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LHON/MELAS overlap mutation in ND1 subunit of mitochondrial complex I affects ubiquinone binding as revealed by modeling in *Escherichia coli* NDH-1

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ABSTRACT

Defects in complex I due to mutations in mitochondrial DNA are associated with clinical features ranging from single organ manifestation like Leber hereditary optic neuropathy (LHON) to multiorgan disorders like mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome. Specific mutations cause overlap syndromes combining several phenotypes, but the mechanisms of their biochemical effects are largely unknown. The m.3376G>A transition leading to p.E24K substitution in ND1 with LHON/MELAS phenotype was modeled here in a homologous position (NuoH-E36K) in the *Escherichia coli* enzyme and it almost totally abolished complex I activity. The more conservative mutation NuoH-E36Q resulted in higher apparent K_m for ubiquinone and diminished inhibitor sensitivity. A NuoH homolog of the m.3865A>G transition, which has been found concomitantly in the overlap syndrome patient with the m.3376G>A, had only a minor effect. Consequences of a primary LHON-mutation m.3460G>A affecting the same extramembrane loop as the m.3376G>A substitution were also studied in the *E. coli* model and were found to be mild. The results indicate that the overlap syndrome-associated m.3376G>A transition in *MTND1* is the pathogenic mutation and m.3865A>G transition has minor, if any, effect on presentation of the disease. The kinetic effects of the NuoH-E36Q mutation suggest its proximity to the putative ubiquinone binding domain in 49 kD/PSST subunits. In all, m.3376G>A perturbs ubiquinone binding, a phenomenon found in LHON, and decreases the activity of fully assembled complex I as in MELAS.

1. Introduction

NADH-ubiquinone oxidoreductase (Complex I, EC 1.6.5.3) is by far the most complicated enzyme of the mitochondrial respiratory chain. It is composed of 45 different polypeptides and a set of redox centers resulting in a total molecular mass of about 1000 kDa in mammals [1]. Seven of the complex I subunits (ND1–ND6 and ND4L) are encoded in mitochondrial genome (mtDNA) while the rest of the genes are located in the nucleus. Fourteen of the subunits, including all the NDs, have their homologs in NDH-1, the bacterial counterparts of the enzyme, and are considered to comprise the catalytic core of complex I (for a review, see [2]).

Regardless of increasing knowledge of the structure of complex I [3–6], the number and exact position of ubiquinone binding sites are still ambiguous. The iron–sulphur cluster N2 in the PSST subunit is thought to be the final electron donor to ubiquinone (UQ). Based on extensive mutational analysis of the PSST and 49 kD subunit homologs in *Yarrowia lipolytica* it has been demonstrated that many conserved residues lining the cavity between these peptides in the proximity of N2 are critical for the catalytic activity and inhibitor binding. It has therefore been suggested that a UQ binding domain is located within this pocket, although apart from Tyr-144 mutations in 49 kD subunit, most mutations with appreciable changes in inhibitor sensitivity affect very little the apparent K_m value for UQ [7–11]. A second puzzling

Abbreviations: d-NADH, deamino-NADH; DB, decylubiquinone; HAR, hexaammineruthenium; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome; mtDNA, mitochondrial DNA; NDH-1, bacterial proton pumping NADH-ubiquinone oxidoreductase; NDH-2, bacterial non-proton pumping NADH-ubiquinone oxidoreductase; Q1, ubiquinone-1; Q2, ubiquinone-2; UQ, ubiquinone; VNA, N-vanillylnonanamide

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feature of UQ and inhibitor binding is that binding equilibrium data indicate only partially competitive binding for classical, quinone site-binding inhibitors like piericidin A. Some others, like rotenone, show a more mixed (non-competitive) type of inhibition [12], although all these inhibitors are regarded to bind to separate partially overlapping sites in the same domain [13].

ND1 as one of the core subunits in the membrane domain of complex I is apparently involved in attaching the hydrophilic part of the enzyme to the membrane [6,14]. Based on the locations of the helices of the membrane arm of the holoenzyme in *Thermus thermophilus*, an essential role has been proposed for ND1 in coupling the electron and proton transfer reactions of complex I [6]. Some evidence points to its involvement in UQ-binding, too. Structure of the membranous domain of *Escherichia coli* and *T. thermophilus* NDH-1 in combination with previous biochemical evidence has revealed a putative ubiquinone entry channel or binding site in NuoH/Nqo8, which are homologs of the mitochondrial ND1 subunit [6,15].

