The mitochondrial-encoded subunits of respiratory complex I (NADH:ubiquinone oxidoreductase): identifying residues important in mechanism and disease

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Abstract
Complex I (NADH:ubiquinone oxidoreductase) is crucial to respiration in many aerobic organisms. The hydrophilic domain of complex I, containing nine or more redox cofactors, and comprising seven conserved core subunits, protrudes into the mitochondrial matrix or bacterial cytoplasm. The α-helical membrane-bound hydrophobic domain contains a further seven core subunits that are mitochondrial-encoded in eukaryotes and named the ND subunits (ND1–ND6 and ND4L). Complex I couples the oxidation of NADH in the hydrophilic domain to ubiquinone reduction and proton translocation in the hydrophobic domain. Although the mechanisms of NADH oxidation and intramolecular electron transfer are increasingly well understood, the mechanisms of ubiquinone reduction and proton translocation remain only poorly defined. Recently, an α-helical model of the hydrophobic domain of bacterial complex I [Efremov, Baradaran and Sazanov (2010) Nature 465, 441–447] revealed how the 63 transmembrane helices of the seven core subunits are arranged, and thus laid a foundation for the interpretation of functional data and the formulation of mechanistic proposals. In the present paper, we aim to correlate information from sequence analyses, site-directed mutagenesis studies and mutations that have been linked to human diseases, with information from the recent structural model. Thus we aim to identify and discuss residues in the ND subunits of mammalian complex I which are important in catalysis and for maintaining the enzyme’s structural and functional integrity.

The membrane-bound domain of complex I
Complex I (NADH:ubiquinone oxidoreductase) is a complicated multisubunit enzyme found in the mitochondrial inner membrane of many eukaryotes and in the cytoplasmic membrane of many aerobic bacteria. It couples the transfer of two electrons, from NADH to ubiquinone, to the translocation of four protons across the membrane. NADH is oxidized by an FMN in the hydrophilic domain of the enzyme (that protrudes into the mitochondrial matrix or bacterial cytoplasm), then the electrons are transferred along a series of iron–sulfur clusters to the highly hydrophobic ubiquinone acceptor, bound in, or close to, the membrane domain [1–3]. Structural models for the hydrophilic domain of complex I from Thermus thermophilus, containing the flavin and FeS clusters, have been determined [4], and the electron-transfer reactions within this domain are increasingly well understood [1]; conversely, the mechanisms of ubiquinone reduction and proton translocation remain only poorly understood.

Complex I from Bos taurus is closely related to the human enzyme, and is known to contain 45 different subunits [5]. A total of 14 core subunits are conserved in all complexes I; the additional supernumerary subunits are generally specific to eukaryotes and vary between species [6]. There are seven core subunits in the hydrophilic domain, and seven in the hydrophobic domain. The hydrophobic core subunits (named ND1–ND6 and 4L) are encoded by the mitochondrial genome in mammals [7] and most eukaryotes (Chlamydomonas reinhardtii is an exception that encodes ND3 and ND4L in its nuclear genome [8]). Recently, new structural information about the membrane domains of the complexes I from Escherichia coli, T. thermophilus [2] and Yarrowia lipolytica [3] (see Figure 1) has been described, forming a foundation for the interpretation of functional data and the formulation of mechanistic proposals for ubiquinone reduction and proton translocation. The present review focuses on the seven mitochondrial-encoded subunits in mammalian complex I, aiming to relate information from sequence analyses, site-directed mutagenesis and disease-linked mutations to recent structural data.
The arrangement of transmembrane helices in the membrane domain of complex I from *T. thermophilus*

The subunits are ND1 (magenta, eight helices), ND2 (green, 14 helices), ND4 (blue, 14 helices), ND5 (red, 16 transmembrane helices plus lateral helix), ND3+ND6+ND4L (grey, 11 helices). In the lower panel, the hydrophilic domain (yellow) is protruding from the page and the molecule is viewed from above the membrane, showing that many of the helices are tilted. In the upper panel, the molecule has been rotated to display more clearly the structural similarity of ND2, ND4 and ND5. Taken from PDB code 3I9V [2].

