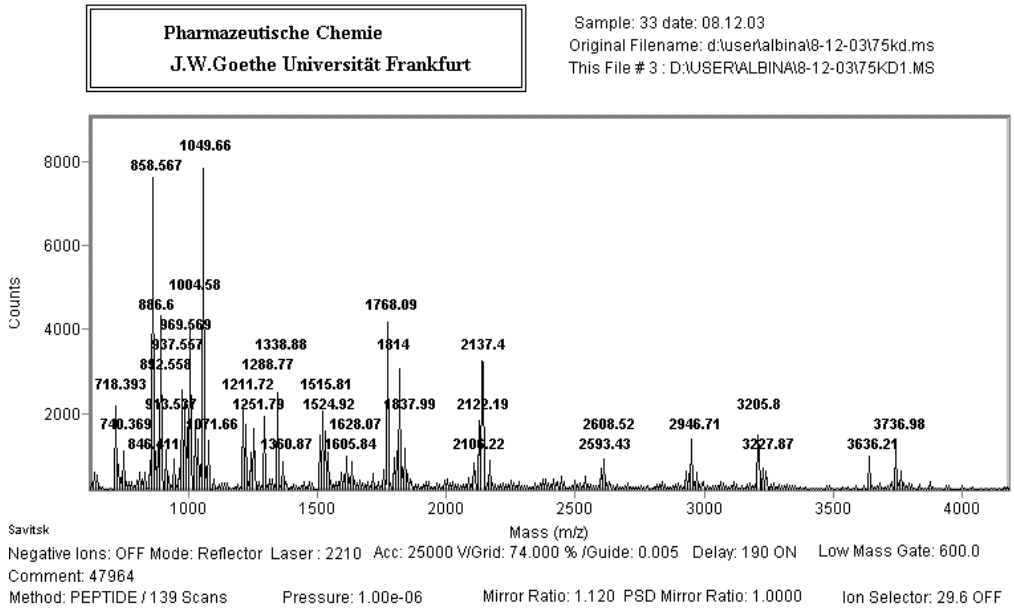


Figure 9.2 MALDI-TOF mass spectra of in-gel V8 (DE) digested NUAM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. The found sequence was compared with N-termini obtained by Edman degradation.

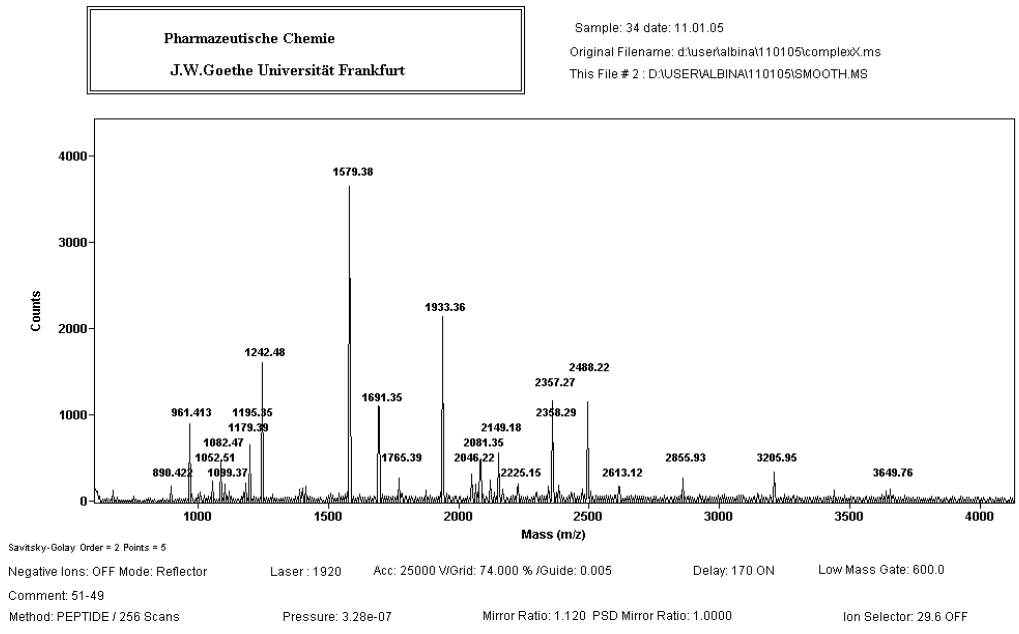


Figure 9.3 MALDI-TOF mass spectra of in-gel trypsin digested NUBM and NUCM subunits.

They are mixture from two proteins NUCM and NUBM. The NUCM subunit was identified using Protein Prospector and NUBM using Prowl programm package. The found sequences were compared with N-termini obtained by Edman degradation.

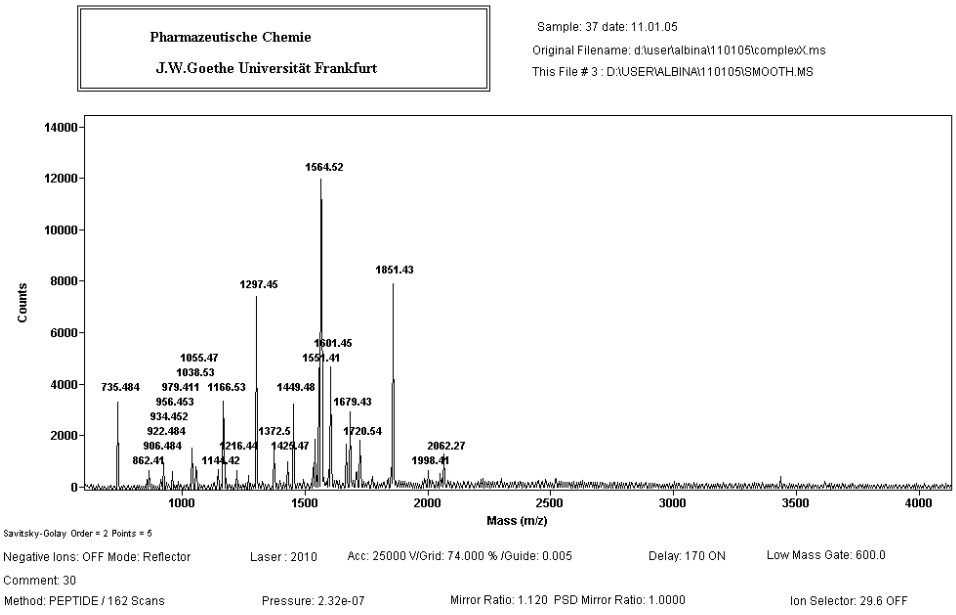


Figure 9.4 MALDI-TOF mass spectra of in-gel trypsin digested NUGM subunit

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. The found sequence was compared with N-termini obtained by Edman degradation.

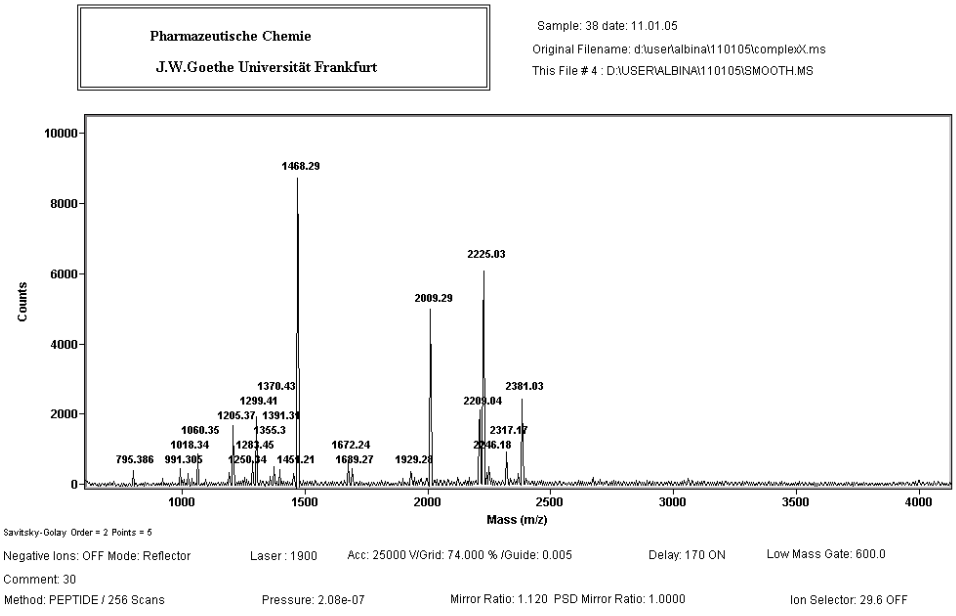


Figure 9.5 MALDI-TOF mass spectra of in-gel trypsin digested NUHM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. The found sequence was compared with N-termini obtained by Edman degradation.