Complex I dysfunction is a common cause of mitochondrial disorders such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) and Leber hereditary optic neuropathy (LHON). At least 33 disease-associated substitutions have been reported so far in the *MTND1* gene (Mitomap, available from http://mitomap.org/MITOMAP, on April 14th 2011) emphasizing the importance of ND1 in complex I function. Similarly to the common m.3243A>G MELAS mutation [16–18], a decrease in amount of active complex I or its stability has been reported in transmitochondrial cybrid cell lines derived from patient fibroblasts and in bacterial models displaying ND1-subunit MELAS mutations [19–21]. In the case of ND1-affecting m.3460G>A mutation, one of the primary mutations associated with non-syndromic LHON, the in vitro electron transfer activity of the enzyme complex is strongly reduced and resistance to rotenone has been documented (for a review, see e.g. [22]). *MTND1* is a target for less common disease-associated mtDNA mutations as well. A potentially pathogenic, heteroplasmic m.3376G>A mutation in *MTND1* has been discovered in a patient suffering from the LHON/MELAS overlap syndrome [23]. The proband carried also an m.3865A>G transition affecting the same subunit, but it was not considered to be pathogenic since it was also present in the unaffected mother of the proband. In skeletal muscle, the citrate synthase-normalized complex I activity was 36% of control [23]. Effects of this mutation on complex I have not been scrutinized any further. Interestingly, the m.3376G>A transition results in replacement of the highly conserved glutamate residue (Glu-24) by lysine in ND1 in an extramembranous loop, which is also a target of the common m.3460G>A LHON mutation as well as many other disease-associated substitutions (see Fig. 1).

The m.3376G>A mutation does not fulfill all the commonly accepted criteria for a pathogenic mtDNA mutation [24] and also the role of the m.3865A>G transition in the formation of the disease phenotype has remained unproven. We show here by employing an *E. coli* model that m.3376G>A mutation is clearly pathogenic, whereas m.3865A>G has only minor, if any, effect. Interestingly, m.3376G>A decreases enzyme amount similarly to other MELAS mutations affecting the same gene [19] and also disturbs the ubiquinone binding domain, which has been previously shown to be associated with LHON mutations [22,25,26]. To our knowledge, this is the first time the biochemical consequences of an mtDNA mutation associated with an LHON/MELAS overlap syndrome is analyzed on a stable, bacterial application framework. Because of the relative paucity of the mtDNA mutations, their metabolic link to human disease is difficult to confirm on basis of biopsy samples. Therefore, their investigation by means of site-directed mutagenesis in bacterial models is gaining increasing practice, exemplified by studies on LHON, [25–28] and MELAS [19]. Although limited to the structural genes of the core subunits and to the effects at enzymatic level, a bacterial model for an mtDNA mutation enables introduction of mutations not occurring naturally, to better analyze the consequences of a single amino acid substitution.

2. Materials and methods

2.1. Chemicals

Decylubiquinone (DB), ubiquinone-1 (Q_1), ubiquinone-2 (Q_2), NADH, deamino-NADH (d-NADH), DL-malate, N-vanillylnonanamide (VNA), soya bean phospholipids (Asolectin) and p-Nitro-Blue tetrazolium chloride (NBT) were from Sigma. Hexaammineruthenium (HAR) was purchased from Aldrich, Hepes and Mes from AppliChem, and

