FEBS Letters 352 (1994) 375–379

FEBS 14584

Functional alterations of the mitochondrially encoded ND4 subunit associated with Leber's hereditary optic neuropathy

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Received 22 July 1994

Abstract Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease associated with point mutations in mitochondrial DNA. The most frequent of these mutations is the G-to-A substitution at nucleotide position 11,778 which changes an evolutionarily conserved arginine with a histidine at position 340 in subunit ND4 of NADH:ubiquinone reductase (respiratory complex I). We report that this amino acid substitution alters the affinity of complex I for the ubiquinone substrate and induces resistance towards its potent inhibitor rotenone in mitochondria of LHON patients. Such changes could reflect a substantial loss in the energy conserving function of NADH:ubiquinone reductase and thus explain the pathological effect of the ND4/11,778 mutation.

Key words: LHON; Mitochondrial DNA; Ubiquinone; NADH:ubiquinone reductase

1. Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease which is clinically characterized by the sudden loss of central vision and affects prevalently young males [1]. To date, several point mutations in mitochondrial DNA (mtDNA) have been associated with LHON [1–3]. The most frequent of these mutations [1–3] is the substitution at nucleotide position (np) 11,778 in the ND4 subunit of complex I [4–6]. The functional defect that arises from the amino acid substitution ($Arg^{340} \rightarrow His$ [4]) caused by this mutation is not clear yet [4,6–8], even if a recent report has shown a 25% decrease in complex I activity measured with a short analogue of ubiquinone [9]. In contrast, the LHON mutation ND1/3460 has been shown to induce a strong decrease in the activity of complex I [7,9,10].

We report here that the ND4/11,778 mutation induces resistance to rotenone, which is the classical potent inhibitor of complex I [11–13], and alters the reaction of the complex with the ubiquinone substrate. The alteration in both rotenone potency and ubiquinone affinity indicate that the major functional defect in patients with this LHON mutation is at the energy conserving site of complex I.

2. Materials and methods

Platelet mitochondria were isolated from several unrelated control individuals and three affected members (Fig. 1A) of a large LHON family from Northern Italy [6]. The platelets were purified from 100 ml of venous blood [14], and mitochondrial particles were prepared by sonication in 0.1 M Tris-HCl, pH 7.4, containing 1 mM EDTA. After centrifugation at 10,000 rpm for 10 min in an Eppendorf microcentrifuge, the supernatant was diluted threefold with 0.25 M sucrose, 50 mM Tricine-HCl, pH 7.6, containing 30 mM KCl, 5 mM $MgCl_2$ and 0.5% bovine serum albumin, and centrifuged at $100,000 \times g$ to precipitate mitochondrial particles, which were resuspended in the same sucrose-Tricine buffer at 6–12 mg/ml of protein, measured by the Lowry method. The preparation was immediately used in the biochemical assays.

The NADH:quinone reductase activity was measured at 37°C with 150 μM NADH and 30 μM 2,3-dimethoxy-5-methyl-6-*n*-undecyl-*p*-benzoquinone (UBQ [15], a generous gift of E. Berry, University of California at Berkeley, USA) or ubiquinone-2 (Q_2, a gift from Eisai Co., Tokyo, Japan) as detailed recently [16]. The activities of ubiquinol-2:cytchrome *c* reductase and NADH:ferricyanide reductase were assayed at 30°C in the same buffer used for the NADH:quinone reductase assay (50 mM K-phosphate, pH 7.6, containing 10 mM KCN and 1 mM EDTA) as described in [16]. The inhibitory effect of rotenone was assayed by incremental amounts of a concentrated solution to the mitochondrial suspension as in [16]. The potency of rotenone and other inhibitors was expressed as the final concentration of inhibitor in the assay that yielded 50% inhibition of NADH oxidation by UBQ (I_{50}).

mtDNA was extracted from the same platelet samples utilized to prepare the mitochondrial particles by the standard phenol/chloroform method [6]. A 119 bp mtDNA fragment encompassing the np 11,778 site was amplified by PCR using primers L11720–11740 and H11820–11839 [4,6,17]. The presence of the 11778 G-to-A transition [4] was detected with 100% specificity by the combined use of the restriction enzymes *Sfa*NI (loss of a restriction site) and *Mae*III (creation of a new restriction site) [17]. mtDNA fragments were separated by 3% Nu-Sieve and 0.5% agarose gels (Figs 1B and C).

