Investigation of Conserved Amino Acids in the PSST and TYKY Subunits of Complex I from *Yarrowia lipolytica*

Dissertation

zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich Chemische und pharmazeutische Wissenschaften
der Johann Wolfgang Goethe Universität
in Frankfurt am Main

von

Aurelio Garofano

aus Sacile (Italien)

Frankfurt am Main 2004
(DF1)

vom Fachbereich Chemische und pharmazeutische Wissenschaften der Johann Wolfgang Goethe Universität als Dissertation angenommen.

Dekan : Prof. Schwalbe

Gutachter: Prof. Ludwig

Prof. Brandt

Datum der Disputation: .....

Teile der vorliegenden Arbeit wurden veröffentlicht:

P. Ahlers, A.Garofano, S.Kerscher, and U. Brandt. 2000a. Application of the Obligate Aerobic Yeast *Yarrowia lipolytica* as a Eucaryotic Model to Analyze Leigh Syndrome Mutations in the Complex I Core Subunits PSST and TYKY. *Biochim. Biophys. Acta - Bioenerg.* 1459:258-265.

Garofano A., Zwicker K., Kerscher S., Okun P. and Brandt U. 2003. Two Aspartic Acid Residues in the PSST-Homologous NUKM Subunit of Complex I from *Yarrowia lipolytica* Are Essential for Catalytic Activity. *J. Biol. Chem.* 278:42435-42440.

Stefan Kerscher, Ljuban Grgic, Aurelio Garofano and Ulrich Brandt. Application of the Yeast *Yarrowia lipolytica* as a Model to Analyse Human Pathogenic Mutations in Mitochondrial Complex I (NADH:Ubiquinone Oxidoreductase), BBA in press.

R. A. Duval, G. Lewin, E. Peris, A. Garofano, D. Cortes, U. Brandt and Reynald Hocquemiller. First Heterocyclic Analogs of Squamocin as Inhibitors of Mitochondrial Complexes I and III. On the Role of the Terminal Lactone of Annonaceous Acetogenins, in preparation.

A. Garofano, S. Mauesberger, G. Barth, U. Brandt and S. Kerscher. Realisation of a Substrate Inducible NDH2i in the Yeast *Y. lipolytica*, in preparation.

Die vorliegende Arbeit wurde in der Zeit von Februar 2000 bis Juni 2004 am Institut für Biochemie I - Molekulare Bioenergetik des Gustav Embden-Zentrums der Biologischen Chemie des Universitätsklinikums der Johann Wolfgang Goethe-Universität in Frankfurt am Main unter Anleitung von Prof. Dr. Ulrich Brandt durchgeführt.

Besonders möchte ich mich bei Prof. Dr. Ulrich Brandt für die Überlassung des Themas, die Betreuung und Förderung meiner Dissertation und die Vielzahl an anregenden Diskussionen, die zum Gelingen dieser Arbeit beigetragen haben bedanken.

Herrn Prof. Dr. Bernd Ludwig möchte ich für die Übernahme der externen Betreuung danken, die es mir ermöglichte, diese Arbeit am Institut für Biochemie I durchzuführen.

Mein weiterer Dank gilt :

Herrn Dr. Stefan Kerscher, der immer hilfsbereit war und stets mit mir wissenschaftliche Fragestellungen diskutierte, sowie für das kritische Lesen dieser Arbeit.

Herrn Dr. Klaus Zwicker für die Aufnahme von EPR-Spektren.

Herrn Prof. Dr. Hermann Schägger gilt mein Dank für die hervorragenden experimentellen Bedingungen bei der Durchführung dieser Arbeit und eine stete Diskussionsbereitschaft.

Frau Dipl.-Ing. Gudrun Beyer für die kompetente Einführung in die Welt der molekularen Biologie.

Herrn Dr. Stefan Dröse für viele wertvolle Tipps und Diskussionen, sowie für das kritische Lesen eines Teiles dieser Arbeit..

Frau Jolanta Maria Brzoska für die Mitochondrienpräparationen.

Frau Andrea Böttcher für ihre wertvolle Hilfe bei der Sequenzierung und ihre Betreuung von PCs.

Ilka Siebels, Karin Siegmund und Franz- Josef Streb für die Hilfsbereitschaft und die netten Kaffeepausen.

Allen Laborkollegen besonders meinen Mitdoktoranden Albina Abdrakhmanova, Andrea Eschemann, Ljuban Grgic und Anke Stroh für die stete Unterstützung sowie die freundliche und lockere Arbeitsatmosphäre.

Andrea und Domenica für die Hilfe beim Schreiben des deutschen Textes.

Un „Grazie“ particolare ai miei genitori per il sostegno e l´aiuto amorevolmente datomi duranti gli anni di studio. (Schließlich möchte ich meinen Eltern danken, die mir diese Ausbildung ermöglicht haben).

1 INTRODUCTION

1.1 The Mitochondrial Respiratory Chain

The mitochondrial respiratory chain transfers electrons through a redox potential span of 1.14 V ($E_{m,7}$), from the NAD^+ / NADH couple to the O_2 / $2H_2O$ couple (Nicholls and Ferguson, 2001). Four enzymes (complexes) are responsible for the electron transfer:

- NADH:Ubiquinone Oxidoreductase (complex I)
- Succinate:Ubiquinone Oxidoreductase (complex II)
- Ubiquinol:Cytochrome *c*-Oxidoreductase (complex III)
- Cytochrome *c*-Oxidase (complex IV)

and two types of electron carriers

- Ubiquinone (UQ)
- Cytochrome c.

The respiratory chain and the ATP-synthase constitute the oxidative phosphorylation system (OXPHOS), that produces most of the ATP in aerobic organisms. The ATP production begins with the oxidation of NADH (produced mostly in the citric acid cycle and β-oxidation of fatty acids) in the mitochondrial matrix. The resulting electrons are transported to oxygen through the respiratory chain in the mitochondrial inner membrane. During this transfer protons are translocated across the membrane producing an electrochemical potential. This protonmotive force is used for the synthesis of ATP from ADP and inorganic phosphate by the ATP-synthase, as first proposed by Mitchell (1961).

1.2 Complex I

1.2.1 General Overview

Proton-translocating NADH:ubiquinone oxidoreductase (complex I, EC 1.6.99.3) transports two electrons from NADH to membrane bound ubiquinone: in this process four protons are translocated across the membrane, producing 40% of the total proton gradient between matrix side and intermembrane space (Wikström, 1984).

Complex I is present in many eucaryotes and procaryotes. Bacterial enzymes have a molecular mass of around 500 kDa and consist of 14 subunits (Yagi et al., 1998; Dupuis et al., 1998). Mitochondrial complex I has a molecular weight of around 1000 kDa (Hirst et al., 2003), contains more than 35 subunits in fungi and plants (Leif et al., 1993) and at least 46 in mammals. Most of the genes for mammalian complex I are located in the nuclear genome and seven in the mitochondrial genome. This dual coding system and the size of this multi-protein enzyme cause a number of difficulties in studying function and mechanism. Furthermore, an X-ray structure is not available.

On the other hand, complex I raises interest not only in the bioenergetic field as the least understood enzyme of the OXPHOS system (X-ray structures are available for the other complexes), but also in the medical field and as target for insecticides and acaricides (see 1.4 and 1.5).

1.2.2 Complex I Structure

The subunits that form the bacterial proton-translocating NADH-quinone oxidoreductase (NDH-1) in organisms like *Escherichia coli, Thermus thermophilus, Paracoccus denitrificans* and *Rhodobacter capsulatus*, have homologous subunits in mitochondrial complex I (Yagi et al., 1998). Therefore, the bacterial enzyme is considered as the „minimal form" of complex I with regard to eucaryotic enzyme, and the bacterial subunits are defined as "core" subunits.

For simplicity, in this work the nomenclature of *E. coli* NDH-1 and of *Bos taurus* NADH-ubiquinone oxidoreductase are used for bacterial and mitochondrial complex I, respectively. Table 1.1 compares nomenclatures of homologous subunits between bacterial and mitochondrial enzymes. A comparison between nomenclatures and subunit composition of complex I from different organisms is shown in appendix 9.1.

The bacterial enzyme from *E. coli* and the eucaryotic enzyme from the filamentous fungus *Neurospora crassa*, the mammal *B. taurus* and from the yeast *Yarrowia lipolytica* were investigated by electronic microscopy. They showed an L-form, which consists of two domains: a peripheral "arm" (hydrophilic domain) and a membrane "arm" (hydrophobic domain) (Djafarzadeh et al., 2000).

Treatment with the non-denaturing detergent *N,N*-dimethyl-dodecylamine *N*-oxide allowed the dissociation of the bovine enzyme into two subcomplexes called Iα and Iβ (Finel et al., 1992). Characterization of these subcomplexes revealed that Iα consists of mostly hydrophilic subunits and contains all the known redox centres and has NADH:ferricyanide oxidoreductase activity. Most likely this fragment constitutes the peripheral and part of the membrane arm (Walker et al., 1995). The peripheral arm, which is the hydrophilic part of the protein and protrudes into the matrix, is constituted of seven subunits (NUOB, C-D, E, F, G, I) in bacteria. The same domain in eucaryotes contains the 7 nuclear coded subunits 20 kDa (or TYKY), 23 kDa (or PSST), 24 kDa, 30 kDa, 49 kDa, 51 kDa, and 75 kDa that are homologous to the bacterial ones. The Iβ fragment contains hydrophobic subunits, does not have prosthetic groups and did not show NADH:ferricyanide oxidoreductase activity (Sazanov et al., 2000). This domain constitutes the membrane arm and is embedded in the lipid bilayer of the membrane by virtue of its hydrophobicity: NUOA, NUOK, NUOL, NUOJ, NUOH, NUOM, NUON are the seven subunits which form this domain in *E. coli*. ND1-6 and ND4L, encoded by the mitochondrial genome, are their homologous in eukaryotes.

Little is known about the function of the remaining subunits in the eucaryotic complex I: in this work only the core subunits are considered.

Mitochondrial Complex I (*B. taurus*)		Bacterial Complex I (*E. coli*)	Subunit Location (L-shaped form)
Name of the Subunit	Gene Localization	Name of the Subunit	Domain
NUAM or 75 kDa	Nucleus	Nuo B	Peripheral domain
NUBM or 51 kDa	Nucleus	Nuo C*	Peripheral domain
NUCM or 49 kDa	Nucleus	Nuo D*	Peripheral domain
NUGM or 30 kDa	Nucleus	Nuo E	Peripheral domain
NUHM or 24 kDa	Nucleus	Nuo F	Peripheral domain
NUIM or TYKY	Nucleus	Nuo G	Peripheral domain
NUKM or PSST	Nucleus	Nuo I	Peripheral domain
ND1	Mitochondria	Nuo H	Membrane domain
ND2	Mitochondria	Nuo N	Membrane domain
ND3	Mitochondria	Nuo A	Membrane domain
ND4	Mitochondria	Nuo M	Membrane domain
ND4L	Mitochondria	Nuo K	Membrane domain
ND5	Mitochondria	Nuo L	Membrane domain
ND6	Mitochondria	Nuo J	Membrane domain

Table 1.1 ***B. taurus (bovine) and E. coli nomenclatures of the 14 core subunits of complex I***

*In *E. coli*, Nuo C and D are fused

1.2.3 Complex I Redox Groups

Complex I contains one non-covalently bound flavin mononucleotide (FMN) and many iron-sulfur clusters as redox active groups. They transport electrons from NADH to ubiquinone. FMN oxidises NADH by uptake of two electrons and acts as a converter between the two-electron donor NADH and the one-electron acceptors, the iron-sulfur clusters. There are bi- and tetra–nuclear clusters in complex I. Binuclear clusters are composed of two iron ions, which are bridged by two inorganic sulfides and ligated to four cysteinyl sulfurs that belong to the polypeptide chain of the protein. Tetranuclear clusters contain four iron and four inorganic sulfur ions arranged in a distorted cube structure in which the iron ions are bound to the polypeptide chain via four cysteine sulfur ligands (Ohnishi, 1998). The number of iron-sulfur clusters in complex I is still unclear and depends on the origin of the enzyme under study (Meinhardt et al., 1990). In bovine complex I (the best characterised mammalian enzyme) six iron-sulfur clusters were identified by EPR spectroscopy: $N1_a$, $N1_b$, N2, N3, N4 and N5 (Walker, 1992). Cluster $N1_a$ and $N1_b$ are binuclear and N2, N3, N4 and N5 are tetranuclear. Controversial and still discussed is the subunit location of the redox centers (Ohnishi, 1998). The latest commonly accepted hypotheses about type and location of the EPR detectable redox centers has been summarised in Fig. 1.1. The first event is the oxidation of NADH by FMN. Successively, electrons are transported through the clusters $N1_a$, $N1_b$, N3, N4 and N5. The exact order of electron transport trough the iron-sulfur clusters is still unknown. Cluster N2, having a pH dependent redox midpoint potential, is most likely the last step to ubiquinone.

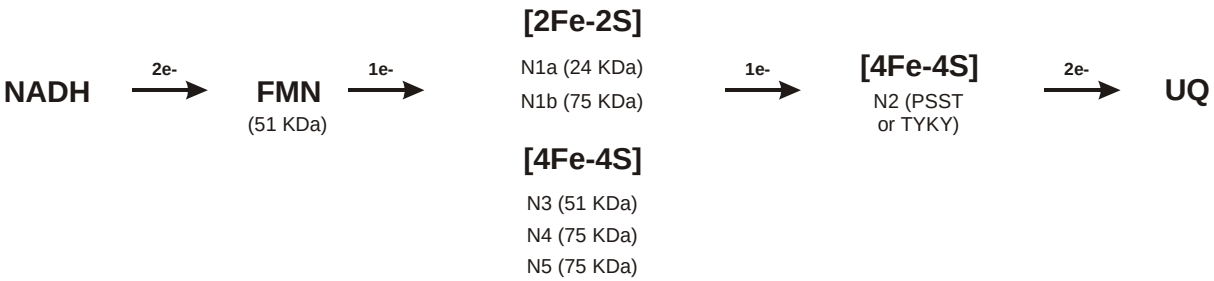

Figure 1.1 ***-Electron transport in complex I-***

For simplicity, the bovine nomenclature has been used. Ubiquinone (UQ) is the final electron acceptor.

Cluster N2

Cluster N2 is supposed to be the immediate electron donor to ubiquinone by virtue of its highest and pH dependent redox midpoint potential ($E_{m,7}$ −150 mV), which distinguishes it from the other clusters. Ohnishi and coworkers showed an EPR detectable magnetic interaction between cluster N2 and semiquinone radicals and predicted their distance to be 8-11 Å, suggesting cluster N2 as immediate electron donor to ubiquinone (Ohnishi et al., 1999). Since in bovine complex I the redox midpoint potential of N2 was found to be dependent on the pH value (Ingledew and Ohnishi, 1980), cluster N2 has been considered to be involved in the proton translocation mechanism (Brandt and Trumpower, 1994).

The exact location of the tetra-nuclear cluster N2 is still a matter of debate. The TYKY subunit contains two canonical binding motifs for tetranuclear clusters (CxxCxxCxxxCP – $(x)_{27}$ – CxxCxxCxxxCP) that contain eight cysteins as possible ligands. The PSST subunit contains one non canonical motif CC$(x)_{63}$ - C$(x)_{28}$ - GCPP in which two cysteins are contiguous in the amino acids sequence. For steric reasons it seems unlikely that both of them ligate cluster N2, posing the question about the fourth ligand. Site directed mutagenesis in the PSST homologous subunits from *E. coli* (Friedrich, 1998), *N. crassa* (Dupuis et al., 1998), and *Yarrowia lipolytica* (see 1.3.4) did not solve this question unequivocally.

1.2.4 Functional Modules of Complex I

As previously described, 14 genes constitute the "minimal form" of a proton-pumping respiratory NADH:ubiquinone oxidoreductase in bacteria. This "minimal form" can be functionally sub-divided into three modules (Friedrich and Scheide, 2000):

1. An electron transfer module, formed by the 24, 51 and 75 kDa subunits that oxidizes NADH via FMN.
2. A proton translocation module, constituted by ND4/5 and ND2 subunits.
3. A ubiquinone reduction module, formed by the 49 kDa, PSST and TYKY subunits.

Complex I evolved from the above described pre-existing modules:

1. The ability to transfer electrons by FMN and Fe-S clusters has been found in many bacterial hydrogenases: they oxidize hydrogen, reduce NAD^+ and contain non-covalently bound FMN and several Fe-S clusters. These hydrogenases show homology to parts of the 24, 51 and 75 kDa subunits from complex I (Friedrich and Weiss, 1997).
2. The ability to pump protons seems to be related to a module present in bacterial K^+ / H^+ or Na^+ / H^+ antiporters (Pinner et al., 1992). Genes coding for this module show homology to ND4 and ND5 in complex I, (Friedrich and Weiss, 1997; Mathiesen and Hägerhäll, 2002).
3. A common evolutionary origin of complex I and [Ni-Fe] hydrogenases was suggested (Friedrich and Scheide, 2000; Böhm et al., 1990). Assuming a similar mechanism for the electron transport in the two enzyme classes, knowledge about hydrogenases could be a help for understanding the ubiquinone reduction module of complex I (see below).

1.2.5 [Ni-Fe] Hydrogenases: a Model for the Ubiquinone Reduction Module in Complex I

Hydrogenases catalyse either cleavage or production of molecular hydrogen, following the reaction: $H_2 \rightleftharpoons 2H^+ + 2e^-$. For ours purposes we distinguish between water-soluble and membrane-bound hydrogenases.

The water soluble [Ni-Fe] hydrogenases from *Desulfovibrio gigas* (Volbeda et al., 1995) and *Desulfovibrio fructosovorans* (Montet et al., 1997) are composed of a large subunit which contains the [Ni-Fe] cluster coordinated by four cysteines and a small subunit which contains three iron-sulfur clusters, one [3Fe-4S] and two [4Fe-4S] in a linear arrangement. Sequence comparison suggests that the large subunit corresponds to the 49 kDa and that the small one corresponds to the PSST subunit of mitochondrial complex I (Fig. 1.2A-C). According to this theory, the proximal tetranuclear cluster in the small subunit corresponds to cluster N2 in complex I (Kashani-Poor et al., 2001b). Three of the four cystein ligands of the proximal cluster, which form a conserved motif $CxxCn_nGxCxxxGx_mCPP$, have corresponding cysteines in the PSST subunit, but the first cysteine of this motif is missing in complex I (see 1.2.3). The proximal cluster is close to the interface between small and large subunit (Fig. 1.2A). The [Ni-Fe] cluster is the active site, in which hydrogen production takes place, and donates electrons to the proximal cluster. Four cysteines are ligands for the [Ni-Fe] cluster: C72, C75, C543 and C546 (*D. fructosovorans*). These positions are not conserved in complex I and the [Ni-Fe] cluster is missing. It has been proposed that the catalytic centre of complex I evolved from the hydrogenases catalytic centre and that the [Ni-Fe] site became the ubiquinone-binding site (Kashani-Poor et al., 2001b). With respect to this model, it is predicted that the fold around cluster N2 is partially conserved, that the PSST and the 49 kDa subunits in complex I are close to each other like the small and large subunits in hydrogenases, and that cluster N2 is located near the interface between these two subunits (Kerscher et al., 2001b). Figure 1.2C represents schematically complex I subunits that are homologous to subunits in [Ni-Fe] hydrogenases from *D. fructosovorans* and *M. barkeri*.

Interestingly, a random mutagenesis study in the 49 kDa homologous subunit in complex I from *Rhodobacter capsulatus* revealed that exchange of valine 407 into methionine causes resistance against quinone-analogous complex I inhibitors (Darrouzet et al., 1998). This residue corresponds to the [Ni-Fe] cluster ligand C543 in *D. fructosovorans*, indicating that inhibitor resistance is caused by a mutation in the region of the 49 kDa subunit that originated

from the [Ni-Fe] site of hydrogenases. These observations are in line with the results of a site directed mutagenesis study in *Y. lipolytica*. Aspartate 458, corresponding to position D541 in the large subunit from *D. fructosovorans* (located about 8 Å away from the [Ni-Fe] site and only two residues upstream from the ligand C453) was changed into alanine. Strain D458A showed I_{50} values for quinone-analogous inhibitors up to 30 times higher than the parental one. In addition, site directed mutagenesis of each residue that corresponds to the cysteine ligands of the hydrogenase [Ni-Fe] cluster has been carried out in *Y. lipolytica*. These mutations had dramatic effects on complex I NADH:ubiquinone oxidoreductase activity. Overall, there is substantial evidence that the fold around the [Ni-Fe] site in hydrogenases has been conserved and evolved into part of the quinone binding region of complex I (Kashani-Poor et al., 2001b).

The membrane-bound [Ni-Fe] hydrogenase from *Methanosarcina barkeri* consists of five subunits: EchA, EchB, EchC, EchE and EchF. The [Ni-Fe] cluster is located in EchE, which is homologous to the 49 kDa subunit, EchC corresponds to PSST and EchF corresponds to TYKY (Fig. 1.2B-C). Two four-cysteine motifs for the binding of two [4Fe-4S] clusters were identified in the amino acid sequence of the EchF subunit and one four-cysteines motif in the EchC subunit, as illustrated in figure 1.2B. EPR analysis indicated the presence of a tetranuclear cluster in the EchC subunit, which by virtue of its magnetic interaction with the [Ni-Fe] site is supposed to be close to the interface between the two subunits. Both other clusters were consequently assigned to EchF (Kurkin et al., 2002). In membrane-bound [Ni-Fe] hydrogenases, the homologue of the small subunit of water-soluble enzymes has suffered a C-terminal deletion, removing the binding motifs of two iron-sulfur clusters. Apparently, these have been replaced by a novel, ferrodoxin-like subunit in membrane bound hydrogenases. Both EchA and EchB are supposed to be involved in the transfer of proton across the membrane, similarly to ND2/5/4 and ND1 in complex I.

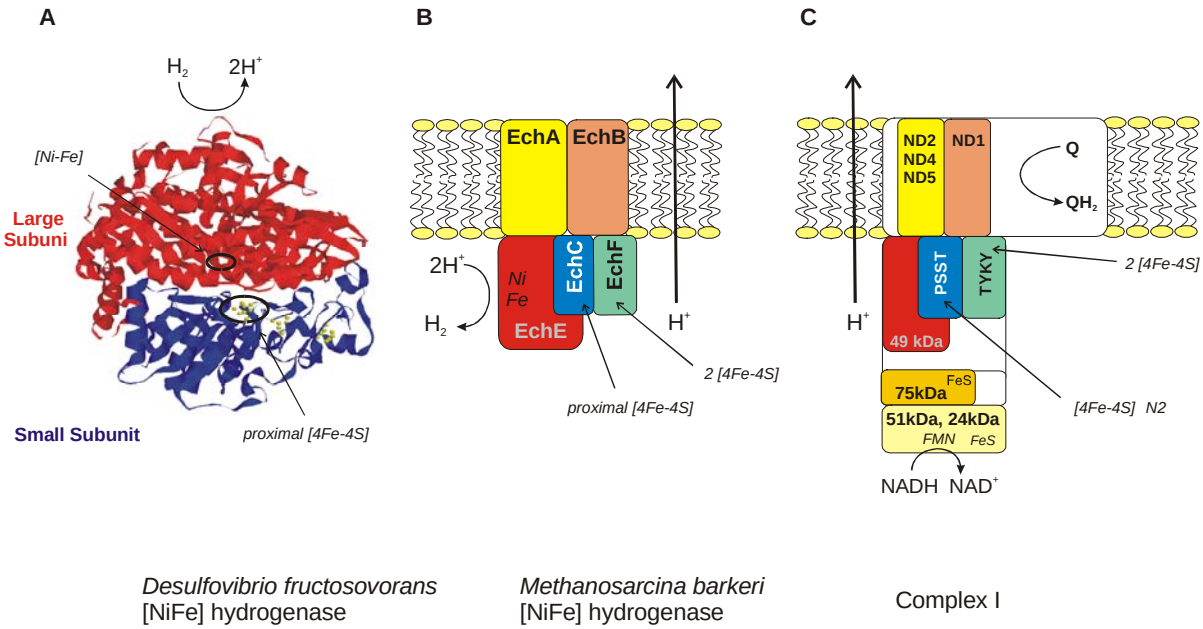

Figure 1.2 ***-Homology between [Ni-Fe] hydrogenases and complex I-***

A. X-ray representation of the [Ni-Fe] hydrogenase from *Desulfovibrio fructosovorans*; **B.** [Ni-Fe] hydrogenase from *Methanosarcina barkeri*; **C.** Schematic representation of NADH:ubiquinone oxidoreductase (Complex I). Subunits that are homologous between the three classes of enzymes are indicated by the same colour. The direction of the proton transport is indicated by arrows.

1.3 Yarrowia lipolytica

1.3.1 Yarrowia lipolytica as a Model Organism for Studying Complex I

Y. lipolytica is a strictly aerobic yeast which grows on different carbon sources like alkanes, fatty acids, ethanol and acetate (Barth and Gaillardin, 1996). Its growth-temperature optimum is at 28°C and its biomass yield is up to 100 g/l (wet weight).

Y. lipolytica offers the advantage of an eucaryotic organism containing complex I, which is amenable to straightforward genetic manipulation (Kerscher et al., 2001b). This organism has a simple haplo/diplontic life cycle, two naturally stable mating types (Mat A and B), a high frequency of homologous recombination and a relatively small genome for which a draft

sequence has been completed (Casaregola et al., 2000). These features have allowed the cloning, sequencing, and generation of point mutations in the seven nuclear genes *NUAM, NUBM, NUCM, NUGM, NUHM, NUIM, NUKM* (corresponding to the 75, 51, 49, 30, 24 kDa, TYKY and PSST subunits from bovine complex I, respectively) that encode the seven highly conserved subunits of complex I of the peripheral arm (Djafarzadeh et al., 2000).

As an obligate aerobic organism, *Y. lipolytica* expresses complex I even when mutations compromise its enzyme activity, allowing study of defective complex I.

1.3.2 The Respiratory Chain of *Yarrowia lipolytica*

The respiratory chain of *Y. lipolytica* contains all complexes that make up the respiratory chain in mammals. In addition, the yeast *Y. lipolytica* has one NADH:ubiquinone oxidoreductase (NDH2 or alternative dehydrogenase) that is also able to oxidise NADH and transfer electrons to ubiquinone, but without translocating protons.

NDH2

NDH2, being located at the external side of the inner mitochondrial membrane, cannot contribute to the oxidation of NADH in the mitochondrial matrix. This enzyme, whose physiological function is unclear, consists of a single water soluble subunit that carries a non-covalently bound flavin dinucleotide (FAD) as unique prosthetic group (de Vries and Marres, 1987). The gene for the alternative NADH dehydrogenase of *Y. lipolytica* was identified (Kerscher et al., 1999) and fused with the sequence which is responsible for the import of the 75 kDa subunit of complex I into the mitochondrial matrix. This fusion allows the redirection of NDH2 to the internal face of the mitochondrial inner membrane (NDH2i) (Kerscher et al., 2001a). NDH2i, by virtue of its ability to transfer electrons from NADH in the mitochondrial matrix to the ubiquinone pool of the respiratory chain, rescues complex I deficiency making complex I dispensable for cell survival. In fact, complex I defects causing loss of catalytic activity higher than ~70% are not compatible with survival of *Y. lipolytica* without NDH2i (Ahlers, data not published). NDH2i allows the study of complex I defects even if complex I activity is strongly compromised or completely absent, as in the case of complex I assembly defects. Taking advantage of NDH2i, each of the genes for the seven highly conserved and nuclear coded subunits of complex I was deleted from the *Y. lipolytica* genome, allowing a

simple, direct mutagenesis of every single subunit via complementation on a replicative plasmid (Kerscher et al., 2001b). The introduction of NDH2i represented a key contribution to the development of *Y. lipolytica* as a genetic system for studying complex I.

1.3.3 Complex I from *Yarrowia lipolytica*

In terms of function, structure, number and type of redox centres, the features of complex I from *Y. lipolytica* are very similar to the features of the traditionally well characterised complexes I from *E. coli*, *N. crassa* and bovine heart mitochondria (Djafarzadeh et al., 2000). In addition, complex I from *Y. lipolytica* is very stable and purification can be achieved by affinity chromatography (for more details see 2.3.10). Attaching a six histidine tag to the C-terminus of the 30 kDa subunit allowed a His-tag affinity purification (Kashani-Poor et al., 2001a). This method produces enzyme with a purity of around 95% and a yield of 38% for the parental strain. Two-dimensional electron microscopic analysis of single particles showed that complex I from *Y. lipolytica* has an L-shaped structure with a membrane and a peripheral domain perpendicular to each other, similar to the enzymes from *E. coli* and *N. crassa* (Djafarzadeh et al., 2000).

1.3.4 Mutants in the NUKM Subunit of Complex I from *Yarrowia lipolytica*

Site directed mutagenesis of the PSST homologous subunit (NUKM) in complex I from *Y. lipolytica* had been carried out in this laboratory previously, in an effort to investigate the role of this key subunit and the position of cluster N2 (Ahlers et al., 2000b).

It had been speculated that the conserved glutamic acid in position 89 in the NUKM subunit of *Y. lipolytica* might act as fourth ligand for cluster N2 (Ohnishi, 1993). In fact, this position is in proximity to cysteines 85-86 that belong to the non canonical binding motif (see 1.2.3). Glutamic acid 89 was mutagenised into glutamine, cysteine and alanine. EPR spectra from isolated complex I of mutants had excluded the hypothesis that this glutamate could act as ligand for cluster N2. On the other hand, the slight shift to lower values of the N2 EPR specific g_z signal observed in all three mutant complexes strongly suggested that position 89 is located in the proximity of N2 cluster (Ahlers et al., 2000b).

Other conserved acidic residues had been mutagenised in the NUKM subunit from *Y. lipolytica*: D136, E140 and D168 (Ahlers et al., 2000b). When positions D136 and E140 were changed into their corresponding amides, ubiquinone reductase activity of complex I was found to be moderately decreased and hypersensitivity to rotenone and slight resistance to DQA were observed. These data strongly suggested that the conserved acidic residues in the NUKM subunit play a role in complex I catalytic activity. Differently, mutant D168N showed essentially the same properties as the parental strain. Data from EPR spectra revealed that none of these mutagenised amino acids could be identified as the fourth ligand for N2 cluster, leaving this question open.

1.4 Complex I in Disease

In general, dysfunction of complex I can cause three different kinds of problems:

1. Reduced ability to oxidise NADH, which results in lactic acidosis;
2. Reduced ability to pump protons, which decreases the rate of ATP synthesis;
3. Enhanced production of superoxide radicals, which could cause DNA mutations, lipid peroxidation and protein denaturation (Kitajima-Ihara and Yagi, 1998).

Actually, the reduced proton pumping capacity as such does not appear to be a serious health issue: complex III and IV could rescue this complex I defect (Seo et al., 1998). In contrast, the inability of mitochondria to oxidize NADH and damages provoked by superoxide radical production cause severe health problems. It has been hypothesized that oxygen radical production is increased when complex I activity is compromised (Robinson, 1998). Different studies suggest a direct correlation between myopathies (Bentlage et al., 1995), familial cardiomyopathy (Pitkanen et al., 1996), neurodegenerative disorders (Schapira, 1998), Parkinson´s disease (Hartley et al., 1994), and complex I defects.

Due to the above described dual coding system of complex I, human diseases connected with complex I defects can be caused by mutations in the mitochondrial or in the nuclear DNA. While the role of mutations in mtDNA has been recognised as a major contributor to complex I deficiency (Wallace, 1999), recent findings have shown that mutations in nuclear encoded subunits of complex I can also result in diverse pathologies. Among these, a severe progressive neurodegenerative disorder called “Leigh syndrome”, is the most common

clinical manifestation. Mutations in two nuclear coded complex I subunits that result in Leigh syndrome are described below.

Leigh Syndrome

Recently, three missense mutations in two nuclear coded subunits of mitochondrial complex I have been identified as the causative genetic defects in two cases of neuropathologically proven Leigh syndrome (MIM 25600), that resulted in death in early infancy. Homozygosity for a V122M substitution in the human *NDUFS7* (PSST homologous) subunit has been found in two siblings (Triepels et al., 1999); and heterozygosity for mutations P79L and R102H in the human *NDUFS8* (TYKY homologous) subunit has been found in another patient (Loeffen et al., 1998).

NADH:ubiquinone oxidoreductase activity was low in all tissues examined, suggesting that a reduction in the electron transfer activity of complex I could be one cause of the Leigh syndrome.

1.5 Complex I as a Target for Insecticides and Acaricides

Complex I is also a target for certain insecticides and acaricides: same of them have been shown to inhibit its catalytic activity (Hollingworth and Ahammadsahib, 1995; Hollingworth et al., 1994). The natural compound piericidin A was isolated from cultures of *Streptomyces mobaraensis* (S.Yoshida, 1978) and rotenone is the active component in the insecticidal and fish-poisonous extract of *Derris* (*Leguminosae*) roots. Both compounds are known as high affinity inhibitors for complex I. Synthetic insecticides and acaricides that inhibit complex I can be grouped in two main classes: pyrazoles and substituted pyrimidines in the first class, pyridines and quinazolines in the second class (Lümmen, 1998). Both classes of compounds are of commercial interest.

1.6 Aims

1.6.1 Site Directed Mutagenesis of NUIM (TYKY) and NUKM (PSST) Subunits from *Yarrowia lipolytica*

In an effort to investigate the **"**ubiquinone reduction module" of complex I, in the first part of this work site directed mutagenesis of the NUIM (TYKY) and NUKM (PSST) subunits has been carried out.

Mutagenesis of Conserved Cysteine Residues

The bovine TYKY and PSST subunits have been discussed as candidates for carrying cluster N2 by virtue of their three conserved binding motifs for tetra-nuclear iron sulfur clusters (see 1.2.3).

The first goal of this work was to carry out a systematic mutagenesis of all cysteines that belong to these motifs in the NUIM (TYKY) and NUKM (PSST) subunits of *Y. lipolytica*.

Mutagenesis of Conserved Acidic Residues in the PSST Subunit

The two adjacent cysteines in the binding motif of the PSST subunit had posed the question about the existence of a non-cysteine fourth ligand in this subunit (Ahlers et al., 2000b). In addition, some mutations affecting conserved acidic residues had been found to result in inhibitor resistance and to affect catalytic activity of complex I (see 1.3.4). These results corroborated the hypothesis that this subunit plays a role in complex I catalytic activity. In an effort to investigate the position of the fourth ligand and the involvement of the PSST subunit in complex I catalytic activity, the second goal of this work was to carry out site directed mutagenesis of each conserved acidic amino acid in the NUKM (PSST) subunit of *Y. lipolytica*.

In Vitro Characterization of Leigh Mutations

In the nuclear coded subunits of mitochondrial complex I homologous to TYKY and PSST, three missense mutations had been identified as causative genetic defects of Leigh syndrome (see 1.4). The three point mutations were reconstituted in *Y. lipolytica*. The third goal of the present work was the characterisation of these mutants with intent to provide a first clue towards a molecular understanding of the aetiology of this syndrome.

Characterization of Mutant Strains

Mutant strains were characterised in terms of:

1. Complex I content;
2. Catalytic activity and inhibitors resistance;
3. EPR signature of cluster N2.

1. Complex I content

   NADH:HAR activity was used to estimate complex I content in mitochondrial membrane preparations. This activity is based on the ability of complex I to oxidize NADH in the presence of the electron acceptor HAR (hexammineruthenium-III). Being dependent only on FMN in the 51 kDa subunit, but independent of the ubiquinone oxidoreductase activity or of proton pumping, the NADH:HAR activity essentially reflects the amount of complex I in the membrane preparation. By virtue of these peculiarities, NADH:HAR ubiquinone oxidoreductase activity can be used to quantify and compare complex I contents of different membrane preparations from parental or mutant strains, even if the NADH:ubiquinone oxidoreductase activity of complex I is compromised by mutations.