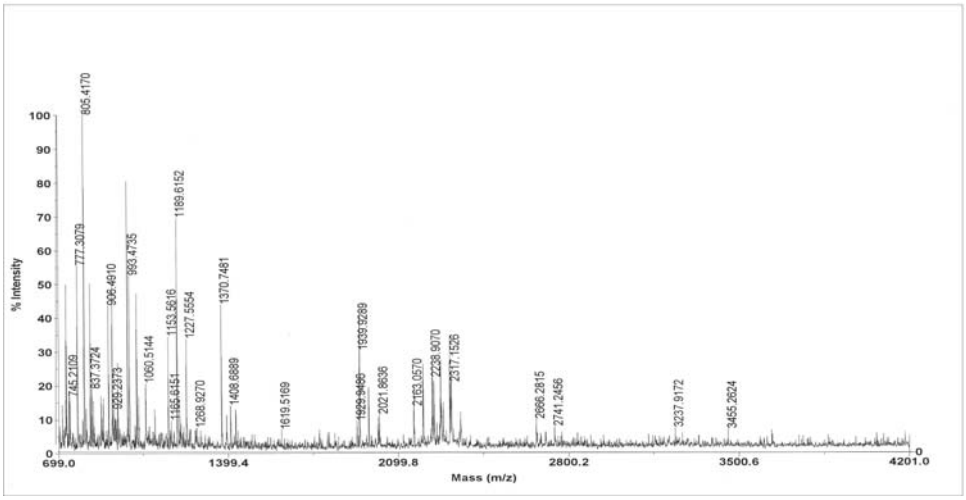


Figure 9.6 MALDI-TOF mass spectra of in-gel trypsin digested NUIM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database.

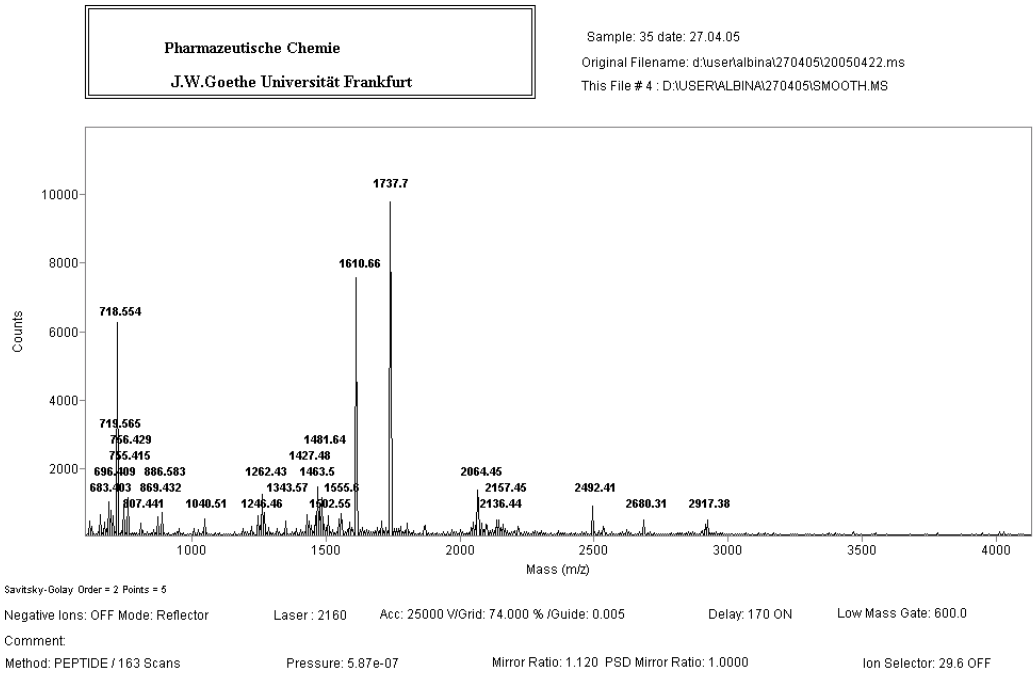


Figure 9.7 MALDI-TOF mass spectra of in-gel trypsin digested PSST and NUPM subunits.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database.

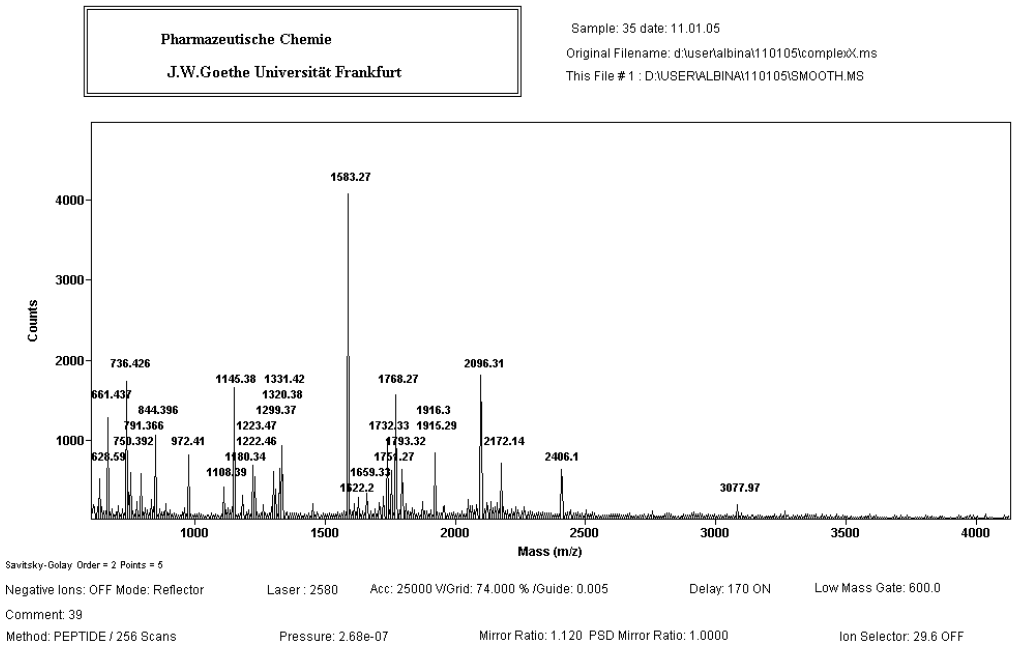


Figure 9.8 MALDI-TOF mass spectra of in-gel trypsin digested NUEM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. The found sequence was compared with N-termini obtained by Edman degradation.

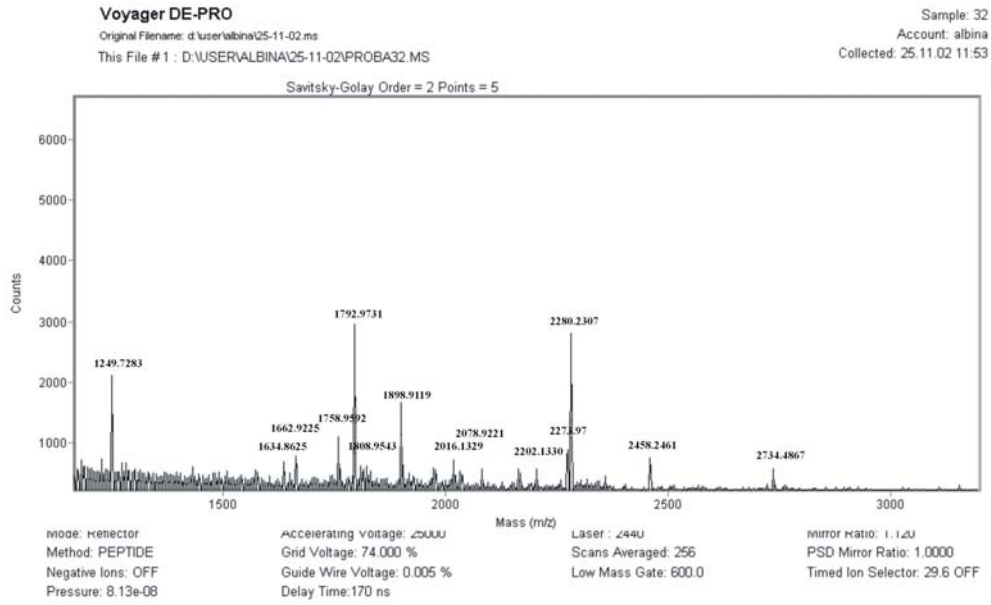


Figure 9.9 MALDI-TOF mass spectra of in-gel trypsin digested "X" protein.

