Electron and Proton Transfer in NADH:Ubiquinone Oxidoreductase (Complex I) from *Escherichia coli*

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Cover art: The figure represents the overall structure of Complex I, the electron transfer pathway within the protein and the scheme of proposed mechanism explaining proton pumping by this energy transducing enzyme.

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To my family

The number of different hypotheses erected to explain a given biological phenomenon is inversely proportional to the available knowledge.

Eddington's Theory

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-VI):

I **Sinegina L.**, Wikström M., Verkhovsky M.I., and Verkhovskaya M.L. (2005) Activation of isolated NADH:ubiquinone reductase I (complex I) from *Escherichia coli* by detergent and phospholipids. Recovery of ubiquinone reductase activity and changes in EPR signals of iron-sulfur clusters, *Biochemistry 44*, 8500-6.

II Belevich G., **Euro L.**, Wikström M., and Verkhovskaya M. (2007) Role of the conserved arginine 274 and histidine 224 and 228 residues in the NuoCD subunit of complex I from *Escherichia coli*, *Biochemistry 46*, 526-33.

III **Euro L.**, Bloch D.A., Wikström M., Verkhovsky M.I., Verkhovskaya M. (2008) Electrostatic interactions between FeS clusters in NADH:ubiquinone oxidoreductase (Complex I) from *Escherichia coli*, *Biochemistry 47*, 3185-93.

IV Verkhovskaya M.L., Belevich N., **Euro L.**, Wikström M., Verkhovsky M.I. (2008) Real-time electron transfer in respiratory complex I, *Proc. Natl. Acad. Sci U. S. A 105*, 3763-7.

V **Euro L.**, Belevich G., Verkhovsky M.I., Wikström M., Verkhovskaya M. (2008) Conserved lysine residues of the membrane subunit NuoM are involved in energy conversion by the proton-pumping NADH:ubiquinone oxidoreductase (Complex I), *Biochim. Biophys. Acta 1777*, 1166-72.

VI **Euro L**., Belevich G., Verkhovsky M.I., Wikström M., and Verkhovskaya M. (2009) The role of the invariant glutamate in the catalytic site of Complex I from *Escherichia coli*, *Biochim. Biophys. Acta 1787*, 68-73.

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Helsinki, April 2009 Liliya Euro

ABBREVIATIONS AND NOMENCLATURE

ADP-ribose	adenosine 5′-diphosphoribose sodium salt
DDM	n-dodecyl β-D-maltopyranoside
DMSO	dimethyl sulfoxide
dNADH	nicotinamide hypoxanthine dinucleotide reduced sodium salt
DQ	decylubiquinone
$\Delta\psi$	electric membrane potential
$\Delta\mu_{H+}$	electrochemical proton gradient across the membrane
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
FeCy	ferricyanide
FMN	flavin mononucleotide
HAR	hexaammineruthenium (III) chloride
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IC_{50}	half maximal inhibitory concentration
K_m	Michaelis-Menten constant
LB	Luria-Bertani broth
MES	2-(N-morpholino)ethanesulfonic acid
NAD	β-nicotinamide adenine dinucleotide sodium salt from yeast
NADH	β-nicotinamide adenine dinucleotide reduced disodium salt
NDH-I	NADH dehydrogenase Type-I (proton-pumping NADH:ubiquinone oxidoreductase)
NDH-II	NADH dehydrogenase Type-II (non-proton-pumping NADH:ubiquinone oxidoreductase)
N-side	negatively charged side of the (inner mitochondrial or bacterial) membrane
P-side	positively charged side of the (inner mitochondrial or bacterial) membrane
pK_a	a logarithmic measure of the acid dissociation constant
Q	ubiquinone
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris/HCl	Tris (hydroxymethyl)aminomethane hydrochloride

The amino acid numbering and subunit nomenclature are given for *Escherichia coli* Complex I, if not mentioned otherwise.

1. INTRODUCTION

Life on our planet is powered by the energy of sunlight. Phototrophs, plants, algae and cyanobacteria, absorb it directly and convert it into the energy of chemical bonds during photosynthesis. In this complex stepwise process, photon energy is used for electron transfer from water to carbon dioxide, which results in the formation of energy-rich carbohydrates, mainly glucose, sucrose and starch, and the release of molecular oxygen. Photosynthesis is one of the most important biochemical processes in the biosphere, since it conserves solar energy in the form of organic matter. Living cells have a number of powerful catabolic pathways for the conversion of energy from this store into a primary metabolically usable form, namely a transmembrane proton electrochemical gradient. The energy released upon the controlled flow of protons down their electrochemical gradient is used for many energy-consuming processes, such as active transport of molecules across the membrane and production of ATP, the main energy intermediate in the cell.

Utilization of food molecules in order to form the electrochemical proton gradient is referred to as cellular respiration. This process of energy conversion starts from stepwise dehydrogenation of sugars, fatty acids and proteins in correspondent catabolic pathways accompanied by simultaneous reduction of several intermediate electron carriers in the cell, such as NAD^+ to NADH, quinone to quinol, and release of carbon dioxide as a final product of the disintegration of nutrients. Further, the electrons from these electron donors are transferred through the respiratory or electron transport chain, consisting of a series of transmembrane proteins, to a final electron acceptor, such as molecular oxygen. This multi-step redox reaction releasing free energy is coupled with a vectorial proton transfer across the membrane by the respiratory enzymes. In this way the proton electrochemical gradient, $\Delta\mu_{H+}$, is formed.

This concept of energy conservation in the form of a proton gradient across the membrane was proposed by Peter Mitchell in 1961 in his chemiosmotic theory and nowadays has become the paradigm in bioenergetics (Mitchell, 1961).

1.1. The respiratory chain of eukaryotes

The respiratory chain of eukaryotes is located in mitochondria and in most cases consists of four multisubunit enzymes embedded into the inner mitochondrial membrane and ubiquinone and cytochrome *c* (see for review (Saraste, 1999)) (Fig. 1). The electron passage from NADH to oxygen starts from the NADH:ubiquinone oxidoreductase or Complex I. This enzyme catalyzes the transfer of two electrons from NADH to ubiquinone and utilizes free energy released in this redox reaction for the translocation of four protons across the membrane, from the matrix to the intermembrane space (from the N-side to the P-side of the membrane). Hence Complex I serves as a proton pump with an $H^+/2e^-$ stoichiometry of 4. So far the mechanism of energy transduction by Complex I is unknown. It should also be noted, that some aerobic organisms, such as yeast *Saccharomyces cerevisiae*, have instead of Complex I an alternative NADH dehydrogenase, which also reduces ubiquinone, but does not contribute to formation of $\Delta\mu_{H+}$ (Joseph-Horne *et al.*, 2001). Ubiquinone can also be reduced by another respiratory enzyme, succinate dehydrogenase or Complex II, which serves as a 'funnel' for the electrons into the respiratory chain. Complex II accepts electrons from succinate, an intermediate product of the Krebs cycle, and transfers them to ubiquinone without coupling this reaction with proton translocation across the membrane (see for reviews (Cecchini, 2003; Horsefield *et al.*, 2004)). Ubiquinone reduced by Complex I or Complex II donates electrons to the ubiquinol:cytochrome c oxidoreductase (bc_1 complex) or Complex III, which utilizes them for the reduction of cytochrome *c*. Complex III contributes to formation of $\Delta\mu_{H+}$ and works as a Q-loop. This means that a proton uptake from the N-side and its release on the P-side is based on the redox chemistry of quinone, but not on direct translocation of a proton across the membrane part of the protein. Complex III has two quinone-binding sites located on opposite sides of the membrane. Oxidation of quinol accompanied by a release of two protons takes place on the P-side of the membrane. Complex III splits two electrons accepted from one quinol molecule. One of these electrons is used for the reduction of a molecule of water-soluble one-electron carrier cytochrome *c*, and another is transferred through cofactors of the enzyme to the opposite site of the membrane where it is used for the reduction of another molecule of quinone to semiquinone.

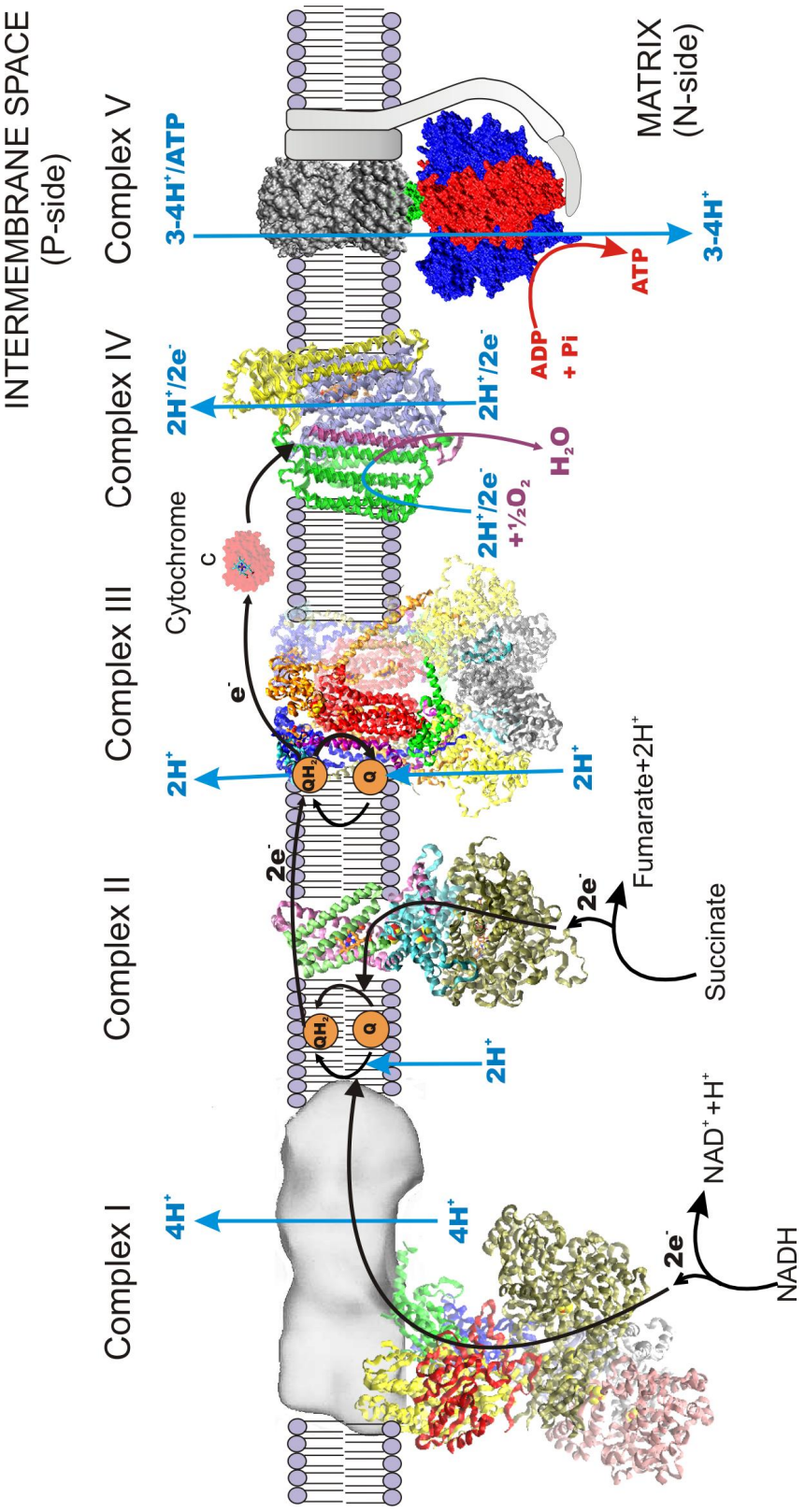

Figure 1. Schematic representation of the mitochondrial respiratory chain and ATP syntase. The picture is based on the 3D-structures 2FUG, 1YQ3, 1BGY, 2B4Z, 1QLE, 1BMF, 1YCE. The image of the hydrophobic domain of Complex I comes from the electron microscopy images of the intact enzyme from *N. crassa* (Guenebaut, 1997). Ubiquinone is depicted as an orange circle.

Oxidation of the next molecule of quinol on the P-side provides a second electron to complete the reduction of semiquinone with simultaneous uptake of two protons from the N-side of the membrane. Thus, proton translocation by Complex III is based on vectorial chemistry and has a stoichiometry of $2H^+/2e^-$ (for reviews see (Crofts, 2004; Osyczka *et al.*, 2005)). The reduced cytochrome *c* donates an electron to the last enzyme in the respiratory chain, cytochrome *c* oxidase or Complex IV, which then reduces the final electron acceptor, molecular oxygen, into water. Cytochrome *c* oxidase works as a proton pump since it translocates two protons across the membrane per electron accepted from cytochrome *c* (reviewed in (Wikström and Verkhovsky, 2006; Zaslavsky and Gennis, 2000). The redox-driven proton transfer by complexes I, III and IV generates the electrochemical proton gradient across the membrane, which has two components: electrical, $\Delta\psi$, and osmotic, ΔpH. In mitochondria the energy stored in the form of an electrochemical proton gradient is used presumably by ATP synthase for ATP synthesis from ADP and inorganic phosphate (for the review Walker and Dickson, 2006). This process is known as oxidative phosphorylation. ATP serves as the molecular 'currency' of the energy in the cell. However, one has to keep in mind that ATP hydrolysis is accompanied by the release of free energy only because the living cell keeps the mass–action ratio between the substrate, ATP, and products, ADP and phosphate, shifted by several orders of magnitude from the equilibrium through constant synthesis of ATP (Nicholls, 1992). Although some fraction of ATP is available from substate-level phosphorylation (for example, in glycolysis), the energy for the synthesis of the major fraction is produced by the respiratory chain.