**Correlations between structurally observed transmembrane helices and the protein sequences**

Figure 1 shows the currently available α-helical model of the membrane domain of complex I from *T. thermophilus* [2]. There are two striking features: the structural symmetry between three groups of 14 helices that comprise the distal section of the domain, and the lateral (transverse) helix that runs in the plane of the membrane, suggestive of a mechanical linkage. The three 14-helix units are assigned to subunits ND2, ND4 and ND5, which are all related to one another, and to proteins from the Mrp Na⁺/H⁺ antiporter family [9]. Two ‘broken’ helices, indicative of proton-translocation loci, were observed in each of the three subunits; in each subunit, one of them contacts the lateral helix. Subunit ND5 is the terminal subunit; it has a C-terminal sequence extension, relative to ND2 and ND4, consistent with two additional helices and the lateral helix. Subunits ND4 and ND5 form the core of subcomplex Iβ from *B. taurus* complex I [6], and can be resolved together from *E. coli* complex I [10], so subunit ND4 is the central subunit, and ND2 is closest to the hydrophilic arm. The eight helices that comprise subunit ND1 were identified by comparing the data from *T. thermophilus* complex I (Figure 1) with data from the membrane domain of *E. coli* complex I lacking ND1 [2]. The remaining 11 helices in Figure 1 cannot be assigned to specific subunits at present; they are formed together by subunits ND3, ND6 and ND4L. The hydrophilic domain interacts predominantly with ND1, but also with the ND3, ND6 and ND4L bundle; the binding site for the quinone headgroup is probably located close to the interfacial region between the domains.

Figure 2 summarizes information from our analyses of the sequences of the seven ND subunits, based on the sequences of the human subunits. An alignment was generated for each subunit, using the sequences of 40 organisms chosen to represent all parts of the complex I-encoding phylogenetic tree. Then, the positions of the transmembrane helices in each individual sequence were predicted, and the consensuses shown in Figure 2 were generated by counting the number of sequences for which a helix was predicted at each position (see [11] for details). There is a clear consensus for eight helices in ND1, consistent with previous proposals [12–14] and with the structural analysis described above; the long loop region between helices I and II is thought to be on the cytoplasmic (matrix) side [13]. A total of 11 helices are predicted clearly for ND2; the lack of the three N-terminal helices in bilaterian complexes I (including mammals), relative to the 14-helix subunit in fungi, bacteria...
Figure 2 | Summary of information about the sequences of the human ND subunits from sequence alignments

![Diagram of the sequences of human ND subunits from sequence alignments.](image-url)
and lower eukaryotes (consistent with the structural data and as proposed previously [11]). The 14-helix homologue is thought to begin on the periplasmic side [15], so, assuming that the orientation is conserved, the 11-helix homologue begins on the cytoplasmic (matrix) side. The truncation may represent a loss or change in function for ND2 in the bilaterian complex, or it may suggest that the three N-terminal helices of ND2 in lower organisms (and, by extension, the corresponding helices in ND4 and ND5) are not essential for function. Alternatively, the truncation of ND2 may have facilitated its recruitment to another role in the cell; ND2 has been shown to form a complex with Src kinase and the NDMA (N-methyl-D-aspartate) receptor at synaptic membranes in the brain, a function that requires its export from the mitochondrion [16]. There are 14 helices predicted for ND4, consistent with the structural data; previously, 14 helices were proposed, starting on the periplasmic side, but it was not clear whether helices XI and XII were transmembrane helices or not [9,17].

A total of 16 transmembrane helices can be identified for ND5, again consistent with the structural data and previous proposals, and starting on the periplasmic side [9,18]; the lateral helix between transmembrane helices XV and XVI is an unusual feature that is not expected to be analysed meaningfully by transmembrane helix predictions. The three remaining subunits, ND3, ND4L and ND6, are the smallest subunits, and they are predicted to have three, three and five transmembrane helices respectively, consistent with previous proposals [19–25] and with the 11 helices observed in the structural analysis (the predicted topologies are marked on Figure 2).

Sequence conservation in the ND subunits

The ND subunits from very few species have been characterized directly (they are difficult to identify and characterize by MS owing to their hydrophobicity and lack of protease cleavage sites [26]), so the majority of the sequences compared in Figure 2 are translations of genome sequences: they rely on identification of the relevant stretches of sequence by homology searches, use of the appropriate translation code and (particularly in fungi and plants) the correct identification of introns [27]. The sequence alignments reveal a general trend for shorter sequences in the higher eukaryotes; the insertions in the sequences of the lower organisms are not included in Figure 2, but they lend flexibility to the alignments (aiding ‘identification’ of conserved residues, particularly in loop regions). With this in mind, the conserved residues marked on Figure 2 are those that are identical in at least 38 of the 40 sequences analysed.