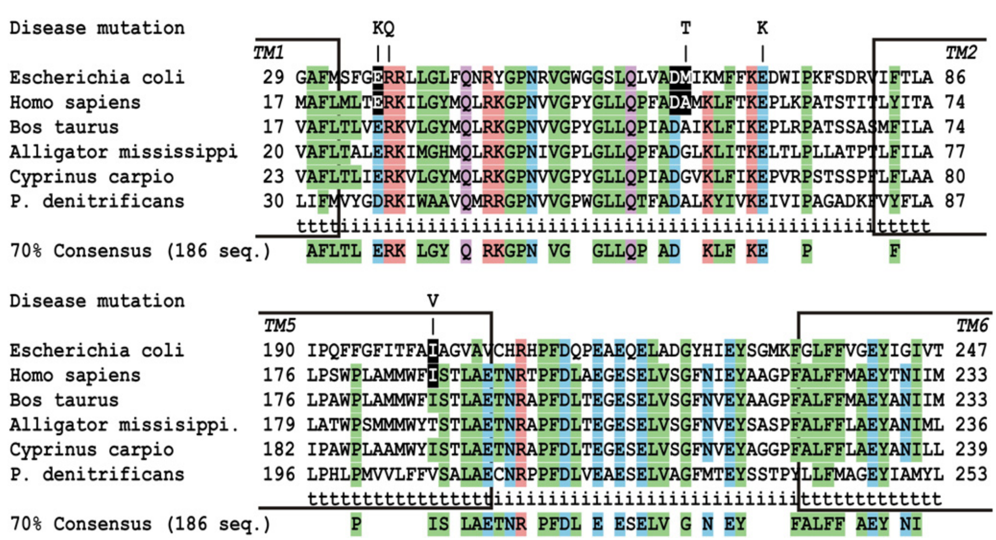

Fig. 1. The first and the third inside loops of human ND1 and *E. coli* NuoH with parts of bordering transmembrane helices 1 and 2, 5 and 6. Disease-associated mtDNA mutations and the corresponding amino acid changes p.E24K (m.3376G>A) [23], p.R25Q (m.3380G>A) [53], p.Y30H (m.3394T>C) [54,55], p.M31V (m.3397A>G) [56], p.A52T (m.3460G>A) [57], p.E59K (m.3481G>A) [21,58] and p.I187V (m.3865A>G) [23] are indicated. The residues undergoing site-directed mutagenesis are shown in white font on black background. An alignment of homologous complex I subunits from human (GenBank ID: AEO90223.1), bovine (GenBank ID: ADF49509.1), carp (NCBI ID: NP_007082.1), alligator (UniProt ID: O47868), *E. coli* (GenBank: AAC75342.1) and *P. denitrificans* (GenBank ID: AAA25592.1) was performed by means of ClustalW, and the transmembrane topology by means of the TMHMM program [40]. "t" denotes transmembrane helix and "i" inside loop. The 70% consensus sequence was compiled by the PredictProt server from an alignment of 186 homologous sequences.

potassium cyanide and PMSF from Merck. Piericidin A was from Fluka. Oligonucleotides were purchased from Sigma-Genosys or Oligomer. The QuikChange® XL mutagenesis kit was from Stratagene, BCA kit from Pierce and Phusion DNA polymerase from Finnzymes. Bis–Tris gradient gels for Blue native electrophoresis were from Invitrogen. An annonaceous acetogenin mixture (*Annona squamosa* seed extract) was a generous gift from Dr. Y. K. Gupta, Varanasi, India. Stigmatellin was from Sigma except for the inhibition type analysis, wherein it was kindly provided by Professor U. Brandt, Frankfurt am Main, Germany.

2.2. Site-directed mutagenesis and NuoH expression

NuoH point mutations listed in Table 1 were introduced into an expression plasmid pHwt carrying the wild type *nuoH* under a T7 *lac* promoter as previously described [19]. The oligonucleotides used in mutagenesis are listed in Supplementary Table S1. Correctness of the constructs was verified by DNA sequencing of the mutant gene. *In-trans* complementation of the *nuoH* deletion was done by transforming a *nuoH* knock-out derivative (HK18) of *E. coli* strain GV102 [19] with plasmids expressing either wild type or mutated *nuoH*.

To evaluate the performance of the *in-trans* complementation system, genomic complementation of the *nuoH-E36Q* mutant allele was accomplished as follows. The recipient strain for mutant allele was prepared from MG1655 wild type laboratory strain by deleting the whole *nuoH* gene and replacing it with a Zeocin resistance gene according to [19] except that the Zeo^R gene was used instead of the Sm^R gene in the deletion construct according to [29]. For the mutant allele, Nhe1–Xho1 restriction sites were introduced in front of the streptomycin resistance gene in pLitmus39 containing *nuoH* flanking regions with streptomycin resistance gene in between UF + Sm + LF [19] by QuikChange® XL mutagenesis kit according to the manufacturer's instructions and oligonucleotides GstopF and GstopR listed in Supplementary Table S1. The flanking regions were sequenced in order to exclude unintentional mutations in the construct. Nhe1-Sal1 fragment containing the whole *nuoH* gene from NuoH-E36Q expression plasmid was cloned into the Nhe1–Xho1 site in pLitmus39-UF + Sm + LF. Sal1-Stu1 fragment from this plasmid was used to transform JW2277(*ΔnuoH::kan*) strain [30] expressing phage λ Red recombinase from pKD46 plasmid [31]. A streptomycin resistant and kanamycin sensitive colony was selected and the mutant allele was transferred to the recipient strain (MG1655 *ΔnuoH::Zeo* described earlier) by standard P1 transduction and selected for streptomycin resistance. The correct strain was verified by loss of Zeocin resistance and PCR. In order to obtain the same composition of *E. coli* respiratory chain as in plasmid complementation strains, *Δcyo::kan* allele was transferred from GV102 into MG1655 (resulting in MKG311 strain, used as a control) and into NuoH-E36Q strain (MKGnuoH36-31 strain).

The MKG51-32 strain lacking the whole *nuo* operon was generated as follows. BW25113 strain [31] expressing phage λ Red recombinase from pKD46 plasmid was transformed with PCR product containing recombination sequences up- and downstream of *nuo* genes according to [32] with chloramphenicol resistance marker in between amplified from pKD3 plasmid [31]. The deletion and *Δcyo::kan* alleles were transferred into MG1655 by P1 transduction as stated earlier. The resulting strain was verified for the loss of *nuo* operon by PCR and absence of d-NADH oxidation and HAR reductase capacities.

Table 1
Mutations introduced into the NuoH subunit of *Escherichia coli* NDH-1 and corresponding disease-associated substitutions in the ND1 subunit of mitochondrial complex I.

Mitochondrial mutation in ND1 subunit gene				Mutations introduced into *E. coli* NuoH
Nucleotide change	Amino acid change	Resulting disease	Reference	
m.3376G > A/ m.3865A > G	E24K/I187V	LHON/MELAS	[23]	E36K, E36Q, E36D I201V
m.3460G > A	A52T	LHON	[57]	M64T
Not applicable				D63E

2.3. Bacterial growth and membrane preparation

Bacteria were routinely grown in LB medium supplemented with appropriate antibiotics. Collection of cells and preparation of membrane samples were performed as described before [19]. Protein concentrations of the membrane samples were determined according to Lowry [33] or by BCA kit from Pierce.