MALDI spectra were analysed by the Mascot software package.

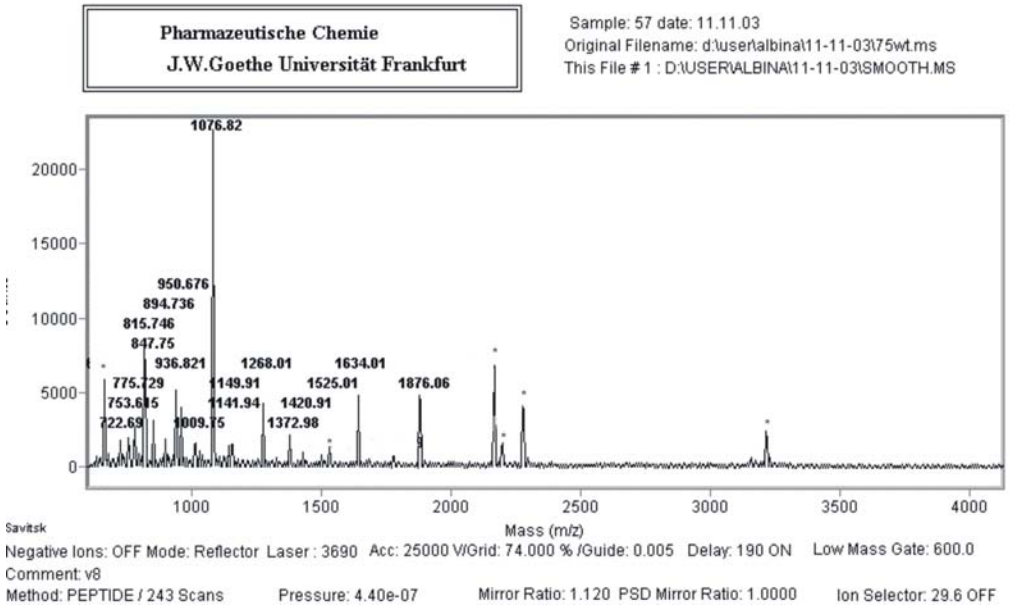


Figure 9.10 MALDI-TOF mass spectra of in-gel trypsin digested NUWM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

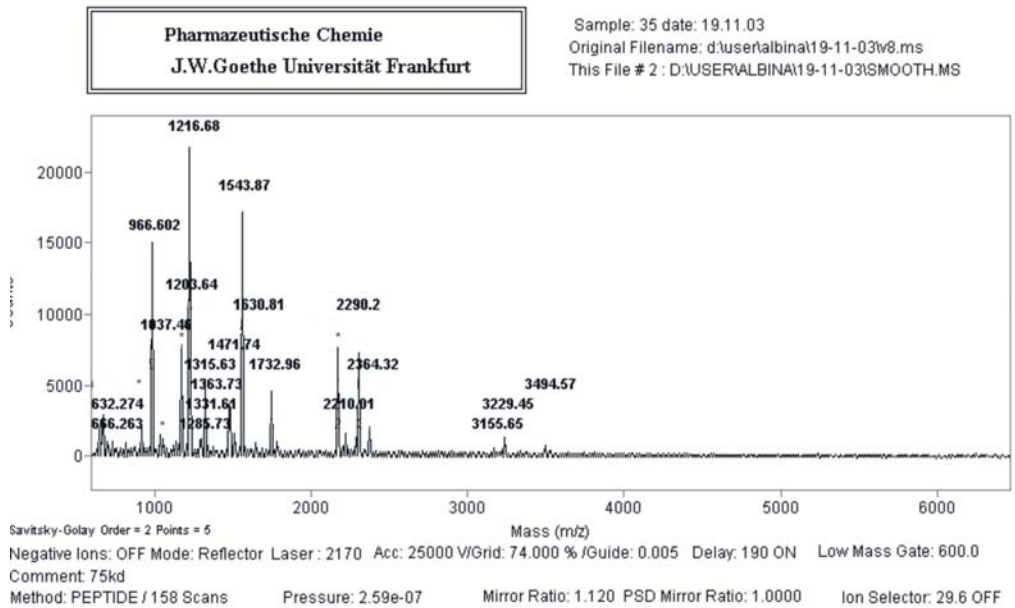


Figure 9.11 MALDI-TOF mass spectra of in-gel trypsin digested NUZM subunit

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

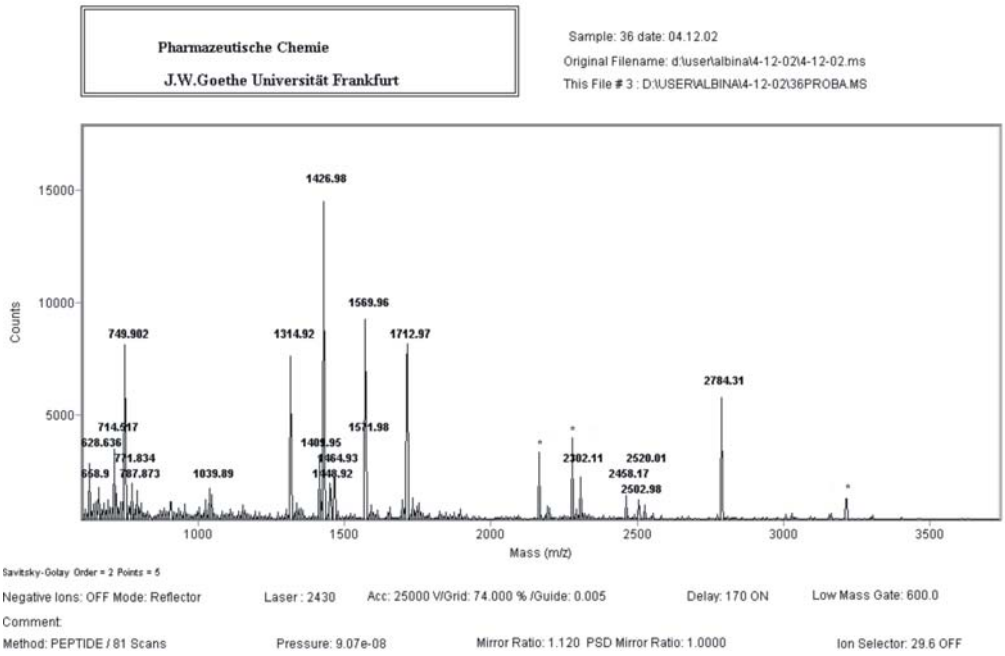


Figure 9.12 MALDI-TOF mass spectra of in-gel trypsin digested NUYM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

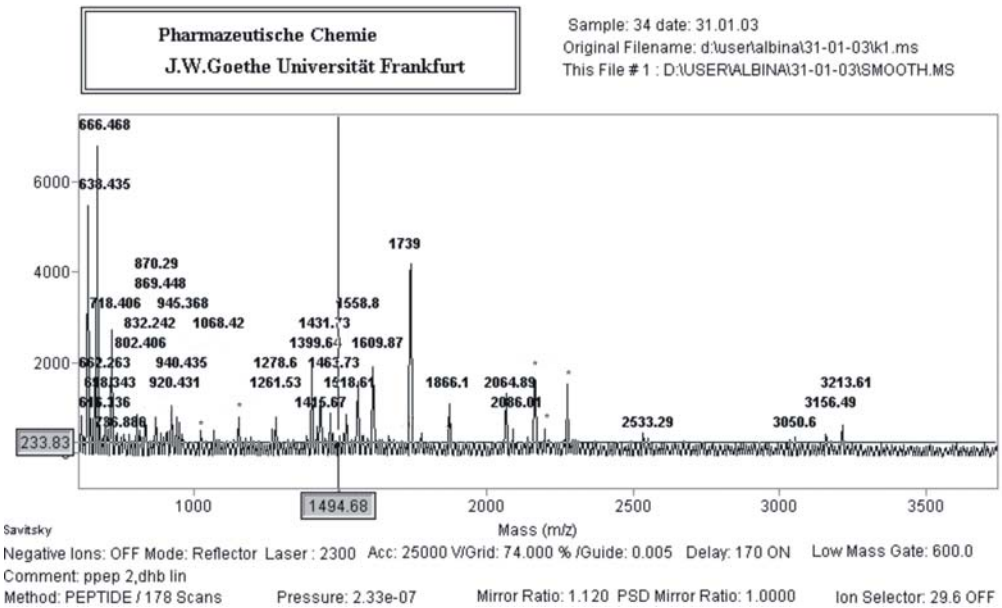


Figure 9.13 MALDI-TOF mass spectra of in-gel trypsin digested NUPM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments.