2. Catalytic activity and inhibitors resistance

   The physiological activity of complex I (NADH:ubiquinone oxidoreduction) is measured in vitro as deamino-NADH:n-decyl-ubiquinone oxidase activity (dNADH:DBQ). The substrate deamino-NADH, in contrast to NADH, offers the advantage to react specifically with complex I and not with the alternative NADH:ubiquinone oxidoreductase. Decyl-ubiquinone is an artificial hydrophobic substrate that resembles the physiological Ubiquinone (Q_9) of *Y. lipolytica* inner

mitochondrial membranes. Thus, dNADH:DBQ activity mimics the physiological NADH:ubiquinone oxidoreductase activity of complex I. Furthermore, investigating the effect of mutations on complex I sensitivity for quinone-analogous inhibitors, I_{50} for rotenone and DQA have been measured.

3. EPR signature of cluster N2

Most of the iron-sulfur clusters of complex I are detectable by Electron Paramagnetic Resonance spectroscopy (EPR, see appendix 9.2). The EPR signal of an iron-sulfur cluster depends on the geometry and on the microenvironment of the cluster. A point mutation can directly (in the case of a ligand) or indirectly influence this microenvironment. In an effort to investigate the position of cluster N2, EPR spectroscopy is a powerful method for analysing the effects of point mutations.

1.6.2 Making a Substrate Inducible NDH2i

The second part of this work was aimed at developing a substrate inducible version of the internal alternative NADH:ubiquinone oxidoreductase (NDH2i, see 1.3.2). The substrate inducible NDH2i is supposed to offer a mechanism for "switching" between complex I activity dependent (no NDH2i activity) and independent (NDH2i activity) cell growth, by changing between activating and non-activating substrates. This strategy would allow screening for mutants with and without complex I catalytic activity. Complex I deficient mutants could be easily identified, since *Y. lipolytica* mutant strains with residual complex I catalytic activity lower than ~ 30% are not able to survive under complex I dependent growth conditions (no NDH2i activity). Amino acids, which have an important role for complex I structure or function, could then be identified by sequence analysis. Mutant strains that survive under complex I dependent growth conditions could be tested for resistance to complex I specific inhibitors. This screening system is a prerequisite for realising a random PCR mutagenesis of single subunits of complex I. Random PCR mutagenesis is a technique that allows the production of a high number of point mutations in relatively short time. By contrast, the generation and analysis of a large number of site-directed mutants would be a very time consuming task. In an effort to study structure and function of complex I, this part

of the present work was directed towards developing an efficient screening system for selecting mutations which affect activity or inhibitor resistance of complex I.

The promoter for the isocitrate lyase (pICL1) was used for realising a substrate inducible version of the NDH2i. The ICL1 gene (X72848) encodes the enzyme isocitrate lyase, which is one of the key enzymes of the glyoxylate pathway (Vanni et al., 1990). Yeast growth on acetate or ethanol as a sole carbon source requires this pathway as anaplerotic cycle for producing C_4 compounds that are needed for the tricarboxylic acid cycle, and builds blocks for a large number of biosynthetic pathways. The isocitrate lyase gene is induced in the presence of n-alkanes, fatty acids, ethanol or acetate as carbon sources but not by glucose. Northern blot analysis of *Saccharomyces cerevisiae* cells that were grown on different carbon sources (ethanol and glucose) confirmed substrate dependent regulation of the isocitrate lyase mRNA (Fernandez E, 1993). Studies carried out with *lacZ* as reporter gene indicated that the ICL1 promoter of *Y. lipolytica* is up regulated by transferring cells from glucose to acetate media (Juretzek et al., 2001).

2 Materials and Methods

2.1 Materials

2.1.1 List of Chemicals

Ethanol (J.T. Baker, Deventer-Nederland); 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^{TM} 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 (Merck, Darmstadt-Germany); ammonium sulfate, EDTA, glass pearls (0.25 – 0.5 mm), KCl, KOH, KH_2PO_4, sodium acetate, sodium citrate, NaCl, NaOH, $NiSO_4$, NaH_2PO_4, saccharose, X-Gal (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, dodecylsulfate N-salt (SDS), trichine, agarose, amino caproic acid, amino acids, ampicilline, DMSO, ethidium bromide, glucose, glycerine (Pharmacia); hexaminruthenuim(III) chloride (HAR), hepes, KCN, lithium acetate, mercapto ethanol, mops, d-NADH, NADH, NaN_3, nystatine, PMSF, TEMED, tris, asolectin, oligonucleotides (Sigma Chemie GmbH, Deisenhofen-Germany), oligonucleotides (ARK Scientific GmbH Biosystems, Darmstadt-Germany) or MWG-Biotech Ebersberg-Germany).

2.1.2 Inhibitors

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

2.1.3 Media and Buffers

Media for *Escherichia coli*:

LB-Medium: 1% NaCl, 0.5% yeast extract, 1% tryptone, pH 7.5

Media for *Yarrowia lipolytica*:

Sporulation-medium (CSM): 0.17% yeast nitrogen base without $(NH_4)_2SO_4$ and amino acids, 0.5% $(NH_4)_2SO_4$, 50 mM natrium citrate

YM-medium: 0.5% Bacto™Peptone, 0.3% yeast extract, 0.3% malt extract

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

YPAc-medium: 2% Bacto™Peptone, 1% yeast extract, 0.4% acetate

Glycerol stocks: YPD-medium + 40% glycerine

Minimal synthetic medium (10 × S): 1.7% yeast nitrogen base without $(NH_4)_2SO_4$ and amino acids plus 5% $(NH_4)_2SO_4$, pH 5.0, were prepared as a 10 time stock solution (10 X S) and sterilefiltered. Carbon sources (0.4% acetate or 2% glucose solutions) were autoclaved and added to the 10 × S medium. Depending on the type of auxotrophie selection, one or several of the following components were added: 130 µM histidin, 200 µM lysine, 460 µM leucin 180 µM uracil.

Buffers and solutions:

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

10 × TBE-buffer: 890 mM Tris / borate, 890 mM boric acid, 20 mM EDTA, pH 8.3

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

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

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

2.1.4 Strains

Table 2.1 ***-Escherichia coli Strains-***

Strain	Genotype
DH5α	supE44 ΔlacU169(ϕ80 lacI^{q}ZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1
XL10-Gold	Tetr D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacIqZDM15 Tn10 (Tetr) Amy Camr]a

Table 2.2 ***-Yarrowia lipolytica strains-***

*Kerscher et al., 2002

Strain	Genotype
E129	MatA, lys11-23, ura3-302, leu2-270, xpr2-322
E150	MatB, his-1, ura3-302, leu2-270, xpr2-322
GB9	MatA, lys-1, ura3-302, leu2-270, xpr2-322, nugm::LEU2, NDH2i,30Htg2 in pUB4
PIPO*	30 Htg pop-in-pop-out MatA, lys-1, ura3-302, leu2-270

2.1.5 Plasmids

Table 2.3 ***-E .coli and Y. lipolytica plasmids-***

Name	Properties	Producer
pCR2.1	see description of the product	Invitrogen Groningen (Netherlands)
pBluescript SK^-	see description of the product	Stratagene (Heidelberg)
pINA443	Yarrowia lipolytica Shuttle“ Vector, 2,3 kb fragment, LEU 2 gene	Prof. Gaillardin, Paris (France)
P67JP	ICL1 promoter	Prof. Barth, Dresden (Germany)
pUB4	Yarrowia lipolytica “shuttle”-vector containing 1.6 kb fragment with Hyg B^R gene	Dr. Stefan Kerscher, Frankfurt/Main, Germany
pUB26	modified pUB4 in ARS68 base changed (T737A)	Dr. Stefan Kerscher, Frankfurt/Main, Germany

2.1.6 Instruments

Centrifugues:

Heraeus Biofuge A

Heraeus Labofuge 400

Heraeus Minifuge GL

Cryofuge 8500 Sorvall Heraeus (Osterode) -Germany

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

Ultracentrifugues L7-65 und L8-70M, Beckman Instruments GmbH (München) -Germany.

Rotors:

JA-10, JA-20, JS13.1 Beckman Instruments GmbH (München) -Germany

Ti 45, Ti 70.1, Beckman Instruments GmbH (München) -Germany

Fluorimeter:

Hoefer® DyNA Quant® 200, Pharmacia Biotech

Photometers:

UV 300, Shimadzu (Düsseldorf) -Germany

U-3210, Hitachi (Düsseldorf)–Germany

Microplate spectrophotometer SPECTRA MAX^{384} plus, Molecular Devices (Sunnyvale)-CA-USA

Thermocyclers:

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

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

Cyclone gradient, Biotechnologir Gmbh-England

Elektrotransformation Device:

E. coli Pulser Bio-Rad (Hercules USA)

EPR-Spectrometer:

Bruker ESP 300E spectrometer equipped with a liquid helium continuos flow cryostat, ESR 900 from Oxford Instruments-United Kingdom

EPR-tubes:

Quarz glass Nr.: 707-SQ-250M (lenght: 250 mm, diameter: 4 mm), Spintec (Remshalden)-Germany

DNA Sequencer:

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

Other instruments:

10 l fermenter, Biostat E; Braun (Melsungen)-Germany

Bead-Beater, Biospec (Bartlesville)-USA

Cell-disintegrator-C, Bernd Euler (Frankfurt am Main)-Germany

BioSys 2000 protein-isolation-workstation®, Beckman Instruments GmbH (München)-Germany

camera MP4 land camera, Polaroid

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

Microscope, Leitz (Wetzlar)

TSKgel G 4000 SW column (21.5 mm × 600 mm), TosoHaas GmbH (Stuttgart)-Germany

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

UV-Stratalinker 1800/254 nm, Stratagene (Heidelberg)

UV-transluminator TF 20M, 312 nm, Herolab

Software:

DNA-analysis-program:

Mac Vector 3.5, IBI

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

Sequence Navigator, Applied Biosystems

Husar, DKFZ of Heidelberg, Germany

Enzfitter, Biosoft, Cambrige (UK)

PROF (Secondary Structure Prediction System), Department of Computer Science, Aberystwyth SY23 3DB, Copyright 2000-2003, Wales, UK.

Swiss Pdb Viewer V3.7b2, Glaxo Wellcome Experimental Research

RasWin molecular Grafics, Windows Version 2.7.1, Copyright R. Sayle 1992-1999.

2.2 Protocols of Molecular Biology

2.2.1 DNA Gel Electrophoresis

DNA was separated according to standard procedures (Sambrook et al., 1989) in the presence of ethidium bromide (0.5 µg/ml). The agarose concentration was 1.0% in 1×TEA buffer. A TEA buffer with extra additive (UV-safe TAE, MWG-Biotech, Ebersberg-Germany) was used for extracting DNA fragments from the gel. DNA molecular weight standards: 1 kb Ladder, 100 bp Ladder plus (MBI Fermentas, St. Leon-Rot).

2.2.2 Fill-in Reaction of 5`-Overhang

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

2.2.3 DNA-Vector Dephosphorylation

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

2.2.4 Phosphorylation of PCR-Products

The phosphorylation of both fragment ends of PCR products was made by T4 polynucleotide kinase (New England Biolabs) as described by Ausubel (2000). Alternatively, primers were phosphorylated before PCR.

2.2.5 DNA Extraction from Agarose Gels

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

2.2.6 Ligation

T4 DNA-ligase and provided buffer (Gibco BRL Life Technologies) were used to ligate DNA fragments. Ligation was usually carried out over night at 14°C in 5µl volume.

2.2.7 Preparation of Electro-Competent *Escherichia coli* Cells

Electro competent *E. coli* cells were made according the procedure from Current Protocols (2000). Transformation efficiency was up to $2 \cdot 10^9$ colonies/µg pSK^-.

2.2.8 Transformation into *Escherichia coli* (Electro Competent Cells)

For the transformation of plasmid (with ampicillin resistance gene) into *E. coli* electro competent cells an *E. coli* Pulser (Biorad) was used as described in "Current Protocols In Molecular Biology (2000)". Transformants were successively grown over night on LB solid medium with ampicillin (50 µg/ml) and clones were replated using the same ampicillin concentration.

2.2.9 Isolation of Plasmid-DNA from *Escherichia coli*

Plasmid-DNA was prepared according to (Zhou et al., 1990) from small amount of cultures (1,5-3 ml). Plasmid DNA for sequencing reaction was prepared using the QIAprep® Spin Miniprep Kit (Qiagen).

2.2.10 DNA Sequencing

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

2.2.11 Polymerase Chain Reaction (PCR)

10 ng of plasmid-DNA or 100 ng of genomic DNA were combined in a total reaction volume of 50 µl with 5 µl of each oligonucleotide (6.4 pmol solution, c_{end} = 128 pM) and 5 µl of provided 10x reaction buffer. To avoid dimerisation of oligonucleotides as well as non-specific binding of oligonucleotides 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-Germany) as well as *Taq* DNA polymerase from Sigma Chemie GmbH (Deisenhofen-Germany).

2.2.12 Generation of Point Mutations

The shuttle-vectors pINA443 or pUB4 (carrying the *Hyg*^{R} resistance gene) with a 2,3 kb insert (*EcoR*I fragment coding for the NUKM subunit and 2.8 Kb *Hind*III fragment coding for the NUIM subunit) were used as template for site directed mutagenesis. The point mutation was introduced by PCR with the "QuikChange™ site-directed mutagenesis kit" (Stratagene, Heidelberg-Germany). Phosphorylated primers were used to amplify the plasmid carrying the wild type copy of the gene (isolated from *E. coli*). The reaction mix was digested by *Dpn*I for eliminating template plasmid. Phosphorylated PCR products were ligated and the so obtained circular plasmids were transformed into electro-competent cells. The open reading frame (ORF) of mutagenised plasmids was sequenced and compared to the wild type ORF to confirm the sequence changes.

2.2.13 Southern Blot

Digested DNA (genomic DNA: 500 ng; plasmid DNA: 50 ng) was separated using agarose gel electrophoresis. The DNA was transferred over night to Hybond N^+-membrane (Amersham, Braunschweig-Germany). A Stratalinker (Stratagene, Heidelberg-Germany) was used to crosslink DNA to the membrane by UV-light radiation.

2.2.14 ^{32}P DNA Labelling

DNA fragments were labelled with [α-^{32}P] dCTP (25 µCi for 25 ng DNA) using the "Random primer labelling kit – Prime-It® II Kit" (Stratagene, Heidelberg-Germany). Efficiency check of radioactive labelling was done by adding 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.

2.2.15 Hybridisation of Radio Active Labelled DNA Probes

Hybridisation took place in a glass tube in a hybridisation oven (HB-1D, Techne). A pre-hybridisation was made for 15 minutes at 68°C and the main hybridisation for 60 minutes at 68°C with "QuikHyb® hybridisation solution" (Stratagene, Heidelberg-Germany). For the main hybridisation, ^{32}P-labelled DNA fragment was used in the presence of 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 an Kodak X-Omat AR films with an amplifier-sheet over night at –80°C.

2.2.16 Transformation of *Yarrowia lipolytica*

Competent cells were made according to the one-step transformation protocol by Chen et al., 1997 (Chen et al., 1997). One ml of overnight culture in YPD or YPAc was spun down and resuspended in 100 µl one step buffer. 200 µg of linear DNA fragment solution (1-5 µl) were added for each transformation. The transformation cocktail was thoroughly vortexed and incubated at 39°C for 60 minutes. The mixture was spread on a selective plate and incubated at 27°C for 48-56 h.

2.2.17 Conjugation, Sporulation and Random Spore Isolation

Strains of opposite mating type were separately inoculated into 10 ml of YPD and grown overnight. 0.1 ml of these cultures were transferred into fresh 10 ml YPD and grown for 18-21 h. Cells were centrifuged (6000 *g* for 3 minutes) and resuspended in 1 ml YM (about 2-10 • 10^8 cells/ml). Strains of opposite mating type were then mixed together with 8 ml YM in a flask, shaken at 28°C for 16-24 h and plated on selective medium to isolate diploid cells. A single colony from the plate was inoculated into 10 ml YPD and grown overnight. Cells were centrifuged and resuspended in CSM to a concentration of around 2-5 X 10^7 cells/ml and sporulated for 4 days in flasks at 220 rpm and 23°C. After 4 days the culture was stored at 4°C for 1 day. 5 ml of the culture were centrifuged and cells resuspended in 10 ml YPD and shaken for 2 h at 28°C. The pH was adjusted to 4.5-5.0 and 0.25 ml of a 1 mg/ml nystatin solution were added. The incubation at 28°C was continued for another 1.5 h. Cells were washed twice with water, resuspended in SD and portioned into 6 aliquots. Aliquots were incubated with 0.5 ml of a 12.5% ethanol solution and incubated for a series of different times (0-120 minutes). After each incubation time, cells were washed and plated on selective medium.

2.2.18 Isolation of Total DNA of *Yarrowia lipolytica*

Total DNA isolation was carried out according to the protocol “Rapid Isolation of Yeast Chromosomal DNA” (2000). Plasmid DNA was obtained by transformation of 100 ng of total DNA into *E. coli* “electro competent cells”.

2.3 Protocols of Protein Chemistry

2.3.1 Growth of *Yarrowia lipolytica*

Yarrowia lipolytica strains were grown in YPD media at 220 rpm and 28°C in flasks. A single colony of *Y. lipolytica* from an agarose YPD plate was inoculated into a pre-culture and grown in a flask for 12-24 hours (depending on the strain). One ml of the pre-culture was inoculated into 500 - 1000 ml (also depending of the strain), shaken for 12-24 h and transferred into a 10 l fermenter (Biostat E; Braun, Melsungen). The fermentation lasted for 12 - 18 hours at 28°C, 400 rpm stirring, and on air stream of 16 l/min. The yield was up to 90 g cells/l (wet/weight) for the wild type and 30 g cells/l (wet/weight) for "complex I inactive strains".

Mutant strains (*nukm*/*nuim* subunit in pINA 443 plasmid) were grown with the same conditions as the wild type in a pre-culture (Synthetic Dextrose medium with lysine (30 mg /l)). Fermentation was throughout in YPD medium.

Mutant strains (*nukm* subunit in pUB4 plasmid) were grown with the same conditions as described for the parental strain in a pre-culture (YPD medium with Hygromycine B® (100 mg /l). Fermentation was carried out in the absence of Hygromycine B®.

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 (Bend Euler Biotecnologie, Frankfurt-Germany) were used in a cell disintegrator. 300 – 500 g of cells were suspended in the same amount of buffer (600 mM saccharose, 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 absorption spectra (530-630 nm) of the reduced minus oxidised forms of the heme groups contained in the respiratory chain. Mitochondrial

membranes were reduced by addition of dithionite and oxidised by addition of ferricyanide. Content of heme b and $a+a_3$ was measured at 562-575 nm (reduced minus oxidized form, heme b: $\varepsilon_{562\text{-}575}$ 28.5 $mM^{-1}cm^{-1}$ heme $a+a_3$: $\varepsilon_{605\text{-}650}$ 24 $mM^{-1}cm^{-1}$) and at 605-630 nm, respectively. Heme concentration was usually 1-10 µM and the ratio heme b: heme $a+a_3$ around 3:1.

2.3.3 Preparation of Mitochondrial Membranes in Small Amounts

Freshly harvested cells (4 - 8 g) were mixed at a 1:1:1 ratio of cells to buffer (same as in 2.3.2) to glass beads. Cell breakage was carried out by vortexing the mixture in a falcon tube for 10 X 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 Quantification

Protein determination was conducted after the procedure of (Lowry et al., 1951), modified after (Helenius and Simons, 1972). Calibration was carried out with bovine serum albumin (BSA), in a dilution series from 0.1 – 2.0 mg/ml.

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 and the resulting solubilised mitochondrial membranes were put on 4 / 4 → 13% gradient gels.

2.3.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Tricine SDS-PAGE was used to check the composition and purity of complex I preparations (Schägger and von Jagow, 1987).

2.3.7 Activity Measurements

Deamino-NADH:n-decyl-ubiquinone (dNADH:DBQ) oxidase activity was assayed at 30°C using a Shimadzu UV-300 spectrophotometer as dNADH oxidation rate ($\varepsilon_{340\text{-}400\ nm}$ = 6.22 mM^{-1} cm^{-1}) in the presence of 100 µM dNADH in 50 mM Tris/HCl, pH 7.4, 2 mM KCN. The reaction was started by the addition of 60 µM DBQ. Inhibitors were added from stock solutions (10 mM in ethanol or in DMSO) prior to DBQ. Michaelis-Menten parameters were determined by varying the concentration of DBQ (2-100 µM) and data were analyzed with the Enzfitter software-package (Version 2.0.16.0, Biosoft, Cambridge). Detergent- and inhibitor-insensitive NADH:HAR (HAR: hexaammineruthenium(III) chloride) activity was measured using 200 µM NADH and 2 mM HAR, 2 mM NaN_3 in 20 mM Na^+/Hepes, pH 8.0 at 30°C (Sled and Vinogradov, 1993), and used to quantify the complex I content in the membranes. This activity depends only on FMN and possibly on Fe-S cluster N3 (Gavrikova et al., 1995). The reaction was started by the addition of 50 µg (total protein) of unsealed mitochondrial membranes. The complex I specific substrate dNADH was used instead of NADH for measuring NADH:HAR activities of cysteines-alanines mutants. Using dNADH avoids influence of non specific NADH oxidase activity of NDH2 of mitochondrial membranes. Both (d)NADH:HAR and dNADH:DBQ are expressed as specific activities in Units (U: µmol min^{-1} mg^{-1}) per mg of total protein.

To test for temperature stability, mitochondrial membranes were incubated for five minutes at increasing temperatures (30-35-40-45-48-50-53-55-60°C) before NADH:HAR activity was tested as described above.

2.3.8 Determination of I_{50} Values for Inhibitors

The I_{50} value is defined as the inhibitor concentration required for a 50% reduction in catalytic rate of complex I.

The dNADH:DBQ activity test was done as described in 2.3.8, but mitochondrial membranes were incubated with different inhibitor concentrations (0-2000 µM for DQA and 0-20 µM for rotenone) for 10 seconds and the reaction was started by adding 60 µM DBQ. The residual activity, defined as the dNADH:DBQ membrane activity which is not inhibited by high concentrations of inhibitor (2 µM for DQA and 40 µM for rotenone) and not dependent on complex I, were subtracted when calculating the I_{50} values. For the parental strain the residual activity was between 0 and 5% (depending on the membrane preparation).

2.3.9a Purification of Complex I (by His-Tag Affinity and Size Exclusion Chromatography)

Unsealed mitochondrial membranes were prepared from plasmid complemented wild type and mutant haploid strains (*nukm*::*LEU2*, *ura3, leu2, lys*, pUB4-*nukm*$_{mut}$/*Hyg*) as described in 2.3.2. Complex I was purified by extraction of mitochondrial membranes with dodecyl maltoside (1g dodecyl maltoside per g total protein content in the mitochondrial membrane) according to a modified protocol described by (Kashani-Poor et al., 2001a). All procedures were carried out on ice or at 4°C. The protein concentration of the membranes was diluted to 20 mg/ml and the salt concentration was adjusted to 50 mM NaCl and 20 mM Na_3BO_3 at pH 7.2. After addition of PMSF (2 mM final concentration) and dodecyl maltoside (1 gram detergent per gram total protein) under stirring, the suspension was centrifuged at 140,000 • g for one hour. The supernatant was loaded on a Ni^{2+}-agarose column equilibrated with buffer A. After washing with 3 column-volumes of buffer A followed an elution step with one column-volume of buffer B (160 ml/h maximal velocity). The eluate was collected in 4 ml fractions. Fractions which showed NADH:HAR activity higher than 15 U/ml were pooled and concentrated down to 10 ml. This sample was loaded on a TSK-gel column (size exclusion chromatography using a BioSys 2000 protein-isolation-workstation®) equilibrated with buffer D. The elution step was carried out with the same buffer at 120 ml/h. Fractions with higher activity were concentrated, shock-frozen and stored in liquid nitrogen. This protocol produces complex I with a purity about 95%, and a total yield of 38% for the PIPO strain.

Table 2.4 ***-Scheme of buffers used for isolation of complex I-***

* Buffer C was used for regeneration of the Ni^{2+}-agarose column.

Buffer		A	B	C*	D
NaCl	mM	400	400	500	100
L dodecyl-maltoside	%	0.1	0.1	0.1	0.05
Na-phosphate	mM	20	20		
Imidazol	mM	55	140		
pH	mM	7.2	7.2	7.2	7.2
Na/Mops	mM				25
EDTA	mM			50	1

2.3.9b Purification of Complex I (by Ion Exchange and Size Exclusion Chromatography)

Unsealed mitochondrial membranes were prepared from plasmid complemented wild type and mutant haploid strains (*nukm*::*LEU2*, *ura3*$^-$, *lys*$^-$, pINA443) as described in 2.3.2. Complex I was purified by extraction of mitochondrial membranes with dodecyl maltoside according to the protocol described by (Kashani-Poor et al., 2001a) All procedures were carried out on ice or at 4°C. At first, membrane were diluted to a protein concentration of 20 mg/ml with buffer A and the salt concentration was adjusted to 50 mM NaCl. After addition of PMSF (2 mM final concentration) and dodecyl maltoside (0.2 gram detergent per gram total protein) under stirring, the suspension was centrifuged at 140,000 • g for 90 minutes. The pellet was homogenised after dilution with puffer A to 20 mg/ml. For extracting complex I, the supernatant was adjusted to 75 mM NaCl and the procedure described above was repeated using 0.75 g LM / g total protein. The supernatant was loaded on a 200 ml DEAE Bio-Gel A agarose column (ion exchange chromatography) equilibrated with buffer B. After washing with two column-volumes of buffer C protein was eluted in two column-volumes of buffer D (160 ml/h maximal velocity). The eluate was collected in 4 ml fractions. Fractions which showed NADH:HAR activity higher than 15 U/ml were pooled and concentrated down to 10

ml and loaded on a TSK-gel column (size exclusion chromatography using a BioSys 2000 protein-isolation-workstation®) equilibrated with buffer E. The elution step was carried out with the same buffer at 120 ml/h. Fractions with high NADH:HAR activity were concentrated, shock-frozen and stored in liquid nitrogen. This protocol allows isolation of complex I with a purity of about 95% and a total yield of 10% for the parental strain.

Table 2.5 ***-Scheme of buffer used for isolation of complex I-***

For using description of the buffers, see text.

Buffer		A	B	C	D	E
NaCl	mM		75	100	200	100
Dodecyl maltoside	%		0.05	0.1	0.25	0.1
Na/Mops	mM	20	25	25	25	25
EDTA	mM	2	1	1	1	1
pH	mM	7.0	7.0	7.0	7.0	7.0

2.3.10 EPR-Spectra

EPR Spectroscopy - Low temperature EPR spectra were obtained on a Bruker ESP 300E spectrometer equipped with a liquid helium continuos 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 millitesla. Under these conditions spectra show contributions from clusters N1, N2, N3 and N4 (Djafarzadeh et al., 2000). Spectra were recorded and analysed by Dr. Klaus Zwicker.

2.3.11 Redox Titrations

For redox titrations mitochondrial membranes (~200 mg protein) were sedimented by centrifugation for 1 h at 48,000 X g. The pellet was resuspended in 30 ml of buffer containing 30 mM each of sodium-acetate, Mes, Mops, Tris, glycine, pH 7.0, 100 mM NaCl and 1 mM EDTA and centrifuged as before. After one additional washing step the resulting pellet was suspended in 4–5 ml of the above buffer yielding a final protein concentration of 25–30 mg/ml. The following redox mediators were added to a final concentration of 30 μM each: tetramethyl-phenylenediamine, phenazine-methosulfate, methylene blue, menadione, resorufin, indigotrisulfonate, 1,2-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, phenosafranine, benzyl viologen, and methyl viologen. Redox titrations were performed anaerobically as described by (Dutton, 1978). The membrane suspension was poised at appropriate potential values by small additions of freshly prepared 50 mM dithionite. Aliquots were anaerobically transferred into an EPR tube, frozen rapidly in cold isopentane/methylcyclohexane (5:1), and stored in liquid nitrogen. Cluster N2 reduction rates in the frozen samples were then determined by recording EPR spectra at 12 K from samples poised at redox potentials between +100 and -500 mV. After subtraction of the oxidized spectrum recorded at a redox poise between +30 and -30 mV, to eliminate signal contributions from components with higher redox potential, the intensity of the N2 EPR signal was calculated by scaling it to a simulated N2 spectrum. The resulting N2 reduction rates were fitted to the Nernst equation using PSI Plot (Poly Software International, Salt Lake City, UT). Redox titrations were performed by Dr. Klaus Zwicker.

3 Results

3.1 Site Directed Mutagenesis in the TYKY (NUIM) and PSST (NUKM) Subunits

A 2.3 kb *Eco*RI genomic fragment from the *NUKM* locus and a 2.8 kb *Hind*III genomic fragment from the *NUIM* locus have been cloned into the replicative vector pUB4 (carrying the Hyg^R resistance gene) and pINA443 (carrying the *URA3* resistance gene) respectively, and used as template for the mutagenesis (see 2.2.12). The mutagenised plasmids were used for transformation into the deletion strain Δ*nuim, ndh2i, leu*$^-$, *lys*$^-$, *Mat A* using plasmid pINA443 and Δ*nukm, ndh2i, 30Htg, leu*$^-$, *his*$^-$, *Mat A* using plasmid pUB4 (Table 3.1).
Transformants were selected on minimal media without uracil (pINA443) or on complete media containing Hygromycin B (pUB4). Sequencing of the entire open reading frame on the plasmids reconfirmed the mutations.

TYKY		PSST	
Mutation	Vector	Mutation	Vector
C110A	pINA443	C85A/S	pUB4
C113A	pINA443	C86A/S	pUB4
C116A	pINA443	C150A/S	pUB4
C120A	pINA443	C180A/S	pUB4
C149A	pINA443	S180C	pUB4
C152A	pINA443	D99N/E/G	pUB4
C155A	pINA443	D115N/E/G	pUB4
C159A	pINA443	D174N	pUB4
		E185Q	pUB4

Table 3.1: ***-Point mutations-***

3.1.1 Characterisation of Mutants in the TYKY (NUIM) Subunit

Strain Δnuim

Mitochondrial membranes were prepared as described in 2.3.3 and analysed by BNP (Blue-Native Polyacrylamide gel electrophoresis, see 2.3.5). This method allows separation of the multiprotein complexes of the oxidative phosphorylation system after their solubilization by n-dodecyl-β-D-maltoside (Lauryl Maltoside, LM). Membranes of mutant strains were solubilised by 1g/g LM and loaded on the SDS-free gel. The complex I band was missing in the case of strain Δ*nuim*, whereas the bands of the complexes V and III of the respiratory chain were comparable to the wild type (E150) strain (Fig. 3.1). In E150 strain the complex I specific band (C I) is located between the complex V dimer (V_{DIM}) and monomer ($V_{MON.}$) bands. The presence of both $V_{DIM.}$ and $V_{MON.}$ bands indicated that the concentration of LM used corresponded to the opportune concentration for solubilising complex I. Data from BNP analysis and NADH:HAR activity measurements (see 2.3.8) reported in table 3.2 indicated that the mitochondrial membrane preparation of strain Δ*nuim* did not contain any relevant quantity of assembled complex I.

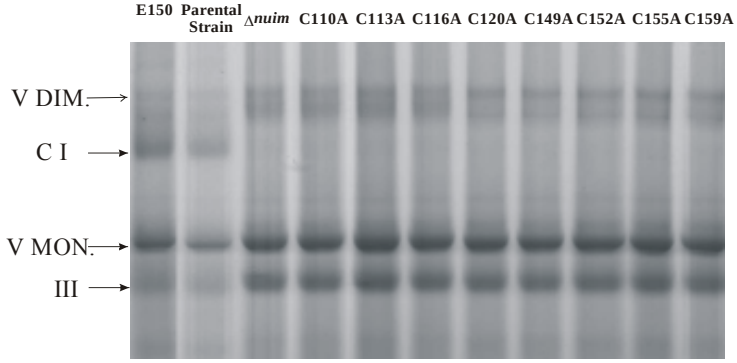

Figure 3.1 ***-Blue native gel of strains E150 (wilde type), parental, and C110A, C113A, C116A, C120A, C149A, C152A, C155A, C159A-***

Mitochondrial membranes were loaded on a blue native gel after solubilisation with 1 g n-dodecyl-β-D-maltoside (LM) per g total protein. The figure shows the first dimension of blue native page (BNP): E150 (wild type, cf. 2.1.4), Parental strain (Δ*nuim* strain plasmid complemented with a wild type copy of *NUIM* gene), Δ*nuim* and Cys mutant strains.

Strain	Complex I band in BN-Page	Complex I content (%)
E150	yes	110
Parental	yes	100
Δnuim	no	20
C110A	no	20
C113A	no	20
C116A	no	20
C120A	no	20
C149A	no	30
C152A	no	20
C155A	no	20
C159A	no	30

Table 3.2: ***-Characterization of E150, parental, Δnuim and cysteine-mutant strains in the TYKY homologous NUIM subunit-***

* Complex I content in mitochondrial membranes was extimated by measuring the dNADH:HAR activity that is not affected by mutations in the NUKM subunit (cf. 2.3.7). 100% = 1.0 μmol min^{-1} mg^{-1}.

Complemented NUIM Deletion Strain

An unmodified copy of the *NUIM* gene was subcloned into plasmid pINA443 as described in 2.2.12 and transformed into the Δ*nuim* strain. The resulting strain was defined as complemented *NUIM* deletion and compared to the wild type (E150) strain in terms of complex I content and catalytic activity. Mitochondrial membranes loaded on a blue native gel showed almost identical patterns (Fig. 3.1) and similar complex I contents were measured (Table 3.2). dNADH:DBQ oxidoreductase activity (0.35 U/mg for the complemented *NUIM* deletion and 0.42 U/mg for the E150 strain) indicated that the catalytic activities of both complexes were comparable.

Since both strains showed essentially the same characteristics with respect to complex I, the complemented *NUIM* deletion strain is referred to as the parental strain.

Cysteine Mutants

Every single of the eight fully conserved cysteines shaded in Fig. 3.2 were mutagenized into alanine. As a qualitative test for complex I assembly, mitochondrial membranes from each mutant were tested by BNP and compared to the wild type and to the parental strain (Fig. 3.1). Both V_{DIM} and V_{MON} bands were visible in each line, but complex I band was missing in the case of Δ*nuim* and Cys mutant strains.

Successively, bands were resolved into the individual polypeptides by a second-dimension SDS-PAGE (Fig. 3.3): no mutant showed the specific complex I bands. Complex I content in mitochondrial membranes was quantified by NADH:HAR activity (cf. 2.3.8): Δ*nuim* strains and all mutants showed comparable basal activities that are not caused by assembled complex I (Table 3.2).