2.4. Enzyme activity measurements and inhibitor sensitivity tests

E. coli and some other bacteria express two NADH-ubiquinone oxidoreductases, NDH-1 and NDH-2. The former is a homolog of complex I and is capable of oxidizing both d-NADH and NADH, while the single subunit, non-proton pumping NDH-2 purified from *E. coli* is able to oxidize only NADH [34]. Therefore, d-NADH was used instead of NADH throughout this study to measure NDH-1-dependent activities.

d-NADH:DB, d-NADH:Q_1, d-NADH:Q_2, d-NADH:O_2, d-NADH:HAR and NADH:O_2 oxidoreductase activities in cytoplasmic membranes were assayed spectrophotometrically as described by us earlier [19]. Sensitivity of d-NADH:DB activity to piericidin A, stigmatellin, VNA and annonaceous acetogenins was tested as previously [25]. Curve fitting to a kinetic equation was performed with Sigmaplot 9.0 (Systat Software, Erkrath, Germany). Enzyme activities or inhibitor sensitivities of the mutants and control in the present study are measured from *in-trans* complemented strains if not otherwise specified.

To determine the inhibition type of piericidin A, VNA and stigmatellin, d-NADH:DB activity was assayed basically as described earlier. Initial velocities were measured with 52, 100 and 200 μM DB and reaction progress curves run with 25 μM or 41 μM initial DB concentration in the absence and presence of an inhibitor. 50 and 150 μM VNA, 9, 18 and 36 μM piericidin A or 5, 10, 20 and 33 μM stigmatellin were used to inhibit the NDH-1 activity, and control reactions without inhibitors were run in the presence of equal volume of ethanol used as a solvent for the inhibitors. Global fit of the data to the general equation $v = V_{max} \cdot [S]/\{K_m \cdot (1 + [i]/K_{ic}) + [S] \cdot (1 + [i]/K_{iu})\}$ for mixed inhibition [35] was performed for each of the inhibitors with non-linear regression in Microsoft Excel to solve the inhibitor constants for free enzyme (K_{ic}) and the enzyme–substrate complex (K_{iu}). Concentration of the ethanol stock solution of stigmatellin used for the analysis of its inhibition type was determined spectrophotometrically by measuring its absorbance at 270 nm in 50 mM potassium phosphate buffer, pH 7.4, and using an extinction coefficient 48.9 $mM^{-1}\,cm^{-1}$ calculated from the data presented by Gerencsér and co-workers [36].

2.5. Analytical methods

To investigate the assembly of NDH-1 in the E36Q mutant produced by genomic complementation, proteins from cytoplasmic membranes were solubilized with 0.2% n-dodecyl-β-D-maltoside in ε-aminocaproate Bis–Tris buffer as described earlier [37]. Subsequent Blue native PAGE (BN-PAGE) was performed in 3 to 12% Novex NativePAGE Bis–Tris 8 cm × 8 cm gel (Invitrogen) essentially according to Schägger [38], followed by staining with NADH and p-nitro blue tetrazolium [14].

2.6. Statistical methods

Student's *t* test for independent samples was used to compare two groups. For multiple comparisons of the statistical significance between different mutants or control, one-way ANOVA followed by the Dunnett's

test was used. Sequence similarities were evaluated with Clustal W [39] or the PredictProtein server (http://www.predictprotein.org/), the conservation scores with the Scorecons server (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl), and transmembrane topology by means of the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/) [40].

3. Results

3.1. NuoH effects of mutations corresponding to the LHON/MELAS overlap syndrome-associated m.3376G>A and m.3865A>G

The m.3376G > A and m.3865A > G transitions in human lead to p.E24K and p.I187V substitutions in ND1. Corresponding residues in the homologous NuoH subunit of *E. coli* are Glu-36 and Ile-201 (Fig. 1). Replacement of the evolutionally conserved residue Glu-36 with lysine in *E. coli* NuoH had a drastic effect on NDH-1 activity. The d-NADH:DB oxidoreductase activity (at 100 μM DB) in membranes from the E36K mutant was almost totally abolished (Table 2) and could not be enhanced by increasing the DB concentration (data not shown) preventing further analysis of the effects of this mutation on NDH-1 function. The mutation effect was similar when Q_2 or Q_1 was the substrate, although the latter showed slight increase of NDH-1 activity with increasing Q_1 concentration, but this was not different from the *nuoH* deletion strain (data not shown). Interestingly, the d-NADH:O_2 activity, which utilizes endogenous UQ, was more severely affected than the d-NADH:UQ reductase activity with short-chained DB, presenting 4% and 13% of control activities (all normalized for enzyme amount), respectively (Table 2). Also the d-NADH:HAR reductase activity was decreased, suggesting a decrease in the amount of fully assembled NDH-1 in cytoplasmic membranes (Fig. 2).