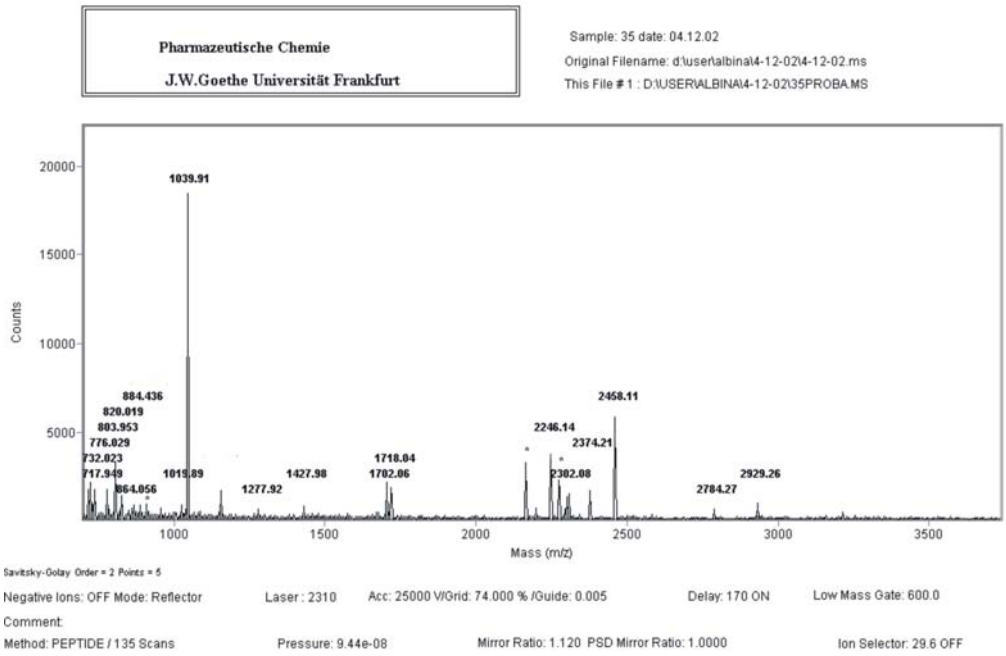


Figure 9.14 MALDI-TOF mass spectra of in-gel trypsin digested NUXM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments.

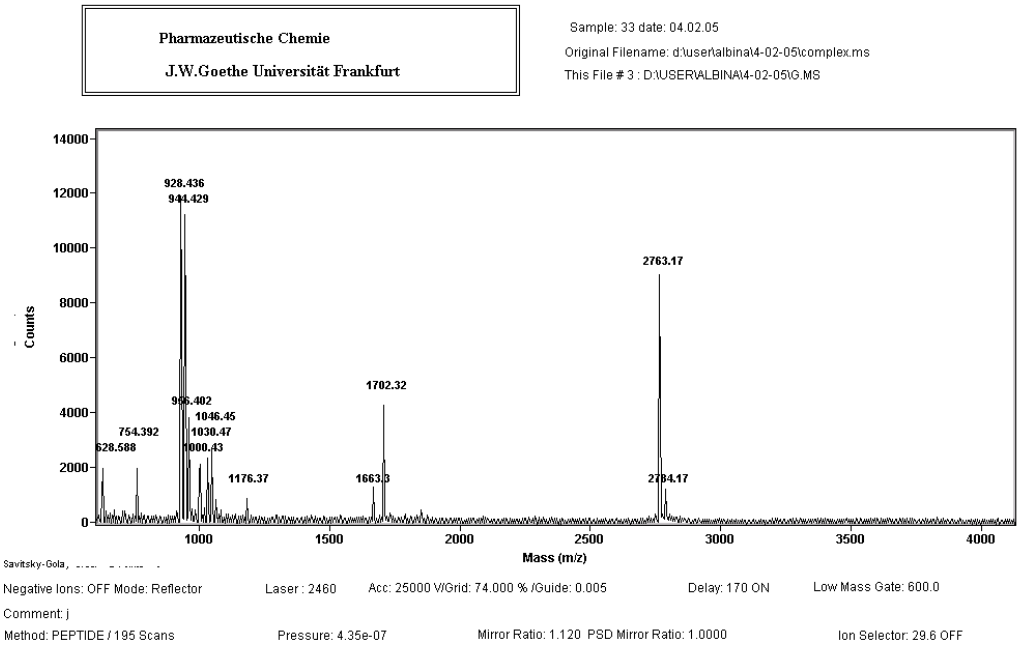


Figure 9.15 MALDI-TOF mass spectra of in-gel trypsin digested NB7M subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database.

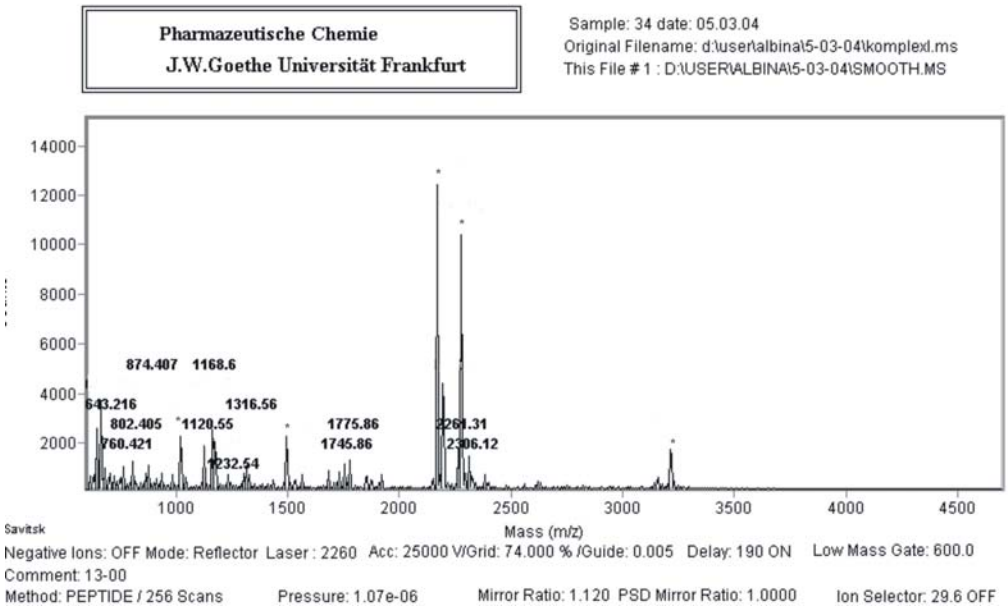


Figure 9.16 MALDI-TOF mass spectra of in-gel trypsin digested NUFM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

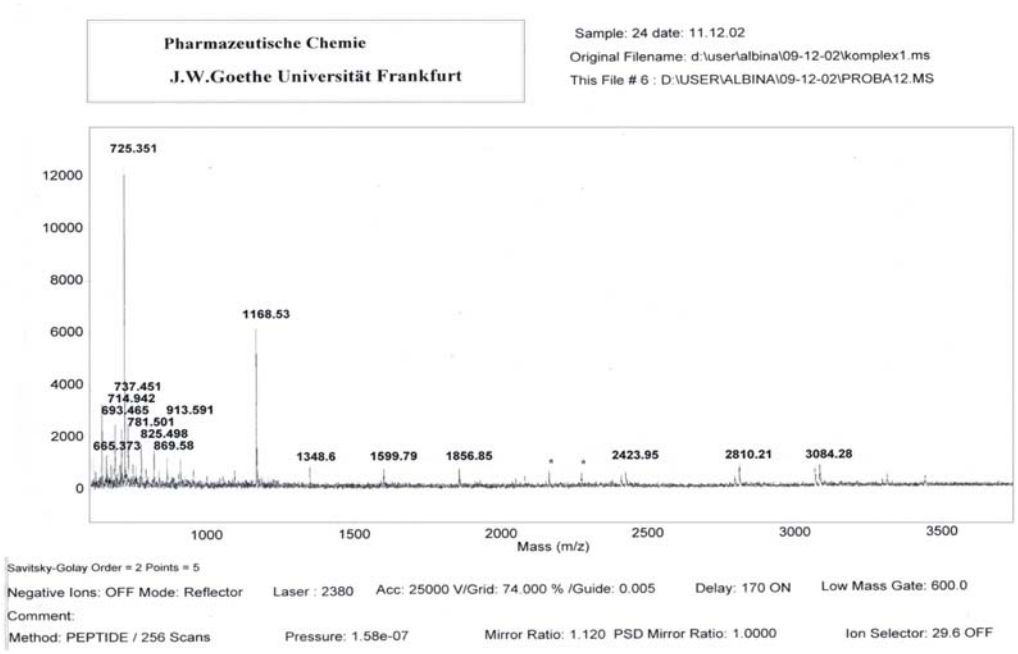


Figure 9.17 MALDI-TOF mass spectra of in-gel trypsin digested NIAM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