The analysis of the deduced amino acid sequences of the ND4 subunit has been carried out with the methods utilized previously for cytochrome *b* [18,19]. 34 complete sequences from mitochondrial, plastidial and bacterial species were taken from recent versions of the EMBL data banks and aligned as in [19]. The average hydropathy and amphipathy profile of the aligned sequences confirmed the analysis in [20] except that the region corresponding to the previously proposed helix H [20] was not predicted to be transmembranous.

The consensus sequences of the ND4 subunit and cytochrome *b* were obtained by analyzing 20 mitochondrial sequences from the same set of species plus that of *E. coli* for ND4 and that of *R. rubrum* for cytochrome *b*. Sequence similarity with a database of quinone-reacting peptides was evaluated as described previously [18]. The complete alignments are available from the Authors.

3. Results and discussion

3.1. Complex I of LHON patients is resistant to rotenone

The biochemical defect in complex I consequent on the ND4/11778 mutation was determined by the specific activity of NADH:ubiquinone (Q) reductase in platelet mito-

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SSDI 0014-5793(94)00971-6

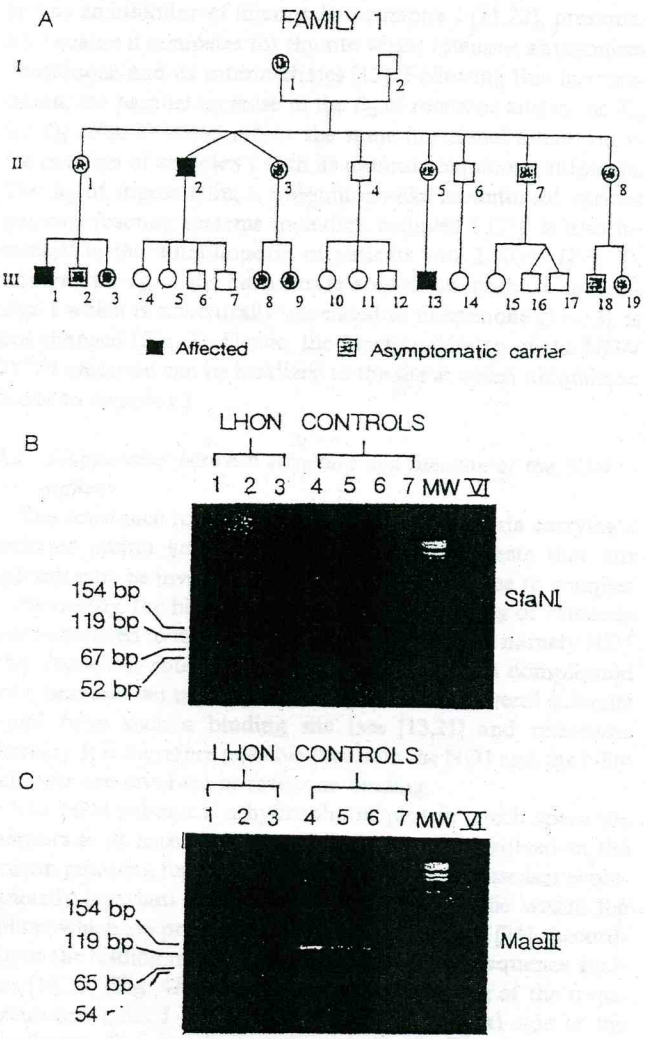

Fig. 1. (A) Pedigree of the Italian LHON family [6] investigated. The symbol * marks the three affected individuals studied. Twelve asymptomatic family members, all along the matrilinear linage, also harbor the ND4/11778 mutation, while four children of an affected male, as expected, lack the mutation. Molecular investigation has not been performed on individuals II-4 and II-6. (B and C) Molecular confirmation of the virtually homoplasmic ND4/11778 mutation in all three affected individuals. The amplified fragment of 119 bp remains uncut with *Sfa*NI (site loss) and is cut with *Mae*III (new site creation) in LHON individuals. Note that *Sfa*NI and *Mae*III cut, respectively, wild-type (controls) and mutated (LHON) mtDNA in two different sites separated by 13 nucleotides.

chondrial particles from the affected members of an Italian family carrying this mutation [4] (Fig. 1A). The mtDNA of the three individuals with LHON investigated was virtually homoplasmic for the ND4/11778 mutation (Fig. 1B and C) and no other known LHON mutation has been detected in the mtDNA of this family (data not shown).