1.2. The respiratory chain of prokaryotes

In prokaryotes the respiratory chain is located in the cytoplasmic membrane. The main difference between mitochondrial and bacterial respiratory chains is that the latter is branched, variable and inducible by growth conditions. Bacteria have a wide diversity of the membrane-bound dehydrogenases through which electrons can enter the respiratory chain (Anraku, 1988; Ingledew and Poole, 1984). For example, under aerobic conditions they could be: NADH:ubiquinone oxidoreductase and succinate dehydrogenase, counterparts of mitochondrial Complex I and Complex II; lactate dehydrogenase,

glycerophosphate dehydrogenase. All mentioned dehydrogenases reduce the membrane quinone pool, but only Complex I contributes to the formation of $\Delta\mu_{H+}$. Quinol is a crossroad for the electron flux in bacterial respiratory chains, because it can be oxidized not only by an analog of mitochondrial bc_1 complex (Complex III), but also by the enzymes catalyzing the final electron transfer step. Under aerobic conditions these are terminal oxidases, which catalyse reduction of oxygen to water. Under anaerobic conditions a bacterial cell utilizes other final electron acceptors, such as dimethyl sulfoxide, fumarate, trimethylamine N-oxide, nitrite or nitrate, which are reduced by the enzymes designated as terminal reductases (Anraku, 1988; Ingledew and Poole, 1984).

The present work focuses on the study of Complex I. Since the enzyme used for the research was isolated from aerobically grown *Escherichia coli* cells, it is worth describing the organization of the respiratory chain of this bacterium in more detail. The oxygen-dependent respiratory chain of *E. coli* has two distinct NADH:ubiquinone oxidoreductases: NDH-I or Complex I and NDH-II or NADH dehydrogenase type-II (Calhoun and Gennis, 1993; Matsushita *et al.*, 1987). NDH-II consists of one polypeptide with noncovalently bound FAD as a redox cofactor (Jaworowski *et al.*, 1981a; Jaworowski *et al.*, 1981b). This enzyme catalyses ubiquinone reduction but does not couple this reaction to proton translocation. Electrons can also enter the respiratory chain of *E. coli* through Complex II or succinate:ubiquinone oxidoreductase, which consists of four subunits containing five redox groups: covalently bound FAD, three Fe-S clusters and B-type heme (Kita *et al.*, 1989). The respiratory chain of *E. coli* is simplified because of the absence of an analog of mitochondrial bc_1 complex or Complex III (Ingledew and Poole, 1984). The reduced ubiquinone donates the electrons directly to an oxidase catalyzing reduction of oxygen to water. *E. coli* has two types of terminal oxidases, namely cytochrome *bd* quinol oxidase and cytochrome bo_3 quinol oxidase, which have different affinities to oxygen (Ingledew and Poole, 1984). Under high tension of oxygen presumably bo_3 quinol oxidase is expressed, which works as a proton pump with a stoichiometry of $2H^+/e^-$. This enzyme consists of four polypeptides and contains copper (Cu_B), B-type low-spin heme and O-type high-spin heme, the latter of which reacts with oxygen (Matsushita *et al.*, 1983; Salerno *et al.*, 1990). Under low concentration of oxygen in the medium *bd* quinol oxidase is expressed. This enzyme consists of two subunits and has B- and D-type high-spin hemes in addition to B-type low-spin heme,

which accepts electrons from quinol (Green *et al.*, 1986; Miller and Gennis, 1983). The *bd* quinol oxidase has a higher affinity to oxygen than bo_3 type oxidase, but does not pump protons (Miller and Gennis, 1985; Puustinen *et al.*, 1991), though it contributes to the generation of an electrochemical proton gradient due to the operation of the redox-loop mechanism.

2. THE STRUCTURE AND FUNCTION OF COMPLEX I

Complex I is the first member in the respiratory chain of mitochondria and many aerobic bacteria. The enzyme catalyses the transfer of two electrons from NADH to ubiquinone coupled with the translocation of four protons across the membrane, from the matrix side of mitochondria or cytoplasm of bacteria (negative side of the membrane) to the intermembrane space or bacterial periplasm (positive side of the membrane). This reaction contributes to the formation of the proton motive force generated by the respiratory chain. The overall reaction catalyzed by Complex I can be described by the following equation:

$$NADH_{(N\text{-}side)} + H^+ + Q + 4H^+_{(N\text{-}side)} \rightarrow NAD^+_{(N\text{-}side)} + QH_2 + 4H^+_{(P\text{-}side)}$$

2.1. The overall structure of Complex I

Complex I is the largest and the most complicated of all respiratory enzymes. In mammalian mitochondria the enzyme is composed of 45 different subunits with a total molecular mass of 980 kDa (Carroll *et al.*, 2006). In contrast bacterial Complex I or NDH-1 is two times smaller in size and has 13-14 subunits with a total mass of approx. 550 kDa, but what is important, all of them have counterparts in mitochondrial enzyme (Friedrich *et al.*, 1995; Leif *et al.*, 1993; Leif *et al.*, 1995; Yagi *et al.*, 1998). Seven of these subunits are highly hydrophobic and in eukaryotes they are encoded by mitochondrial genome (Weidner *et al.*, 1993). Another seven subunits are hydrophilic and contain all redox groups of Complex I, namely the flavin and a number of iron-sulfur clusters (Finel *et al.*, 1992; Wang *et al.*, 1991; Weiss *et al.*, 1991) (Table 1). In eukaryotes hydrophilic subunits are nuclear encoded (Carroll *et al.*, 2002; Hirst *et al.*, 2003). Thus, in both mitochondrial and bacterial Complex I the catalytic core is composed of 14 'minimal' subunits which are sufficient for energy transduction (Leif *et al.*, 1993). The remaining up to 31 subunits in eukaryotic Complex I are called additional or accessory and encoded by the nucleus (Carroll *et al.*, 2006; Fearnley *et al.*, 2007). Their function is not yet clear. In bovine enzyme, 14 of accessory subunits, classified as single transmembrane domain proteins, are probably needed for assembly of the membrane

subunits of Complex I and the stability of the enzyme (Abdrakhmanova *et al.*, 2004). In the yeast *Y. lipolytica* one of the accessory subunits tightly binds NADPH, which is needed for the stability of the enzyme (Abdrakhmanova *et al.*, 2006). In addition, for several subunits a regulatory role has been proposed (Fearnley *et al.*, 2001; Papa *et al.*, 2002).

Single particle analysis of Complex I isolated from a number of eukaryotic (Guenebaut *et al.*, 1997; Djafarzadeh *et al.*, 2000; Grigorieff, 1998; Sazanov and Walker, 2000; Zickermann *et al.*, 2003) and prokaryotic (Peng *et al.*, 2003; Sazanov *et al.*, 2003) sources revealed the unusual L-shape structure of the enzyme, which is composed of two nearly perpendicular standing domains, one hydrophilic and another hydrophobic. The former one contains all redox cofactors and protrudes into the mitochondrial matrix or cytoplasm in bacteria. The latter one is embedded into the membrane. It was noted that the particle size of a bacterial enzyme is smaller in comparison to that of the mitochondrial enzyme due to the absence of additional subunits (Guenebaut *et al.*, 1998).

Since Complex I from bacteria and Complex I from mitochondria are structurally and functionally similar in terms of catalytic core and contain equivalent redox groups, the bacterial Complex I with its 14 subunits is considered a useful simple model for structure-functional studies of mitochondrial enzyme.

Complex I has been under investigation for more than 40 years (Hatefi *et al.*, 1962). For a long time bovine heart mitochondria or the fungus *Neurospora crassa* were used as a source for purification of mitochondrial enzyme. Recently, the yeast *Yarrowia lipolytica* was introduced as a model organism in studies of eukaryotic Complex I (Kerscher *et al.*, 2002). Complex I is present in bacteria, for example, in *Escherichia coli*, *Thermus thermophilus*, *Paraccocus denitrificans* and *Rhodobacter capsulatus* (Yagi *et al.*, 1998). The advantage of bacterial systems for studying Complex I is the relative simplicity of genetic manipulation of prokaryotes. However, the main obstacle in studies of the most bacterial NDH-I is the instability of the protein upon solubilization with detergent (Dupuis *et al.*, 1995; Herter *et al.*, 1997; Yagi, 1986).

Table 1

Central subunits of Complex I (NDH-I in bacteria)

Bovine Complex I	*P. denitrificans* and *T. thermophilus* NDH-I	*E. coli* and *R. capsulatus* NDH-I	Domain	Sub-complex	Cofactors	Names of Fe-S centers
51-kDa	Nqo1	NuoF	Hydrophilic domain	DF	FMN [4Fe-4S]	N3
24-kDa	Nqo2	NuoE			[2Fe-2S]	N1a
75-kDa	Nqo3	NuoG			[2Fe-2S] 2x[4Fe-4S] [4Fe-4S]	N1b N4, N5 N7[b]
49-kDa	Nqo4	NuoD[α]		CF		
30-kDa	Nqo5	NuoC[α]				
PSST	Nqo6	NuoB			[4Fe-4S]	N2
TYKY	Nqo9	NuoI			2x[4Fe-4S]	N6a, N6b
ND1	Nqo8	NuoH	Hydrophobic domain	MF		
ND2	Nqo14	NuoN				
ND3	Nqo7	NuoA				
ND4	Nqo13	NuoM				
ND4L	Nqo11	NuoK				
ND5	Nqo12	NuoL				
ND6	Nqo10	NuoJ				

[α]In some bacteria, NuoC and D are fused, [b] this cluster present only in some bacteria

Abbreviations: DF, dehydrogenase fragment; CF, connecting fragment; MF, membrane fragment

So far the intact bacterial enzyme has been purified only from *Escherichia coli* (David *et al.*, 2002; Holt *et al.*, 2003; Leif *et al.*, 1995) (**Paper I, II**), the closely related *Klebsiella pneumoniae* (Krebs *et al.*, 1999), and the extreme thermophile *Aquifex aeolicus* (Peng *et al.*, 2003), while in other bacteria Complex I has been studied in situ (Di Bernardo and Yagi, 2001; Kao *et al.*, 2004b), or in fragments (Sazanov and Hinchliffe,

2006). Although all studied prokaryotic and eukaryotic forms of Complex I have similar core subunits, there is no unified nomenclature established for them. In mitochondrial enzyme hydrophilic nuclear encoded subunits are named according to their molecular masses while hydrophobic mitochondrial DNA-encoded subunits are named 'ND' with a corresponding number. Subunits of bacterial NDH-I from *Paracoccus denitrificans* and *Thermus thermophilus* are named as Nqo, from *Escherichia coli* and *Rhodobacter sphaeroides* – as Nuo (Table 1).

It is worth noting that structure-functional studies of Complex I also have medical importance since numerous neurodegenerative disorders in humans, such as Lebers Hereditary Optic Neuropathy (LHON), Leigh syndrome and Parkinson's disease, are associated with mutations in Complex I or its deficiency (Robinson, 1998; Loeffen *et al.*, 2000; Triepels *et al.*, 2001). Pathogenic mutations in Complex I have been found in all fourteen "core" subunits as well as in accessory nuclear encoded subunits (Lebon *et al.*, 2003; Lebon *et al.*, 2007; Loeffen *et al.*, 1998; Martin *et al.*, 2005; McFarland *et al.*, 2004; Ugalde *et al.*, 2007; van del Heuvel *et al.*, 1998; Petruzzella and Papa, 2002).

2.1.1. The hydrophilic domain

The NDH-I from *E. coli* is commonly used as a minimal model to study Complex I. The genes encoding all subunits of *E. coli* Complex I named *nuo*A-N are organized in the *nuo* operon (Weidner *et al.*, 1993). The same order of genes is found in other species of bacteria (Friedrich *et al.*, 1995; Weidner *et al.*, 1993; Xu *et al.*, 1993). It should also be noted that in *E. coli* Complex I two genes — *nuo*C and *nuo*D — are fused (Blattner *et al.*, 1997; Braun *et al.*, 1998), which results in a 13 subunit enzyme. Anyhow, this fusion has been found only in a few bacterial species.

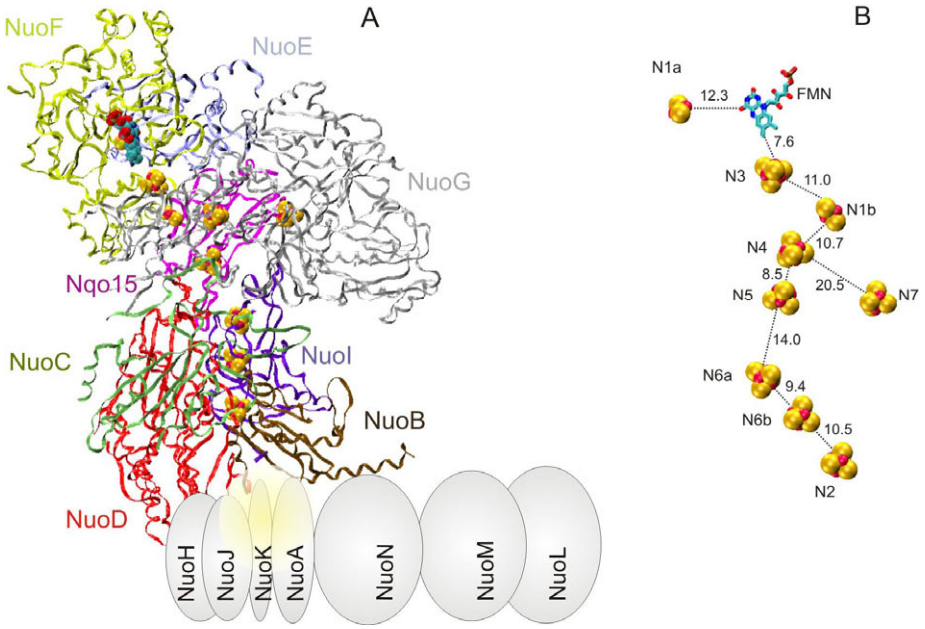

Figure 2. A. The overall structure of the catalytic core of Complex I. The picture was prepared based on the crystal structure of the hydrophilic domain of *T. thermophilus* Complex I (Protein Data Bank entry 2FUG (Sazanov and Hinchliffe, 2006)). The arrangement of subunits within the hydrophobic domain is based on currently available data (see text). Subunits are marked according to *E. coli* nomenclature except for the subunit Nqo15, the presence of which could be specific only for thermophiles. The yellow transparent circle marks the place of the tentative quinone-binding site. **B.** Arrangement of redox groups within the hydrophilic domain. Edge-to-edge distances are given in Å. The picture was prepared using VMD molecular visualization software (Humphrey *et al.*, 1996).