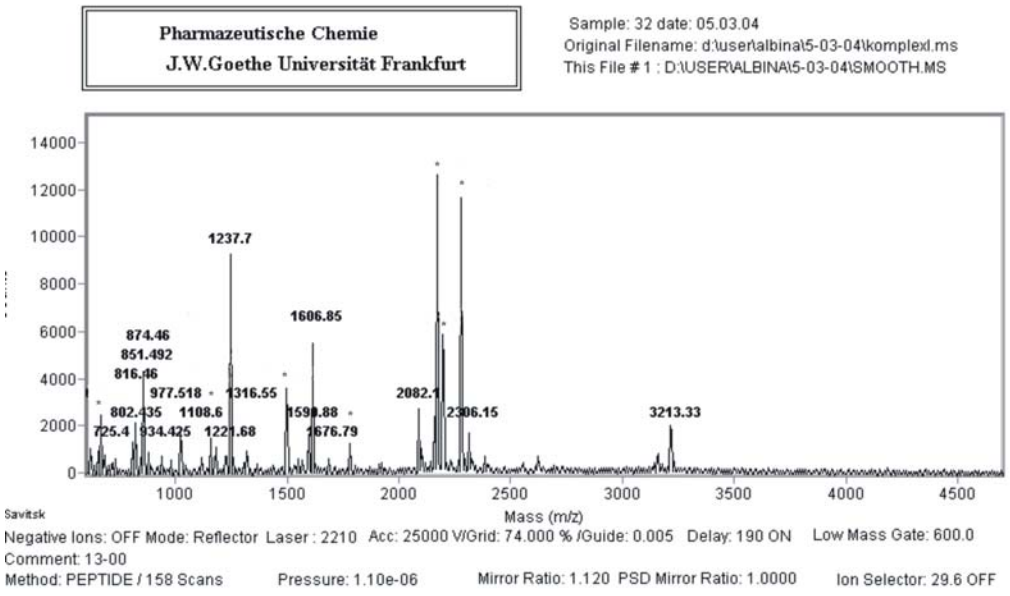


Figure 9.18 MALDI-TOF mass spectra of in-gel trypsin digested NB6M subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments.

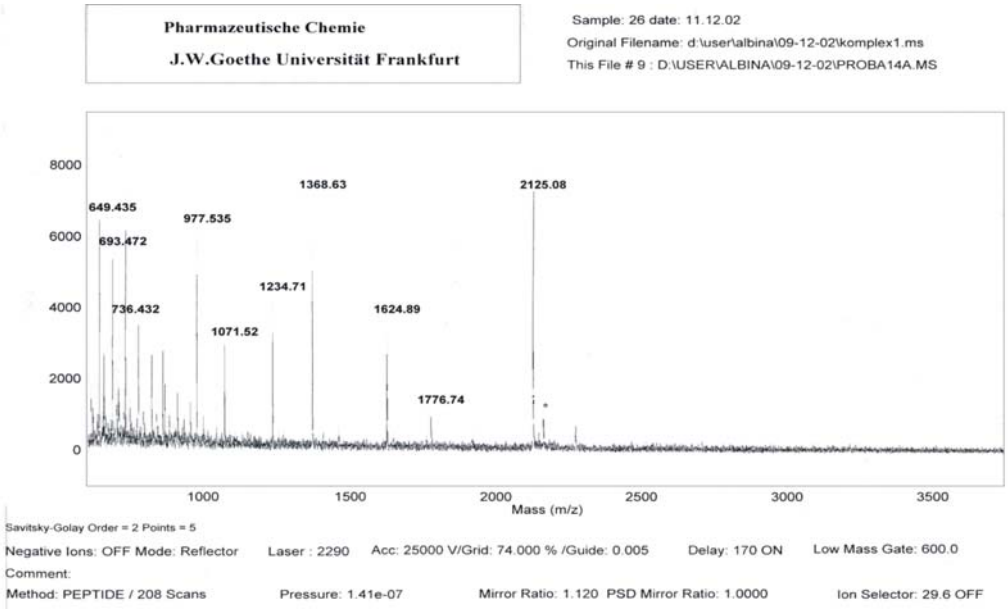


Figure 9.19 MALDI-TOF mass spectra of in-gel trypsin digested NB4M subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

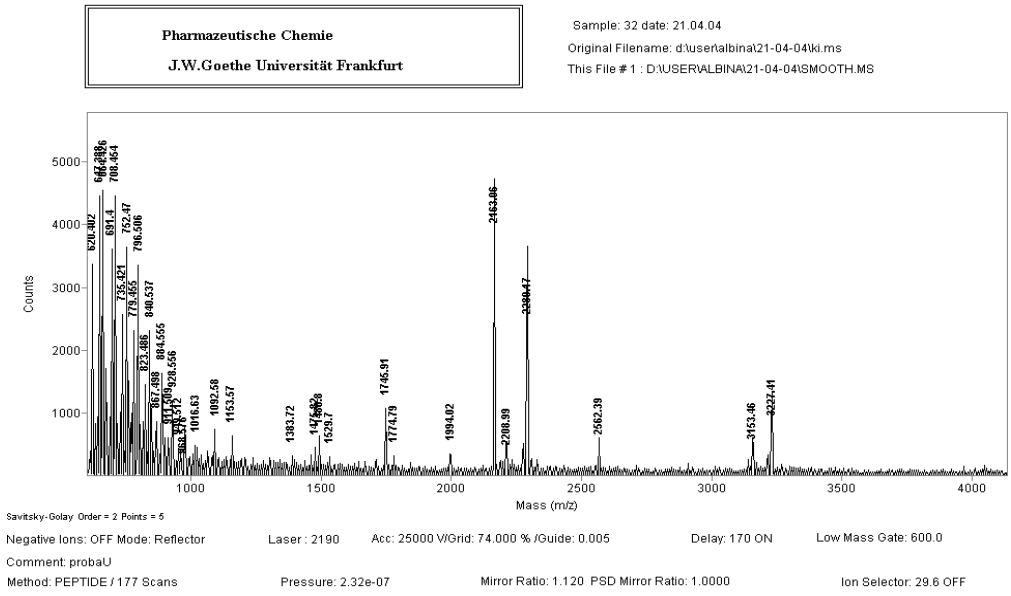


Figure 9.20 MALDI-TOF mass spectra of in-gel trypsin digested ACPM 1 subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. The picks in lower m/z range (600-1000) corresponded to polymers dissolved from the reaction vessels.

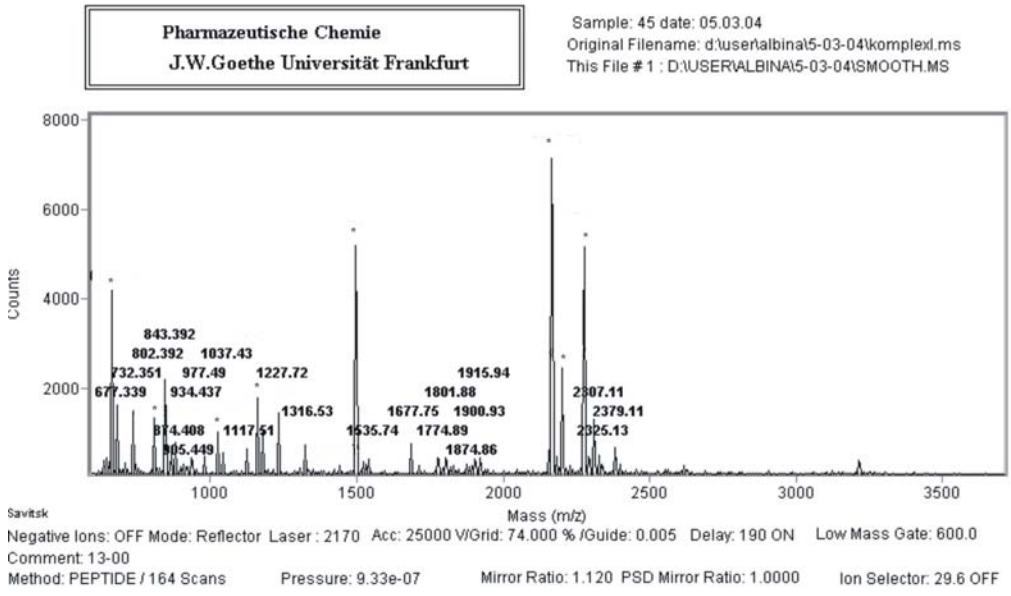


Figure 9.21 MALDI-TOF mass spectra of in-gel trypsin digested NUVM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments. The found sequence was compared with N-termini obtained by Edman degradation.

