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CATALYTIC CORE OF RESPIRATORY CHAIN NADH-UBIQUINONE OXIDOREDUCTASE

ROLES OF THE ND1, ND6 AND ND4L SUBUNITS AND MITOCHONDRIAL DISEASE MODELLING IN ESCHERICHIA COLI

UNIVERSITY OF OULU,
FACULTY OF MEDICINE, INSTITUTE OF BIOMEDICINE,
DEPARTMENT OF MEDICAL BIOCHEMISTRY AND MOLECULAR BIOLOGY

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Roles of the ND1, ND6 and ND4L subunits and mitochondrial disease modelling in *Escherichia coli*

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium F101 of the Department of Physiology (Aapistie 7), on 10 June 2011, at 12 noon.

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Abstract

NADH-ubiquinone oxidoreductase (complex I) is one of the largest enzymes in mammals. Seven (ND1-ND6 and ND4L) of its 45 subunits are encoded in mitochondrial DNA, mutations of which are usually behind mitochondrial diseases such as Leber hereditary optic neuropathy (LHON) and MELAS-syndrome. The rest of the genes are located in the nucleus. Bacterial homologs of complex I (NDH-1) consist of only 13–14 subunits, comprising the catalytic core of the enzyme. These complexes are simpler but perform a similar function.

Escherichia coli NDH-1 was employed here to generate amino acid replacements at conserved sites in NuoH, NuoJ and NuoK, counterparts of ND1, ND6 and ND4L, to elucidate their role in complex I. Consequences of homologous amino acid substitutions brought about by ND1-affecting LHON/MELAS-overlap syndrome-associated m.3376G>A and m.3865A>G mutations and the ND6-affecting m.14498T>C substitution associated with LHON were also studied to validate their pathogenicity. Effects of the site-directed mutations were evaluated on the basis of enzyme activity, inhibitor sensitivity and growth phenotype.

Highly conserved glutamate-residues 36 and 72 within transmembrane helices of NuoK in positions similar to proton translocating transmembrane proteins were found essential for electron transfer to ubiquinone and growth on medium necessitating normal proton transfer by NDH-1. NuoH and NuoJ replacements at sites corresponding to targets of m.3376G>A and m.14498T>C decreased ubiquinone reductase activity and altered the ubiquinone binding site, while the counterpart of m.3865A>G was without a major effect. Other NuoH and NuoJ mutations studied also affected the interactions of ubiquinone and inhibitors with NDH-1.

The results corroborate the pathogenicity of the m.14498T>C and m.3376G>A mutations and demonstrate that the overlap syndrome-associated modification affects complex I in a pattern which appears to combine the effects of separate mutations responsible for LHON and MELAS. Change in ubiquinone binding affinity is a likely pathomechanism of all LHON-associated mutations. Effects of the NuoH, NuoJ and NuoK subunit substitutions also indicate that ND1 and ND6 subunits contribute to the ubiquinone-interacting site of complex I and the site is located in the vicinity of the membrane surface, while ND4L is likely involved in proton pumping activity of the enzyme.

Keywords: Leber hereditary optic neuropathy, MELAS syndrome, mitochondrial diseases, mitochondrial DNA, NADH-ubiquinone oxidoreductase, oxidative phosphorylation, site-directed mutagenesis, ubiquinone

Pätsi, Jukka, Hengitysketjun NADH-ubikinoni oksidoreduktaasin toiminnallisen ytimen ND1, ND6 ja ND4L alayksiköiden tehtävän selvittäminen ja mitokondriotautien mallintaminen *Escherichia colissa*.

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Tiivistelmä

45 alayksiköstä muodostuva NADH-ubikinoni oksidoreduktaasi (kompleksi I) on nisäkkäiden suurimpia entsyymejä. Sen mitokondriaalisessa DNA:ssa koodattujen alayksiköiden ND1-ND6 ja ND4L geeneihin liittyvät mutaatiot ovat yleisiä mitokondriosairauksien, kuten Leberin perinnöllisen näköhermoatrofian (LHON) ja MELAS-oireyhtymän, syitä. Bakteerien vastaava entsyymi (NDH-1) koostuu vain 13–14 alayksiköstä. Tästä huolimatta sen katalysoima reaktio on samankaltainen kuin kompleksi I:n. NDH-1:n katsotaankin edustavan entsyymin katalyyttistä ydintä.

Tässä työssä tutkittiin ND1, ND6 ja ND4L alayksiköiden tehtävää kompleksi I:ssä niiden *Escherichia coli* bakteerissa olevien vastineiden (NuoH, NuoJ ja NuoK) kohdennetun mutageneesin avulla. Samaa lähestymistapaa käytettiin LHON/MELAS-oireyhtymässä todettujen ND1 alayksikön mutaatioiden, m.3376G>A ja m.3865A>G, ja LHON:ssa havaitun ND6:n m.14498T>C mutaation aiheuttamien aminohappomuutosten seurauksien selvittämiseen. Tehtyjen mutaatioiden vaikutuksia arvioitiin entsyymiaktiivisuus-mittauksin ja kasvukokein.

NuoK:n solukalvon läpäisevissä rakenteissa olevien kahden glutamaatti-aminohappotähteen sijainti muistuttaa protoneita kalvon läpi kuljettavissa proteiineissa todettua. NuoK:n glutamaattien havaittiinkin olevan tärkeitä elektronien ja protonien kuljetukselle kompleksi I:ssä. m.3376G>A ja m.14498T>C mutaatioiden aiheuttamien aminohappomuutosten vastineet NDH-1:ssä alensivat NDH-1:n elektroninsiirtoaktiivisuutta ja heikensivät ubikinonin sitoutumista, kun taas m.3865A>G mutaatiolla ei ollut vaikutusta. Muut NuoH ja NuoJ alayksiköihin tehdyt aminohappovaihdokset johtivat huonontuneeseen ubikinonin ja kompleksi I:n inhibiittoreiden sitoutumiseen.

Saadut tulokset vahvistavat m.3376G>A ja m.14498T>C mutaatioiden patogeenisyyden. Ne myös osoittavat, että LHON/MELAS-oireyhtymään liitetyn mutaation biokemiallisissa vaikutuksissa yhdistyvät sekä LHON:ssa että MELAS-oireyhtymässä todettujen mutaatioiden seuraukset. Esitetyt tulokset tukevat näkemystä siitä, että ubikinonin ja kompleksi I:n välisessä vuorovaikutuksessa tapahtuva muutos on kaikille LHON:aan liitetyille mutaatioille yhteinen vaikutusmekanismi. NuoH:n, NuoJ:n ja NuoK:n kohdennetusta mutageneesista saatujen tulosten perusteella ND1 ja ND6 alayksiköt ovat osa ubikinonin sitoutumispaikkaa entsyymikompleksissa, kun taas ND4L osallistuu protoninkuljetukseen.

Asiasanat: Leberin hereditaarinen optikusneuropatia, MELAS-oireyhtymä, mitokondrio-DNA, mitokondriotaudit, NADH-ubikinoni oksidoreduktaasi, oksidatiivinen fosforylaatio, suunnattu mutageneesi, ubikinoni

To my family

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Kiiminki, May 2011 Jukka Pätsi

Abbreviations

BBB	blood-brain barrier
DB	decylubiquinone
DCCD	*N,N'*-dicyclohexylcarbodiimide
DPI	diphenyliodonium
DQA	2-decyl-4-quinazolinyl amine
d-NADH	nicotinamide hypoxantine dinucleotide, reduced form
EPR	electron paramagnetic resonance
Fe-S cluster	iron-sulphur cluster
FMN	flavin mononucleotide
HAR	hexaammineruthenium(III) chloride
LHON	Leber hereditary optic neuropathy
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy associated with ragged-red fibers
mtDNA	mitochondrial DNA
NAD^+	β-nicotinamide adenine dinucleotide, oxidized form
NADH	β-nicotinamide adenine dinucleotide, reduced form
NDH-1	bacterial proton-pumping NADH:ubiquinone oxidoreductase
NDH-2	bacterial non-proton-pumping NADH:ubiquinone oxidoreductase
Q_1	ubiquinone-1
Q_2	ubiquinone-2
QH_2	ubiquinol
RNFL	retinal nerve fiber layer
ROS	reactive oxygen species
SQ_{Nf}	fast relaxing semiquinone
SQNs	slow relaxing semiquinone
UQ	ubiquinone
VNA	*N*-vanillylnonanamide
V_{max}/HAR	ratio of enzyme's V_{max} to HAR-reductase activity

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Kervinen M, Pätsi J, Finel M & Hassinen IE (2004) A pair of membrane-embedded acidic residues in the NuoK subunit of Escherichia coli NDH-1, a counterpart of the ND4L subunit of the mitochondrial complex I, are required for high ubiquinone reductase activity. Biochemistry 43(3): 773–781.

II Pätsi J, Kervinen M, Finel M & Hassinen IE (2008) Leber hereditary optic neuropathy mutations in the ND6 subunit of mitochondrial complex I affect ubiquinone reduction kinetics in a bacterial model of the enzyme. Biochemical Journal 409(1): 129–137.

III Pätsi J, Maliniemi P, Pakanen S, Hinttala R, Uusimaa J, Majamaa K, Nyström T, Kervinen M & Hassinen IE (2011) LHON/MELAS overlap mutation in ND1 subunit of mitochondrial complex I has distinct effect on ubiquinone binding. Manuscript.

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1 Introduction

Life in every form depends on the availability of energy. In order for an organism to carry out its tasks and maintain an organized state, energy is needed to perform the work of metabolism and transport of cellular components. Power needed to maintain homeostasis originates from the environment. Organisms classified as autotrophs, like plants, are themselves capable to generate energy by means of photo- or chemosynthesis. Contrary to that, energy supply in heterotrophs, like animals, relies on the breakdown of organic molecules taken from their surroundings, initially produced by the autotrophs. Part of the energy released during the decomposition of nutrients in heterotrophs is transformed to molecules that can be used to synthesize other compounds. ATP is an example of such universal energy currency that drives cellular reactions.

Combustion of dietary carbohydrates, proteins, and lipids yields reducing equivalents that are finally used for ATP synthesis in oxidative phosphorylation by the mitochondrial respiratory chain. Consecutive oxidation-reduction reactions catalyzed by the respiratory chain enzymes lead to formation of a proton and electric gradient (electrochemical membrane potential) across the inner mitochondrial membrane that is used to generate ATP (Figure 1). In addition to this oxygen-demanding pathway, ATP is produced anaerobically in glycolysis, but the yield is far less than in oxidative phosphorylation and may lead to formation of lactic acidosis due to accumulation of lactate. Some cells and tissues, like mammalian erythrocytes that are devoid of mitochondria, rely on substrate-level phosphorylation in their energy production even under aerobic conditions. During oxygen limitation (hypoxia) all cells and tissues become dependent on anaerobic ATP synthesis, but most are able to sustain it only briefly.

Respiratory chain dysfunction due to a mutation in one of its constituent genes is a common cause of inborn heritable metabolic disorders. It is not surprising that tissues with high energy demand, like the nervous system, heart and skeletal muscle, are most vulnerable to defects in oxidative phosphorylation. Respiratory diseases caused by mutations in the mitochondria's own genome, mtDNA, are expressed with variable symptoms. This is due not only to the complexity of mitochondrial genetics, but also the characteristics of the cells and tissues affected. Lack of knowledge of the normal structure and function of the enzyme affected, especially in the case of complex I, limits the evaluation of the effects of the mutations.

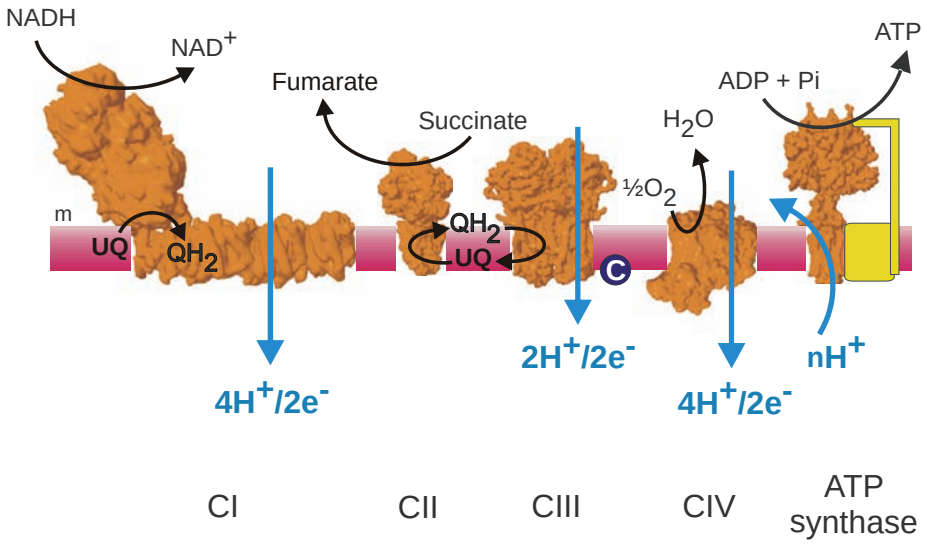

Fig. 1. Overview of the reactions catalyzed by the mitochondrial oxidative phosphorylation enzymes located in the inner mitochondrial membrane. Presentation of each of the enzyme complexes is based on crystallographic structures of complex I from *Thermus thermophilus* (protein data bank code 3M9S), avian complex II (1YQ3), bovine complex III dimer (1BGY), complex IV from *Paracoccus denitrificans* (1QLE) and bovine ATP synthase (2XND) viewed with Jmol (Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/). H^+/e^- ratios indicate the number of charges translocated across the membrane upon electron (e^-) transfer in complexes I, III and IV. n denotes the number of protons needed to synthesize an ATP molecule and is variable depending on the number of *c*-subunits present in the membranous part of the ATP synthase in a given species. The matrix side of the membrane is marked by the letter m.

This study focuses on the function of respiratory chain NADH-ubiquinone oxidoreductase (complex I, EC 1.6.5.3). The role of three complex I subunits in the membranous core of the enzyme is investigated using site-directed mutagenesis of their homologs in *Escherichia coli*. Biochemical effects of disease-associated mtDNA substitutions within two of the three studied subunits are evaluated in the bacterial model to gain more knowledge on their pathogenicity and on the pathogenesis of the diseases involved. The use of *E. coli* as a model organism to study the effects of mtDNA mutations is also addressed.

2 Review of the literature

2.1 Subunits and structure of complex I

Membrane embedded NADH-ubiquinone oxidoreductase (complex I, EC 1.6.5.3) is by far the largest and most complicated enzyme of the respiratory chain. It is composed of 45 different polypeptides and a set of redox-active cofactors resulting in a total molecular mass of about 1000 kDa in mammals (Carroll *et al.* 2006). Seven of the subunits (ND1-ND6 and ND4L) are encoded in the mitochondrial genome (mtDNA), while the rest are nuclear-encoded. NDH-1, the bacterial counterparts of the enzyme, are simpler, being comprised of only 13–14 subunits, seven of which are homologs of the NDs. The NDH-1s are considered to comprise the catalytic core of complex I. (Brandt 2006, Leif *et al.* 1993, Yagi *et al.* 1998). A list of the core subunits is presented in Table 1. The significance of the additional or accessory subunits in complex I is not fully understood. Some of them may be involved in protection against reactive oxygen species generation (Friedrich & Weiss 1997) and some, like GRIM-19 and mitochondrial acyl carrier protein, are needed for proper assembly and stability of the enzyme (Fearnley *et al.* 2001, Huang *et al.* 2004, Schneider *et al.* 1995).

Table 1. Core subunits of complex I in bovine mitochondria and their homologs in *Escherichia coli*.

Complex I domain	Bovine complex I	*E. coli* NDH-1	Redox-active cofactors[1]
Hydrophilic	51 kDa	NuoF	FMN, Fe-S cluster N3
	24 kDa	NuoE	Fe-Scluster N1a
	75 kDa	NuoG	Fe-S clusters N1b, N4, N5
	49 kDa	NuoD[2]	
	PSST	NuoB	Fe-S cluster N2
	30 kDa	NuoC[2]	
	TYKY	NuoI	Fe-S clusters N6a, N6b
Hydrophobic	ND1	NuoH	
	ND2	NuoN	
	ND3	NuoA	
	ND4	NuoM	
	ND5	NuoL	
	ND6	NuoJ	
	ND4L	NuoK	

[1]Assignment of the prosthetic groups into individual subunits according to (Sazanov & Hinchliffe 2006).

[2] Genes of NuoC and NuoD are fused in *E. coli* into a single subunit NUOCD.

Electron microscopic single particle analysis of complex I from fungus *Neurospora crassa* (Guenebaut *et al.* 1997), yeast *Yarrowia lipolytica* (Djafarzadeh *et al.* 2000), mammalian *Bos taurus* (Grigorieff 1998), and from the bacteria *Aquifex aeolicus* (Peng *et al.* 2003) and *Escherichia coli* (Sazanov *et al.* 2003) has revealed that complex I possesses an evolutionarily conserved L-shaped structure with an arm embedded in and a hydrophilic arm protruding out of the hydrophobic membrane. These findings are supported by the recent results of X-ray crystallographic analysis of complete complex I from *Y. lipolytica* (Hunte *et al.* 2010) and *Thermus thermophilus* (Efremov *et al.* 2010). All the redox-active cofactors are located in the hydrophilic part of the enzyme (Sazanov & Hinchliffe 2006), which can be subdivided into electron input and ubiquinone (UQ) reduction modules based on the modular evolution scheme of complex I (Friedrich & Weiss 1997, Friedrich & Scheide 2000, Mathiesen & Hägerhäll 2003). The transfer of electrons that are delivered to complex I by NADH to ubiquinone (UQ) results in proton translocation across the membrane. Vectorial proton transfer reactions take place in the membrane arm, which is composed of the seven core subunits encoded by mtDNA in mammals. For clarity, description of the core structure of complex I in the following sections is based on the division of the enzyme into a hydrophilic promontory arm and the distal and proximal parts of the membranous fragment, exemplifying division of the enzyme complex into different functional domains.

2.1.1 Molecular structure of complex I

More detailed but still incomplete structural data on complex I is currently available only for its promontory and membranous domains separately (see sections 2.1.2 and 2.1.4 below). X-ray crystallographic analyses of complete complex I have been presented recently at 6.3 Å for *Y. lipolytica* and at 4.5 Å resolution for *T. thermophilus* (Figure 2). It was shown that the enzyme is L-shaped, as depicted earlier by the single particle analysis of fungal, bacterial, yeast and mammalian complex I, and the angle between the two arms is about 100^{o}. The membrane arm, slightly curved both in plane and perpendicular to membrane surface views, was shown to have a length of about 180 Å and a thickness of 40 Å. The tip of the hydrophilic protrusion resides about 130 Å over the lipid bilayer. Contact between these two domains is likely to be mediated by homologs of 49 kDa, PSST and ND1 together with one of the ND3, ND6 and

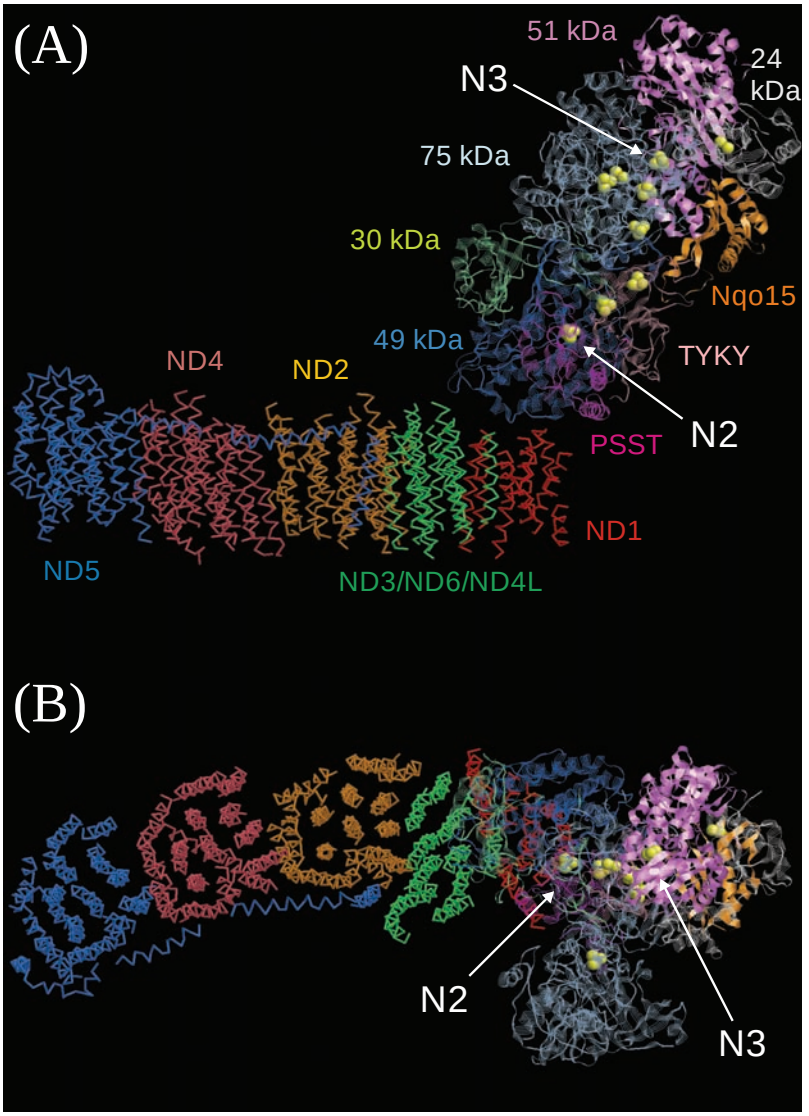

Fig. 2. Molecular structure of the entire complex I from *T. thermophilus* (protein data bank code 3M9S) viewed with RasMol program. Panel (A) shows a side view of the molecule, view in (B) is from above the hydrophilic arm. Subunits are marked according to bovine nomenclature apart from Nqo15, which does not have a homolog in bovine complex I. Fe-S clusters are denoted by yellow-filled spheres and the positions of clusters N2 and N3 are indicated with arrows to aid orientation within the structure.

ND4L subunits, and the pocket formed by the same subunits probably also houses the ubiquinone binding site. Fe-S cluster N2, which is thought to act as an electron donor to ubiquinone, is located in the PSST subunit approximately 20–30 Å away from the expected surface of the lipid bilayer. (Efremov *et al.* 2010, Hunte *et al.* 2010). Although the resolution in both of these studies was sufficient for determining the overall arrangement of the α-helices of the membrane domain, the extramembrane loops were not resolved nor could a second quinone binding site in the distal part of the membrane arm as suggested by Nakamura-Ogiso *et al.* (2003) and Gong *et al.* (2003) be verified or ruled out.