The function of complex I has been measured with the undecyl analog of ubiquinone [15] which, contrary to other quinones [16], elicits a reaction that is fully sensitive to rotenone (Fig. 2), the classical potent inhibitor of complex I that interacts as a ubiquinone antagonist [11–13]. Previous studies on mitochondria isolated from patients having the ND4/11778 mutation found a substantial decrease in respiratory rates of NAD-linked substrates such as glutamate, but showed little [7,8] or only 25% [9] decrease in the NADH:Q reductase activity of complex I. We also did not observe significant changes of complex I activity in LHON patients with respect to control individuals (results not shown). However, we found that the NADH:Q reductase activity in LHON patient has a clearly altered sensitivity to rotenone (Fig. 2). The I_{50} of rotenone is dependent upon the protein concentration in the assay [13,14,16] and has an average value of 35 pmol per mg of protein in platelet mitochondria of healthy individuals. This I_{50} of rotenone increases over threefold (Figs. 2 and 3) in the patients with the ND4/11778 mutation. In addition to the decreased sensitivity to rotenone, patients with the ND4/11778 mutation also show a change in affinity for the ubiquinone substrates (Fig. 3). The K_m for undecyl-ubiquinone decreases from an average of 17 μM in the controls to an average of 8 μM in the LHON patients, whereas the K_m for Q_2 increases from 2 to 7 μM (Fig. 3). Unlike UBQ and other quinones with saturated hydrocarbon chains, Q_2 functions both as a substrate

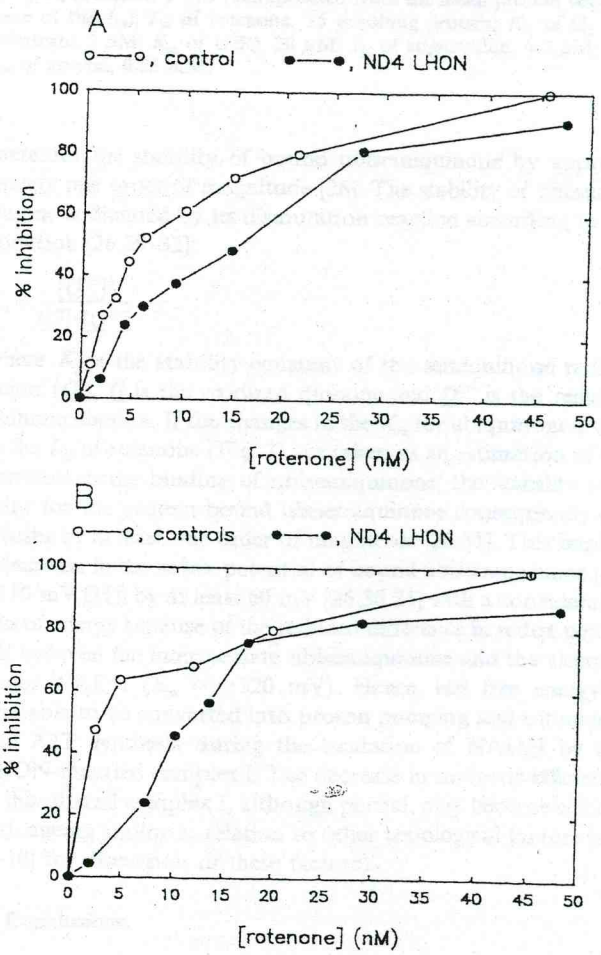

Fig. 2. Rotenone titration of NADH:ubiquinone reductase activity of platelet mitochondria from normal individuals (open circles) and LHON patients (fill circles). (A) 0.12 mg/ml of mitochondrial protein for one control and 0.1 mg/ml of mitochondrial protein of one LHON patient 1 [4]. (B) Average data with 0.07 mg/ml of mitochondrial protein from two normal individuals and 0.08 mg/ml of mitochondrial protein from patient 2 of the same LHON family [6]. The specific activity of NADH:UBQ reductase (average rate of 15 nmol/min per mg protein, cf. [10]), NADH:ferricyanide and ubiquinol:cytochrome *c* reductase [16] were comparable in control individuals and in the members of the LHON family (cf. [6]).