The hydrophilic domain of *E. coli* Complex I is composed of six subunits, namely NuoE, NuoF, NuoG, NuoCD, NuoI and NuoB, which contain all redox groups of the enzyme (Table 1). Recently, the 3D crystal structure of this domain from *T. thermophilus* enzyme has been resolved by X-ray analysis (Sazanov and Hinchliffe, 2006) (Fig. 2A). The resolved structure shows the arrangement of the core subunits within the hydrophilic domain and redox groups that they contain. The iron-sulfur clusters form an intramolecular electron transport chain, which spans through the whole length of the hydrophilic domain and provides a pathway for the electron transfer from the flavin, located on the top of the peripheral arm to the ubiquinone bound in the membrane domain of the enzyme. Thus, the function of the hydrophilic domain is the oxidation of the NADH

and electron delivery to the quinone-binding site. It should also be noted that the crystal structure revealed the presence of an additional subunit in the hydrophilic arm of *T. thermophilus* Complex I (Hinchliffe *et al.*, 2006; Sazanov and Hinchliffe, 2006). This new subunit was designated as Nqo15, according to *T. thermophilus* nomenclature. Although Nqo15 interacts with subunits Nqo3, Nqo2, Nqo1, Nqo9 and Nqo4 (NuoG, NuoE, NuoF, NuoI and NuoCD in *E. coli*) it is not a part of the operon encoding the bacterial enzyme. It has been suggested that permanent binding of Nqo15 to Complex I is needed for the stability of the enzyme in thermophiles (Sazanov and Hinchliffe, 2006). Since this subunit has homology to the frataxin protein family, it can also be involved in storage of iron, which is used for reconstitution of the neighboring iron-sulfur clusters N3 and N1a (Hinchliffe *et al.*, 2006; Sazanov and Hinchliffe, 2006).

Sequence analysis of the 'central' subunits of Complex I has prompted the suggestion that this protein has evolved from the preexisting modules (Friedrich and Scheide, 2000; Friedrich and Weiss, 1997). This conclusion correlates with the experimental observation that upon treatment with a detergent the isolated bacterial Complex I can be cleaved into three big fragments (Leif *et al.*, 1995). Three hydrophilic subunits, NuoE, NuoF and NuoG, are similar to non-energy converting NAD^+-reducing hydrogenases (Pilkington *et al.*, 1991) and comprise the NADH-dehydrogenase fragment of the hydrophilic domain of Complex I (Leif *et al.*, 1995) (Table 1). This three subunit fragment serves as an electron input of the protein (Braun *et al.*, 1998). It is also capable of catalyzing an artificial reaction of electron transfer from NADH to a number of water-soluble electron acceptors, such as FeCy and HAR (Vinogradov, 1998), which are bound at a specific site close to the flavin and accept electrons most probably from this redox cofactor (Dooijewaard and Slater, 1976; Gavrikova *et al.*, 1995; Sled' and Vinogradov, 1993; Zickermann *et al.*, 2000). The reduction of artificial electron acceptors is insensitive to specific inhibitors of Complex I and not coupled to proton transfer. The connecting fragment of the hydrophilic domain consists of NuoCD, NuoB and NuoI subunits, and is located between the NADH-dehydrogenase and membrane fragments. These subunits have homologues in the family of membrane-bound dehydrogenases (Friedrich *et al.*, 1993; Weidner *et al.*, 1993). NuoB and NuoD have an intrinsic homology with small and big subunits of bacterial [NiFe] hydrogenases (Albracht, 1993; Volbeda *et al.*, 1995). Two fragments comprising the hydrophilic domain can be

easily distinguished on the resolved crystal structure (Fig. 2A) (Sazanov and Hinchliffe, 2006). The third fragment in Complex I is the membrane domain which serves as a transporter module. It contains seven subunits three of which are homologues to cation/proton antiporters (Friedrich and Scheide, 2000) (see below).

2.1.2. The hydrophobic domain

Present knowledge of the structure of the hydrophobic domain is limited, but this part of the enzyme must be involved in quinone binding and also be responsible for proton pumping. The membrane arm of *E. coli* Complex I is composed of seven hydrophobic subunits, small NuoA, NuoH, NuoJ, NuoK and large NuoL, NuoM and NuoN, which have been predicted to form approximately 60 α-helices spanning the membrane (Bernardo *et al.*, 2000; Kao *et al.*, 2002; Kao *et al.*, 2003; Mathiesen and Hagerhall, 2002; Roth and Hagerhall, 2001). The 3D-structure of the membrane domain is not yet known. Tentative assignment of subunit localization within the membrane domain has been done based on the following: the results of cross-linking experiments (Di Bernardo and Yagi, 2001; Kao *et al.*, 2004b); mutagenesis (Bai and Attardi, 1998; Cardol *et al.*, 2002; Sazanov *et al.*, 2000); fragmentation of Complex I with detergents (Holt *et al.*, 2003; Sazanov *et al.*, 2000); cryo-electron microscopy of two-dimensional crystals of the membrane part of Complex I (Baranova *et al.*, 2007a; Sazanov and Walker, 2000); and single-particle analysis of the protein fragmentized by detergent (Baranova *et al.*, 2007b) (Fig. 2A). It has been suggested that subunits NuoA, H, J and K form the proximal part of the membrane arm. In other words, they are located in the area of the junction between two domains where the quinone-binding site has been proposed to reside (Sazanov and Hinchliffe, 2006; Sazanov, 2007). The site-directed mutagenesis of the conserved intramembrane acidic amino acid residues in NuoA (Kao *et al.*, 2004a), NuoJ (Kao *et al.*, 2005a), NuoH (Kurki *et al.*, 2000) and NuoK (Kao *et al.*, 2005b; Kervinen *et al.*, 2004) has revealed that these subunits are involved in the process of quinone reduction. Two of the large subunits, NuoM and NuoL, are located in the distal part of the membrane domain and clearly separated from the others (Baranova *et al.*, 2007a; Baranova *et al.*, 2007b). The remaining NuoN, even if it could be considered to be located in the middle of the membrane domain, tends to be adjacent more to the proximal than to the distal part

of the membrane domain (Baranova *et al.*, 2007a). Sequence analysis of the NuoM, N and L has revealed that they are similar to each other and related to the family of multisubunit K^+ or Na^+/H^+ antiporters (Fearnley and Walker, 1992; Hiramatsu *et al.*, 1998; Kikuno and Miyata, 1985; Mathiesen and Hagerhall, 2002). It is very probable that these subunits are involved in proton translocation by Complex I. This suggestion is also favored by the results of site-directed mutagenesis of the NuoM and NuoN subunits (Amarneh and Vik, 2003; Torres-Bacete *et al.*, 2007).

Native inhibitors of Complex I are being widely used for probing the structure and function of the membrane domain of the protein. They are hydrophobic or amphipathic naturally occurring compounds which occur naturally and interact within the hydrophobic part of the enzyme and compete with the native electron acceptor for the binding site. Inhibitors were divided into three groups according to their steady-state inhibition kinetics (Degli Esposti, 1998). The first group is comprised of antagonists of ubiquinone (type A). Members of the second group displace ubisemiquinone (type B) and members of the third (type C) are antagonists of the formed ubiquinol (Degli Esposti, 1998). For example, piericidin A belongs to type A inhibitors; the classical inhibitor of mitochondrial Complex I, rotenone – to type B, and capsaicin A – to type C. However, it should be noted that in contrast to mitochondrial Complex I some of its bacterial analogs are not sensitive to rotenone (Friedrich *et al.*, 1994). The most potent competitive inhibitors of quinone reductase activity are the members of the annonaceous acetogenin family (type A) (Degli Esposti *et al.*, 1994; Shimada *et al.*, 1998), which inhibit mitochondrial and bacterial Complex I in the nanomolar concentration range (Degli Esposti *et al.*, 1994) (**Paper II**). One of the commonly used representatives of this family is rolliniastatin (Hui *et al.*, 1989). Studies of the direct competition between inhibitors of different types have revealed that they have overlapping binding sites (Friedrich *et al.*, 1994; Okun *et al.*, 1999; Tormo and Estornell, 2000). It has been concluded that Complex I has a single pocket in the membrane domain which accommodates the quinone-binding site as well as binding sites for specific inhibitors with a different structure. Thus far there is no consensus on subunits comprising the quinone- and inhibitor-binding sites. Based on the resolved crystal structure of the hydrophilic domain of *T. thermophilus* Complex I it has been suggested that subunits Nqo4 and Nqo6 (NuoB and NuoD in *E. coli*) from the connecting fragment accommodate the head group

of quinone (Sazanov and Hinchliffe, 2006), while subunits located in the proximal part of the membrane arm should accommodate its hydrophobic tail. This suggestion is in line with results of the mutagenesis work on *R. capsulatus* Complex I which revealed several mutations in the NuoD subunit that cause resistance towards potent inhibitors of the quinone-binding site, piericidin and rotenone, and decrease the rate of quinone reduction (Prieur *et al.*, 2001; Dupuis *et al.*, 2001). Photolabelling of Complex I showed that the NuoB (Schuler and Casida, 2001) and NuoH (Murai *et al.*, 2007; Schuler and Casida, 2001) subunits are indeed involved in the binding of quinone and inhibitors. Unexpectedly, NuoL (Nakamaru-Ogiso *et al.*, 2003) and NuoM (Gong *et al.*, 2003), located in the distal part of the hydrophobic domain were also labeled. These results imply the presence of at least two quinone-binding sites in Complex I. Since these findings are controversial, further experiments are clearly needed.

2.2. Redox groups

2.2.1. Flavin

Flavin mononucleotide is the primary electron acceptor in Complex I. It simultaneously accepts two electrons and a proton in the form of a hydride from NADH (Ghisla and Massey, 1989) and serves as a converter between strictly two-electron donor NADH and strictly one-electron carriers, iron-sulfur clusters (Sled' *et al.*, 1994). Flavin is non–covalently bound (Rao *et al.*, 1963) to the NuoF subunit (Table 1) (Pilkington *et al.*, 1991) which is located at the top of the hydrophilic domain (Fig. 2) (Sazanov and Hinchliffe, 2006). Using a photoactive arylazido derivative of NAD^+ it was shown that the NADH-binding site also resides within the same subunit (Chen and Guillory, 1981; Chen and Guillory, 1984). Indeed, the crystal structure of the hydrophilic domain of *T. thermophilus* Complex I showed that the FMN is located in a cavity which is large enough to accommodate one molecule of NADH (Sazanov and Hinchliffe, 2006).

Studies have also been made into the thermodynamic properties of the flavin in mitochondrial Complex I. The midpoint redox potentials of two one-electron transitions of the flavin, $E_{m,7.5}{}^{1/0}$ and $E_{m,7.5}{}^{2/1}$, were found to have values of -415 mV and -336 mV,

respectively (Sled' *et al.*, 1994). The midpoint potential of the two-electron reduction of the flavin, $E_{m,7.0}^{2/0}$, was determined to be about – 340 mV (Barker *et al.*, 2007; Esterhazy *et al.*, 2008; Sled' *et al.*, 1994). We obtained a similar value for the flavin in *E. coli* Complex I (– 350 mV, pH 7.5) (**Paper III**). The midpoint potential of FMN in Complex I is approx. 130-140 mV lower than that of the free flavin in an aqueous solution (– 207 mV, pH 7.0) (Draper and Ingraham, 1968). This downshift is probably needed for matching the midpoint potential of a donor, NADH, which has the E_m value of – 335 mV, pH 7.5 (Zu *et al.*, 2003; Clark, 1960). A close inspection of the flavin-binding site revealed that the FMN is surrounded by four strictly conserved acidic amino acid residues, which could be responsible for maintaining its low redox potential (PDB entry 2FUG, chain 1). A similar case of downshifting of the redox potential of non-covalently bound flavin by acidic amino acid residues has been described for flavodoxin from *Clostridium beijerinckii* (Bradley and Swenson, 1999).

2.2.2. Iron-sulfur clusters

Complex I derived from most sources contains eight iron-sulfur clusters (Fearnley and Walker, 1992; Weidner *et al.*, 1993; Yagi *et al.*, 1993), in accordance with the number of conserved sequence motifs. However, in some bacteria, such as *E. coli* and *T. thermophilus*, Complex I carries one additional iron-sulfur cluster (Yano *et al.*, 1997; Friedrich, 1998). The most reliable methodology for detecting iron-sulfur clusters and studying their properties is EPR spectroscopy at low temperatures (Palmer, 1985). Using this method, individual spectra of five Fe-S centers have been resolved in bovine enzyme reduced with NADH (Ohnishi, 1998). They have been designated as N1, N2, N3, N4, and N5, according to their spin relaxation rates (the higher the cluster number, the lower the optimal temperature at which the spectrum of the cluster can be seen). Complex I has two binuclear clusters named N1a and N1b. In mitochondrial enzyme they have different redox properties (see below) and only N1b can be detected after reduction of the protein with NADH, whereas in bacterial enzyme signals derived from both clusters can be observed. Since binuclear clusters have slow spin relaxation rates, their spectra can be detected and studied separately from tetranuclear centers at higher temperatures (>30K). All other clusters in Complex I are tetranuclear, and can be seen at temperatures lower

than 20 K and high microwave power. In principle, all iron-sulfur clusters present in a protein should be EPR visible when they are reduced — present in the paramagnetic state — but for some unknown reasons, this is not the case for Complex I (Ohnishi, 1998). Moreover, the number of EPR detectable clusters varies with the source of the enzyme. Thus, five clusters were determined in *Y. lipolytica* (Djafarzadeh *et al.*, 2000) and *E. coli* Complex I (Leif *et al.*, 1995), and only four in the enzyme from *N. crassa* (Wang *et al.*, 1991). Identification of the subunit localization of individual Fe-S clusters (Table 1) has been done on the basis of EPR analysis of a) the protein with disrupted subunits, b) subcomplexes of Complex I and c) its overexpressed and purified individual subunits (see below).

It has been shown that the N1a cluster is located in the NuoE (Nqo2/24 kDa) subunit (Yano *et al.*, 1994a; Yano *et al.*, 1994b; Yano *et al.*, 1995); N3 resides in the NuoF subunit (Nqo1/51kDa) where the NADH- and FMN-binding sites are located (Fecke *et al.*, 1994; Yano *et al.*, 1996); and that the NuoG (Nqo3/75kDa) contains one binuclear center N1b (Yano *et al.*, 1995), and two tetranuclear clusters, N4 (Yano *et al.*, 1995) and N5 (Yano *et al.*, 2003).

