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Electron transfer properties of NADH:ubiquinone reductase in the ND1/3460 and the ND4/11778 mutations of the Leber hereditary optic neuroretinopathy (LHON)

Anna Majander[1], Kirsi Huoponen[2], Marja-Liisa Savontaus[2], Eeva Nikoskelainen[3] and Mårten Wikström[1]

[1]*Helsinki Bioenergetics Group, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki, Finland,* [2]*Department of Medical Genetics, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, SF-20520 Turku, Finland* and [3]*Department of Ophthalmology, Turku University Central Hospital, Kiinamyllynkatu 4-8, SF-20520 Turku, Finland*

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We report the electron transfer properties of the NADH:ubiquinone oxidoreductase complex of the respiratory chain (Complex I) in mitochondria of cells derived from LHON patients with two different mutations in mitochondrial DNA (mtDNA). The mutations occur in the mtDNA genes coding for the ND1 and ND4 subunits of Complex I. The *ND1*/3460 mutation exhibits 80% reduction in rotenone-sensitive and ubiquinone-dependent electron transfer activity, whereas the proximal NADH dehydrogenase activity of the Complex is unaffected. This is in accordance with the proposal that the ND1 subunit interacts with rotenone and ubiquinone. In contrast, the *ND4*/11778 mutation had no effect on electron transfer activity of the Complex in inner mitochondrial membrane preparations; also K_m for NADH and NADH dehydrogenase activity were unaffected. However, in isolated mitochondria with the *ND4* mutation, the rate of oxidation of NAD-linked substrates, but not of succinate, was significantly decreased. This suggests that the ND4 subunit might be involved in specific aggregation of NADH-dependent dehydrogenases and Complex I, which may result in fast ('solid state') electron transfer from the former to the latter.

LHON; *ND1*/3460 mutation; *ND4*/11778 mutation; Electron transfer; NADH:ubiquinone reductase; Complex I

1. INTRODUCTION

The Leber hereditary optic neuroretinopathy (LHON) is a maternally inherited disease resulting in acute bilateral blindness due to retinal degeneration. Three replacement mutations in 2 mitochondrial genes of the NADH:ubiquinone reductase complex of the respiratory chain have been identified in LHON pedigrees. Wallace et al. found a nucleotide change at nt11778 in *ND4* converting a conserved Arg to His [1]. However, genetic heterogeneity was evident, since only about half of the LHON families appeared to have this mutation [2,3]. This concept was confirmed by Howell et al. who identified a mutation at nt4160 of the *ND1* gene resulting in a substitution of Pro for the conserved Leu in a large LHON family [4]. Huoponen et al. identified a third LHON mutation, also in *ND1*, in 3 independent families: a G to A change at nt3460 converting Ala to Thr [5].

Although the maternal inheritance of LHON strongly suggests a pathogenic role of these mutations, there is still little known about the energy metabolism in LHON and its possible role in the pathogenesis of this disease. So far, only Parker et al. has reported reduced NADH:ubiquinone reductase activity in an LHON family with several neurological symptoms, which later was shown to have the *ND1*/4160 mutation [4,6].

We analysed Complex I electron transfer activity in mitochondria and inner mitochondrial membranes derived from peripheral lymphocytes of patients with the *ND1*/3460 and *ND4*/11778 mutations. In the *ND1* mutations we report a clearcut blockade of rotenone-sensitive electron transfer activity of the Complex. In the *ND4* mutation both electron transfer and K_m for NADH were found to be unaffected in the Complex. However, in intact mitochondria with the *ND4* mutation we found decreased rates of oxidation of NADH-dependent substrates, but not of succinate. The nature of the enzymatic defects in the 2 mutants and their possible relation to the pathogenesis of LHON are discussed.

Abbreviations: EBV, Epstein-Barr virus; EDTA, ethylenedinitrilo tetraacetic acid; DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; LHON, Leber hereditary optic neuroretinopathy; MOPS, 3-[*N*-morpholino]propanesulfonate; PCR, polymerase chain reaction; SDH, succinate dehydrogenase; Tris, tris[hydroxymethyl]aminomethane; UQ-10, ubiquinone-10.

Correspondence address: A. Majander, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki, Finland. Fax: (358) (0) 1918276.

2. MATERIALS AND METHODS

The ubiquinone analogue, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB), was synthesized by Dr. Hase (Department of Organic Chemistry, University of Helsinki) [7].