Subunit ND1 contains 37 conserved residues (11.6%), located predominantly in the loop regions on the cytoplasmic (matrix) side; these loops are probably important in forming contacts with the hydrophilic domain. There are conserved residues in the interfacial regions also, but relatively few in the helical regions; of these, only one is charged (Glu227 in helix VI). There are only 12 conserved residues (3.5%) in subunit ND2; most notable are Glu14 in helix II and Lys129 in helix IV. Subunits ND4 and ND5 contain 24 and 54 conserved residues respectively (5.1% and 9.0%); the lack of conservation in the first three helices (homologous with the three helices absent from ND2) is notable, and consistent with the suggestion that these helices are not essential functionally. In contrast with ND1, most of the conserved residues in ND2, ND4 and ND5 fall within helices, supporting their proposed roles in proton translocation. Two conserved charged residues in ND4 and ND5, Glu123 and Glu145 in helix V (Glu145 is not marked as conserved on Figure 2, but there is always an acidic residue (glutamate or aspartate) at this position, and Lys223 in helix VII, are equivalent to the two conserved residues in helices II and IV in ND2, and they are conserved also in MrpA and MrpD of the Mrp Na+ /H+ antiporters [9]. It is possible that these residues produce the two broken helices observed in the structural analysis. There are further conserved charged residues in the helical regions in ND2 (Lys135 in helix V), ND4 (Lys137 in helix VIII, analogous to Lys135 in ND2) and ND5 (Lys336 in helix XI). Interestingly, although there are several highly conserved regions in ND5 (notably helices V, VIII, X and XI, and the loop between VII and VIII), there is no apparent conservation within the lateral helix, or in helices XV or XVI. Subunits ND3 and ND4L are poorly conserved [four and two conserved residues (3.5% and 2.1%) respectively], but there are two charged conserved residues in helices in each subunit, suggestive of possible roles in proton translocation: Asp66 and Glu68 are conserved in helix II in ND3 (plus Glu30 in the periplasmic loop between helices I and II) and Glu14 and Glu120 are conserved in helices II and III in ND4L. There are no conserved residues in subunit ND6.
Studies of site-directed mutations in bacterial model systems

The difficulty of genetically manipulating the mitochondrial DNA in eukaryotic systems means that mutagenesis studies on the ND subunits are usually carried out in bacterial model systems: often in *E. coli*, but also in *Paracoccus denitrificans* and *Rhodobacter capsulatus*. Figure 2 indicates those residues for which at least one mutation that decreases the catalytic activity to less than 50% of the wild-type value has been studied. Here, we refer to the NADH:quinone (or NADH:O₂) oxidoreduction rate, normalized for the amount of complex I present, as the ‘catalytic activity’ or ‘activity’. Typically, the amount of complex I is quantified via the rate of NADH oxidation (catalysed in the hydrophilic domain) induced by a hydrophilic artificial electron acceptor such as K₃[Fe(CN)₆]. Only residues which are the same in humans and in the model system are included in Figure 2, and conserved residues for which all the mutants studied so far have relative catalytic activities above 50% are marked with crosses. In the following sections, residue numbers are given for the human sequences, with the bacterial positions in parentheses for cross-referencing.

Several studies have described mutations of residues in the bacterial homologues of subunit ND1 [14,28,29]. Recently, Sinha and coworkers identified a set of residues (Arg²⁵ (37), Arg³⁴ (46), Asp⁵¹ (63), Gly¹²⁰ (134), Gly¹³¹ (145), Arg¹³⁴ (148), Glu²⁵⁶ (220) and Glu²¹⁴ (228)) [14] that, when mutated, disrupt the structural integrity of the enzyme: the enzymes exhibit low NADH:ferricyanide oxidoreduction activities and are not assembled fully. These residues (marked by open green triangles in Figure 2) are located in the cytoplasmic (matrix) loops of ND1, supporting their proposed importance for the connection with the hydrophilic domain. Further mutations in ND1 that compromise catalysis are shown on Figure 2: they are also located in the cytoplasmic loops, and it has been suggested that some of them affect quinone binding [28]. No significant effect on catalysis was observed from mutating Glu²⁵⁶ in helix VI [14].