The NuoB (Nqo6/PSST) subunit has a unique sequence motif for binding of the tetranuclear cluster N2 (Ahlers *et al.*, 2000; Duarte *et al.*, 2002; Flemming *et al.*, 2003). The EPR spectra of two tetranuclear clusters have been detected in the overexpressed and purified subunit NuoI from *P. denitrificans* (Ohnishi, 1998). These clusters have also been studied using UV/Vis spectroscopy, and have been designated as N6a and N6b (Rasmussen *et al.*, 2001).

All these studies were the basis for the assignment of Fe-S clusters in the resolved crystal structure of the hydrophilic domain of *T. thermophilus* Complex I (Sazanov and Hinchliffe, 2006). The 3D-structure also showed the position of additional cluster ligated by the NuoG subunit. This center was named as N7. At the present time it is clear that seven clusters, N3, N1b, N4, N5, N6a, N6b and N2 compose a chain through which electrons are transferred from the flavin to the ubiquinone, while clusters N1a and N7 are placed outside of this chain (Fig. 2B) (Sazanov and Hinchliffe, 2006). Recently it has been shown that cluster N7 is not involved in electron transfer, but needed for stability of the enzyme (Pohl *et al.*, 2007). Considering redox properties of iron-sulfur clusters, it is widely accepted that most of them, N1b, N3, N4, N5, N6a and N6b, have a

pH-independent midpoint potential of about – 250mV (pH 7.0) (Ingledew and Ohnishi, 1980; Ohnishi, 1998; Rasmussen *et al.*, 2001). Therefore these centers are called isopotential. The midpoint potential of the binuclear cluster N1a in mitochondrial Complex I was found to have the very low value of – 400 mV (Ohnishi, 1998; Zu *et al.*, 2002) and also to be pH-dependent (– 60 mV/pH) (Ingledew and Ohnishi, 1980; Meinhardt *et al.*, 1987; Zu *et al.*, 2002). However, in bacterial enzyme this cluster has a higher value of E_m, such as – 150 mV and – 250 mV in Complex I from *P. denitrificans* (Meinhardt *et al.*, 1987) and *E. coli* (Leif *et al.*, 1995), respectively. Clusters N1a and N3 are the closest to the flavin. Considering the order in which the flavin donates electrons to them, it was suggested that the first electron is going to N3, because it is located at a distance of 7.6 Å from the flavin (Sazanov and Hinchliffe, 2006) and has strong spin-spin interaction with semireduced flavin (Sled' *et al.*, 1994). The N1a cluster is more distal (Fig. 2B) and it accepts the second electron from the reduced flavin. It has been suggested that this cluster may play the role of an antioxidant in Complex I, preventing accumulation of the flavosemiquinone radical which can be the source of ROS production (Sazanov, 2007).

The last center in the chain is tetranuclear N2. The midpoint potential of N2 is pH-dependent (– 60 mV/pH) (Ingledew and Ohnishi, 1980; Meinhardt *et al.*, 1987) and has the highest value among all iron-sulfur clusters in Complex I. The E_m value for N2 was reported within the range from – 30 mV (Krishnamoorthy and Hinkle, 1988) to – 220 mV (Leif *et al.*, 1995). The EPR studies of bovine enzyme revealed a magnetic interaction of N2 with ubisemiquinone radicals bound to Complex I (see below) (Magnitsky *et al.*, 2002; Ohnishi *et al.*, 1998a; Vinogradov *et al.*, 1995). A distance of 12Å has been calculated between N2 and fast relaxing ubisemiquinone (Vinogradov *et al.*, 1995). On the basis of all these findings, cluster N2 is considered an immediate electron donor for ubiquinone.

2.3. Semiquinones

Thus far EPR signals of ubisemiquinone radicals associated with Complex I have been observed in isolated bovine enzyme (Suzuki and King, 1983) and in submitochon-

drial particles derived from bovine heart (De Jong *et al.*, 1994; De Jong and Albracht, 1994; Kotlyar *et al.*, 1990; Magnitsky *et al.*, 2002; Salerno *et al.*, 1979; van Belzen *et al.*, 1997; Vinogradov *et al.*, 1995). Two distinct species of ubisemiquinone have been identified, named SQN_{fast} and SQN_{slow}, based on their spin relaxation rate (Vinogradov *et al.*, 1995; Magnitsky *et al.*, 2002). Extremely fast relaxing SQN_{fast} can be detected only in tightly coupled membrane particles. It is sensitive to the electrochemical proton gradient across the membrane and specific inhibitors of Complex I (Magnitsky *et al.*, 2002; Vinogradov *et al.*, 1995). A fast rate of relaxation of SQN_{fast} indicates that this radical magnetically interacts with a neighboring paramagnetic center. Indeed, analysis of the effect of membrane energization on the saturation of SQN_{fast} signal and properties of the spectra of N2 cluster revealed that they are correlated with each other (De Jong *et al.*, 1994). Splitting of the characteristic signal of N2 cluster was interpreted as a spin-spin interaction with SQN_{fast}. Considering dipolar coupling between spins which leads to broadening and splitting of the signal, the distance between N2 and the fast relaxing semiquinone radical was calculated as 12 Å (Vinogradov *et al.*, 1995; Yano *et al.*, 2005).

In contrast to the fast relaxing semiquinone radical, the SQN_{slow} is not sensitive to $\Delta\mu_{H+}$ and can be detected in uncoupled SMP. Another characteristic of the slow relaxing ubisemiquinone radical is its low sensivity to Complex I inhibitors (Magnitsky *et al.*, 2002; Ohnishi *et al.*, 2005; Yano *et al.*, 2005). The SQN_{slow} also magnetically interacts with the N2 cluster, but this interaction is weak. The distance between these two centers was estimated to be about 30 Å (Ohnishi, 1998; Vinogradov *et al.*, 1995).

2.4. ROS production by Complex I

Since the redox cofactors of Complex I have the lowest midpoint potentials within the respiratory chain, it is widely considered that Complex I is one of the main sources of reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, in mitochondria (Brand *et al.*, 2004; Raha and Robinson, 2000; Turrens, 2003). Studies of the mechanism of ROS production by Complex I are of particular interest, because of the biological significance of ROS. Reactive oxygen species damage cell structures, DNA and RNA and are involved in the processes of oxidative stress and aging (Balaban *et al.*, 2005). There are two tentative sites for ROS production in Complex I: the flavin and the

ubiquinone, since both of them have been detected in the radical form (Sled' *et al.*, 1994; Yano *et al.*, 2005). There are several lines of evidence suggesting that most probably fully reduced or semireduced flavin is the source of reactive oxygen species in Complex I. It has been shown that the dependence of ROS production on the pre-set redox potential can be described as a Nernstian curve with a midpoint potential of – 360 mV (pH 7.5), which corresponds to the potential of a two-electron transition of the flavin (Kussmaul and Hirst, 2006). The pH optimum for ubiquinone reduction differs from that for superoxide generation and the latter's rate is not sensitive to the inhibitors of the quinone-binding site (Galkin and Brandt, 2005). Remarkably, it was found also that mitochondrial Complex I presumably generates $O_2^{\bullet-}$, while its bacterial counterpart – H_2O_2 (Esterhazy *et al.*, 2008).

2.5. Proton translocation stoichiometry

The first determinations of proton translocation stoichiometry of Complex I were done using the oxidant 'pulse' method introduced by Mitchell and Moyle (Mitchell and Moyle, 1967). In this technique, a defined quantity of oxygen is added into a suspension of anaerobic mitochondria. This initiates a brief respiratory burst accompanied by proton ejection from the mitochondrial matrix, which can be measured with a pH-sensitive electrode. Using selective conditions and modifications of the oxidant 'pulse' method, it was reported that Complex I translocates one proton per electron (Lawford and Garland, 1972a; Lawford and Garland, 1972b; Mitchell and Moyle, 1967; Ragan and Hinkle, 1975). Therefore it was suggested that Complex I works as a Q-loop (Lawford and Garland, 1972b; Mitchell and Moyle, 1967). However, due to the technical limitations of the used oxidant pulse approach the determined H^+/e^- ratio was underestimated (Lawford and Garland, 1973; Brand *et al.*, 1976). Later, Pozzan and co-workers based on their comparative studies of H^+/e^- stoichiometries of Complex I and Complex III, suggested that the former pumps 2 protons per electron (Pozzan *et al.*, 1979). The first direct evidence that Complex I works as a proton pump was published by Wikström (Wikström, 1984b). The experiment was simple and elegant. The intact anaerobic mitochondria were incubated with either hydroxybutyrate or succinate. The alkalinization of the matrix after the pulse of ferricyanide as an oxidant was followed

spectrophotometrically using a pH sensitive membrane permeable probe, neutral red (Junge *et al.*, 1979). Optical absorption of this probe changes proportionally in response to the change of proton concentration in the mitochondrial matrix (Wikström, 1984a). As a result, when hydroxybutyrate was used as a substrate, the linear dependence of the extent of alkalinization on the amount of added oxidant had a 2.8-times greater slope than in the case of succinate. The same result was obtained in a set of similar experiments where charge displacement was measured in response to oxidation by ferricyanide using potential sensitive dye safranine, which had been reported as a probe for measurements of mitochondrial membrane potential (Akerman and Wikström, 1976). Since at that time it was already known that the q^+/e^- ratio for the span between succinate and cytochrome c (or ferricyanide) is one (Leung and Hinkle, 1975), the obtained results indicated that the H^+/e^- and q^+/e^- ratio for the span between NADH and ubiquinone is 2. Thus it was shown that Complex I translocates 2 protons per electron (Wikström, 1984b). Later, the proton pumping stoichiometry of Complex I was measured using submitochondrial particles derived from bovine heart mitochondria (Galkin *et al.*, 1999) and finally in proteoliposomes with a reconstituted enzyme isolated from *Yarrowia lipolytica* (Galkin *et al.*, 2006). These works further supported the conclusion that the H^+/e^- stoichiometry of mitochondrial Complex I has the value of 2. Unfortunately, for bacterial Complex I the proton pumping stoichiometry is not so well defined as it is for its mitochondrial analog. The main obstacle for measurement of H^+/e^- ratio for NDH-I in the intact bacterial cells is the presence of other membrane-bound respiratory chain linked NADH-dehydrogenases (see Chapter 1.2), which do not pump protons. Their activity decreases the H^+/e^- ratio measured for Complex I. So far, there is only one report on proton pumping stoichiometry in bacterial Complex I (Bogachev *et al.*, 1996). For these measurements the authors used *E. coli* cells grown anaerobically in the presence of dimethyl sulfoxide (DMSO). Under these conditions the respiratory chain of this bacteria consists of NDH-I, terminal cytochrome *bd* oxidase and dimethyl sulfoxide reductase. On the basis of the extent of acidification in the media in response to a pulse of a defined amount of either O_2 or DMSO, the H^+/e^- stoichiometry for Complex I and Complex I *plus bd* oxidase was determined as 1.5 and 2.5, respectively. Therefore, it was concluded that Complex I pumps at least 1.5 protons per electron (Bogachev *et al.*, 1996). Low stoichiometry was explained by the fact that in anaerobic conditions menaquinone is used

in the *E. coli* respiratory chain as an electron acceptor. Since it has a more negative redox potential than ubiquinone, the energy released upon its reduction with NADH by Complex I may not be enough for the translocation of four protons across the membrane (Bongaerts *et al.*, 1995). The obtained H^+/e^- stoichiometry does not exclude the possibility that in aerobically grown *E. coli* cells this ratio is higher, but this hypothesis has not been tested yet.

2.6. Proposed mechanism of proton pumping

At the present time two kinds of hypotheses explaining the molecular mechanism of Complex I are being discussed. The first one is referred to as direct (redox-driven) coupling (Dutton *et al.*, 1998; Ohnishi and Salerno, 2005). It is based on the observation of two distinct species of ubisemiquinone associated with Complex I (Vinogradov *et al.*, 1995; Yano *et al.*, 2005) and therefore employs two types of quinone molecules, which reside in membrane dielectric. One of them is exchangeable with the membrane quinone pool, while another is tightly bound to the protein and serves as the gate and the coupling site. According to this model, tightly bound quinone accepts electrons from the N2 cluster with simultaneous acquisition of two protons from the matrix side of the membrane. The energy released upon this redox reaction is used to convert formed ubiquinol into a state in which it can release protons to the cytoplasmic side of the membrane with simultaneous transfer of electrons to the molecule of the exchangeable quinone. Then tightly bound quinone returns to the initial 'input' state in which it can be again reduced by N2 and the exchangeable quinone, after uptake of two protons from the matrix side in order to accomplish redox chemistry, thereby dissociating from the protein. Thus the proton pumping stoichiometry approaches the value of $4H^+/2e^-$ (Ohnishi and Salerno, 2005)

Another proposed mechanism of Complex I is called indirect conformation-driven coupling (Brandt *et al.*, 2003; Brandt *et al.*, 2005; Zickermann *et al.*, 2003; Yagi and Matsuno-Yagi, 2003). It hypothesizes that only one molecule of quinone is involved in the mechanism and it is exchangeable with the membrane quinone pool. The essence of this hypothesis is that the reduction of the quinone by the N2 cluster results in long-range conformational changes which lead to proton translocation by distantly located antiporter-like subunits NuoN, NuoM and NuoL. Several lines of evidence favour this

hypothesis. For example, comparison of two-dimensional crystals of *E. coli* Complex I obtained in the presence and absence of nucleotides revealed conformational rearrangements in the protein structure upon reduction with NADH (Mamedova *et al.*, 2004; Morgan and Sazanov, 2008). Large reorganization in the structure upon reduction was also detected in FT-IR experiments on Complex I (Hellwig *et al.*, 2000). It is also possible that a combination of direct and indirect mechanisms occurs in Complex I (Baranova *et al.*, 2007b; Friedrich, 2001; Sazanov *et al.*, 2000), but in any case neither of the proposed models provides a comprehensive explanation of the mechanism of proton translocation by the membrane part of this respiratory enzyme.