2.1.2 The hydrophilic domain

Half of the core subunits of complex I are constituents of its hydrophilic arm and contain all the redox-active components needed for NADH oxidation and electron transfer reactions to ubiquinone (see Table 1 and Figure 2). The atomic level molecular structure for this enzyme domain has been solved for *T. thermophilus* at 3.3 Å resolution. It was shown that the about 140 Å high molecule is Y-shaped with the lower part, consisting of homologs of PSST and 49 kDa subunits, forming the interface with the membrane arm (Sazanov & Hinchliffe 2006). The molecule can be divided into two functional modules connected to each other by a chain of Fe-S clusters. The NADH oxidation domain is composed of 51 kDa, 24 kDa and 75 kDa subunits, although only the 51 kDa and 24 kDa subunits are sufficient for the oxidation activity (Barker *et al.* 2007). The other domain formed by the 30 kDa, 49 kDa, PSST and TYKY subunits is involved in ubiquinone reduction. This modular classification of complex I reflects its evolutionary origin. (Brandt 2006). The 49 kDa and PSST subunits interact extensively with each other. A cavity at the interface of the 49 kDa and PSST subunits leading to the proximity of Fe-S cluster N2 in PSST has been observed and proposed to be the ubiquinone binding site. (Sazanov & Hinchliffe 2006). A more detailed description of the NADH and ubiquinone binding sites in the hydrophilic domain will be presented in sections 2.3.1 and 2.3.2. The 30 kDa subunit in the hydrophilic part of the enzyme is the only one not involved in cofactor binding or electron transfer reactions. However, it has been shown in *E. coli* that the highly conserved acidic residues Glu-138, Glu-140 and Asp-143 of the 30 kDa subunit (in *E. coli* numbering) are essential for the structural integrity of the enzyme complex. This suggests it has a significant role in anchoring the hydrophilic arm into the membrane domain. (Castro-Guerrero *et al.* 2010).

2.1.3 Subunits in the proximal part of the hydrophobic domain

ND1

ND1 is a polypeptide of 318 amino acids in humans and is one of the most conserved subunits of the membranous core of complex I. Topological analysis of alkaline phosphatase fusion constructs of its homolog from *Rhodobacter capsulatus* in *E. coli* showed that it contains eight transmembrane helices and four relatively large hydrophilic domains facing the bacterial cytoplasm (Roth & Hägerhäll 2001). Contrary to other core subunits in the membrane arm of complex I, the most conserved regions of ND1 are found in the relatively large hydrophilic sequence motifs between the transmembrane helices facing the cytoplasmic (matrix) side of the membrane (Fearnley & Walker 1992, Sinha *et al.* 2009).

ND1 has been shown to be centrally located with respect to the hydrophilic subunits and close to the provisional ubiquinone binding site at the interface of the 49 kDa and PSST subunits, see Figure 2 (Efremov *et al.* 2010, Sazanov *et al.* 2000, Schuler & Casida 2001). Site-directed mutagenesis of its homolog in *E. coli* led to a suggestion that many conserved residues within its matrix-side loops are essential for the attachment of the peripheral arm to the membrane domain of complex I (Sinha *et al.* 2009). Furthermore, ND1 seems to contribute to the ubiquinone binding site(s) of complex I, since it is labeled with photoaffinity derivatives of the inhibitors rotenone and acetogenins. Binding of the acetogenin derivatives to ND1 is suppressed by UQ analogs in a concentration-dependent manner or by the inhibitors rotenone and piericidin A. (Earley *et al.* 1987, Kakutani *et al.* 2010, Murai *et al.* 2007, Sekiguchi *et al.* 2009). Moreover, the m.3460G>A mutation in the *MTND1* gene resulting in replacement of p.Ala-52 by Thr has been shown to alter complex I interaction with the inhibitor rotenone and also with the substrate ubiquinone both in human and bacterial cells (Majander *et al.* 1996, Zickermann *et al.* 1998). Also the substitutions of the highly conserved glutamate residues 158, 212 and 247 in *P. denitrificans* in the region of transmembrane helices four to six altered complex I interaction with ubiquinone (Kurki *et al.* 2000). It has also been postulated that ND1 may be important even for coupling the electron and proton transfer reactions of the enzyme (Efremov *et al.* 2010). Finally, the *MTND1* gene has been shown to be a mutational hot spot for many LHON- and MELAS-associated substitutions,

emphasizing the importance of ND1 to complex I function (Kirby *et al.* 2004, Valentino *et al.* 2004).

ND4L

With only 98 amino acids in humans, ND4L is the smallest polypeptide of the membranous core of complex I. Consistent with hydropathy analysis of the subunit, topological study of its homolog in *P. denitrificans* using maltose binding protein fusions showed that ND4L has three transmembrane helices, and its cytoplasmically located C-terminus is not covered by other subunits (Kao *et al.* 2002).

It has been shown that ND4L displays sequence similarity to a certain class of Mrp antiporters involved in monovalent cation/H^+ antiporter activity in diverse organisms. Thus, ND4L likely has a role in proton translocation by complex I. (Mathiesen & Hägerhäll 2003). Of the few conserved amino acid residues in its sequence, glutamates 36 and 72 (*E. coli* numbering) are located within the provisional transmembrane helices two and three of ND4L (see Figure 5 in original publication I) and are candidates involved in proton pumping (Fearnley & Walker 1992, Kao *et al.* 2002). Site-directed mutagenesis of these two conserved glutamates in the *E. coli* homolog showed that they are essential for the proper insertion of the subunit into the membranes aided by the protein YidC, a chaperone involved in complex I assembly in *E. coli* (Price & Driessen 2010). ND4L seems to be essential for the assembly of the entire complex I as well, since disruption of its gene in *R. capsulatus* leads to a suppression of complex I activity and disappearance of the Fe-S clusters, although individual subunits are still immunodetectable in the membranes (Dupuis *et al.* 1998). Similarly, suppression of ND4L expression by RNA interference in *Chlamydomonas reinhardtii* leads to a complete complex I assembly defect (Cardol *et al.* 2006).

ND6

ND6 contains 174 amino acid residues in humans and is intermediate in size among the subunits of the hydrophobic core of complex I. Although it is one of the least conserved proteins of the enzyme, its N-terminal part shows relatively high homology between species (Fearnley & Walker 1992). Topology of the subunit has been determined for its counterpart in *P. denitrificans*. In accordance with its hydropathy profile, ND6 was shown to contain five transmembrane

segments with N- and C-termini facing the periplasmic and cytoplasmic sides of the membrane, respectively (Kao *et al.* 2003).

Based on gene disruption experiments in *R. capsulatus*, ND6 has been shown to have a role in biosynthesis of complex I (Dupuis *et al.* 1998). Lack of ND6 may cause defective assembly of the other mtDNA-encoded subunits of complex I, since a single frameshift mutation in its gene leading to expression of an unstable, truncated form of ND6 has been shown to reduce expression of other ND-subunits in mouse fibroblast cells (Bai & Attardi 1998). A more detailed description of the role of ND6 in complex I activity was gained by site-directed mutagenesis of its homolog in *E. coli* (Kao *et al.* 2005). Tyr-59, Gly-61, Met-64, Val-65, Phe-67 and Glu-80 (in *E. coli* numbering) were found to be important for the NADH-ubiquinone activity. Although no change in sensitivity towards complex I inhibitor capsaicin-40 was observed in any of these mutants (Kao *et al.* 2005), ND6 seems to contribute to the ubiquinone binding site of complex I on the grounds of the results of inhibitor and quinone binding studies of the disease-associated substitutions in its gene (Carelli *et al.* 1999, Jun *et al.* 1996).

ND3

The ND3 subunit is composed of 115 amino acids in humans and is one of the smallest subunits in the membranous core of complex I. Its membrane topology has been determined in *P. denitrficans* using immunochemical and cysteine modification techniques. The results were suggestive of three transmembrane segments with the N-terminus of the polypeptide facing the cytoplasmic and the C-terminus facing periplasmic side of the membrane (Bernardo *et al.* 2000). Although the primary sequence of ND3 is not highly conserved, hydropathy profiles of the homologs are similar to each other (Bernardo *et al.* 2000).

Close location of ND3 with respect to a potential ubiquinone binding site at the interface of the 49 kDa and PSST subunits has been demonstrated with crosslinking experiments in *P. denitrificans* membranes (Di Bernardo & Yagi 2001, Kao *et al.* 2004b). Based on primary sequence comparison of the ND3 subunit and its homologs and the proposed topological model for its counterpart in *P. denitrificans*, it was suggested that the highly conserved Asp-74 and Glu-76 residues (*P. denitrificans* numbering) in the middle of the second transmembrane helix may be structurally or functionally essential. Indeed, site-directed mutagenesis of the corresponding residues in the *E. coli* homolog demonstrated that these two acidic residues are important for complex I activity, but have no

major impact on the architecture of complex I (Kao *et al.* 2004a). However, ND3 seems to be essential for the proper assembly of intact complex I, since it was shown by RNA interference technique in green alga *C. reinhardtii* that absence of ND3 prevents complex I assembly entirely (Cardol *et al.* 2006).

2.1.4 Subunits in the distal part of the hydrophobic domain

X-ray crystallographic analysis of the membrane domain of *E. coli* complex I at 3.9 Å has demonstrated that the distal part of this domain is composed of three repeating modules with 14 transmembrane helices in each. Four of the helices form a core of each module surrounded by a ring of ten helices, two of which are discontinuous. The most distally located module contains an additional transmembrane helix, which continues as an amphipathic α-helix that extends a distance of about 110 Å towards the proximal part of the enzyme and ends in a sixteenth transmembrane helix for that subunit (Figure 2). (Efremov *et al.* 2010). Based on fragmentation and single particle analysis studies on isolated complex I from *E. coli* (Baranova *et al.* 2007, Holt *et al.* 2003) and sequence comparison (Efremov *et al.* 2010), this subunit at the tip of the membrane domain must be ND5, and the one residing next to it is ND4, while the most proximal one is ND2.

ND2, ND4 and ND5 show sequence similarity to each other and are also homologous to Mrp family antiporters, suggesting that these subunits may be operating in the vectorial proton translocation by complex I (Fearnley & Walker 1992, Hamamoto *et al.* 1994, Mathiesen & Hägerhäll 2003). In addition to their provisional role in proton pumping activity, the ND2, ND4 and ND5 subunits have been proposed to be involved in quinone binding, too (Gong *et al.* 2003, Nakamaru-Ogiso *et al.* 2003, Nakamaru-Ogiso *et al.* 2010a).

Sequence comparison of ND2, ND4 and ND5 proteins has demonstrated the presence of conserved glutamate and lysine residues (Glu-144 and Lys-234 of *E. coli* NuoM (homolog of the ND4 subunit)) in corresponding positions of the transmembrane helices of these three complex I subunits, and the glutamate is conserved even in the Mrp antiporters (Euro *et al.* 2008a, Torres-Bacete *et al.* 2007, Torres-Bacete *et al.* 2009). Based on their location in the transmembrane helices, these residues were proposed to have an essential role in proton pumping (Efremov *et al.* 2010). The Glu-144 and Lys-229 residues of the ND5 subunit homolog in *E. coli* have been subjected to site-directed mutagenesis and were found to be essential for the catalytic activity (Nakamaru-Ogiso *et al.* 2010b). Similarly, Glu-144 and Lys-234 in ND4 and Glu-133 and Lys-217 in ND2 have

been shown to be essential for quinone reductase activity in *E. coli* (Amarneh & Vik 2003, Euro *et al.* 2008a, Torres-Bacete *et al.* 2007). Unfortunately, no detailed analysis of the proton pumping efficiency of complex I was performed in any of these studies for the mutagenized residues. However, for ND5 it was found that replacement of Asp-178, Asp-303 and Asp-400 residues by non-carboxylic amino acids impairs the H^+/e^- efficiency to about 50% of the control, suggestive of involvement of this subunit in indirect proton translocation (Nakamaru-Ogiso *et al.* 2010b). Clearly, further studies are needed to clarify the role of these subunits in complex I function.

2.2 Complex I redox-groups

Complex I contains a total of eight iron-sulfur clusters and one FMN that take part in the electron transfer activity of complex I (Ohnishi 1998). Some bacteria, like *E. coli* and *T. thermophilus,* possess one additional iron-sulfur cluster that has shown to be essential for the stability of the enzyme (Friedrich 1998, Pohl *et al.* 2007, Yano *et al.* 1997). All the redox-groups of complex I are localized in the core subunits of the hydrophilic part of the enzyme with less than the 14 Å distance apart that is a prerequisite for physiological electron transfer (Page *et al.* 2003, Sazanov & Hinchliffe 2006). Energy released in the consecutive oxidoreduction reactions along the way of this intramolecular electron transport chain to ubiquinone is used to drive the proton pumping activity of complex I.

2.2.1 Flavin (FMN)

Flavin mononucleotide (FMN), a derivative of B2 vitamin riboflavin, is the immediate electron acceptor from NADH in complex I. Based on the molecular structure of the hydrophilic domain of complex I from *T. thermophilus* at 3.3 Å, it is located at the end of a solvent-accessible cavity in the 51 kDa subunit at the top of the hydrophilic domain at a distance of about 90 Å from the membrane plane. Edge-to-edge distances from FMN to nearby iron-sulfur clusters N3 and N1a are 7.6 and 12.3 Å, respectively. (Sazanov & Hinchliffe 2006). FMN is non-covalently bound to complex I and is held in place through hydrogen bonds (Sazanov & Hinchliffe 2006). Dissociation of FMN from the enzyme seems to be dependent also on the redox state of complex I, since reduction of dilute samples with NADH under alkaline conditions results in reversible dissociation of FMN from the enzyme complex in bovine heart submitochondrial particles

(Gostimskaya *et al.* 2007), but this probably has no major significance under physiological conditions.

FMN can occur in three different redox states; oxidized, fully reduced or in semireduced radical form. Potentiometric analysis of the thermodynamic properties of FMN in isolated bovine heart complex I has shown that the midpoint redox potentials at pH 7.5 for the one-electron reduction of oxidized and semireduced FMN are -415mV and -336mV, respectively. The midpoint potential of the two-electron reduction of enzyme-bound FMN was also determined in the same study, and found to be -340mV at pH 7.0. (Sled *et al.* 1994).

Being capable of accepting/donating one or two electrons at a time forming a reduced or semireduced state implies that FMN can serve complex I as an electron flow splitter from two- to one-electron carriers NADH and iron-sulfur clusters, respectively. Occasionally, electrons may escape from reduced FMN forming oxygen radicals, and both semiflavin and fully reduced FMN have been suggested to be a source of complex I-derived superoxide anions (Galkin & Brandt 2005, Kussmaul & Hirst 2006, Ohnishi *et al.* 2010b). FMN can react with other substrates as well. The inhibitor-insensitive ubiquinone reduction by complex I has been localized to its NADH binding site and shown to be catalyzed by FMN (King *et al.* 2009). Complex I also oxidises NADH with some artificial electron acceptors like hexaammineruthenium (III) and ferricyanide in a diaphorase-type reaction (Sled & Vinogradov 1993). It is generally accepted that in this reaction the reductant is the flavin in an enzyme-NAD^+-FMN_{red} intermediate (Vinogradov 2008). This implies that the reaction occurs at or near the NADH-binding site, as evidenced by the high K_m for NADH in the diaphorase reaction as compared with the physiological reaction with ubiquinone (for a review, see Vinogradov (2008)). This also means that this reaction is not dependent on the function of more distal redox centers or subunits and that this shortcut reaction can be used to estimate the amount of complex I in a membrane preparation under investigation.

2.2.2 Iron-sulfur clusters

There are three different types of Fe-S clusters in the respiratory chain, two of which, the binuclear [2Fe-2S] and tetranuclear [4Fe-4S] clusters, are found in complex I. The binuclear clusters are composed of two iron atoms that function as one electron redox couple bridged to each other by two sulphur atoms, and each iron is coordinated by an additional two cysteinyl sulphurs from the protein

polypeptide chain (Ohnishi 1998). In the [4Fe-4S] Fe-S clusters, four iron atoms and four sulphur atoms are in cubical arrangement with each iron ligated to the apoprotein by a cysteine-residue sulphur. These tetranuclear clusters of complex I are of the ferredoxin-type being capable of transferring one electron at a time. (Ohnishi 1998).

Complex I contains two [2Fe-2S] clusters, namely N1a and N1b, while the rest, N2, N3, N4, N5, N6a and N6b as well as N7, which is present only in some bacteria, are tetranuclear. The location of each of these clusters within the enzyme (see Table 1) has been finally determined by resolution of the crystal structure of the hydrophilic domain of complex I in *T. thermophilus*. The edge-to-edge distance between the Fe-S clusters varies from about 8 to about 20 Å, although the distance between the clusters in the order the electrons are believed to flow are within a maximum of 14 Å (Hunte *et al.* 2010, Sazanov & Hinchliffe 2006). It has been widely accepted that cluster N1a has the lowest midpoint potential of about -400mV (Ohnishi 1998, Zu *et al.* 2002), which is even lower than for the two-electron reduction of FMN (see above). The rest of the Fe-S clusters, apart from high-potential N2 with an E_m of about –100mV on average (Garofano *et al.* 2003, Ohnishi 1998, Zwicker *et al.* 2006), have their midpoint potentials in the range from -240mV to -270mV and are classified as isopotential clusters (Ohnishi 1998). However, these redox-potentials of Fe-S clusters have been questioned by the results of EPR spectroscopy-based redox titration of isolated *E.coli* complex I. It was shown that the E_m of cluster N1a is -235mV, while the E_m of -315mV was assigned to N3 or N7 and -330 mV to a cluster N4 or N5. Tetranuclear cluster Nx with a yet unknown identity showed E_m of about -365mV. Redox potentials of the rest of the EPR-detectable clusters (N1b -245/-320mV, N2 -200/-300mV, N6b -235/-315mV) was presented as a sum of at least two components due to intrinsic electrostatic interactions. (Euro *et al.* 2008b).

The NADH oxidation site of complex I is located about 100 Å away from cluster N2, which is thought to be the final electron donor to ubiquinone (Efremov *et al.* 2010, Hunte *et al.* 2010, Sazanov & Hinchliffe 2006). The wire formed by the Fe-S clusters serves the enzyme as an electron transferring pathway from NADH to ubiquinone. Cluster N1a may exhibit an antioxidative role by diminishing the oxygen radical production by reduced flavin (Sazanov & Hinchliffe 2006).

2.3 Substrate-binding sites of complex I

Complex I has two natural substrates, NADH and ubiquinone, which bind at distinct domains of the enzyme. Details of these substrate-binding sites will be presented below.

2.3.1 NADH

Photoaffinity labeling experiments of isolated complex I of bovine and bacterial origin with derivatives of NAD^+ have shown that NADH is presumably bound to the 51 kDa subunit (Chen & Guillory 1981, Deng *et al.* 1990, Yagi & Dinh 1990). When the atomic level molecular structure of the hydrophilic part of complex I with nucleotides bound was resolved for *T. thermophilus*, it appeared that NADH is lined up with its nicotinamide ring adjacent to the isoalloxane ring of FMN coordinated by the 51 kDa subunit (Sazanov & Hinchliffe 2006). Homologous position of this NADH binding cavity in *E. coli* has been subjected to site-directed mutagenesis. It was found that replacement of the 51 kDa subunit's invariant Tyr-97 residue (*T. thermophilus* numbering), predicted to interact with the nicotinamide group of NADH, with glutamine decreased complex I affinity to NADH but increased the affinity to NAD^+. Furthermore, it was proposed that the negative charge of this amino acid residue is essential for propulsion of the oxidized NADH from the active site. (Euro *et al.* 2009).

2.3.2 Ubiquinone

It is generally accepted that the Fe-S cluster N2 of complex I is the immediate electron donor to ubiquinone that serves the respiratory chain as a mobile electron carrier. This is supported by the findings of the direct spin-spin interaction of the cluster N2 and fast relaxing semiquinone observed in the presence of membrane potential in bovine complex I (Magnitsky *et al.* 2002). When the molecular structure of the hydrophilic domain of complex I from *T. thermophilus* was resolved, Fe-S cluster N2 was assigned to the PSST subunit next to a cavity formed at the interface of the 49 kDa and PSST subunits (Sazanov & Hinchliffe 2006). This is in line with earlier findings of a photoaffinity labeling of the PSST subunit with a derivative of complex I inhibitor pyridaben (Schuler *et al.* 1999), and of inhibitor resistance induced by the amino acid substitutions of the 49 kDa subunit (Darrouzet *et al.* 1998, Prieur *et al.* 2001). Later on, it was shown by site-

directed mutagenesis of the 49 kDa and PSST subunits in *Y. lipolytica* that many residues lining the cavity between them in the proximity of cluster N2 are essential for complex I activity and inhibitor binding. The data suggest that the hydroxyl-group of the Tyr-144 residue (*Y. lipolytica* numbering) in the 49 kDa subunit is involved in binding of the head group of the ubiquinone molecule. (Fendel *et al.* 2008, Tocilescu *et al.* 2007, Tocilescu *et al.* 2010). However, apart from Tyr-144 in the 49 kDa subunit, most mutations with appreciable changes in inhibitor affinity had little effect on ubiquinone binding affinity. It was suggested that this could mean that the interaction of complex I with ubiquinone is partly under diffusional control, since the site-directed mutations with inhibitor resistance had lowered ubiquinone reductase activities and mutations with lowered activities tend to have a lowered apparent K_m for ubiquinone (Fendel *et al.* 2008).

The cavity formed by the 49 kDa and PSST subunits close to the Fe-S cluster N2 seems to be involved in inhibitor and ubiquinone binding. Based on the molecular structure of the entire complex I from *T. thermophilus* at 4.5 Å resolution, subunit ND1 together with one of the ND3, ND6 and ND4L subunits contribute to this ubiquinone binding site (Efremov *et al.* 2010). Of these polypeptides in the membranous core of the enzyme, the most extensive evidence in favour of its involvement in ubiquinone binding has been presented for the ND1 subunit (see section 2.1.3 above).

Whether a second ubiquinone binding site in addition to the one described above exists is unclear. Based on the different mode of action of molecules belonging to different classes of complex I inhibitors, like piericidin A and rotenone, it was suggested that two or more distinct inhibitor (and ubiquinone) binding sites exist (Friedrich *et al.* 1994). However, this has been disproved by competition binding assays of different types of inhibitors (Okun *et al.* 1999) and by site-directed mutagenesis studies in *Y. lipolytica* (Fendel *et al.* 2008) demonstrating a single, partially overlapping inhibitor binding site for all inhibitors acting at the electron transfer step to ubiquinone. On the other hand, identification of two different semiquinone species has been used as an indication of two separate quinone binding sites (Magnitsky *et al.* 2002), but this has also been questioned (Brandt 2006, Zickermann *et al.* 2009). As long as the detailed molecular structure of the entire complex I with substrates bound is unsolved, the number and location of the ubiquinone binding sites is only provisional.