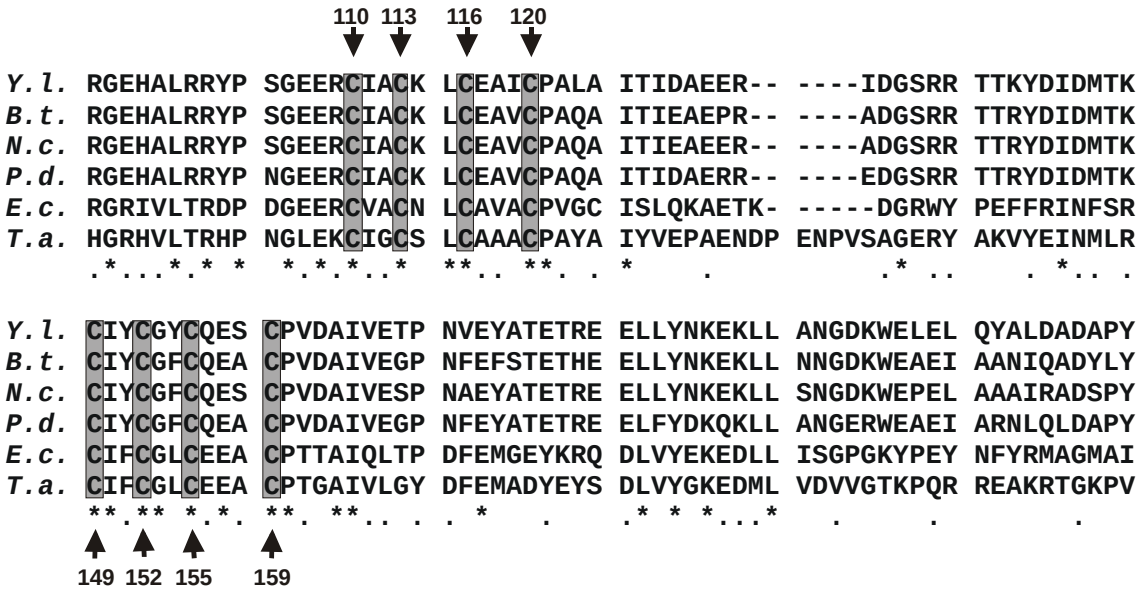

Figure 3.2 ***-Alignment of TYKY-homologous subunits of complex I-***

Identical amino acids are marked by *asterisks*, similar residues by *dots*. Cysteins that form the canonical binding-motifs for two Fe_4S_4 clusters in a ferrodoxin-like arrangement are shaded in grey and labelled with their number in the sequence of the *Y. lipolytica* protein. *Y.l., Yarrowia lipolytica; B.t., Bos taurus; N.c., Neurospora crassa; E.c., Escherichia coli; P.d., Paracoccus denitrificans; T.a., Thermus aquaticus*.

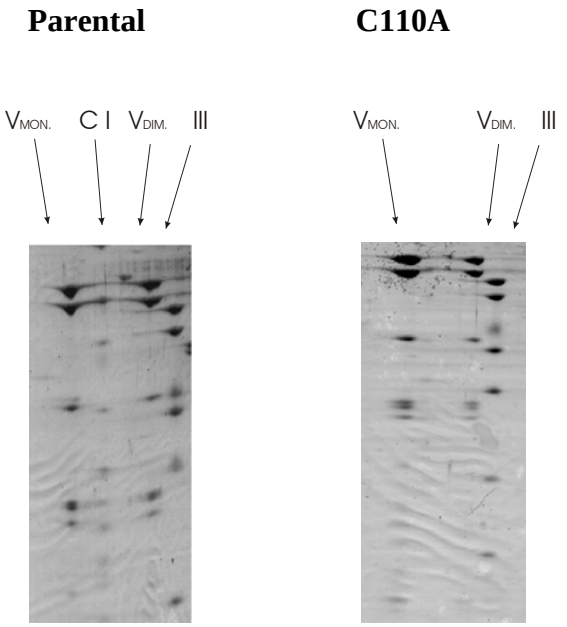

Figure 3.3 ***-Second dimension-***

Parental strain and mutant C110A are shown as examples: the other mutants, which exhibited the same pattern, are not shown.

3.1.2 Characterisation of Mutants in the PSST (NUKM) Subunit

Δnukm

Similar to the Δ*nuim* strain, mitochondrial membranes of the Δ*nukm* strain were tested by Blue Native Polyacrylamide gel electrophoresis (BNP) for complex I assembly, after solubilisation with 1 g/g LM. As in the case of strain Δ*nuim*, a complex I specific band was missing and the pattern of complexes V and III of the respiratory chain were comparable to the pattern of the wild type (E150), as shown in Fig. 3.4a. Data from BNP analysis and dNADH:HAR activity measurements (Table 3.3) indicated that the mitochondrial membrane preparation of strain Δ*nukm* did not contain relevant quantities of assembled complex I.

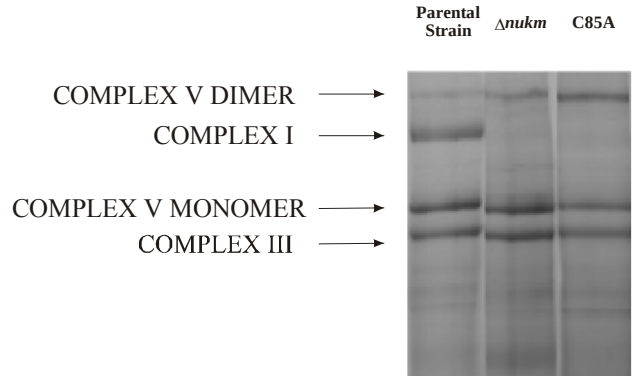

Figure 3.4a -***Blue native gel of the parental, Δnukm and C85A strains***-

Mitochondrial membranes were loaded on a blue native gel after solubilisation with 1 gram n-dodecyl-β-D-maltoside (LM) per gram total protein. **Parental strain** (Δ*nukm* strain plasmid complemented with a wild type copy of *NUKM* gene), **Δ*nukm*** (Δ*nukm* strain), **C85A** (cysteine-alanine mutant strain).

Complemented NUKM Deletion Strain

An unmodified copy of the *NUKM* gene was subcloned into the plasmid pUB4 as described in 2.2.12 and transformed into the Δ*nukm* strain. The resulting strain was defined as complemented *NUKM* deletion strain. Mitochondrial membranes from the complemented *NUKM* deletion and the wild type (E150) strain were characterised for comparison. Mitochondrial membranes are loaded on a blue native gel (Fig. 3.4b), and complex I content (estimated by measuring the NADH:HAR activity), NADH:ubiquinone activity, Michaelis-Menten parameters, and I_{50} for specific complex I inhibitor like DQA and rotenone were determined and are reported in table 3.3. Both strains showed, essentially, the same characteristics. Therefore, complemented deletion strain is referred to as the parental strain.

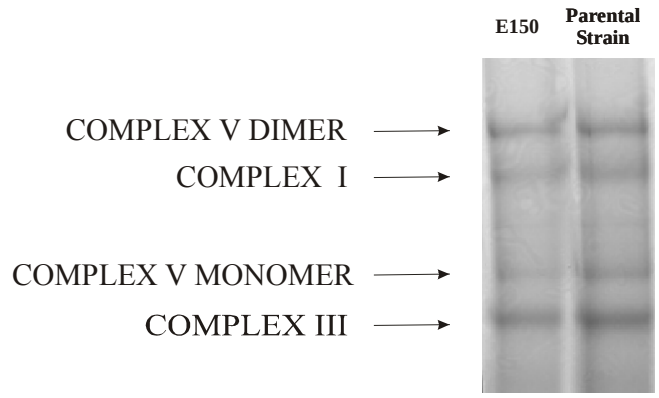

Figure 3.4b ***-Blue native gel of the wild type (E150) and parental strains-***

Mitochondrial membranes were loaded on a blue native gel after solubilisation with 1 g n-dodecyl-β-D-maltoside (LM) per g total protein. E150 (wild type, cf. 2.1.4), parental strain.

Strain	Complex I content (%)[*1]	Complex I band in BN-page	Vmax[*3] µM min^{-1} mg^{-1}	Km µM	I_{50}[*4] rotenone nM	I_{50}[*4] DQA nM
E150	110	yes	0.42 ± 0.02	32 ± 6	500	20
Parental	100	yes	0.40 ± 0.02	22 ± 2	500	20
Δnukm	20	no				

Table 3.3 ***-Characterization of strains E150 (wild type), parental and Δnukm-***

[*1] Complex I content in mitochondrial membranes was extimated by measuring the dNADH:HAR activity that is not affected by mutations in the NUKM subunit (100% = 1.0 µmol min^{-1} mg^{-1}).

[*2] Complex I assembly was determined by Blue Native Page.

[*3] To account for variations in complex I content in different batches of mitochondrial membranes, dNADH:DBQ activity was normalized to complex I content expressed as specific NADH:HAR activity.

[*4] I_{50} is the inhibitor concentration required for a 50% reduction in catalytic rate (dNADH:DBQ µM min^{-1} mg^{-1}) of complex I.

Cysteine Mutants

Every single of the four fully conserved cysteines (Fig. 3.5) were mutagenised into alanine and serine.

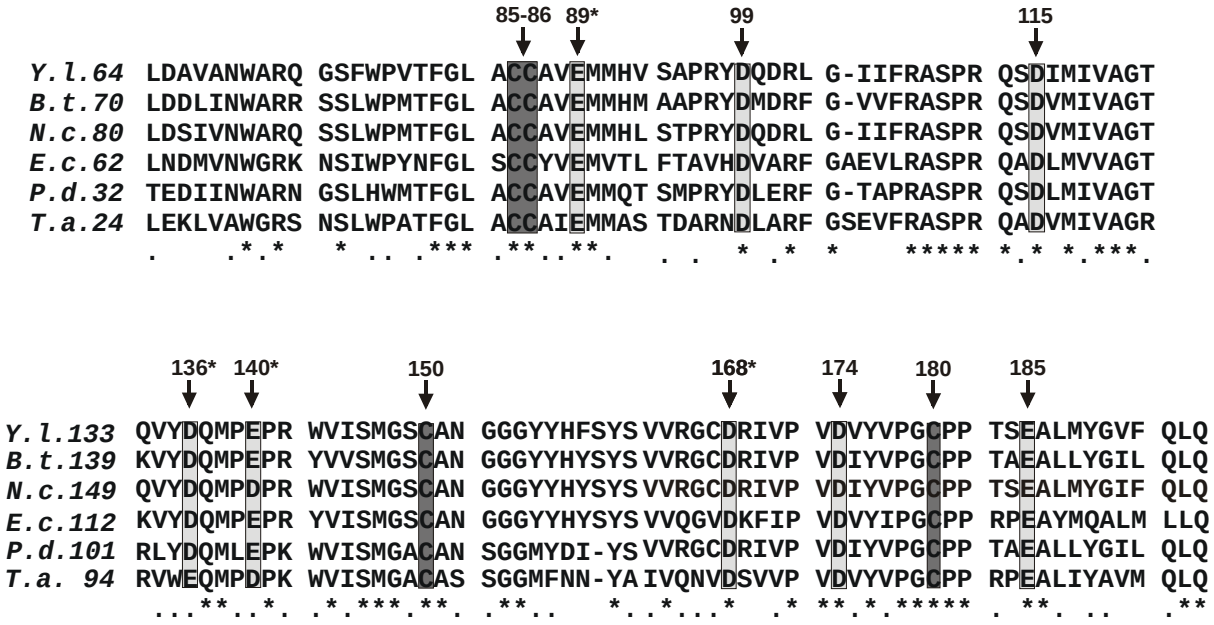

Figure 3.5 ***-Alignment of PSST-homologous subunits of complex I-***

Identical amino acids are marked by *asterisks*, similar residues by *dots*. Proposed ligands for Fe_4S_4 cluster N2 are shaded in dark grey and labelled with their number in the sequence of the *Y. lipolytica* protein. Conserved acidic amino acids are shaded in light grey and labelled with their number in the sequence of the *Y. lipolytica* protein. *Y.l., Yarrowia lipolytica; B.t., Bos taurus; N.c., Neurospora crassa; E.c., Escherichia coli; P.d., Paracoccus denitrificans; T.a., Thermus aquaticus*.

*Mutants in positions E89, D136, E140 and D168 were analysed by (Ahlers et al., 2000b) and results are summarised in 1.3.4.

To verify the mutagenesis system used, mutant C180S was reverted to the original cysteine. The resulting strain S180C was supposed to show the same characteristics as the parental strain. This expectation was confirmed by dNADH:HAR activity measurement (Table 3.4).

Strain	Complex I content (%)[*1]	Complex I band in BN-Page
Parental	100	yes
Δnukm	20	no
C85A	20	no
C85S	20	no
C86A	30	no
C86S	30	no
C150A	20	no
C150S	20	no
C180A	20	no
C180S	20	no
S180C*[2]	100	yes

Table 3.4 -Characterization of cystein-mutants in the PSST homologous NUKM subunit-

[*1] Complex I content in mitochondrial membranes was extimated by measuring the dNADH:HAR activity that is not affected by mutations in the NUKM subunit (100% = 1.0 μmol min^{-1} mg^{-1}).

[*2] Mutation *nukm*-C180S was changed back into the original *nukm*-S180C.

Mitochondrial membranes from the parental, Δ*nukm* and mutant strains C-85, 86, 150, 180-A and C-85, 86, 150, 180–S, were solubilised with 1 g/g LM and loaded on a native gel (BNP). Figure 3.4 shows the native gel of the parental, Δ*nukm* and C85A strain. The other mutants had a similar pattern as the C85A strain and are not shown. A complex I specific band was visible only in the case of the parental strain. Similarly as for the *nuim* mutants, complex V dimer and monomer bands are visible: this was taken as an indicator that the optimal LM concentration needed for solubilising complex I was used. Figure 3.6 shows the second dimensions (SDS-PAGE) from the bands of the parental and the C85A strains: again, only the parental strain showed complex I bands. In addition, dNADH:HAR membrane activities of all cysteine mutants were comparable to the activity of the Δ*nukm* strain (Table 3.4).

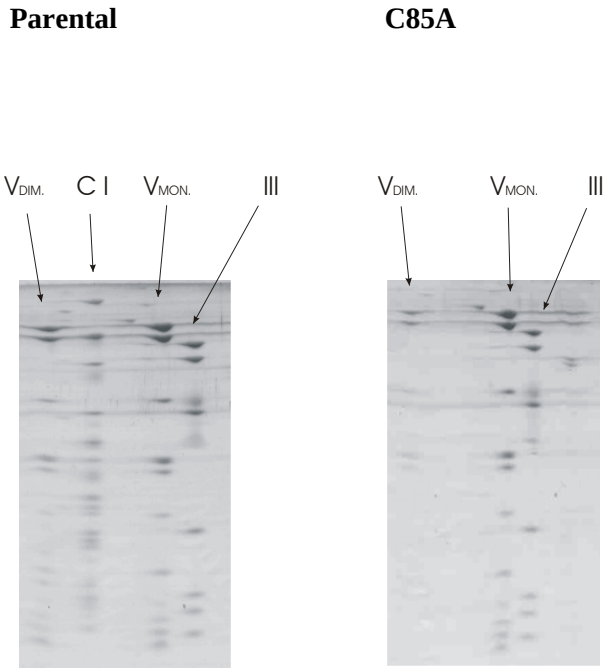

Figure 3.6 ***-Second dimension of Blue Native gel shown in figure 3.5-***

Parental strain and C85A mutant are shown as examples: other mutants which showed the same BNP pattern are not reported for brevity. $\mathbf{V_{DIM.}}$, complex V in dimeric form; **CI**, complex I; $\mathbf{V_{MON.}}$, complex V in monomeric form; **III**, complex III.

EPR spectra from mitochondrial membranes of parental and Cys-Ala mutant strains were recorded. To compensate for different complex I concentrations in different batches of membrane preparations and to allow direct comparison of signal intensities between the different spectra reported in figure 3.7, spectra were normalised to the signal intensity of iron-sulfur cluster S3 from complex II. The spectrum of the parental strain clearly showed the g_z signal of cluster N2 and the typical signals in the middle region of the spectrum that are due to the contributions of clusters N1, N2, N3 and N4. None of the mutants showed any signal that could be assigned to a complex I cluster. The low signals visible between the dotted lines are most likely due to the clusters of complex II and to the Rieske protein.

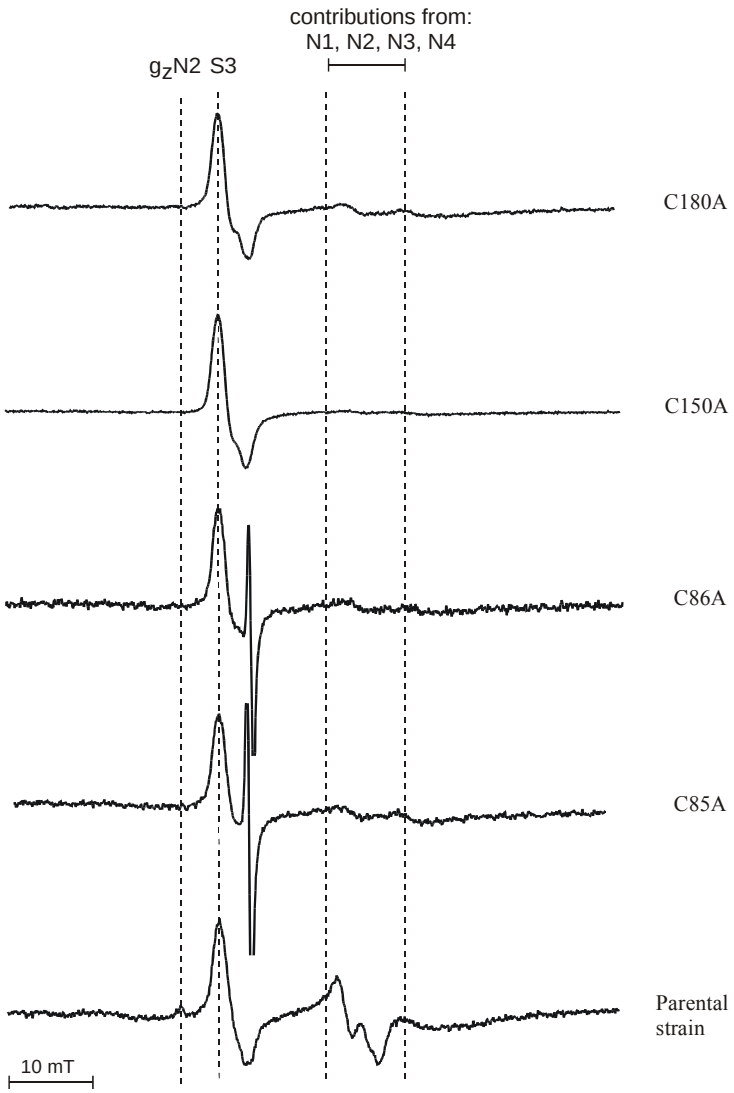

Figure 3.7 ***-EPR spectra of mitochondrial membranes from parental and C-85, 86, 150, 180-A mutant strains reduced by NADH–***

The intensities of the spectra were normalised to the signal intensity of the Fe-S cluster S3 from complex II. *Dotted lines* indicate field positions of the g_z signal of cluster N2 (2.051) and the field region where the contributions from clusters N1, N2, N3, and N4 are detectable. EPR conditions: microwave frequency 9.475 GHz, modulation amplitude 0.63 mT, microwave power 1 mW, temperature 12 K.

- *Mutations of Acidic Residues*

Eight fully conserved acidic residues in PSST subunit, E89, D99, D115, D136, E140, D168, D174 and E185 (shaded in light grey in Fig. 3.5) were hypothetical candidates for the fourth ligand for N2 cluster: positions E89, D136, E140 and D168 were analysed in a previous study (Ahlers et al., 2000b) and results are briefly reported in 1.3.4.

In the present work, site directed mutagenesis in positions D99, D115, E185 and D174 was carried out. Mutant membranes were characterised in terms of complex I assembly and content, catalytic activity and sensitivity toward quinone-analogous inhibitors. EPR spectra were recorded from isolated complex I and/or from membrane preparations.

Mutant D99N, D99E and D99G

Codon 99 in the *NUKM* open reading frame translates into a fully conserved aspartic acid. Mitochondrial membranes from mutants D99N, D99E and D99G were prepared in small amounts (2.3.3) from cells grown in YPD medium containing hygromycin B (Hyg). The complex I content of all three strains was in the same range as the parental strain (Table 3.5). Membranes of mutant D99N were solubilised with 1 g/g LM and loaded on a blue native gel (BNP). The second dimension is shown in figure 3.8: complex I bands were clearly visible and comparable to the complex I bands of the parental strain.

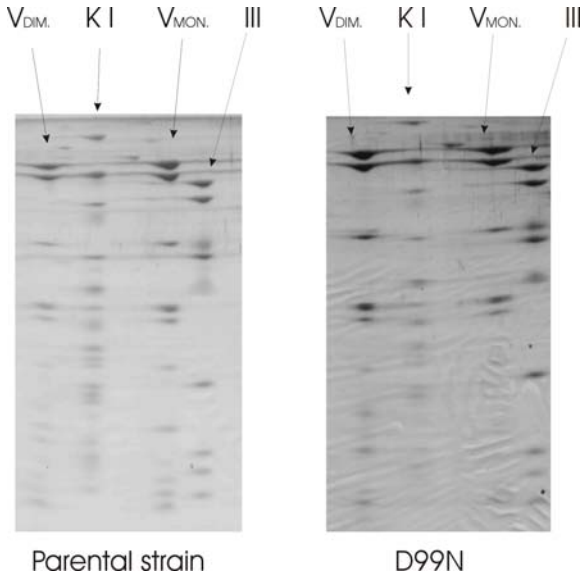

Figure 3.8 ***-BNP second dimensioN–***

$V_{DIM.}$, complex V in dimeric form; **CI**, complex I; **$V_{MON.}$**, complex V in monomeric form; **III**, complex III.

Strain	Complex I Content (%)[*1]	V_{Max}[*2] µmol min^{-1} mg^{-1}	K_M µM	I_{50} Rotenone[*3] nM	I_{50} DQA[*3] nM
E150	110	0.42 ± 0.02	32 ± 6	500	20
Parental	100	0.40 ± 0.02	22 ± 2	500	20
D99N	90	0.02	-	-	-
D99E	90	0.02	-	-	-
D99G	90	0.02	-	-	-
D115N	100	0.02	-	-	-
D115E	100	0.02	-	-	-
D115G	100	0.02	-	-	-
D174N	90	0.40 ± 0.05	35 ± 5	500	20
E185Q	110	0.14 ± 0.01	50 ± 4	500	20

Table 3.5: ***-Characterization of mutants in the PSST homologous NUKM subunit-***
Mitochondrial membranes (prepared as described in 2.3.2) from different strains were characterised and compared in the table.

[*1]Complex I content in mitochondrial membranes was extimated by measuring the dNADH:HAR activity that is not affected by mutations in the NUKM subunit (100% = 1.0 µmol min^{-1} mg^{-1}).

[*2]To account for variations in complex I content in different batches of mitochondrial membranes, dNADH:DBQ activity was normalized to complex I content expressed as specific NADH:HAR activity. Determination of V_{Max}, K_M, and I_{50} values for mutants in positions D99 and D115 was not possible. dNADH:DBQ activities of these two mutants were determined at 100 µM dNADH and 60 µM DBQ whereby the parental strain showed 0.3 µmol min^{-1} mg^{-1}.

[*3]I_{50} is the inhibitor concentration required for a 50% reduction of the dNADH:DBQ catalytic rate of complex I.

Changing the carboxylate residue into its corresponding amide asparagine resulted in almost complete loss (< 5% residual activity) of dNADH:DBQ oxidoreductase activity, measured in the presence of saturating amounts of both substrates. Due to this very low activity, determination of Michaelis-Menten parameters like K_M and V_{Max}, and also the determination of I_{50} values for complex I inhibitors was not possible for this mutant. To isolate complex I, a

second batch of mitochondrial membranes was prepared from cells grown in 10 l of YPD medium (without Hygromycin) as described in 2.3.1-2. Complex I content as estimated by NADH:HAR activity in this second mitochondrial preparation (60%) was lower than in the first preparation. This phenomenon could find an explanation in plasmid loss during growth in media without selective pressure by the antibiotic. In fact, the mutagenised *nukm* copy carried on the plasmid, providing assembly of complex I but no NADH:ubiquinone oxidoreductase activity, does not constitute an advantage for cell growth. To investigate loss of plasmid in absence of Hyg pressure, cells were grown 12 hours in YPD and plated on YPD and YPD-Hyg agar media. After 48 hours incubation, cells were counted and the survival ratio YPD/YPD-Hyg was calculated. Only cells that still have the plasmid (containing a copy of the gene for Hyg resistance and for the PSST subunit) are able to survive on medium with Hyg and to produce an assembled complex I. Consequently, the ability to survive in YPD-Hyg medium is taken as indicator for the ability to produce an assembled complex I. The same experiment was carried out growing cells 12 hours in YPD-Hyg medium, to avoid loss of plasmid. As expected, parental strain showed essentially the same survival ratio when cells were incubated in the presence and in the absence of antibioticum (Table 3.6). Differently, the survival ratio for mutant D99N decrease significantly when cells were grown without Hyg (see table 3.6 for more details). This suggested that a significant portion of the cells had lost the plasmid (and the ability to form complex I) in the absence of Hyg selective pressure, and explained the observed decrease of NADH:HAR activity in the second membrane preparation. For strain D99N, the presence of a fully-assembled complex I, does not constitute an advantage.

	Cells grown in YPD			Cells grown in YPD-Hyg		
	Agar medium			Agar medium		
	YPD	YPD-Hyg	Survival rate (%)	YPD	YPD-Hyg	Survival rate (%)
Parental	93	83	89%	90	81	90%
D99N	87	43	49%	85	70	82%

Table 3.6 ***-Survival rate (YPD/YPD-Hyg agar medium) of parental and D99N strains -***

Cells were grown for 12 hours in YPD medium (with and without Hyg). 100 cells were plated on YDP and YPD-Hyg agar medium and incubated for 48 hours. In the table the number of cells counted on the plates after 48 hours growth time are indicated. Survival rates (YPD/YPD-Hyg agar medium) are expressed in %.

Affinity purification of complex I from mitochondrial membranes (see 2.3.9a) of mutant D99N was carried out twice: in both experiments the yield was about 8% of the complex I in mitochondrial membrane (about one fourth of the yield usually obtained from the parental strain). Table 3.7 compares the two purification experiments of complex I from D99N and parental strain.

Strain	Solubilized membranes (1g/g LM)	Purified enzyme	
	Units	Units	%
D99N (I exp.)	4920	409	8
D99N (II exp.)	5012	501	8
Parental	7051	2115	30

Table 3.7 ***-Complex I purification experiments-***

Two different complex I purification experiments for mutant D99N and one purification experiment for the parental strain are summarised. Units = μmol min^{-1}.

The low purification yield suggests a somewhat lower stability of complex I in mutant D99N. Data obtained by comparing temperature stability of NADH:HAR oxidoreductase activity of mutant and parental membranes were consistent with this assumption (Fig. 3.9). Membranes were incubated at different temperatures for five minutes and their NADH:HAR activity was measured at 30°C (see 2.3.7). For this experiment mitochondrial membrane preparations from the parental and the D99N mutant strain with comparable specific NADH:HAR activities were used.

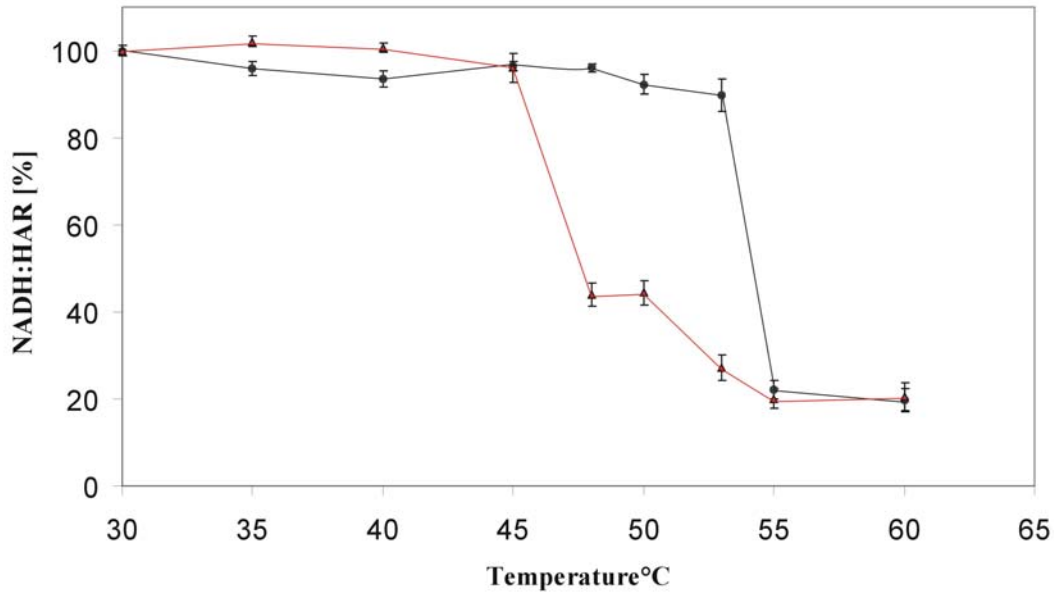

Figure 3.9 ***-NADH:HAR activity as a function of incubation temperature-***

- Parental strain; ▲ Mutant D99N

  NADH:HAR activity at each temperature point is reported as average of eight measurements.

NADH:HAR activity of the mitochondrial membranes after incubation at 30°C was taken as 100%. The activity from both the parental and the mutant strain was quite stable up to 45°C, as shown in figure 3.9 (13% reduction). By contrast, at 48°C D99N showed a decrease by about 57%, whereas the activity of the parental strain did not change substantially up to 53°C (96%). Since the NADH:HAR oxidoreductase activity depends only on the FMN and the 51 kDa subunit, the different NADH:HAR temperature dependence of mutant and parental strains seems to be linked to a destabilization effect on the enzyme due to the mutation, and not to the specific effect of the mutation on the NADH:ubiquinone activity of complex I.

The proposed enzyme instability was also confirmed by EPR spectroscopy. In fact, in the first complex I preparation from this mutant, cluster N2 was not clearly detectable by EPR spectroscopy, whereas in the second preparation the presence of a N2 g_z signal is clearly evident, and the spectra of all other visible clusters in both cases seemed to be essentially unchanged when compared to the parental strain (Fig. 3.10). In the first isolation, due to the low yield obtained from mutant enzyme purification, the protein concentration of the mutant sample (2.4 mg/ml) was considerably lower compared to the parental sample concentration (3.4 mg/ml). This caused the lower signal/noise ratio of the mutant spectrum. To compensate

for different complex I concentrations and to allow direct comparison of signal intensities between the different spectra reported in this figure, spectra were normalised to the N1 signal intensity of the parental enzyme at 40 K. At this temperature cluster N1 can be measured selectively avoiding background effects from other overlapping iron-sulfur clusters, like iron–sulfur cluster S3 of complex II. The smaller peak identifiable at the N2 g_z position in the first isolation is presumably due to a reduced amount of this cluster.

In the second batch of complex I from mutant D99N shown in Fig 3.10 (8.5 mg/ml), the presence of iron-sulfur cluster N2 was clearly evident from the EPR spectrum recorded at 12 K and the g_z value for iron-sulfur cluster N2 was not shifted, indicating that the mutation had not influenced ligand geometry. The g values and relative intensities of the signals for clusters N4 and N3 were comparable to the parental signals whereas the middle region (contributions of all clusters to the EPR signal) showed different intensities and line shapes, and the N1 g_z signal seemed to be sfifted. From the spectra recorded at 40 K became clear that the signal of the iron–sulfur cluster S3 of complex II contributed significantly to the slightly shifted peak at the N1 g_z field position. This indicated that significant amounts of complex II were present as an impurity in the complex I preparations from mutant D99N. It follows that cluster N2 remains the only possible candidate for causing the different patterns in the middle region of the spectra, but since signals originating from all iron-sulfur clusters overlap in this field position this signal could not be used for quantification. Observing the N2 g_z signal it can be concluded that in the second batch of complex I from mutant D99N the amount of N2 was decreased by about 50%.

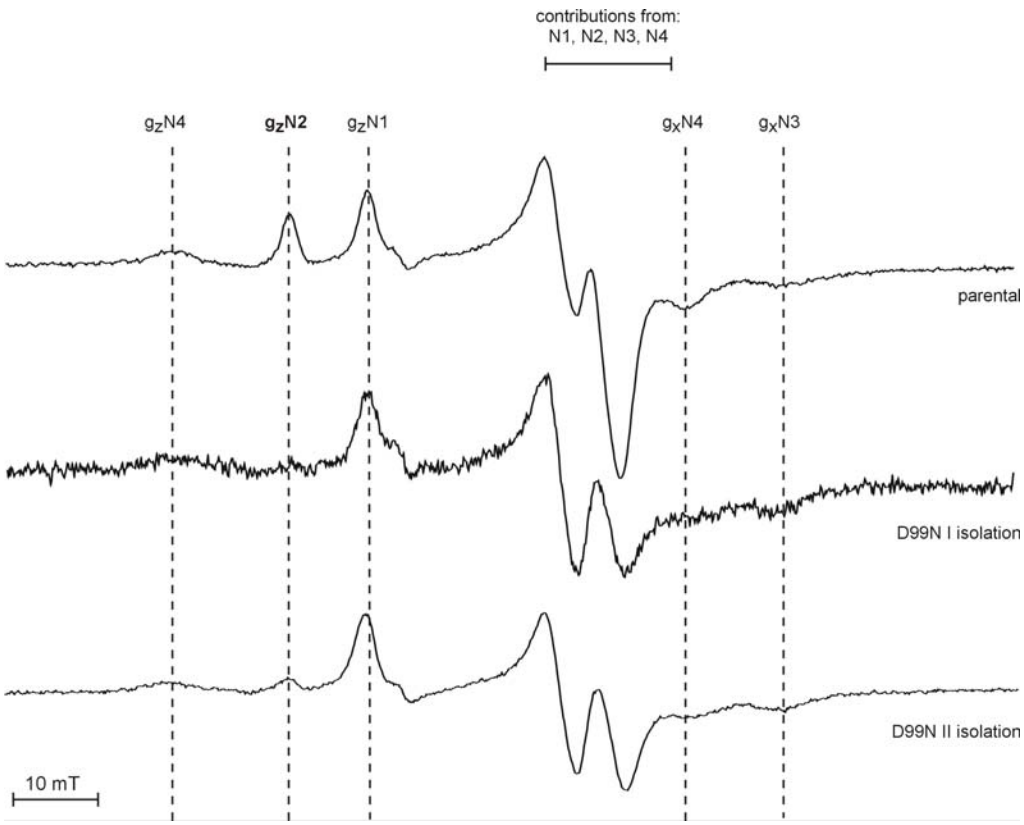

Figure 3.10 ***-EPR spectra of isolated complex I from the parental strain and the D99N mutant strain reduced with NADH-***

The intensities of the spectra were scaled to the signal intensity of cluster N1 recorded separately at 40 K for each sample. *Dotted lines* indicate field positions for g values of individual clusters: g_zN4, 2.104; g_zN2; 2.051; g_zN1, 2.018; g_xN4, 1.892; g_xN3, 1.861. EPR conditions: microwave frequency 9.475 GHz, modulation amplitude 0.64 mT, microwave power 1 mW, temperature 12 K.

On the basis of the EPR spectra the function of aspartate 99 as a direct ligand for cluster N2 seems to be implausible but an indirect role of this amino acid in cluster binding cannot be excluded. In fact, cysteines ligate an iron-sulfur cluster by binding the iron ions of the cluster to their thiol group (1.2.3). The cysteinyl sulfur of a ligand offers two electron pairs for hydrogen bound. It is reasonable to suppose that the carboxylic group of the aspartate 99 may form a hydrogen bond to this sulfur, influencing with its charge the ability of the cluster to accept electrons. In this case, the elimination of the residue may still allow assembly of the cluster, but on the other hand its removal should result in a change of the midpoint potential of cluster N2. In this working hypothesis, an expected consequence of the changed midpoint

potential of redox centre N2 in D99N might be an incomplete reduction of N2 clusters in the EPR sample. The observed 50% reduction in N2 signal intensity would fit to this scenario. To investigate this possibility the midpoint potential of cluster N2 in D99N was determined in membranes (Fig. 3.11). The measured value of –125 mV for the mutant strain was not significantly different from that obtained for the parental strain, demonstrating that the reduction in N2 g_z signal intensity reflects a reduction in N2 content and definitively excluding D99 as ligand for cluster N2.