3. AIMS OF THE PRESENT STUDY

The main focus of this thesis was an extensive study of the electron transfer reactions in Complex I upon its reduction with NADH and an assessment of the mechanistic principle of the proton pumping by this energy transducing enzyme.

- Since the techniques used in this study require a big amount of pure and active enzyme, the first objective was to establish a conventional protocol for purification of Complex I from *E. coli* and optimize the conditions for observation of its enzymatic activity.

- The second objective was to determine accurately the midpoint potentials of the redox centers of Complex I by means of spectroelectrochemical and EPR redox titrations.

- The third objective was to resolve real time electron transfer kinetics through the chain of iron-sulfur clusters, thereby providing answers to some questions on the principles of the molecular mechanism of coupled proton translocation by Complex I.

- The fourth objective was to validate the involvement of the membrane subunit NuoM in the proton pumping process and propose the mechanism for energy coupling between the spatially separated Q-binding site and proton pumping subunits.

- The fifth objective was to assess the role of invariant Glu95 from the NADH-binding site (electron input in Complex I) and three conserved amino acid residues, Arg274, His224 and His228, located in the vicinity of the N2 cluster, the immediate electron donor for ubiquinone.

4. METHODOLOGY

4.1. Isolation of Complex I

For isolation of wild type Complex I, the *E. coli* strain MWC215 (Calhoun and Gennis, 1993) was used. It has a knockout of NDH-II that yields a 2-fold increase of the Complex I content in cytoplasmic membranes.

For the membrane preparation and further purification of Complex I, the bacterial cells were grown aerobically in a 25 L fermentor in LB medium at 37 ºC for approx. 4h until the late exponential growth phase. The detailed description of the membrane preparation can be found in **Papers II** and **VI**.

Since Complex I is stable only in a narrow pH range and sensitive to detergent concentration, its purification should be done in mild conditions. The developed protocol for Complex I purification consists of two ion-exchange chromatographies on the weak anion exchanger DEAE-Trisacryl M (BioSepra) and centrifugation in a sucrose density gradient (for details see **Papers II** and **VI)**. However, the last step has recently been replaced by gel filtration on Superdex 200 prep grade (GE Healthcare). For a single gel filtration chromatography 1ml of protein solution (10mg/ml) after two previous steps of ion exchange chromatography on DEAE-Trisacryl was applied onto a self-packed 130 ml bed volume of Superdex 200 in an XK16/70 column (GE Healthcare), equilibrated with 50 mM MES/NaOH, pH6.0, containing 150 mM NaCl, 10% sucrose, 2.5% glycerol and 0.025% DDM. The elution was performed at 0.5 ml/min flow rate in the equilibration buffer.

For site-directed mutagenesis of Complex I, the parental strain GR70N containing both NDH-I and NDH-II (Green *et al.*, 1984) was used. The details of the genetic manipulations can be found in **Papers II, V** and **VI**. Complex I from the parent GR70N strain was used as a control for the characterization of obtained mutations. In order to estimate the content of Complex I in membranes of GR70N and mutated strains and avoid interference from NDH-II, an analog of NADH, namely dNADH, was used for the activity measurements, because it is specific only for Complex I (Matsushita *et al.*, 1987).

4.2. Measurements of the enzymatic activity

The ability of Complex I to catalyze the electron transfer from NADH to an electron acceptor is considered as its enzymatic activity. It can be measured with both membrane-bound and solubilized enzymes. The assay is based on spectrophotometrical measurement of the rate of NADH oxidation by following the decrease of absorption at 340 nm where the spectrum of reduced NADH has a maximum. Therefore the activity is usually expressed in μmol of NADH oxidized by mg of the protein per minute. In vivo *E. coli* Complex I catalyzes reduction of ubiquinone-8. This activity is referred to as native. For in vitro measurements an analog of the natural electron acceptor, decylubiquinone, was usually used. Its reduction also requires the entire enzyme and is sensitive to inhibitors of Complex I. However, the measurement of the native activity can not be used for quantitative determinations of Complex I, since it is always limited by the low solubility of the acceptor in an aqueous solution and therefore a low rate of quinone exchange in the catalytic site of Complex I. In the present study we partially overcame this obstacle by adding into the reaction a mixture of purified ubiquinol bo_3 oxidase, which facilitates turnover of Complex I by fast oxidation of ubiquinol dissociating from the catalytic site.

In vitro, a number of artificial electron acceptors, such as FeCy and HAR (Vinogradov, 1998), can be utilized besides DQ. Their reduction by Complex I is much faster than that of quinones, because it employs only FMN and the NADH-binding site that are located within the NADH-dehydrogenase fragment. Therefore, the rate of such artificial reactions virtually does not change upon isolation of Complex I from the membranes, and can be used as a measure of the enzyme purity. In the present study HAR was routinely used as an artificial electron acceptor, because, in contrast to FeCy, its reduction has no effect on the optical changes at 340 nm and it does not compete with NADH for the nucleotide-binding site (Gavrikova *et al.*, 1995; Sled' and Vinogradov, 1993). In the present study we also found that a low concentration of potassium ions facilitates artificial activity of Complex I. Measurements of artificial and native enzymatic activities are described in detail in **Papers I** and **VI.**

4.3. Monitoring of proton pumping

Proton pumping activity of Complex I coupled with NADH:ubiquinone oxidoreduction can be registered either in membrane vesicles obtained from bacterial cells or in liposomes with reconstituted purified enzyme. In the former case one can follow energy dependent acidification of the interior of membrane particles by Complex I by means of the pH-sensitive fluorescent probe, acridine orange (λ_{ex}= 493 nm, λ_{em}=530 nm) and estimate the efficiency of the proton pump by comparing the initial rate of acidification with ubiquinone reductase activity of the enzyme. Besides Complex I, native *E. coli* membrane vesicles contain NDH-II and terminal bo_3 oxidase. In order to observe exclusively proton pumping activity of Complex I, dNADH was used as an electron donor, which cannot be utilized by an alternative NDH-II (Matsushita *et al.*, 1987). Also, KCN was added in order to inactivate the terminal oxidase. The measurements were performed with ionophore valinomycin and potassium ions present, in order to maximize the ΔpH component of the proton motive force generated by Complex I and reduce formation of the transmembrane potential, which inhibits proton pumping. Details of the assay are discussed in **Paper V**.

In proteoliposomes the proton pumping activity of Complex I was monitored by registration of the Δψ component of the proton motive force by means of an electric potential-sensitive dye, Oxonol VI. At physiological pH oxonols are present in an anionic water-soluble form (Smith *et al.*, 1976) which is characterized by an absorption maximum at approx. 600 nm (Bashford and Thayer, 1977). Generation of electric potential across the membrane in a way that the inside is more positive than the outside, results in Oxonol VI neutralization with simultaneous shift of its absorption maximum to 625 nm (Bashford and Thayer, 1977). Upon neutralization, Oxonol VI becomes insoluble and starts to precipitate, which results in quenching of its absorbance at 625 nm. In comparison to other oxonols, Oxonol VI is a more sensitive probe because of its short response time and large amplitude of the red shift in response to the formed electric potential (Smith *et al.*, 1976). The reconstitution of the isolated enzyme into proteoliposomes was completed in two steps. During the first one the protein was equilibrated with the mixture of soybean asolectin solubilized by sodium cholate. The formation of closed lipid bilayer vesicles containing the reconstituted protein was achieved by sequential additions of SM-2

Bio-Beads (Bio-Rad Laboratories) which slowly absorb the detergent from the protein-lipid mixture (Jasaitis *et al.*, 1999; Rigaud *et al.*, 1995). During this process Complex I is incorporated into liposomes randomly in both orientations. Estimation of the ratio between the two orientations was based on NADH:HAR oxidoreductase activity of Complex I in proteoliposomes measured with and without alamethicine. Molecules of this antibiotic form channels in biological membranes thereby providing an access for the water-soluble NADH to the liposome interior where it can be oxidized by Complex I with the hydrophilic domain oriented inwards. Description of Complex I reconstitution into liposomes and details of the assay can be found in **Paper II**.

4.4. EPR spectroscopy

Properties of iron-sulfur clusters in Complex I were studied by means of EPR spectroscopy. Each of the Fe-S centers in reduced state has an individual EPR spectrum characterized by the special parameters of the magnetic field g_x, g_y and g_z, and unique EPR property, namely relaxation time. EPR studies of Complex I are complicated since the spectra of iron-sulfur clusters overlap and have very close g-values. In order to investigate the properties of a particular cluster separately from the others one has to use selective temperature and microwave power conditions under which the signal from the center of interest will dominate in the overall spectrum of Complex I.

We used EPR spectroscopy for several purposes. First, we detected and characterized the signals of iron-sulfur clusters of Complex I reduced by an excess of NADH. This is the so-called steady-state condition of the reduced enzyme (**Paper I** and **II**). Second, we resolved the kinetics of electron transfer through the chain of iron-sulfur clusters. This was done by ultra-fast freezing of the sample in the time range from tens of microseconds to tens of milliseconds after mixing it with NADH. EPR spectra of frozen samples were analyzed to follow the redox changes of individual Fe-S clusters, which allowed us to monitor the movement of two electrons delivered by NADH in real time (**Paper IV**). Third, we determined the midpoint potentials of Fe-S clusters in the intact enzyme by means of electrochemical redox titration in the presence of mediators. In this study we used methyl viologen (E_m= − 455 mV), benzyl viologen (E_m= −360 mV), hexaammineruthenium (E_m= 50 mV) and pentaammineruthenium (E_m= −130 mV). This

choice was based on the fact that these mediators do not interact with each other and provide fast equilibration between an electrode poised to a certain potential and protein in solution. The details of the equilibrium redox titration of iron-sulfur clusters can be found in **Paper III**.

4.5. Optical spectroscopy

In order to determine the midpoint potential of the flavin, we monitored changes of its optical spectra during redox titration of purified intact Complex I. The potential within the range from −500 to − 40 mV was applied with steps of ± 20 mV for both oxidative and reductive titrations using a potentiostat. To accelerate equilibrium between the electrode and the solubilized protein a set of redox mediators was added as for titration in the EPR cell. Absorption of the oxidized flavin has a pronounced maximum at 450 nm characterized by a high extinction coefficient (ε_{450}=12.8 $mM^{-1}*cm^{-1}$) (Massey and Palmer, 1962). Iron-sulfur clusters also absorb in this region, but their contribution to the spectra is small because of their low extinction coefficients (ε_{450}= 4.1 $mM^{-1}*cm^{-1}$) (Fu *et al.*, 1994; Mayhew *et al.*, 1969). Analysis of the redox changes at 450 nm reveals the potential area where the slope of the titration curve is maximal, which corresponds to oxidation/reduction of the flavin. The first derivative from this titration curve gives a set of small overlapped peaks, corresponding to the midpoint potentials of the iron-sulfur clusters, and one sharp peak characterizing the midpoint potential of the flavin. Details of the experiment and data treatment are described in **Papers III** and **VI**.

4.6. Homology modeling of Complex I from *E. coli*

In the present study we probed the role of several conserved amino acid residues in electron and proton transfer reactions catalysed by Complex I. In order to verify the position of selected residues and explain the effect of the site-directed mutagenesis we made a prediction of molecular structure for the subunits where the selected amino acids are located. We made the homology modeling of the NuoF and NuoE subunits from *E. coli* Complex I using as templates the sequences of homologues subunits (Nqo1, Nqo2) from *T. thermophilis* enzyme with resolved structure (PDB entry 2FUG, chains 1, 2)

(Sazanov and Hinchliffe, 2006). A detailed description of the homology modeling can be found in **Paper VI**.

5. RESULTS AND DISCUSSION

5.1. Purification of Complex I

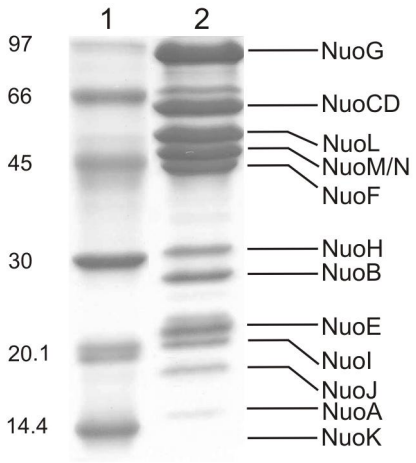

Figure 3. Coomassie Blue-stained 10-20% SDS-PAGE gel of purified wild type Complex I (lane 2, 30 μg) Lane 1: molecular mass markers (kDa).

The purification procedure for *E. coli* Complex I was developed on the basis of previously published protocols (Leif *et al.*, 1995; David *et al.*, 2002). Initially our protocol consisted of two chromatography steps on the weak anion-exchanger DEAE-Trisacril M (BioSepra) followed by centrifugation in a sucrose density gradient. Recently, the last step has been replaced by gel filtration on Superdex 200 prep grade (GE Healthcare), which has the same resolution as sucrose gradient but takes less time and is more reproducible. In contrast to published protocols (for example, Sazanov *et al.*, 2003; Flemming *et al.*, 2003) we decreased the detergent concentration during all chromatography steps, which resulted in an increased yield and higher ubiquinone reductase activity of the purified enzyme. The SDS–PAGE of the standard preparation of Complex I containing all subunits is shown in Figure 3. The assignment of the subunits is based on previously published studies (David *et al.*, 2002; Sazanov *et al.*, 2003) and our results from his-tag labeling of several subunits in wild type Complex I (our unpublished data).

5.2. Recovery of the native activity of purified Complex I

For a long time the main obstacle in research of Complex I has been the low ubiquinone reductase activity of the purified enzyme. The intactness of the enzyme can be estimated by the ratio between its artificial and natural activities (see 2.1.1 and 4.2).