2.4 Function of complex I

The main purpose of complex I is to oxidize NADH, recycling it for use as a cosubstrate in cellular metabolism, mainly in the citric acid cycle (Krebs cycle) and β-oxidation of fatty acids in the mitochondrial matrix, and to transfer the electrons (e^-) to ubiquinone. Energy released in this process is used to drive proton pumping across the inner mitochondrial membrane to generate the proton (H^+) gradient needed for ATP synthesis by the mitochondrial ATP synthase. The H^+/e^- ratio for complex I is close to 2 (Galkin *et al.* 1999, Wikström 1984), meaning the transfer of four protons across the membrane per each molecule of NADH oxidized. The overall reaction catalyzed by complex I is summarized by the following equation:

$$NADH + H^+ + UQ + 4H^+_{in} \rightarrow NAD^+ + QH_2 + 4H^+_{out}$$

where *in* denotes the matrix and *out* the cytosolic side of the mitochondrial membrane.

2.4.1 Electron transfer

NADH oxidation by complex I is initiated by its binding to the active site of the 51 kDa subunit. Electron transfer from NADH to FMN is considered to proceed by hydride transfer rather than by transfer of two electrons and a proton separately to avoid formation of an unstable radical form of NAD^+ (Hirst 2009). From FMN, electrons are passed on to the ubiquinone reduction site one by one through the Fe-S clusters in the order of N3-N1b-N4-N5-N6a-N6b-N2 based on the structural data for the hydrophilic domain of *T. thermophilus* (Sazanov & Hinchliffe 2006). Cluster N1a, located outside the chain formed by the other Fe-S clusters, has no role in energy transduction, but is considered to be important in preventing the formation of oxygen radicals by the flavosemiquinone (Hirst 2009, Sazanov & Hinchliffe 2006, Verkhovskaya *et al.* 2008). A model of a specific electron wiring pathway has been suggested, which consists of cysteine residues liganding with the Fe-S clusters and other conserved amino acid residues together with internal water molecules between protein subunits, mediating the electron transfer reactions between neighbouring Fe-S clusters (Hayashi & Stuchebrukhov 2010). The advantage of such an arrangement could be enhancement of the electron transfer rates of complex I (Hayashi & Stuchebrukhov 2010).

Based on an ultrafast freeze-quench approach for purified *E. coli* complex I, it has been demonstrated that electron transfer from FMN to Fe-S cluster N2 is fast and occurs within a time scale of less than 100 µs, which is close to that of the reduction of cluster N1a that occurs within the same time constant as well, and is unlikely to be coupled to proton translocation or conformational changes of complex I (Verkhovskaya *et al.* 2008). Moreover, it seems very likely that intramolecular electron transfer is not rate limiting in complex I catalysis (Hirst 2009, Verkhovskaya *et al.* 2008).

2.4.2 Semiquinones

Semiquinone is a product of one electron reduction of ubiquinone and can exist in neutral or anionic form. Due to the presence of an unpaired electron, semiquinones can be studied with EPR spectroscopy. Formation of semiquinones during catalysis has been demonstrated for many respiratory enzymes, e.g. succinate dehydrogenase (complex II) and ubiquinone-cytochrome *c* oxidoreductase (complex III) of the mitochondrial respiratory chain (Ingledew & Ohnishi 1977, Ohnishi & Trumpower 1980). For complex I, two separate semiquinone species, namely SQ_{Nf} and SQ_{Ns}, have been identified on the basis of their EPR properties (Ohnishi *et al.* 2005, Ohnishi & Salerno 2005, Yano *et al.* 2005). SQ_{Nf} has been detected only in the presence of an electrochemical proton gradient across the membrane and is sensitive to the complex I inhibitors rotenone and piericidin A, while the latter, SQ_{Ns}, is insensitive to membrane potential. Based on its spin-spin interaction with Fe-S cluster N2, SQ_{Nf} has been suggested to be located close to it, and the estimated distance between N2 and SQ_{Nf} is about 12 Å. Contrary to that, SQ_{Ns} is more distally located with at least 30 Å separating it from N2. (Magnitsky *et al.* 2002, Ohnishi 1998, Ohnishi *et al.* 2008, Yano *et al.* 2005).

The occurrence of two semiquinones has been suggested to be indicative of two separate quinone binding sites in complex I. The SQ_{Nf} site serves the enzyme as a tightly bound cofactor site that mediates one electron transfer to the other site presenting a binding pocket for the SQ_{Ns} in equilibrium with the ubiquinone pool of the membrane. SQ_{Nf} may also be involved in proton pumping. (Dutton *et al.* 1998, Ohnishi *et al.* 2010a, Ohnishi 1998, Ohnishi & Salerno 2005). The existence of a tightly bound ubiquinone as a cofactor in complex I is supported by the findings from isolated complex I from bovine heart mitochondria and *E. coli* membranes, which were shown to contain one ubiquinone molecule per each

holo-enzyme (Shinzawa-Itoh *et al.* 2010, Verkhovskaya *et al.* 2008). This does not rule out the possibility of two separate quinone binding sites nor verify the number of semiquinones formed during catalysis. On the other hand, it has been argued that the observed SQ_{Nf} and SQ_{Ns} signals arise from the same semiquinone species located at different sites and/or present in different states of the catalysis along the quinone binding pocket of complex I (Brandt 2006, Zickermann *et al.* 2009). Whether two separate positions for ubiquinone binding really exist remains to be solved.

2.4.3 Proton pumping

Contrary to recent progress on the intramolecular electron transfer reactions, there is less solid understanding of the mechanism of proton pumping in complex I. Several hypotheses regarding the mechanism of proton translocation driven by the redox chemistry of complex I have been presented (for reviews, see Brandt (1997) and Hirst (2009)). Only the outlines of the current views will be presented here.

A consensus H^+/e^- ratio of 2 for complex I has been reached based on experiments with bovine and yeast mitochondria, tightly coupled submitochondrial particles, and proteoliposomes containing reconstituted isolated complex I from yeast (Galkin *et al.* 2006, Galkin *et al.* 1999, Honkakoski & Hassinen 1986, Wikström 1984). For bacterial complex I, anaerobically grown *E. coli* cells were shown to have a stoichiometry of at least 1.5 (Bogachev *et al.* 1996), but more extensive evidence on the H^+/e^- ratio in prokaryotes is still lacking.

A number of subunits in the membranous core of complex I have been postulated to be involved in proton pumping. Based on analysis of other enzymes involved in proton and ion transfer across cell membranes, like ATP synthase and Na^+/H^+ antiporters, the presence of highly conserved protonable residues in the middle of a transmembrane helix has been shown to be essential for the activity (Inoue *et al.* 1995, Kajiyama *et al.* 2009, Miller *et al.* 1990, Padan 2008). For complex I, such extraordinarily positioned, functionally important glutamate, aspartate and lysine residues in highly hydrophobic surroundings within transmembrane helices have been demonstrated for subunits ND4L, ND2, ND3, ND4 and ND5 by site-directed mutagenesis of their bacterial homologs (Amarneh & Vik 2003, Euro *et al.* 2008a, Kao *et al.* 2004a, Kao *et al.* 2005, Nakamaru-Ogiso *et al.* 2010b, Torres-Bacete *et al.* 2007), and suggested to be present even in subunit ND1 (Euro *et al.* 2008a). The H^+/e^- efficiency has been estimated only

for ND4 and ND5 subunit mutations and was found to be affected solely in the latter case (Euro *et al.* 2008a, Nakamaru-Ogiso *et al.* 2010b). However, due to their intrinsic sequence similarity and homology to Mrp-like Na^+/H^+ antiporters, ND2, ND4 and ND5 have been proposed to be the strongest candidates for the proton translocation machinery of complex I (Hamamoto *et al.* 1994, Mathiesen & Hägerhäll 2002, Mathiesen & Hägerhäll 2003).

Mechanism of proton pumping

At present, two different coupling mechanisms of electron and proton transfer activity in complex I have been put forward to explicate the observed H^+/e^- ratio. In the direct coupling originally presented by Ohnishi and Salerno (Ohnishi & Salerno 2005), proton pumping is driven by the redox-chemistry of two separate ubiquinone species allocated to two separate binding sites and involves semiquinones without participation of any other coupling site or long-range conformational changes of the enzyme. Of the semiquinones involved, SQ_{Nf} located at a site next to Fe-S cluster N2, accepts two protons from the matrix side of the membrane upon reduction by an electron from N2, and this leads to conversion of the ubiquinol into a state in which the protons are released to the cytoplasmic side with simultaneous transfer of an electron to the ubiquinone in equilibrium the membrane pool (SQ_{Ns} -site). The remaining semiquinone at the SQ_{Nf} –site accepts the other NADH-derived electron from N2, and the cycle is re-rotated resulting in a vectorial transfer of $4H^+$ per each electron pair. The most compelling evidence for this hypothesis comes from the findings of two complex I-associated semiquinone species. (Ohnishi & Salerno 2005).

The other indirect conformational change-driven proton pumping hypothesis for complex I is based on the distal location of the ND4 and ND5 subunits with respect to the ubiquinone reduction site. They are homologs of the Na^+/H^+ antiporters and are believed to be important for proton translocation across the membrane. Based on that and the redox-dependent changes in the crosslinking pattern of different complex I subunits (Belogrudov & Hatefi 1994, Mamedova *et al.* 2004) and even over the entire complex I structure (Mamedova *et al.* 2004), long-range structural rearrangements have been suggested to occur during catalysis (Brandt *et al.* 2003, Friedrich 2001, Zickermann *et al.* 2009), but these observations have been questioned later by other studies (Morgan & Sazanov 2008, Pohl *et al.* 2010). In fact, the current view of possible conformational changes during catalysis is that redox energy liberated upon ubiquinone reduction

at the interface of the hydrophilic and hydrophobic domains of complex I is transferred to proton pumping subunits in the distal part of the membranous core through a 60–100 Å long α-helix observed parallel to the long axis of the membrane arm (Efremov *et al.* 2010, Hunte *et al.* 2010).

In addition to these two different approaches, a hypothesis combining the direct and indirect coupling mechanisms has been presented recently by Ohnishi and co-workers (Ohnishi *et al.* 2010a). According to their suggestions, for each pair of electrons two protons are translocated through the direct proton pump, while the other two are pumped across the membrane in the distal part of the membranous core through redox-driven conformational changes brought about by the redox chemistry by the ubiquinones. The recent findings of Yagi's group on proton pumping efficiency in *E. coli* with mutations in the distally located complex I subunit homologous to ND5 (Nakamaru-Ogiso *et al.* 2010b) would fit this model well. Finally, a model involving changes in properties of key amino acid residues within the membrane upon electron transfer to ubiquinone has been presented by Wikström's group. It was proposed that reduction of ubiquinone in the proximal part of the membrane domain drives proton pumping by a series of electrostatic interactions of key lysine residues in the ND1, ND2, ND4 and ND5 subunits (Euro *et al.* 2008a).

2.4.4 Inhibitors of complex I activity

A variety of complex I inhibitors, both naturally occurring, like rotenone (from *Derris scandens*), piericidin A (from *Streptomyces mobaraensis*), acetogenins (from various *Annonaceae* species) and their derivatives, and synthetically manufactured like pyridaben and various piperazin derivatives (Ichimaru *et al.* 2008) have been used to probe the catalytic activity of complex I especially in order to clarify its ubiquinone binding site. Most of these compounds act at the electron transfer step from the Fe-S clusters to ubiquinone (Friedrich *et al.* 1994).

The prevailing classification of complex I inhibitors is based on their types of action. Type A inhibitors, like piericidin A, 2-decyl-4-quinazolinyl amine (DQA), annonin VI and rolliniastatin-1 and -2, are considered to be antagonists of the ubiquinone substrate. For piericidin A, it has been shown that it inhibits NADH:Q_2 activity in a partially competitive manner. Contrary to type A, type B inhibitors, like the commonly used rotenone, have hydrogen-bonding acceptors only in the cyclic head of the molecule and are non-competitive towards UQ, but are believed to displace the semiquinone intermediate during the catalysis. Finally,

inhibitors classified as type C, like myxothiazol, stigmatellin, *N*-vanillylnonanamide (VNA) and capsaicin, form a third group of hydrophobic complex I inhibitors and are believed to act as antagonists of reduced ubiquinone. (Degli Esposti 1998, Friedrich *et al.* 1994). Some inhibitors affect outside the ubiquinone reduction site and do not fit the preceding classification. Examples of such compounds are ADP-ribose, which competes for substrate binding at the NADH binding site (Zharova & Vinogradov 1997), and diphenyleneiodonium (DPI) that covalently attacks reduced FMN in the hydrophilic part of the enzyme (Majander *et al.* 1994) blocking the electron transfer to the Fe-S clusters.

Based on fluorescence quench titration experiments of bovine complex I with DQA, it has been shown that inhibition involves one inhibitor molecule per one complex I. Competition experiments with representatives of all three different types of inhibitors revealed that type A and B and type B and C, but not type A and C, compete with each other for binding. This led to a suggestion that all complex I inhibitors acting at the ubiquinone binding pocket share a common binding domain with partially overlapping sites. (Okun *et al.* 1999). Several site-directed mutagenesis studies and labeling experiments with a variety of photoreactive derivatives of complex I inhibitors have been conducted to locate the inhibitor binding pocket of complex I. For example, missense mutations in the 49 kDa subunit of *R. capsulatus* were shown to render complex I resistant to piericidin A, rotenone and pyridaben (Darrouzet *et al.* 1998, Prieur *et al.* 2001), and binding of a photoaffinity derivative of pyridaben to PSST has been demonstrated (Schuler *et al.* 1999). Photoreactive azidoquinazoline, another complex I inhibitor, crosslinks with the 49 kDa subunit (Murai *et al.* 2009). Stimulated by these findings and homology of the 49 kDa and PSST subunits to the [NiFe] hydrogenases, the most extensive study of the complex I inhibitor binding site has been carried out in *Y. lipolytica*. Using this model organism, it has been shown that several amino acid residues at the interface of the 49 kDa and PSST subunits are essential for the sensitivity of complex I activity to all three different types of inhibitors acting at the potential ubiquinone binding pocket with partially overlapping sites (Fendel *et al.* 2008).

The 49 kDa and PSST subunits, proposed to contribute to the inhibitor binding domain of complex I, are constituents of the hydrophilic part of the enzyme. Whether an inhibitor (and ubiquinone) binding site is present also in the membranous core is ambiguous. Based on inhibitor experiments with photoaffinity derivatives of rotenone (Earley *et al.* 1987), azidoquinazoline (Murai *et al.* 2009) and acetogenin (Murai *et al.* 2007, Sekiguchi *et al.* 2009), and

with radioactively labeled *N,N´*-dicyclohexylcarbodiimide (DCCD) (Yagi & Hatefi 1988), and due to rotenone resistance of mitochondria from LHON patients with an ND1 subunit-affecting m.3460G>A substitution in mtDNA (Ghelli *et al.* 1997), it appears that the ND1 subunit contributes to the inhibitor binding site of complex I as well. DCCD binding to the ASHI subunit of complex I has also been demonstrated (Hassinen & Vuokila 1993). Photoaffinity labeling of ND2 and ND5 subunits with complex I inhibitors (Nakamaru-Ogiso *et al.* 2003, Nakamaru-Ogiso *et al.* 2010a) and changes in inhibitor sensitivity in mitochondria from LHON patients with m.11778G>A and m.14484T>C substitutions in the ND4 and ND6 subunits (for a review, see (Lenaz *et al.* 2004)), suggest that these proteins also contribute to the inhibitor binding site(s) of complex I. Nonetheless, it is noteworthy that labeling of a single subunit does not necessarily indicate the actual inhibitor binding site, since the label could also be incorporated a distance away depending on the location and orientation of the photoactive group in the molecule. In any case, it is obvious that many of the core subunits contribute to the inhibitor binding site.

2.5 Complex I-associated diseases

Oxidative phosphorylation catalyzed by the mitochondrial respiratory chain is the terminal metabolic pathway to capture the combustion energy of dietary lipids, carbohydrates and proteins into ATP that is used as a driving force in many cellular reactions. Aerobic energy production via these consecutive redox reaction-catalyzing enzymes is energetically much more efficient than anaerobic metabolism, since e.g. 38 moles of ATP are produced per one mole of glucose in aerobic respiration (according to the common textbook views, although differing stoichiometries can be reached, depending on the values adopted for the H^+/e^- and ATP/H^+ ratios), while anaerobic fermentation of glucose yields only 2 moles of ATP. Therefore, it is not surprising that many diseases affecting the mitochondrial respiratory chain enzymes affect mostly tissues with high energy demand, like the nervous system, eye, heart, kidney and skeletal muscle.

Respiratory dysfunction has a prevalence of at least 1:10000 (Skladal *et al.* 2003) and complex I deficiency is one of the most commonly encountered diseases of the respiratory chain (Loeffen *et al.* 2000). Genetically heterogeneous complex I disorders present themselves with variable clinical manifestations ranging from tissue-selective damage in Leber hereditary optic neuropathy (LHON) to multi-organ syndromes like mitochondrial encephalomyopathy, lactic

acidosis and stroke-like episodes syndrome (MELAS) in adults and severe disorders like Leigh's disease in childhood. Even the more common neurodegenerative diseases like Parkinson's disease have been linked to complex I deficiency (Schapira *et al.* 1989).

Like all other respiratory complexes apart from succinate dehydrogenase, complex I is under the control of both the nuclear and mitochondrial genomes, meaning that inherited complex I-associated diseases follow either autosomal dominant or recessive, X-linked or mitochondrial inheritance, depending on the location of the underlying genetic cause. Diagnosis of complex I associated diseases is based on various tests. Failure to oxidize NADH leads to a change in cellular redox state which will ultimately cause elevation of blood lactate and ketone body levels, and the lactate/pyruvate ratio is a frequently used laboratory tests for screening especially when a syndromic respiratory disorder is suspected (Munnich *et al.* 1992). More precise analysis is based on enzymatic, genetic and histopathological examination of patient samples. No curative treatment is available.

In the following sections, two complex I-associated non-syndromic and syndromic diseases, LHON and MELAS, will be described in more detail and examples of overlap syndromes with features of two or more distinct diseases are included.

2.5.1 Leber hereditary optic neuropathy (LHON)

In 1871, German ophthalmologist Theodor Leber reported on a hereditary optic atrophy affecting young men. Since then, the disease has been known as LHON. It was as late as 1988, when this maternally inherited disease was shown to be due to a mitochondrial DNA mutation m.11778G>A affecting the MTND4 subunit of complex I (Wallace *et al.* 1988), and indeed, this was the first study to link mtDNA substitution with a human disease. Later on, numerous other complex I-affecting mtDNA substitutions have been shown to be associated with LHON (see Table 2), although many of them are sporadic or found only in single families.

LHON is one of the most common mitochondrial disorders. Estimates of its prevalence vary between 2/100000 in Finland (Puomila *et al.* 2007) to about 3.22/100000 in the North East of England (Man *et al.* 2003). Interestingly, not all individuals with pathogenic mutations become affected, since the prevalence of LHON mutations was shown to be as high as about 1 in 10000 for the three most common LHON mutations (Man *et al.* 2003, Puomila *et al.* 2007). Indeed, only

about 50% of men and 10% of women carrying an LHON mutation develop the disease (Yen *et al.* 2006). This incomplete penetrance and gender bias has led to a suggestion of involvement of additional factors in the pathogenesis of LHON. Linkage analysis of the nuclear genes has identified two different loci on the X-chromosome as candidates for nuclear genetic factors that could explain the sex bias (Hudson *et al.* 2005, Shankar *et al.* 2008). Involvement of other genetic factors in LHON is also presented by mtDNA haplogroup analysis that shows increased penetrance of m.11778G>A and m.14484T>C mutations in haplogroup J and m.3460G>A in haplogroup K (Hudson *et al.* 2007). In addition to these genetic factors, exposure to certain environmental factors, like tobacco smoking, affects the clinical manifestation of LHON (Kirkman *et al.* 2009).

Table 2. LHON-associated mtDNA mutations and the resulting amino acid changes affecting complex I (modified from Yu-Wai-Man *et al.* (2009)).

Complex I subunit	mtDNA mutation	Amino acid change
ND1	m.3460G>A[1]	p.Ala52Thr
	m.3697G>A	p.Gly131 Ser
	m.3733G>A	p.Glu143 Lys
	m.4160T>C	p.Leu285 Pro
	m.4171C>A	p.Leu289 Met
ND4	m.11253T>C	p.Ile165 Thr
	m.11696G>A	p.Val312 Ile
	m.11778G>A[1]	p.Arg340 His
ND4L	m.10663T>C	p.Val65 Ala
ND5	m.12848C>T	p.Ala171 Val
	m.13730G>A	p.Gly465 Glu
ND6	m.14596A>T	p.Ile26 Met
	m.14568C>T	p.Gly36 Ser
	m.14498C>T	p.Tyr59 Cys
	m.14495A>G	p.Leu60 Ser
	m.14484T>C[1]	p.Met64 Val
	m.14482C>G	p.Met64 Ile
	m.14459G>A	p.Ala72 Val
	m.14279G>A	p.Ser132 Leu

[1] Most common mutations.

Clinical course of LHON

The onset of symptoms of LHON is typically between the ages of 15 and 35 years, with 95% of patients having become ill by the age of 50 years (Harding *et al.* 1995). Most of the patients are men. The clinical course of LHON includes painless, subacute loss of central vision and impairment of color perception in one eye usually followed by the other eye with an average time interval of about two months (Yen *et al.* 2006). Initial signs show circumpapillary teleangiectatic microangiopathy and papillary pseudoedema (Nikoskelainen *et al.* 1983, Smith *et al.* 1973). Thickening of the retinal nerve fiber layer (RNFL) has been observed during the acute phase using optical coherence tomography (Barboni *et al.* 2005). As the disease progresses, RNFL degenerates with a preferential loss of the small-caliber fibers of retinal ganglion cells forming the papillomacular bundle, and optic atrophy develops (for a review, see (Fraser *et al.* 2010)). On visual field testing, a typical dense central scotoma is found. Pupillary reflexes are spared. Final visual acuity varies, but is mostly severely reduced and permanent. (Carelli *et al.* 2009, Yu-Wai-Man *et al.* 2009). Prognostic factors for better recovery include a mutation type (e.g. m.14484T>C carriers having 37–58% and m.11778G>A carriers 4–25% recovery rates), onset of the symptoms before the age of 20, and thicker RNFL and larger optic disk diameter as analyzed with optical coherence tomography (for a review, see Fraser *et al.* (2010) and Yu-Wai-Man *et al.* (2009)).

Pathophysiology of LHON

Selective loss of the retinal ganglion cells in LHON occurs by apoptosis (Danielson *et al.* 2002, Ghelli *et al.* 2003). Hypotheses on the primary cause of this phenomenon include deficient energy metabolism and involvement of reactive oxygen species, since all known mutations associated with LHON affect the respiratory chain that is the main source of oxygen radicals in cells.