and as an inhibitor of mammalian complex I [21,22], presumably because it competes for the site where rotenone antagonizes ubiquinone and its intermediates [12]. Following this interpretation, the parallel increase in the I_{50} of rotenone and in the K_m for Q_2 (Fig. 3) could reflect the same functional alteration in the reaction of complex I with its natural ubiquinone substrate. The I_{50} of stigmatellin, a ubiquinone-like inhibitor of various quinone reacting systems including complex I [23], is also increased in the mitochondria of patients with LHON (Fig. 3), whereas the I_{50} of the barbiturate amytal, an inhibitor of complex I which is structurally unrelated to ubiquinone [11–13], is not changed (Fig. 3). Hence, the functional lesion of the ND4/11778 mutation can be localized to the site at which ubiquinone binds to complex I.

3.2. Relationship between structure and function of the ND4 subunit

The resistance towards rotenone in mitochondria carrying a mutated amino acid in the ND4 subunit suggests that this subunit may be involved in the binding of rotenone to complex I. Previously, the binding of a radiolabelled analog of rotenone was associated to another subunit of the complex, namely ND1 [20]. However, rotenone binds to complex I in a complicated way, because two binding sites are present and several subunits could form such a binding site (see [13,21] and references therein). It is therefore possible that both the ND1 and the ND4 subunits are involved in rotenone binding.

The ND4 subunit is a hydrophobic protein which spans the membrane at least ten times [21]. It may be involved in the proton pumping function of complex I since it possesses evolutionarily invariant residues with ionizable that lie within the helices which are predicted to cross the membrane [21]. According to the folding model elaborated by multiple sequence analysis [18,19] (Fig. 4A), Arg^{340} lies at the beginning of the transmembrane helix J [21] near the negative (matrix) side of the membrane. The protein region preceding Arg^{340} at the negative side of the membrane shows some sequence similarity with quinone-reacting peptides of membrane complexes such as cytochrome *b* of ubiquinol:cytochrome *c* reductase [18,19] (Fig. 4B). Within this region an interesting sequence match is seen between the highly conserved His^{319} of ND4 and the invariant His^{202} (yeast numbering [19]) of mitochondrial cytochrome *b*, the latter being involved in the interaction with the ubisemiquinone intermediate that is formed during ubiquinol oxidation [25,26]. A clear sequence similarity is also found between the region following His^{319} in ND4 and that following His^{202} in cytochrome *b*, which are both predicted to lie at the negative side of the membrane (Fig. 4). This section of cytochrome *b* contains several point mutations conferring resistance to ubiquinone antagonists such as antimycin [19,25–27] (Fig. 4B). Structural similarities of this kind suggest common functional properties for the ND4 subunit and cytochrome *b*, which are both encoded by mtDNA. It is thus inferred that ND4 may be involved in the binding of the ubisemiquinone intermediate that is also formed in the oxidation of NADH by complex I [28,29].

Protein-bound ubisemiquinone anions are stabilized by positively charged amino acids such as arginine [26,30,31], and the replacement of an arginine with a less basic histidine, as arises from the ND4/11778 mutation, may reduce the stability of ubisemiquinone bound to complex I [30,31]. Indeed, the replacement of His^{202} with an Arg in bacterial cytochrome *b*

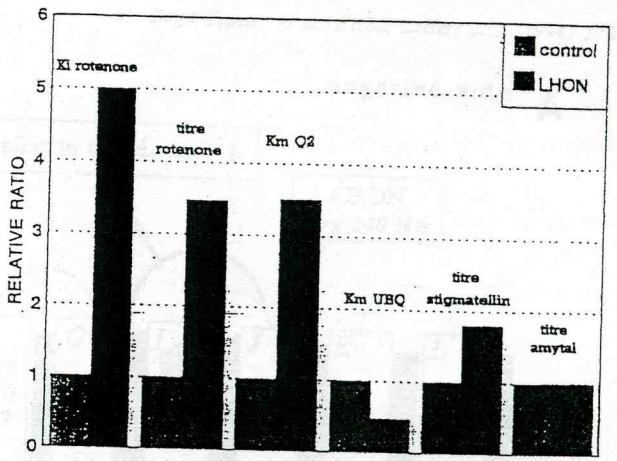

Fig. 3. Relative ratio of affinities for ubiquinone substrates and inhibitors of complex I in control individuals and LHON patients. The average values of the parameters in 8 control individuals were as follows. K_i rotenone, 1 nM (extrapolated from the mean protein dependence of the I_{50}); I_{50} of rotenone, 35 pmol/mg protein; K_m of Q_2 as a substrate, 2 μM; K_m of UBQ, 20 μM; I_{50} of stigmatellin, 4.2 μM; and I_{50} of amytal, 0.25 mM.

