Mutations in subunit 6 of the F_1F_0-ATP synthase cause two entirely different diseases

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Abstract A lowered efficiency of oxidative phosphorylation was recently found in a Leber hereditary optic neuropathy (LHON) proband carrying a mutation in the *mtDNA* gene for subunit 6 of the membrane-bound F_0 segment of the F_1F_0-ATP synthase [9]. This phenotype was transferred to cytoplasmic hybrid cells together with the mutation, proving its functional significance. Increasing the respiratory rate in the mitochondria from this mutant raised the ATP/2e$^-$ ratio back to normal values. A different mutation in the same *mtDNA* gene has been found in patients with the NARP syndrome [10]. Although the ATP/2e$^-$ ratio is also decreased in this mutant, in this case an increase in the respiratory rate could not compensate for it. Whilst both mutations affect subunit 6 of the proton-translocating F_0 segment, the LHON mutation induces a proton leak whereas the NARP mutation blocks proton translocation. Hence, the latter will have much more destructive metabolic consequences in agreement with the large clinical differences between the two diseases.
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1. Introduction

Mitochondrial oxidative phosphorylation fulfils most of the energy requirement of eukaryotic cells. Cell respiration generates an electrochemical proton gradient across the inner mitochondrial membrane, and the F_1F_0-ATP synthase utilizes this proton gradient by catalysing ATP synthesis from ADP *plus* inorganic phosphate coupled to influx of protons. Thirteen polypeptides of the respiratory chain complexes, and of the F_1F_0-ATP synthase, are encoded by human mitochondrial DNA (mtDNA; [1]).

The Leber hereditary optic neuropathy (LHON) is a mitochondrial disease that results in bilateral optic nerve atrophy in young, usually male, adults. The condition appears quite specific, although cardiac and neurological symptoms are observed in some patients [2]. Several homoplasmic mutations in the structural genes of mtDNA have been associated with

LHON, suggesting a bioenergetic basis for its pathogenesis. Until recently, all these mutations have been found in the respiratory chain complexes. In these cases the rate of respiration and, consequently, that of oxidative ATP synthesis is reduced, but only with limited severity [3–8]. Notably, the efficiency of oxidative phosphorylation (ATP/2e$^-$ ratio) is unaltered.

Recently, a point mutation at nucleotide (nt) 9101 of the mtDNA gene that encodes subunit 6 of the F_1F_0-ATP synthase was found in one LHON family [9]. This was associated with a lowered efficiency of oxidative phosphorylation in lymphoblast mitochondria, which is consistent with a defect in the F_1F_0-ATP synthase. Except for general polymorphisms, no other protein-encoding mutations were found in the mtDNA of the LHON patients [9].

Interestingly, mutation of another site in subunit 6 of the ATP synthase has also been linked to mitochondrial disease. Thus, the nt 8993 mutations T→C and T→G have been associated with Leigh's disease, and with a syndrome termed NARP (neurogenic weakness, ataxia and retinitis pigmentosa; [10–13]). The clinical manifestations of the T→G mutation have been studied in four generations of a Finnish family, in which a wide range of presentations from suboptimal dark adaptation to severe encephalopathy was observed, depending on the degree of heteroplasmy [12]. In complete contrast to this, the patient with the nt 9101 mutation represents a typical case of LOHN.

Here we show by the cytoplasmic hybrid (cybrid) cell approach [14,15] that the defect in the LHON case is truly carried by the mitochondrial DNA. Comparison of the functional defects due to the two mutations in subunit 6 of the ATP synthase reveal that they are completely different in nature, which may help to explain why they lead to very different diseases.

2. Materials and methods

Mitochondria were isolated and uncoupler-sensitive ATP synthesis and electron transfer determined as in [5].

An *rho*^{0} cell line devoid of mtDNA (143BTK; provided by I. Trounce) was produced as in [14]. Cybrid cells were made from enucleated LHON patient or control lymphoblasts (cytoplasts) and *rho*^{0} cells, essentially as described by Trounce et al. [15]. Screening for the nt 9101 mutation in cybrid cells was done after amplification with primers nt 8973–8991 and nt 9292–9276. After digestion with *Hph*I, the mtDNA fragments were separated on agarose gels and visualised under UV light. Samples with the nt 9101 mutation yielded fragments of 128 and 192 basepairs (bp). Samples without the muta-

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tion remained uncut, producing a single 320 bp fragment. All single cybrid colonies derived from LHON cytoplasts were homoplasmic for the nt 9101 mutation. All control cybrids lacked this mutation.