Studies of the biochemical deficit in LHON have demonstrated a variable degree of complex I activity decline. In lymphoblasts and cybrids the most pronounced reduction of activity is found in m.3460G>A mutation carriers, where the NADH-ubiquinone activity is only about 20% of control. On the contrary, the other two common LHON mutations show less severe loss of complex I activity with only about 20% decline for the m.11778G>A mutation, while in m.14484T>C it was comparable to controls. (Brown *et al.* 2000). Other studies

have shown significant reduction of complex I activity only for the m.3460G>A mutation (Carelli *et al.* 1997, Carelli *et al.* 1999, Majander *et al.* 1991). Despite the fact that a definitive complex I activity deficiency has been observed only for one of the common LHON mutations, they all seem to affect complex I interaction with ubiquinone. Mitochondria with m.3460G>A or m.11778G>A mutations have been shown to render complex I resistant to its inhibitor rotenone (Carelli *et al.* 1997, Ghelli *et al.* 1997), while in m.14484T>C cybrids an increased sensivity to myxothiazol, another complex I inhibitor, has been observed (Carelli *et al.* 1999). More explicit evidence of a change in quinone binding in LHON is provided by an increase in the apparent K_m for ubiquinone in mitochondria with the m.11778G>A mutation (Ghelli *et al.* 1997). Although the electron transfer activity decline in m.11778G>A and m.14484T>C mutants with exogenous ubiquinone is at most only moderate, they have been shown to significantly impair complex I-driven respiration and ATP production with endogenous ubiquinone (Baracca *et al.* 2005, Brown *et al.* 2000, Zanna *et al.* 2003).

It has become evident that reactive oxygen species (ROS) have a crucial role in the pathogenesis of LHON in addition to imbalanced cellular energy state brought about by the complex I defect. Cybrid cells harbouring m.11778G>A have been shown to be sensitized to oxidative stress (Wong & Cortopassi 1997). Measurements of cellular antioxidative defense system activities have given indirect evidence of increased oxygen radical production in cybrids with any of the three common LHON-mutations (Floreani *et al.* 2005), as well as increased levels of 8-hydroxy-2'-deoxyguanosine in leukocyte DNA from patients with the m.11778G>A substitution (Yen *et al.* 2004). Finally, increased levels of superoxide production have been documented for the m.3460G>A, m.11778G>A and m.14484T>C mutations (Beretta *et al.* 2004, Wong *et al.* 2002). Moreover, inactivation of the mitochondrial superoxide dismutase enzyme in an animal model brought histopathological findings similar to those observed in LHON (Qi *et al.* 2003).

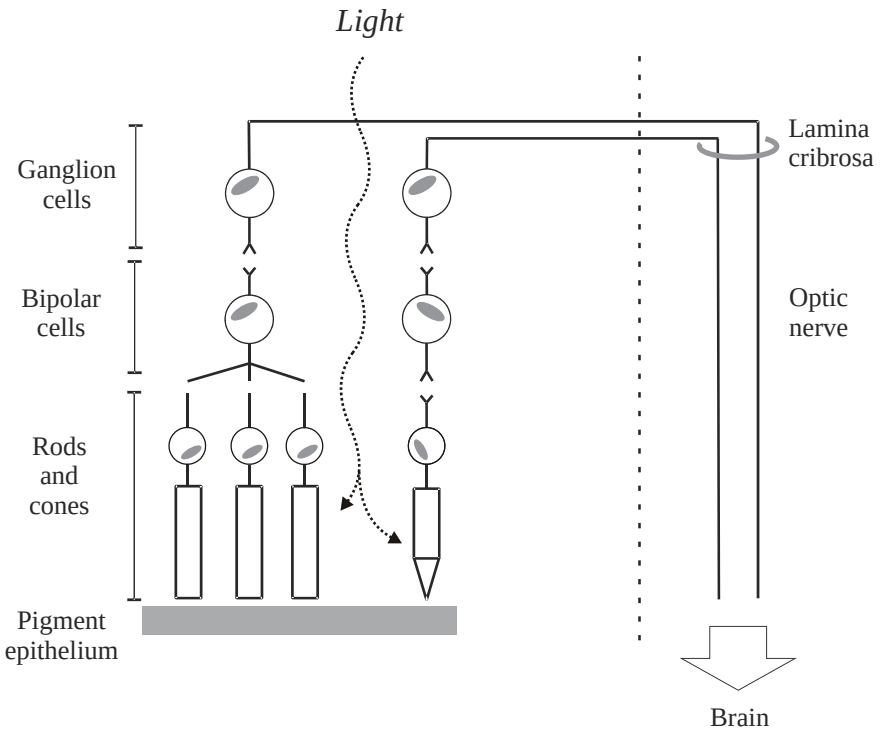

Fig. 3. Schematic presentation of the cells involved in the phototransduction and signal trasmission of an eye. Light stimulus induces changes in visual pigments of the rods and cones and causes hyperpolarization of their cell membranes. Upon hyperpolarization, reduced synaptic neurotransmitter release from the photoreceptors activates bipolar cells, which in turn leads to generation of an action potential in ganglion cells. The ganglion cell action potential is transmitted through the ganglion cell axons into the thalamus and from there to the visual cortex of the brain. The ganglion cells are affected in LHON. Amacrine and horizontal cells involved in modulation of phototransduction in retina are omitted from the drawing.

Gradual degeneration of retinal ganglion cells with sparing of pigment epithelium and photoreceptors and the fact that almost all LHON mutations are nearly homoplasmic have raised the question of why the defect is so selective. Cell bodies of the retinal ganglion cells reside in the inner retina and their long axons enter the retinal nerve fiber layer to travel into the optic nerve head, where they traverse the lamina cribrosa and form the optic nerve transferring the visual stimulus into the brain (see Figure 3). Contrary to the optic nerve, where action potential conduction is saltatory (i.e. hopping from one node of Ranvier to the next for higher velocity) in the myelin-isolated axons, transmission of visual

stimuli in the intraocular part of the axons is more energy-dependent, since the axons are unmyelinated and the volume to surface ratio of the small axons in the papillo-macular bundle is more unfavourable (Carelli *et al.* 2002). This skewed energy demand is demonstrated by uneven distribution of mitochondria in the retinal ganglion cell axons which are more abundant in mitochondria in the unmyelinated intraocular part (Bristow *et al.* 2002, Hollander *et al.* 1995). However, impaired energy production alone does not explain the selective loss of retinal ganglion cells, since photoreceptors have even higher oxidative activity (Lowry *et al.* 1956). Optical coherence tomography findings of axonal swelling in the retina of LHON-patients point to impaired axonal transport that is essential also for bi-directional transport of mitochondria in the axon (Barboni *et al.* 2005, Barboni *et al.* 2010, Hollenbeck & Saxton 2005). In addition, the common m.3460G>A, m.11778G>A and m.14484T>C LHON-mutations have been shown to impair removal of extracellular glutamate in osteosarcoma cybrid cells, suggesting that in LHON the retinal ganglion cells would be more vulnerable to excitotoxic injury caused by the neurotransmitter glutamate. This glutamate transport deficiency was shown to correlate with mitochondrial ROS production but not with cellular ATP levels. (Beretta *et al.* 2004). To conclude, selective retinal ganglion cell deterioration in LHON is clearly a multifactorial process, wherein disturbed energy metabolism and ROS generation together with unique features of the cell type involved have a significant role.

2.5.2 Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)

Patients with normal early development but later presenting with lactic acidemia, short stature, seizures, ragged red fibers in skeletal muscle and hemiparesis, hemianopia or cortical blindness suggestive of a mitochondrial myopathy were first reported more than two decades ago (Pavlakis *et al.* 1984). This maternally inherited disease with myopathy, encephalopathy, lactic acidosis and recurrent stroke-like episodes became known as MELAS syndrome. On the grounds of active research on the subject, the clinical spectrum of the symptoms associated with MELAS has expanded substantially over the decades, demonstrating the complexity of the underlying pathophysiology.

Maternal inheritance of the disease together with other findings was suggestive of mitochondrial abnormality caused by an mtDNA mutation. In 1990, Goto and co-workers were able to show that in about 80% of MELAS-patients the disease is associated with a m.3243A>G transition in the mtDNA $tRNA^{Leu(UUR)}$ gene (Goto *et al.* 1990). Later on, nearly half of the remaining patients were shown to harbour an m.3271T>C transition (Goto *et al.* 1991), while less common MELAS mutations are found for example in *ND1* (Kirby *et al.* 2004), *ND5* (Liolitsa *et al.* 2003) and *ND6* (Ravn *et al.* 2001) as well as in several mitochondrial tRNA genes.

MELAS is probably the most frequent hereditary respiratory chain disorder. A population-based study in Finland gave an estimate of 16.3/100000 for the prevalence of the common MELAS-mutation m.3243A>G (Majamaa *et al.* 1998). A more recent report from Australia on a Caucasian-based population reported a much higher prevalence of 236/100000 (Manwaring *et al.* 2007), emphasizing that the prevalence of MELAS-associated mutations is much higher than previously considered.

The age of onset of the MELAS symptoms is highly variable, but initial signs of the disease present in nearly 70% of the patients between the ages of 2 and 20 years (Pavlakis *et al.* 1984). Original criteria for the diagnosis include stroke-like episodes before the age of 40 years, encephalopathy (seizures or dementia or both), and ragged-red fibers on muscle biopsy or lactic acidosis (measured either from serum or cerebrospinal fluid) or both (Hirano *et al.* 1992, Sproule & Kaufmann 2008). Later on, several other symptoms and findings have been linked to MELAS (see Table 3).

Recurrent, fluctuating, asymmetric and predominantly cortical and subcortical ischemia-like lesions in the occipital and parietal lobes that do not follow the vascular distribution are seen on magnetic resonance imaging (MRI) of the brain during the stroke-like episodes (Sproule & Kaufmann 2008). Calcification of basal ganglia, and cerebral and cerebellar atrophy have also been documented (Sue *et al.* 1998). Immunohistochemistry of biopsy samples frequently shows blood vessels staining strongly for succinate dehydrogenase activity, indicative of mitochondrial proliferation (Sproule & Kaufmann 2008). Mitochondrial angiopathy seen in cerebral blood vessels of autopsied MELAS-patients (Ohama *et al.* 1987) has been considered to be suggestive of dysfunctional blood-brain barrier (BBB). Indeed, increased permeability of BBB in MELAS has been observed (Tanji *et al.* 2001).

Table 3. Common clinical symptoms and findings in MELAS patients according to Sproule & Kaufmann (2008).

Symptoms and findings	Frequency/ incidence (%)
Cognitive impairment	70–90
Sensorineural hearing loss	74
Migraine or other headache	77–91
Peripheral neuropathy	22–95
Stocking-glove like loss of proprioseption and vibration	
Abnormal reflexes and sensory examination	
Short stature	35–50
Cardiac abnormalities	15–30
Cardiomyopathy	
Congestive heart failure	
Conduction defects	
WPW-syndrome	
Myopathy	50–89
Exercise intolerance	
Limb weakness	
Gastrointestinal disturbances	64
Diabetes	50

Pathophysiology of MELAS

Although inclusive pathophysiology of MELAS is largely obscure, relatively much is known about the molecular level changes. Biochemical analyses of MELAS cybrid cells has pointed to a complex I activity deficiency and impaired mitochondrial protein synthesis (Dunbar *et al.* 1996). The number of studies on the biochemical consequences of MELAS mutations is highest for the common m.3243A>G mutation. It has been shown to result in a taurine modification defect at the wobble position of $tRNA^{Leu(UUR)}$ (Yasukawa *et al.* 2000), which specifically impairs the translation of the UUG codon (Kirino *et al.* 2004). The UUG codon is clearly most often used in translation of the ND6 gene of complex I and its decoding defect explains the complex I deficiency observed in the cybrid cells (Dunbar *et al.* 1996). Both in patients and bacterial models, decrease in the amount or stability of active complex I has been reported for the less common MELAS-associated ND1-subunit mutations as well (Kervinen *et al.* 2006, Kirby *et al.* 2004, Malfatti *et al.* 2007). Respiratory chain deficiency in MELAS is demonstrated also by the impaired mitochondrial ATP synthesis (DiFrancesco *et*

al. 2008, Rusanen *et al.* 2000). Induction of antioxidative defense systems suggestive of increased oxidative stress has also been observed in MELAS myoblasts (Rusanen *et al.* 2000). How these molecular level changes lead to the presentation of the disease is unclear. One possibility is neuronal excitotoxic injury caused by the neurotransmitter glutamate, since the m.3243A>G mutation has been shown to impair glutamate uptake in cybrid cells, which is energy-dependent (DiFrancesco *et al.* 2008). On the other hand, dysfunctional blood-brain barrier due to mitochondrial angiopathy could lead to neuronal hyperexcitability in the brain (Iizuka *et al.* 2007, Sproule & Kaufmann 2008).

2.5.3 Overlap syndromes

Overlap syndromes are a rare form of complex I-associated respiratory chain disorders that are characterized by features of two or more distinct diseases. The underlying genetic cause can be a single mutation that produces a phenotype that presents with symptoms of various different diseases, or much more rarely, co-occurrence of two or more common primary disease-associated substitutions.

A recent work by Nakamura and co-workers reported on a family with members presenting symptoms of MERRF or MERFF and MELAS. Analysis of the mitochondrial genome in this family revealed the presence of both m.3243G>A and m.8356T>C transitions that have been causative for MELAS and MERRF, respectively. (Nakamura *et al.* 2010). Interestingly, a similar overlapping phenotype has also been seen in some patients with either of the substitutions alone (Mongini *et al.* 2002, Sano *et al.* 1996). Other single mtDNA substitutions that have been associated with an overlap syndrome include m.3376G>A in *MTND1* in a patient with symptoms of LHON and MELAS (Blakely *et al.* 2005), an m.13084A>T missense mutation in *MTND5* in a proband with Leigh-MELAS overlap syndrome (Crimi *et al.* 2003) and an m.13513G>A transition in *MTND5* in patients with clinical findings of LHON and MELAS (Pulkes *et al.* 1999). Common to all these overlap syndromes is that they are sporadic or found only in small pedigrees. Whether they truly present an overlap phenotype or just a modification of one of the diseases involved is unclear, since symptoms of a respiratory chain disorder can be versatile as discussed above. Evaluation of these rare forms of oxidative phosphorylation linked defiences is also restricted by the lack of detailed analyses of the biochemical effects of the underlying genetic changes.

2.6 Evaluation of the pathogenicity of mtDNA mutations

Mitochondrial DNA has many special features that differ from nuclear genes, making mitochondrial genetics a challenging task. Firstly, mtDNA is maternally inherited (Hutchison *et al.* 1974). Although sperm mitochondria enter the egg during fertilization, their mtDNA is degraded and the mitochondria are eliminated (Nishimura *et al.* 2006, Sutovsky *et al.* 2000). Secondly, accumulation of mutations in the mtDNA is at least ten times faster than for nuclear DNA (Brown *et al.* 1979). Since each human cell contains hundreds or even thousands of mtDNA copies (Satoh & Kuroiwa 1991) and mutations of mtDNA occur independently in each of the mtDNA molecules, a mixture of mutant and unmutated mtDNA is present in a cell. This is known as a heteroplasmy. However, mtDNA mutation does not manifest as a clinical disease before reaching a certain degree of heteroplasmy, a phenomenon that is known as the threshold effect (for a review, see e.g. (Rossignol *et al.* 2003)). Fourthly, mtDNA molecules are randomly distributed to daughter cells at cell division resulting in a change of mutant mtDNA content between cells and even at a tissue or organ level, a property known as mitotic segregation (DiMauro & Schon 2003). Moreover, it is not unusual that an individual carrying an mtDNA substitution is not affected (Montoya *et al.* 2009). This incomplete penetrance highlights the significance of other genetic or environmental factors affecting the expression of the mtDNA mutation.

Because of the unique properties of mitochondrial genetics, mtDNA variants are abundant and not easy to distinguish as a polymorphism or truly pathogenic mutation. Therefore, several classification schemes and scoring systems have been presented to evaluate the pathogenic role of a novel mtDNA mutation. The earlier criteria of DiMauro and Schon (2001) were much dependent on the heteroplasmy of the mutation, but the increasing number of homoplasmic pathogenic mutation discoveries led to a revision of the criteria and presentation of a scoring system to determine the likelihood of the pathogenicity of the mutation (Mitchell *et al.* 2006). Outlines of the classification are presented in Table 4. Based on the scoring system, the pathogenicity of many of the mutations affecting *MTND* genes has been questioned, mainly because of a lack of functional studies (Mitchell *et al.* 2006, Wong 2007). Compared to earlier criteria, much more emphasis is now put on studying the functional consequences of a putatively pathogenic mtDNA mutation. However, detailed analyses of the biochemical effects are often limited due to the scarcity of patient-derived

material and difficulties in modelling of the mtDNA mutations in mammalian cells. Although very useful, various criteria for pathogenicity should still be used with cautiousness (Montoya *et al.* 2009).

Table 4. Summary of the scoring system presented by Mitchell and co-workers to assess the pathogenicity of an mtDNA substitution (Mitchell *et al.* 2006).

Criteria	Maximum score[1]
Demonstration of a biochemical deficit in affected or multiple tissues	10
Functional studies with single fiber PCR and/or cybrid cells	7
Degree of heteroplasmy	5
Evolutionary conservation of the affected amino acid	10
Segregation of the mutation with the disease within a family	3
Reports on the mutation from two or more independent laboratories	5

[1]Candidate mutations scoring 30 or more of the maximum score total of 40 are classified as pathogenic, those with a score between 21 and 29 as probably pathogenic and mutations with total score from 10 to 20 as possibly pathogenic.

3 Outlines of the present study

PSST and 49 kDa subunits in the hydrophilic part of complex I are involved in ubiquinone and inhibitor binding by complex I. ND1, ND3, ND4L and ND6 subunits in the proximal part of the membranous core of the enzyme are in close contact with or in the immediate vicinity of these functionally important proteins, and therefore some of them may be involved in the interaction of complex I with ubiquinone, a lipophilic electron carrier in the membrane, or are otherwise essential for the activity. The functional importance of the ND1 and ND6 subunits is also emphasized by the clustering of disease-associated substitutions within their genes in mtDNA. However, the biochemical effects of many of the disease-associated mtDNA alterations are unknown, hampering also the evaluation of their pathogenicity.

The purpose of this thesis study was to focus on the role of the ND1, ND4L and ND6 subunits in complex I activity by analysing the biochemical effects of disease-associated and other selected substitutions within their primary sequence in an *Escherichia coli* model. The specific aims were:

1. to elucidate the role of the two highly conserved glutamate residues within transmembrane helices two and three of ND4L subunit homolog NuoK in complex I function,
2. to analyze the biochemical effects of LHON-associated m.14498C>T, m.14484T>C, m.14459G>A and m.3460G>A transitions in mtDNA in order to validate the pathogenicity of the m.14498C>T transition and to get more insight into the pathomechanism of LHON,
3. to evaluate the effects of LHON/MELAS overlap syndrome-associated m.3376G>A and m.3865A>G substitutions to discriminate between their pathogenicity, and finally,
4. to clarify the role of ND1 and ND6 subunits in the interaction between ubiquinone and complex I.

4 Materials and methods

A summary of the methods used in this thesis is shown in Table 5. Description of the experimental procedures with references and list of materials used are presented in detail in the original publications (I-III).

Table 5. Summary of methods used in the original publications.

Method	Used in
Protein homology analysis	
Amino acid sequence alignment	I, II, III
Conservation score calculus	III
DNA cloning	I, III
Site directed mutagenesis of a gene of interest	I, II, III
DNA sequencing	I, II, III
Knockout of a gene of interest by homologous recombination	I, III
Expression of a mutant allele of a gene of interest	
Complementation of a knockout strain by protein expression from a plasmid	I, II, III
Genomic complementation	III
Bacterial cell membrane isolation	
Lysozyme treatment with osmotic shock	I
French Press treatment	I, II, III
Protein concentration determination	I, II, III
Western blotting	I
Bacterial cell growth phenotype analyses	I, II, III
Spectrophotometric enzyme activity assays	I, II, III
Analysis of ubiquinone reduction kinetics	II, III
Inhibitor sensitivity test	I
Enzyme kinetic analysis of inhibition types	II, III
Statistical analyses	
Student's t test	II, III
One-way ANOVA followed by Dunnett's test	II, III

5 Results

5.1 Mutation dependence of growth phenotype

Growth medium with malate as the main carbon source was initially selected for culturing the *E. coli* cell lines for sample preparation as in the earlier work with *P. denitrificans* NDH-1 mutants (Kurki *et al.* 2000, Zickermann *et al.* 1998, Zickermann *et al.* 2000). Later on, the growth ability of the *E. coli* cells harbouring amino acid substitutions in the NuoK subunit of NDH-1 on malate turned out to be a useful way to initially screen the mutants, and was also used thereafter to characterize the NuoJ (II) and NuoH (III) subunit substitutions.

The initial finding of the growth phenotype analysis of the constructed mutants was the inability of the *nuoJK* knockout derivative of the *E. coli* strain GV102, namely GVnuoJK in (I), to grow on malate. When the *nuoJK* knockout was introduced into another strain, GO103, the cells were able to grow to higher densities (Figure 4 in I). The growth defect in GVnuoJK was also resolved by growing the cells in a medium with pyruvate or glycerol but not with succinate as the carbon source, or by transforming the GVnuoJK strain with an expression plasmid expressing wild-type NuoJ and NuoK (GVcJK) (Figure 3 in I). Overexpression of the alternative non-proton pumping NADH-UQ oxidoreductase (NDH-2) in the GVnuoJK strain did not improve the growth ability on malate (Figure 3 in I) indicating that NADH oxidation capacity in the *nuoJK* knockout was not a limiting factor. Taken together, these results are suggestive of impaired NDH-1 proton translocation activity in cell lines that fail to grow on malate (discussed in more detail in section 6.1).

5.2 Mutagenesis of the two highly conserved glutamate residues within the transmembrane helices of the ND4L subunit impairs complex I activity

The ND4L subunit displays two highly conserved glutamate residues (Glu-36 and Glu-72 in *E. coli* numbering) within consecutive transmembrane helices (see Figure 5 in I). Such a location of protonable residues in a hydrophobic surrounding is common in many proteins involved in proton translocation across a membrane like e.g. in the *c* subunit of ATP synthase (Miller *et al.* 1990). To investigate the role of the ND4L subunit glutamates in complex I function, site-

directed mutagenesis of them and some nearby amino acid residues was performed using *E. coli* as a model organism.