The turnover of purified bovine Complex I was found to be of 900 s^{-1} and 30 s^{-1} with FeCy and ubiquinone, respectively (Finel *et al.*, 1994). So, the ratio artificial/natural activity was 30. Almost the same value of 30-40 was obtained for purified *E. coli* Complex I (David *et al.*, 2002). However, this ratio is 1.5-2.5 for the membrane-bound either mitochondrial or bacterial Complex I (Prieur *et al.*, 2001; Sled' and Vinogradov, 1993). Recently it was concluded that the quinone-reductase activity of Complex I decreases during purification due to delipidation of this membrane protein (Dröse *et al.*, 2002; Sazanov *et al.*, 2003). For example, the turnover of quinone reduction catalyzed by purified *Y. lipolytica* and *E. coli* Complex I reached the values of 120 s^{-1} (Dröse *et al.*, 2002) and 200 s^{-1} (Sazanov *et al.*, 2003) after treatment with phospholipids. It is worth noting that lipids used for the recovery of the native activity of the protein were solubilized with detergent, which might also have an effect on the activity.

In **Paper I** we studied separately the effects of DDM and phospholipids on the quinone-reductase activity of isolated Complex I.

5.2.1. Activation of purified Complex I by detergent and phospholipids

DDM itself strongly stimulated quinone reductase activity of the purified Complex I. The dependence of the activity on detergent concentration had a bell shape with a maximum at 0.005%. At this concentration the total increase of the activity was over 10-fold from the value without added DDM into the assay. The higher concentrations of the detergent most probably caused the cleavage of the protein, which explains the descending part of the dependence curve. Incubation with the phospholipids resulted in an additional 2-fold increase of the quinone reductase activity, but in this case the optimal detergent concentration in the assay decreased to a lower value of 0.0025%. As a result the native activity of the isolated Complex I can reach the value of 8 or 18 μmol NADH $mg^{-1}*min^{-1}$ (corresponding to turnovers 130 and 300 s^{-1} when calculated per flavin) in the assay with the detergent or after the activation, respectively. Since HAR reductase activity remained the same regardless the activation, of the order of 80-90 μmol of NADH oxidised by mg of protein per min, the ratios between the artificial and the native activities were found to be 10 and 4 for DDM and lipid treated enzyme, respectively. For the membrane-bound *E. coli* Complex I this value was determined as

1.5-2. The higher artificial/native activity ratio for purified activated enzyme could be explained by different properties of the natural substrate, Q8, and DQ, the artificial electron acceptor that was used for the activity measurements.

We explained the activation of the isolated Complex I by conformational rearrangements in the junction between the hydrophilic and hydrophobic domains. The binding of additional detergent and/or phospholipid molecules to the surface of the delipidated enzyme helps the protein to adopt its native state, which it has in the membrane. This might result in an increase of the accessibility of the quinone-binding site for the electron acceptor and/or improvement of the electron transfer from the N2 cluster to quinone. This premise is based on the analysis of EPR spectra (see below) and kinetic parameters of the activated enzyme. Neither different DDM concentrations nor the activation procedure changed the K_m of Complex I for ubiquinone. The activation by phospholipids resulted in only a 2-fold increase of V_{max} in comparison with DDM treated enzyme. To exclude the possibility that DDM or phospholipids altered the structure of the native DQ-binding site, we titrated the native activity of Complex I with rolliniastatin (Okun *et al.*, 1999; Tormo and Estornell, 2000) and found that affinity of this inhibitor did not change due to detergent and lipid binding.

5.2.2. Changes in EPR signals of Fe-S clusters

The paramagnetic properties of the Fe-S clusters are affected by immediate protein surroundings; therefore any structural rearrangements may modify the EPR signals of these cofactors. Since the activation of Complex I likely affects the junction between hydrophilic and hydrophobic domains, one might expect to see changes in the EPR signals from clusters residing in the connecting fragment. Indeed, the treatment of Complex I with lipids resulted in an increase in the amplitude of the signals from two fast-relaxing tetranuclear clusters. One of them can be simulated with parameters close to those reported for N2 in *E. coli* (Leif *et al.*, 1995). The other is most likely cluster N6b, which also resides in the connection fragment close to the membrane plane. The increase in these EPR signals could be due to positive shift of the values of the redox potentials of these clusters and/or magnetic decoupling of their spins from each other, or from another EPR-silent paramagnetic species.

5.3. Electron transfer kinetics in Complex I

The best way to reveal a molecular mechanism of the energy converting enzyme is to develop an ultra-fast kinetic teqnique allowing measurement single events comprising its catalytic cycle. The catalytic reaction of Complex I is extremely fast (k_{cat}~300s^{-1}), so it can not be captured by conventional techniques.

Paper IV describes the resolved dynamics of the electron transfer through the chain of iron-sulfur clusters in Complex I by following the changes in their EPR signals upon reduction of the enzyme with NADH. We trapped the reaction in the time range from tens of microseconds to tens of milliseconds by freezing the sample using a specially developed ultra-fast freeze-quenching setup (Fig. 5 in **Paper IV**). Further EPR analysis of obtained samples revealed a time dependent order of the appearance of the signals derived from individual clusters. First, we observed an appearance of the signals from clusters N1a and N2. Then the features from other clusters started to appear in the spectra in addition to an increase in the N2 and N1a signals. Since the EPR spectra of [4Fe-4S] iron-sulfur clusters overlap we chose for the analysis only those clusters, which have well defined prominent signals. We analyzed the rate of the reduction of N1a, N1b, N2 and N6b centers. Their spectra were simulated with parameters which are very close to those reported elsewhere (Friedrich, 1998; Rasmussen *et al.*, 2001; Uhlmann and Friedrich, 2005; Yano *et al.*, 1999). The signal from N6b was simulated based on our own assignment described in **Paper II** and **III**.

The EPR spectra were collected from the samples frozen in time from 90 μs to 8 ms after mixing the enzyme with NADH. The kinetic spectra of selected iron-sulfur clusters were fitted with simulated signals and their amplitudes were plotted against time (Fig. 4). The data analysis showed that the major fraction of clusters N1a and N2 undergoes a very fast reduction with a time constant (τ) of ~90 μs, implying that these clusters have the highest redox potentials. This conclusion is in line with previously determined E_m for these centers (see Chapter 2.2.2) (Brandt, 2006; Friedrich, 1998; Leif *et al.*, 1995; Ohnishi *et al.*, 1998b; Uhlmann and Friedrich, 2005). A different behavior was observed for N1b and N6b clusters. The major fraction of these clusters was reduced slowly with the time constant of τ ~1.2 ms.

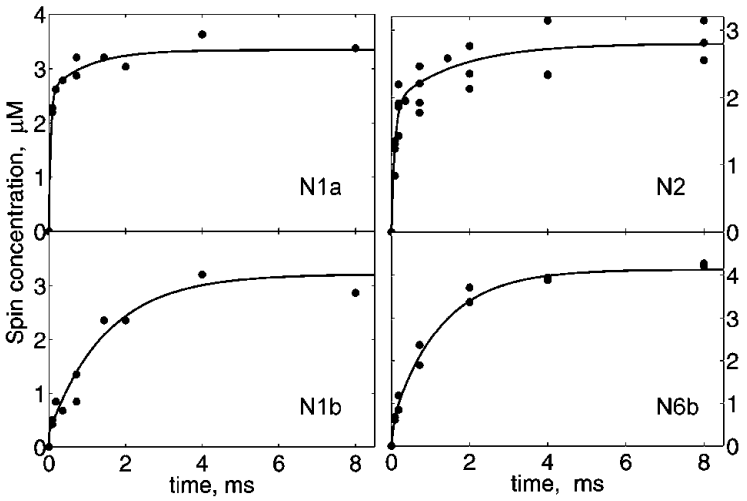

Figure 4. Kinetics of the redox changes of clusters N1a, N1b, N2 and N6b after mixing of Complex I with NADH. The spin concentration of the individual FeS clusters was plotted against time and fitted with an exponential curve. Reproduced from **Paper IV**.

According to the electron tunneling theory (Moser *et al.*, 2006) and determined distances between FeS centers in Complex I (Sazanov and Hinchliffe, 2006) the electron transfer from FMN to N2 through the entire chain of iron-sulfur clusters is expected to proceed with a time constant of ~100 µs. Since the observed rate is close to the theoretical one, it is obvious that the electron transfer in Complex I is unlikely to be coupled with other reactions such as proton transfer or conformational changes, otherwise the latter ones would slow down the process and result in a larger time constant. Since the rate of N1b and N6b reduction (τ ~1.2 ms) is several orders of magnitude lower than the rate of electron transfer between clusters, we concluded that the reduction of these clusters is limited by a single process – dissociation of the product NAD^+ from the nucleotide-binding site, where it prevents entry for the next NADH molecule, needed for further reduction of the enzyme.

Using the method of an organic extraction of quinones we found that in our preparation each molecule of Complex I contains one tightly bound quinone. However, we did not observe formation of ubisemiquinone, which might imply a high thermodynamic barrier for ubiquinone reduction to semiquinone radical. Possible implications of these findings for the proton translocation mechanism are discussed in Chapter 5.6.

5.4. Determination of the midpoint potentials of the redox centers in Complex I

The characterization of the redox properties of the electron carriers in Complex I can provide a thermodynamic basis for an understanding of the mechanism of energy transduction by this enzyme. Nowadays, it is widely believed that all of them are isopotential with the E_m value of about – 250 mV (Hirst, 2005; Ingledew and Ohnishi, 1980; Ohnishi, 1998), except for N2 and N1a. The former has the highest redox potential among all clusters while the latter - the lowest, although the values of their potentials vary with the source of the enzyme (see Chapter 2.2.2). Moreover, not all iron-sulfur clusters in Complex I have a visible EPR signal after reduction with NADH (Ingledew *et al.*, 1980; Ohnishi, 1998). Thus far the reason for this phenomenon is unclear.

The recently resolved crystal structure of the hydrophilic domain of *T. thermophilus* Complex I (Sazanov and Hinchliffe, 2006) showed that the distances between iron-sulfur clusters in Complex I are in the range of 10.7–16.9 Å (Fig. 2B). Considering that the dielectric constant inside the protein has a typical value of ≤20 (Laurents *et al.*, 2003; Schutz and Warshel, 2001), one might predict an electrostatically induced negative cooperativity between iron-sulfur clusters upon reduction. This physical phenomenon can affect the redox properties of the clusters, for example, by decreasing the value of their midpoint potentials.

To date, the values of the midpoint potentials of the redox cofactors in Complex I were determined using submitochondrial particles (Ingledew and Ohnishi, 1980; Zwicker *et al.*, 2006), bacterial membranes (Leif *et al.*, 1995; Meinhardt *et al.*, 1987; Yano *et al.*, 2003), fragments of Complex I (Barker *et al.*, 2007) or overexpressed individual subunits (Yano *et al.*, 2003; Zu *et al.*, 2002). There is only one work where the intact isolated mitochondrial Complex I was used for redox titration of binuclear clusters (Ohnishi *et al.*, 1981). Any electrochemical or potentiometric titration of Complex I is a complicated task. Firstly, it should be done in strictly anaerobic conditions. Secondly, selected mediators should facilitate equilibrium between the protein and an electrode poised to a certain potential, but should not interact with each other or with the protein. Thirdly, there are only two methods, optical and EPR spectroscopy, by which one can follow the redox changes of the flavin and iron-sulfur clusters. In order to fulfil the listed

demands we used a special EPR and optical cells for continuous electrochemical redox titration of the redox active proteins. In this study, we for the first time presented titrations of purified intact bacterial Complex I. In order to keep the protein in a state close to that which it has in the membrane, we performed redox titration in the presence of lipids. In our potentiometric redox titrations we applied potential in a broad range from – 500 to – 140 mV (**Paper III**). Using optical redox titration we determined the midpoint potential of the flavin. It has a value of – 350 mV (pH 7.5), that is in agreement with the value reported for the mitochondrial enzyme (Sled' *et al.*, 1994). Redox properties of iron-sulfur clusters were studied using EPR spectroscopy. Using selective temperature and microwave power conditions we distinguished the signals of two binuclear clusters, N1a and N1b, from all the tetranuclear centers. Each binuclear Fe-S cluster has a well characterized spectrum that was used in its entity for the determination of the dependence of the spin concentration on the redox potential. In contrast, spectra of tetranuclear clusters are more complicated for decomposition, therefore the most prominent characteristic features, peaks and troughs, that belong to known [4Fe-4S] centers were used to obtain the titration curves (Fig. 4 in **Paper III**).

The analysis of the EPR redox titration showed that the majority of clusters have complicated titration curves that could not be fitted with a simple one-electron Nernstian curve but only with the sum of at least two of them (see for example Fig. 5 in **Paper III**) (Table 2). These clusters are N1b, N3(7), N6b, N2 and one tetranuclear cluster, temporarily designated Nx, which had not been detected previously. Only the binuclear cluster N1a had a titration curve with a very clear one-electron transition. Its midpoint potential was determined to have a value of about – 235 mV, which was close to that published for *E. coli* enzyme by Leif and co-workers (Leif *et al.*, 1995). However, in the mitochondrial counterpart of Complex I binuclear cluster N1a has a much lower midpoint potential of about – 400 mV (Ohnishi, 1998; Zu *et al.*, 2002). Due to such a low E_m value center N1a could not be EPR detected in the eukaryotic Complex I reduced with NADH. The reason for such a difference in redox properties between corresponding clusters from bacterial and mitochondrial enzyme is not yet clear.

The titration of a very low potential Nx cluster was fitted with a one-electron Nernstian curve with an E_m~ – 365 mV. Since the spin concentration of this cluster was estimated as one half in comparison to the other well characterized and determined

tetranuclear centers, we concluded that cluster Nx could have another redox transition, possibly at even lower potentials, which could not be achieved in our study. At slightly higher potential ranges we observed a signal of small amplitude from another, yet unidentified, tetranuclear cluster. This cluster is a very fast-relaxing one, since it could be seen at a temperature lower than 10 K and high microwave power. The titration of the prominent band in its spectra had a single transition with an E_m~ – 330 mV. This signal has not yet been attributed to a particular iron-sulfur center in Complex I. A summary of our findings is presented in Table 2.