Cell lines from one normal control individual, two LHON patients with the *ND1*/3460 mutation and one LHON patient with the *ND4*/11778 mutation were established by Epstein-Barr virus (EBV) transformation of peripheral lymphocytes.

For detection of *ND1* and *ND4* mutations from the 4 cell lines the *Aha*II- and *Sfa*NI-RFLP analyses were performed as described elsewhere [1,5]. Before analyses the cells were lysed and the DNA fragments spanning the mutation sites were amplified by PCR according to Gyllensten [8].

Mitochondria were isolated from EBV-transformed cell lines. Cells were sedimented by centrifugation and the pellet was resuspended at 250 mg wet weight/ml in isolation buffer (0.25 M sucrose, 25 mM MOPS/KOH, pH 7.4, 1 mM EDTA). The cell suspension was treated with 0.12% (w/v) digitonin for 20 s, diluted with 3 vols. of isolation buffer and centrifuged at 10 000 × *g* for 2 min. The pellet was resuspended in isolation buffer and centrifuged at 800 × *g* for 5 min. The resulting supernatant was centrifuged at 10 000 × *g* for 5 min. All the centrifugations were performed at +4°C and the incubations in an ice-bath. An inner mitochondrial membrane preparation was further isolated from the mitochondrial pellet as previously described [9].

The NADH:ubiquinone reductase activity was assayed by following 2,6-dichlorophenolindophenol (DCIP) reduction at 600 nm [10]. The rate of reduction increased linearly with increasing DCIP concentration. The assays were performed in 25 mM MOPS/KOH, pH 7.4, 1 mM EDTA, 25 μM DCIP, 2 mM KCN, 62.5 μM DB, and 5 μg of mitochondrial protein in a 0.8 ml vol. The effect of the ubiquinone analogue, DB [7], on the NADH:DCIP reductase activity was tested with 150 μM NADH, by varying DB concentration from 1.25 μM to 62.5 μM. Rotenone (7.5 μM) was used to determine the rotenone-sensitive fraction of activity. NADH:$K_3Fe(CN)_6$ reductase indicating NADH dehydrogenase activity was assayed in 25 mM MOPS/KOH, pH 7.4, and 1 mM EDTA by following the reduction of 0.875 mM $K_3Fe(CN)_6$ at 410 nm by 1.3–400 μM NADH. Succinate:DCIP reductase indicating succinate dehydrogenase activity was assayed as previously described [11]. Rotenone-sensitive α-ketoglutarate:$K_3Fe(CN)_6$ reductase and antimycin-sensitive succinate:$K_3Fe(CN)_6$ reductase were analysed immediately from isolated mitochondria derived from control cells and cells with the *ND4* mutation. The assays were performed in 0.25 M mannitol, 10 mM KCl, 10 mM potassium phosphate, pH 7.2, 0.2 mM EDTA, 1 mM $MgCl_2$, 2 mM KCN and 0.2 mM ADP. Reduction of potassium ferricyanide was followed at 420–500 nm, either in the presence of 25 μM $K_3Fe(CN)_6$, 3–15 mM α-ketoglutarate, 0.63 mM L-malate, or with 6 μM $K_3Fe(CN)_6$, 6.3 mM succinate, and 7.5 μM rotenone. The oxidation of NAD-linked substrates was corrected for rotenone-insensitive activity. Oxidation of succinate was corrected for antimycin-insensitive activity by addition of 0.3 μg/ml antimycin. All the activities are expressed as nmol/min·mg mitochondrial protein. Protein concentrations were assayed with the BCA Protein Assay Reagent (Pierce).

3. RESULTS

The control cell line was confirmed not to have either *ND1* or *ND4* mutations while the cell lines ND1 and ND4 were homoplasmic for nt3460 and nt11778 mutations, respectively (see Materials and Methods).

The inner mitochondrial membrane fraction from controls and cells with the *ND1*/3460 and *ND4*/11778 mutation all showed a very similar specific succinate dehydrogenase activity suggesting that these preparations were of similar purity. Thus also other specific enzyme activities should be comparable (Table I).