increases the stability of bound ubisemiquinone by approximately one order of magnitude [26]. The stability of ubisemiquinone is dictated by its dismutation reaction according to the equation [26,29–32]:

$$K_s = \frac{[Q^{\cdot-}]^2}{[Q]\cdot[Q^{2-}]}$$

where K_s is the stability constant of the semiquinone radical anion ($Q^{\cdot-}$), Q is the oxidized quinone and Q^{2-} is the reduced quinone dianion. If the changes in the K_m for ubiquinone-2 and in the I_{50} of rotenone (Fig. 3) are taken as an estimation of the decrease in the binding of ubisemiquinone, the stability constant for the protein-bound ubisemiquinone consequently decreases by at least one order of magnitude [26,31]. This implies a decrease in the redox potential of bound ubisemiquinone (ca. −110 mV [31]) by at least 60 mV [26,30,31] with a considerable loss of energy because of the reduced difference in redox potential between the intermediate ubisemiquinone and the electron donor NADH (E_m = −320 mV). Hence, less free energy is available to be converted into proton pumping and ultimately into ATP synthesis during the oxidation of NADH by the LHON-mutated complex I. The decrease in energetic efficiency of this altered complex I, although partial, may become critical with ageing and/or in relation to other aetiological factors (see [1–10] for discussion of these factors).

4. Conclusions

The present results and their interpretation suggest that the usual ND4/11778 mutation causing Leber's hereditary optic neuropathy [4,5] produces a loss of the redox energy developed by the reaction of complex I with ubiquinone. A reduced stability of the ubisemiquinone intermediate due to the replacement of Arg^{340} with His could be responsible for the decrease in the energy-conserving function of complex I. The less stable ubisemiquinone radical dismutates rapidly and reacts directly with oxygen to form radicals that may contribute to further