One by one removal of the Glu-36 and Glu-72 residues from the ND4L subunit homolog NuoK in *E. coli* by substituting them with a glutamine residue (NuoK-E36Q and NuoK-E72Q mutations in I) led to expression of membrane-bound NDH-1 that showed reduced inhibitor-sensitive NADH:UQ activity with Q_2 as a substrate. Activity decline in E36Q was more pronounced than with the E72Q mutation with 8% and 76% residual activities, respectively (Table 4 in I). Moreover, E36Q or E72Q mutation-carrying cells were unable to grow to higher densities in a medium with malate as the main carbon source. When the glutamates were replaced by aspartate instead of glutamine, the effect on NDH-1 activity was mild and the growth ability in malate medium was not compromized (Table 4 in I).

When the acidic residues were placed on the same helix about one helix turn apart, i.e. E36Q/A69D and E72Q/I39D mutations in (I), NDH-1 activity was almost normal (Table 4 in I). However, placing either of the acidic residues about one helix turn towards the periplasmic side of the membrane within the same transmembrane helix led to about 20% residual NDH-1 activity and growth failure in malate in the case of E36Q/I39D, while with the E72Q/A69D mutation NDH-1 activity was spared at higher levels (76% of control) and the cells were able to grow on malate (Table 4 in I).

Replacement of both of the glutamates by glutamine (E36Q/E72Q in (I)) led to almost complete loss of NDH-1 activity (Table 4 in I). When the acidic residues were shifted about one helix-turn (three amino acids) towards the periplasmic side of the membrane, i.e. the quadruple mutant E36Q/I39D/A69D/E72Q (QDDQ) in (I), NDH-1 activity was not affected. However, the cells were not able to grow on malate or succinate as the main carbon source, although they grew considerably well in medium supplemented with pyruvate (Figure 3 in I).

To test if the poor growth capacity on malate or a decrease in NDH-1 activity in the mutants analysed was due to a reduced amount of NDH-1 in the membranes, their hexaammineruthenium(III)chloride(HAR)-reductase activities were determined. It turned out that some mutants, like QDDQ and E36Q, that failed to grow on malate with or without normal NDH-1 activity exhibited HAR-reductase activities comparable to or even higher than those with normal NDH-1 activity and growth phenotype in malate, like the E72D and E36Q/A69D substitutions (Table 5 in I).

5.3 Effects of the LHON-associated ND1 and ND6 subunit mutations on complex I activity in *Escherichia coli*

Of the LHON-associated *MTND6* and *MTND1* substitutions (see Table 2), biochemical effects have been studied in more detail only for the common m.14484T>C and m.3460G>A, and the rare m.14459G>A transitions, while a partial analysis of the enzymatic defect has been performed only for the m.3697G>A, m.3733G>A and m.14596A>T mutations. In order to evaluate in more detail the biochemical effects of the m.14498C>T transition, the role of the ND1 and ND6 subunits in complex I activity, and the biochemical consequences of LHON-associated mtDNA substitutions affecting the complex I, a series of amino acid substitutions in NuoJ and NuoH, *E. coli* homologs of the ND6 and ND1 subunits, were constructed and analysed (II, III).

5.3.1 Biochemical effects of the m.14498C>T mutation homolog in Escherichia coli NuoJ

The m.14498C>T transition in *MTND6* leads to a replacement of the highly conserved Tyr-59 by a cysteine residue. An *E. coli* equivalent of this substitution, namely NuoJ-Y59C, produced only a moderate NDH-1 activity deficiency. HAR-reductase-normalised V_{max} of NADH:UQ activity with DB in NuoJ-Y59C was 73% of control, while with the endogenous ubiquinone it was less severely affected, presenting 93% residual activity as estimated by the d-NADH-oxidation rate. Kinetic analysis of DB binding in NuoJ-Y59C did not reveal any change in its apparent K_m. However, marked substrate inhibition by DB at concentrations above 100µM DB was observed (Figure 4).

Replacement of the invariant Tyr-59 by phenylalanine instead of a cysteine residue had more drastic effects on NDH-1 activity. The V_{max}/HAR-reductase ratio of NADH:UQ activity with DB showed about 60% activity decline, and the d-NADH oxidation activity with endogenous ubiquinone encompassing the entire length of the respiratory chain was reduced to 45% of control. As in NuoJ-Y59C, the K_m for DB did not differ from control in NuoJ-Y59F, but some changes in UQ-binding properties of NDH-1 did occur as a slight tendency for substrate inhibition by DB was observed. Neither NuoJ-Y59F nor NuoJ-Y59C affected NDH-1 sensitivity to VNA, which has been classified as a C-type inhibitor of complex I (Degli Esposti 1998).

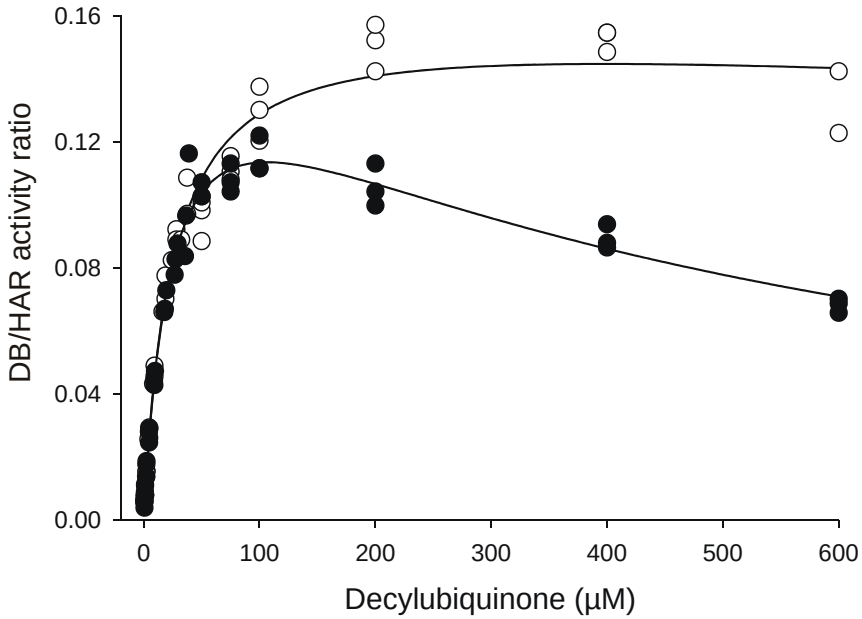

Fig. 4. Substrate inhibition of NADH:UQ activity in *E. coli* NuoJ-Y59C mutation. The activity was normalized to HAR-reductase activity to account for variation in expression levels. The filled circles represent mutant cells and the open circles wild-type controls.

5.3.2 Effects of the m.14484T>C and m.14459G>A transition homologs in NuoJ

Amino acid residues Met-64 and Ala-72 of the ND6 subunit affected by the m.14484T>C and m.14459G>A transitions reside in the same transmembrane helix as the one affected by the m.14498C>T substitution. Therefore, mutagenesis of the homologous positions in *E. coli* NuoJ was included to investigate their effects in the model utilized to analyze mtDNA substitutions.

Enzymatic effects of the substitutions at NuoJ-Met64 were moderate. The V_{max}/HAR-reductase ratio of NADH:UQ activity with DB was reduced to 83% of control in M64V, while M64C had a residual activity of 56%. Contrary to Tyr-59 position substitutions, both M64V and M64C showed a change in NDH-1 affinity to DB with the apparent K_m being increased to about 1.4- fold in M64V and 1.6-fold in M64C compared to control. Sensitivity of NDH-1 activity to VNA in M64V and M64C substitutions was comparable to that of control.

The position homologous to Ala-72 of ND6 is occupied by a more bulky methionine in *E. coli*. Replacement of this Met-72 in NuoJ by valine (M72V), alanine (M72A) or cysteine (M72C) residues reduced the HAR-reductase activity normalised V_{max} of NADH:UQ activity with DB to 53%, 79% and 68% of control, respectively. Analysis of ubiquinone reduction kinetics in these NDH-1 mutants showed a clear increase in the apparent K_m for DB up to two-fold compared to control. Moreover, all Met-72 mutants were less sensitive to substrate inhibition by DB than any other mutation generated and analysed. As it showed the largest decrease in DB affinity, the NuoJ-M72V substitution was subjected to more detailed analysis of inhibitor sensitivity, and resistance to annonin and VNA, representatives of A- and C-type inhibitors, was observed.

5.3.3 Effects of the m.3460G>A transition equivalent in NuoH

The *E. coli* homolog of the ND1 subunit Ala-52 residue affected by the m.3460G>A substitution is Met-64 in NuoH. Replacement of this methionine by a threonine residue had only a minor effect on *E. coli* NDH-1 activity. Although the NADH:UQ activity with DB was comparable to that of control, ubiquinone reductase activity utilizing endogenous ubiquinone (demonstrated by the d-NADH oxidation activity utilizing the entire span of the respiratory chain) was reduced to 70% of control. NuoH-M64T did not show any change in ubiquinone binding properties of NDH-1, since the apparent K_m for DB was similar to that of a control, nor was substrate inhibition observed.

Replacement of the adjacent more conserved aspartate-63 residue by glutamate had more drastic effects on NDH-1 activity. This D63E mutant displayed reduced HAR reductase activity indicative of a decrease in the amount of NDH-1 in the membranes. Additionally, the V_{max}/HAR reductase ratio of NADH-UQ activity with DB as a substrate showed about a 34% decline, and was similar with endogenous ubiquinone as a substrate. D63E also seemed to interfere with the NDH-1 interaction with ubiquinone, since the apparent K_m for DB was about half of that of control.

5.4 Enzymatic effects of the LHON/MELAS overlap syndrome-associated m.3376G>A and m.3865A>G substitution homologs in *E. coli*

Overlap syndromes presenting features of two or more different phenotypes are a rare form of respiratory chain disorder. The m.3376G>A and m.3865A>G transitions in *MTND1* are associated with an LHON/MELAS overlap syndrome and lead to replacement of Glu-24 by lysine and Ile-187 by valine in the ND1 subunit of complex I. The corresponding residues in *E. coli* NuoH are Glu-36 and Ile-201, which were subjected to site-directed mutagenesis to clarify the biochemical effects and pathogenity of the disease-associated mtDNA transitions.

The *E. coli* version of the LHON/MELAS overlap syndrome-associated p.E24K substitution in ND1, namely NuoH-E36K, showed a marked decrease in HAR-reductase activity, indicating a reduced amount of NDH-1 in the membranes, and also severely hampered specific NDH-1 activity. HAR-reductase normalized NADH:UQ activity with 100µM DB in NuoH-E36K was 12% of control and could not be enhanced with higher DB concentrations, preventing further analysis of the NDH-1 interaction with ubiquinone. Activity with endogenous ubiquinone was even more affected, showing only about 3% residual activity, and results with other ubiquinone analogs, Q_1 and Q_2, were similar to that with DB as a substrate. The effect of a NuoH-E36Q replacement on NDH-1 activity was milder than that of the NuoH-E36K replacement. The V_{max}/HAR-reductase ratio of NADH:UQ activity in NuoH-E36Q was 60% of control with DB and 74% of control with Q_1 as a substrate, and the apparent K_m for DB and Q_1 was increased more than four-fold compared to control. Accordingly, NuoH-E36Q rendered resistance to the complex I inhibitors annonin, piericidin A and stigmatellin with their I_{50} values being increased 2.7-, 1.5- and 3.3-fold, respectively, whereas sensitivity to VNA was unaltered. These changes in quinone binding brought about by the NuoH-E36Q substitution in the *in trans* complemented deletion strain were verified by genomic complementation of the mutant allele. The apparent K_m for DB was 135µM and 33µM in E36Q and the control strain, respectively, and the I_{50} for piericidin A was 16µM in the E36Q mutant and 13µM in the control. The role of the Glu-36 residue in NDH-1 activity was examined with one additional replacement by substituting it with an aspartate residue. Apart from a slight decrease in its d-NADH oxidation activity (Figure 2 in III), the NuoH-E36D mutant did not differ from the control.

Introduction of a mutation homologous to p.I187V in ND1 into the *E. coli* NuoH subunit, namely I201V, had only a minor effect on NDH-1. HAR-reductase activity in NuoH-I201V was slightly decreased, although the difference to control was not statistically significant, suggestive of a slight reduction in the amount of NDH-1 in the membranes, but the NDH-1 activity and the apparent K_m for DB in I201V were similar to that of control (Figure 2 and Table 2 in III).

6 Discussion

6.1 Significance of the conserved glutamate residues within the transmembrane helices of the ND4L subunit

A sequence motif containing only a highly conserved acidic amino acid in the middle of a transmembrane α-helix may be associated with proton translocation across biological membranes. The ND4L/NuoK subunit was selected for a study on the functional significance of dicarboxylic amino acids on the basis of a sequence motif search amongst the complex I subunits (I).

Replacement of the two highly conserved glutamate residues 36 and 72 of the NuoK subunit of *E. coli* complex I by a corresponding amide (glutamine), either one at a time or simultaneously, was harmful for the enzyme function. These results have been reproduced later by another research group utilizing a different approach (Kao *et al.* 2005), supporting the view that Glu-36 and Glu-72 of NuoK are important for the catalytic activity of the enzyme. Some freedom regarding the exact location of the residues became apparent. Placement of the two acidic residues about one helix turn apart within either of the helices, but not the simultaneous shifting towards the periplasmic side of the membrane within the original helices (the QDDQ mutant in I), was tolerated well. Similar flexibility in the position of a functionally important acidic residue within a transmembrane segment of a protein has been demonstrated for example for the proton conducting *c* subunit of the *E. coli* F_1F_o ATP synthase, wherein relocation of Asp-61 to a corresponding site in an adjacent helix partially retained the enzyme function (Miller *et al.* 1990). Compared to the *c* subunit of the F_1F_o ATP synthase, the exact position of the carboxyl groups of the NuoK residues 36 and 72 is not equally critical, since their replacement by aspartate retained the complex I activity, while substitution of glutamate for the Asp-61 in the *c* subunit of F_1F_o ATP synthase led to severe loss of activity. This discrepancy between these two proteins may be due to a difference in the number of acidic residues present. The glutamates in NuoK might function as a pair in the vicinity of each other and therefore tolerate more drastic changes than the *c* subunit of F_1F_o ATP synthase, in which the exact orientation of the only carboxyl group in the transmembrane segment is more essential.

The Glu-36 and Glu-72 residues of NuoK are located within two consecutive transmembrane helices of the subunit at approximately equal depth from the

surface of the membrane. Based on the results of the present work, they are proposed to be involved in the proton pumping activity of the enzyme. This is analogous to other proton transferring respiratory complexes, such as the F_1F_o ATP synthase as well as cytochrome *c* oxidase and cytochrome bo_3 quinol oxidase, which have acidic residues involved in proton translocation deeply buried within transmembrane helices (Hoppe *et al.* 1982, Verkhovskaya *et al.* 1997, Ädelroth *et al.* 1997). Such an arrangement is not typical only for the respiratory complexes. *E. coli* lactose permease, a representative of large major facilitator superfamily membrane transporter proteins and involved in galactoside/H^+ symport activity, displays a conserved glutamate within a transmembrane segment and the residue is directly involved in H^+ translocation (for a review, see Kaback (2005)).

NuoK's Glu-36 and Glu-72 involvement in proton translocation is supported by the results of the growth phenotype experiments performed, although the growth defect on malate in complex I mutants from *R. capsulatus*, for example, has not been linked to faulty proton translocation function (Lunardi *et al.* 1998). When the *nuoJK* knockout was introduced into *E. coli* strain GO103 instead of GV102, the growth defect in a medium with malate as the main carbon source was partially resolved (Figure 4 in I). The main difference between these two bacterial strains is the type of terminal oxidase in their respiratory chains. GO103 carries a bo_3-type terminal ubiquinol oxidase whose H^+/e- stoichiometry is 2, while in GV102 that expresses *bd*-type terminal oxidase it is 1 (Puustinen *et al.* 1991). Strains with deficient energy conservation efficiency of complex I together with a terminal oxidase with lower H^+/e^- ratio would lead to a lower proton gradient across the membrane on equal electron transfer, which may impair ATP synthesis. Deprivation of cellular ATP has been shown to repress dicarboxylate transporter expression and this in turn leads to insufficient transport capacity of malate and succinate into the cells (Boogerd *et al.* 1998). On the other hand, it has been shown that uptake of succinate and malate into cells is coupled to proton translocation (Gutowski & Rosenberg 1975), and a defective proton gradient due to deficient respiratory energy conservation could cause their improper transport for energy metabolism. Both of these possibilities are dependent on proton motive force generated by the respiratory complexes and could explain the growth defects that were observed in the NuoK subunit substitutions on succinate and malate medium (I).

NuoK could be the fourth subunit involved in the conformation-driven proton translocation mechanism suggested on the grounds of the findings of the molecular structure of complex I (Efremov *et al.* 2010). The results of the present

work are also congruent with the suggestions of Ohnishi *et al.* (2010a), combining the direct and conformation-driven proton translocation mechanisms. NuoK locates close to the interface of the hydrophilic and hydrophilic arm of complex I, where the quinone-gated direct proton pump of the combined mechanism is assumed to operate, and NuoK glutamates 36 and 72 could be part of the proton well involved in SQ_{Nf}-mediated proton translocation. Whether the comformation-driven or combined mechanism accounts for the proton translocation by complex I, our hypothesis about the involvement of NuoK in proton pumping presented in (I) is reasonable.

6.2 Pathogenicity of the LHON-associated m.14498C>T mutation

The m.14498C>T transition in *MTND6* has been proposed to account for LHON in a multigeneration family with four affected members and leads to a non-conservative replacement of the highly conserved Tyr-59 by a cysteine residue in the ND6 subunit of complex I (Wissinger *et al.* 1997). The affected family members suffer from a severe type of LHON with poor visual acuity, although the eldest patient shows only about 50% mutation load (Leo-Kottler *et al.* 1996, Wissinger *et al.* 1997). The mutation has not been reported in other families yet, nor have any studies of its biochemical consequences been performed; therefore, it is classified as possibly pathogenic (Mitchell *et al.* 2006).

Pathogenicity of the m.14498C>T transition is supported by the results of the present work. The *E. coli* equivalent of the amino acid substitution brought about by the mtDNA mutation led to a moderate loss of NDH-1 activity, and removal of the hydroxyl-side chain only of the benzene-ring in Tyr-59 (Y59F mutation in I) was even more deleterious. Similar results have been obtained with a different approach (Kao *et al.* 2005). Moreover, this domain of the NuoJ subunit where Tyr-59 is located seems to contribute to ubiquinone binding by NDH-1. Substrate inhibition by the short-chained ubiquinone analog observed in the NuoJ-Y59C mutation has earlier been reported for the LHON-associated m.14459G>A transition affecting the same domain of the ND6 subunit as the m.14498T>C transition (Jun *et al.* 1996) and also for the primary LHON mutation m.3460G>A in *MTND1* (Majander *et al.* 1996). Furthermore, other LHON-associated substitutions within the same domain of the ND6 subunit also affect UQ and/ or inhibitor binding (II, Carelli *et al.* 1999).

6.3 Implication of the LHON mutation effects on the pathomechanism of the disease

An increasing amount of evidence provided by several reports on the biochemical effects of the LHON-associated mtDNA substitutions points to a common pathomechanism in LHON. It seems obvious that a change in ubiquinone interaction with complex I is involved in the pathogenesis of the disease. But how could this enzymatic deficiency lead to the development of the disease? If complex I operates at a ubiquinone concentration range close to its apparent K_m for UQ, as it seems at least in the case of beef heart mitochondria (Fato *et al.* 1997), then a small change in K_m would result in a relatively large change in activity, which could finally lead to altered ATP production. Conversely, higher apparent K_m for UQ means lower occupancy of the actual ubiquinone reduction site if the ubiquinone concentration remains unchanged, and this results in a situation where other redox-active groups of complex I are in a more reduced state, which could promote reactive oxygen species (ROS) generation. Both ATP depletion and increased ROS generation have been demonstrated for LHON, and the specific sensitivity of the retinal ganglion cells to these changes could be caused by other properties of this cell type (see section 2.5.1). Increased generation of complex I-derived reactive oxygen species can lead to increased risk of the retinal ganglion cells to excitotoxic injury caused by defective removal of the neurotransmitter glutamate by the retinal Müller cells (Beretta *et al.* 2004). Vulnerability of the retinal ganglion cells to glutamate can be explained by their greater sensitivity to excitotoxicity compared with other retinal cell types (Luo *et al.* 2001), which promotes retinal ganglion cell selectivity.

6.4 Biochemical effects and pathogenicity of the m.3376G>A transition associated with LHON/MELAS overlap syndrome

A single patient with clinical features of both LHON and MELAS has been reported to carry an m.3376G>A transition in *MTND1* (Blakely *et al.* 2005). The mutation leads to a replacement of an evolutionarily conserved glutamate-24 by lysine in the first matrix-side loop of the ND1 subunit and results in obvious complex I deficiency with 36% residual activity. No more detailed data about its biochemical consequences are available. Although m.3376G>A was originally regarded as pathogenic, its role in the development of the disease has been lately

questioned mainly because of its sporadic nature, high tolerance of complex I for ND1 subunit mutations, and paucity of functional studies (Wong 2007).

Introduction into the *E. coli* NuoH subunit of an E36K mutation homologous to the one brought about by the m.3376G>A transition led to expression of NDH-1 that was practically devoid of NADH:UQ activity. Substitution by alanine instead of lysine has similar effects (Sinha *et al.* 2009), although the activity decline with the NuoH-E36K mutation is more distinct. The results of the present work suggest that the Glu-36 residue of the NuoH subunit is also essential for the assembly of the enzyme and ubiquinone and inhibitor binding. The *E. coli* equivalent of the m.3865A>G transition occurring concomitantly in the patient with the m.3376G>A transition had less severe effects than the substitution homologous to m.3376G>A. Additionally, the consequences of the m.3866T>C mutation affecting the same amino acid as m.3865A>G, but resulting in less conservative substitution of p.I187 by threonine, are mild (Hinttala *et al.* 2010). These findings strongly argue for the pathogenicity of the m.3376G>A transition and emphasize its implication to the disease development when compared to the m.3865A>G transition.

Mitochondrial overlap syndromes associated with distinct mtDNA mutations are rare. There have not been any detailed studies about their biochemical consequences so far, but relatively much is known on the effects of isolated phenotypes. For example in LHON, complex I activity decline is usually mild, but many mutations, including the m.3460G>A substitution in the first matrix side loop of ND1, affect the interaction between ubiquinone and complex I (Ghelli *et al.* 1997, Lenaz *et al.* 2004, Pätsi *et al.* 2008, Zickermann *et al.* 1998). Contrary to that, MELAS mutations usually show more drastic effects on the enzyme activity and a decrease in the amount or stability of the active complex I is not uncommon (Kervinen *et al.* 2006, Kirby *et al.* 2004, Malfatti *et al.* 2007). An *E. coli* mutation equivalent to the m.3376G>A substitution associated with LHON/MELAS overlap syndrome produced a biochemical defect similar to that commonly observed in MELAS. Interestingly, the affected amino acid was also found to contribute to ubiquinone binding by complex I. Overall, the *E.coli* equivalent of the LHON/MELAS overlap syndrome-associated mtDNA mutation affects complex I activity and its co-substrate and inhibitor affinity in a pattern which appears to combine the effects of the separate mutations responsible for LHON and MELAS.