Table I

Complex I activity in inner mitochondrial membranes

	Control	*ND4*	*ND1*/A	*ND1*/B
NADH:DCIP				
R+ V	87	100	29	17
K_m	2.6	2.1	ND	ND
R− V	44	19	40	39
K_m	1.8	2.4	2.0	2.1
T V	125	119	63	50
K_m	2.6	2.0	1.7	1.8
NADH:$K_3Fe(CN)_6$				
V	2036	2933	2773	2476
K_m	7.1	10.0	8.0	6.7
SDH	298	258	315	257

V_{max} (V) and K_m for NADH of rotenone-sensitive (R+), rotenone-insensitive (R−) and total (T) (NADH:DCIP) reductase, and of NADH:$K_3Fe(CN)_6$ reductase and succinate dehydrogenase (SDH) activity in control, *ND4*/11778 and two *ND1*/3460 mutations (*ND1*/A, *ND1*/B). V_{max} and SDH are expressed as nmol/min·mg, and K_m as μM NADH. ND = not determined.

All samples exhibited NADH:DCIP reductase activity, which in these assay conditions had both rotenone-sensitive and rotenone-insensitive components (Table I). The total NADH:DCIP reductase activities were nearly identical in the control and the *ND4*/11778 mutation, whereas in the *ND1*/3460 mutations it was about 50% of the contol. In the control the rotenone-sensitive fraction was 70% of total activity, in the *ND4*/11778 mutation 85%, and in the *ND1*/3460 mutations about 40%. In the *ND1*/3460 mutations the rotenone-sensitive activity was only about 20–35% of the control, whereas the rotenone-insensitive activity was normal.

The K_m for NADH and V_{max} were analysed in all samples and determined by the Lineweaver-Burk method for the total, the rotenone-sensitive and the rotenone-insensitive activities (Table I). All K_m values ranged from 1.7 μM to 2.6 μM, except the rotenone-sensitive activity in the *ND1* mutations, which was not linear enough for the estimation of K_m. This result suggests that NADH binding to the enzyme is undisturbed in both LHON mutations. It also suggests that both rotenone-sensitive and rotenone-insensitive activity in these assay conditions may involve the same NADH binding site.

The proximal NADH dehydrogenase segment of the NADH:ubiquinone reductase was analysed by NADH:ferricyanide reductase activity. All samples showed a similar V_{max} with K_m ranging from 6.7–10.0 μM (Table I). This strengthens the view that the proximal part of the enzyme is unaffected in both LHON mutations.

As shown in Table II, in mitochondrial membranes from control cells the rotenone-sensitive NADH:DCIP

Table II

Complex I activity in inner mitochondrial membranes

		Control	*ND4*	*ND1*/A	*ND1*/B
R+	DB−	1	7	5	1
	DB+	83	99	29	14
R−	DB−	37	18	53	41
	DB+	33	21	33	38
T	DB−	38	25	58	42
	DB+	116	120	62	53

Total (T), rotenone-sensitive (R+) and rotenone-insensitive (R−) reduction of DCIP by NADH in the absence (DB−) and presence (DB+) of 62.5 μM ubiquinone analogue DB. Activities are expressed as nmol/min·mg. (For experimental conditions, see Materials and Methods.) *ND4*, *ND4*/11778 mutation. *ND1*/A and *ND1*/B, mutation in *ND1*/3460.

reductase activity depended almost completely on addition of the ubiquinone analogue DB. In contrast, rotenone-insensitive activity was unaffected by DB. This indicates that the water-soluble redox mediator DCIP is unable, on its own, to accept electrons from endogenous ubiquinone in the membrane, so that a proximal, rotenone-insensitive, activity is measured. DB, which is more hydrophilic than ubiquinone-10 (UQ-10), has been shown to readily accept electrons from the UQ-10 pool in a rotenone-sensitive fashion [7,12]. Thus, in the conditions employed here, DB mediates electron transfer between this pool and the water-soluble DCIP.

Table II also shows that the defect in the *ND1* mutants is specifically related to the failure of DB to induce rotenone-sensitive activity. On the other hand, the DB-induced enhancement is similar to the control in the *ND4* mutant. This is in accordance with the data of Table I, and indicates that in the *ND1* mutant the defect is located in the distal rotenone-sensitive ubiquinone-reducing part of Complex I.

Whereas the *ND1* mutation thus exhibits a clearcut enzymatic defect in Complex I, the *ND4* mutation is more problematic as it shows no significant deficiency in the inner mitochondrial membrane preparations. We therefore studied rotenone-sensitive oxidation of NAD-dependent substrates, and of succinate, in intact mitochondria from ND4 and control cells. Table III shows that oxidation of NAD-linked substrates is substantially depressed in ND4 mitochondria relative to controls, whilst the succinate oxidation rates are, in fact, somewhat enhanced. The latter difference could well relate to a difference in protein composition of the mitochondria of controls and ND4 mutants. If, therefore, the NADH oxidase activities are normalised with respect to succinate oxidase activity, it follows that NADH-oxidase activity is depressed about 7-fold in the ND4 mitochondria relative to the control.