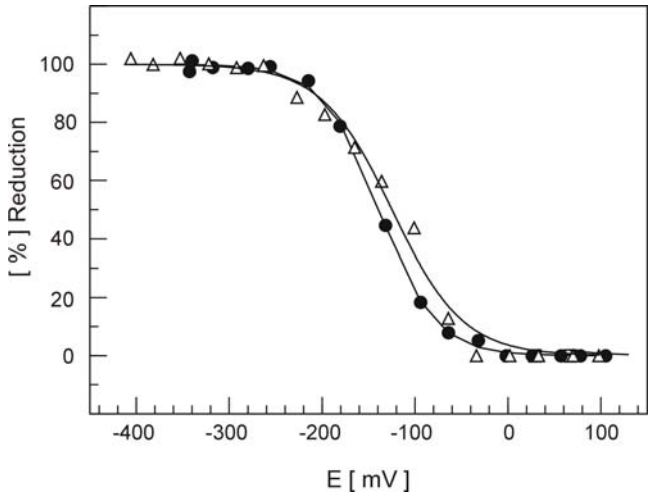

Figure 3.11 ***-Redox titrations of iron-sulfur cluster N2 monitored by EPR spectroscopy-***

•, parental mitochondrial membranes, $Em_{,7}$ = -140 mV; Δ, mutant D99N, $Em_{,7}$ =-125 mV.

To test a more conservative mutation that left intact the carboxylic acid function at position 99, the aspartic acid residue was replaced with a glutamic acid residue. In terms of specific NADH:HAR activity of mitochondrial membranes, D99E did not show any significant difference compared to the parental strain or mutant D99N (Table 3.5). Also dNADH:DBQ activities of D99E and D99N were comparable (< 5% of the wild type rate). As a more drastic mutation aspartic acid 99 was changed into glycine. Again, specific NADH:HAR activity of the mutant membranes was comparable to the parental strain (Table 3.5) and the dNADH:DBQ specific activity was as low as for mutants D99N and D99E. Summarising,

mutants D99 N/E/G were indistinguishable in terms of complex I content and catalytic activity.

Mutants D115N, D115E and D115G

A second fully conserved aspartic acid residue in the PSST sequence, D115 (Fig. 3.5) was mutagenised to give asparagine, glutamic acid or glycine. Mitochondrial membranes from each mutant were prepared in small amounts (cf. 2.3.3) from cells grown in YPD medium with hygromycin B. The complex I content of all three mutant preparations was in the same range as the parental strain. Membranes were solubilized with 1g/g LM and loaded on a blue native gel (BNP). The second dimension of parental and D115N strains is shown in figure 3.12: specific complex I bands were clearly visible and comparable to the complex I bands of the parental strain.

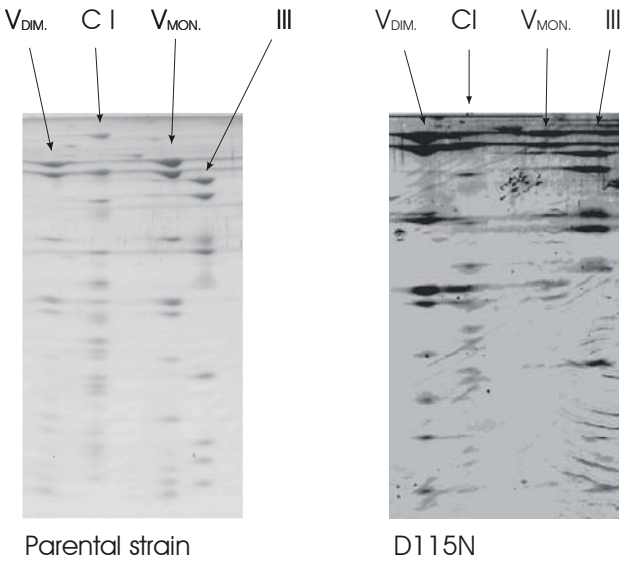

Figure 3.12 ***-BNP second dimension –***

$V_{DIM.}$, complex V in dimeric form; **CI**, complex I; **$V_{MON.}$**, complex V in monomeric form; **III**, complex III.

dNADH:DBQ activities were found to be only ~8% of the wild type rate in all three mutant preparations. As for the D99 mutants, this low activity made the determination of K_M , V_{Max}

and I_{50} values for complex I inhibitors impossible. For isolating complex I, a second batch of mitochondrial membranes was prepared from cells grown in 10 l YPD medium (without hygromycine) as described in 2.3.1-2. Complex I content estimated by NADH:HAR activity (70%) was lower than in membranes obtained from cells grown in the presence of hygromycine. As in the case of the D99 mutants, the explanation of this phenomenon could be found in plasmid loss by growth in media without antibiotic pressure, when assembly of complex I does not constitute a growth advantage. Loss of plasmid in the absence of hygromycine (Hyg) pressure was investigated by plating cells on YPD and YPD-Hyg plates after 12 hours growing-time in YPD medium. This experiment was carried out for the mutant D115N and the parental strain as described for mutant D99 (see above). The survival rate on YPD-Hyg / YPD agar media for the D115N mutant strain was only around 45% (Table 3.8). This fact suggested that a significant portion of the cells had lost the plasmid (and the ability to form complex I) in the absence of Hyg selective pressure, and explained the observed decrease of NADH:HAR activity in two different membrane preparations.

	Cells grown in YPD			Cells grown in YPD-Hyg		
	Agar medium			Agar medium		
	YPD	YPD-Hyg	Survival rate (%)	YPD	YPD-Hyg	Survival rate (%)
Parental	95	86	90%	95	86	91%
D115N	91	41	45%	87	70	80%

Table 3.8 ***-Survival rate (YPD/YPD-Hyg agar medium) of parental and D115N strains -***

Cells were grown for 12 hours in YPD (with and without Hyg) medium. 100 cells were plated on YDP and YPD-Hyg agar medium and incubated for 48 hours. In the table are indicated the number of colonies counted on the plates after 48 hours growth time. Survival rate (YPD/YPD-Hyg agar medium) are expressed in %.

Purification of complex I from both mutants gave a yield of 12% for D115N and 10% for D115E, that is about one third of the parental strain yield (Table 3.9).

Solubilized membranes (1g/g LM)			Purified enzyme	
	Units	%	Units	%
D115N	3781	100	454	12
D115E	4587	100	459	10
Parental	7051	100	2115	30

Table 3.9 ***-Complex I purification experiments-***

Two different complex I purification experiments for mutants at position D115 and one purification experiment for the parental strain are summarised. Units = µmol min^{-1}.

As in the case of mutant D99N, this low yield suggested some destabilisation of complex I. Membranes of both strains were incubated at different temperatures for five minutes, followed by NADH:HAR activity measurements. The experimental conditions were the same as for mutant D99N (described above) and results are shown in figure 3.13: mutant D115N showed different temperature stability when compared to the parental strain.

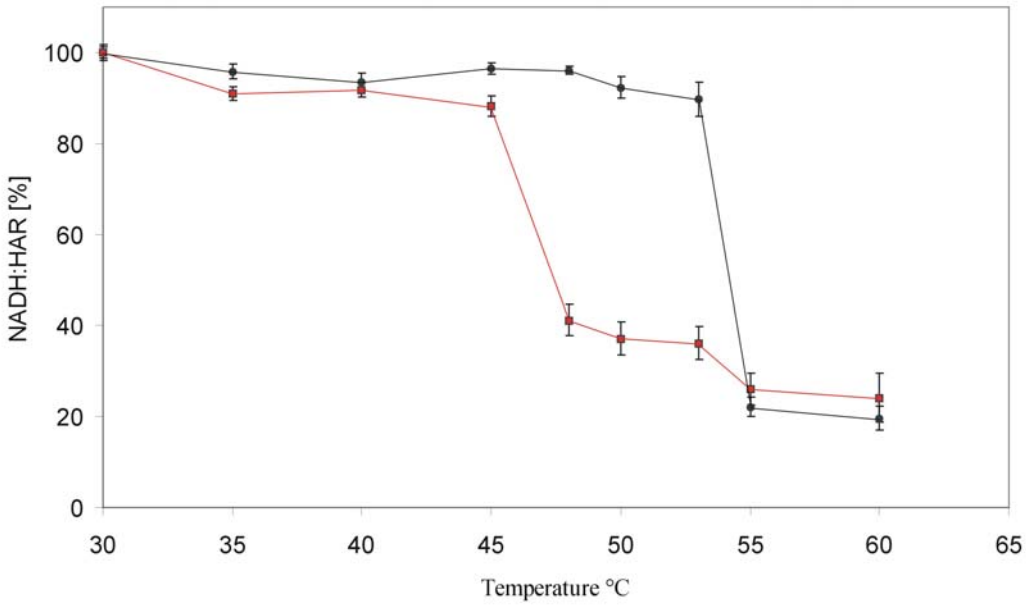

Figure 3.13 ***-NADH:HAR activity as a function of incubation temperature-***

• Parental strain; ■ Mutant D115N

NADH:HAR activity at each temperature point is reported as average of eight measurements.

Incubation at temperatures below 45°C did not change substantially the NADH:HAR oxidoreductase activity of both strains, whereas incubation at higher temperatures up to 53°C reduced the activity of mutant mitochondrial membranes, but not from the parental strain.
EPR spectroscopy of complex I isolated from D115N (Fig. 3.14) showed that the g values and relative signal intensities for clusters N1, N3 and N4 were not changed and that their relative amplitudes were comparable to complex I from the parental strain.

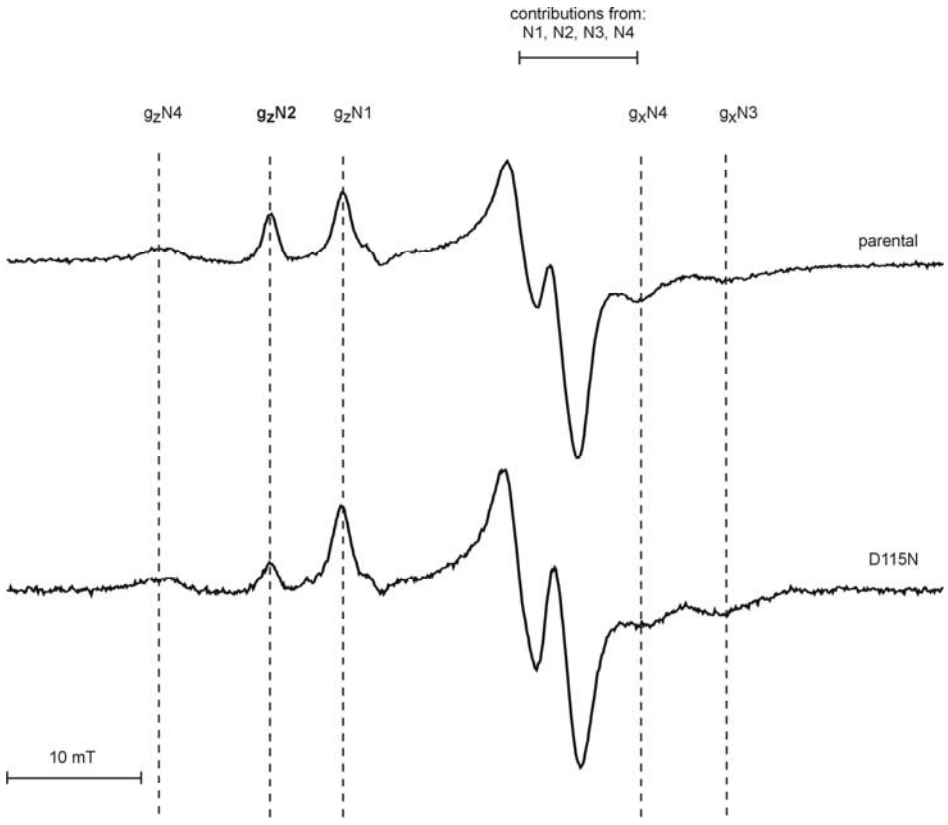

Figure 3.14 ***-EPR spectra of isolated complex I from the parental and the D115N mutant strains reduced with NADH-***

The intensities of the spectra were scaled to the signal intensity of cluster N1 recorded separately at 40 K for each sample. *Dotted lines* indicate field positions for characteristic g values of individual clusters: g_zN4, 2.104; g_zN2; 2.051; g_zN1, 2.018;

g_xN4, 1.892; g_xN3, 1.861. EPR conditions: microwave frequency 9.475 GHz, modulation amplitude 0.64 mT, microwave power 1 mW, temperature 12 K.

As in the case of mutant D99N, the g_z signal for cluster N2 was not shifted but a reduction in signal intensity by about 50% was observed. As for D99, the possibility that this amino acid forms hydrogen bound to cluster N2, thereby influencing the redox midpoint potential, was checked. The determined value of –120 mV for D115N was not significantly different from that obtained for the parental strain (Fig. 3.15).

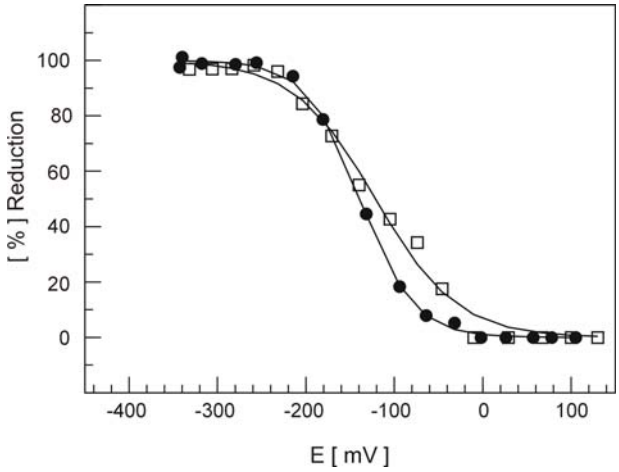

Figure 3.15 ***-Redox titrations of iron-sulfur cluster N2 monitored by EPR spectroscopy-***

•, parental mitochondrial membranes, Em,7 = -140 mV; □ mutant D115N, Em,7 = -120 mV.

The aspartic acid in position 115 was also changed into asparagine and glycine. Again, mitochondrial membranes from D115 N/E/G mutants were indistinguishable in terms of complex I content and catalytic activity (Table 3.5).

Mutant D174N

A third fully conserved aspartate is located at position 174 (Fig. 3.5). This position was changed into asparagine. Complex I content in membranes from mutant D174N was within the range commonly observed for the parental strain (Table 3.5). For the determination of Michaelis-Menten parameters V_{Max} and K_M for DBQ, complex I activity of mitochondrial membranes was assayed as electron transfer from dNADH to DBQ and catalytic rates were normalized for NADH:HAR activity. V_{Max} for DBQ and I_{50} values for rotenone and DQA were not significantly changed, whereas the K_M value for ubiquinone was slightly increased (Table 3.5). Except for the K_M value, the catalytic activity of complex I is not substantially modified by this mutation (Fig. 3.16). This fact allows the conclusion that the aspartate in position 174 does not play an important role in complex I function. This hypothesis is confirmed by EPR spectra of mitochondrial membranes: mutant D174N and the parental strain were indistinguishable, indicating that iron-sulfur cluster N2 content and line shape were not significantly affected by the mutation (Fig 3.17).

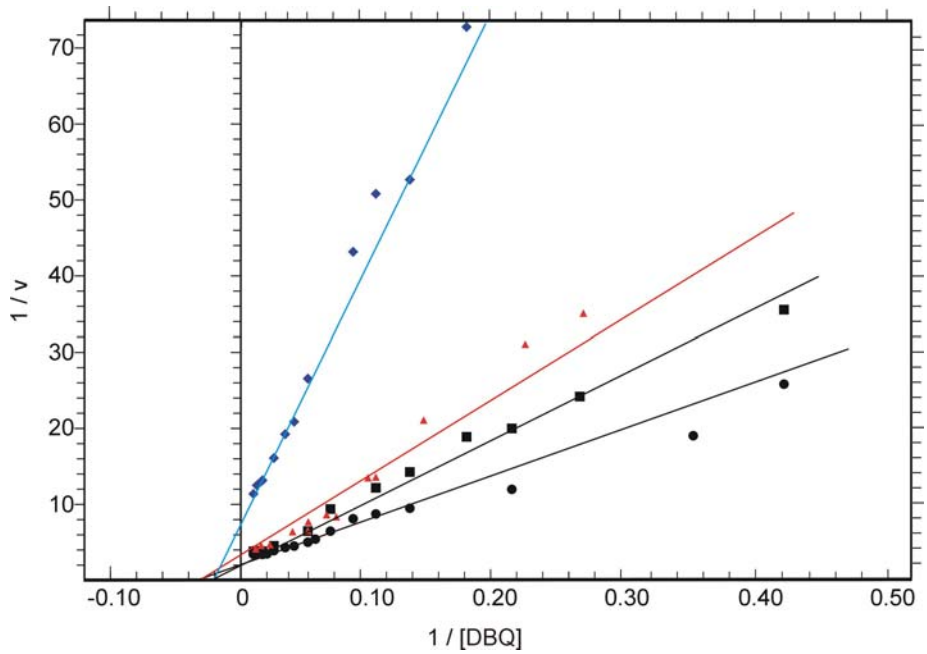

Figure 3.16 ***-Kinetic characterization of dNADH:DBQ activity-***

K_M and V_{Max} values were determined by direct fit of the data to the standard Michaelis-Menten equation.

The K_M and V_{Max} values were determined by direct fit of the data to the standard Michaelis-Menten equation. These parameters (cf. Table 3.5) were used to construct

the shown double-reciprocal plots of the parental strain and the mutants. ■, Wild type; ●, Parental strain; ♦, mutant *nukm* E185Q; ▲ mutant *nukm* D174N.

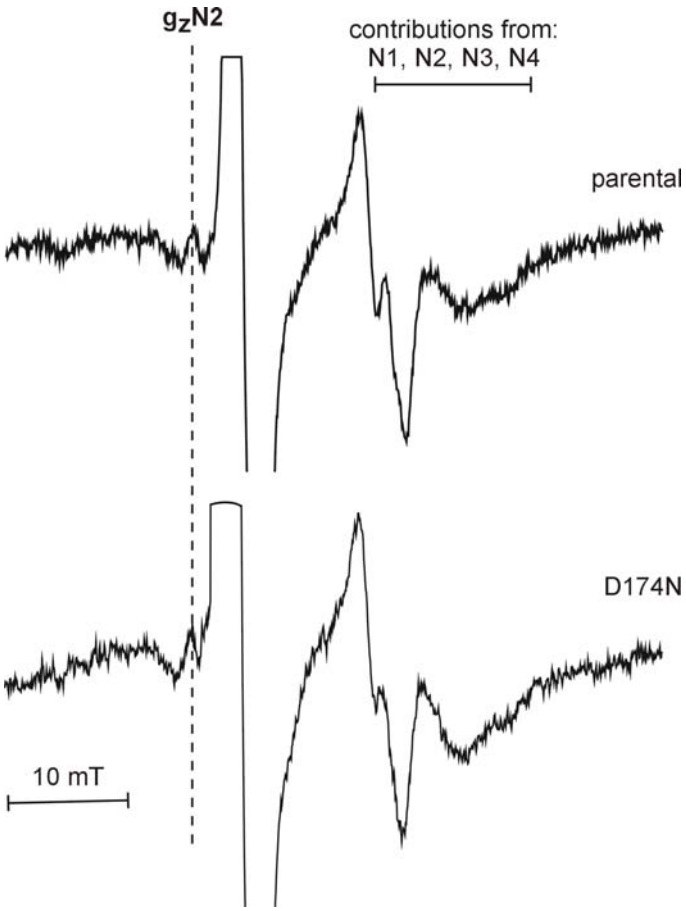

Figure 3.17 ***-EPR spectra of mitochondrial membranes from the parental and the D174N mutant strains reduced with NADH –***

The intensities of the spectra were scaled to the signal intensity of the middle field region of the spectra (marked as contribution from N1, N2, N3, N4). The *dotted line* indicates field positions for g values of g_zN2 (2.051). EPR conditions: microwave frequency 9.475 GHz, modulation amplitude 0.64 mT, microwave power 1 mW, temperature 12 K.

Mutant E185Q

Two fully conserved glutamic acid residues can be identified in the sequence of the complex I PSST subunit (Fig. 3.5): E89 and E185. Glutamate 89 (previously described in 1.3.4) has a position close to cysteine 86 and glutamate 185 is located near cysteine 180. Similarly as E89, E185 is candidate as ligand for cluster N2 by virtue of its position in the PSST sequence. Mutant E185Q exhibited a substantial decrease in dNADH:DBQ activity of up to 65%, the K_M value for DBQ was increased about twofold whereas I_{50} values for rotenone and DQA were the same as for the parental strain (Table 3.5, Fig. 3.16). The purification of complex I from mutant E185Q was successful and the yield obtained from membranes of this mutant was comparable to the yield normally obtained for the parental strain. The EPR spectrum of E185Q was virtually indistinguishable from spectra of parental complex I: there were no effects on the positions of the g_z signals of clusters N1, N2, N3, N4 and their intensities were also unaffected (Fig. 3.18).

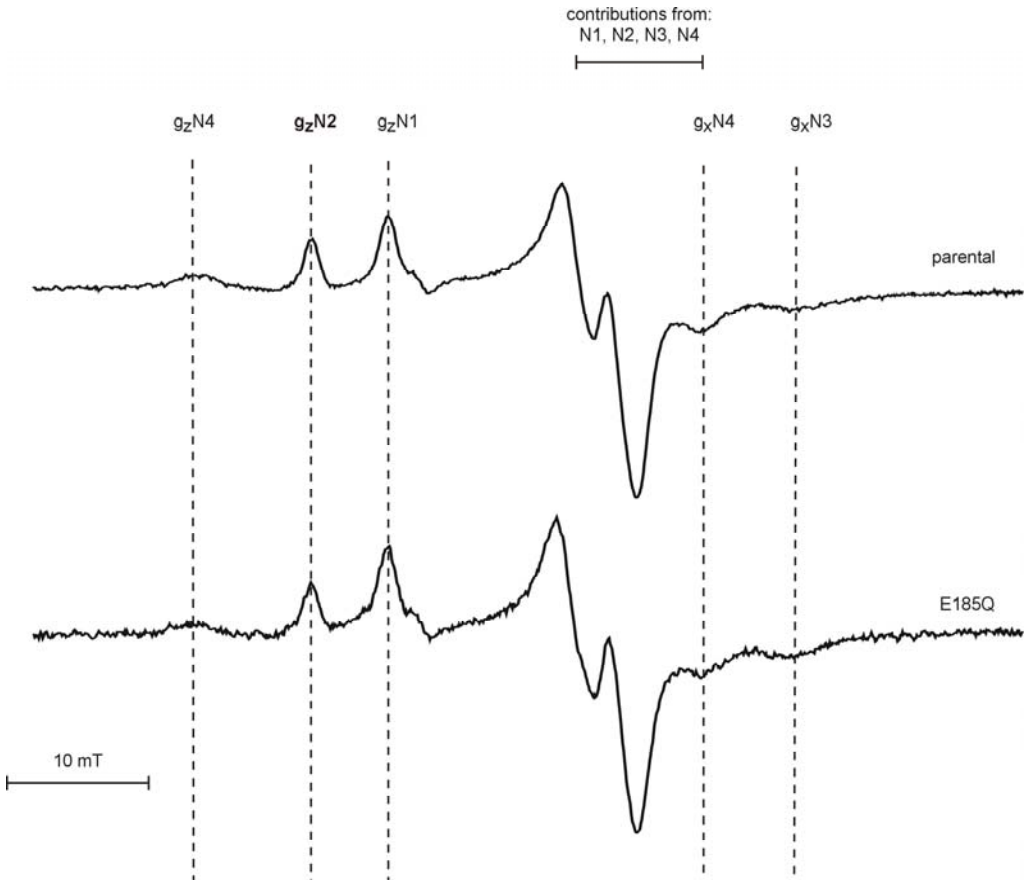

Figure 3.18 ***- EPR spectra of isolated complex I from the parental and the E185Q mutant strains reduced by NADH-***

The intensities of the spectra were scaled to the signal intensity of cluster N1 recorded separately at 40 K for each sample. *Dotted lines* indicate field positions for characteristic g values of individual clusters: g_zN4, 2.104; g_zN2; 2.051; g_zN1, 2.018; g_xN4, 1.892; g_xN3, 1.861. EPR conditions: microwave frequency 9.475 GHz, modulation amplitude 0.64 mT, microwave power 1 mW, temperature 12 K.

3.1.3 Characterisation of the Leigh Mutations

Since researchers at the Nijmegen Center for Mitochondrial Diseases had identified point mutations in subunits *NDUFS7* and *NDUFS8* of complex I (homologous to PSST and TYKY from *Y. lipolytica*, respectively) as the causative genetic defects in two cases of neuropathologically proven Leigh syndrome (cf. 1.4), the corresponding positions were mutagenised in *Y. lipolytica* in an effort to analyse in vitro the effects of these mutations on complex I. Mutagenesis was carried out in a previous work by transforming a modified copy of the *nukm* or *nuim* gene on plasmid pINA443 in Δ*nukm, ndh2i (lys⁻ ura⁻ nukm::leu2 Mat A)* and Δ*nuim, ndh2i (lys⁻ ura⁻ nuim::leu2 Mat A)* strains, respectively (Ahlers et al., 2000a). In the present work, mitochondrial membranes of the mutant strains were prepared and characterised in terms of complex I content and catalytic activity.

Valine 122 in *NDUFS7* corresponds to V119 in the PSST protein of *Y. lipolytica*. This position is fully conserved in the alignment of the PSST homologous subunits from different organisms shown in figure 3.19, and is part of a well-conserved region, even in prokaryotes like *Paraccocus denitrificans* and *E. coli*.

P78 and R101 in the TYKY protein correspond to P79 and R102 in *NDUFS8*. In contrast to position 119 in NUKM, sequence homology in the region between both residues is high only for eucaryotes.

Figure 3.19 ***-Positions of Leigh mutations in the PSST- and TYKY- homologues of Y. lipolytica-***

The protein sequences from various organisms were aligned using the CLUSTAL program of the HUSAR 4.0 package, DKFZ Heidelberg, Germany. Identical amino acids are marked by asterisks. Residues forming the first half of the ferredoxin-type binding motif in TYKY and conserved cysteins in PSST are shaded in gray. Leigh mutations are marked with arrows and the positions of the mutated residues in the *Y. lipolytica* protein are indicated.

Mitochondrial membranes were prepared from cells grown in minimal medium to avoid loss of plasmid, and NADH:HAR oxidoreductase activity was measured for each mutant. V119M showed an estimated complex I content of about 110% of the parental strain content, whereas mutants R101H and P78L showed about 80 and 60%, respectively (Table 3.10).

Strain (subunit)	Complex I content (%)*[1]	V_{Max}*[2] µmol min^{-1} mg^{-1}	V_{Max} (%)	K_M (DBQ) µM	I_{50} Rotenone*[4] nM	I_{50} DQA*[4] nM
Parental (PSST)*[3]	100	0.40 ± 0.02	100	22 ± 2	500	20
V119M (PSST)	110	0.22 ± 0.01	55	12 ± 1	700	10
Parental (TYKY)*[3]	100	0.44 ± 0.02	100	23 ± 2	600	25
P78L (TYKY)	60	0.25 ± 0.01	57	12 ± 1	700	20
R101H (TYKY)	80	0.20 ± 0.01	45	19 ± 2	500	10

Table 3.10 ***-Characterization of the Leigh mutantions in the PSST and TYKY homologous subunits-***

Mitochondrial membranes (prepared as described in 2.3.2) from different strains were characterised and compared in the table.

*[1] Complex I content in mitochondrial membranes was extimated by measuring the dNADH:HAR activity that is not affected by mutations in the PSST and TYKY subunit (100% = 1.0 µmol min^{-1} mg^{-1}).

*2 To account for variations in complex I content in different batches of mitochondrial membranes, dNADH:DBQ activity was normalized to complex I content expressed as specific NADH:HAR activity. V_{Max} was set at 100% for parental strains.

*3 *Δnukm, ndh2i (lys⁻ ura⁻ nukm::leu2 Mat A)* complemented by an unmodified copy of the *NUKM* gene carried on plasmid pINA443.

Δnuim, ndh2i (lys⁻ ura⁻ nuim::leu2 Mat A) complemented by an unmodified copy of the *NUIM* gene carried on plasmid pINA443.

*4 I_{50} is the inhibitor concentration required for a 50% reduction of the dNADH:DBQ catalytic rate of complex I.

In all three mutants, V_{Max} was found to be significantly reduced by about 50%. The K_M for DBQ was unaffected in the R101H mutant (TYKY), while it was reduced from around 20 µM to 12 µM in the V119M (PSST) and the P78L (TYKY) mutants. These two mutants also exhibited a tendency to higher I_{50} values for inhibitor rotenone. As indicated by a somewhat lowered I_{50}, the R101H and the V119M mutants exhibited a slight hypersensitivity to DQA (Table 3.10). Figures 3.20 and 3.21 show Lineaver-Burk representations of mutant and parental strains kinetic characterization.

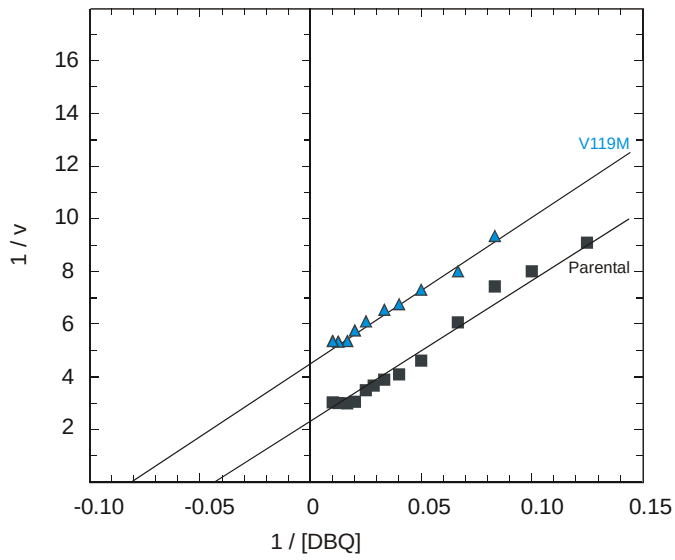

Figure 3.20 ***- Kinetic characterization of the V119M and parental (NUKM) strains-***

The K_M and V_{Max} values were determined by direct fit of the data to the standard Michaelis-Menten equation. These parameters (Table 3.10) were used to construct the shown double-reciprocal plots of the parental strain and the Leigh mutants. ■, Parental (PSST) strain; ▲, mutant V119M (PSST).

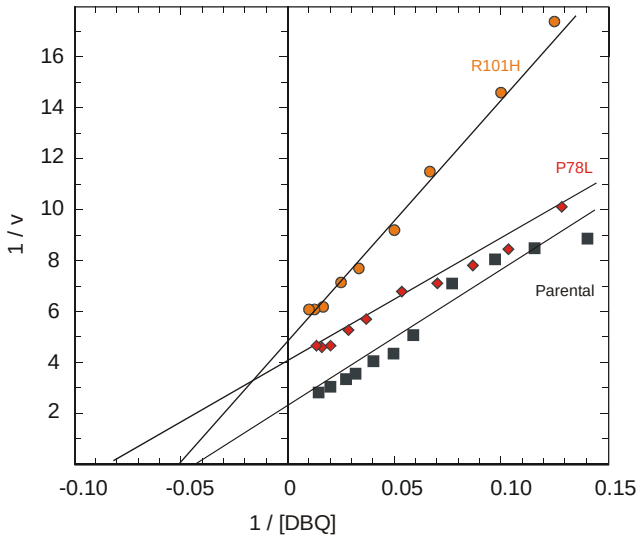

Figure 3.21 - ***Kinetic characterization of the P78L, R101H and parental (TYKY) strains*** -

The K_M and V_{Max} values were determined by direct fit of the data to the standard Michaelis-Menten equation. These parameters (Table 3.10) were used to construct the shown double-reciprocal plots of the parental strain and the three Leigh mutants. ■, Parental (TYKY) strain; ●, mutant R101H (TYKY), ♦, mutant P78L (TYKY).

The particular position of V119, separated by 30 and 32 amino acids from the closest conserved cysteine that could serve as ligands for iron-sulfur cluster N2 and located in a highly conserved region, posed the question about a possible interaction with cluster N2. In fact, the mutant had effects not only on catalytic activity (V_{Max} and K_M), but also on I_{50} for quinone analogous inhibitors like rotenone and DQA.

For analysing possible effects of the mutation on cluster N2, complex I was isolated from mutant mitochondrial membranes by ion exchange and size exclusion chromatography (2.3.9b). Enzyme purification from the mutant strain gave a yield of 8% whereas the normal yield for the parental strain enzyme obtained by the same procedure was about 10%. The EPR spectra from mutant and parental strains are shown in figure 3.22.

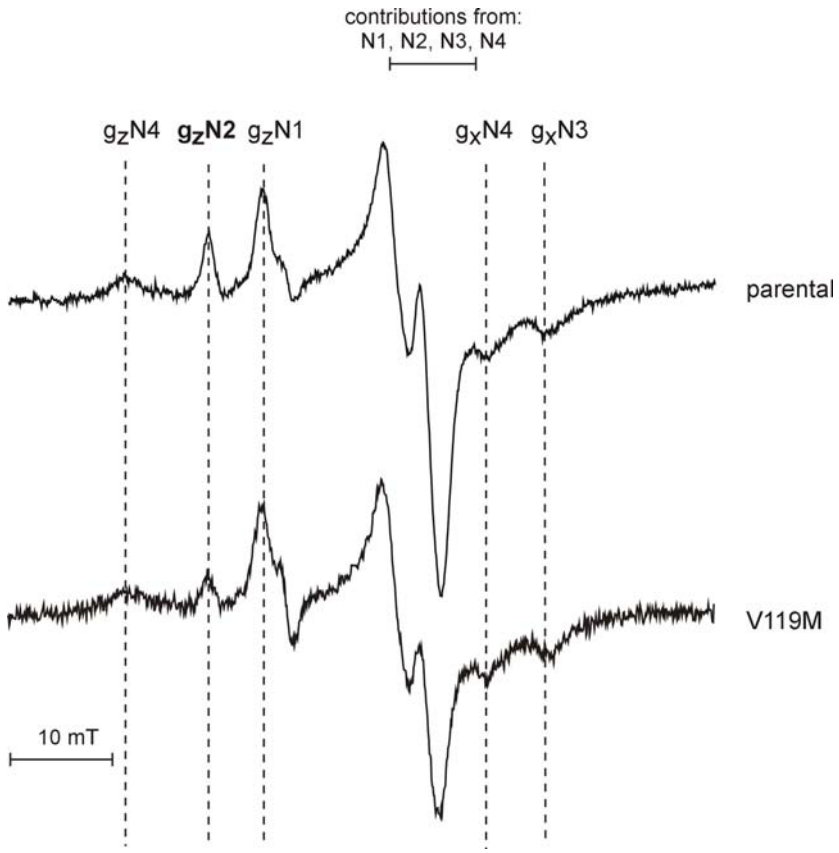

Figure 3.22 ***-EPR spectra of isolated complex I from the parental (*PSST*) and the V119M mutant strains reduced by NADH-***

Dotted lines indicate field positions for characteristic g values of individual clusters: g_zN4, 2.104; g_zN2; 2.051; g_zN1, 2.018; g_xN4, 1.892; g_xN3, 1.861. EPR conditions: microwave frequency 9.475 GHz, modulation amplitude 0.64 mT, microwave power 1 mW, temperature 12 K.

To compensate for different protein concentrations of the samples, spectra were scaled to the g_x signals intensities of clusters N3, N4 and the g_z intensity of cluster N4. The spectrum of V119M did not show shifts of the g values of the EPR detectable clusters. Also, the intensities of the signals of clusters N1, N3 and N4 were unchanged in the mutant sample. By contrast, the amplitude of N2 signal in V119M mutant complex I was decreased by about 40%, when compared to the amplitude of the g_z N2 signal in complex I from the parental strain. Summarizing, mutation V119M showed a reduced N2 content in isolated enzyme, whereas the spectra of all other visible clusters were essentially unchanged compared to the parental strain.

3.2 Construction of a Δ*nukm*-Δ*nucm* Strain

To obtain a strain with deletions of the genes for both PSST (*NUKM*) and 49 kDa (*NUCM*) subunits, two haploid *Y. lipolytica* strains were mated: *nucm*::*URA3*, *Mat* A, *ndh2i*, *30Htg*, lys11-23, ura3-302, xpr2-322, leu$^-$, his$^-$ and *nukm*::*LEU2*, *Mat* B, *ndh2i*, *30Htg*, leu2-270, xpr2-322, ura$^-$, lys$^-$. After sporulation haploid strains carrying the appropriate genetic markers were selected.