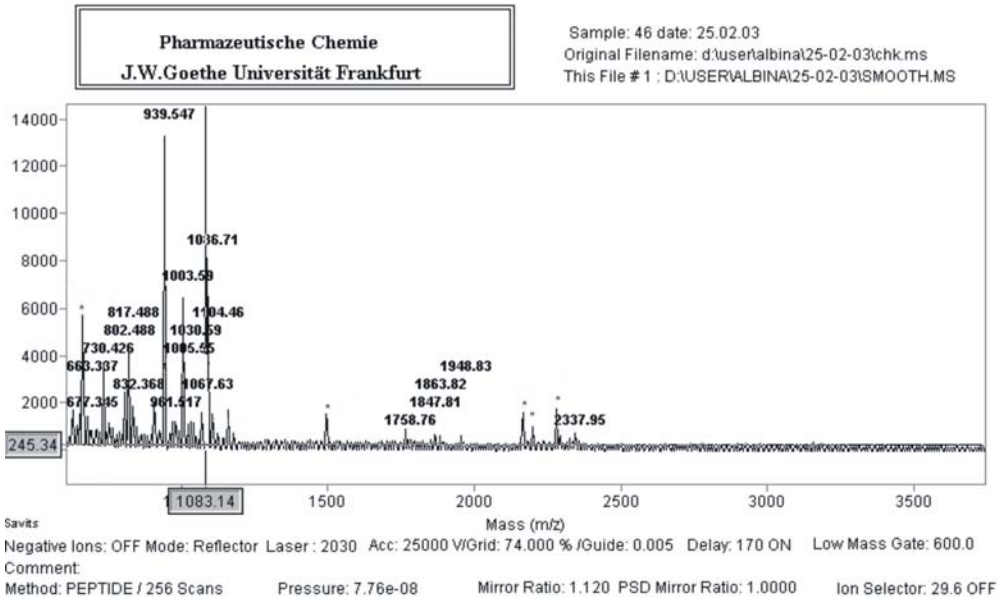


Figure 9.22 MALDI-TOF mass spectra of in-gel trypsin digested of NIPM subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments.

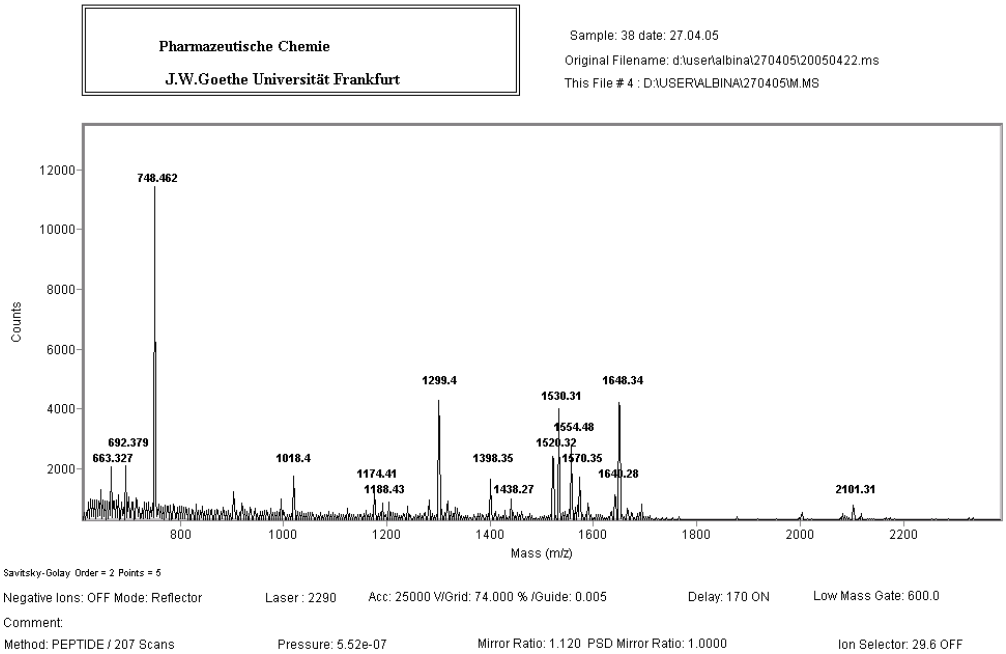


Figure 9.23 MALDI-TOF mass spectra of in-gel trypsin digested of NI8M subunit.

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database.

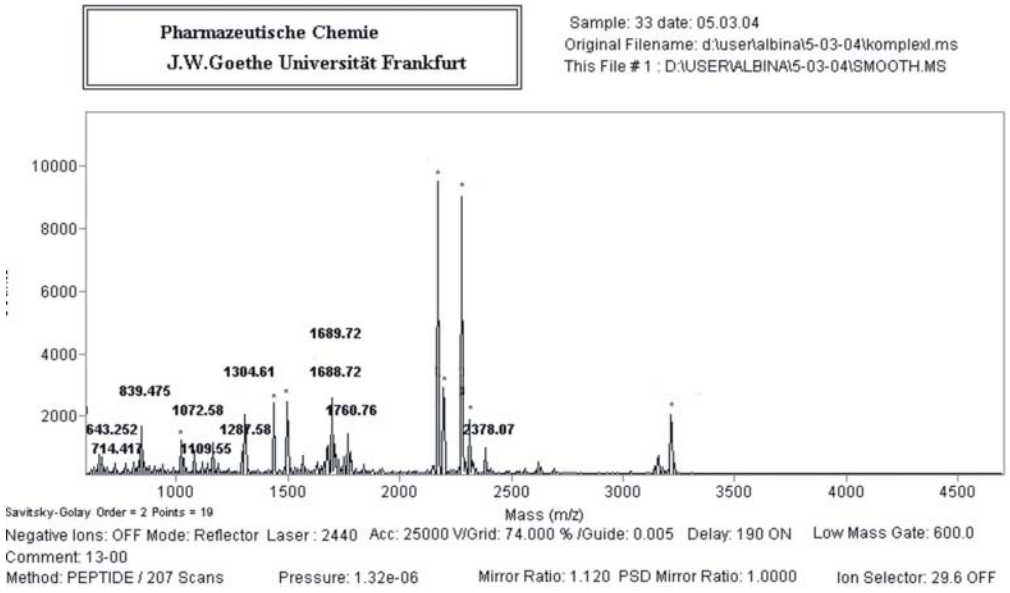


Figure 9.24 MALDI-TOF mass spectra of in-gel trypsin digested NIMM subunit

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments.