Table 2

Electrochemical and EPR spectral properties of the redox groups of Complex I from *E. coli*. Reproduced from **Paper III**.

redox center	E_m (mV)	The basis of titration profiles	g_{xyz}	temperature (K)	microwave power (mW)
FMN	–350 (n=2)[a]				
			2Fe-2S[b]		
N1a	–235	simulated spectrum	1.920, 1.950, 2.00	45	2
N1b	–320, –245	simulated spectrum	1.930, 1.938, 2.021		
			4Fe-4S[b]		
N2	–300, –200	1.895, 2.045	1.985, 1.904, 2.045	10	10
N6b	–315, –235	1.877, 2.089	1.887, 1.894, 2.089		2, 10
N3 or N7	–315	1.956 minus 1.947	1.90, 1.947, 2.046		2, 10
Nx	–365	1.932, 2.022	1.931, 1.935, 2.022		10
N4 or N5	–330	1.929	1.929	5.5	10

[a] Optical titration. [b] EPR titration

We explained the complicated titration curves of the iron-sulfur clusters in terms of intrinsic electrostatic interactions between them in the entire Complex I during reduction of the protein under equilibrium conditions. This phenomenon has important

implications. First, negative repulsion between neighboring clusters results in a shift of their midpoint potentials to more negative values. The value of the shift(s) for a particular cluster is determined by: 1) the number of neighboring clusters, 2) their midpoint redox potentials, 3) distances between them and 4) dielectric constant in the protein. These factors together determine the energy of interaction between clusters. In order to illustrate the effect of electrostatic repulsion between neighbouring redox groups on the value of their midpoint potentials we developed a model that considers interaction between clusters N3, N1b, N4 and N5 located in the middle of the internal electron transport chain of Complex I during the reduction of the protein under equilibrium conditions (**Paper III**). Second, the fact that some iron-sulfur clusters in Complex I are EPR-invisible could be explained by the negative repulsion from neigbouring redox active centers that strongly decreases their E_m values. In other words, some clusters cannot be reduced by conventional reductants in order to become EPR detectable.

5.5. The role of the conserved amino acid residues in the NADH- and quinone-binding sites

In the present work we were interested in examining the conserved amino acid residues of Complex I that are involved in the reactions of electron and proton transfer. In this respect we studied the role of invariant glutamate 95 from the NADH-binding site (**Paper VI**) and conserved arginine 274, histidines 224 and 228 located in the vicinity of the quinone-binding site of Complex I (**Paper II**).

5.5.1. The role of Glu95 in the process of substrate binding

The flavin-binding cavity in the Nqo1 subunit (NuoF in *E. coli*) has a very prominent feature – the presence of two invariant amino acid residues, glutamate 97 and tyrosine 180 that are exposed to the solvent and located in the vicinity of the FMN. It was suggested that they might be involved in the binding of NADH through interaction with its nicotinamide moiety (Sazanov and Hinchliffe, 2006). For Tyr180 this suggestion is in line with the results of the analysis of the double mutation Y204C/C206G in human

Complex I (Y204 is the counterpart of Y180 in *T. thermophilus*) which causes severe symptoms (Benit *et al.*, 2001) that could be explained by interaction of these residues with FMN or/and NADH, but no such proof was available for Glu97. In order to assess its function we mutated the correspondent glutamate 95 in the NuoF subunit of *E. coli* Complex I for glutamine.

If anionic Glu95 does indeed participate in the interaction with the nicotinamide moiety of the nucleotide, then the removal of its negative charge from the NADH-binding site would decrease affinity to the reaction product, NAD^+, since the latter carries a positive charge on its pyridine ring. However, the opposite effect was observed. The affinity for NAD^+ was increased 2.4-fold in the mutated enzyme in comparison to the wild type. The mutation also caused a 2-fold increase in K_m^{NADH}. In order to explain the effects of this mutation one should take into account that at neutral pH the overall charge of NAD^+ is negative because of the two negative phosphate groups. We therefore suggested that the main role of Glu95 is not in the binding of nucleotide, but rather in the electrostatic repulsion of its negatively charged phosphates. This process facilitates release of the product NAD^+ and, as a result, accelerates turnover of Complex I. This is in line with the finding that the affinity of the mutated Complex I for ADP-ribose, which differs from NAD^+ by the absence of the positively charged nicotinamide moiety, was increased 7.5-times in comparison to the wild type enzyme (**Paper VI**).

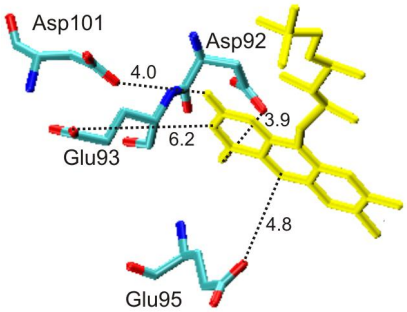

Figure 5. Conserved negatively charged amino acid residues surrounding the FMN in the modeled NuoF subunit of *E. coli* Complex I. Atomic distances are represented in Å. Structures are drawn using the VMD program (Humphrey *et al.*, 1996). Reproduced from **Paper VI**.

Glutamate 95 is one of four conserved negatively charged amino acid residues surrounding the flavin at a distance of 4-6 Å from its isoalloxazine ring (Fig. 5), each of which contributes to the overall 130 mV negative shift in the redox potential of the protein-bound flavin in comparison to the free FMN (E_m = – 220 mV, pH 7.5) in solution (see also Chapter 2.2.1). Optical redox titration of the purified E95Q mutant showed that the midpoint potential of the flavin shifted to positive values by 40 mV and resulted in a value of – 310 mV (versus –350 mV in the wild type as determined in **Paper III**).

Therefore, we postulated that another role of the negatively charged glutamate is to decrease the midpoint redox potential of the primary electron acceptor in Complex I.

5.5.2. The role of conserved Arg274 and His224, His228 from the NuoCD subunit in the final step of electron transfer

According to the hypothesis of the conformational driven coupling (Zickermann *et al.*, 2003) (see Chapter 2.5) the amino acid residues surrounding the N2 cluster have a critical role in energy transduction. The role of several conserved amino acid residues located in the vicinity of N2 and the proposed quinone-binding site was probed by site-directed mutagenesis of the 49kDa subunit of Complex I from *Yarrowia lipolytica* (Brandt *et al.*, 2005; Tocilescu *et al.*, 2007). Two conserved histidines, H91 and H95 were found to be essential for the ubiquinone reductase activity, but their replacement did not affect the EPR spectra of N2, an immediate electron donor for ubiquinone (Grgic *et al.*, 2004). These histidine residues are located at the edge of the "funnel" formed by the 49 kDa subunit (the Nqo4 in *T. thermophilus*, the NuoCD in *E. coli*) and the PSST subunit (the Nqo6 in *T. thermophilus*, the NuoB in *E. coli*), where it was postulated that binding of the quinone head group occurred (Sazanov and Hinchliffe, 2006). Another highly conserved residue in the 49 kDa subunit (the NuoCD in *E. coli*), R141, was thought to reside close to N2 based on the analysis of the conserved structural fold of the [NiFe] dehydrogenase from which most probably the quinone-reducing part of the connecting fragment of Complex I had been evolved (Kashani-Poor *et al.*, 2001). The recently resolved structure of the hydrophilic domain of *T. thermophilus* Complex I (Sazanov and Hinchliffe, 2006) showed that the distance between cluster N2 and R141 (R84 in *T. thermophilus*) is only 5 Å. The replacement of R141 with alanine, methionine and lysine was reported to cause the loss of the N2 signal in the EPR spectra of Complex I reduced with NADH and dithionite, although the quinone reductase activity in the mutants remained significant (Grgic *et al.*, 2004). On the basis of these results it was suggested that the N2 cluster was lost due to the mutation and cluster that should reside at a distance of ca. 5 Å from N2 became the immediate electron donor for the quinone. The best candidate for such a role could be cluster N6b. However, crystallographic data shows that the distance between N2 and N6b is 10.5 Å (PDB entry 2FUG), which means

that N6b is removed from quinone at approx. 22.5 Å and therefore could not be an efficient immediate electron donor for it.

Since the particular features of Complex I might be organism specific, we made the mutations of corresponding amino acid residues, R274, H224, H228 from the NuoCD subunit of *E. coli* Complex I in order to reveal their relevance for the function of the enzyme.

The H224A and H228A (H91 and H95 in *Y. lipolytica*) mutations did not affect the expression level of the enzyme, but caused decrease of the ubiquinone reductase activity by 50 % in comparison with the wild type. The reduction of ubiquinone by these mutants was specific, since their affinity to rolliniastatin did not change. Comparison of EPR spectra of wild type enzyme and these mutants showed no difference in the signals of binuclear and tetranuclear iron-sulfur clusters. These results showed that H224 and H228 are important for ubiquinone reduction but their presence is not an obligatory requirement. Mutation of arginine 274 for alanine caused more dramatic effects than mutations of histidines. Quinone reductase activity catalyzed by the R274A mutant dropped significantly, although the affinity of this enzyme to rolliniastatin, IC_{50}, was approximately the same as in wild type, in the range of 1.4-2.4 nM. Analysis of the EPR spectra of the isolated mutated enzyme reduced with NADH showed the loss of a signal at g-values corresponding to the N2 spectrum, which was in agreement with the previously reported data on a corresponding mutation in *Y. lipolytica* (Grgic *et al.*, 2004) Complex I. Further reduction of the mutant with dithionite at alkaline pH (E_m = – 500 mV) in the presence of methyl viologen caused the appearance of a signal similar to that of N2 (with the parameters g_{xyz} = 1.900, 1.901, 2.045) but slightly broader and with smaller amplitude. Unexpectedly, the reduction with dithionite also caused an increase in the signal from another fast-relaxing tetranuclear iron-sulfur cluster. This signal with parameters g_{xyz} = 1.889, 1.905, 2.087 we assigned to N6b. Since the distance between the counterpart of R274 in *T. thermophilus* and N6b is 8 Å (PDB entry 2FUG) and the removal of the positive charge could affect the cluster located at a distance less than 10 Å, cluster N6b was suggested to be the best candidate for such an assignment. In addition, it is also possible that in the entire enzyme the distance between arginine 274 from the NuoCD subunit and cluster N6b from NuoI could be even shorter than 8 Å, as determined based on crystal structure of the hydrophilic domain of *T. thermophilus* Complex I,

because the connecting fragment with part of the postulated quinone-binding site could be distorted upon fragmentation of the enzyme.

We explained the re-appearance of the signal from N2 in the mutant after the reduction with dithionite by a downshift of its redox potential caused by the removal of the neighbouring positive charge. Hence arginine274 is one of the amino acids in the vicinity of N2 which maintain the high redox potential of this cluster.

We also tested the effect of the mutations on the proton-pumping activity of Complex I reconstituted into liposomes by monitoring $\Delta\psi$ generation driven by NADH:quinone oxidoreduction. Electric potential was generated by all mutated enzymes, suggesting that none of the three amino acid residues, R274, H224, or H228, are involved in proton pumping directly.

5.6. Proposed mechanism of proton pumping by Complex I

Presently, three of the distantly located membrane subunits, NuoM, NuoN and NuoL, are considered to be involved directly in proton translocation across the membrane. This assumption is based on the relation of these subunits to a family of proton/cation antiporters (Hiramatsu *et al.*, 1998; Mathiesen and Hagerhall, 2002). However, there is no hypothesis which satisfactory explains how the free energy released during quinone reduction is transferred at distance of 60-100 Å from the quinone-binding site to the distal part of the membrane domain.

We found that conserved amino acid residues from the NuoN and NuoM subunits and their homologues in $H^+/K(Na)^+$ antiporters share a common pattern (Fig. 1 in **Paper V**). Prediction of its membrane topology revealed one very prominent feature – the presence of two lysines located within the membrane dielectric (Fig. 6). Such localization of charged amino acid residues is rare in membrane proteins. Site-directed mutagenesis of the conserved lysines, K217 and K247, from the NuoN subunit showed that they are essential for the enzyme activity (Amarneh and Vik, 2003).

In order to verify the role of corresponding lysines from the NuoM subunit, K234 and K265, we used site-directed mutagenesis (**Paper V**). We found that lysine 234 is essential for quinone reductase activity. Furthermore, we found that not only the charge is important, but also the properties of the side chain of the amino acid, since the

replacement of K234 by arginine resulted in an inactive enzyme as in the case of K234A mutation.

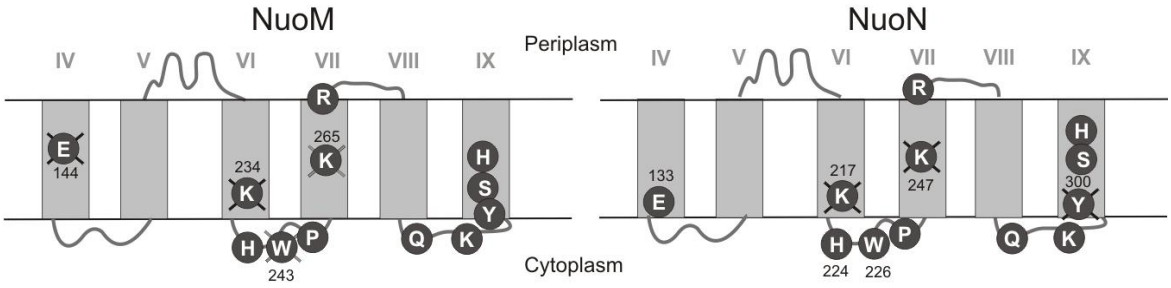

Figure 6. Predicted membrane topology of the highly conserved residues comprising the common pattern found in the NuoN and NuoM subunits. Black crosses indicate residues, which are found to be essential for quinone reductase activity; grey crosses indicate residues, the replacement of which to alanine yielded only partial loss of quinone reductase activity. Results of site-directed mutagenesis of the NuoN subunit are published in (Amarneh and Vik, 2003). Reproduced from **Paper V**.