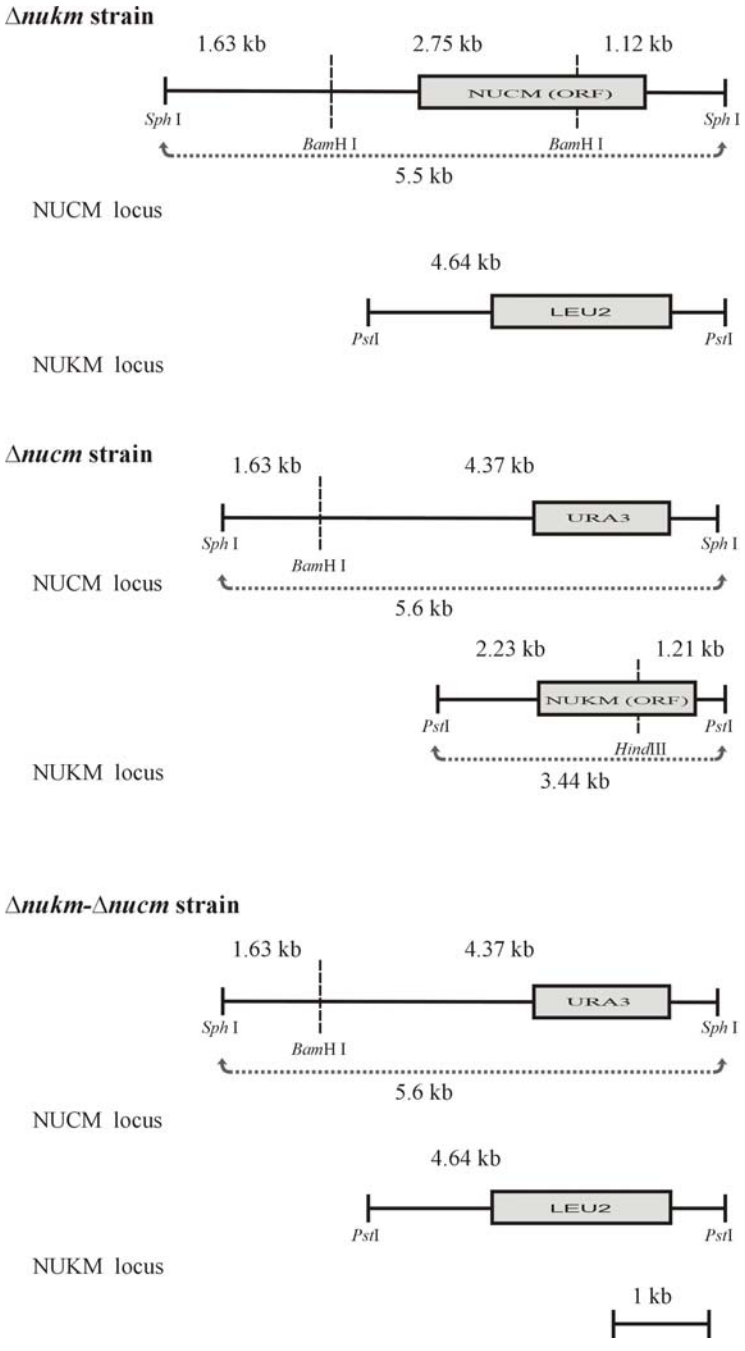

Figure 3.23 ***–Strategy to obtain a Δnukm-Δnucm strain-***

Strains Δ*nucm* and Δ*nukm* were mated and sporulated. The desired double deletion mutant Δ*nucm*-Δ*nukm* should contain both *URA3* and *LEU2* alleles.
Open Reading Fream (ORF) of *NUCM* and *NUKM* genes have been partially substituted by URA3 and LEU2, respectively (Ahlers et al., 2000b).

Different spores were investigated by PCR for deletion of both genes. Primers SAPA seq (5´- CCC TGA CCA CTC TGG ATG C -3´) and PSST mut (5´- GTT TCG CAT CTT TCG CTG G -3´) were used for the *NUKM* gene and primers 49i2 (5´- GCG TAC GTG CGA CCT GGA -3´) and 49i1 (5´- GCC TCC ATA TCC TCC –3´) for the *NUCM* gene. A 450 bp PCR product for both genes was visible only for the wild type strain, which was used as a control. Southern Blot confirmed results obtained by PCR. Genomic DNA from spores and WT was digested with *Hind*III. The Southern Blot probe used was the 3.44 kb *Pst*1 fragment from the NUKM locus, which gave two bands for the WT (2.23 kb and 1.21 kb, as shown in figure 3.23). Strains Δ*nukm* and the eight spores analysed did not shown the characteristic wild type bands. To detect the *NUCM* gene genomic DNA from spores and WT was digested with *Bam*HI. The Southern Blot probe used was the 5.5 kb *Sph*I fragment from the NUCM locus, which gave three bands for the WT (1.63 kb, 2.75 and 1.12 kb, Fig. 3.23). Strains Δ*nucm* and the eight spores analysed (AGΔKC 1-8) did not shown the characteristic wild type bands.
The spore AGΔKC1 was grown for preparing mitochondrial membranes: the measured NADH:HAR activity was 0.2 U/mg, suggesting that no assembled complex I was present. NADH:HAR and dNADH:DBQ activities of the plasmid complemented parental strain (*NUCM-NUKM* wild type copy carried on the pUB4 plasmid in the AGΔKC strain) were comparable to the wild type activities, as reported in table 3.11.

Strain	Complex I content (%)[*2]	dNADH:DBQ[*1] U/mg
E150	100	0.35
AGΔKC1	20	-
Parental[*]	80	0,47

Table 3.11 ***-Characterization of the E150 (Wild Type), AGΔKC1 and parental (NUCM-NUKM) strains-***

*[1]To account for variations in complex I content in different batches of mitochondrial membranes, dNADH:DBQ activity was normalized to complex I content expressed as specific NADH:HAR activity.

*[2]Complex I content in mitochondrial membranes was extimated by measuring the dNADH:HAR activity that is not affected by mutations in the NUKM subunit (100% = 1.0 μmol min^{-1} mg^{-1}).

3.3 Realisation of a pICL1-NDH2i Construct

The NDH2i, oxidizing matrix NADH and transferring electrons into the respiratory chain quinone pool, can rescue the NADH:oxidoreductase function of complex I (see 1.3.2). In this part of the work, a construct in which the NDH2i open reading frame is fused with the promoter for the isocitrate lyase (pICL1-NDH2i) was realised as described below. The pICL1-NDH2i construct should allow to conveniently switch between complex I dependent (glucose as sole carbon source) and independent (acetate as sole carbon source) growth, for selecting complex I activity deficient mutants (1.6.2).

A pBluescriptSK$^-$ derivate, the pUB5 plasmid, was used as starting clone. It contains a 4.7 kb *Sal*I fragment that consists of the NUAM (75 kDa subunit) promoter, the NUAM pre-sequence (1-34), the truncated (101-582) version of the NDH2 open reading frame, and 3´sequence from the NUAM locus, as shown in figure 3.24A (Kerscher et al., 2001a). The ATG codon underlined in figure 3.24A is the start codon for the NUAM presequence (cf. appendix 9.4). The promoter for the NUAM subunit was deleted by inverse PCR using primers 75AGf (5´-CTC TCG AGA AAC CTC AGC AAG-3´) and 75AGr (5´-CAT CAG GAA GAG GAG TGT CC-3´), whose position is shown in the figure. Both primers were designed for deleting the NUAM promoter, but not the NUAM 1-34 import-sequence, and also the *Sal* I site located upstream of the NUAM promoter was conserved. The pICL1 promoter (shown in figure 3.24B) was amplified from plasmid pINA354b (kind gift of Prof. Barth) using primers pICL1f1 (5´-AAG TGC GGC CGC GGT ACC G-3´) and pICL1r (5´-CAC TGG GTT AGT ACG GGA C-3´), whose position is shown in the figure. The intron is necessary for the functionality of this promoter: the first methionine of the NUAM import-sequence is coded by the triplet ATG (underline in Fig. 3.24A). The pICL1 intron interrupts this triplet, but still allows the expression of the methionine (Fig. 3.24B). Cloning the pICL1 in front of the NUAM pre-

sequence deleting the NUAM promoter resulted in the desired regulation of the NDH2i under the control of the pICL1 (Fig. 3.25).

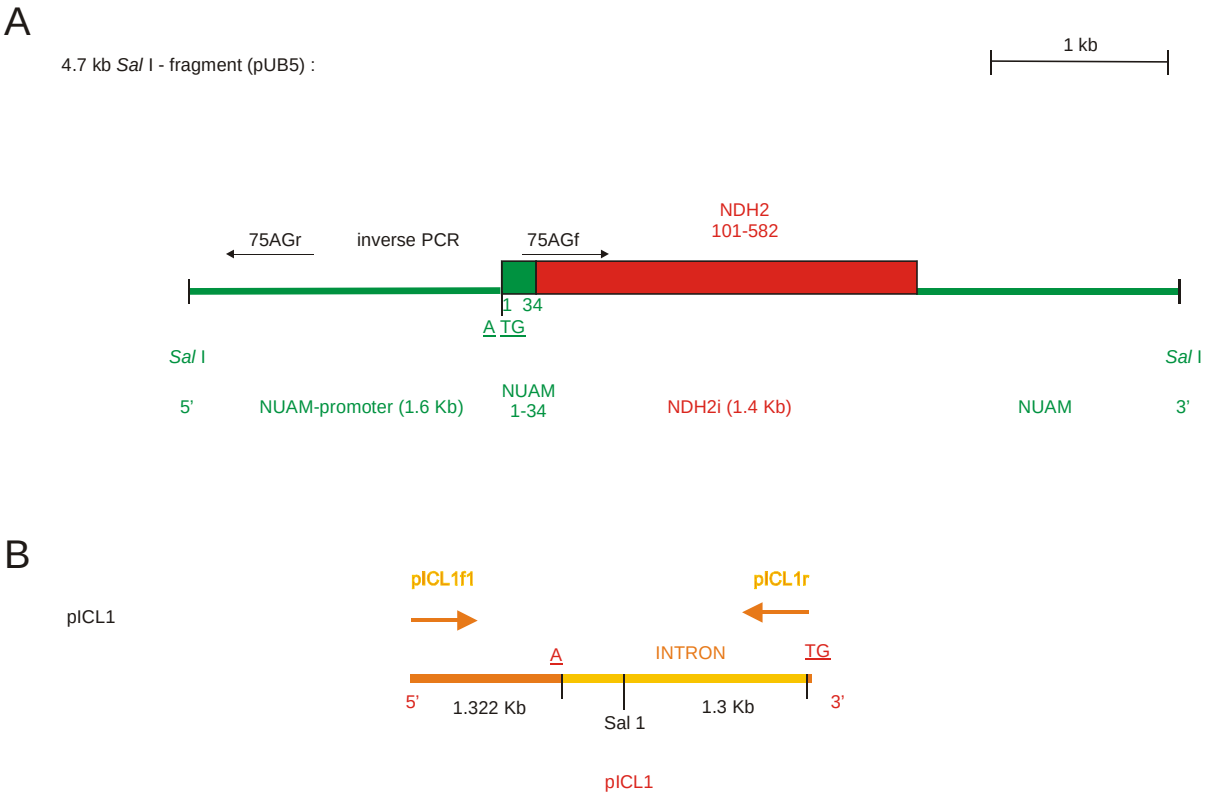

Figure 3.24 ***-Subcloning strategy for the pICL1-NDH2i construct -***

A. *Sal*I fragment of the replicative vector pUB5. Arrows indicate the approximate position and direction of the primers.

B. PCR product derived from plasmid pINA 354b. Arrows indicate the approximate position and direction of the primers used.

Primers pICL1f1 and pICL1r were phosphorylated at the 5´ ends (see. 2.2.4) to provide a blunt-ends ligation between the amplified pICL1 fragment and the product of the inverse PCR. Figure 3.25 shows the obtained ligation product (pUB5S) in its linear form.

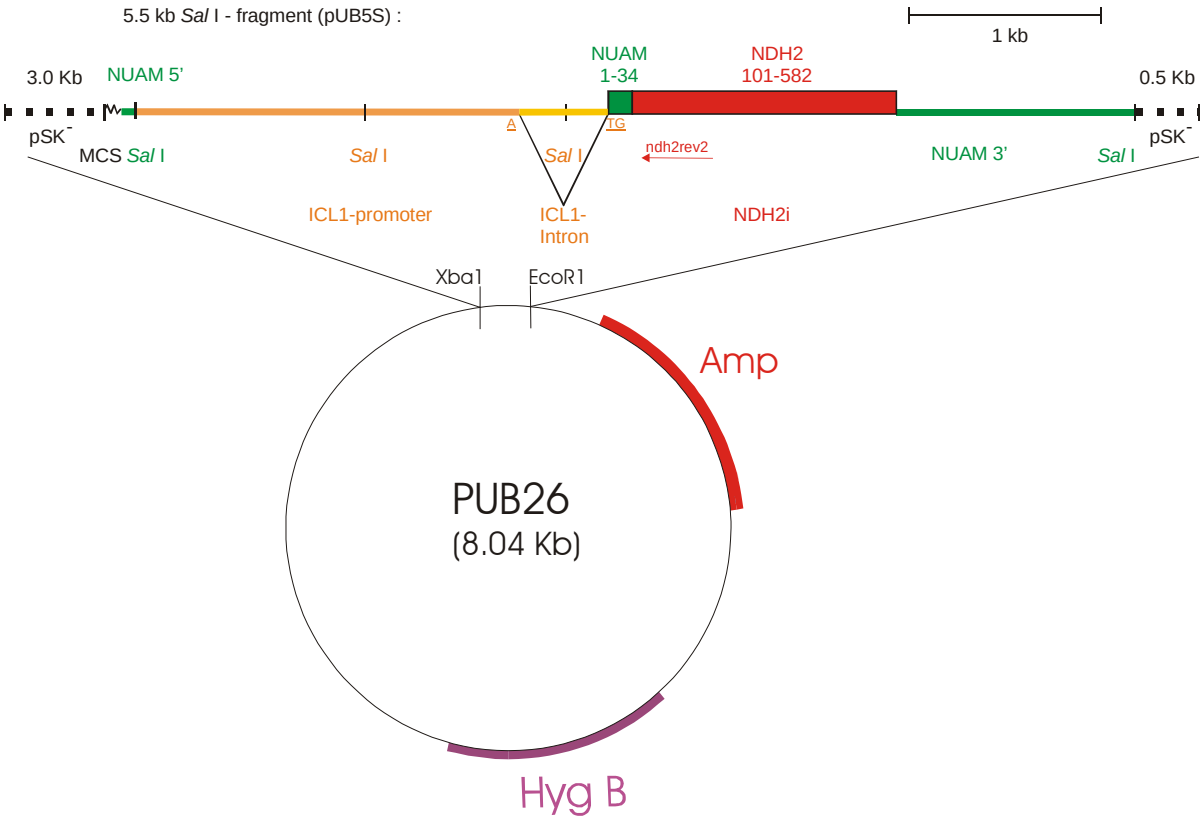

Figure 3.25 ***-Subcloning strategy used for making pUB26SC plasmid–***

A 7.2 kb fragment from pUB5S was cloned into pUB26. *Dotted lines* were not drawn to scale. An arrow indicates the approximate position and direction of primer ndh2rev2. *Sal*I sites are indicated.

Plasmid pUB5S contains the pICL1 sequence that is blunt-end ligated to the presequence (import sequence) of the NUAM subunit of complex I (amino acid 1 to 34). This is fused to the truncated version of the NDH2 open reading frame (from amino acid 101 to 582). Different clones were tested by *Sal*I digestion for selecting the right ligation products, and the correct orientation of pICL1 was verified by sequencing using the primer ndh2rev2 (5´-GTG TCA ATG GTT CCG GTG- 3´), which position is reported in Fig. 3.25. The sequence of the shown 5.2 kb *Sal*I fragment is reported in the appendix 9.4. A 7.2 kb pUB5S fragment was cloned into plasmid pUB26 (see 2.1.5) by cutting with *Nhe*I and *Eco*RI. PUB26 contains the Amp^R gene, the $HygB^R$ gene and a unique *Nhe*I site (compatible with *Xba*I site). The subcloning strategy is shown in figure 3.25. The resulting plasmid (pUB26SC) was amplified in *E. coli* and transformed into *Y. lipolytica*, as described below.

3.3.1 Function of the pICL1-NDH2i Construct in Vivo

Plasmid pUB26SC was transformed into strain PIPO (see 2.1.4). Transformants were selected for growth on YPAc (see 2.1.3) agar medium with hygromycin B. Subsequently, the ability of NDH2i to rescue NADH oxidation in the mitochondrial matrix and electron transfer into the respiratory chain quinone pool was checked by inhibition of complex I with increasing concentrations of 2-decyl-4-quinazolinyl amine (DQA) in the presence of acetate or glucose. After growth in glucose or in acetate minimal media (both adjusted to pH 7.0), cells were plated on the appropriate agar medium. Plates were prepared with glucose or acetate as sole carbon source (pH 7.0) and different concentration of DQA (0-10 µM) as selective inhibitor for complex I. Concentrations of DQA higher than 1.5 µM inhibited growth of cells on glucose, but not on acetate, as shown in figure 3.26. From these data it can be concluded that NDH2i is under the substrate dependent control of the ICL1 promoter.

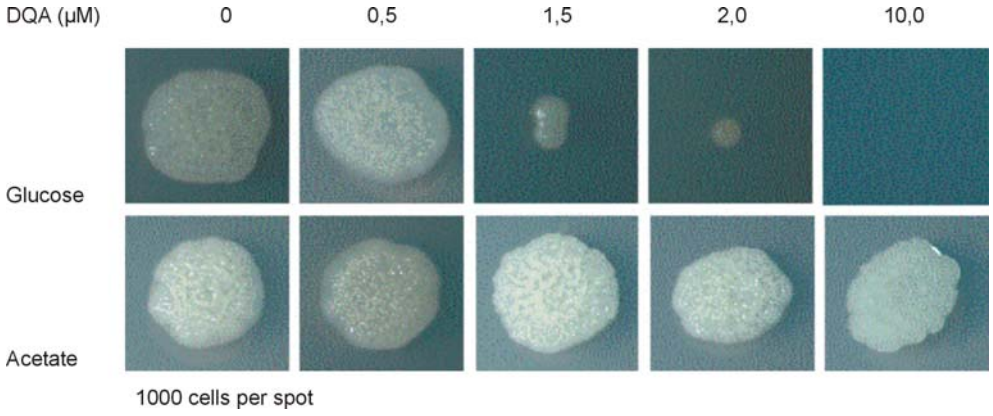

Figure 3.26 ***-PIPO strain complemented with pUB26SC on glucose and acetate as sole carbon source-***

NDH2i is under the control of pICL1. DQA concentrations > 1.5 µM inhibit complex I. The pICL1-NDH2i gene functionally replaces complex I activity on acetate, whereas on glucose complex I inhibition is not compatible with survival of *Y. lipolytica*.

4 DISCUSSION

4.1 Site Directed Mutagenesis of Conserved Cysteines in the TYKY and PSST Subunits of Complex I

The exact path of electron transport from NADH to ubiquinone in complex I is still unknown: the number and location of iron-sulfur clusters are matters of discussion. The tetranuclear iron-sulfur cluster N2 is considered to be one of the central players for understanding the complicated complex I machinery (see 1.2.3). At the beginning of this work, subunits PSST and TYKY were the most likely candidates for harbouring this cluster.

In the present work, a mutagenesis study has been carried out on all the twelve conserved cysteines that form the three binding motifs for iron-sulfur clusters in subunits PSST and TYKY (see 1.2.2). Mitochondrial membranes from strains Δ*nukm*, Δ*nuim* and cysteine mutants in both subunits were analysed by Blue Native Page: no complex I band was visible, whereas the bands of complex V and III of the OXPHOS could be identified. This result was also confirmed by dNADH:HAR activity measurement: only 0.2-0.3 U/mg was the observed activity in these strains (see 3.1), whereas the activity of the parental strain was about 1 U/mg. From these results it can be concluded that the deletion of subunits PSST and TYKY and the mutagenesis of any cysteine residue in the conserved motifs for tetranuclear iron-sulfur clusters affected the stability of complex I. The interpretation of the residual dNADH:HAR activity in the membrane preparations from these strains is unclear. From these experiments is not possible to establish if the residual 0.2-0.3 U/mg of activity are due to other membrane proteins, to complex I fragments (of the peripheral arm, containing the 51 kDa subunit and FMN, cf. 2.3.7), or to a small amount of assembled complex I which is not detectable by BNP. Consequently, failure to detect mutant complex I could be ascribed to misfolding or to structural destabilisation caused by the mutations. Assuming that mutations destabilise protein folding, one would expect a small amount of enzyme in the mutant membrane preparations. Along the same line, it is reasonable to consider that detergent solubilisation by 1g/g LM, which works efficiently for parental strain complex I, would not be gentle enough for the "less stable" mutant complexes. For detecting small amounts of complex I in membranes avoiding the solubilisation step, mitochondrial membranes from mutant and parental strains reduced with NADH were analysed by EPR spectroscopy. None of the cysteine-alanine mutants in subunit PSST showed any signal that could be assigned to a complex I cluster. It follows that the residual dNADH:HAR activity measured from these membranes was either

due to small fragments of complex I (most likely containing the FMN and the 51 kDa subunit and no EPR visible clusters), or is due to the activity of other membrane proteins. On the basis of these results it can be concluded that there is no relevant quantity of assembled complex I in the mitochondrial membranes of strains in which the putative ligands of cluster N2 were mutagenised.

In an effort to selectively modify the microenvironment of the cluster N2, maintaining its structure intact, more conservative mutations have been carried out. By changing each single cysteine into serine, the function of the thiol group of cysteine was replaced with a hydroxyl group of a serine side chain, thereby providing an oxygen ligand for the iron ions of the cluster. Again, assembled complex I could be not detected in mitochondrial membranes of Cys-Ser mutants. From these data it is not possible to conclude unambiguously if the above mentioned amino acids are the ligands. In fact, it is plausible to reason that the mutagenised cysteines play an important role for complex I stability, or that formation of the affected iron-sulfur cluster is a prerequisite for complex I assembly in *Y. lipolytica*.

In the last four years, mutagenesis studies have been carried out in *Rhodobacter capsulatus*, *N. crassa* and *E. coli* (summarised in appendix 9.3). The results obtained from mutagenesis in *N. crassa* agree with the results of the present work. Three cysteines, corresponding to C113, C152 and C155 in the TYKY subunit from *Y. lipolytica*, were mutagenised into alanine or serine: no assembled complex I was found in membrane preparations of the mutant strains. The authors concluded that the TYKY subunit and most likely the iron sulfur clusters are important elements for assembly of complex I (Duarte and Videira, 2000). In contrast, mutagenesis of the same residues in the *Rh. capsulatus* TYKY homologous subunit (corresponding to C113 and C152 in *Y. lipolytica*) gave an assembled complex I, which showed altered N2 signals in EPR spectroscopy and in which content and catalytic activity were lower than in the parental strain. On the basis of these results, authors concluded that there are two N2 type clusters in TYKY (Chevallet et al., 2003). In strong contradiction to this work, a combination of UV/vis and EPR spectroscopy study in the TYKY homologous subunit detected two novel 4Fe-4S redox groups named $N6_a$ and $N6_b$ (Friedrich et al., 2000), which are invisible under standard EPR conditions.

More recently, all eight conserved cysteines in the TYKY homologous subunit from *E. coli* were mutagenised into cysteines or alanines. In contrast to eucaryotic organisms like *N. crassa* and *Y. lipolytica*, and similar as in *Rh. capsulatus*, the bacterial complex I from *E. coli* was assembled but did not show any catalytic activity (for an overview about mutagenesis of subunits PSST and TYKY of complex I from different organisms and relative references, see

appendix 9.3). The complex I content in the membrane preparations from both bacterial organisms was significantly decreased, suggesting a somehow compromised protein stability. Enzyme preparation of one mutant from *E. coli* was unstable and aggregated upon concentration and could therefore not be used for EPR spectroscopy. This observation corroborates the theory that these cysteines, and most likely the iron-sulfur clusters, play an important role for complex I stability (Flemming et al., 2003).

In the same work all four conserved cysteines in the PSST subunit were also mutagenised into alanine or serine. Mutants showed an assembled complex I (unable to transport electrons to quinone) whose EPR properties were changed. The conclusion of this work was that cluster N2 is located in the PSST subunit. However, it is still unclear whether both adjacent cysteines in PSST are ligands or if the fourth ligand has still to be identified (Flemming et al., 2003). These data agree with data from a previous work in *N. crassa*: cysteines 85 and 86 (in subunit PSST) were changed into serine and alanine, respectively. Mutants were assembled, complex I content in membranes was comparable to the parental strain and the specific EPR signal of cluster N2 was absent (Duarte et al., 2002). Yield and purity of these mutant complex I preparations were lower when compared to the parental one and no specific N2 signal, recorded under standard condition, was identified from these preparations.

Thus, the stability of cysteine-mutated complex I seems to depend on the organism. The data obtained from the experiments presented in this work and from the literature do not allow a satisfactory explanation for these phenomena. Nevertheless these results stress the important role of TYKY and PSST subunits for folding and assembly of complex I.

4.1.2 Site Directed Mutagenesis of Conserved Acidic Residues in the NUKM Subunit

To obtain more information about the role of the conserved amino acid in the PSST subunit and to search for the still unknown fourth ligand of iron-sulfur cluster N2, mutants of the highly conserved acidic residues in the PSST homologous NUKM protein from *Y. lipolytica* were constructed and characterized. These results, in addition to the data of a previous work in *Y. lipolytica* (Ahlers et al., 2000b), constitute complete mutagenesis study on the conserved acidic residues in this subunit and led to the identification of functionally important amino acids.

EPR spectra of complex I from strains mutated in positions E89, D99, D115, D136, E140, D168, D174 and E185 showed normal or in one case (E89) somewhat shifted g_z signals of iron-sulfur cluster N2. The slightly shifted N2 signals in mutants E89C, E89A and E89Q were

interpreted as a consequence of an altered electromagnetic environment of the cluster. On the basis of these results, residue E89 is most likely located in the vicinity of cluster N2, but definitely can be excluded as a ligand (Ahlers et al., 2000b).

Two aspartates, Asp-99 and Asp-115, were found to be essential for complex I catalytic activity, but in contrast to mutant E89, the N2 g_z positions of D99N and D115N were not shifted. This indicated that the geometry of the cluster was not substantially changed. On the other hand, the intensity of the g_z signal was reduced by about 50% in both mutants. These results can be explained by a decreased protein stability, which became more evident from lower yields during protein purification and may have resulted in a partial loss of iron-sulfur cluster N2, or in a modified redox midpoint potential of the cluster. A putative formation of hydrogen bonds by Asp-99 and/or Asp-115 to the cysteinyl sulfur of one ligand of cluster N2, thereby influencing the ability of the cluster to accept electrons (defined by the redox midpoint potential), is a possible explanation for the reduced intensity of the N2 signal observed in EPR spectra of both D99N and D115N mutants. The resulting shifted redox potential would not allow the complete reduction of clusters N2 in the EPR sample. This could be excluded by titrating the redox potential and monitoring the reduction of N2 by EPR spectroscopy. These titrations demonstrated that the redox midpoint potential of this cluster was unchanged when compared to membranes of the parental strain (cf. 3.1.2). This also indicated that the electron transfer to N2 was not blocked and implied that the loss of catalytic activity, as well as the reduction of the g_z signal intensities, were not due to a change in the redox properties of the cluster. Consequently, the modified N2 signal intensity is most likely due to a destabilisation effect caused by the mutations.

One explanation for the loss of dNADH:DBQ activity caused by removing the carboxylic groups in positions 99 and 115 could be their role as proton donor/acceptors. In fact, two protons are required for the reduction of ubiquinone to ubihydroquinone. Assuming the hypothesis that both carboxylic groups act as proton acceptor-donor in the ubiquinone oxidoreduction process, the carboxylic groups were conserved introducing an additional methylene group by changing the aspartates to glutamates. The complete loss of ubiquinone reductase activity in both D99E and D115E lowered this theory. Analysis of purified complex I from mutant and parental strains by FTIR (Fourier Transform Infrared) spectroscopy, a technique that can detect whether an acidic residue is in the protonated or no-protonated state during the oxidoreduction, are currently under way in the group of Professor Peter Rich (London, UK). Also removing the side chain completely by changing the aspartates into glycines had the same effects as the more conservative mutations. Mitochondrial membranes

from all six mutants contained virtually normal amounts of complex I: even the rather drastic exchange of each of the aspartates to glycine still allowed normal assembly of complex I and its residual activity was as low as for the more conservative exchanges. The very similar effect of rather different amino acid exchanges in these two positions makes it tempting to speculate that in all cases a similar local change of a structural configuration occurred and that the resulting fold was incompatible with ubiquinone reductase activity of complex I. Further experiments will be required for checking this hypothesis (see 5). From these data it can be concluded that D99 and D115 play a vital role for complex I NADH:ubiquinone reductase activity, but are not ligands for N2 cluster. In addition, their position seems to be not close enough to the cluster to influence directly its electromagnetic environment.

In contrast to aspartates 99 and 115 in the PSST subunit, changing other acidic amino acids that are fully conserved between complex I from different organisms but not between complex I and [Ni-Fe] hydrogenases, had only moderate effects on NADH:ubiquinone oxidoreductase activity or on inhibitor resistance (Fig. 4.1A). Results from mutagenesis that have been carried out until now in *Y. lipolytica* are briefly summarised below.

Mutations D136N and E140Q have significant effects on inhibitor binding, whereas changing asparagine 168 to the corresponding amide had little or no effect on inhibitor resistance (Ahlers et al., 2000b). Aspartate 174, when changed to its corresponding amide, had little or no effect on the Michaelis-Menten parameters and the N2 EPR signal from the membrane did not show any difference when compared to the parental strain. Mutant E185Q showed a significantly reduced V_{Max}, but EPR spectra of isolated complex I showed unequivocally that not only the specific N2 signal, but also the signals of the other iron-sulfur clusters were not different from those of the parental strain. This definitely excludes a role of this residue as ligand of cluster N2. Both amino acid residues are located in the vicinity of C180, one of the candidates for ligating this cluster (Fig. 4.1A). The modification of these positions, changing the carboxylic groups of the side chains into amide groups, did not have any consequence on the micro-environment of the N2 cluster. Considering the result obtained from changing glutamate 89 into glutamine (see 1.3.4), it can be concluded that both residues D174 and E185 are not ligands of cluster N2, even though their positions in the primary structure suggest them as possible fourth ligand. A more detailed interpretation of the observed effects of the mutations on complex I catalytic activity and inhibitor sensitivity is not possible without a high-resolution structure of complex I. Nevertheless, looking at the alignment between the small subunit of the [Ni-Fe] hydrogenase from *D. fructosovorans* and the PSST subunit from complex I (Fig. 4.1A), and using the X-ray structure of the [Ni-Fe] hydrogenase as a model,

preliminary conclusions can be drawn on the basis of the homology between the two enzyme classes (see below).

4.1.3 The Structure of [Ni-Fe] Hydrogenases as a Model for Complex I

Structures of homologous proteins can be helpful for understanding the machinery of complex I. In particular, the molecular structure of the water soluble [Ni-Fe] hydrogenases from *D. fructosovorans* offers a useful model for studying subunits PSST and 49 kDa. In fact, not only homology studies (cf. 1.2.4) but also experimental evidences (cf. 1.3.4), strongly suggest that both subunits are structurally and functionally connected, constituting part of the "module" which is responsible for the ubiquinone reductase activity and which has evolved from the hydrogenases structure (cf. 1.2.5).

A sequence alignment between the [Ni-Fe] hydrogenase small subunit from *D. fructosovorans* and the PSST homologous subunit from *Y. lipolytica* is shown in Fig. 4.1A. Sequence conservation is rather weak, but three of the four cysteines that ligate the proximal cluster in the small hydrogenase subunit are conserved in subunit PSST, allowing a reasonable alignment of the sequences. Figure 4.1B shows the X-ray structure of the [Ni-Fe] hydrogenase of *D. fructosovorans*. Subunit PSST is much smaller than the small hydrogenase subunit because the C-terminal half that forms the domain harbouring two of the three iron-sulfur clusters of the hydrogenase is missing (marked in grey) and the N-terminal part up to the first cysteine ligating cluster N2 is partially missing in the small subunit (marked in yellow). The domain marked in blue represents the region between cysteines 86 and 150: this region in complex I is shortened by 30 amino acids in comparison to the corresponding region of the small hydrogenase subunit. Changing glutamate 89 in *Y. lipolytica* PSST subunit has an effect on the N2 signal in EPR spectroscopy. In addition, the fully conserved position 99 is vital for complex I physiological activity. Also aspartate 115 was found to be essential for complex I activity, even if apparently this position is not fully conserved in the alignment in Fig. 4.1A. Looking at the sequence of the small subunit there is no amino acid corresponding to this position in the alignment, whereas the adjacent position is an aspartate. In addition, the two above mentioned aspartates are separated by 35 amino acids in the small subunit from *D. fructosovorans* whereas only 16 amino acids are present in PSST. It follows that the 35 amino acids which separate both positions form a connecting loop in the [Ni-Fe] hydrogenase of *D. fructosovorans*, which is supposed to be much smaller in complex I. The two aspartates D33

and D68 (supposed to be homologous to D99 and D115 in NUKM, respectively) are drawn as space fill models and the connecting loop in between is marked in grey (Fig. 4.1B).

All this suggests that the position of cluster N2 and the folding of its environment are essentially conserved, although the sequence homology between PSST and small subunit is rather weak.