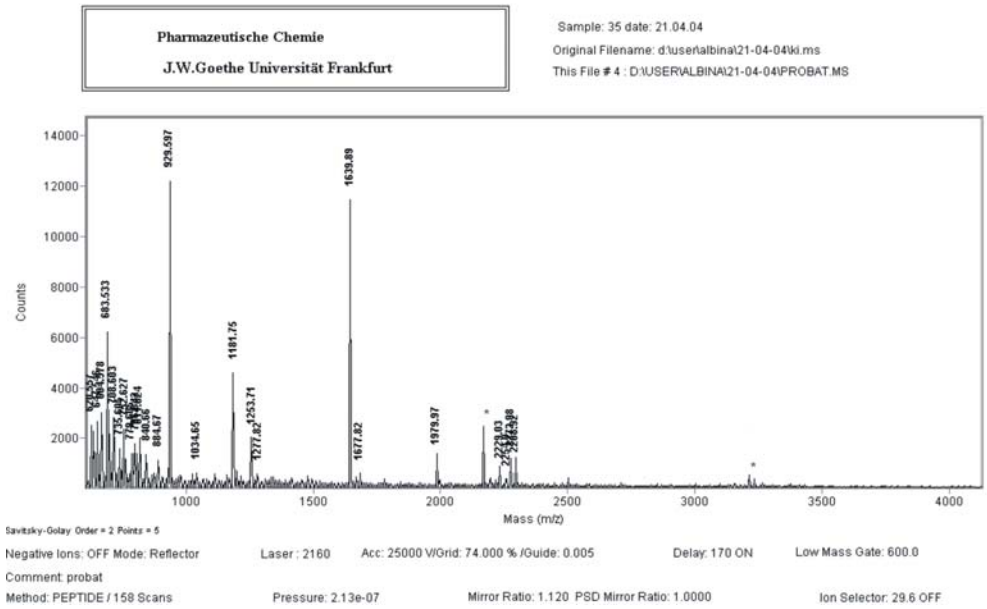
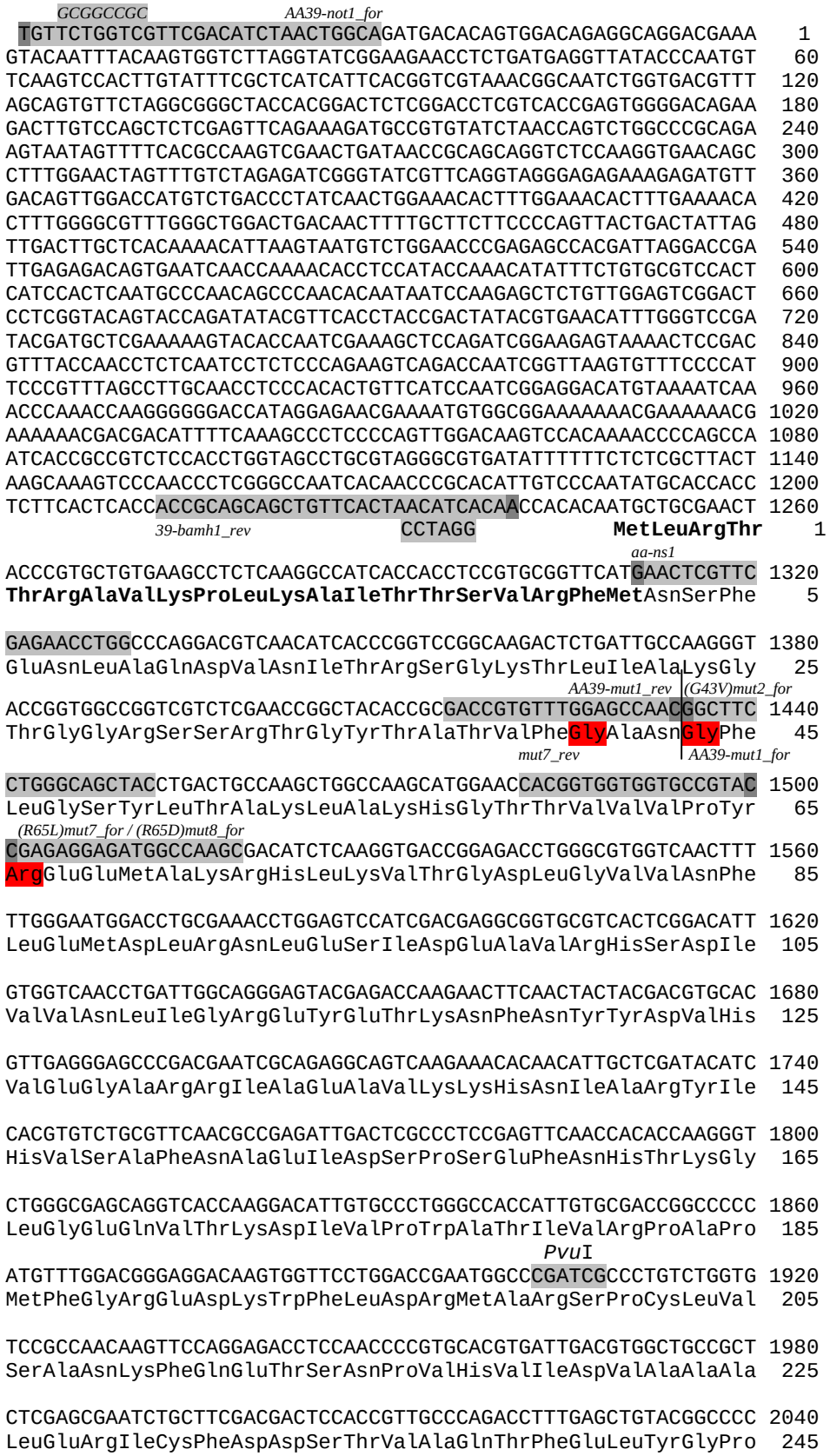


Figure 9.24 MALDI-TOF mass spectra of in-gel trypsin digested NB2M subunit

MALDI spectra were analysed by the Protein Prospector software package using a proprietary *Y. lipolytica* genomic database. Peaks marked by asteriks correspond to the trypsin autolysis fragments.

9.3 NUEM Gene

9.3.1 NUEM Gene encoding 39-kDa subunit, ACC. No. YALI0D24585g ID 2910508



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CGACACATTGAGCTGCCCAAGGCTCTGTACCAGGCTTACACCAAGGCCACCCAGGCCATC 2160
ArgHisIleGluLeuProLysAlaLeuTyrGlnAlaTyrThrLysAlaThrGlnAlaIle  285

TGGTGGCCCACCTACTCCCCCGACCAGGTCGAGCGACAGTTCCTGAGCCAGAAGATTGAT 2220
TrpTrpProThrTyrSerProAspGlnValGluArgGlnPheLeuSerGlnLysIleAsp  305

CCTTCTGCTAAGACCTTCAACGATCTGGACCTGACCCCCATGGAGCTGCCCGATCTCATG 2280
ProSerAlaLysThrPheAsnAspLeuAspLeuThrProMetGluLeuProAspLeuMet  325
                                                    aa-ns2
TTCAAGCTGATTCGACCCTACCGAGTCAACACCTTCCAGCATGATGTGTCGCAGCTGGAG 2340
PheLysLeuIleArgProTyrArgValAsnThrPheGlnHisAspValSerGlnLeuGlu  345