6.5 Role of the ND1 and ND6 subunits in the interaction between ubiquinone and complex I

Reports on the number and location of ubiquinone binding sites in complex I are inconsistent. Various inhibitors have been used to probe for this domain of the enzyme, and competition experiments with representatives of all three types of inhibitors show that they share a common binding site with partially overlapping positions (Okun *et al.* 1999). Results of the extensive site-directed mutagenesis of the 49 kDa and PSST subunits in *Y. lipolytica* demonstrate that many residues lining the cavity between them in the proximity of cluster N2 are essential for inhibitor, and possibly also for quinone, binding with partially overlapping sites (Fendel *et al.* 2008, Tocilescu *et al.* 2007, Tocilescu *et al.* 2010). It is still unknown which of the subunits in the membranous core of the enzyme, if any, contribute to UQ binding.

Mutations within the first matrix side loop of NuoH, homolog of ND1, and on the matrix side of the third transmembrane helix of the NuoJ subunit, counterpart of ND6, were found to affect ubiquinone binding and inhibitor sensitivity of NDH-1. Substitutions in NuoH were found more deleterious than those in NuoJ. The molecular structure of complex I shows that ND1 is the most proximally located subunit of the enzyme's membrane arm and interacts with the PSST and 49 kDa subunits that constitute the UQ reduction site around the Fe-S cluster N2 (Figures 2 and 5), while assignment of individual subunits in the domain containing the ND3, ND6 and ND4L proteins next to ND1 could not be performed (Efremov *et al.* 2010). However, the following observations of components of the ND3, ND6 and ND4L subunit bundle give a tentative location for each of them. Namely, the C-terminus of the ND4L subunit is accessible from the cytoplasmic side of the membrane in *P. denitrificans* (Kao *et al.* 2002) and therefore cannot be located underneath the hydrophilic arm of the enzyme, but rather it occupies a distal location within the three subunit unit. On the other hand, the ND3 subunit has been crosslinked with the PSST and 49 kDa subunits (Di Bernardo & Yagi 2001, Kao *et al.* 2004a), but unlike for the ND6 subunit, there are no reports about its contribution to ubiquinone and/or inhibitor binding. Hence, ND6 must be located closer to the quinone reduction site than ND3 and also be in the vicinity of the PSST and 49 kDa subunits.

The results of the present work and earlier photoaffinity labeling studies and site-directed mutageneses suggest that the ND1 subunit houses two distinct regions that contribute to the ubiquinone binding site of complex I. One is located

in the first matrix side loop of the subunit and involves at least the residues Glu-24, Qln-47, Asp-51, Ala-52 and Lys-54 (III, Zickermann *et al.* 1998), while the other one is in the matrix side region of transmembrane helices four, five and six of the subunit (Kakutani *et al.* 2010, Kurki *et al.* 2000, Sekiguchi *et al.* 2009). Although located rather apart from each other in the primary structure of the subunit, these two domains could be brought together in the UQ-binding site of complex I by the quaternary structure of the enzyme-oligomer.

The effects of the mutations analyzed in the present work provide important evidence on the UQ binding domain of complex I enzymes. Mutations in the Tyr-144 locus of the 49 kDa subunit of *Y. lipolytica* and the Glu-36 residue of the NuoH subunit in *E. coli* decrease the apparent affinity of the enzyme to ubiquinone and to partially competitive type A inhibitors DQA or piericidin A, and also modify resistance to inhibitors of a different, less competitive class such as rotenone or stigmatellin. Similarly, substitution of valine for the Met-72 residue in NuoJ of *E. coli* NDH-1 affected UQ binding and sensitivity to inhibitors that belong to the same class as piericidin A and stigmatellin. Contrary to *Y. lipolytica* 49 kDa Tyr-144 mutations, the *E. coli* NuoH-E36Q mutation affected the kinetics of all ubiquinone analogs tested and quinone and inhibitor binding to a similar extent. So the UQ-binding site around Glu-36 of the NuoH subunit appears to be in better equilibrium with the substrate UQ pool within the membrane, but the UQ molecule is not recognized in a strict manner in this site, as the NuoH-E36D mutant was highly active and there was no loss of binding affinity. Noteworthy, the changes in affinity to the tested inhibitors were relatively small but reproducible. These factors point to a rather loose or even transitory binding to this site.

To conclude, analogous kinetic effects on the enzyme suggest that the above-mentioned amino acid residues in the 49 kDa, ND1 and ND6 subunits are located in the same functional domain of complex I. Furthermore, the UQ-binding site comprised of the 49 kDa, PSST, ND1 and ND6 subunits (Figure 5) resides in the immediate proximity of the membrane surface. Whether two separate ubiquinone molecules function cooperatively during catalysis, as suggested by the discovery of two complex I-associated semiquinone species, cannot be judged from the results presented here.

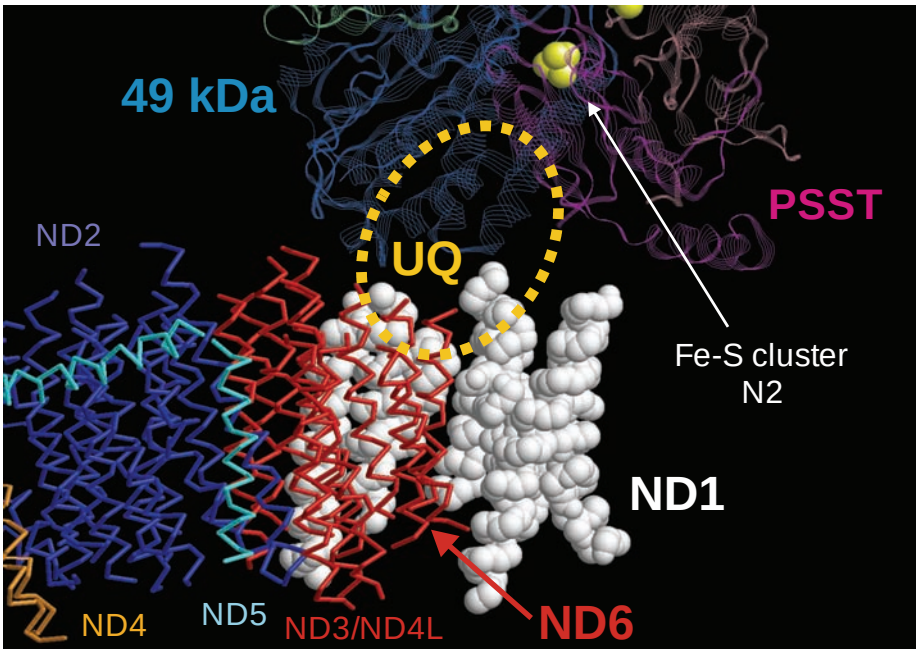

Fig. 5. Interface of the hydrophilic and hydrophobic arms in the molecular structure of *T.thermophilus* complex I (protein data bank code 3M9S) viewed with RasMol program. Extramembrane loops of the helices in the ND-subunits are not shown since they were not unravelled in the structure solved by Efremov *et al.* (2010). Proposed domain for the ubiquinone (UQ) interaction is marked with an orange ellipse. The location of the ND6 subunit in the ND3/ND4L/ND6 bundle of the structure is tentative.

6.6 mtDNA mutation modelling in *Escherichia coli*

Accumulation of mitochondrial DNA mutations is relatively fast. Therefore, classification of a novel mtDNA substitution as a neutral polymorphism or pathogenic mutation is often troublesome and even the application of criteria for the assessment of pathogenicity is not always straightforward.

Detailed studies about the biochemical effects of an mtDNA mutation are often limited due to scarcity of patient-derived material, since the majority of the cases are sporadic or the syndromes occur in small pedigrees. Furthermore, it is not always possible to get a sample from the affected tissue for analysis. One way to solve this problem could be experimental modelling of mitochondrial disorders in mammalian cells. Several experimental approaches have been applied to introduce exogenous DNA into mitochondria of a mammalian cell, but none of

these has led to a major breakthrough in generating a mitochondrial network with a modified genome (Yoon *et al.* 2010). mtDNA-mutator mice that have defective proofreading activity of the mitochondrial DNA polymerase accumulate mtDNA point mutations at a high rate but in a random mode (Trifunovic *et al.* 2004) and cannot be used to generate site-specific substitutions into the mtDNA-encoded respiratory chain subunits. Similarly, application of selective growth conditions results in randomly distributed mtDNA substitutions as demonstrated e.g. by Bai & Attardi (1998) and Acin-Perez *et al.* (2004). Allotopic gene expression (expression from the nucleus of a gene that normally resides in mtDNA) studied as a potential gene therapy for mtDNA mutation-associated diseases could be used to express mutated respiratory chain components, but has proven to be impractical thus far because of lack of integration into mitochondria of a translation product equipped with a mitochondrial import signal sequence (Figueroa-Martinez *et al.* 2011, Perales-Clemente *et al.* 2011). Clearly, experimental manipulation of mtDNA is possible but technically very difficult and not applicable to site-directed mutagenesis at the moment.

The accepted modular evolution scheme of complex I enzymes suggests that they all have a common ancestor. Phylogenetic analysis of the core subunits show that the mitochondrial complexes are closely related to the bacterial ones. (Friedrich & Weiss 1997, Friedrich & Scheide 2000). Moreover, all the bacterial subunits have homologs in mitochondria and the structure and function of the enzymes are similar (for a review, see e.g. Hirst (2009)). Therefore, the bacterial counterparts of the enzyme are considered as a minimal form of complex I and are a useful tool to analyze it. Application of bacteria to model complex I function has several advantages. Sufficient amount of material for analysis is gathered relatively fast and generation of a vast number of mutations with molecular biology techniques in the absence of confounding substitutions in the genome is easy. Moreover, many bacteria, like *E. coli*, have a branched metabolic network that enables utilization of various metabolic substrates and viable cell lines with otherwise lethal point mutations in complex I.

When the effects of an mtDNA mutation are studied in a model organism, one of the main concerns is the relevance of the results in a clinical setting. A summary of the biochemical effects of the diseases-associated mtDNA mutations modeled in the present work on complex I function in mitochondria and bacteria is presented in Table 6. This comparison demonstrates that the observed changes in bacteria are fairly congruent with the results of the analysis in patients. Similar

Table 6. Comparison of the biochemical effects of the amino acid replacements in complex I between mitochondria with a disease-associated mtDNA substitution and *E.coli* model.

Mitochondrion			*Escherichia coli*		References
Mutation	Amino acid change	Effect on complex I	Mutation introduced	Effect on NDH-1	
m.3376G>A	p.E24K	36% residual activity	NuoH-E36K	3% residual activity, residue affected contributes to UQ and inhibitor binding	III, (Blakely *et al.* 2005)
m.3460G>A	p.A52T	30% residual activity, inhibitor resistance	NuoH-M64T	87% residual activity, K_m for DB normal	III, (Brown *et al.* 2000, Lenaz *et al.* 2004)
m.14498C>T	p.Y59C	No data available	NuoJ-Y59C	73% residual activity, substrate inhibition by DB	II
m.14484T>C	p.M64V	90% residual activity, inhibitor resistance	NuoJ-M64V	83% residual activity, K_m for DB increased	II, (Brown *et al.* 2000, Carelli *et al.* 1999)
m.14459G>A	p.A72V	40% residual activity, substrate inhibition by UQ	NuoJ-M72V	53% residual activity, inhibitor resistance, K_m for DB increased	II, (Jun *et al.* 1996)

results have been obtained for MELAS-associated *MTND1* transitions using the same approach (Kervinen *et al.* 2006). Nonetheless, the discrepancy of the effects of the m.3460G>A transition in patient samples and the *E. coli* model show that use of bacteria also has some limitations. The amino acid residue affected by the mtDNA substitution may not always be fully conserved. Secondly, sequence alignment with different algorithms may give different results. Moreover, although the bacterial and mitochondrial enzymes are very similar, some differences between them still exist (Kurki *et al.* 2000, Zickermann *et al.* 2000). Furthermore, the potential modulatory effect of the additional subunits present in the mitochondrial enzyme is absent in bacteria. When these limitations are considered, bacteria provide a feasible tool to study the biochemical effects of an mtDNA mutation.

7 Conclusions

The following conclusions can be drawn from the findings of the present work regarding the role of ND1, ND6 and ND4L subunits in complex I function, biochemical effects of the disease-associated substitutions within their genes, and pathogenicity of the rare disease-associated *MTND1* and *MTND6* substitutions.

1. The results of site-directed mutagenesis of the highly conserved glutamate residues 36 and 72 located within two consecutive transmembrane helices in the *Escherichia coli* homolog of ND4L subunit demonstrate their importance for the catalytic activity of complex I. It seems likely that they are involved in the proton translocation activity of the enzyme, but further experiments are needed to clarify it.
2. Exploration of the biochemical effects of the LHON-associated m.14498T>C transition equivalent in *E. coli* showed reduced complex I activity and substrate inhibition by the ubiquinone analog DB. The observed defects support the pathogenicity of this mutation. Introduction of other equivalents of LHON-associated mtDNA substitutions within the same domain of the NuoJ subunit (ND6 homolog) had similar effects. All this suggests that the primary fault underlying the biochemical deficit in LHON implies a change in complex I interaction with ubiquinone.
3. Of the complex I ND1 subunit-affecting LHON/MELAS overlap syndrome-associated substitutions m.3376G>A and m.3865A>G, only the former had significant effects on the enzyme. This indicates that m.3376G>A is the pathogenic mutation. The results also demonstrate that the overlap syndrome-associated mtDNA mutation affects complex I activity in a pattern which appears to combine the effects of the separate mutations responsible for LHON and MELAS.
4. Mutations within the third transmembrane helix of the NuoJ subunit and in the first matrix side loop of NuoH (homolog of the ND1 subunit) affected the interaction of NDH-1 with ubiquinone and its sensitivity to complex I inhibitors. The similarity of these effects to those of the 49 kDa and PSST subunit changes in *Yarrowia lipolytica* suggests their close proximity to the ubiquinone binding site of complex I located in the vicinity of the membrane surface.

Furthermore, the enzymatic effects of the mtDNA mutations in the *E. coli* model were found to be in accord with those of mitochondria from patients. This

strengthens the view that the *E. coli* system used here is a powerful tool to model the effects of other mtDNA replacements as well.

References

Acin-Perez R, Bayona-Bafaluy MP, Fernandez-Silva P, Moreno-Loshuertos R, Perez-Martos A, Bruno C, Moraes CT & Enriquez JA (2004) Respiratory complex III is required to maintain complex I in mammalian mitochondria. Mol Cell 13(6): 805–815.

Ädelroth P, Ek MS, Mitchell DM, Gennis RB & Brzezinski P (1997) Glutamate 286 in cytochrome aa3 from Rhodobacter sphaeroides is involved in proton uptake during the reaction of the fully-reduced enzyme with dioxygen. Biochemistry 36(45): 13824–13829.

Amarneh B & Vik SB (2003) Mutagenesis of subunit N of the Escherichia coli complex I. Identification of the initiation codon and the sensitivity of mutants to decylubiquinone. Biochemistry 42(17): 4800–4808.

Bai Y & Attardi G (1998) The mtDNA-encoded ND6 subunit of mitochondrial NADH dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme. EMBO J 17(16): 4848–4858.

Baracca A, Solaini G, Sgarbi G, Lenaz G, Baruzzi A, Schapira AH, Martinuzzi A & Carelli V (2005) Severe impairment of complex I-driven adenosine triphosphate synthesis in leber hereditary optic neuropathy cybrids. Arch Neurol 62(5): 730–736.

Baranova EA, Morgan DJ & Sazanov LA (2007) Single particle analysis confirms distal location of subunits NuoL and NuoM in Escherichia coli complex I. J Struct Biol 159(2): 238–242.

Barboni P, Carbonelli M, Savini G, Ramos Cdo V, Carta A, Berezovsky A, Salomao SR, Carelli V & Sadun AA (2010) Natural history of Leber's hereditary optic neuropathy: longitudinal analysis of the retinal nerve fiber layer by optical coherence tomography. Ophthalmology 117(3): 623–627.

Barboni P, Savini G, Valentino ML, Montagna P, Cortelli P, De Negri AM, Sadun F, Bianchi S, Longanesi L, Zanini M, de Vivo A & Carelli V (2005) Retinal nerve fiber layer evaluation by optical coherence tomography in Leber's hereditary optic neuropathy. Ophthalmology 112(1): 120–126.

Barker CD, Reda T & Hirst J (2007) The flavoprotein subcomplex of complex I (NADH:ubiquinone oxidoreductase) from bovine heart mitochondria: insights into the mechanisms of NADH oxidation and NAD+ reduction from protein film voltammetry. Biochemistry 46(11): 3454–3464.

Belogrudov G & Hatefi Y (1994) Catalytic sector of complex I (NADH:ubiquinone oxidoreductase): subunit stoichiometry and substrate-induced conformation changes. Biochemistry 33(15): 4571–4576.

Beretta S, Mattavelli L, Sala G, Tremolizzo L, Schapira AH, Martinuzzi A, Carelli V & Ferrarese C (2004) Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines. Brain 127(Pt 10): 2183–2192.

Bernardo SD, Yano T & Yagi T (2000) Exploring the membrane domain of the reduced nicotinamide adenine dinucleotide-quinone oxidoreductase of Paracoccus denitrificans: characterization of the NQO7 subunit. Biochemistry 39(31): 9411–9418.

Blakely EL, de Silva R, King A, Schwarzer V, Harrower T, Dawidek G, Turnbull DM & Taylor RW (2005) LHON/MELAS overlap syndrome associated with a mitochondrial MTND1 gene mutation. Eur J Hum Genet 13(5): 623–627.

Bogachev AV, Murtazina RA & Skulachev VP (1996) H+/e- stoichiometry for NADH dehydrogenase I and dimethyl sulfoxide reductase in anaerobically grown Escherichia coli cells. J Bacteriol 178(21): 6233–6237.

Boogerd FC, Boe L, Michelsen O & Jensen PR (1998) atp Mutants of Escherichia coli fail to grow on succinate due to a transport deficiency. J Bacteriol 180(22): 5855–5859.

Brandt U (1997) Proton-translocation by membrane-bound NADH:ubiquinone-oxidoreductase (complex I) through redox-gated ligand conduction. Biochim Biophys Acta 1318(1–2): 79–91.

Brandt U (2006) Energy converting NADH:quinone oxidoreductase (complex I). Annu Rev Biochem 75: 69–92.

Brandt U, Kerscher S, Dröse S, Zwicker K & Zickermann V (2003) Proton pumping by NADH:ubiquinone oxidoreductase. A redox driven conformational change mechanism? FEBS Lett 545(1): 9–17.

Bristow EA, Griffiths PG, Andrews RM, Johnson MA & Turnbull DM (2002) The distribution of mitochondrial activity in relation to optic nerve structure. Arch Ophthalmol 120(6): 791–796.

Brown MD, Trounce IA, Jun AS, Allen JC & Wallace DC (2000) Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation. J Biol Chem 275(51): 39831–39836.

Brown WM, George M,Jr & Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76(4): 1967–1971.

Cardol P, Lapaille M, Minet P, Franck F, Matagne RF & Remacle C (2006) ND3 and ND4L subunits of mitochondrial complex I, both nucleus encoded in Chlamydomonas reinhardtii, are required for activity and assembly of the enzyme. Eukaryot Cell 5(9): 1460–1467.

Carelli V, Ghelli A, Bucchi L, Montagna P, De Negri A, Leuzzi V, Carducci C, Lenaz G, Lugaresi E & Degli Esposti M (1999) Biochemical features of mtDNA 14484 (ND6/M64V) point mutation associated with Leber's hereditary optic neuropathy. Ann Neurol 45(3): 320–328.

Carelli V, Ghelli A, Ratta M, Bacchilega E, Sangiorgi S, Mancini R, Leuzzi V, Cortelli P, Montagna P, Lugaresi E & Degli Esposti M (1997) Leber's hereditary optic neuropathy: biochemical effect of 11778/ND4 and 3460/ND1 mutations and correlation with the mitochondrial genotype. Neurology 48(6): 1623–1632.

Carelli V, La Morgia C, Valentino ML, Barboni P, Ross-Cisneros FN & Sadun AA (2009) Retinal ganglion cell neurodegeneration in mitochondrial inherited disorders. Biochim Biophys Acta 1787(5): 518–528.

Carelli V, Ross-Cisneros FN & Sadun AA (2002) Optic nerve degeneration and mitochondrial dysfunction: genetic and acquired optic neuropathies. Neurochem Int 40(6): 573–584.

Carroll J, Fearnley IM, Skehel JM, Shannon RJ, Hirst J & Walker JE (2006) Bovine complex I is a complex of 45 different subunits. J Biol Chem 281(43): 32724–32727.

Castro-Guerrero N, Sinha PK, Torres-Bacete J, Matsuno-Yagi A & Yagi T (2010) Pivotal Roles of Three Conserved Carboxyl Residues of the NuoC (30k) Segment in the Structural Integrity of Proton-Translocating NADH-Quinone Oxidoreductase from Escherichia coli. Biochemistry.

Chen S & Guillory RJ (1981) Studies on the interaction of arylazido-beta-alanyl NAD+ with the mitochondrial NADH dehydrogenase. J Biol Chem 256(16): 8318–8323.

Crimi M, Galbiati S, Moroni I, Bordoni A, Perini MP, Lamantea E, Sciacco M, Zeviani M, Biunno I, Moggio M, Scarlato G & Comi GP (2003) A missense mutation in the mitochondrial ND5 gene associated with a Leigh-MELAS overlap syndrome. Neurology 60(11): 1857–1861.

Danielson SR, Wong A, Carelli V, Martinuzzi A, Schapira AH & Cortopassi GA (2002) Cells bearing mutations causing Leber's hereditary optic neuropathy are sensitized to Fas-Induced apoptosis. J Biol Chem 277(8): 5810–5815.

Darrouzet E, Issartel JP, Lunardi J & Dupuis A (1998) The 49-kDa subunit of NADH-ubiquinone oxidoreductase (Complex I) is involved in the binding of piericidin and rotenone, two quinone-related inhibitors. FEBS Lett 431(1): 34–38.

Degli Esposti M (1998) Inhibitors of NADH-ubiquinone reductase: an overview. Biochim Biophys Acta 1364(2): 222–235.

Deng PS, Hatefi Y & Chen S (1990) N-arylazido-beta-alanyl-NAD+, a new NAD+ photoaffinity analogue. Synthesis and labeling of mitochondrial NADH dehydrogenase. Biochemistry 29(4): 1094–1098.

Di Bernardo S & Yagi T (2001) Direct interaction between a membrane domain subunit and a connector subunit in the H(+)-translocating NADH-quinone oxidoreductase. FEBS Lett 508(3): 385–388.