A

```
                                 86  89          99                115  119                          136  140
yl      nukm    82   GLACCAVEMMHVSAPRYD        SPRQSD..IMIVAGSLTN    KMAPVLRQVYDQMPEPRWVIS
human   nukm    85   GLACCAVEMMHMAAPRYD        SPRQSD..VMIVAGSLTN    KMAPALRKVYDQMPEPRYVVS
ECHC            16   SCNGCDIEVVACLTPLYD        TPKQAD..IMVVTGSVNY    KNVNVLKNIYNQIPDPKVVLA
dfss            16   ECTGCTEAAIRTIKPKID        LEGKD.GYYLVVEGGLPT    AGHPMIETCKKAAAKAKGIIC
                        17  20          33            68

                                 150                                  174   180  185
yl      nukm   146                MGSCANGGGYYHFSYS..VVRGCDRIVPVDVY.VPGCPPTSEALMYG
human   nukm   149                MGSCANGGGYYHYSYS..VVRGCDRIVPVDIY.IPGCPPTAEALLYG
ECHC           92                 VGACASTGGIFHDCN...VIGGVDQVIPVDAY.VPGCCPRPEAILDG
dfss           111                IGTCSAYGGVQKAKPNPSQAKGVSEALGVKTINIPGCPPNPINFVGA
                                     114                                  147
```

B

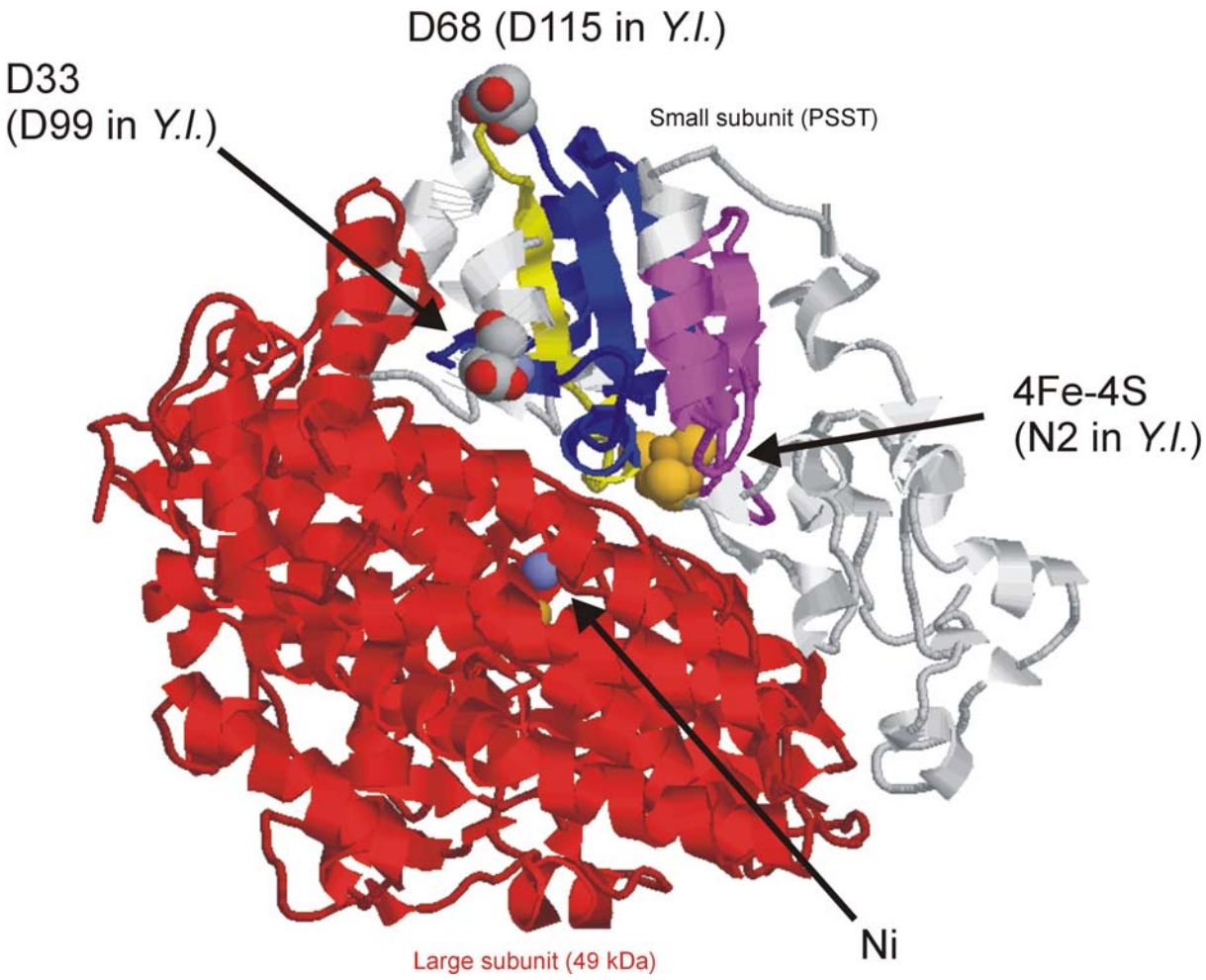

Figure 4.1 ***-Alignment between domains of the small subunit of [NiFe] hydrogenase and correspondent domains of subunit PSST of complex I-***

A Sequences of PSST homologous subunits of complex I from Yarrowia lipolytica (yl) and human (human) mitochondria have been aligned with the small subunits of [NiFe] hydrogenases from Methanosarcina barkeri (ECHE) and Desulfovibrio fructosovorans (dfss). C17, C20, C114, and C147 which ligate the proximal iron-sulfur cluster in hydrogenase and their homologues C86, C150, and C180 in Y. lipolytica are marked in red, conserved residues in yellow, similar residues in grey, and mutagenised residues which have been characterised in pink.

B Ribbon representation of the X-ray structure of the [Ni-Fe] hydrogenase. The domain marked in blue is the region between the second and third cysteine of the small subunit, magenta is the region downstream of the third cysteine which is retained in subunit PSST of complex I, grey is the C-terminal domain of the small subunit that carries two iron-sulfur clusters and that is missing in subunit PSST of complex I. Mutations in subunit PSST of complex I that have significant effects are clustered in the region corresponding to the domain shown in blue. The proximal iron-sulfur cluster is shown in yellow space fill representation. D33 and D68 (corresponding to D99 and D115 in complex I, respectively) are represented in space fill. The large subunit, homologous to the 49 kDa subunit in complex I, is marked in red. See text for detailed discussion.

A domain formed by three β-strands in the [Ni-Fe] hydrogenase secondary structure is shown in figure 4.2. Two of the three β-strands are predicted to be conserved in complex I folding (cf. 9.7): cysteine 114 (C150 in *Y.l.*), V74 (V119 in *Y.l.*) and D68 (D115 in *Y.l.*) belong to this conserved domain (Fig. 4.2).

With respect to this assumption, all the fully conserved residues that have been mutagenised showed dramatic effects on complex I, in terms of assembly (cysteine mutants) or catalytic activity (D99-D115). Mutant V119M, which also affects a residue fully conserved in the alignment shown in figure 4.1A, showed 50% reduction of V_{Max} *in vitro* when mitochondrial membranes were prepared from *Y. lipolytica* and had a lethal effect in two patients that were homozygous for this mutation.

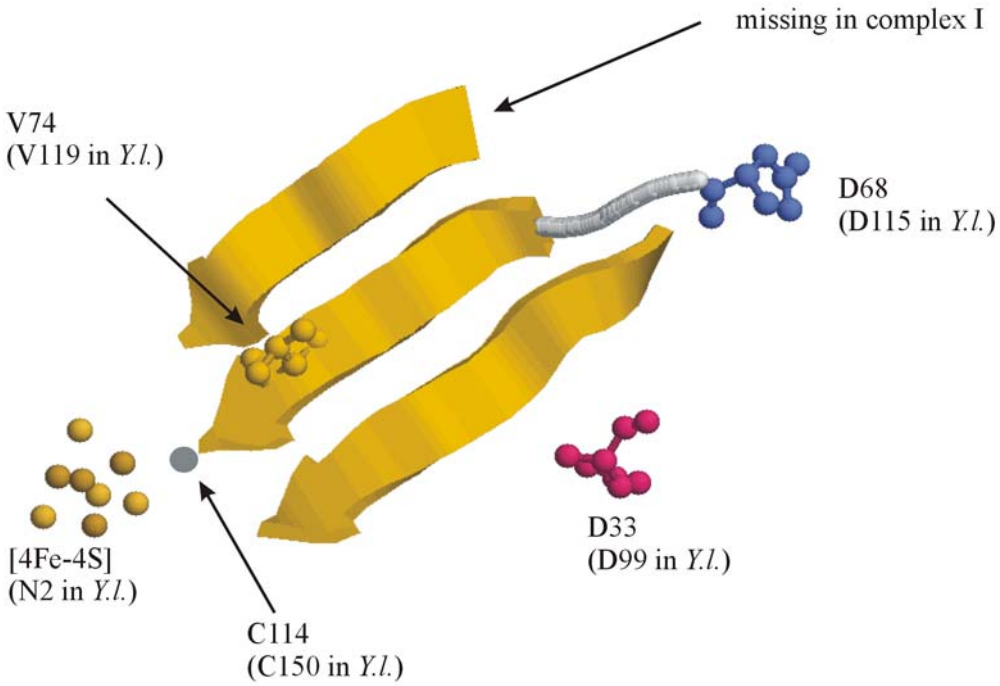

Figure 4.2 ***-A domain in the small subunit of [Ni-Fe] hydrogenase corresponds to a functional relevant domain within subunit PSST-***

The shown domain formed by 3 β-strands is predicted (by the software "PROF") to be partially conserved in complex I secondary structure (bottom two β-strands in the figure). Position corresponding to mutagenised residues in the PSST subunit of *Y. lipolytica* are indicated.

Mutations at positions E89, D136, E140, D174 and E185, which are conserved between complex I subunits of eucaryotes but not between complex I and [Ni-Fe] hydrogenases, showed milder effects.

In the 49 kDa subunit of *Y. lipolytica*, two strictly conserved histidines (91 and 95) have been mutagenised. Likely as D99 and D115, both of them were found to be essential for complex I NADH:oxidoreductase activity (Grgic et al., 2004), whereas they are not conserved in the hydrogenases from *D. fructosovorans* (only histidine 91 is conserved in the [Ni-Fe] hydrogenase from *M. barkeri*). Replacing each histidine by alanine causes a drastic reduction (~95%) of the complex I catalytic activity but had no effect on complex I content in the membrane and on the EPR signals of the iron-sulfur clusters. In the hydrogenase structure, both residues are near (10-20 Å) to the proximal iron-sulfur cluster and the prosthetic Ni. Histidine 228 in the large subunit of the [Ni-Fe] hydrogenase from *D. fructosovorans*, which has a position near to both Ni site and iron-sulfur cluster (~10 Å and 6 Å, respectively), is

conserved between complex I and [Ni-Fe] hydrogenases. Mutant H226A in *Y. lipolytica* (Kashani-Poor et al., 2001b) showed 25% of the wild type catalytic activity and no N2 signal in EPR spectroscopy, indicating that the mutation in the 49 kDa subunit has an effect on cluster N2. These results support the suggestion that the "catalytic core" (N2 cluster and quinone binding site) of complex I has been evolved from the electron transfer module of the [Ni-Fe] hydrogenases. Aspartates 99 and 115, being somehow necessary for the electron transport from N2 to quinone, directed the attention to a conserved region in the PSST subunit, which is predicted to be located at the interface of two key subunits.

4.1.4 *Yarrowia lipolytica* as a Eucaryotic Model to Analyse Leigh Syndrome Mutations in the Complex I Subunits PSST and TYKY

The effects of three mutations identified in the PSST and TYKY homologous subunits of patients affected with Leigh syndrome were reconstructed in the obligate aerobic yeast *Y. lipolytica* (cf. 1.4 and 3.1.3). This approach may help to understand the aetiology of the Leigh syndrome, in terms of the ability of complex I to oxidize NADH and to transport electrons (cf. 1.6). The kinetics of mutant complexes was analysed in vitro with mitochondrial membranes of each mutant strain. The purpose of these experiments was to detect alterations in the NADH:ubiquinone oxidoreductase activity of complex I. In fact, the mutations are located in two subunits that belong to the "ubiquinone reduction" module of complex I. All three mutations showed effects on electron transport: V_{Max} was reduced by about 50% in all three mutants (Table 3.6). The NADH:HAR activity of V119M and R101H was comparable to the parental strain implying a normal complex I content in the membranes. On the other hand, P78L showed a significant reduction in NADH:HAR activity (~60% of the parental strain activity, Table 3.10). The lower complex I content in the membrane was most likely due to a somehow reduced stability of the enzyme: as complex I is essential for survival of *Y. lipolytica* its expression cannot be selectively down regulated. Since the TYKY subunit is not directly involved in NADH oxidation, it can be also excluded that this mutation has some effects on the NADH:HAR activity (cf. 2.3.7). Consequently, it can be speculated that the proline has an important role in the secondary structure of the protein, and that a lysine is not able to fulfill its role. The previously described hypothesis (3.1.1) that the TYKY subunit may play an important structural role was supported by a reduced complex I content in the membrane preparation caused from the change of a single conserved residue. In addition, the inhibitory potency of the ubiquinone analogues DQA and rotenone was also altered in all

three mutant strains. These facts suggested a somehow modified interaction between the ubiquinone binding site and substrates and/or inhibitors of complex I.

Residue V119 is located in a high conserved region of eucaryotic PSST subunits, which is also highly conserved when procaryotes are included into the alignment (Fig. 3.19). Position D115, which has been demonstrated to play a vital role for complex I catalytic activity, is only four residues away. Investigating the effect of this Leigh mutation on the microenvironment of the N2 cluster, complex I from mutant V119M was isolated and the EPR spectrum was recorded. No significant differences were noticed comparing this spectrum to the parental one, except for a reduction of the N2 signals (Fig. 3.22). The unshifted N2 g_z signal excluded definitely a modification of the geometry of this cluster. The interpretation of the about 40% reduction of the g_z signal intensity is more ambiguous. Differently to D99N and D115N, mutating valine 119 into methionine no carboxylic group able to form hydrogen bonds to the cluster had been removed. Therefore, the most plausible explanation of the reduced g_z signal intensity in the V119M spectrum is a folding destabilization due to the mutation.

4.2 The pICL1-NDH2i Construct Rescues Complex I Deficiency Selectively on Acetate (or Ethanol) Synthetic Medium

Recently *Y. lipolytica* has been developed as a yeast genetic system to study mitochondrial complex I (for an overview see Kerscher et al., 2002).

The present work introduced the promoter for the Isocitrate lyase (pICL1) as a useful tool for the substrate selective expression of the internal version of the alternative NADH:ubiquinone oxidoreductase (NDH2i under the control of the pICL1), which allows the "*in vivo*" rescue of complex I deficiency selectively on acetate (or ethanol) medium (cf. 3.3.1).

The integration of the pICL1-NDH2i construct into the genome of *Y. lipolytica* deletion strains for nuclear-coded subunits (like Δ*nukm*, Δ*nucm* and Δ*nuim*) would contribute to develop *Y. lipolytica* as a useful genetic device for studying mitochondrial complex I (cf. 5.2). This strategy presents the advantage to produce a high number of point mutations in a specific subunit, by using a "degenerate PCR" technique. Transformation of these products into the complex I subunit deletion strains, which carry the pICL1-NDH2i construct integrated into the genome, would allow the screening of the resulting transformants by simply "switching" between glucose and acetate as sole carbon source. In fact, mutant strains in which complex I activity is compromised by the mutation could be easily screened by plating mutants on

acetate (complex I independent growth) and on glucose (complex I dependent growth) media. In addition, resistance against complex I inhibitors could be screened in a similar way, by adding different concentrations of inhibitor to the agar media.

In an effort to understand the complex I mechanism without an X-ray structure, and in particularly being interested in the “ubiquinone reduction” module (PSST, TYKY, 49 kDa), the possibility to screen for a large number of activity deficient or inhibitor resistant point mutations following region specific random mutagenesis constitutes an attractive perspective for complex I research.

5 Outlook

5.1 Site Directed Mutagenesis of both 49 kDa and PSST Subunits of Complex I

Results from mutagenesis studies in both PSST and 49 kDa subunits pointed out that residues which are conserved between hydrogenases and complex I play a important role for the catalytic activity of this complex protein. At the time of writing this thesis, important information about structure and mechanism of complex I was still missing. Therefore an exhaustive interpretation of the mutagenesis results would be premature. However, assuming that the folding of the [Ni-Fe] hydrogenase from *D. fructosovorans* has been partially conserved during evolution helped to find a reasonable interpretation for experimental data. Using the X-ray structure of the [Ni-Fe] hydrogenase as a model, the aspartate 99 in PSST and the histidines 91 or 95 in the 49 kDa subunit are predicted to be located at the interface between the two proteins. Consequently, they can be speculated to form a specific ionic bond between the two subunits, which may play an important role in protein folding or enzyme activity. Analysis of double PSST/49 kDa-mutants, in which an aspartate in the PSST is changed into histidine and the histidine in 49 kDa subunit is changed into aspartate, will test this hypothesis. In this respect and in an effort to investigate the interaction between the two subunits, a Δ*nukm*/*nucm* (PSST/49 kDa) strain has been constructed (cf. 3.2).

5.2 Integration of the pICL1-NDH2i Construct into the Genome

Deletion strains of the subunits that form the "ubiquinone reduction" module (Δ*nukm,* Δ*nucm* or Δ*nuim*) containing a chromosomal-integrated copy of the pICL1-NDH2i construct would allow the analysis of plasmid encoded mutants of the corresponding deleted subunit. Furthermore, these strains could be used for mutant screens with complex I dependent versus independent growth, allowing the identification of essential amino acid residues or of inhibitor resistance mutations.

Two strategies for the integration of the pICL1-NDH2i construct could be proposed:

1. Integration of the pICL1-NDH2i into the *Y. lipolytica* genome and subsequent deletion of the desired subunit
2. Changing NDH2i with the pICL1-NDH2i by POP-IN-POP-OUT in each deletion strain (Kerscher et al., 2002).

The first strategy presents the advantage to require relatively short time and to give a strain that can be used as starting point for creating deletion strains of all seven nuclear coded subunits of complex I. The disadvantage is that the pICL1-NDH2i would be randomly integrated into the *Y. lipolytica* genome and the integrity and functionality of the resulting strain must be checked.

The second strategy offers the advantage to integrate the construct into the background of an existing and characterised locus of the NDH2i, but the entire procedure has to be repeated for each deletion strain.

6 Summary

Proton-translocating NADH:ubiquinone oxidoreductase (complex I) transports two electrons from NADH to membranal ubiquinone: in this process protons are translocated across the membrane, producing 40% of the total proton gradient between matrix side and intermembrane space. Mitochondrial complex I contains at least 46 subunits in mammals, and has a molecular weight of around 1000 kDa. Electronic microscopy analysis showed that complex I has an L-form, which consists of two domains: a peripheral "arm" (hydrophilic domain) and a membrane "arm" (hydrophobic domain). The peripheral domain, which protrudes into the matrix, contains one non-covalently bound flavin mononucleotide (FMN) and the iron-sulfur clusters $N1_a$, $N1_b$, N2, N3, N4 and N5 as redox active groups. They transport electrons from NADH to ubiquinone. Cluster N2 is supposed to be the immediate electron donor to ubiquinone by virtue of its highest and pH dependent redox midpoint potential ($E_{m,7}$ –150 mV). The exact location of the tetra-nuclear cluster N2 is still object of discussion. The TYKY and the PSST subunits contain three binding motifs for tetranuclear clusters which are formed by twelve cysteins. In an effort to investigate the **"**ubiquinone reduction module" of complex I, in the first part of this work site directed mutagenesis of the TYKY and PSST subunits has been carried out. Mutant strains were characterised in terms of complex I content, catalytic activity and EPR signature of cluster N2. The second part of this work was aimed at developing a substrate inducible version of the internal alternative NADH:ubiquinone oxidoreductase (NDH2i). A substrate inducible NDH2i is expected to offer a "switch" between complex I activity dependent (no NDH2i activity) and independent (NDH2i activity) cell growth, by changing between activating and non-activating substrates. This strategy would allow the screening for two types of complex I mutants, which is a prerequisite for realising a random PCR mutagenesis of single subunits of complex I, that allows the production of a high number of point mutations in relatively short time. *Y. lipolytica* complex I deficiency mutant strains could be easily identified, by virtue of their inability to survive under complex I dependent growth conditions (no NDH2i activity). By this way, amino acids that have an important role for complex I structure or function could be identified by subsequent sequence analysis.

Each of the twelve cysteines that form the above mentioned three binding motifs for iron-sulfur cluster have been mutagenised. In mutant mitochondrial membranes, no assembled complex I could be detected. From these data one may conclude that the mutagenised

cysteines play an important role for complex I stability, or that are a prerequisite for complex I assembly in *Y. lipolytica*, but there is not direct evidence indicating that any of the four mutagenised residues acts as a ligand. Two aspartates in the PSST subunit, Asp-99 and Asp-115, were found to be essential for complex I catalytic activity. EPR spectroscopic analysis indicated that the electron transfer to N2 cluster was not blocked and implied that this was not the reason for the loss of catalytic activity. From these data it can be concluded that D99 and D115 play a vital role for complex I NADH:ubiquinone reductase activity, but are not ligands for cluster N2 and that their position is not close enough to the cluster to influence directly its electromagnetic environment.

Three mutations, identified in the PSST and TYKY homologous subunits of patients affected with Leigh syndrome (V119M in PSST, P78L and R101H in TYKY) were reconstructed in the obligate aerobic yeast *Y. lipolytica*. This approach may help to understand the aetiology of the Leigh syndrome, in terms of the ability of complex I to oxidize NADH and to transport electrons. In fact, all three mutations showed effects on electron transport, reducing the V_{Max} by about 50%. Mutant V119M in the PSST subunit, which had a lethal effect in two patients that were homozygous for this mutation, affects a fully conserved residue. Overall, the results from site directed mutagenesis carried out so far support the theory that the "catalytic core " (N2 cluster and quinone binding site) of complex I has been evolved from the electron transfer module of the [Ni-Fe] hydrogenases. In fact, mutagenesis of residues that are fully conserved between complex I and [Ni-Fe] hydrogenases, showed dramatic effects on complex I in terms of assembly (cysteine mutants) or catalytic activity (D99-D115). Differently, changing aspartate 174 and glutamic acid 185 (not fully conserved, Fig 4.1A) had little or no effect on the Michaelis-Menten parameters and N2 EPR signal.

In recent years *Y. lipolytica* has been developed as a yeast genetic system to study mitochondrial complex I. The present work introduced the promoter for the isocitrate lyase (pICL1) as a useful tool for the substrate selective expression of the internal version of the alternative NADH:ubiquinone oxidoreductase (pICL1-NDH2i). This allows to rescue complex I deficiencies "*in vivo*" selectively by growth on acetate (or ethanol) medium. The integration of the pICL1-NDH2i construct into the genome of *Y. lipolytica* and subsequent deletion of nuclear-coded subunits like PSST, TYKY and 49 kDa, would contribute to further develop this organism as a useful genetic model for studying subunits of mitochondrial complex I by site directed mutagenesis.

7 References

1. Current protocols in molecular biology. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 2000. New York.
   Ref Type: Serial (Book, Monograph).

2. Ahlers, P., A.Garofano, S.Kerscher, and U.Brandt. 2000a. Application of the Obligate Aerobic Yeast *Yarrowia lipolytica* as a Eucaryotic Model to Analyze Leigh Syndrome Mutations in the Complex I Core Subunits PSST and TYKY. *Biochim. Biophys. Acta - Bioenerg.* 1459:258-265.

3. Ahlers, P., K.Zwicker, S.Kerscher, and U.Brandt. 2000b. Function of conserved acidic residues in the PSST-homologue of complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica*. *J. Biol. Chem.* 275:23577-23582.

4. Barth, G. and C.Gaillardin. 1996. *Yarrowia lipolytica*. *In* Non-conventional yeasts in biotechnology. K.Wolf, editor. Springer, Berlin-Heidelberg. 313-388.

5. Bentlage, H.A.C.M., R.de Coo, H.J.Ter Laak, R.C.A.Sengers, F.J.M.Trijbels, W.Ruitenbeek, W.Schlote, K.Pfeiffer, S.Gencic, G.von Jagow, and H.Schägger. 1995. Human diseases with defects in oxidative phosphorylation: I. Decreased amounts of assembled oxidative phosphorylation complexes in mitochondrial encephalomyopathies. *Eur. J. Biochem.* 227:909-915.

6. Böhm, R., M.Sauter, and A.Böck. 1990. Nucleotide sequence and expression of an operon in Escherichia coli coding for formate hydrogenlyase components. *Mol. Microbiol.* 4:231-243.

7. Brandt, U. and B.L.Trumpower. 1994. The protonmotive Q cycle in mitochondria and bacteria. *CRC Crit. Rev. Biochem.* 29:165-197.

8. Casaregola, S., C.Neuveglise, A.Lepingle, E.Bon, C.Feynerol, F.Artiguenave, P.Wincker, and C.Gaillardin. 2000. Genomic exploration of the hemiascomycetous yeasts: 17. *Yarrowia lipolytica*. *FEBS Lett.* 487:95-100.

9. Chen, D.-C., J.-M.Beckerich, and C.Gaillardin. 1997. One-step tranformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl. Biochem. Biotechnol.* 48:232-235.

10. Chevallet, M., A.Dupuis, J.P.Issartel, J.L.Lunardi, R.van Belzen, and S.P.J.Albracht. 2003. Two EPR-detectable [4Fe-4S] clusters, N2a and N2b, are bound to the NuoI (TYKY) subunit of NADH: ubiquinone oxidoreductase (Complex I) from Rhodobacter capsulatus. *Biochimica et Biophysica Acta-Bioenergetics* 1557:51-66.

11. Darrouzet, E., J.P.Issartel, J.Lunardi, and A.Dupuis. 1998. The 49-kDa subunit of NADH-ubiquinone oxidoreductase (Complex I) is involved in the binding of piericidin and rotenone, two quinone-related inhibitors. *FEBS Lett.* 431:34-38.

12. de Vries, S. and C.A.M.Marres. 1987. The mitochondrial respiratory chain of yeast. Structure and biosynthesis and the role in cellular metabolism. *Biochim. Biophys. Acta* 895:205-239.

13. Djafarzadeh, R., S.Kerscher, K.Zwicker, M.Radermacher, M.Lindahl, H.Schägger, and U.Brandt. 2000. Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*. *Biochim. Biophys. Acta - Bioenerg.* 1459:230-238.

14. Duarte, M., H.Populo, A.Videira, T.Friedrich, and U.Schulte. 2002. Disruption of iron-sulphur cluster N2 from NADH:ubiquinone oxidoreductase by site-directed mutagenesis. *Biochem J* 364:833-839.

15. Duarte, M. and A.Videira. 2000. Respiratory chain complex I is essential for sexual development in neurospora and binding of iron sulfur clusters are required for enzyme assembly. *Genet.* 156:607-615.

16. Dupuis, A., M.Chevallet, E.Darrouzet, H.Duborjal, J.Lunardi, and J.P.Issartel. 1998. The Complex I from *Rhodobacter capsulatus*. *Biochim. Biophys. Acta* 1364:147-165.

17. Dutton, P.L. 1978. Redox potentiometry: Determination of midpoint potentials of oxidation-reduction components of biological electron-transfer systems. *Methods Enzymol.* LIV:411-435.

18. Fernandez E, F.M.M.F.R.R. 1993. Transcriptional regulation of the isocitrate lyase encoding gene in Saccharomyces cerevisiae. *FEBS Lett.*

19. Finel, M., J.M.Skehel, S.P.J.Albracht, I.M.Fearnley, and J.E.Walker. 1992. Resolution of NADH:ubiquinone oxidoreductase from bovine heart mitochondria into two subcomplexes, one of which contains the redox centers of the enzyme. *Biochem.* 31:11425-11434.

20. Flemming, D., A.Schlitt, V.Spehr, T.Bischof, and T.Friedrich. 2003. Iron-sulfur cluster N2 of the Escherichia coli NADH:ubiquinone oxidoreductase (complex I) is located on subunit NuoB. *J. Biol. Chem.* 278:47602-47609.

21. Friedrich, T. 1998. The NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli*. *Biochim. Biophys. Acta* 1364:134-146.

22. Friedrich, T., B.Brors, P.Hellwig, L.Kintscher, T.Rasmussen, D.Scheide, U.Schulte, W.Mäntele, and H.Weiss. 2000. Characterization of two novel redox groups in the respiratory NADH:ubiquinone oxidoreductase (complex I). *Biochim. Biophys. Acta* 1459:305-309.

23. Friedrich, T. and D.Scheide. 2000. The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. *FEBS Lett.* 479:1-5.

24. Friedrich, T. and H.Weiss. 1997. Modular evolution of the respiratory NADH:Ubiquinone oxidoreductase and the origin of its modules. *J. theor. Biol.* 187:529-540.

25. Gavrikova, E.V., V.G.Grivennikova, V.D.Sled, T.Ohnishi, and A.D.Vinogradov. 1995. Kinetics of the mitochondrial three-subunit NADH dehydrogenase interaction with hexammineruthenium(III). *Biochim. Biophys. Acta* 1230:23-30.

26. Hartley, A., J.M.Stone, C.Heron, J.M.Cooper, and A.H.V.Schapira. 1994. Complex I Inhibitors Induce Dose-Dependent Apoptosis in PC12 Cells: Relevance to Parkinson's Disease. *J. Neurochem.* 63:1987-1990.

27. Helenius, A. and K.Simons. 1972. The binding of detergents to lipophilic and hydrophilic proteins. *J. Biol. Chem.* 247:3656-3661.

28. Hirst, J., J.Carroll, I.M.Fearnley, R.J.Shannon, and J.E.Walker. 2003. The nuclear encoded subunits of complex I from bovine heart mitochondria. *Biochim. Biophys. Acta* 1604:135-150.

29. Hollingworth, R.M. and K.I.Ahammadsahib. 1995. Inhibitors of Respiratory Complex I: Mechanisms, Pesticidal Actions and Toxicology. *Rev. Pestic. Toxicol.* 3:277-302.

30. Hollingworth, R.M., K.I.Ahammadsahib, G.Gadelhak, and J.L.McLaughlin. 1994. New inhibitors of complex I of the mitochondrial electron transport chain with activity as pesticides. *Biochem. Soc. Trans.* 22:230-233.

31. Ingledew, W.J. and T.Ohnishi. 1980. An analysis of some thermodynamic properties of iron-sulfur centres in site I of mitochondria. *Biochem. J.* 186:111-117.

32. Juretzek, T., M.T.Le Dall, S.Mauersberger, C.Gaillardin, G.Barth, and J.M.Nicaud. 2001. Vectors for gene expression and amplification in the yeast *Yarrowia lipolytica*. *Yeast* 18:97-113.

33. Kashani-Poor, N., S.Kerscher, V.Zickermann, and U.Brandt. 2001a. Efficient large scale purification of his-tagged proton translocating NADH:ubiquinone oxidoreductase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*. *Biochim. Biophys. Acta - Bioenerg.* 1504:363-370.

34. Kashani-Poor, N., K.Zwicker, S.Kerscher, and U.Brandt. 2001b. A central functional role for the 49-kDa subunit within the catalytic core of mitochondrial complex I. *J. Biol. Chem.* 276:24082-24087.

35. Kerscher, S., S.Dröse, K.Zwicker, V.Zickermann, and U.Brandt. 2002. *Yarrowia lipolytica*, a yeast genetic system to study mitochondrial complex I. *Biochim. Biophys. Acta - Bioenerg.* 1555:83-91.

36. Kerscher, S., A.Eschemann, P.M.Okun, and U.Brandt. 2001a. External alternative NADH:ubiquinone oxidoreductase redirected to the internal face of the mitochondrial

inner membrane rescues complex I deficiency in *Yarrowia lipolytica*. *J. Cell Sci.* 114:3915-3921.

37. Kerscher, S., N.Kashani-Poor, K.Zwicker, V.Zickermann, and U.Brandt. 2001b. Exploring the catalytic core of complex I by *Yarrowia lipolytica* yeast genetics. *J. Bioenerg. Biomembr.* 33:187-196.

38. Kerscher, S., J.G.Okun, and U.Brandt. 1999. A single external enzyme confers alternative NADH:ubiquinone oxidoreductase activity in *Yarrowia lipolytica*. *J. Cell Sci.* 112:2347-2354.

39. Kitajima-Ihara, T. and T.Yagi. 1998. Rotenone-insensitive internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* mitochondria: the enzyme expressed in *Escherichia coli* acts as a member of the respiratoy chain in the host cells. *FEBS Lett.* 421:37-40.

40. Kurkin, S., J.Meuer, J.Koch, R.Hedderich, and S.P.J.Albracht. 2002. The membrane-bound [NiFe]-hydrogenase (Ech) from Methanosarcina barkeri : unusual properties of the iron-sulphur clusters. *Eur. J. Biochem.* 269:6101-6111.

41. Leif, H., U.Weidner, A.Berger, V.Spehr, P.van Heek, T.Friedrich, T.Ohnishi, and H.Weiss. 1993. *Escherichia coli* NADH dehydrogenase I, a minimal form of the mitochondrial complex I. *Biochem. Soc. Trans.* 21:998-1001.

42. Ljuban Grgic, K.Z.N.K.-P.S.K.a.U. 2004. Functional Significance of Conserved Histidines and Arginines in the 49 kDa
Subunit of Mitochondrial Complex I. *J. Biol. Chem.* in press.

43. Loeffen, J., J.Smeitink, R.Triepels, R.Smeets, M.Schuelke, R.Sengers, F.Trijbels, B.Hamel, R.Mullaart, and L.Van den Heuvel. 1998. The first nuclear-encoded complex I mutation in a patient with Leigh syndrome. *Am. J. Hum. Genet.* 63:1598-1608.

44. Lowry, O.H., N.R.Rosebrough, A.L.Farr, and R.J.Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.

45. Lümmen, P. 1998. Complex I inhibitors as insecticides and acaricides. *Biochim. Biophys. Acta* 1364:287-296.

46. Mathiesen, C. and C.Hägerhäll. 2002. Transmembrane topology of the NuoL, M and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and bona fide antiporters . *Biochim. Biophys. Acta* 1556:121-132.

47. Meinhardt, S.W., D.-C.Wang, K.Hon-nami, T.Yagi, T.Oshima, and T.Ohnishi. 1990. Studies on the NADH-Menaquinone Oxidoreductase Segment of the Respiratory Chain in *Thermus thermophilus* HB-8. *J. Biol. Chem.* 265:1360-1368.

48. Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* 191:144-148.

49. Montet, Y., P.Amara, A.Volbeda, X.Vernede, E.C.Hatchikian, M.J.Field, M.Frey, and J.C.Fontecilla-Camps. 1997. Gas access to the active site of Ni-Fe hydrogenases probed by X-ray crystallography and molecular dynamics. *Nature Struct. Biol.* 4:523-526.

50. Nicholls, D.G. and S.J.Ferguson. 2001. respiratory chains. *In* bioenergetics3. Elsevier Science, editor. 89-154.

51. Ohnishi, T. 1993. NADH-Quinone Oxidoreductase, the Most Complex Complex. *J. Bioenerg. Biomembr.* 25:325-329.

52. Ohnishi, T. 1998. Iron-sulfur clusters semiquinones in Complex I. *Biochim. Biophys. Acta* 1364:186-206.

53. Ohnishi, T., S.Magnitsky, L.Toulokhonova, T.Yano, T.Yagi, D.S.Burbaev, and A.D.Vinogradov. 1999. EPR studies of the possible binding sites of the cluster N2, semiquinones, and specific inhibitors of the NADH:quinone oxidoreductase (complex I). *Biochem. Soc. Trans.* 27:586-591.

54. Pinner, E., E.Padan, and S.Schuldiner. 1992. Cloning, Sequencing, and Expression of the Nhab Gene, Encoding A Na+/H+ Antiporter in Escherichia-Coli. *J. Biol. Chem.* 267:11064-11068.

55. Pitkanen, S., F.Merante, D.R.Mcleod, D.Applegarth, T.Tong, and B.H.Robinson. 1996. Familial cardiomyopathy with cataracts and lactic acidosis: A defect in complex I (NADH-Dehydrogenase) of the mitochondria respiratory chain. *Pediatric Research* 39:513-521.

56. Robinson, B.H. 1998. Human Complex I deficiency: Clinical spectrum and involvement of oxygen free radicals in the pathogenicity of the defect. *Biochim. Biophys. Acta* 1364:271-286.

57. S.Yoshida, N.T. 1978. *Heterocycles* 10 :425-467.

58. Sambrook, J., E.F.Fritsch, and T.Maniatis. 1989. Molecular cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

59. Sazanov, L.A., S.Y.Peak-Chew, I.M.Fearnley, and J.E.Walker. 2000. Resolution of the Membrane Domain of Bovine complex I into Subcomplexes: Implications for the Structural Organization of the Enzyme. *Biochem.* 39:7229-7235.

60. Schägger, H. 2003. Blue Native Electrophoresis. *In* Membrane Protein Purification and Crystallization: A Practical Guide. C.Hunte, G.von Jagow, and H.Schägger, editors. Academic Press, San Diego. 105-130.

61. Schägger, H. and G.von Jagow. 1987. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-100 kDalton. *Anal. Biochem.* 166:368-379.

62. Schapira, A.H. 1998. Human complex I defects in neurodegenerative diseases. *Biochim. Biophys. Acta* 1364:261-270.

63. Seo, B.B., T.Kitajima-Ihara, E.K.L.Chan, I.E.Scheffler, A.Matsuno-Yagi, and T.Yagi. 1998. Molecular remedy of complex I defects: Rotenone-insensitive internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells. *Proc. Natl. Acad. Sci. USA* 95:9167-9171.

64. Sled, V.D. and A.D.Vinogradov. 1993. Kinetics of the mitochondrial NADH-ubiquinone oxidoreductase interaction with hexammineruthenium(III). *Biochim. Biophys. Acta* 1141:262-268.