The I201V substitution, analogous to human p.I187V, had a small effect on the amount of NDH-1 in the membranes but the activity with DB or the apparent K_m for DB were not markedly decreased (Fig. 2 and Table 2).

3.2. Glu-36 residue of NuoH subunit in E. coli NDH-1 contributes to ubiquinone binding

Since the E36K mutant was devoid of NDH-1 activity, more conservative changes were tested by replacing Glu-36 with aspartate or glutamine. Replacement of the glutamate side chain with the amido group-bearing glutamine decreased the d-NADH:O_2 and d-NADH:DB activities to about 32% of controls when normalized for the amount of enzyme in the membrane (Table 2). Using Q_1 instead of DB showed a less severe loss of NDH-1 activity, the V_{max}/HAR reductase-ratio being about 74% of control (0.431 *vs* 0.581 in the mutant and control, respectively). Surprisingly, the E36Q mutant also showed decreased affinity to DB (Table 2), and the apparent K_m for Q_1 was almost 4-fold higher, being 151 μM in E36Q and 42 μM in the control. To evaluate this change in UQ interaction with NDH-1 in more detail, sensitivity towards various complex I inhibitors acting at or near the UQ binding site(s) was also tested for the E36Q mutant and control. Congruent with changes in apparent K_m for UQ, the E36Q mutant was found more resistant to piericidin-A, stigmatellin and annonaceous acetogenins but not to VNA (Table 3). These findings of the effects of the *in-trans* complemented E36Q mutant on inhibitor sensitivity and kinetic parameters were verified by introducing the E36Q mutation to the original position in *nuo*-operon. As with complementation *in trans*, the chromosomal mutation affected the apparent K_m for DB to similar extent (133 ± 4 μM in the E36Q strain and 38 ± 13 μM in the control strain). In the chromosomal E36Q mutant the I_{50} value for piericidin A was 16 μM and 13 μM in the control.

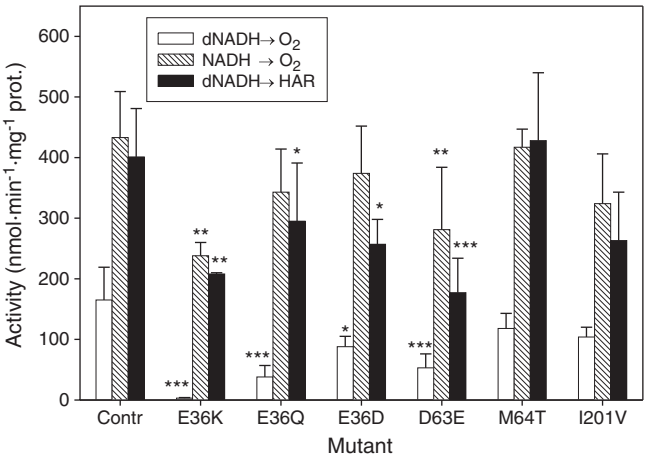

Fig. 2. Respiratory activities in NuoH mutants and control. HAR reductase activity is 13.0 ± 0.2 nmol min^{-1} mg^{-1} prot in the *nuo* operon deletion strain. The values are means ± S.D from two to nineteen independent membrane preparations. *p<0.05, **p<0.01, ***p<0.001.

In the case of the genomic E36Q mutant, the amount or activity of the assembled NDH-1 in the membranes was evaluated by means of BN-PAGE followed by NADH:nitro blue tetrazolium reductase activity staining using *nuo*-operon knock-out to confirm the identity of the observed NDH-1 band. The results were in accord with the d-NADH:HAR reductase activity (see Fig. 2), indicating that these shortcut activities migrate with the assembled enzyme (Fig. 3).

Replacement of glutamate-36 with the less bulky aspartate was better tolerated than the loss of the acidic group by the E36Q mutation. When normalized for the amount of the enzyme in the membranes, the d-NADH:DB activity in the E36D mutant was normal as well as was the DB binding affinity (Table 2). Nevertheless, despite the normal DB reductase moiety in E36D, its d-NADH:O_2 activity was slightly reduced (Table 2 and Fig. 2).

Table 2
Analysis of d-NADH:UQ oxidoreductase activity with DB (d-NADH → DB) and endogenous ubiquinone (d-NADH → O_2) as a UQ-substrate in control and NuoH-mutants E36K, E36Q, E36D, D63E, M64T and I201V.
Activity at 100 μM DB and V_{max} as well as d-NADH → O_2 activity were normalized to d-NADH → HAR activities to account for variation in expression level. Values are means ± S.D., numbers in parenthesis indicate the number of independent membrane preparations analyzed.