Table III

Substrate oxidation in mitochondria

	Control	*ND4*
α-Ketoglutarate + L-malate	44.4 ± 3.4 (3)	10.2 ± 2.6 (3)
Succinate	22.1 ± 6.4 (4)	36.4 ± 6.7 (4)

Reduction of $K_3Fe(CN)_6$ by α-ketoglutarate + L-malate, and by succinate, in intact mitochondria from cells of controls and with the *ND4*/11778 mutation. Activities are expressed as nmol/min·mg; mean ± SD, number of assays in parentheses. (For experimental conditions, see Materials and Methods.)

4. DISCUSSION

In this report we show that electron transfer of the NADH:ubiquinone reductase complex (Complex I) was dramatically reduced in the *ND1*/3460 mutation. The *ND1* gene product has been identified to bind rotenone and to interact with ubiquinone [13,14]. This is in good agreement with our results, since in the *ND1*/3460 mutation only rotenone-sensitive electron transfer was blocked. Whether this is due to a defect in ubiquinone interaction with the complex or a reduction of electron transfer velocity proximal to the ubiquinone interaction remains to be clarified.

The *ND4* gene product is suggested to be an M_r 39 000 membrane-spanning protein of the hydrophobic fragment of Complex I [14]. So far, there is nothing known about its function, which also applies to most of the 30 subunits of the complex. We could not show any significant reduction of Complex I electron transfer activity in the *ND4*/11778 mutation, neither were K_m for NADH nor NADH dehydrogenase activity affected.

The maternal inheritance of LHON, together with the observed mutations in mtDNA-encoded subunits of the NADH:ubiquinone reductase complex, suggest that a defect in this enzyme complex is likely to be central in the pathogenesis of this disease. In this sense the clear defect attributable to the function of the *ND1* gene product in LHON patients with the *ND1*/3460 mutation is interesting. It is also interesting that the LHON family with reduced Complex I activity reported by Parker et al. [6] has a mutation in the *ND1* gene though at a site different from the *ND1*/3460 mutation studied here [4]. On the other hand, we also show here that in an LHON patient with the *ND4*/11778 mutation confirmed in the lymphoblastoid cell line, the corresponding enzyme complex has normal electron transfer activity. The conservative view that an NADH:ubiquinone reductase defect is the pathogenic basis for LHON would thus require the hypothesis that the *ND4* mutation results in Complex I being affected in some other function than electron transfer between NADH and ubiquinone. If this other function were energy conservation (proton translocation [15,16]), the overall pathophysiological effect may well be analogous to that in the *ND1* mutations, i.e. a decrease in ATP synthesis. How-

ever, during the course of this work Larsson et al. [17] have reported that the *ND4*/11778 mutation does not significantly affect the yield of ATP synthesis (P/O ratio) in isolated mitochondria from muscles of LHON patients. This argues against a defect in energy conservation. Larsson et al. [17] also reported unaffected electron transfer activity of Complex I itself, but lowered activities of oxidation of NADH-linked substrates in mitochondria, in good agreement with the present data.

It is usually thought (but see [18,19]) that NADH-linked dehydrogenases (e.g. those of the Krebs cycle), which are located in the mitochondrial matrix, communicate with the NADH-oxidizing Complex I by means of diffusion of the dinucleotide. However, many of these dehydrogenases have been shown to associate to Complex I [18,19], and this may have kinetically important implications. The dissociation of NAD(H) from the enzymes, and/or its diffusion between binding sites of Complex I and dehydrogenases, may be significantly slower than direct 'solid state' transfer or 'substrate channeling' [19] of reducing equivalents within an enzyme–enzyme complex. If this were indeed the case, there would have to be specific association domains in Complex I for binding of the dehydrogenases. If the function of the ND4 subunit were to form such a domain, its mutation could lead to depressed activities of substrate oxidation without a discernible change in the activity of Complex I itself, as observed here (and see [17]).

It is probably essential that Complex I electron transfer activity is not reduced by more than about 80% in the mutations described here; complete inhibition would most likely be lethal. Thus, most tissues may function virtually normally at Complex I activities well below V_{max} and/or by compensating the defect by secondary mechanisms. Only a few tissues may be dependent on the maximal kinetic capacity of Complex I, or may lack sufficient compensatory mechanisms for the primary defect. This may explain the unique tissue specificity of LHON.

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