65. Triepels, R., L.P.Van den Heuvel, J.L.Loeffen, C.A.Buskens, R.J.P.Smeers, M.E.Rubio-Gozalbo, S.M.S.Budde, E.C.M.Mariman, F.A.Wijburg, P.G.Barth, J.M.Trijbels, and J.A.Smeitink. 1999. Leigh Syndrome Associated with a Mutation in the NDUFS7 (PSST) Nuclear Encoded Subunit of Complex I. *Ann. Neurol.* 45:787-790.

66. Vanni, P., E.Giachetti, G.Pinzauti, and B.A.Mcfadden. 1990. Comparative Structure, Function and Regulation of Isocitrate Lyase, An Important Assimilatory Enzyme. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 95:431-458.

67. Volbeda, A., M.H.Charon, C.Piras, E.C.Hatchikian, M.Frey, and J.C.Fontecilla-Camps. 1995. Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. *Nature* 373:580-587.

68. Walker, J.E. 1992. The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. *Q. Rev. Biophys.* 25:253-324.

69. Walker, J.E., J.M.Skehel, and S.K.Buchanan. 1995. Structural analysis of NADH : ubiquinone oxidoreductase from bovine heart mitochondria Structural analysis of NADH: ubiquinone oxidoreductase from bovine heart mitochondria. *Methods Enzymol.* 260:14-34.

70. Wallace, D.C. 1999. Mitochondrial diseases in man and mouse. *Science* 283:1482-1488.

71. Wikström, M.K.F. 1984. Two protons are pumped from the mitochondrial matrix per electron transferred between NADH and ubiquinone. *FEBS Lett.* 169:300-304.

72. Yagi, T., T.Yano, S.Di Bernardo, and A.Matsuno-Yagi. 1998. Procaryotic complex I (NDH-1), an overview. *Biochim. Biophys. Acta* 1364:125-133.

73. Zhou, C., Y.Yang, and A.Y.Jong. 1990. Mini-prep in ten minutes. *BioTechniques* 8:172-173.

8 Zusammenfassung

8.1 Die Mitochondriale Atmungskette

Die mitochondriale Atmungskette transportiert Elektronen über ein Potenzialgefälle von 1,14 V ($E_{m,7}$) von NADH zum Sauerstoff und pumpt Protonen über die innere mitochondriale Membran in den Zwischenmembranraum. Dabei wird ein Protonengradient erzeugt. Vier Enzyme (Komplexe) und zwei Elektronüberträger gehören zu der Atmungskette: NADH:Ubichinon Oxidoreduktase (Komplex I), Succinat:Ubichinon Oxidoreductase (Komplex II), Ubihydrochinon:Cytochrom *c*-Oxidoreduktase (Komplex III), Cytochrom *c*-Oxidase (Komplex IV), Ubichinon (UQ), Cytochrom *c*. Die ATP-Synthase (Komplex V) verwendet diesen Protonengradienten um ATP zu synthetisieren.

8.2 Der Komplex I

Die protonenpumpende NADH:Ubichinon Oxidoreduktase (Komplex I) oxidiert NADH und überträgt zwei Elektronen auf Ubichinon. Dabei werden zwei Protonen pro Elektron über die Membran transportiert. Komplex I, vorhanden in vielen Prokaryonten und Eukaryonten, trägt zu ca. 40% des Protonengradientes zwischen Matrix und Zwischenmembranraum bei. Das bakterielle Enzym besteht aus 13 Untereinheiten (in *E. coli*) und hat eine Masse von ca. 500 kDa. Das mitochondriale Enzym besteht aus mindestens 46 Untereinheiten in Säugetieren und hat eine molekular Masse von ca. 1000 kDa.

- Komplex I Struktur

Die 13 Untereinheiten, aus denen der bakterielle Komplex I aufgebaut ist, sind zu den mitochondrialen Untereinheiten homolog. Aus diesem Grund wird der bakterielle Komplex I aus *E. coli* als „minimale Form“ bezeichnet. Elektronmikroskopische Untersuchungen haben gezeigt, dass der Komplex I eine L-förmige Gestallt aufweist, wobei der sogenannte Membranarm (Membrandomäne) in die innere mitochondriale Membran eingebettet ist und der periphere Arm (Peripherendomäne) in die mitochondriale Matrix hinein ragt. In der hydrophilen, Peripherendomäne befinden sich 7 kernkodierte Untereinheiten: 20 kDa (oder TYKY), 23 kDa (oder PSST), 24 kDa, 30 kDa, 49 kDa, 51 kDa, und 75 kDa. Diese enthalten

alle bekannten Redoxgruppen des Komplex I: FMN und die Eisen-Schwefel Cluster $N1_a$, $N1_b$, N2, N3, N4 und N5. Die Membrandomäne enthält 7 hydrophobe Untereinheiten: ND1-6 und ND4L, die in Eukaryonten mitochondrial kodiert sind.

- Eisen-Schwefel Zentrum N2

Das tetranukleare (4Fe-4S) Zentrum N2 wird als letztes Redox-Zentrum in einer Kette von Eisen-Schwefel Zentren vor dem Ubichinon vermutet. Außerdem könnte N2 wegen seines pH-abhängigen Mittelpunktpotenziales eine wesentliche Rolle bei der Protonentranslokation spielen. Drei konservierten Cystein-Bindungsmotive für tetranukleare Eisen-Schwefel Cluster wurden in zwei kernkodierten, hydrophilen Untereinheiten identifiziert: PSST (NUKM) und TYKY (NUIM).

8.3 Zielsetzungen

- Mutagenese aller zwölf Cysteine, die zu den drei oben-genanten Bindungsmotiven gehören, um die N2 Liganden zu identifizieren.
- Mutagenese von konservierten sauren Resten in der PSST Untereinheit.
- Erzeugen einer Substrat induzierbaren Version der alternativen nicht protonenpumpenden NADH:Ubichinon Oxidoreduktase, um *Y. lipolytica* als hefegenetisches Modellsystem für Komplex I Untersuchungen weiter zu entwickeln.

8.4 Ergebnisse

- TYKY (NUIM)

Alle konservierten Cysteinreste, die zu dem Bindungsmotiv (CxxCxxCxxxCP $-(x)_{27}-$ CxxCxxCxxxCP) für zwei tetranukleare Eisen-Schwefel Cluster gehören, wurden zu Alaninreste mutiert. Es wurde, weder in den Cys-Ala Mutanten noch in dem ΔNUIM Deletionsstamm, ein assemblierter Komplex I gefunden.

- PSST (NUKM)

Alle konservierten Cysteine, die zu dem Bindungsmotiv ($\underline{CC}(x)_{63}$ - $\underline{C}(x)_{28}$ – G$\underline{C}$PP) für ein tetranukleares Eisen-Schwefel Cluster gehören, wurden zu Alaninresten und Serinresten mutiert. Auch in diesem Fall, war weder in ΔNUKM noch in den Cys-Mutanten assemblierter Komplex I vorhanden.

- Saure Reste

Vier konservierte saure Reste in der PSST Untereinheit D99, D115, D174 und E185 könnten als vierter Ligand für das Cluster N2 dienen. Mutagenese dieser Reste wurde durchgeführt und Komplex I Assemblierung, dessen Gehalt in mitochondrialen Membranen und dessen katalytische Aktivität in dem Elternstamm und in den Mutanten wurden gemessen.
Die Aspartatreste 99 und 115 wurden durch Asparaginreste, Glutaminreste und Glycinreste ersetzt. Alle Mutanten hatten einen assemblierten Komplex I und die Membranpräparationen zeigten einen normalen Gehalt an Komplex I. Die NADH:Ubichinon oxidoreductase Aktivität war jedoch auf 5% der wildtypischen Aktivität reduziert. Im EPR Spektren mit isoliertem Komplex I aus beiden Stämmen war das g_z Signal nicht verschoben. Redoxtitrationen von Cluster N2, die mit der Hilfe der EPR Spektroskopie gemessen wurden, schlossen beiden Reste als vierten Liganden aus.
Aspartat 174 wurde zu Asparagin mutiert und Glutamat 185 zu Glutamin. Der Komplex I Gehalt war in beiden Mutanten mit dem wildtypischen vergleichbar. Die katalytische Aktivität war jedoch im Stamm E185Q deutlich reduziert und in Stamm D174N unverändert. Auch in diesem Fall konnten die beiden sauren Reste, anhand von EPR-Spektren, als Liganden für Cluster N2 ausgeschlossen werden.

- Leigh Mutanten

Zwei Punktmutationen in der TYKY (P78L, R101H) und PSST (V119M) homologe Untereinheiten wurden mit der Leigh Syndrome in Verbindung gebracht. Diese drei Mutationen wurden in *Y. lipolytica* rekonstruiert. Alle drei Mutanten zeigten ca. 50% reduzierte Aktivität und veränderte I_{50} Werte für Rotenon und DQA.

- NDH2i-pICL1

Das Konstrukt für die interne Version der alternativen NADH:Ubichinon Oxidoreduktase (NDH2i) besteht aus dem NUAM Promoter, der NUAM Pre-Sequenz, die für den Import des Proteins auf die innere Seite der Membran verantwortlich ist, und einer verkürzen Version des ORFs der NDH2. Der NUAM Promoter wurde gegen den pICL1 Promoter ausgetauscht. Das resultierende Konstrukt (NDH2i-pICL1) wurde in *Y. lipolytica* transformiert. Transformanten wurden auf synthetischem Glucose Medium (SD) und synthetischem Acetat Medium (SAc) entweder mit oder ohne den Komplex I spezifischen Hemmstoff DQA ausplattiert. Das Wachstum der Transformanten zeigte, dass die NDH2i selektiv unter der Kontrolle des pICL1 Promoters nur auf SAc eprimiert wird, aber nicht auf SD.

- Erzeugung eines Δ*nucm* -Δ*nukm* Stammes

Zur Untersuchung von Doppel-Punktmutanten in der PSST und 49 kDa Untereinheiten, die zu einem besseren Verständnis von Struktur und Funktion des „Ubiquinone Oxidoreduktase" Modules beitragen sollen, war es zunächst notwendig einen haploiden Stamm zu erzeugen, in dem die beiden Allele für die PSST (NUKM) und 49 kDa (NUCM) Untereinheiten deletiert sind. Die Deletion beider Allele entstand durch Kreuzung der NUCM und NUKM Stämme und wurde durch PCR und Southern Blot bestätigt.

8.5 Fazit

Die Mutation von zwei Aspartatresten in der PSST Untereinheit führte zu einen assemblierten Komplex I, dessen katalytische Aktivität weniger als 5% des Elternstammes betrug. EPR Spektroskopische Untersuchungen schlossen eine direkte Beteiligung der beiden Reste als N2 Liganden und eine Blockierung des Elektronentransportes zu Cluster N2 durch die Mutationen aus. Die mögliche Rolle der beiden Reste wurde anhand der Homologie zu den [Ni-Fe] Hydrogenasen diskutiert. Punktmutationen zeigten, dass Aminosäuren, die zwischen [Ni-Fe] Hydrogenasen und Komplex I konserviert sind, eine zentrale Rolle für die Struktur oder die Funktion des Komplex I spielen. Trotz der niedrigen Homologie zwischen den zwei Enzymklassen, weisen die Ergebnisse dieser Arbeit darauf hin, dass die allgemeine Faltung des Komplex I und der [Ni-Fe] Hydrogenase in der Nähe des Cluster N2 im Laufe der Evolution konserviert geblieben ist.

In den letzen Jahren, wurde *Y. lipolytica* als hefegenetisches Modellsystem entwickelt, um den mitochondrialen Komplex I zu untersuchen. In dieser Arbeit wurde der Promoter für die Isocitrat lyase als genetisches „Werkzeug“ für die „*in vivo*“ Substrat-abhängige Expression der NDH2i eingeführt. Diese ermöglicht, für Komplex I inaktive Mutanten, das selektive Wachstum auf Acetat Medium.

8.6 Ausblick

Die Integration des pICL1-NDH2i Konstruktes in das *Y. lipolytica* Genom ist der nächste notwendige Schritt für eine Zufallsregiospezifische Mutagenese auf einzelne Untereinheiten von Komplex I. Der Stamm mit einer genomischen Version des pICL1-NDH2i Konstruktes und die genomische Deletion der gewünschten Untereinheit sollte mit der mutagenisierten Kopie des Genes für die deletierte Untereinheit transformiert werden. Zwei Integrationsstrategien wurden in dieser Arbeit vorgeschlagen:

- Zufalls-Integration des Konstruktes in das *Y. lipolytica* Genom und eine darauffolgende Deletion der gewünschten Untereinheit
- Austausch der NDH2i Version in den Delektionsstämme gegen das pICL1-NDH2i Konstrukt, mit der Hilfe der POP-IN-POP-OUT Strategie.

9 Appendix

9.1 Nomenclature Comparison Between Complex I Subunits from *E. coli* and the Homologous Subunits from Different Organisms

E. coli NUO	*Bos taurus*	*Y. lipolytica*	*H. sapiens*	*N. crassa* NUO	*P. denitrificans* *Th. thermophilus* NQO
B	20 kDa or PSST	NUKM	NDUFS7	19.3 kDa.	6
I	23 kDa or TYKY	NUIM	NDUFS8	21.3c.	9
E	24 kDa	NUHM	NDUFV2	24 kDa	2
C*	30 kDa	NUGM	NDUFS3	31 kDa	5
D*	49 kDa	NUCM	NDUFS2	49 kDa	4
F	51 kDa	NUBM	NDUFV1	51 kDa	1
G	75 kDa	NUAM	NDUFS1	78 kDa	3
H	ND1	NU1M	MTND1	ND1	8
N	ND2	NU2M	MTND2	ND2	14
A	ND3	NU3M	MTND3	ND3	7
M	ND4	NU4M	MTND4	ND4	13
K	ND4L	NULM	MTND4L	ND4L	11
L	ND5	NU5M	MTND5	ND5	12
J	ND6	NU6M	MTND6	ND6	10

Table 9.1 ***-Nomenclature of complex I core subunits in different organisms-***

The different nomenclatures for the homologous subunits that constitute the "minimal form" of complex I are compared (life Science Division CEA, 2004)

*Subunits C and D are fused in *E. coli*.

9.2 A brief Summary of EPR Spectroscopy of Iron-Sulfur Clusters in Complex I from *Y. lipolytica*

Two forms of iron-sulfur clusters are found in complex I, $[2Fe\text{-}2S]^{(ox.:\ 2+,\ red.:\ 1+)}$ and $[4Fe\text{-}4S]^{(ox.:\ 2+,\ red.:\ 1+)}$, which function as single electron transferring redox cofactors. In the oxidized state binuclear $[2Fe\text{-}2S]^{2+}$ clusters contain two high spin ferric ions (S=5/2 spin state), which are antiferromagnetically coupled resulting in a S=0 ground state. Upon electron uptake, one high spin ferric ion becomes antiferromagnetically coupled with one high spin ferrous ion (S=2) producing a S=1/2 ground state. The same assumption can be made for tetranuclear [4Fe-4S] clusters. In complex I these occur in two forms with the formal oxidation states of the irons (+3, +3, +2, +2) and (+3, +2, +2, +2). The latter, reduced state possesses an S=1/2 ground state resulting from antiferromagnetic coupling between two pairs of iron ions. The S=1/2 spin state is responsible for the paramagnetism of the reduced iron sulfur clusters in complex I making them detectable by electron paramagnetic resonance spectroscopy (EPR).

So far five iron-sulfur clusters have been detected in complex I from *Y. lipolytica* by EPR spectroscopy (Fig. 9.2.1). These are designated in the order of increasing spin relaxation as N1, N2, N3, N4, and N5. Due to its slower relaxation rate the binuclear cluster N1 is detectable at higher temperatures (> 30 K) than the tetranuclear clusters, which need temperatures below 20 K.

Usually continuous wave X-band EPR spectra are recorded as first derivative spectra (Fig. 9.2.1 column B), which also allow a better assignment of characteristic g-values. But it is worthwhile to keep in mind that the spin concentration and consequently the concentration of an individual specie in the sample is reflected by the area under the "absorption type" spectra (Fig. 9.2.1 column A). Hence the amplitude of a signal in the derivative spectrum can only give an estimate of the concentration of a paramagnetic species and the exact concentration can only be obtained by double integration of the EPR spectrum.

Spectrum B.1 in the figure, recorded at 12 K from isolated NADH reduced complex I from *Y. lipolytica*, shows contributions from the binuclear cluster N1 as well as from the tetranuclear clusters N2, N3 and N4. Under the experimental conditions used for this spectrum N5 is not detectable due to its very fast spin relaxation. The experimental spectrum can be simulated by addition of individually simulated EPR spectra for each iron sulfur cluster in an approximate 1:1:1:1 stoichiometry (A.2 to A.6 and B.2 to B.6, respectively) The g-values which were used for the simulations are given in Fig. 9.2.1 B.2 - B.5.

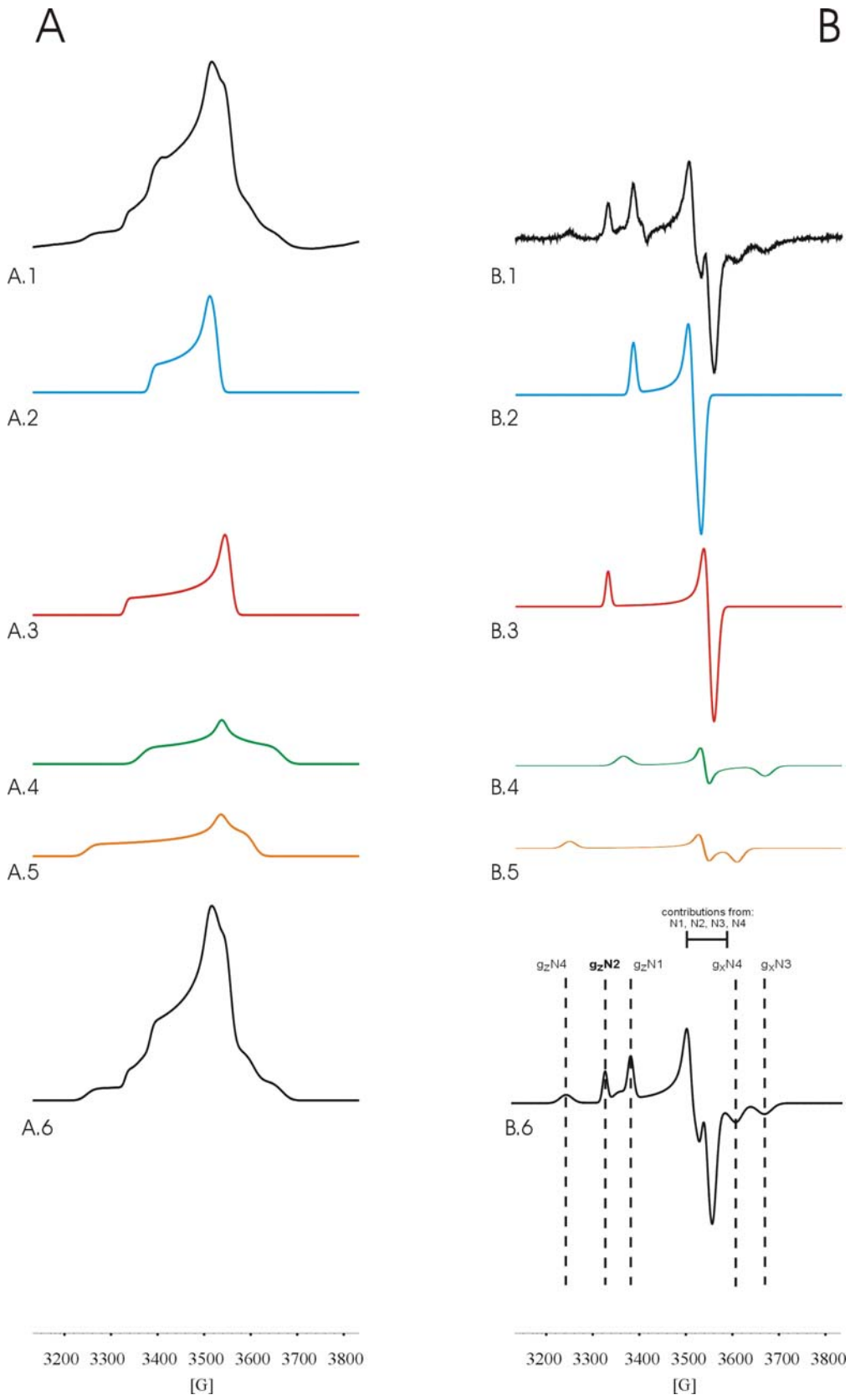

Figure 9.2.1 ***–Experimental and simulated EPR spectra of iron-sulfur clusters from Y. lipolytica complex I -***

Column A: absorption spectra; Column B: Derivative spectra. For details see text.

For a more detailed description of EPR spectroscopy of iron sulfur clusters in complex I see:

Ohnishi, T. (1998), "Iron-sulfur clusters / semiquinones in Complex I", Biochim. Biophys. Acta, 1364, 186 – 206

and for a more general view of EPR spectroscopy with biological samples see:

Weil, J. A., Bolton, J. R., Wertz, J. E. (1994) "Electron Paramagnetic Resonance (Elementary Theory and Practical Applications)", John Wiley & Sons, Inc., New York

Smith, T. D. and Pilbrow, J. R. (1980) "ESR of Iron Proteins", in "Biological Magnetic Resonance", Vol. 2, Berliner, L. J., Reuben, J. (eds.), Plenum Press, New York

9.3 Mutagenised Cysteines which Belong to the Tetranuclear Iron-Sulfur Cluster Binding Motifs in TYKY and PSST Subunits

9.3.1 Mutagenesis in TYKY Homologous Subunits from Different Organisms

Organism	Mutant[*1]	Assembly	Content[*2] (%)[*]	Activity[*3] (%)[*]	EPR spectroscopy	Reference	Authors conclusion
N. crassa	113A-S	no				Duarte (2000)	TYKY is essential for assembly
N. crassa	152 A-S	no				Duarte (2000)	TYKY is essential for assembly
N. crassa	155 S	no				Duarte (2000)	TYKY is essential for assembly
N. crassa	113-152 A	no				Duarte (2000)	TYKY is essential for assembly
R. capsulatus[1]	110 S	no				Chevallet (2003)	No complex I
R. capsulatus[1]	110 R	no				Chevallet (2003)	No complex I
R. capsulatus[1]	113 S	yes	17	43	altered	Chevallet (2003)	Two N2 cluster in TYKY
R. capsulatus[1]	113 R	no		n.d.	No N1-N3-N4	Chevallet (2003)	No complex I
R. capsulatus[1]	116 S	yes	8	28	n.d.	Chevallet (2003)	Two N2 cluster in TYKY
R. capsulatus[1]	120 S	no			No N1-N3-N4	Chevallet (1997-2003)	No complex I
R. capsulatus[1]	152 S	yes	34	71	altered	Chevallet (2003)	Two N2 cluster in TYKY
E. coli[2]	110 A	yes	125	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	113 A	yes	100	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	116 A	yes	62	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	120 A	yes	100	n.d.	No N2 (membrane)	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	149 A	yes	75	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	152 A	yes	87	6	Slight shift	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	155 A	yes	112	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	155 S	yes	75	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly
E. coli[2]	159 A	yes	112	n.d.	n.d.	Flemming (2003)	[Fe-S] cluster are essential for assembly

Table 9.2 ***-TYKY Subunit-***

[*1] The *Y. lipolytica* nomenclature is used

[*2] measured as NADH:UB rate, % of parental strain activity

[*3] measured as NADH:O_2 rate (piericidine sensitive), % of parental strain activity

nd: not determined

9.3.2 Mutagenesis in PSST Homologous Subunits from Different Organisms

Organism	Mutant[*1]	Assembly	Content[*2] (%)[*]	Activity[*3] (%)[*]	EPR	Reference	Authors conclusion
***N. crassa*[1]**	85 S	Yes	100	~10	No N2	Duarte (2002)	N2 in PSST only 1 C is ligand
***N. crassa*[1]**	86 A	Yes	100	~10	No N2	Duarte (2002)	N2 in PSST only 1 C is ligand
***E. coli*[*1]**	85 A	Yes	37	0	No N2	Flemming (2003)	N2 in PSST fourth ligand ?
***E. coli*[*1]**	85 S	Yes	37	0	No N2	Flemming (2003)	N2 in PSST fourth ligand ?
***E. coli*[*1]**	86 A	Yes	25	0	No N2	Flemming (2003)	N2 in PSST fourth ligand ?
***E. coli*[*1]**	150 A	Yes	25	0	No N2	Flemming (2003)	N2 in PSST fourth ligand ?
***E. coli*[*1]**	180 A	Yes	25	0	No N2	Flemming (2003)	N2 in PSST fourth ligand ?

Table 9.3 ***-PSST Subunit-***

[*1] The *Y. lipolytica* nomenclature is used

[*2] measured as NADH:UB rate, % of parental strain activity

[*3] measured as NADH:O_2 rate (piericidine sensitive), % of parental strain activity

nd: not determined

9.4 Sequence of the Construct pICL1-NDH2i

```
Nuam                                                          pICL1-intron
     GTCGACGGTCTCTCCCAGGACACTCCTCTTCCTCATGAAGTGCGGCCGCGGTACCGGGCC      60
     CGTCGAGATGGACATACTTGTATCGTCGCCCTATGTACTCGTAATGCAAGGGATTCCACC     120
     AGACATTCCTGCCACAATGGCAGGGTCCGTGAAAACGCCGACCACTGACAAGATGCCTTG     180
     TTCGTCTTGACCACGGACTAACTGGCACAAGCGAGATTAACGTCGTCGGAGACTATTCGG     240
     CACACAAGGCCAGACTGTGTGGCACTTCTCATCTCTCGTACCGACCTCTGTCAACAGTCT     300
     AACCGATTTTTAATGCTCGATATTACCAATGTTTCTTTGTGTCTTTTAACCAGAACAACC     360
     GAGCAGACCCGAACAGGTGCCGAACATGTGAATAGCAGTGCTGGAGCTCCATCAGTAAGC     420
     ATAATAACACAGCTGCCCAGCGACCTCCGCCCAGCGACCTCTACCCAGCGACCTCGGGCA     480
     CGTGACTATCTGCTCCGTTCCTCGCGGTCGCTGGCACGCTGGCAAATCTGGGGTCTCCAC     540
     ATTTTCCCCCGGATGTCTTGTTCCGTAGCGTGACTCATGCGGAATGACGTGAATGTAGGA     600
     GGGGCTGAGAATGGGGTCCGCAGTTGATAACCGGGGATTATTGGCCGGCGGCATTGTCAA     660
     CCAGGTGTTTTCACTGGCGTTCCTAGAATAAAAAGAAATAGGCGACCCCCTTGAGCGAGT     720
     TCAGCGGCGGCAAAATGCCTGTTGAAACACCTACTTTGTTCCCAGCACCCCCATCGGATA     780
     AATGGAGACGCATACATCGGCTATGTTTGGATACGATCTTGGGCCGGTGTGCGTGGTGTG     840
     CGCGGTCATTTTGTTCTCCTTTTGGACCCACGCAAGGTTCAACCGAACCCCGGATTCGAC     900
     TGTGAAAACGAACAACGGTTTAGTGCGGTTTAAAAAGTATCAAGTTCAGGGAGGGAAGCG     960
     ATCCAGGCCAACAGCTATGACCAAGAAACCAAGCGACCAAGACATCTGAAGACCAACAAA    1020
     ACCAATAATCGCTCACCAGATGCTCCCCAAACACTAACGGCAGACTCTACTCCAGATTTG    1080
     CACTTGTAGGACCCCGATATCGGGTTGCAGATCATGGTGTCATAATCTCTGAACGTGAAG    1140
     GTTAGGTGGAGGGGATGTTTTGGCCAGAAATGAGCGGTTTTGTGAGCTTGGAGACGGTAA    1200
     ATCGGATACGCCCAGCGTGAGGATTCCATAGACCCCCTCCTTTTGCCAGTATATCCACCG    1260
     CAACACCCACCATGAGCGACATCTGATACCGTGCCGCGACCACTACCCCAAATAAGCTCC    1320
     AACTAATATGCCGAGGCAGGTGGGAAACTATGCACTCCAGTCGACGCTGTAGAAGCACAT    1380
     GGAAGGTGCGGAGGCGGTGGCAACGAGGGGCATGAGCCATCAACGAGTAACCACAGACAA    1440
     GGCAAGGGGGGAAACGCGACCGGAATCTCTCGCGGTCACGTGACCCGCCCGGGTTCCACT    1500
     CGTCCATGTTGTGTCTCTGGTGTCTTCGGCCGACTCGCATTGGTTAAACTTCCACCACCG    1560
     CAATCACGTCCCACTGGCCAAACTTTTTCTGCTTTCTCTGACTTTTTCTGGCCAAAAGGC    1620
     AACGTCGGAAAGGGTCGGGAGGATTCGGAACCGACGAAAATCGGCCGGCTCCAGCGGGGG    1680
     TAGTTCGGCAGTCCTGGTGGGAGCTCTAGGGGAGCTGTGGTCTGTGTAGGGCGCGGGTCC    1740
     GGGTTTGTTGGGTGTCAAATCACGTGTTTTTGCCCCCCCGCTGAGCCGGACTCCGACAAC    1800
     CGTGTCTCCAACGGCCTGACTAAGCTGCTCCCAGCACTCTGCCGTAGCGTTGGTCTGTCC    1860
     TGTCGCACTCTGTTCAAAGACAGAAGAAAGAAAAAGCTAACCTCCACGTCAGAGACAATG    1920
     GTAGAAGGCTTGTTCCTTGCAACCGAGGAGAGTGAGTGTTCTCGGCACGAGCATCATGGG    1980
     CGATCTGGAGGGTATTTTTGAGGGGAAAAAACGGGATCAGGACAAACAGAGGCCACAGAC    2060
     CGGGAATCTGGGCCCCAAAACGGCCTTTTCCCGTCGCAAAACCGGTCTACATACACCCCT    2120
     TCGGCCCGCCACAGGCCGGTGTGAAAAACCCTAAAGCTTGCTTCAAACCAGACGGACGCA    2180
     CAGCAAGACACATCATGAAGAGTCACCTGCAGTATATATAGATCTGGGGATCCCCAGTAG    2240
     ACTGACCAAGCATACAAAAAGTGAGTATCCAACAGCGACACGTGAGATGGCAGAGACACA    2240
     GAGACGTGTCTACATGGTTGGACAAGTCTCCACATTCGCCAGAGACGTATCCACATACAA    2300
     ACACAATCTCACAGCTGATCTGCTCCTGTGACAGCACAGTACATGTTAGTGGATGAGGTG    2360
     TTGTGTAGTGGGTTAAATGGGTGGACTGATTCAGTGGCATCGGTGGCGACACCCTCTACT    2420
     CTTCATGTCGTCACCTACCGTTCGGAATCCCAATTATCTGATGAACTAAACGATTTCTGG    2480
     CCAAAACACAATTTTGCCAAAGAAGTCGGTCTCACCAATGCAAGTGTCACATCAAACATC    2420
     TGTCCCGTACTAACCCAG TGCTCTCGAGAAACCTCAGCAAGTTTGCTCGAGCCGGTCTC    1619
                       MetLeuSerArgAsnLeuSerLysPheAlaArgAlaGlyLeu      14

     ATCCGGCCAGCAACCACATCCACACACACCCGACTATTCAGCGTCTCCGCCCGACGTCTC ▼  1679
     IleArgProAlaThrThrSerThrHisThrArgLeuPheSerValSerAlaArgArgLeu      34

Ndh2i
   940 GACCCCTCCGACCAGTTGCCCGCCGACCCCTCCAAGAAGACCCTGGTGGTGCTGGGTTCC    1739
       AspProSerAspGlnLeuProAlaAspProSerLysLysThrLeuValValLeuGlySer      54

       GGCTGGGGCTCCGTCTCCTTCCTCAAGAAGCTCGATACTTCTAACTACAACGTCATTGTC    1799
       GlyTrpGlySerValSerPheLeuLysLysLeuAspThrSerAsnTyrAsnValIleVal      74

       GTCTCTCCCCGAAACTACTTCCTGTTCACCCCTCTACTGCCCTCTTGTCCCACCGGAACC    1859
       ValSerProArgAsnTyrPheLeuPheThrProLeuLeuProSerCysProThrGlyThr      94

       ATTGAGCACCGATCCATTATGGAGCCCATCCGAGGCATCATTCGACACAAGCAGGCCGAA    1919
       IleGluHisArgSerIleMetGluProIleArgGlyIleIleArgHisLysGlnAlaGlu     114

       TGCCAGTACCTCGAGGCTGATGCTACCAAGATTGACCACGAGAAGCGAATTGTGACCATC    1979
       CysGlnTyrLeuGluAlaAspAlaThrLysIleAspHisGluLysArgIleValThrIle     134

       CGATCCGCCGTCTCTGAGAACTCCAAGGAGGAGGTCATCAAGGAGATCCCCTTCGACTAT    2039
       ArgSerAlaValSerGluAsnSerLysGluGluValIleLysGluIleProPheAspTyr     154

       CTTGTTGTCGGTGTTGGCGCCATGTCCTCCACCTTTGGTATCCCCGGTGTCCAGGAGAAC    2099
       LeuValValGlyValGlyAlaMetSerSerThrPheGlyIleProGlyValGlnGluAsn     174

       GCTTGCTTCCTCAAGGAGATCCCCGACGCCCAGCAGATTCGACGAACCCTCATGGATTGC    2159
       AlaCysPheLeuLysGluIleProAspAlaGlnGlnIleArgArgThrLeuMetAspCys     194
```

```
ATTGAGAAGGCCCAATTCGAGAAGGACCCTGAGGTCCGAAAGCGACTTCTGCACACTGTC     2219
IleGluLysAlaGlnPheGluLysAspProGluValArgLysArgLeuLeuHisThrVal      214

GTTGTTGGAGGTGGCCCTACCGGTGTCGAGTTCGCTGCCGAGCTCCAGGACTTCTTCGAG     2279
ValValGlyGlyGlyProThrGlyValGluPheAlaAlaGluLeuGlnAspPhePheGlu      234

GACGATCTCCGAAAGTGGATCCCCGATATCCGAGACGATTTCAAGGTTACTCTTGTCGAG     2339
AspAspLeuArgLysTrpIleProAspIleArgAspAspPheLysValThrLeuValGlu      254

GCTCTCCCCAACGTTCTGCCCTCTTTCTCCAAGAAGCTCATTGACTACACCGAGAAAACC     2399
AlaLeuProAsnValLeuProSerPheSerLysLysLeuIleAspTyrThrGluLysThr      274

TTCTCTGACGAGAAGATCTCCATTCTGACCAAGACCATGGTTAAGTCTGTTGACGAGAAT     2459
PheSerAspGluLysIleSerIleLeuThrLysThrMetValLysSerValAspGluAsn      294

GTGATCCGAGCCGAGCAGACCAAGGGTGACGGTACTAAGGAAACCCTTGAGATGCCTTAC     2519
ValIleArgAlaGluGlnThrLysGlyAspGlyThrLysGluThrLeuGluMetProTyr      314

GGAACTCTTGTGTGGGCCACCGGTAACACTGTGCGACCTGTTGTTCGAGAGCTCATGTCC     2579
GlyThrLeuValTrpAlaThrGlyAsnThrValArgProValValArgGluLeuMetSer      334

AAGATCCCTGCTCAGAAGGGCTCCCGACGAGGTCTTCTTGTCAACGAGTACCTTGTTGTT     2639
LysIleProAlaGlnLysGlySerArgArgGlyLeuLeuValAsnGluTyrLeuValVal      354

GAGGGTACCGAGGGCATCTGGGCTCTTGGTGACTGTTCTGCCACCAAGTACGCACCCACT     2699
GluGlyThrGluGlyIleTrpAlaLeuGlyAspCysSerAlaThrLysTyrAlaProThr      374

GCCCAGGTTGCCTCCCAGGAGGGATCCTACCTTGCTAACCTGCTCAACGGCATTGCTAAG     2759
AlaGlnValAlaSerGlnGluGlySerTyrLeuAlaAsnLeuLeuAsnGlyIleAlaLys      394

ACCGAGGACCTCAACAACGAGATCACCAACCTCGAGAAGCAGTCGGAGCACACCTTTGAC     2819
ThrGluAspLeuAsnAsnGluIleThrAsnLeuGluLysGlnSerGluHisThrPheAsp      414

GAGCAGGAGCGAAAGAACATCTTTGCTCAACTCGAGTCCAAGTCCCGAAAGCTGCGACGA     2879
GluGlnGluArgLysAsnIlePheAlaGlnLeuGluSerLysSerArgLysLeuArgArg      434

TCCAGAGCCATGCTGCCCTTCGAGTACTCTCACCAGGGTTCTCTGGCCTACATTGGTTCC     2939
SerArgAlaMetLeuProPheGluTyrSerHisGlnGlySerLeuAlaTyrIleGlySer      454

GACCGAGCCGTTGCCGACCTGTCCTTCAACTTCTGGGGTATCATGAACTGGTCTTCCGGA     2999
AspArgAlaValAlaAspLeuSerPheAsnPheTrpGlyIleMetAsnTrpSerSerGly      474

GGAACCATGACCTACTACTTCTGGCGATCCGCCTACGTGTCCATGTGCTTCTCCATGCGA     3059
GlyThrMetThrTyrTyrPheTrpArgSerAlaTyrValSerMetCysPheSerMetArg      494

AACAAGATTCTTGTTTGCATTGATTGGATGAAGGTCCGAGTCTTCGGCCGTGATATCTCT     3119
AsnLysIleLeuValCysIleAspTrpMetLysValArgValPheGlyArgAspIleSer      514

CGAGAATAACAATTAGATGTTATATGAAAACCCGAGTACAGCATGTACTGGTAGAGGAGT     3179
ArgGlu***

AGGGATGATTCGAGGATAGTGGAGCTATTGTGAAGTGACCGGGTGGGTTAACAAAAGTAT     3239
TGCTTTGAATTAGTTTGACGATATAGATGTATATGACTACTGTATGTACTTGTAGTTGCG     3299
AGCTCAGAACGAATGAGACAGAGCAGCTCAGCCATTTACAGTACAGTATGTGCTTGCTCA     3359
TGGGCAGTTACAAGTTCCTACTTGTAATACGGAGTCTGGTTTTTCAGTTGACATGGATTA     3419
TACAACTTTAAGGCGCTCGAAAAGCGTTGTACCACTTTCTATCCACTAAGATATGTTTGA     3479
ACCAACCAGAGCTATCTACTTGTAGCTACACGGCTGACCACCAAACTCTTGGTCAACTAA     3539
TGTCACCAGCAACAACTGATGAACTCCAAAGGGTGAACCATAGACTCCACGCTGTGGAAG     3599
GATATGTAAATAAAGACAACCATAAAACAGAACTTCAGACACTTGGCAAGGAAAAAGATT     3659
ACAGCACCTAGGATTCTCGTATGGTCTCCCACTACAATACTAACTAGGCTCTCTGGTGCT     3719
TGACTATGGCTGATCGGACGGGAAGCCGTATTTTCACCAGGATATGGCCGTAACCAAGAC     3779
TCCGATACGGGGAATCGAACCCCGGTCTCCACGGTTCTCAACATGAGAGCGTGATGTGAT     3839
AGCCCCTACACTATATCGGAAATGTGCAAGACACGGTGTTTTTGGCGTCACGCATCTGAT     3899
TCGCCAAATAAGGTTTAATTAAAAAGTTAACGAATATTAAAATAGTTAGAATAAAAATAA     4059
TTTGTCAAGCGTTTGTATCGGCTCAAGAAAAGAAAAATAAGGGAACCGGGGAGTGTGGAA     4119
TTTTCAAACTGTTCGGAGAAAAACTATTACGGGTCTATTGCACGAGATTTCTCCCTGTTT     4179
TATGGTCGTTTAGTTGCTGAGATGCATTGGGCAAGAATCACAAAATAAAATGACTCGTGA     4239
AATGCGCACAATGCGACCTTAATGCACGTTTAGCCAGTAGTGTGCATGATAATCCACATA     4299
CAAAGTTAAGATCCGATGTTAGCCTACGTGTATTTACTATAGCTGAAATGTCTACCTGTA     4359
CTCATTTAGTGCGAAAAAATTTAACCACCTCTAGCGCCCGGTCTTGTCATGTCTTGGCTC     4419
ATGTACATGAGATGAGAGTGTATGTGATCTGACGGAAGTGGGCACATACACGCTGATAAC     4579
ATTTCAGGTGCGTTCCTGCGGTTGATATCACAAGCATCTCCGAGCACACGAAAATGAGAT     4539
GGTATGCTGAAGTCTCCACCTTTAGTCGAC
```