Mutation	d-NADH → DB at 90 μM d-NADH and 100 μM DB		K_m for DB	V_{max}/HAR reductase	d-NADH → O_2/HAR reductase-ratio
	nmol min^{-1} mg^{-1}	DB/HAR reductase-ratio	(μM)		
Control	184 ± 32 (14)	0.456 ± 0.053 (14)	38 ± 13 (12)	0.642 ± 0.042 (12)	0.398 ± 0.093 (14)
E36K	12 ± 2 (3)[a]	0.057 ± 0.013 (3)[a]	n.a.[d]	n.a.	0.014 ± 0.004 (3)[a]
E36Q	34 ± 10 (4)[a]	0.147 ± 0.035 (4)[a]	182 ± 97 (3) [a]	0.386 ± 0.087 (3)[a]	0.123 ± 0.036 (5)[a]
E36D	106 ± 22 (3)[b]	0.414 ± 0.084 (3)	38 ± 17 (3)	0.564 ± 0.077 (3)	0.341 ± 0.038 (3)
D63E	65 ± 29 (6)[a]	0.357 ± 0.051 (6)[b]	17 ± 6 (3)	0.429 ± 0.107 (3)[a]	0.292 ± 0.025 (4)[c]
M64T	166 ± 36 (5)	0.392 ± 0.032 (5)	43 ± 12 (5)	0.562 ± 0.072 (5)	0.281 ± 0.033 (5)[c]
I201V	124 ± 24 (2)	0.481 ± 0.054 (2)	29 ± 7 (5)	0.628 ± 0.016 (2)	0.407 ± 0.062 (2)

[a]p<0.001; [b]p<0.01; [c]p<0.05; [d]n.a., not applicable.

Table 3
Inhibitor sensitivity of control and NuoH-E36Q mutant NDH-1. I_{50}, the inhibitor concentration that produced 50% inhibition of the d-NADH:DB activity, was measured for each of the inhibitors at 100 μM DB. Values are means ± S.D, numbers in parenthesis indicate the number of independent membrane preparations analysed.

	I_{50}	
	Control	NuoH-E36Q
VNA (μM)	52 ± 10 (3)	34 ± 1 (2)
Acetogenins [a] (μg/ml)	0.51 ± 0.06 (2)	1.41 ± 0.22 (2)[b]
Piericidin A (μM)	8 ± 1 (2)	12 ± 0.05 (2)[b]
Stigmatellin (μM)	13 ± 2 (3)	44 ± 14 (3)[b]

[a] Annona squamosa extract.
[b] $p<0.05$.

3.3. Effects of a NuoH homolog of the m.3460G>A mutation associated with non-syndromic LHON

The amino acid residue affected by the LHON-associated m.3460G > A mutation (Ala-52 in ND1) is located within the same extramembrane loop as the one affected by the overlap syndrome mutation m.3376G > A, and although this residue is not conserved in *E. coli*, mutagenesis of the equivalent residue Met-64 in NuoH was included in the present work. The M64T mutation decreased the d-NADH:O_2 but not the d-NADH:DB reductase activity when normalized for the amount of NDH-1 in the membrane preparation (Table 2). However, there was some tendency towards reduced V_{max}/HAR reductase ratio (87% of control) in d-NADH:DB oxidoreductase activity, but this difference was not statistically significant (Table 2). Moreover, affinity to DB was not affected by the M64T mutation. Because it has previously been shown that the mitochondrial p.A52T mutation carrying complex I displays substrate inhibition by different UQ analogs [41], the NADH-ubiquinone reductase activity of M64T mutant *E. coli* NDH-1 was also tested for substrate inhibition. Titration of the d-NADH:DB reductase activity within the 0.5–400 μM DB concentration range did not, however, reveal any tendency for substrate inhibition by DB in the M64T mutant, nor was it observed in any other *E. coli* mutations listed in Table 1 (data not shown).

Although the effects of M64T substitution were mild, mutagenesis of the adjacent highly conserved Asp-63 residue had more drastic effects on NDH-1 activity. Replacement of this aspartate with a more bulky glutamate resulted in decreased d-NADH:HAR reductase activity (Fig. 2), indicative of a reduction in the amount of NDH-1 in the membranes. The HAR reductase-normalized V_{max} of d-NADH:DB reductase was only 78% of that in controls and the d-NADH:O_2 activity was similarly affected (Table 2). Because a homologous substitution in *Paracoccus denitrificans* results in substrate inhibition of the d-NADH:Q_2 activity by Q_2 [26] this was also tested in the D63E. Indeed, substrate inhibition by Q_2 at concentrations above 200 μM was observed in the *E. coli* D63E mutant, but the same phenomenon occurred also in controls (data not shown). However, some change in the UQ binding properties of D63E mutant enzyme did occur, as the apparent K_m for DB was only about half of that in the control (Table 2).