AACAAGGAGAAGACTTTTGTTCATATTCTTGACTAGTGTGTGAGCGCACCGGGCGAAATG 2400
AsnLysGluLysThrPheValHisIleLeuAsp***                          365
        GAATTC                  39-ecor1_for
AACACAAGTTCGTGATTGAGCGAGCACACTGCATCACAGGACGACGACTGTGGGCTGTCG 2460
AAGAATATAAAGTATTAGAAGTTGTATTAACCAGCAGTGCAGCGCTGCAAGTGTAGTGGG 2520
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AGCAAGTACCCCAGGGCGTATCCTTCCTGAAGGATTCCTGAGACGAGGCCTCTGG      3660
                          39-sal1_rev          GTCGAC
```

Figure 9.25 The fragment of NUEM gene encoding 39 kDa subunit used for generating the mutants.

Oligonucleotides used for generating the ⊿39 kDa strain, point mutants G43A, G43V, R65L, R65D and, restriction sites for PvuI are marked with grey. Darker grey indicates direction of the primer. The amino acids which were mutated are marked with red.

9.3.2 Used oligonucleotides in NUEM gene

name	sequence	binding site
39-not_for	5'-TGTGCGGCCGCCGCTCGACATCTAACTGGCA- 3'	1-29
39-ecor1_for	5'-AAGGAATTCATTGAGCGAGCACACTGCAT-3'	2465-2494
39-sal1_rev	5'-AGACAGCTGGTCTCAGGAATCCTTCA-3'	3713-3687
39-bamh1_rev	5'-TTGGGATCCTAGTGAACAGCTGCTGCGGT-3'	1301-1272

Table 9.1 Oligonucleotides for generation the deletion strain of NUEM gene

name	sequence	binding site
AA39-ü_for	5'-GAGCGTATACAAGGAAGCCA-3'	outside of sequenced area
AA39-ü_rev	5'-GTCACCACCTACAAGCAGTT-3'	
aa-ns1	5' –GAACTCGTTCGAGAACCTGG-3'	1369-1389

Table 9.2 Oligonucleotides for deletion checking via PCR

name	sequence	binding site
AA39-mut1_for	5'-GCATTCCTGGGCAGCTAC-3'	1493-1511
(G43V)mut2_for	5'- GTCTTCCTGGGCAGCTAC-3'	
(G43L)mut3_for	5'- CTCTTCCTGGGCAGCTAC-3'	
(G43W)mut4_for	5'- TGGTTCCTGGGCAGCTAC-3'	
(G43Y)mut5_for	5'- TACTTCCTGGGCAGCTAC-3'	
aa-mut6(G40A-G43A)2_for	5'- GCCAACGCATTCCTGGGCAG-3'	1487-1506
(R65L)mut7_for	5'- CTAGAGGAGATGGCCAAGC-3'	1560-1578
(R65D)mut8_for	5'- GACGAGGAGATGGCCAAGC-3'	
aa-mut6(G40A+G43A)2_rev	5'- TGCAAACACGGTCGCGGTGT-3'	1488-1468
mut7_rev	5'- GTACGGCACCACCACCGTG-3'	1541-1560

Table 9.3 Oligonucleotides for creating the point mutants of NUEM gene

name	sequence	binding site
39-seq1_for	5'- GAGCGTATACAAGGAAGCCA-3'	outside of sequenced area
39-seq2_for	5'- GCAGCAGGTCTCCAAGGTGA-3'	335-355
39-seq3_for	5'- CGTTCACCTACCGACTATAC-3'	802-822
39-seq4_for	5'- TTGCCGTGAGCGTCAGTCAG-3'	2983-2963
39-seq_for	5'- CGAGTTCAACCACACCAAGG-3'	1837-1857

Table 9.4 Oligonucleotides for checking NUEM gene

9.3.3 Alignment of NUEM (39 kDa) subunit

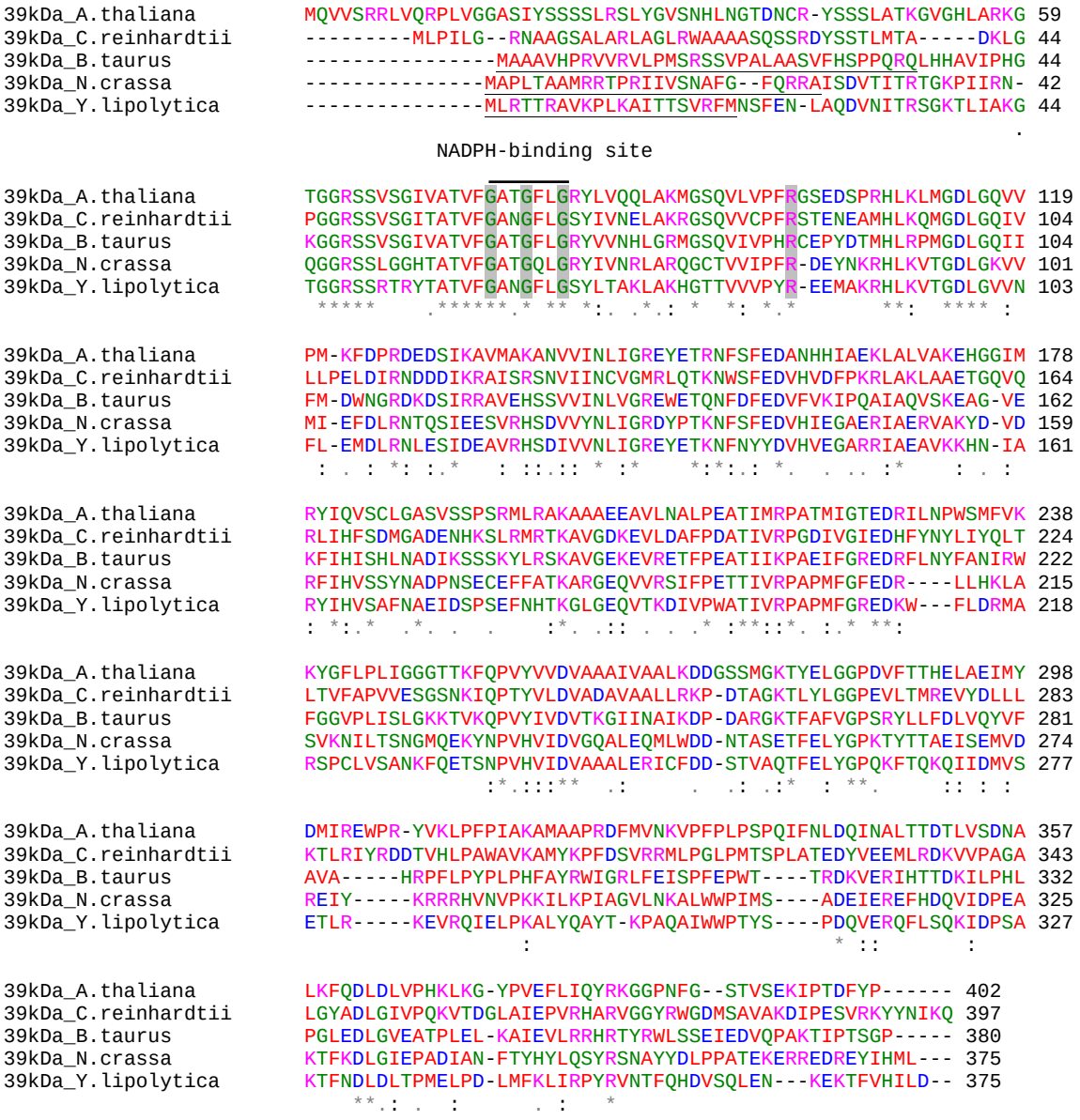

Figure 9.26 Alignment of NUEM subunits from different species.

The sequences were aligned using the CLUSTALW program (http://www.ebi.ac.uk/clustalw/index.html). * - conserved amino acids; **:** - conserved substitutions; **.** - semi-conserved substitutions. Small and hydrophobic amino acids are red, acidic are blue, basic are magenta; hydroxyl + amine + basic are green. The presequences are underlinded. The amino acids, which compose the NADPH-binding domain are marked with grey .

10 ABBREVIATIONS

cAMP	cyclic Adenosine MonoPosphate
(mt)ACP	(mitochondrial) Acyl carrier protein
(mt)DNA	(mitochondrial) Deoxy Ribonucleic Acid
17βHSD3	17beta-Hydroxysteroid Dehydrogenase type 3
ACC. No.	Accession Number
ATP	Adenosine Triphosphate
BN-PAGE	Blue-Native Polyacryl Amide Gel Electrophoresis
bp	base pair
BSA	Bovine Serum Albumin
2,5-DHB	2,5-dihydroxybenzoic acid
dSDS-PAGE	doubled Sodium Dodecylsulphate Polyacryl Gel Electrophoresis
DBQ	n-Decyl-Benzoquinone
dNADH	deamino Hydronicotineamide Adenine Dinucleotide (reduced form)
DQA	2-decyl-4-quinazolinyl amine
EPR	Electron Paramagnetic Resonance
ESI-MS	Electrospray Ionization Mass Spectrometry
FAD	Flavin Adenine Dinucleotide
$FADH_2$	Flavin Adenine Dinucleotide reduced form
FeS	Iron-Sulphur Cluster
FMN	Flavin Mononucleotide
FP	Flavo Protein
HCCA	alpha-cyano-4-hydroxycinnamic acid
HAR	Hexaammine ruthenuim(III) chloride
kb	kilobase
MALDI-TOF-	Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass

MS	Spectrometry
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Hydronicotineamide Adenine Dinucleotide (NAD^+ reduced form)
NDH-2(i)	external alternative NADH Dehydrogenase (internal)
OXPHOS	Oxidative Phosphorylation
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl flouride
PSI-BLAST	Position Specific Iterative BLAST
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
SDR	Short chain Dehydrogenase/Reductase
SDS	dodecylsulphate Na-salt
TIM	Translocase of the Inner Membrane
Q	Ubiquinone
Q_1	Quinone with 1 isoprenyl side chain
QH_2	Ubihydroquionone (Ubiquinol)

11 LIST OF FIGURES AND TABLES

11.1 Figures

11.2 Tables

12 LEBENSLAUF

Persönliche Daten

Name: Abdrakhmanova

Vorname: Albina

Geburtsdatum: 13/06/1977

Geburtsort: Almenevo, Russland

Staatsbürgerschaft: Russisch

Familienstand: ledig

Adresse: Goldsteinstrasse 68 - 60528 Frankfurt am Main, Deutschland
Tel. (mobil): 0172 1304955
e-mail: albina_abdr@yahoo.de

Wissenschaftliche Ausbildung

Apr. 2002-Jun. 2005: Doktorarbeit (Fachbereich: Chemische und Pharmazeutische Wissenschaften) am Institut für Biochemie I, Universität Frankfurt am Main, in der Arbeitsgruppe von Prof. Ulrich Brandt.
Thema: “Accessory subunits of complex I from *Yarrowia lipolytica*”.

Jan.2001-Apr.2002: Wissenschaftlicher Mitarbeiter am Institut für Biologie II, Freiburg, Deutschland, in der Arbeitsgruppe von Prof. Peter Nick.
Thema: ”Regulation des mikrotubulaeren Zellskeletts bei der Kälteakklimation von Winterweizen”.

Apr. 2000-Jun. 2000: Diplomarbeit am Lehrstuhl für Pflanzenphysiologie, Universität Kazan, Russland, in der Arbeitsgruppe von Prof. Ludmila Khokhlova.

Thema: “Der Einfluss der cytoskelettmodifizierenden Faktoren auf die Aktivität der Atmungskette der Blätter verschiedener Weizensorten”.

Sep.1995- Jun. 2000: Studium der Biologie an der Universität Kazan, Russland