Figure 9.4 ***-Amino acids sequence of pICL1-NDH2i-***

GTCGAC: *SalI* restriction site

ATG : Start codon

*** : Stop codon

▼: Processing site

Color code

Green: nuam sequence

Orange: pICL1 sequence

Cyan: intron sequence

9.5 Sequence of the *EcoR*I *NUKM* (PSST) Gene Fragment of Complex I in *Y. lipolytica*

```
                                                    GAATTCCCAGAGA     13
CAGTTCTTCTCTGCGAAAACTGATATCCGAGCTGGGCTTTCTGCGGGCTCTGGACACGAG     73
AAACAACCCCGAGACGGAGGAGTTCTATCCGGATCTCATGTCTGTTATGGGCACTCAGGC    133
CGTGAACGGCAAGAGCATCTCGATCCAGTACACCAACACTCAGACGGCCGAGTTTGAGCG    193
GGCATGGCAGGGTACAGACACCAAGTTTGTGCGCAACCTCAAGGCACACAATCGAGAGAC    253
GTACGAGGACAGAATCCTGTACCAGACTCAGGCCATGCTTCGAGTGCATAACAAGGGCGG    313
TCTGCTGTATTGGATGGACGGTGTGTTGCTTGGTAAGGACCCCAAGTACATTGAGTCGTA    373
CAAGAAGCACGCTGCCAAGTGCTGGGCTGCTCGGGAGAAGCGACGCAAGCAGCAAGATGC    433
TGAGGAGAGAGTTGCAGGGCTGTCGAAGGATGTTAACGATACCGCTATGGCTAGTGGAGA    493
AGAGGCTTATGGATTCATTTAATGCTATATTTATTGTATTTTTACCCAAACGAATGAGAG    553
AGCTGAGCTCGAAATGAAAATGAGAATACTGGTACGAGTAACTACAATTACTCGTACCGG    613
TACTTGTGTGTAATTGGAATACTGAGCCAGTGGAGAGTGCGATTCTCCCGTTTTTGTACA    673
TTATCTAGTGGATATAACTAACATTCGAATCAAAAAGCTATCTTGGTTCTGATTTTATAC    733
TGTTTGACACCCCTGTCCATAGTAATATCTTAAGCAGTGGATGGAATCATGATCACTGTA    793
TACTGTATACACCTTCATGATGTTTACAAGCGCTATACTGGTGATGTTGTCCCAACAGGT    853
TAGGGTTTGATCCCTCCTCAGTGTGTTTGCACGTGATCTGGCGTCCAGCCAATAACAGCA    913
ATCACAAGTGGTATTACCAAGAAGCGTGATTTTCGCGCGGTGGCGGTTTTCCCTCGTTGT    973
TGCCGTCGGTTGGCAGTCTCGTTCTCTTCACACCAGGCCAACATTTCTTCAATTGCAACA   1033
TTTTTCTACACTTGTAGGTGCATGTTTGGTGTTTGTCTGAGACGAGGGAGTGAGTCGGAG   1093
ATGTGTTTTTTTTTAGCGAGGAGTGCTCGGAATTGGATATTTAAACACACAAAAACGGTA   1153
ATTTCCGGTGGCTGGTGCGATTAAACACGTCGTCCTGTCACCAGCTTGTCTCGTACGCCC   1213
CTTATATCTCTTCTTACCCCCAAACCACCTCCTCCATTAAGCTTCTGCATTGGCCAATCA   1273
GGGCTTCCCATATTTGGGTAACGAAATTATGGTTGGAGGCTTCCACTATCGTATATTTGC   1333
ACCAGCCAGCAGAGCACCTCACGCTTGTTGTATCGTCACAAAACCACACATCCCTACAGC   1393

ATGCTCAGATCACAGATTGGCCGACTGGCTCTGCGACCGACGCTGGTCCCCGCCACTGTC   1453
 M  L  R  S  Q  I  G  R  L  A  L  R  P  T  L  V  P  A  T  V     20

ATCCCCCAGACCCGAGCATACTCCGCTCCCGCTGGAACCCCCCGAGTGTCGTCCTCCTCC   1513
 I  P  Q  T  R  A  Y  S  A  P  A  G  T  P  R  V  S  S  S  S     40
                     ▲

ATGCCCACCGACTTCCCTCTCCCCTCACAGCAGAAGCCCAACAGCGCCGTCGACTACACC   1573
 M  P  T  D  F  P  L  P  S  Q  Q  K  P  N  S  A  V  D  Y  T     60

CTGACCACTCTGGATGCCGTGGCCAACTGGGCTCGACAGGGCTCTTTCTGGCCCGTGACC   1633
 L  T  T  L  D  A  V  A  N  W  A  R  Q  G  S  F  W  P  V  T     80

TTCGGTCTGGCGTGCTGTGCCGTCGAAATGATGCACGTGTCTGCCCCCCGGTACGATCAG   1693
 F  G  L  A  C  C  A  V  E  M  M  H  V  S  A  P  R  Y  D  Q    100

GATCGTCTGGGTATCATTTTCCGAGCCTCCCCTCGACAGTCCGATATCATGATTGTGGCC   1753
 D  R  L  G  I  I  F  R  A  S  P  R  Q  S  D  I  M  I  V  A    120

GGAACCCTCACAAACAAAATGGCCCCCGTGCTGCGTCAGGTGTACGACCAGATGCCCGAG   1813
 G  T  L  T  N  K  M  A  P  V  L  R  Q  V  Y  D  Q  M  P  E    140

CCCCGATGGGTCATCTCCATGGGCTCCTGCGCTAACGGTGGTGGATACTACCACTTCTCC   1873
 P  R  W  V  I  S  M  G  S  C  A  N  G  G  G  Y  Y  H  F  S    160

TACTCGGTGGTGCGAGGCTGCGACCGAATCGTGCCTGTGGACGTCTACGTTCCCGGATGT   1933
 Y  S  V  V  R  G  C  D  R  I  V  P  V  D  V  Y  V  P  G  C    180

CCCCCCACCTCCGAGGCCCTCATGTACGGCGTCTTCCAGCTCCAGCGAAAGATGCGAAAC   1993
 P  P  T  S  E  A  L  M  Y  G  V  F  Q  L  Q  R  K  M  R  N    200

ACAAAGATTACCCGAATGTGGTACCGAAAGTAAGCATAATAAACGTTTAGATAGTGAGTC   2053
 T  K  I  T  R  M  W  Y  R  K  *                               210
```

```
GGCTGTGTGTAGCCAAAGCTCATCAACGCAGGTCCATTGGTCCGTGTGAGGTACATGTAG   2113
ATCTTTTTACCATGCACCAGGTAGGTCTACGCACCCTCAATTCTGGATGGAGTGATGGTT   2173
ATTGGTACTTCAAGACGCAGTATATTAGCTGTTGATGTTCGTTGTGTTATTCAATCACCT   2233
GTCTACTAGTATCAAATACCGTACTTGTAGTAGAGGAATTC                      2274
```

GAATTC: *Eco*RI resriction site

▲: processing site

* : STOP-codon

Primers used for site directed mutagenesis

Direction	Name	Sequence	Binding site
Forwards	C85S	5'-CTTCGGTCTGGCGTCCTGTGCCGTCGAAATG-3'	1633-1663
	C86S	5'-GGTCTGGCGTGCTCTGCCGTCGAAATG-3′	1636-1666
	C150S	5'-CTCCATGGGCTCCTCCGCTAACGGTGG-3′	1828-1855
	C180S	5'-CTACGTTCCCGGATCTCCCCCCACCTCC-3'	1917-1945
	D99N	5'-GCCCCCCGGTACAATCAGGATCGTCTG-3'	1666-1672
	D99E	5'-CTGCCCCCCGGTACGAGCAGGATCGTCTGGG-3'	1664-1674
	D99G	5'-CTGCCCCCCGGTACGGTCAGGATCGTCTGGG-3'	1664-1674
	D115N	5'-CCTCGACAGTCCAATATCATCATCATTGTGGCC-3'	1054-1073
	D115E	5'-CCTCGACAGTCCGAGATCATGATTGTG-3'	1054-1073
	D115G	5'-CCTCGACAGTCCGGTATCATGATTGTG-3'	1054-1073
	C85S	5'-CATTTCGACGGCACAGGACGCCAGACCGAAG-3'	1663-1633
	C86S	5'-CATTTCGACGGCAGAGCACGCCAGACC-3'	1666-1636
	C150S	5'-CCACCGTTAGCGGAGGAGCCCATGGAG-3'	1855-1828
	C180S	5'-GGAGGTGGGGGGAGATCCGGGAACGTAG-3'	1945-1917
	D174N	5'-GAATCGTGCCTGTGAACGTCTACGTTCCC-3'	1899-1927
	E185Q	5'-CCCCCCACCTCCCAGGCCCTCATGTAC-3'	1994-2021
Reverse	D99N	5'-CCCAGACGATCCTGCTCGTACCGGGGGGCAG-3'	1674-1664
	D99E	5'-CCCAGACGATCCTGACCGTACCGGGGGGCAG-3'	1674-1664
	D99G	5'-CCCAGACGATCCTGCTCGTACCGGGGGGCAG-3'	1674-1664
	D115N	5'-GGCCACAATCATGATATTGGACTGTCGAGG-3'	1073-1054
	D115E	5'-CCTCGACAGTCCGAGATCATGATTGTG-3'	1073-1054
	D115G	5'-CACAATCATGATACCGGACTGTCGAGG-3'	1073-1054
	D174N	5'-GGGAACGTAGACGTTCACAGGCACGATTC-3'	1927-1899
	E185Q	5'-GTACATGAGGGGCTGGGAGGTGGGGGG-3'	2021-1994

9.6 Sequence of the *NUIM* (TYKY) Gene of Complex I in *Y. lipolytica*

```
                                                        TACAGCAGACG     11
GGAGAGGCAAGACAGAGGAATGGATTGAAATGTCCGAGTAACGAAATGAAATAATGTCAG     71
TGACATGTCCGAAAGAAGAGTATAAATACAAATACAAGTACAAGTACAAGTACAGTAGTA    131
ATCTGTCGCCAATATGTCGATCAAATGGCCGTTCGTAGAGCCGTGTTTTAGGATAGCTAA    191
TTACTATTTTATTCGTACTTCTATCATATATTTCTTTATATCCCGAATGACTCAAACTCC    251
CAACCTGAATGCACTGTTGGTACAGTATCACCAAACTCTACTCACAATATCCCCCTTTTC    311
AACCCGCGCCAAACTCGAACCCACACCTTTTCGTCTCCCCATATGCAAACAAAATTTGCG    371
GGGGGGGTGGAATTCCCACCAATAACCTCAGTTACAATCAGCATTCGCTAATGGGGGGTT    431
GTCCGGAGGTTGCAACAAGTAGGTCGAGGGGTGGATAATGTGCAATTTTTGAAATAAATA    491
TATACTAATTTACATGCGGCCAATCATATGCCACGTCTAAAACGAAAATTCACCCACCAA    551
TACACCCACTGTAGACCATTCGTGGCCTGCGGCCACTCATTTCAAGGAACCCTAACCTCG    611
TCCCTCCCAACCGTCAGCCAATGAAAGCAAGCAAAATTACGTGACAAGCTTAAAGGTGTA    671
ATACTGCTTGGAGAGGGTACAACACCACACACATCACCACCACACACAAACCAAAAAAAA    731

ATGTTGAGTCTGGTCCGACCCGCTGTCACCCGATCGATTCTGCGAGGCGCTCCCGGATCC    791
 M  L  S  L  V  R  P  A  V  T  R  S  I  L  R  G  A  P  G  S     20

ATGCGGCTGCTGTCCTCCACAGCCCGGCTCCACGCCCCCGCCACAGACTCCGCCATCAAC    851
 M  R  L  L  S  S  T  A  R  L  H  A  P  A  T  D  S  A  I  N     40

ATCTACGCCGGAGGATCTGCCGCTGCTGCTCCCCCAGCAGGCTTCCGAATTCACAGACCC    911
 I  Y  A  G  G  S  A  A  A  A  P  P  A  G  F  R  I  H  R  P     60

GCCACCTGGGAGGAGAGCGAAGAGGGCGCTCTTTCCAAGGCCACCAAGTACTTTTTGCTG    971
 A  T  W  E  E  S  E  E  G  A  L  S  K  A  T  K  Y  F  L  L     80
                    ▲
GCCGAGATGTTCCGTGGCCTGTACGTTGTGCTGGAACAGTTTTTCCGAGCTCCTTACACC   1031
 A  E  M  F  R  G  L  Y  V  V  L  E  Q  F  F  R  A  P  Y  T    100

ATCTACTACCCGTTCGAGAAGGGACCCGTCTCTCCTCGATTCCGAGGCGAGCACGCCCTG   1091
 I  Y  Y  P  F  E  K  G  P  V  S  P  R  F  R  G  E  H  A  L    120

CGACGTTACCCCAGTGGTGAGGAGCGATGCATTGCCTGCAAGCTGTGTGAGGCCATCTGC   1151
 R  R  Y  P  S  G  E  E  R  C  I  A  C  K  L  C  E  A  I  C    140

CCCGCTCTGGCAATTACCATCGACGCTGAGGAGCGAATCGACGGATCGCGACGAACCACC   1211
 P  A  L  A  I  T  I  D  A  E  E  R  I  D  G  S  R  R  T  T    160

AAGTACGATATCGACATGACCAAGTGCATCTACTGCGGCTACTGCCAGGAGTCGTGCCCC   1271
 K  Y  D  I  D  M  T  K  C  I  Y  C  G  Y  C  Q  E  S  C  P    180

GTGGACGCCATTGTCGAGACTCCCAACGTGGAGTACGCCACCGAGACTCGAGAAGAGCTG   1331
 V  D  A  I  V  E  T  P  N  V  E  Y  A  T  E  T  R  E  E  L    200

CTGTACAACAAGGAAAAGCTGCTTGCTAACGGAGACAAGTGGGAGCTCGAGCTGCAGTAC   1391
 L  Y  N  K  E  K  L  L  A  N  G  D  K  W  E  L  E  L  Q  Y    220

GCTCTGGACGCTGATGCTCCTTACAGATAGAGAGGGGAAATGCATTTTTATTAGGAAAGC   1451
 A  L  D  A  D  A  P  Y  R  *                                  229

GAGCAGTTGGGGTTGCGGAAAATAACAGGACTGTAGCGACTGTTGTTTTACGGCTCCATC   1511
TGCGAACGAGTGTAGCTATCAGTGTCGTAATAAGAGCCAAGCACTACAACCGGAGAGCGC   1571
ATGTGTTGTAGAAAAACACTTTCTTATTATCCGCCCATCTGAGTGTTGTAGGCGCTCTCT   1631
TTACGGTCAATTCAGTTGTAGTGCTTATTGGCGAGCCACATTATGTACGAGTAATGTGCT   1691
CGAGCGAATACATAAATAACTGTGCGGAGACAGTATTCATCATATTAGCACCTTGTACAG   1751
TACAAGTAAAGTCTTGTCCATCCTTTGAGATTGCTTTTGTCGATTCAAGATGGATATTGT   1811
```

```
ATAAGTACTCGCGCAATTGTACACACAGTATTGTAGATCTGCAGGATATGAGTGGATGCA   1871
GTATGTTACGTGTGCTACACATTACCACGTAAATCACGATAATACGGAATCTAAATGGTT   1931
CTGCGGCTCAATAACACTCAATAACGGACAGATTCTGGACGGATTCTGAACACATGTCTT   1991
GCCACATGTCTCTCTTCCCTAGCCTAAGACAACCTCCTGACCTTCAAGTAGAGTGTATAT   2051
CTCATCATCTCAGCATCAAAGTGTAGTATCAACAAGTGGTCTCGATATATTTGCAGCTGT   2111
AGACTTTTGGGCATCTTTGTAATGTCGATTTAAAGCCCCAGATTAGCGAAATCCAATGAT   2171
GTATTTTTCATGTCATTGGACGTTGTATTCGTTGTTCTAGGTCGACGCTGGTTGTGATTA   2231
CTGTAGTCTCTAGTCGACATTTTAGTTGACAACTTGTTGAGGCAAAAATACACCTGGGAG   2291
TTCGTTTGTAGTGTTCCCGCAGGGATGGAGAGCTAATATTTATATTTATAGATACAAAAT   2351
CATCTAAATAAAAGACTTATCTCGGTTCAAATGATCTACATCAAGATCATGGATGAATAT   2411
GTGCACTTCCATGTGTATTCAACCTCCATAGTTAGTTGACAAATTAGTGAAATTGGAATA   2471
CACTTTTTTGGATC                                                 2485
```

AAGCTT: *Hind*III resriction site

▲ : processing site

* : STOP-codon

Primers used for site directed mutagenesis

Direction	Name	Sequence	Binding site
Forwards	C110A	5'-GTGGTGAGGAGCGAGCCATTGCCTGCAAGCTG-3'	1005-1035
	C113A	5'-GAGCGATGCATTGCCGCCAAGCTGTGTGAG-3′	1013-1041
	C116A	5'-GCCTGCAAGCTGGCTGAGGCCATCTGC-3′	1024-1151
	C120A	5'-GTGTGAGGCCATCGCCCCCGCTCTGGC-3'	1134-1161
	C149A	5'-GACATGACCAAGGCCATCTACTGCGGC-3'	1229-1248
	C152A	5'-CCAAGTGCA CTACGCCGGCTACTGCCAG-3'	1231-1259
	C155A	5'-CTACTGCGGCTACGCCCAGGAGTCGTGC-3'	1240-1268
	C159A	5'-CTGCCAGGAGTCGGCCCCGTGGACGCC-3'	1252-1280
Reverse	C110A	5'-GTGGTGAGGAGCGAGCCATTGCCTGCAAGCTG-3'	1035-1005
	C113A	5'-GAGCGATGCATTGCCGCCAAGCTGTGTGAG-3′	1041-1013
	C116A	5'-GCCTGCAAGCTGGCTGAGGCCATCTGC-3′	1151-1024
	C120A	5'-GTGTGAGGCCATCGCCCCCGCTCTGGC-3'	1161-1134
	C149A	5'-GACATGACCAAGGCCATCTACTGCGGC-3'	1248-1229
	C152A	5'-CCAAGTGCA CTACGCCGGCTACTGCCAG-3'	1259-1231
	C155A	5'-CTACTGCGGCTACGCCCAGGAGTCGTGC-3'	1268-1240
	C159A	5'-CTGCCAGGAGTCGGCCCCGTGGACGCC-3'	1280-1252

9.7 PROF (Secondary Structure Prediction System) of the PSST (NUKM) Subunit of Complex I from *Y. lipolytica*

AA	pH	pE	pC	PRED	Nr.
M	0.072	0.009	0.918	C	1
L	0.340	0.016	0.644	C	2
R	0.533	0.015	0.451	H	3
S	0.606	0.023	0.370	H	4
Q	0.549	0.034	0.418	H	5
I	0.427	0.084	0.488	C	6
G	0.339	0.143	0.518	C	7
R	0.315	0.275	0.410	C	8
L	0.309	0.322	0.369	C	9
A	0.346	0.308	0.346	C	10
L	0.267	0.271	0.463	C	11
R	0.170	0.240	0.590	C	12
P	0.124	0.241	0.635	C	13
T	0.163	0.265	0.572	C	14
L	0.181	0.281	0.539	C	15
V	0.152	0.222	0.627	C	16
P	0.156	0.186	0.659	C	17
A	0.204	0.241	0.554	C	18
T	0.220	0.272	0.507	C	19
V	0.201	0.292	0.507	C	20
I	0.172	0.234	0.594	C	21
P	0.171	0.194	0.635	C	22
Q	0.187	0.192	0.620	C	23
T	0.194	0.189	0.617	C	24
R	0.199	0.184	0.617	C	25
A	0.203	0.209	0.589	C	26
Y	0.128	0.170	0.702	C	27
S	0.076	0.112	0.812	C	28
A	0.071	0.075	0.854	C	29
P	0.089	0.054	0.858	C	30
A	0.080	0.045	0.875	C	31
G	0.074	0.039	0.888	C	32
T	0.061	0.047	0.892	C	33
P	0.060	0.069	0.872	C	34
R	0.062	0.100	0.838	C	35
V	0.058	0.111	0.832	C	36
S	0.057	0.084	0.859	C	37
S	0.069	0.056	0.876	C	38
S	0.074	0.044	0.882	C	39
S	0.071	0.038	0.892	C	40
M	0.066	0.043	0.891	C	41
P	0.056	0.046	0.898	C	42
T	0.054	0.049	0.897	C	43
D	0.053	0.048	0.898	C	44
F	0.058	0.041	0.901	C	45
P	0.067	0.032	0.901	C	46
L	0.077	0.029	0.893	C	47
P	0.060	0.025	0.915	C	48
S	0.054	0.029	0.917	C	49
Q	0.050	0.028	0.922	C	50
Q	0.063	0.026	0.911	C	51
K	0.098	0.019	0.883	C	52
P	0.204	0.016	0.780	C	53
N	0.423	0.015	0.562	C	54
S	0.370	0.041	0.588	C	55
A	0.361	0.165	0.474	C	56
V	0.324	0.330	0.346	C	57
D	0.290	0.422	0.288	E	58
Y	0.333	0.469	0.197	E	59
T	0.288	0.591	0.121	E	60
L	0.256	0.564	0.181	E	61
T	0.190	0.400	0.409	C	62
T	0.099	0.224	0.677	C	63
L	0.587	0.059	0.354	H	64
D	0.691	0.030	0.279	H	65
A	0.683	0.027	0.289	H	66
V	0.731	0.023	0.246	H	67
A	0.676	0.024	0.300	H	68
N	0.561	0.027	0.412	H	69
W	0.582	0.032	0.386	H	70
A	0.545	0.053	0.402	H	71
R	0.487	0.073	0.440	H	72
Q	0.335	0.089	0.576	C	73
G	0.188	0.081	0.732	C	74
S	0.122	0.082	0.797	C	75
F	0.120	0.121	0.760	C	76

W	||	0.106	0.212	0.682	C	77
P	||	0.116	0.290	0.594	C	78
V	||	0.149	0.344	0.507	C	79
T	||	0.162	0.286	0.552	C	80
F	||	0.159	0.145	0.696	C	81
G	||	0.182	0.064	0.754	C	82
L	||	0.296	0.095	0.609	C	83
A	||	0.368	0.160	0.472	C	84
C	||	0.383	0.211	0.406	C	85
C	||	0.365	0.216	0.419	C	86
A	||	0.665	0.127	0.208	H	87
V	||	0.730	0.130	0.140	H	88
E	||	0.717	0.148	0.135	H	89
M	||	0.784	0.122	0.093	H	90
M	||	0.820	0.055	0.125	H	91
H	||	0.764	0.026	0.209	H	92
V	||	0.625	0.031	0.343	H	93
S	||	0.378	0.032	0.590	C	94
A	||	0.186	0.043	0.770	C	95
P	||	0.152	0.068	0.781	C	96
R	||	0.219	0.099	0.683	C	97
Y	||	0.185	0.082	0.733	C	98
D	||	0.185	0.069	0.746	C	**99**
Q	||	0.443	0.059	0.498	C	100
D	||	0.447	0.062	0.491	C	101
R	||	0.366	0.133	0.502	C	102
L	||	0.227	0.232	0.541	C	103
G	||	0.082	0.331	0.588	C	104
I	||	0.053	0.458	0.489	C	105
I	||	0.047	0.540	0.413	E	106
F	||	0.056	0.463	0.481	C	107
R	||	0.048	0.325	0.627	C	108
A	||	0.050	0.154	0.796	C	109
S	||	0.060	0.064	0.875	C	110
P	||	0.113	0.051	0.836	C	111
R	||	0.136	0.074	0.791	C	112
Q	||	0.095	0.150	0.756	C	113
S	||	0.047	0.331	0.623	C	114
D	||	0.026	0.663	0.310	E	**115**
I	||	0.016	0.909	0.075	E	116
M	||	0.017	0.947	0.036	E	117
I	||	0.023	0.944	0.033	E	118
V	||	0.023	0.917	0.060	E	**119**
A	||	0.042	0.622	0.336	E	120
G	||	0.042	0.303	0.655	C	121
T	||	0.129	0.150	0.721	C	122
L	||	0.266	0.077	0.657	C	123
T	||	0.338	0.043	0.619	C	124
N	||	0.791	0.008	0.201	H	125
K	||	0.843	0.004	0.153	H	126
M	||	0.869	0.003	0.128	H	127
A	||	0.897	0.003	0.100	H	128
P	||	0.939	0.002	0.059	H	129
V	||	0.947	0.002	0.051	H	130
L	||	0.953	0.002	0.045	H	131
R	||	0.959	0.002	0.039	H	132
Q	||	0.944	0.004	0.052	H	133
V	||	0.931	0.006	0.063	H	134
Y	||	0.903	0.010	0.087	H	135
D	||	0.810	0.012	0.178	H	136
Q	||	0.632	0.018	0.350	H	137
M	||	0.228	0.013	0.760	C	138
P	||	0.052	0.020	0.928	C	139
E	||	0.031	0.029	0.940	C	140
P	||	0.021	0.076	0.903	C	141
R	||	0.021	0.389	0.591	C	142
W	||	0.014	0.866	0.120	E	143
V	||	0.013	0.930	0.056	E	144
I	||	0.018	0.930	0.052	E	145
S	||	0.028	0.867	0.104	E	146
M	||	0.043	0.619	0.338	E	147
G	||	0.064	0.406	0.530	C	148
S	||	0.190	0.279	0.531	C	149
C	||	0.324	0.216	0.460	C	150
A	||	0.299	0.161	0.540	C	151
N	||	0.185	0.100	0.715	C	152
G	||	0.122	0.082	0.796	C	153
G	||	0.084	0.058	0.858	C	154
G	||	0.074	0.101	0.825	C	155
Y	||	0.103	0.237	0.660	C	156
Y	||	0.114	0.362	0.524	C	157
H	||	0.137	0.455	0.408	E	158
F	||	0.148	0.396	0.456	C	159
S	||	0.203	0.307	0.490	C	160
Y	||	0.230	0.316	0.454	C	161
S	||	0.284	0.278	0.438	C	162
V	||	0.312	0.278	0.410	C	163
V	||	0.270	0.278	0.452	C	164
R	||	0.159	0.187	0.654	C	165
G	||	0.071	0.117	0.812	C	166

C	||	0.076	0.123	0.801	C	167
D	||	0.060	0.136	0.804	C	168
R	||	0.041	0.261	0.698	C	169
I	||	0.025	0.507	0.467	E	170
V	||	0.021	0.625	0.354	E	171
P	||	0.015	0.563	0.421	E	172
V	||	0.016	0.677	0.307	E	173
D	||	0.017	0.721	0.262	E	174
V	||	0.023	0.758	0.219	E	175
Y	||	0.030	0.711	0.259	E	176
V	||	0.037	0.469	0.494	C	177
P	||	0.035	0.159	0.806	C	178
G	||	0.047	0.054	0.899	C	179
C	||	0.049	0.043	0.908	C	180
P	||	0.034	0.035	0.930	C	181
P	||	0.051	0.020	0.929	C	182
T	||	0.098	0.011	0.891	C	183
S	||	0.804	0.005	0.191	H	184
E	||	0.869	0.004	0.128	H	185
A	||	0.919	0.003	0.078	H	186
L	||	0.950	0.002	0.049	H	187
M	||	0.956	0.001	0.043	H	188
Y	||	0.958	0.001	0.042	H	189
G	||	0.952	0.001	0.047	H	190
V	||	0.952	0.001	0.047	H	191
F	||	0.955	0.001	0.043	H	192
Q	||	0.959	0.001	0.040	H	193
L	||	0.960	0.002	0.038	H	194
Q	||	0.939	0.004	0.057	H	195
R	||	0.886	0.010	0.104	H	196
K	||	0.854	0.012	0.134	H	197
M	||	0.717	0.013	0.269	H	198
R	||	0.626	0.016	0.357	H	199
N	||	0.426	0.027	0.547	C	200
T	||	0.214	0.046	0.740	C	201
K	||	0.143	0.069	0.788	C	202
I	||	0.163	0.156	0.681	C	203
T	||	0.244	0.289	0.468	C	204
R	||	0.232	0.369	0.400	C	205
M	||	0.276	0.287	0.437	C	206
W	||	0.249	0.312	0.439	C	207
Y	||	0.179	0.320	0.501	C	208
R	||	0.095	0.295	0.610	C	209
K	||	0.020	0.065	0.915	C	210

Table 9.4 ***-A prediction and a confidence value between 0 and 1 for each position in the amino acid sequence is given below-***

AA: amino acids in the NUKM sequence

PRED: Prediction

H: alpha helix, **E**: beta strand, **C**: coil.

10 Abbreviations

Ala	Alanine
Asp	Asparagine
ATP	Adenosine Triphosphate
BN-PAGE	Blue-Native Polyacryl Amide Gel Electrophoresis
bp	base pair
BSA	Bovine Serum Albumin
Cys	Cysteine
DBQ	n-Decyl-Benzoquinone
dNADH	deamino Hydronicotineamide Adenine Dinucleotide (reduced form)
DQA	2-decyl-4-quinazolinyl amine
EPR	Electron Paramagnetic Resonance
FAD	Flavin Adenine Dinucleotide
$FADH_2$	Flavin Adenine Dinucleotide reduced form
FeS	Iron-Sulphur Cluster
FMN	Flavin Mononucleotide
Glu	Glutamate
HAR	Hexaammine ruthenuim(III) chloride
Hyg	Hygromicine
kb	kilobase
LM	Lauryl Maltoside (n-dodecyl-β-D-maltoside)
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Hydronicotineamide Adenine Dinucleotide (NAD^+ reduced form)
NDH2(i)	(internal) version of the alternative NADH Dehydrogenase
OXPHOS	Oxidative Phosphorylation
PCR	Polymerase Chain Reaction
SDS	dodecylsulphate Na-salt

11 Lebenslauf

Vorname:	Aurelio
Name:	Garofano
Adresse:	Stalburgstrasse 32 60318 Frankfurt/Main E-Mail: garofa@zbc.kgu.de, privat: Aurelio.Garofano@poste.it
geboren am	23. Dezember 1973 in Sacile (PN) Italien
Hochschulstudium: 1992-1999	Studium an der Universität in Padua (Italien), "Universitá degli studi di Padova", Facoltá di Farmacia, Studiumsabschluss in Pharmazeutische Chemie.
Diplomarbeit: Nov. 1998-Nov. 1999	Diplomarbeit am CNR (National Centre of Research), Abteilung für Metallproteinen, bei Prof. Dr. Nicolini Marino / Dr. Paolo Zatta. (Padova-Italien): Biochemical effects of Al^{3+}on human platelets: relation with Alzheimer´s disease and other neuropathologies.
Promotionsstudium: Feb. 2000....	seit Juni 2000 angestellt als Doktorand am Zentrum der Biologischen Chemie, Institut für Biochemie I des Universitätsklinikums Frankfurt in der Arbeitsgruppe von Prof. Ulrich Brandt im Rahmen des SFB 472 „Molekulare Bioenergetik" Thema der Arbeit: Untersuchungen am mitochondrialen Komplex I aus Hefe Yarrowia lipolytica