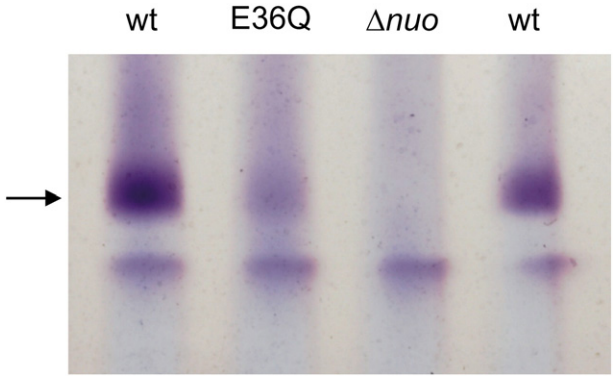

Fig. 3. Blue native PAGE of dodecyl maltoside-solubilized *Escherichia coli* membrane proteins decorated with NADH:nitro blue tetrazolium reductase staining. A *nuo* operon knockout strain was used to determine NDH-1 mobility in the gel. (wt) wild type, (E36Q) genomic NuoH-E36Q mutant, (*Δnuo*) *nuo* operon knockout. The arrow indicates the position of NDH-1-specific band. 2 μg protein was placed in each well.

3.4. Intactness of the respiratory chain downstream of NDH-1 in the NuoH mutants

Some of the mutants analyzed here displayed reduced NADH:O_2 activities (Fig. 2) alongside the changes described earlier. To test whether this was due to NDH-1 defect only or if there were defects in other parts of the respiratory chain, d-NADH:O_2 activities were subtracted from those with NADH as a substrate. This gives an estimate of the NDH-2 linked activity bypassing the NDH-1 coupling site. Fig. 2 shows that NDH-2 accounts for 62% of the total NADH oxidation in the controls and that the absolute contribution of NDH-2 to NADH respiration is not significantly affected by the mutations studied, demonstrating that the respiratory complexes on the oxygen side of NDH-1 were functioning normally.

3.5. Effect of added phospholipids on ubiquinone kinetics

Effects of added phospholipids on d-NADH:DB oxidoreductase were also tested, since in an earlier work [14] the apparent K_m for DB without phospholipids was lower by one order of magnitude than reported here, although no change in K_m for DB has been observed for isolated *E. coli* complex I when phospholipids are omitted from the assay [42]. Unexpectedly, plotting reaction velocity against substrate concentration during reaction progress in the absence of sonicated phospholipids showed that d-NADH:DB activity followed sigmoidal kinetics, which was probably due to inhibition of the enzyme by reduced DB, since changing the initial DB concentration resulted in a pattern suggestive of product inhibition (data not shown). Nevertheless, the apparent K_m value for DB in the absence of extra phospholipids (1.2 ± 1.0 μM, n = 2), was in the range observed previously by Sinha and co-workers [14], when calculated from the initial reaction velocities, validating that our membrane preparation method does not disturb the ubiquinone binding domain.

3.6. Type of inhibition by piericidin A, VNA and stigmatellin

Since the E36Q mutant showed decreased DB affinity and loss of inhibitor sensitivity, we also set out to test the type of inhibition for piericidin A, VNA and stigmatellin in our experimental conditions. As shown in Supplementary Fig. S1, all three showed a mixed (non-competitive) pattern of inhibition. The inhibitor constants (competitive/uncompetitive component) were 17/66, 118/92 and 30/40 μM for piericidin A, VNA and stigmatellin, respectively. Global fits to the inhibitor data are presented in a Supplementary Fig. S1.

4. Discussion

We used here *E. coli* as a model organism to investigate the biochemical effects of the respiratory chain complex I subunit ND1-affecting mutation m.3376G > A. The mutation is associated with the LHON/MELAS overlap syndrome, but is classified as probably pathogenic only [23,43]. In a patient case, mutation heteroplasmy for this mutation has been reported as 18%, 67% and 98% in blood, epithelium and muscle, respectively, and in the latter complex I activity was 36% of normal [23]. When we introduced the corresponding mutation into *E. coli* NDH-1, where it causes a NuoH-E36K replacement homologous to the mitochondrial p.E24K substitution, it led to a 93% loss of enzyme activity. However, it must be borne in mind that the bacterium was

homoplasmic for the mutation and sequence dissimilarity might render the bacterium more sensitive to it. The NuoH-E36 residue modification was also found to affect UQ and inhibitor binding by NDH-1. The results presented here about the biochemical consequences of the glu-36 mutations in *E. coli* strongly argue for the pathogenicity of the m.3376G > A mutation. Moreover, the *E. coli* equivalent of the mitochondrial 3865A > G mutation had less severe effects than the substitution corresponding to m.3376G > A. An m.3866T > C polymorphism has been previously found in a patient with complex I deficiency, and it affects the same amino acid (isoleucine-187) as m.3865A > G but causes a less conservative substitution of isoleucine with threonine. Modeling in *E. coli* has shown that its biochemical consequences are only mild [44], which is in accord with the findings with the adjacent m.3865A > G mutation in the present study. Therefore, it can be concluded that m.3376G > A contributes to the disease development more than m.3865A > G, as suggested previously [23].