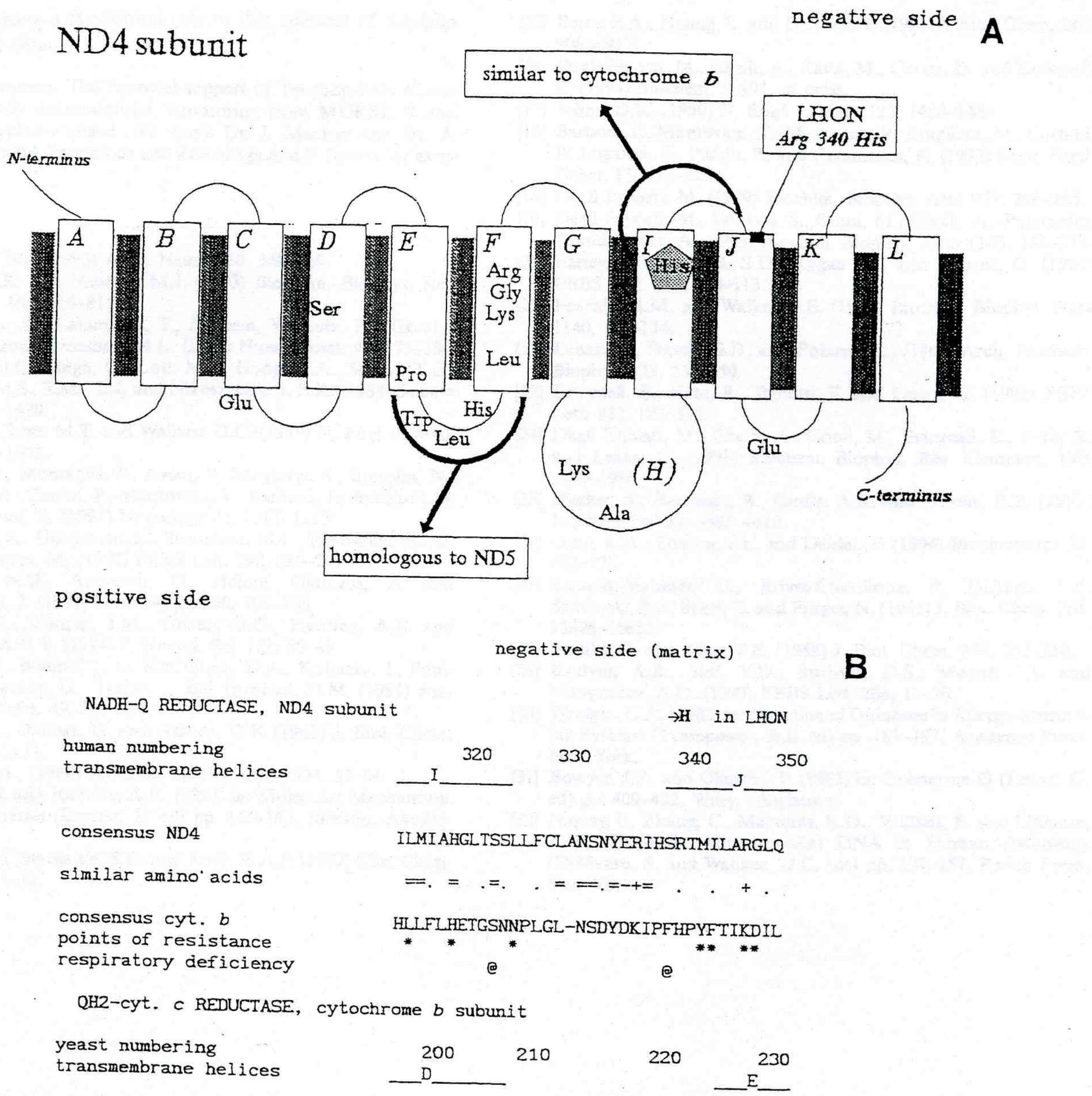

Fig. 4. (A) Hypothetical model for the folding of the ND4 subunit of mitochondrial complex I with the residues that appear to be invariant in all the aligned sequences. The nomenclature of the predicted transmembrane helices conforms to that proposed previously [21]. His^{319} is highlighted by a hatched pentagon. The region which shows the highest sequence homology with the ND5 subunit [21] is also marked. The negative side of the membrane corresponds to the matrix of mitochondria. (B) Alignment of the consensus sequence of mitochondrial and bacterial ND4 proteins to the consensus sequence of mitochondrial and bacterial cytochrome *b* apoprotein in a central region at the negative side of the membrane [19]. The symbol * marks the positions of the mutations which confer resistance towards ubiquinone antagonists such as antimycin, funiculosin and diuron [19]. The symbol @ marks the positions where mutations induce respiratory deficiency [19,27]. Identical amino acids in the two consensus sequences are marked by = , chemically similar amino acids by •, and conserved amino acids with positive or negative charge are marked by + or −, respectively. The gap in the consensus of cytochrome *b* is required for maximal identity also in the aligned sequences of this protein [19].

mitochondrial damage. Such a 'short circuit' in electron transport might not be detectable in the assay of NADH oxidation by exogenous ubiquinones, since rapid autoxidation continuously regenerates the ubiquinone acceptor. It could become evident, however, in the integrated electron transport along the respiratory chain, because rapid autoxidation of ubisemiquinone also implies a decreased efficiency in releasing the ubiquinol product by the mutated complex I. This may explain why NAD-linked substrates are oxidized at lower rates in mitochondria of LHON patients than in those of control individuals [7,8], although other explanations have been advanced [7].

In conclusion, the significance of this work is twofold. First, the functional alteration associated with the most common LHON mutation is shown to lie at the site of interaction between complex I and its substrate ubiquinone, and thus it could be corrected by therapies using appropriate quinones [32]. Second, the domain of the ND4 subunit containing Arg^{340} is likely to be involved in the binding of rotenone and ubiquinone,

thereby assigning a functional role to this subunit of complex I for the first time.

Acknowledgements: The financial support of Telethon-Italy (Grant 391) is gratefully acknowledged. Sponsoring from MURST, Rome, Italy, is also acknowledged. We thank Dr. I. Mackay and Dr. A. Baumer for helpful discussions and Bacchilega and F. Sparla for assistance.

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