Mutations of the two charged residues that are conserved throughout ND2 (Glu¹⁴ in helix II, and Lys¹⁰⁵ in helix IV), ND4 and ND5 (Glu¹²³ and Glu¹⁴⁵ in helix V, and Lys²⁰⁶ and Lys²³⁵ in helix VII) have been studied. In ND2 [15], there was little apparent effect from mutating Glu¹⁴ (133), and the origin of the effect of mutating Lys¹⁰⁵ (217) is unclear because the complex I was not quantified. In ND4, the E123A (144) and E123Q mutations (but not the E123D mutation), and the K206A (234) and K206R mutations, essentially abolished the activity [30,31]. In ND5, the E145A (144) and E145Q variants were ~20% active, and the K223A (229), K223R and K223E variants decreased the activity to 10–25% [32]. Thus these studies are generally consistent with important roles for these residues in catalysis, although an unambiguous picture has yet to emerge. For the conserved lysine residue in ND2 and ND4 (Lys¹⁰⁵ in helix V and Lys²³⁵ in helix VIII) the K135C (247) mutant resulted in very low levels of complex I, and the K135R mutant retained ~80% activity (the complex I was not quantified) [15]; the K237A (237) mutation decreased the activity moderately, to 30–60% [30,31]. These results suggest a secondary role for this residue, perhaps in maintaining structural integrity. Further residues for which mutations decreased the normalized catalytic activity significantly, and conserved residues for which they did not, are indicated in Figure 2 [30–32]. So far, no mutations in the lateral helix of ND5 or the two helices adjacent to it have been studied.

In subunit ND3, the E38A (51), D66A (79), E68Q (81) and E68A mutations retained between 30 and 60% activity, whereas D66N had little effect, and the double mutant D66N/E68Q abolished the activity [20]. In subunit ND4L, the E34A and E34Q (36) mutations abolished the activity, whereas the E70Q (72), E70A, R23A (25) and R23K mutations retained 25–45% activity [22,23]. In subunit ND6, the Y59F (59), V65G (65) and V65L mutations decreased the catalytic activity to between 10 and 45% [25,33]. The results clearly suggest an important role for Glu¹⁴ in ND4L, but, as with many of the mutations described in ND2, ND4 and ND5, the moderate decreases in activity associated with the other mutations are hard to interpret.

Clearly, there are several factors to consider when evaluating studies of site-directed mutations in bacterial complex I model systems.

(i) Mutations may disrupt the assembly or structural integrity of the enzyme. It is common practice to assess the amount of complex I present by measuring the NADH dehydrogenase activity catalysed by the hydrophilic domain, and by using immunodetection to determine whether the full subunit complement is present. Mutations which disrupt the structural integrity may highlight regions that form important structural contacts between domains (as proposed for ND1), but they may also arise from less pertinent effects, such as steric clashes or hydrophobic mismatches. Furthermore, the presence of a subunit in a membrane preparation does not demonstrate that it is properly folded or incorporated into the complex, an issue that can perhaps be addressed by enzyme purification.

(ii) How significant does a decrease in the catalytic activity need to be to define a catalytically ‘critical’ residue (rather than a ‘secondary’ residue that is required to, for example, maintain a structural arrangement or interaction)? Perhaps a cut-off value of 10% activity is reasonable, but then should every mutation of the given residue attain this cut-off? Of course, inappropriate replacements may introduce negative effects on catalysis, rather than remove positive ones. For example, the E123A (144) and E123Q mutations in ND4 essentially abolished the activity, suggesting that the carboxylate functionality is critical [30,31]. On the other hand, the E123D variant retained full activity, and moving the carboxylate residue up or down the helix partially restored the activity [17]. Thus there is considerable flexibility in the position of the carboxylate headgroup, rather suggesting that Glu¹²³ is unlikely to function as a precisely positioned gating residue in proton transfer.
(iii) Equivalent mutations may behave differently in different systems, because of interactions with non-conserved residues. The nuclear-encoded supernumerary MWFE subunit, which is not well conserved and that has no primary role in catalysis, provides a pertinent example. When several residues were mutated to their human counterparts in CHO (Chinese-hamster ovary) cells, the enzyme activity was reduced; furthermore, a single, conservative point mutation (arginine to lysine) produced a catalytically inactive enzyme [34]. These results illustrate both how the same residues can exert very different effects in even closely related systems, dictated by the ‘background’ of the nuclear and/or mitochondrial protein sequences of the other subunits, and how an apparently innocuous change can produce such significant (and potentially misleading) results.

(iv) There is a clear requirement for methods to distinguish which step in the catalytic mechanism of complex I is affected by a mutation [1]. The reactions of the flavin can be studied in isolation, and mutations which affect substrate binding may be tentatively assigned by identifying changes in $K_m$, but otherwise (unless the mutation uncouples the enzyme), all the steps are tightly coupled: inhibiting any one of them inhibits them all. In particular, measurements of proton translocation, often done by using dyes such as oxanol and Acridine Orange, are affected by many different experimental variables and (in our opinion) are not yet sufficiently well developed or understood to provide definitive information.