The same results were obtained by Torres-Bacete and co-workers (Torres-Bacete *et al.*, 2007). In contrast, the replacement of the corresponding lysine from the NuoN subunit, K217, by arginine resulted only in partial inactivation. The results of the replacement of the second conserved lysine from the NuoM subunit, K265, by alanine showed that it is not crucial for proton pumping and the activity of the enzyme, whereas the positive charge of the counterpart of this lysine in the NuoN subunit, K247, was found to be critical for enzyme activity. Substitution of this lysine for cysteine resulted in complete loss of the activity while K247R mutation had a minor effect. Thus one can conclude that in both NuoN and NuoM there is at least one conserved lysine essential for the activity and proton pumping by Complex I. Furthermore, these lysines are likely to be charge-uncompensated since neither NuoM nor NuoN appears to contain conserved acidic amino acid residues adjacent to them. Glutamate 133 from the NuoN subunit is unlikely to interact with one of the intramembrane lysines in this subunit since it is not critical for enzyme activity (Amarneh and Vik, 2003). We probed the role of the corresponding glutamate, E144, from the NuoM subunit by site-directed mutagenesis. Substitution of this glutamate for aspartate, E144D, had no effect on Complex I activity while substitution by hydrophobic uncharged alanine, E144A, resulted in complete loss of quinone reductase activity. However, according to the membrane topology predicted for the above-mentioned common pattern shared by three membrane subunits glutamate 144 from NuoM is unlikely to be adjacent to lysine 234 and is therefore hardly a suitable partner for electrostatic interactions with the latter.

However, one cannot exclude another possibility that lysines from NuoN and NuoM might interact with the conserved acidic amino acid residues from another membrane subunit(s). It was found that uncompensated intramembrane Glu81 and Asp79 from NuoA (Kao *et al.*, 2004a) and two glutamates from NuoK are essential for the activity of Complex I (Kao *et al.*, 2005b; Kervinen *et al.*, 2004). At least two of the mentioned acidic residues could be good candidates for an ion interaction with conserved lysines from the NuoN subunit. In contrast, for the NuoM subunit such a possibility is deemed to be unlikely, since it was suggested that it is located in the distal part of the membrane domain (Sazanov, 2000; Baranova *et al.*, 2007b) and appears not to have extensive contacts with other subunits, except for NuoL (Baranova *et al.*, 2007a).

We also found that other membrane subunits, NuoL and NuoH, are likely to contain conserved lysines in their transmembrane helices. Thus one can assume that the hydrophobic domain contains at least four conserved intramembrane lysines arranged in a row and aligned parallel to the membrane surface. The first lysine in this "chain" is located in the NuoH, the second in the NuoN, the third in the NuoM and the last one in the NuoL subunit. This assumption allowed us to propose a tentative principle governing redox-coupled proton pumping by Complex I (**Paper V**). Recently, Baranova and co-workers (Baranova *et al.*, 2007b) suggested that each of three membrane subunits, NuoN, NuoM and NuoL, could pump one proton, driven by long-range conformational changes. In contrast, we proposed that proton translocation by each of these subunits takes place through protonation and deprotonation of the key intramembrane lysine, which additionally can switch the orientation of its side chain towards the inside and outside of the membrane for proton uptake/release. An example of such an alternative-access mechanism has been previously described for the functionally important protonatable glutamate in membrane H^+/lactose symporter (Mirza *et al.*, 2006) and aspartates in Na^+/H^+ antiporter NhaA (Hunte *et al.*, 2005). Regarding the principle of proton pumping by Complex I an important question can be raised about the state of the key intramembrane lysines since they appear to be electrostatically uncompensated. The predicted position of these residues in the low dielectric core of the membrane is indeed energetically unfavorable. Close inspection of the sequence details and predicted membrane topology of NuoH, NuoN, NuoM and NuoL showed the presence of several unconserved acidic amino acid residues in each of the subunits, some of which may even

be located in the same transmembrane helices where the key lysines reside, but the distances are not favorable for the formation of salt bridges. On the other hand, charge-uncompensated lysine could be placed in the middle of the membrane dielectric in a non-polar environment in case it is deprotonated. If, however, the intramembrane lysine is protonated it could be located at the bottom of a narrow "funnel" or "channel" providing protonic access to the cytoplasmic/periplasmic side of the membrane. It is also possible that both of these suggested states of the key lysines occur during the operation of Complex I, and are altered by binding/reduction of the quinone in the active site. As an example, binding of the substrate might lead to subtle conformational changes in the first proton translocating subunit (NuoH), in its small part above the key lysine, which is located in a non-polar environment and therefore deprotonated. These rearrangements could result in the formation/opening of a temporary narrow "funnel" providing access for water molecules from the cytoplasmic side of the membrane to this lysine, in which way it may become protonated. The latter event might result in charge imbalance and cause two processes. First, in order to release a proton later to the periplasmic side, the first proton translocating subunit might undergo small structural rearrangements resulting in re-orientation of the side chain of the protonated lysine towards the periplasmic side, thereby closing the temporary cytoplasmic "funnel" and opening of another one directed towards the periplasm. An example of such small rearrangements resulting in opening/closing of the channels and change in the orientation of side chains of the key glutamates towards the periplasm or cytoplasm has been described for Na^+/H^+ antiporter NhaA (Hunte *et al.*, 2005). Second, the charge imbalance on the first lysine and structural rearrangements in its vicinity might cause deprotonation of the second lysine in the row (located in the NuoN subunit), which is assumed to be protonated and to have access to the periplasmic side of the membrane (see below). Deprotonation of this lysine could be accompanied by closing of the temporary "funnel" connecting this lysine with the periplasm in a way that the second lysine is left in a non-polar environment in a deprotonated state. Release of the proton by the second lysine and charge imbalance caused by this process in turn could stimulate small conformation rearrangements above the third deprotonated lysine (located in the NuoM subunit) resulting in formation/opening of another temporary "funnel" through which a proton can be acquired from the cytoplasmic side of the membrane in a way described for the first key lysine. Then the

process can propagate further along the membrane domain of the enzyme. It is also worth noting that the structural rearrangements in the proton pumping subunits prompted by quinone binding and reduction should be small in order to maintain a high turnover of Complex I. The described explanation of how the protein can tolerate charge-uncompensated lysines in the membrane dielectric is rather speculative and assumes that two of the four key lysines in the membrane domain of Complex I should be deprotonated and two – protonated in an "as prepared" enzyme. The resolved crystal structure of the entire enzyme can verify whether that is the case or not. However, there are two observations that might be considered to be in line with the proposed principle. First, FT-IR studies on *E. coli* Complex I suggested that the redox transitions of the flavin and iron-sulfur clusters induce conformational change of the protein (Hellwig *et al.*, 2000). Second, the electron microscopy images of the frozen-hydrated crystals of the hydrophobic domain of *E. coli* Complex I (Baranova *et al.*, 2007a) revealed low density areas in the 8 Å resolution projection map that could correspond to the narrow cytoplasmicaly oriented "funnels" above the key lysines.

Ubiquinone binding and reduction likely take place in the proximal part of the membrane domain, formed by the small NuoA, NuoJ, NuoK and NuoH subunits, adjacent to the bottom part of the hydrophilic domain where N2 resides. Thus, the cluster of these subunits could be considered a "coupling domain" which is responsible for transferring free energy from the redox event into the more distant parts of the membrane arm, probably for translocation of protons. We proposed that reduction of the quinone causes a compensatory increase in the pK_a of the key lysine in the NuoH subunit. As a result the first intramembrane lysine becomes protonated. Uptake of the first proton electrostatically affects the pK_a of the next lysine in the row, in the NuoN subunit, causing its deprotonation. Then electrostatic polarization is propagated even further and consequently shifts the pK_a of the following lysines, located in the NuoM and NuoL subunits. After this, the first phase of the electrostatic polarization accompanied by possible changes in the orientation of key lysines is completed (see above). In order to return the protein into the initial state, "input" state, the second phase of polarization may be initiated by the subunit NuoL, in which case the process proceeds in reverse direction, towards the "coupling domain", and finally resultes in neutralization of the negative

charge of the hydroquinone anion (see below) by uptake of the second substrate proton. The schematic representation of the proposed principle is shown in Figure 7.

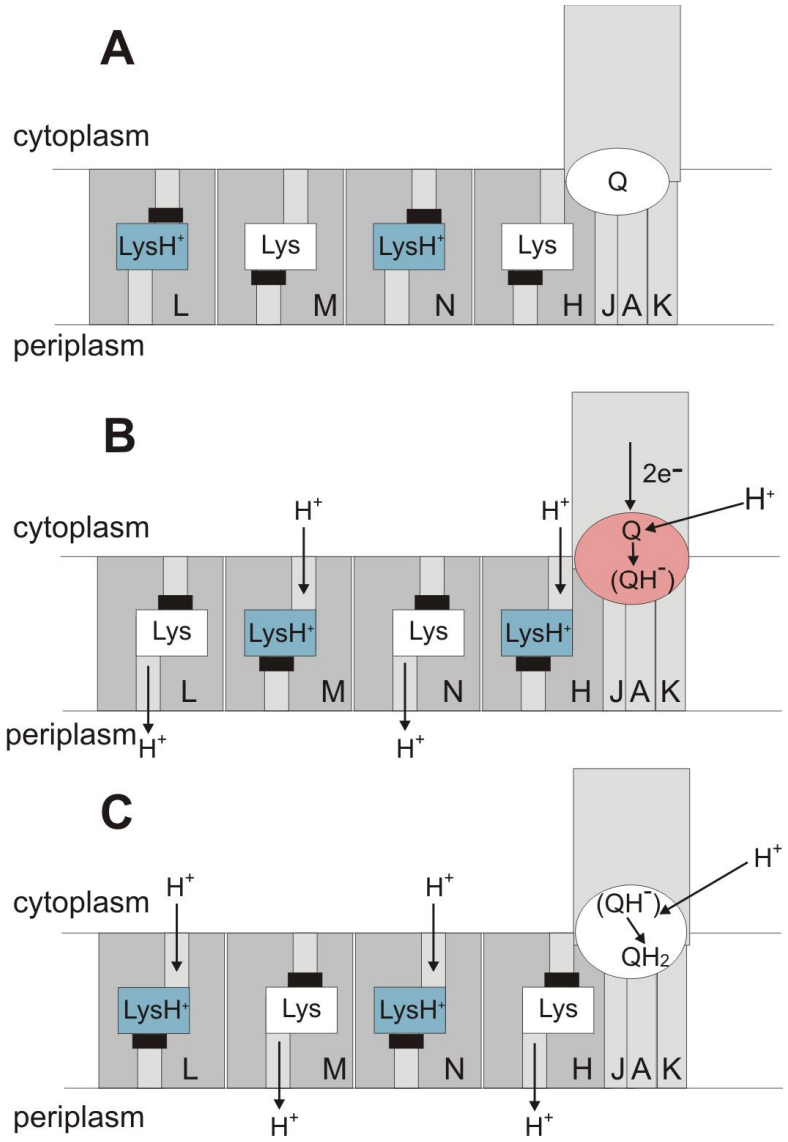

Figure 7. A tentative formulation of the principle governing redox-coupled proton pumping in Complex I

In our studies of the electron transfer kinetics in Complex I using the freeze-quench approach we observed full reduction of the N2 cluster, but no ubisemiquinone was detected. It is worth noting, that the extremely fast relaxing ubisemiquinone radical magnetically interacting with N2 had been detected only in submitochondrial particles and in the presence of high membrane potential (Ohnishi, 1998; Yano *et al.*, 2005). The latter condition might indicate instability of the radical and thus low redox potential of the $Q/Q^{\cdot-}$ couple. If so, this may have important implications for the proton

translocating mechanism. Our failure to detect a signal from semireduced quinone despite full reduction of the N2 cluster could mean that the population of the quinone radical is too small to be detected. Such a low concentration of semiquinone in equilibrium with the reduced center N2 could indicate that the midpoint potential of the $Q/Q^{\cdot-}$ couple is at least 100 mV more negative than that of cluster N2 (i.e. less than −300 mV) and thus close to the E_m of $NADH/NAD^+$ couple. In this case it may happen that the delivery of the first electron from NADH to ubuquinone is not accompanied by any drop of energy whereas the transfer of the second one occurs across a large potential drop and results in release of free energy sufficient to drive translocation of four protons across the membrane. If this is the case, then quinone should be reduced 'virtually' in one step to hydroquinone anion instead of ubisemiquinone, which would explain our failure to detect semiquinone radical.

The suggested mechanism of the energy transduction by Complex I could be classified as direct since quinone reduction is coupled with proton translocation via electrostatic interactions between protonable amino acid residues and conformational changes that are restricted to the switching orientation of the lysines in the membrane dielectric towards the inside or outside of the membrane.

6. SUMMARY

In the present study we applied a set of different methods to study the electron and proton transfer reactions in Complex I from *Escherichia coli*.

We established a conventional protocol for purification of the enzyme and determined the factors, which affect the measurement of the native quinone-reductase activity of the enzyme.

A significant achievement was the experiment where we monitored distribution of two electrons within Complex I upon reduction with NADH using an ultra-fast kinetic technique allowing measurement of electron transfer in the μs time scale. We found that the N2 cluster is reduced in a time close to that predicted by the electron transfer theory, which means that the electron transfer through the chain of Fe-S clusters in Complex I is unlikely to be coupled to proton transfer directly or to conformational changes in the protein.

In this work we have for the first time presented redox titrations of the intact purified bacterial Complex I. We found that clusters N1b, N3(7), N6b, N2 and one tetranuclear cluster, temporarily designated Nx, have complicated titration curves that could be fitted only with the sum of at least two one-electron Nernstian curves. We explained such behavior in terms of electrostatic repulsion between Fe-S clusters within the protein upon reduction under equilibrium conditions. Another manifestation of this phenomenon was a negative shift of the midpoint potentials of the clusters located in the middle of the intramolecular electron transport chain of Complex I

We also studied the role of several conserved amino acid residues in the electron transfer reaction of Complex I. One of them, Glu95 from the NuoF subunit, is located in the NADH-binding site. We found that the negative charge of this residue is needed for repulsion of the product, NAD^+, from the catalytic site, and thus for acceleration of the turnover of Complex I. The other three amino acid residues studied, Arg274, His224 and His228 from the NuoCD subunit, are located in the proximity of the N2 cluster and quinone-binding site, respectively. We demonstrated that histidines are important for quinone reductase activity, but not obligatory, while the positive charge of arginine is critical. It is likely involved in maintaining the high redox potential of N2.

Finally, we found that several membrane subunits share a common pattern of highly conserved amino acid residues, characterized by a very prominent feature – the presence of a few lysines within the membrane dielectric. We probed the role of such lysines in the NuoM subunit and suggested they are involved in the proton translocation process. Based on the experimental data, we tentatively postulated and discussed a principle which we think governing the redox-coupled proton pumping in Complex I.

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