DiFrancesco JC, Cooper JM, Lam A, Hart PE, Tremolizzo L, Ferrarese C & Schapira AH (2008) MELAS mitochondrial DNA mutation A3243G reduces glutamate transport in cybrids cell lines. Exp Neurol 212(1): 152–156.

DiMauro S & Schon EA (2001) Mitochondrial DNA mutations in human disease. Am J Med Genet 106(1): 18–26.

DiMauro S & Schon EA (2003) Mitochondrial respiratory-chain diseases. N Engl J Med 348(26): 2656–2668.

Djafarzadeh R, Kerscher S, Zwicker K, Radermacher M, Lindahl M, Schägger H & Brandt U (2000) Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast Yarrowia lipolytica. Biochim Biophys Acta 1459(1): 230–238.

Dunbar DR, Moonie PA, Zeviani M & Holt IJ (1996) Complex I deficiency is associated with 3243G:C mitochondrial DNA in osteosarcoma cell cybrids. Hum Mol Genet 5(1): 123–129.

Dupuis A, Darrouzet E, Duborjal H, Pierrard B, Chevallet M, van Belzen R, Albracht SP & Lunardi J (1998) Distal genes of the nuo operon of Rhodobacter capsulatus equivalent to the mitochondrial ND subunits are all essential for the biogenesis of the respiratory NADH-ubiquinone oxidoreductase. Mol Microbiol 28(3): 531–541.

Dutton PL, Moser CC, Sled VD, Daldal F & Ohnishi T (1998) A reductant-induced oxidation mechanism for complex I. Biochim Biophys Acta 1364(2): 245–257.

Earley FG, Patel SD, Ragan I & Attardi G (1987) Photolabelling of a mitochondrially encoded subunit of NADH dehydrogenase with [3H]dihydrorotenone. FEBS Lett 219(1): 108–112.

Efremov RG, Baradaran R & Sazanov LA (2010) The architecture of respiratory complex I. Nature 465(7297): 441–445.

Euro L, Belevich G, Bloch DA, Verkhovsky MI, Wikström M & Verkhovskaya M (2009) The role of the invariant glutamate 95 in the catalytic site of Complex I from Escherichia coli. Biochim Biophys Acta 1787(1): 68–73.

Euro L, Belevich G, Verkhovsky MI, Wikström M & Verkhovskaya M (2008a) 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(9): 1166–1172.

Euro L, Bloch DA, Wikström M, Verkhovsky MI & Verkhovskaya M (2008b) Electrostatic interactions between FeS clusters in NADH:ubiquinone oxidoreductase (Complex I) from Escherichia coli. Biochemistry 47(10): 3185–3193.

Fato R, Bernardo SD, Estornell E, Parentic Castelli G & Lenaz G (1997) Saturation kinetics of coenzyme Q in NADH oxidation: rate enhancement by incorporation of excess quinone. Mol Aspects Med 18 Suppl: S269–73.

Fearnley IM, Carroll J, Shannon RJ, Runswick MJ, Walker JE & Hirst J (2001) GRIM-19, a cell death regulatory gene product, is a subunit of bovine mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Biol Chem 276(42): 38345–38348.

Fearnley IM & Walker JE (1992) Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. Biochim Biophys Acta 1140(2): 105–134.

Fendel U, Tocilescu MA, Kerscher S & Brandt U (2008) Exploring the inhibitor binding pocket of respiratory complex I. Biochim Biophys Acta 1777(7–8): 660–665.

Figueroa-Martinez F, Vazquez-Acevedo M, Cortes-Hernandez P, Garcia-Trejo JJ, Davidson E, King MP & Gonzalez-Halphen D (2011) What limits the allotopic expression of nucleus-encoded mitochondrial genes? The case of the chimeric Cox3 and Atp6 genes. Mitochondrion 11(1): 147–154.

Floreani M, Napoli E, Martinuzzi A, Pantano G, De Riva V, Trevisan R, Bisetto E, Valente L, Carelli V & Dabbeni-Sala F (2005) Antioxidant defences in cybrids harboring mtDNA mutations associated with Leber's hereditary optic neuropathy. FEBS J 272(5): 1124–1135.

Fraser JA, Biousse V & Newman NJ (2010) The neuro-ophthalmology of mitochondrial disease. Surv Ophthalmol 55(4): 299–334.

Friedrich T (1998) The NADH:ubiquinone oxidoreductase (complex I) from Escherichia coli. Biochim Biophys Acta 1364(2): 134–146.

Friedrich T (2001) Complex I: a chimaera of a redox and conformation-driven proton pump? J Bioenerg Biomembr 33(3): 169–177.

Friedrich T & Scheide D (2000) The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. FEBS Lett 479(1–2): 1–5.

Friedrich T, van Heek P, Leif H, Ohnishi T, Forche E, Kunze B, Jansen R, Trowitzsch-Kienast W, Hofle G & Reichenbach H (1994) Two binding sites of inhibitors in NADH: ubiquinone oxidoreductase (complex I). Relationship of one site with the ubiquinone-binding site of bacterial glucose:ubiquinone oxidoreductase. Eur J Biochem 219(1–2): 691–698.

Friedrich T & Weiss H (1997) Modular evolution of the respiratory NADH:ubiquinone oxidoreductase and the origin of its modules. J Theor Biol 187(4): 529–540.

Galkin A & Brandt U (2005) Superoxide radical formation by pure complex I (NADH:ubiquinone oxidoreductase) from Yarrowia lipolytica. J Biol Chem 280(34): 30129–30135.

Galkin A, Dröse S & Brandt U (2006) The proton pumping stoichiometry of purified mitochondrial complex I reconstituted into proteoliposomes. Biochim Biophys Acta 1757(12): 1575–1581.

Galkin AS, Grivennikova VG & Vinogradov AD (1999) -->H+/2e- stoichiometry in NADH-quinone reductase reactions catalyzed by bovine heart submitochondrial particles. FEBS Lett 451(2): 157–161.

Garofano A, Zwicker K, Kerscher S, Okun P & Brandt U (2003) Two aspartic acid residues in the PSST-homologous NUKM subunit of complex I from Yarrowia lipolytica are essential for catalytic activity. J Biol Chem 278(43): 42435–42440.

Ghelli A, Degli Esposti M, Carelli V & Lenaz G (1997) Changes in mitochondrial complex I activity and coenzyme Q binding site in Leber's hereditary optic neuropathy (LHON). Mol Aspects Med 18 Suppl: S263–7.

Ghelli A, Zanna C, Porcelli AM, Schapira AH, Martinuzzi A, Carelli V & Rugolo M (2003) Leber's hereditary optic neuropathy (LHON) pathogenic mutations induce mitochondrial-dependent apoptotic death in transmitochondrial cells incubated with galactose medium. J Biol Chem 278(6): 4145–4150.

Gong X, Xie T, Yu L, Hesterberg M, Scheide D, Friedrich T & Yu CA (2003) The ubiquinone-binding site in NADH:ubiquinone oxidoreductase from Escherichia coli. J Biol Chem 278(28): 25731–25737.

Gostimskaya IS, Grivennikova VG, Cecchini G & Vinogradov AD (2007) Reversible dissociation of flavin mononucleotide from the mammalian membrane-bound NADH: ubiquinone oxidoreductase (complex I). FEBS Lett 581(30): 5803–5806.

Goto Y, Nonaka I & Horai S (1990) A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348(6302): 651–653.

Goto Y, Nonaka I & Horai S (1991) A new mtDNA mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Biochim Biophys Acta 1097(3): 238–240.

Grigorieff N (1998) Three-dimensional structure of bovine NADH:ubiquinone oxidoreductase (complex I) at 22 A in ice. J Mol Biol 277(5): 1033–1046.

Guenebaut V, Vincentelli R, Mills D, Weiss H & Leonard KR (1997) Three-dimensional structure of NADH-dehydrogenase from Neurospora crassa by electron microscopy and conical tilt reconstruction. J Mol Biol 265(4): 409–418.

Gutowski SJ & Rosenberg H (1975) Succinate uptake and related proton movements in Escherichia coli K12. Biochem J 152(3): 647–654.

Hamamoto T, Hashimoto M, Hino M, Kitada M, Seto Y, Kudo T & Horikoshi K (1994) Characterization of a gene responsible for the Na+/H+ antiporter system of alkalophilic Bacillus species strain C-125. Mol Microbiol 14(5): 939–946.

Harding AE, Sweeney MG, Govan GG & Riordan-Eva P (1995) Pedigree analysis in Leber hereditary optic neuropathy families with a pathogenic mtDNA mutation. Am J Hum Genet 57(1): 77–86.

Hassinen IE & Vuokila PT (1993) Reaction of dicyclohexylcarbodiimide with mitochondrial proteins. Biochim Biophys Acta 1144(2): 107–124.

Hayashi T & Stuchebrukhov AA (2010) Electron tunneling in respiratory complex I. Proc Natl Acad Sci USA 107(45): 19157–19162.

Hinttala R, Kervinen M, Uusimaa J, Maliniemi P, Finnilä S, Rantala H, Remes AM, Hassinen IE & Majamaa K (2010) Analysis of functional consequences of haplogroup J polymorphisms m.4216T>C and m.3866T>C in human MT-ND1: mutagenesis of homologous positions in Escherichia coli. Mitochondrion 10(4): 358–361.

Hirano M, Ricci E, Koenigsberger MR, Defendini R, Pavlakis SG, DeVivo DC, DiMauro S & Rowland LP (1992) Melas: an original case and clinical criteria for diagnosis. Neuromuscul Disord 2(2): 125–135.

Hirst J (2009) Towards the molecular mechanism of respiratory complex I. Biochem J 425(2): 327–339.

Hollander H, Makarov F, Stefani FH & Stone J (1995) Evidence of constriction of optic nerve axons at the lamina cribrosa in the normotensive eye in humans and other mammals. Ophthalmic Res 27(5): 296–309.

Hollenbeck PJ & Saxton WM (2005) The axonal transport of mitochondria. J Cell Sci 118(Pt 23): 5411–5419.

Holt PJ, Morgan DJ & Sazanov LA (2003) The location of NuoL and NuoM subunits in the membrane domain of the Escherichia coli complex I: implications for the mechanism of proton pumping. J Biol Chem 278(44): 43114–43120.

Honkakoski PJ & Hassinen IE (1986) Sensitivity to NN'-dicyclohexylcarbodi-imide of proton translocation by mitochondrial NADH:ubiquinone oxidoreductase. Biochem J 237(3): 927–930.

Hoppe J, Schairer HU, Friedl P & Sebald W (1982) An Asp-Asn substitution in the proteolipid subunit of the ATP-synthase from Escherichia coli leads to a non-functional proton channel. FEBS Lett 145(1): 21–29.

Huang G, Lu H, Hao A, Ng DC, Ponniah S, Guo K, Lufei C, Zeng Q & Cao X (2004) GRIM-19, a cell death regulatory protein, is essential for assembly and function of mitochondrial complex I. Mol Cell Biol 24(19): 8447–8456.

Hudson G, Carelli V, Spruijt L, Gerards M, Mowbray C, Achilli A, Pyle A, Elson J, Howell N, La Morgia C, Valentino ML, Huoponen K, Savontaus ML, Nikoskelainen E, Sadun AA, Salomao SR, Belfort R,Jr, Griffiths P, Man PY, de Coo RF, Horvath R, Zeviani M, Smeets HJ, Torroni A & Chinnery PF (2007) Clinical expression of Leber hereditary optic neuropathy is affected by the mitochondrial DNA-haplogroup background. Am J Hum Genet 81(2): 228–233.

Hudson G, Keers S, Yu Wai Man P, Griffiths P, Huoponen K, Savontaus ML, Nikoskelainen E, Zeviani M, Carrara F, Horvath R, Karcagi V, Spruijt L, de Coo IF, Smeets HJ & Chinnery PF (2005) Identification of an X-chromosomal locus and haplotype modulating the phenotype of a mitochondrial DNA disorder. Am J Hum Genet 77(6): 1086–1091.

Hunte C, Zickermann V & Brandt U (2010) Functional modules and structural basis of conformational coupling in mitochondrial complex I. Science 329(5990): 448–451.

Hutchison CA III, Newbold JE, Potter SS & Edgell MH (1974) Maternal inheritance of mammalian mitochondrial DNA. Nature 251(5475): 536–538.

Ichimaru N, Murai M, Kakutani N, Kako J, Ishihara A, Nakagawa Y, Nishioka T, Yagi T & Miyoshi H (2008) Synthesis and characterization of new piperazine-type inhibitors for mitochondrial NADH-ubiquinone oxidoreductase (complex I). Biochemistry 47(40): 10816–10826.

Iizuka T, Sakai F, Ide T, Miyakawa S, Sato M & Yoshii S (2007) Regional cerebral blood flow and cerebrovascular reactivity during chronic stage of stroke-like episodes in MELAS – implication of neurovascular cellular mechanism. J Neurol Sci 257(1–2): 126–138.

Ingledew WJ & Ohnishi T (1977) The probable site of action of thenolytrifluoracetone on the respiratory chain. Biochem J 164(3): 617–620.

Inoue H, Noumi T, Tsuchiya T & Kanazawa H (1995) Essential aspartic acid residues, Asp-133, Asp-163 and Asp-164, in the transmembrane helices of a Na+/H+ antiporter (NhaA) from Escherichia coli. FEBS Lett 363(3): 264–268.

Jun AS, Trounce IA, Brown MD, Shoffner JM & Wallace DC (1996) Use of transmitochondrial cybrids to assign a complex I defect to the mitochondrial DNA-encoded NADH dehydrogenase subunit 6 gene mutation at nucleotide pair 14459 that causes Leber hereditary optic neuropathy and dystonia. Mol Cell Biol 16(3): 771–777.

Kaback HR (2005) Structure and mechanism of the lactose permease. C R Biol 328(6): 557–567.

Kajiyama Y, Otagiri M, Sekiguchi J, Kudo T & Kosono S (2009) The MrpA, MrpB and MrpD subunits of the Mrp antiporter complex in Bacillus subtilis contain membrane-embedded and essential acidic residues. Microbiology 155(Pt 7): 2137–2147.

Kakutani N, Murai M, Sakiyama N & Miyoshi H (2010) Exploring the binding site of delta(lac)-acetogenin in bovine heart mitochondrial NADH-ubiquinone oxidoreductase. Biochemistry 49(23): 4794–4803.

Kao MC, Di Bernardo S, Matsuno-Yagi A & Yagi T (2002) Characterization of the membrane domain Nqo11 subunit of the proton-translocating NADH-quinone oxidoreductase of Paracoccus denitrificans. Biochemistry 41(13): 4377–4384.

Kao MC, Di Bernardo S, Matsuno-Yagi A & Yagi T (2003) Characterization and topology of the membrane domain Nqo10 subunit of the proton-translocating NADH-quinone oxidoreductase of Paracoccus denitrificans. Biochemistry 42(15): 4534–4543.

Kao MC, Di Bernardo S, Nakamaru-Ogiso E, Miyoshi H, Matsuno-Yagi A & Yagi T (2005) Characterization of the membrane domain subunit NuoJ (ND6) of the NADH-quinone oxidoreductase from Escherichia coli by chromosomal DNA manipulation. Biochemistry 44(9): 3562–3571.

Kao MC, Di Bernardo S, Perego M, Nakamaru-Ogiso E, Matsuno-Yagi A & Yagi T (2004a) Functional roles of four conserved charged residues in the membrane domain subunit NuoA of the proton-translocating NADH-quinone oxidoreductase from Escherichia coli. J Biol Chem 279(31): 32360–32366.

Kao MC, Matsuno-Yagi A & Yagi T (2004b) Subunit proximity in the H+-translocating NADH-quinone oxidoreductase probed by zero-length cross-linking. Biochemistry 43(12): 3750–3755.

Kao MC, Nakamaru-Ogiso E, Matsuno-Yagi A & Yagi T (2005) Characterization of the membrane domain subunit NuoK (ND4L) of the NADH-quinone oxidoreductase from Escherichia coli. Biochemistry 44(27): 9545–9554.

Kervinen M, Hinttala R, Helander HM, Kurki S, Uusimaa J, Finel M, Majamaa K & Hassinen IE (2006) The MELAS mutations 3946 and 3949 perturb the critical structure in a conserved loop of the ND1 subunit of mitochondrial complex I. Hum Mol Genet 15(17): 2543–2552.

King MS, Sharpley MS & Hirst J (2009) Reduction of hydrophilic ubiquinones by the flavin in mitochondrial NADH:ubiquinone oxidoreductase (Complex I) and production of reactive oxygen species. Biochemistry 48(9): 2053–2062.

Kirby DM, McFarland R, Ohtake A, Dunning C, Ryan MT, Wilson C, Ketteridge D, Turnbull DM, Thorburn DR & Taylor RW (2004) Mutations of the mitochondrial ND1 gene as a cause of MELAS. J Med Genet 41(10): 784–789.

Kirino Y, Yasukawa T, Ohta S, Akira S, Ishihara K, Watanabe K & Suzuki T (2004) Codon-specific translational defect caused by a wobble modification deficiency in mutant tRNA from a human mitochondrial disease. Proc Natl Acad Sci USA 101(42): 15070–15075.

Kirkman MA, Yu-Wai-Man P, Korsten A, Leonhardt M, Dimitriadis K, De Coo IF, Klopstock T & Chinnery PF (2009) Gene-environment interactions in Leber hereditary optic neuropathy. Brain 132(Pt 9): 2317–2326.

Kurki S, Zickermann V, Kervinen M, Hassinen I & Finel M (2000) Mutagenesis of three conserved Glu residues in a bacterial homologue of the ND1 subunit of complex I affects ubiquinone reduction kinetics but not inhibition by dicyclohexylcarbodiimide. Biochemistry 39(44): 13496–13502.

Kussmaul L & Hirst J (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. Proc Natl Acad Sci USA 103(20): 7607–7612.

Leif H, Weidner U, Berger A, Spehr V, Braun M, van Heek P, Friedrich T, Ohnishi T & Weiss H (1993) Escherichia coli NADH dehydrogenase I, a minimal form of the mitochondrial complex I. Biochem Soc Trans 21(4): 998–1001.

Lenaz G, Baracca A, Carelli V, D'Aurelio M, Sgarbi G & Solaini G (2004) Bioenergetics of mitochondrial diseases associated with mtDNA mutations. Biochim Biophys Acta 1658(1–2): 89–94.

Leo-Kottler B, Christ-Adler M, Baumann B, Zrenner E & Wissinger B (1996) Leber's hereditary optic neuropathy: clinical and molecular genetic results obtained in a family with a new point mutation at nucleotide position 14498 in the ND 6 gene. Ger J Ophthalmol 5(4): 233–240.

Liolitsa D, Rahman S, Benton S, Carr LJ & Hanna MG (2003) Is the mitochondrial complex I ND5 gene a hot-spot for MELAS causing mutations? Ann Neurol 53(1): 128–132.

Loeffen JL, Smeitink JA, Trijbels JM, Janssen AJ, Triepels RH, Sengers RC & van den Heuvel LP (2000) Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. Hum Mutat 15(2): 123–134.

Lowry OH, Roberts NR & Lewis C (1956) The quantitative histochemistry of the retina. J Biol Chem 220(2): 879–892.

Lunardi J, Darrouzet E, Dupuis A & Issartel JP (1998) The nuoM arg368his mutation in NADH:ubiquinone oxidoreductase from Rhodobacter capsulatus: a model for the human nd4–11778 mtDNA mutation associated with Leber's hereditary optic neuropathy. Biochim Biophys Acta 1407(2): 114–124.

Luo X, Heidinger V, Picaud S, Lambrou G, Dreyfus H, Sahel J & Hicks D (2001) Selective excitotoxic degeneration of adult pig retinal ganglion cells in vitro. Invest Ophthalmol Vis Sci 42(5): 1096–1106.

Magnitsky S, Toulokhonova L, Yano T, Sled VD, Hägerhäll C, Grivennikova VG, Burbaev DS, Vinogradov AD & Ohnishi T (2002) EPR characterization of ubisemiquinones and iron-sulfur cluster N2, central components of the energy coupling in the NADH-ubiquinone oxidoreductase (complex I) in situ. J Bioenerg Biomembr 34(3): 193–208.

Majamaa K, Moilanen JS, Uimonen S, Remes AM, Salmela PI, Kärppä M, Majamaa-Voltti KA, Rusanen H, Sorri M, Peuhkurinen KJ & Hassinen IE (1998) Epidemiology of A3243G, the mutation for mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes: prevalence of the mutation in an adult population. Am J Hum Genet 63(2): 447–454.

Majander A, Finel M, Savontaus ML, Nikoskelainen E & Wikström M (1996) Catalytic activity of complex I in cell lines that possess replacement mutations in the ND genes in Leber's hereditary optic neuropathy. Eur J Biochem 239(1): 201–207.

Majander A, Finel M & Wikström M (1994) Diphenyleneiodonium inhibits reduction of iron-sulfur clusters in the mitochondrial NADH-ubiquinone oxidoreductase (Complex I). J Biol Chem 269(33): 21037–21042.

Majander A, Huoponen K, Savontaus ML, Nikoskelainen E & Wikström M (1991) Electron transfer properties of NADH:ubiquinone reductase in the ND1/3460 and the ND4/11778 mutations of the Leber hereditary optic neuroretinopathy (LHON). FEBS Lett 292(1–2): 289–292.

Malfatti E, Bugiani M, Invernizzi F, de Souza CF, Farina L, Carrara F, Lamantea E, Antozzi C, Confalonieri P, Sanseverino MT, Giugliani R, Uziel G & Zeviani M (2007) Novel mutations of ND genes in complex I deficiency associated with mitochondrial encephalopathy. Brain 130(Pt 7): 1894–1904.

Mamedova AA, Holt PJ, Carroll J & Sazanov LA (2004) Substrate-induced conformational change in bacterial complex I. J Biol Chem 279(22): 23830–23836.

Man PY, Griffiths PG, Brown DT, Howell N, Turnbull DM & Chinnery PF (2003) The epidemiology of Leber hereditary optic neuropathy in the North East of England. Am J Hum Genet 72(2): 333–339.

Manwaring N, Jones MM, Wang JJ, Rochtchina E, Howard C, Mitchell P & Sue CM (2007) Population prevalence of the MELAS A3243G mutation. Mitochondrion 7(3): 230–233.

Mathiesen C & Hägerhäll C (2002) Transmembrane topology of the NuoL, M and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and bona fide antiporters. Biochim Biophys Acta 1556(2–3): 121–132.

Mathiesen C & Hägerhäll C (2003) The 'antiporter module' of respiratory chain complex I includes the MrpC/NuoK subunit – a revision of the modular evolution scheme. FEBS Lett 549(1–3): 7–13.

Miller MJ, Oldenburg M & Fillingame RH (1990) The essential carboxyl group in subunit c of the F1F0 ATP synthase can be moved and H(+)-translocating function retained. Proc Natl Acad Sci USA 87(13): 4900–4904.