**Mutations implicated as the causes of human diseases**

Mutations in the mitochondrial-encoded subunits of complex I are increasingly implicated in a variety of diseases, including neurodegenerative diseases, optic neuropathies and diabetes [35]. In some cases, for example the 3460 mutation in ND1 and the 11778 mutation in ND4 which cause Leber’s hereditary optic neuropathy, the link between mutation and disease is well established, but in many other cases, distinguishing a cause-and-effect relationship from a polymorphism is difficult. Many patients present multiple deviations from the reference mitochondrial genome sequence, and the relationship is complicated further by variable levels of heteroplasmy. We applied the scoring system of Turnbull and co-workers [35] to the list of disease-related mutations in the MitoMap online database [36]. We discuss mutations which scored above 20 (out of 40) in this evaluation, which uses biochemical deficiencies, number of independent reports, heteroplasmy, matrilineal variant segregation and conservation as its criteria. Turnbull and co-workers analysed the 50 mutations listed in MitoMap in 2006 and found that 16 were very likely to be pathogenic, three probably, ten possibly and the rest almost certainly not [35]: in the present review, we consider these 16 mutations, plus further mutations identified from recent literature. The mutations discussed are summarized in Table 1 and indicated on Figure 2.

First, the majority of the disease-associated residues in Table 1 are not highly conserved; those that are conserved are in ND1 and ND5 only. Thus, although conservation is often taken as an indicator of relevance, it is certainly not a strong determinant. Indeed, mutations of critical catalytic residues that abolish activity may be less likely to present as diseases, and mutations which compromise the activity may be tolerated more easily when the mutation is heteroplasmic. Secondly, the disease-associated residues are distributed throughout the helix and loop regions; in ND1 and ND3 the majority are in the loop regions, whereas in ND5 and ND6, the majority are in the helical regions. Thirdly, equivalent mutations have been made in bacterial model systems for only nine of the mutations listed in Table 1. For ND1-A52T, alanine to methionine and threonine mutations decreased the activity only slightly (80–90%), but they may affect quinone binding [28]; for ND1-E59K, a glutamine to alanine mutation resulted in a decreased level of complex I, and a moderately decreased activity (~60%) [14]; for ND1-G131S, a glycine to valine mutation had a significant effect (decreased enzyme content and very low activity), but a glycine to alanine substitution had less effect (~50% activity) [14]; for ND1-E214A, the direct substitution caused a significant decrease in both enzyme content and catalytic activity, with similarly large effects from other substitutions [14,29]; the direct ND1-Y215H mutation had no apparent effect on the enzyme activity [29]. For ND4, neither the R340H or R340A substitutions had significant effects on catalysis in *R. capsulatus* (although *E. coli* R340H was only ~70% active), but cell growth and metabolism were affected [30,37]. For ND5-D393N/G, substitutions with asparagine, glutamate or alanine produced variable, but limited, changes in enzyme activity (~>80%) [32]. For ND6-A72V, the methionine to valine, alanine, cysteine and isoleucine substitutions had variable effects (the most significant decrease was for M72V, ~40%), but the starting residue in the bacterial system is different [33]; for ND6-M64V, the methionine to isoleucine or valine substitutions had no, or limited, effects [25,33]. Thus in only one case (ND1-E214A) is the direct disease-linked mutation in the bacterial system known to cause a severe defect; for ND1-Gly131, severe effects were observed, but for a different substitution. It is clear that the value of these studies for understanding the mutations in human complex I is compromised unless the same starting and ending residues are applied. Even then, the backgrounds for the mutations differ significantly between the human enzyme and the bacterial homologues, so that observed effects, which may be both very subtle and depend on interactions with other nuclear and mitochondrial-encoded proteins, should not be overinterpreted. It is also important to note that effects that may be considered insignificant in mechanistic studies (for example, retention of 80% of the activity) may be very significant in a physiological context. Finally, simple catalytic rate measurements may not reveal important defects (for example, an enzyme may be compromised only in its ability to work against a substantial protonmotive force, or under certain physiological conditions), underlining
Table 1 | Mutations in the mitochondrial-encoded subunits of complex I that are associated with human disease phenotypes

The human nucleotide and amino acid substitutions for the disease-related mutations are given, along with an example of a disease associated with the mutation. Only mutations which scored at least 20 out of 40 using the evaluation method of Mitchell et al. [35], are included, and one reference for each mutation was chosen from the MitoMap database [36]. The Table is not a complete survey of all disease-associated mutations, but provides a framework for the discussion of point mutations which are likely to have an effect on the enzyme. LHON, Leber’s hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke.

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the requirement for developing improved analytical and mechanistic approaches.

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