The NuoH mutations analyzed in the present study provide important evidence of the UQ binding domain of complex I. Mutations in *E. coli* NuoH-E36 decreased the apparent affinity of the enzyme to ubiquinone and to partially competitive type-A inhibitors and also induce resistance to stigmatellin, an inhibitor of a less competitive class. In *Y. lipolytica*, mutations in 49kD-Y144 cause similar effects [8]. This suggests that the aforementioned amino acid residues in two different subunits are located in the same functional ubiquinone binding domain of the enzyme. Contrary to the *Y. lipolytica* 49kD-Y144 mutations, in the present study the *E. coli* NuoH-E36Q mutation affects the kinetics of all tested UQ species. It also affects UQ and inhibitor binding to similar extent. Although an amino acid with a free carboxyl group seems important at position 36 in NuoH, the interaction of E36 with UQ appears not to be strictly spatially confined, as the shorter side-chained aspartate in this position was functional in terms of enzyme activity and affinity for short-chained DB.

Recent work by Sinha and coworkers [14] demonstrates that many conserved residues within the matrix-side loops of ND1 are essential for the attachment of the peripheral arm to the membrane domain of complex I, but no significant change in UQ-binding was observed in any of their mutants. One difference between that study and ours is that we routinely use externally added sonicated phospholipids in our kinetic assays, a procedure that was found advantageous in our previous experimentation on *P. denitrificans* membranes to enhance reproducibility [26,45]. Phospholipids are known to stabilize and activate isolated complex I of both mammalian and bacterial origin [42,46–48]. They can also diminish the potentially confounding effect of different amount of native lipid in activity assays. For these reasons, we used phospholipids in kinetic assays although they were performed on membrane-attached NDH-1. Added phospholipids affected the apparent K_m for DB, but their use in the assay mixture seemed to be indispensible since they diminished the product inhibition characteristics presented by the d-NADH:DB activity progress curves. The phospholipid effect is most likely due to a solvent effect, which increases the distribution volume of quinone/quinol, thereby resulting in an increase in the apparent K_m. Added phospholipids may also attenuate the product inhibition by facilitating the exchange of quinone and quinol at the active site. One should also note that we interpret the present experiments by comparing the effects of mutations, so that the dilution effect of phospholipids cancels out.

Although being somewhat different from data presented by Sinha et al. [14], our findings are consistent with other observations pointing to the involvement of the first matrix side loop of ND1 in ubiquinone binding. In a patient displaying complex I deficiency an m.3460G > A mutation leading to an A52T replacement in the same extramembranous loop as the m.3376G > A transition has been found. It causes rotenone insensitivity [49] and affects UQ interaction with enzyme in human cell lines [41] and also when modeled in *P. denitrificans* [26]. An *E. coli* mutation corresponding to the m.3460G > A substitution was also generated in our present work. The *E. coli* equivalent, namely M64T, caused only a slight but not statistically significant reduction of NDH-1 activity. As in *P. denitrificans* [26], effects of the adjacent D63E mutation in *E. coli* were found more deleterious than those of M64T. These unexpected consequences of the m.3460G > A mutation in *E. coli* may be due to differences between the mammalian and bacterial complex I [45]. Moreover, the position corresponding to human Ala-52 is occupied by a larger methionine residue in the *E. coli* NuoH subunit, and there is some variation between species, i.e. the site involved is not fully conserved (Fig. 1) in contrast to the neighboring aspartate.

We show here in *E. coli* that a mutation equivalent to the m.3376G > A substitution associated with LHON/MELAS overlap syndrome produces a biochemical defect of similar magnitude to that commonly observed with MELAS mutations. Three-to-four-fold increased apparent K_m for ubiquinones in the NuoH-E36Q mutant and resistance to the type A inhibitors piericidin A and annonins, which bind at or near the UQ binding domain [2,50], suggest that also NuoH-E36K interferes with UQ binding. Piericidin A, VNA and stigmatellin, were found to be mixed type of inhibitors, which is in accord to the view that the domain responsible for substrate and inhibitor-binding is relatively spacious.

It thus appears as if a single LHON/MELAS overlap mutation is able to disturb UQ interaction as found in LHON and strongly decreases the catalytic capacity as observed in MELAS, so that the resulting bacterial phenotype represents an overlap syndrome. If this reflects *in vivo* situation in patients, these two diseases should be considered to arise through different pathogenic mechanisms, although they affect the same enzyme. This view is strengthened by the facts that LHON-like optic neuropathy is less frequently observed in the conjunction of the more severe mutations in terms of enzyme activity decrease, MELAS and Leigh's disease, and that the disease phenotypes associated with complex I affections are indeed linked to specific mutations [51,52].

In conclusion, pathogenicity of the LHON/MELAS overlap syndrome-associated m.3376G > A replacement is corroborated by homologous mutagenesis of *E. coli* NuoH. However, the bacterial counterpart of the m.3865A > G transition, which targets a putative transmembrane helix in the same subunit and has been observed to occur concomitantly with the m.3376G > A substitution, was without a major effect. We propose that residue Glu-36 in the first matrix side loop of NuoH subunit is involved in quinone binding by NDH-1, since its replacement by glutamine lead to decreased UQ binding affinity and inhibitor resistance.

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Appendix A. Supplementary data

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