Mitchell AL, Elson JL, Howell N, Taylor RW & Turnbull DM (2006) Sequence variation in mitochondrial complex I genes: mutation or polymorphism? J Med Genet 43(2): 175–179.

Mongini T, Doriguzzi C, Chiado-Piat L, Silvestri G, Servidei S & Palmucci L (2002) MERRF/MELAS overlap syndrome in a family with A3243G mtDNA mutation. Clin Neuropathol 21(2): 72–76.

Montoya J, Lopez-Gallardo E, Diez-Sanchez C, Lopez-Perez MJ & Ruiz-Pesini E (2009) 20 years of human mtDNA pathologic point mutations: carefully reading the pathogenicity criteria. Biochim Biophys Acta 1787(5): 476–483.

Morgan DJ & Sazanov LA (2008) Three-dimensional structure of respiratory complex I from Escherichia coli in ice in the presence of nucleotides. Biochim Biophys Acta 1777(7–8): 711–718.

Munnich A, Rustin P, Rotig A, Chretien D, Bonnefont JP, Nuttin C, Cormier V, Vassault A, Parvy P & Bardet J (1992) Clinical aspects of mitochondrial disorders. J Inherit Metab Dis 15(4): 448–455.

Murai M, Ishihara A, Nishioka T, Yagi T & Miyoshi H (2007) The ND1 subunit constructs the inhibitor binding domain in bovine heart mitochondrial complex I. Biochemistry 46(21): 6409–6416.

Murai M, Sekiguchi K, Nishioka T & Miyoshi H (2009) Characterization of the inhibitor binding site in mitochondrial NADH-ubiquinone oxidoreductase by photoaffinity labeling using a quinazoline-type inhibitor. Biochemistry 48(4): 688–698.

Nakamaru-Ogiso E, Han H, Matsuno-Yagi A, Keinan E, Sinha SC, Yagi T & Ohnishi T (2010a) The ND2 subunit is labeled by a photoaffinity analogue of asimicin, a potent complex I inhibitor. FEBS Lett 584(5): 883–888.

Nakamaru-Ogiso E, Kao MC, Chen H, Sinha SC, Yagi T & Ohnishi T (2010b) The membrane subunit NuoL(ND5) is involved in the indirect proton pumping mechanism of Escherichia coli complex I. J Biol Chem 285(50): 39070–39078.

Nakamaru-Ogiso E, Sakamoto K, Matsuno-Yagi A, Miyoshi H & Yagi T (2003) The ND5 subunit was labeled by a photoaffinity analogue of fenpyroximate in bovine mitochondrial complex I. Biochemistry 42(3): 746–754.

Nakamura M, Yabe I, Sudo A, Hosoki K, Yaguchi H, Saitoh S & Sasaki H (2010) MERRF/MELAS overlap syndrome: a double pathogenic mutation in mitochondrial tRNA genes. J Med Genet 47(10): 659–664.

Nikoskelainen E, Hoyt WF & Nummelin K (1983) Ophthalmoscopic findings in Leber's hereditary optic neuropathy. II. The fundus findings in the affected family members. Arch Ophthalmol 101(7): 1059–1068.

Nishimura Y, Yoshinari T, Naruse K, Yamada T, Sumi K, Mitani H, Higashiyama T & Kuroiwa T (2006) Active digestion of sperm mitochondrial DNA in single living sperm revealed by optical tweezers. Proc Natl Acad Sci USA 103(5): 1382–1387.

Ohama E, Ohara S, Ikuta F, Tanaka K, Nishizawa M & Miyatake T (1987) Mitochondrial angiopathy in cerebral blood vessels of mitochondrial encephalomyopathy. Acta Neuropathol 74(3): 226–233.

Ohnishi ST, Salerno JC & Ohnishi T (2010a) Possible roles of two quinone molecules in direct and indirect proton pumps of bovine heart NADH-quinone oxidoreductase (complex I). Biochim Biophys Acta 1797(12): 1891–1893.

Ohnishi ST, Shinzawa-Itoh K, Ohta K, Yoshikawa S & Ohnishi T (2010b) New insights into the superoxide generation sites in bovine heart NADH-ubiquinone oxidoreductase (Complex I): The significance of protein-associated ubiquinone and the dynamic shifting of generation sites between semiflavin and semiquinone radicals. Biochim Biophys Acta 1797(12): 1901–1909.

Ohnishi T (1998) Iron-sulfur clusters/semiquinones in complex I. Biochim Biophys Acta 1364(2): 186–206.

Ohnishi T, Johnson JE,Jr, Yano T, Lobrutto R & Widger WR (2005) Thermodynamic and EPR studies of slowly relaxing ubisemiquinone species in the isolated bovine heart complex I. FEBS Lett 579(2): 500–506.

Ohnishi T, Ohnishi ST, Shinzawa-Ito K & Yoshikawa S (2008) Functional role of coenzyme Q in the energy coupling of NADH-CoQ oxidoreductase (Complex I): stabilization of the semiquinone state with the application of inside-positive membrane potential to proteoliposomes. Biofactors 32(1–4): 13–22.

Ohnishi T & Salerno JC (2005) Conformation-driven and semiquinone-gated proton-pump mechanism in the NADH-ubiquinone oxidoreductase (complex I). FEBS Lett 579(21): 4555–4561.

Ohnishi T & Trumpower BL (1980) Differential effects of antimycin on ubisemiquinone bound in different environments in isolated succinate. cytochrome c reductase complex. J Biol Chem 255(8): 3278–3284.

Okun JG, Lummen P & Brandt U (1999) Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase). J Biol Chem 274(5): 2625–2630.

Padan E (2008) The enlightening encounter between structure and function in the NhaA Na+-H+ antiporter. Trends Biochem Sci 33(9): 435–443.

Page CC, Moser CC & Dutton PL (2003) Mechanism for electron transfer within and between proteins. Curr Opin Chem Biol 7(5): 551–556.

Pätsi J, Kervinen M, Finel M & Hassinen IE (2008) Leber hereditary optic neuropathy mutations in the ND6 subunit of mitochondrial complex I affect ubiquinone reduction kinetics in a bacterial model of the enzyme. Biochem J 409(1): 129–137.

Pavlakis SG, Phillips PC, DiMauro S, De Vivo DC & Rowland LP (1984) Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes: a distinctive clinical syndrome. Ann Neurol 16(4): 481–488.

Peng G, Fritzsch G, Zickermann V, Schägger H, Mentele R, Lottspeich F, Bostina M, Radermacher M, Huber R, Stetter KO & Michel H (2003) Isolation, characterization and electron microscopic single particle analysis of the NADH:ubiquinone oxidoreductase (complex I) from the hyperthermophilic eubacterium Aquifex aeolicus. Biochemistry 42(10): 3032–3039.

Perales-Clemente E, Fernandez-Silva P, Acin-Perez R, Perez-Martos A & Enriquez JA (2011) Allotopic expression of mitochondrial-encoded genes in mammals: achieved goal, undemonstrated mechanism or impossible task? Nucleic Acids Res 39(1): 225–234.

Pohl T, Bauer T, Dorner K, Stolpe S, Sell P, Zocher G & Friedrich T (2007) Iron-sulfur cluster N7 of the NADH:ubiquinone oxidoreductase (complex I) is essential for stability but not involved in electron transfer. Biochemistry 46(22): 6588–6596.

Pohl T, Spatzal T, Aksoyoglu M, Schleicher E, Rostas AM, Lay H, Glessner U, Boudon C, Hellwig P, Weber S & Friedrich T (2010) Spin labeling of the Escherichia coli NADH ubiquinone oxidoreductase (complex I). Biochim Biophys Acta 1797(12): 1894–1900.

Price CE & Driessen AJ (2010) Conserved negative charges in the transmembrane segments of subunit K of the NADH:ubiquinone oxidoreductase determine its dependence on YidC for membrane insertion. J Biol Chem 285(6): 3575–3581.

Prieur I, Lunardi J & Dupuis A (2001) Evidence for a quinone binding site close to the interface between NUOD and NUOB subunits of Complex I. Biochim Biophys Acta 1504(2–3): 173–178.

Pulkes T, Eunson L, Patterson V, Siddiqui A, Wood NW, Nelson IP, Morgan-Hughes JA & Hanna MG (1999) The mitochondrial DNA G13513A transition in ND5 is associated with a LHON/MELAS overlap syndrome and may be a frequent cause of MELAS. Ann Neurol 46(6): 916–919.

Puomila A, Hämäläinen P, Kivioja S, Savontaus ML, Koivumäki S, Huoponen K & Nikoskelainen E (2007) Epidemiology and penetrance of Leber hereditary optic neuropathy in Finland. Eur J Hum Genet 15(10): 1079–1089.

Puustinen A, Finel M, Haltia T, Gennis RB & Wikström M (1991) Properties of the two terminal oxidases of Escherichia coli. Biochemistry 30(16): 3936–3942.

Qi X, Lewin AS, Hauswirth WW & Guy J (2003) Optic neuropathy induced by reductions in mitochondrial superoxide dismutase. Invest Ophthalmol Vis Sci 44(3): 1088–1096.

Ravn K, Wibrand F, Hansen FJ, Horn N, Rosenberg T & Schwartz M (2001) An mtDNA mutation, 14453G-->A, in the NADH dehydrogenase subunit 6 associated with severe MELAS syndrome. Eur J Hum Genet 9(10): 805–809.

Rossignol R, Faustin B, Rocher C, Malgat M, Mazat JP & Letellier T (2003) Mitochondrial threshold effects. Biochem J 370(Pt 3): 751–762.

Roth R & Hägerhäll C (2001) Transmembrane orientation and topology of the NADH:quinone oxidoreductase putative quinone binding subunit NuoH. Biochim Biophys Acta 1504(2–3): 352–362.

Rusanen H, Majamaa K & Hassinen IE (2000) Increased activities of antioxidant enzymes and decreased ATP concentration in cultured myoblasts with the 3243A-->G mutation in mitochondrial DNA. Biochim Biophys Acta 1500(1): 10–16.

Sano M, Ozawa M, Shiota S, Momose Y, Uchigata M & Goto Y (1996) The T-C(8356) mitochondrial DNA mutation in a Japanese family. J Neurol 243(6): 441–444.

Satoh M & Kuroiwa T (1991) Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. Exp Cell Res 196(1): 137–140.

Sazanov LA, Carroll J, Holt P, Toime L & Fearnley IM (2003) A role for native lipids in the stabilization and two-dimensional crystallization of the Escherichia coli NADH-ubiquinone oxidoreductase (complex I). J Biol Chem 278(21): 19483–19491.

Sazanov LA & Hinchliffe P (2006) Structure of the hydrophilic domain of respiratory complex I from Thermus thermophilus. Science 311(5766): 1430–1436.

Sazanov LA, Peak-Chew SY, Fearnley IM & Walker JE (2000) Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme. Biochemistry 39(24): 7229–7235.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB & Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1(8649): 1269.

Schneider R, Massow M, Lisowsky T & Weiss H (1995) Different respiratory-defective phenotypes of Neurospora crassa and Saccharomyces cerevisiae after inactivation of the gene encoding the mitochondrial acyl carrier protein. Curr Genet 29(1): 10–17.

Schuler F & Casida JE (2001) Functional coupling of PSST and ND1 subunits in NADH:ubiquinone oxidoreductase established by photoaffinity labeling. Biochim Biophys Acta 1506(1): 79–87.

Schuler F, Yano T, Di Bernardo S, Yagi T, Yankovskaya V, Singer TP & Casida JE (1999) NADH-quinone oxidoreductase: PSST subunit couples electron transfer from iron-sulfur cluster N2 to quinone. Proc Natl Acad Sci USA 96(7): 4149–4153.

Sekiguchi K, Murai M & Miyoshi H (2009) Exploring the binding site of acetogenin in the ND1 subunit of bovine mitochondrial complex I. Biochim Biophys Acta 1787(9): 1106–1111.

Shankar SP, Fingert JH, Carelli V, Valentino ML, King TM, Daiger SP, Salomao SR, Berezovsky A, Belfort R,Jr, Braun TA, Sheffield VC, Sadun AA & Stone EM (2008) Evidence for a novel x-linked modifier locus for leber hereditary optic neuropathy. Ophthalmic Genet 29(1): 17–24.

Shinzawa-Itoh K, Seiyama J, Terada H, Nakatsubo R, Naoki K, Nakashima Y & Yoshikawa S (2010) Bovine heart NADH-ubiquinone oxidoreductase contains one molecule of ubiquinone with ten isoprene units as one of the cofactors. Biochemistry 49(3): 487–492.

Sinha PK, Torres-Bacete J, Nakamaru-Ogiso E, Castro-Guerrero N, Matsuno-Yagi A & Yagi T (2009) Critical roles of subunit NuoH (ND1) in the assembly of peripheral subunits with the membrane domain of Escherichia coli NDH-1. J Biol Chem 284(15): 9814–9823.

Skladal D, Halliday J & Thorburn DR (2003) Minimum birth prevalence of mitochondrial respiratory chain disorders in children. Brain 126(Pt 8): 1905–1912.

Sled VD, Rudnitzky NI, Hatefi Y & Ohnishi T (1994) Thermodynamic analysis of flavin in mitochondrial NADH:ubiquinone oxidoreductase (complex I). Biochemistry 33(33): 10069–10075.

Sled VD & Vinogradov AD (1993) Kinetics of the mitochondrial NADH-ubiquinone oxidoreductase interaction with hexammineruthenium(III). Biochim Biophys Acta 1141(2–3): 262–268.

Smith JL, Hoyt WF & Susac JO (1973) Ocular fundus in acute Leber optic neuropathy. Arch Ophthalmol 90(5): 349–354.

Sproule DM & Kaufmann P (2008) Mitochondrial encephalopathy, lactic acidosis, and strokelike episodes: basic concepts, clinical phenotype, and therapeutic management of MELAS syndrome. Ann N Y Acad Sci 1142: 133–158.

Sue CM, Crimmins DS, Soo YS, Pamphlett R, Presgrave CM, Kotsimbos N, Jean-Francois MJ, Byrne E & Morris JG (1998) Neuroradiological features of six kindreds with MELAS tRNA(Leu) A2343G point mutation: implications for pathogenesis. J Neurol Neurosurg Psychiatry 65(2): 233–240.

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C & Schatten G (2000) Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. Biol Reprod 63(2): 582–590.

Tanji K, Kunimatsu T, Vu TH & Bonilla E (2001) Neuropathological features of mitochondrial disorders. Semin Cell Dev Biol 12(6): 429–439.

Tocilescu MA, Fendel U, Zwicker K, Dröse S, Kerscher S & Brandt U (2010) The role of a conserved tyrosine in the 49-kDa subunit of complex I for ubiquinone binding and reduction. Biochim Biophys Acta 1797(6–7): 625–632.

Tocilescu MA, Fendel U, Zwicker K, Kerscher S & Brandt U (2007) Exploring the ubiquinone binding cavity of respiratory complex I. J Biol Chem 282(40): 29514–29520.

Tocilescu MA, Zickermann V, Zwicker K & Brandt U (2010) Quinone binding and reduction by respiratory complex I. Biochim Biophys Acta 1797(12): 1883–1890.

Torres-Bacete J, Nakamaru-Ogiso E, Matsuno-Yagi A & Yagi T (2007) Characterization of the NuoM (ND4) subunit in Escherichia coli NDH-1: conserved charged residues essential for energy-coupled activities. J Biol Chem 282(51): 36914–36922.

Torres-Bacete J, Sinha PK, Castro-Guerrero N, Matsuno-Yagi A & Yagi T (2009) Features of subunit NuoM (ND4) in Escherichia coli NDH-1: topology and implication of conserved Glu144 for coupling site 1. J Biol Chem 284(48): 33062–33069.

Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlof S, Oldfors A, Wibom R, Tornell J, Jacobs HT & Larsson NG (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429(6990): 417–423.

Valentino ML, Barboni P, Ghelli A, Bucchi L, Rengo C, Achilli A, Torroni A, Lugaresi A, Lodi R, Barbiroli B, Dotti M, Federico A, Baruzzi A & Carelli V (2004) The ND1 gene of complex I is a mutational hot spot for Leber's hereditary optic neuropathy. Ann Neurol 56(5): 631–641.

Verkhovskaya ML, Belevich N, Euro L, Wikström M & Verkhovsky MI (2008) Real-time electron transfer in respiratory complex I. Proc Natl Acad Sci USA 105(10): 3763–3767.

Verkhovskaya ML, Garcia-Horsman A, Puustinen A, Rigaud JL, Morgan JE, Verkhovsky MI & Wikström M (1997) Glutamic acid 286 in subunit I of cytochrome bo3 is involved in proton translocation. Proc Natl Acad Sci USA 94(19): 10128–10131.

Vinogradov AD (2008) NADH/NAD+ interaction with NADH: ubiquinone oxidoreductase (complex I). Biochim Biophys Acta 1777(7–8): 729–734.

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ,2nd & Nikoskelainen EK (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242(4884): 1427–1430.

Wikström M (1984) Two protons are pumped from the mitochondrial matrix per electron transferred between NADH and ubiquinone. FEBS Lett 169(2): 300–304.

Wissinger B, Besch D, Baumann B, Fauser S, Christ-Adler M, Jurklies B, Zrenner E & Leo-Kottler B (1997) Mutation analysis of the ND6 gene in patients with Lebers hereditary optic neuropathy. Biochem Biophys Res Commun 234(2): 511–515.

Wong A, Cavelier L, Collins-Schramm HE, Seldin MF, McGrogan M, Savontaus ML & Cortopassi GA (2002) Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells. Hum Mol Genet 11(4): 431–438.

Wong A & Cortopassi G (1997) mtDNA mutations confer cellular sensitivity to oxidant stress that is partially rescued by calcium depletion and cyclosporin A. Biochem Biophys Res Commun 239(1): 139–145.

Wong LJ (2007) Pathogenic mitochondrial DNA mutations in protein-coding genes. Muscle Nerve 36(3): 279–293.

Yagi T & Dinh TM (1990) Identification of the NADH-binding subunit of NADH-ubiquinone oxidoreductase of Paracoccus denitrificans. Biochemistry 29(23): 5515–5520.

Yagi T & Hatefi Y (1988) Identification of the dicyclohexylcarbodiimide-binding subunit of NADH-ubiquinone oxidoreductase (Complex I). J Biol Chem 263(31): 16150–16155.

Yagi T, Yano T, Di Bernardo S & Matsuno-Yagi A (1998) Procaryotic complex I (NDH-1), an overview. Biochim Biophys Acta 1364(2): 125–133.

Yano T, Chu SS, Sled' VD, Ohnishi T & Yagi T (1997) The proton-translocating NADH-quinone oxidoreductase (NDH-1) of thermophilic bacterium Thermus thermophilus HB-8. Complete DNA sequence of the gene cluster and thermostable properties of the expressed NQO2 subunit. J Biol Chem 272(7): 4201–4211.

Yano T, Dunham WR & Ohnishi T (2005) Characterization of the delta muH+-sensitive ubisemiquinone species (SQ(Nf)) and the interaction with cluster N2: new insight into the energy-coupled electron transfer in complex I. Biochemistry 44(5): 1744–1754.

Yasukawa T, Suzuki T, Ueda T, Ohta S & Watanabe K (2000) Modification defect at anticodon wobble nucleotide of mitochondrial tRNAs(Leu)(UUR) with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. J Biol Chem 275(6): 4251–4257.

Yen MY, Kao SH, Wang AG & Wei YH (2004) Increased 8-hydroxy-2'-deoxyguanosine in leukocyte DNA in Leber's hereditary optic neuropathy. Invest Ophthalmol Vis Sci 45(6): 1688–1691.

Yen MY, Wang AG & Wei YH (2006) Leber's hereditary optic neuropathy: a multifactorial disease. Prog Retin Eye Res 25(4): 381–396.

Yoon YG, Koob MD & Yoo YH (2010) Re-engineering the mitochondrial genomes in mammalian cells. Anat Cell Biol 43(2): 97–109.

Yu-Wai-Man P, Griffiths PG, Hudson G & Chinnery PF (2009) Inherited mitochondrial optic neuropathies. J Med Genet 46(3): 145–158.

Zanna C, Ghelli A, Porcelli AM, Carelli V, Martinuzzi A & Rugolo M (2003) Apoptotic cell death of cybrid cells bearing Leber's hereditary optic neuropathy mutations is caspase independent. Ann N Y Acad Sci 1010: 213–217.

Zharova TV & Vinogradov AD (1997) A competitive inhibition of the mitochondrial NADH-ubiquinone oxidoreductase (complex I) by ADP-ribose. Biochim Biophys Acta 1320(3): 256–264.

Zickermann V, Barquera B, Wikström M & Finel M (1998) Analysis of the pathogenic human mitochondrial mutation ND1/3460, and mutations of strictly conserved residues in its vicinity, using the bacterium Paracoccus denitrificans. Biochemistry 37(34): 11792–11796.

Zickermann V, Kerscher S, Zwicker K, Tocilescu MA, Radermacher M & Brandt U (2009) Architecture of complex I and its implications for electron transfer and proton pumping. Biochim Biophys Acta 1787(6): 574–583.

Zickermann V, Kurki S, Kervinen M, Hassinen I & Finel M (2000) The NADH oxidation domain of complex I: do bacterial and mitochondrial enzymes catalyze ferricyanide reduction similarly? Biochim Biophys Acta 1459(1): 61–68.

Zu Y, Di Bernardo S, Yagi T & Hirst J (2002) Redox properties of the [2Fe-2S] center in the 24 kDa (NQO2) subunit of NADH:ubiquinone oxidoreductase (complex I). Biochemistry 41(31): 10056–10069.

Zwicker K, Galkin A, Dröse S, Grgic L, Kerscher S & Brandt U (2006) The Redox-Bohr group associated with iron-sulfur cluster N2 of complex I. J Biol Chem 281(32): 23013–23017.

Original publications

I Kervinen M, Pätsi J, Finel M & Hassinen IE (2004) A pair of membrane-embedded acidic residues in the NuoK subunit of Escherichia coli NDH-1, a counterpart of the ND4L subunit of the mitochondrial complex I, are required for high ubiquinone reductase activity. Biochemistry 43(3): 773–781.

II Pätsi J, Kervinen M, Finel M & Hassinen IE (2008) Leber hereditary optic neuropathy mutations in the ND6 subunit of mitochondrial complex I affect ubiquinone reduction kinetics in a bacterial model of the enzyme. Biochemical Journal 409(1): 129–137.

III Pätsi J, Maliniemi P, Pakanen S, Hinttala R, Uusimaa J, Majamaa K, Nyström T, Kervinen M & Hassinen IE (2011) LHON/MELAS overlap mutation in ND1 subunit of mitochondrial complex I has distinct effect on ubiquinone binding. Manuscript.

Original publications are not included in the electronic version of the dissertation.

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