3. Results and discussion

To determine whether the functional defect in the LHON case is truly due to a mtDNA mutation, and not to mutations in nuclear genes of the F_1F_0-ATP synthase, we used the cybrid approach [14,15]. Enucleated cells from the patient or from a control were fused with rho^0 cells that lack mtDNA, and oxidative phosphorylation was studied in mitochondria isolated from the resulting cybrid cells. As shown in Table 1, these mitochondria indeed exhibited a lowered efficiency of oxidative phosphorylation, which demonstrates conclusively that the defect is carried with the mutant mitochondria.

A functional defect in the ATP synthase should be independent of the segment of the respiratory chain that generates the electrochemical proton gradient. This is confirmed by the finding that the efficiency of ATP synthesis in the mutant is significantly lowered also when coupled to the oxidation of ferrocytochrome c by O_2 (Table 1).

Since the clinical manifestations of the two ATP synthase subunit 6 mutations are very different, it was of interest to compare their functional consequences. We found that the rates of respiration in the controlled state (state 4), during oxidative phosphorylation (state 3), and in the presence of an uncoupling agent, were essentially normal in nt 9101 and nt 8993 mutant lymphoblast mitochondria (not shown). The only exception was that state 3 respiration was slightly slower in nt 8993 mutant mitochondria. Apparently the lowered ATP synthase activity in this mutant causes some secondary inhibition of coupled respiration, in agreement with the finding of Trounce et al. [13]. Although much weaker, this effect is thus analogous to that of the well-known F_0 inhibitor oligomycin.

An increase of the rate of mitochondrial respiration is normally accompanied by a proportional rise in the rate of ATP synthesis (Fig. 1A). In the control, the ATP/2e$^-$ ratio is close to unity (Fig. 2), which is the known value when ATP synthesis is linked to the cytochrome c-O_2 span of the respiratory chain [16]. Comparison of the rate of ATP synthesis with the rate of respiration revealed a striking difference between the two subunit 6 mutations. In the nt 9101 mutant mitochondria the ATP/2e$^-$ ratio is significantly lower than in the controls at low respiratory rates, but rises towards unity at high respiratory rates (Figs. 1B and 2). In this case, therefore, an elevated rate of respiration is able to compensate for the lowered efficiency of ATP synthesis.

The nt 8993 mutant mitochondria behave very differently.

The ATP/2e$^-$ ratio is almost normal at the lowest respiratory rates, but increasing the respiratory velocity now causes only a very small increase in the rate of ATP synthesis (Fig. 1C). Hence, the ATP/2e$^-$ ratio drops to a low value (cf. [13]), similar to that in the nt 9101 mutant at low respiratory rate (Fig. 2).

Subunit 6 is an essential part of the membrane-bound F_0 domain of the ATP synthase complex, and corresponds to subunit a in the homologous bacterial and chloroplast enzymes. It is a highly hydrophobic polypeptide of unknown structure, and interacts closely with the oligomeric subunit c. Both these subunits have been shown to be intimately involved in proton translocation catalysed by the F_0 domain [17], which drives the synthesis of ATP in the F_1 domain, possibly by a rotational mechanism [18,19].

Positions 156 (affected by the nt 8993 mutation) and 192 (affected by the nt 9101 mutation) are both among the last 80 amino acids in the carboxy-terminus of subunit 6. Mutagenesis work in bacteria has demonstrated that this domain is directly involved in proton translocation through F_0. The nt 8993 T → G (or C) mutation replaces the conserved leu[156] by an arginine at a position close to that of the completely conserved arg[159], which is a key residue for proton translocation [20]. On the other hand, in the nt 9101 mutation a poorly conserved isoleucine at position 192 is replaced by a threonine.

Although both mutations lower the efficiency of oxidative phosphorylation, and both are located in the same region of subunit 6, their effects on ATP synthesis are fundamentally different. The fact that the rate of ATP synthesis was unable to keep up with an increase in respiratory rate in the NARP mutant strongly suggests inhibition of proton translocation by the F_0 segment, as for the classical inhibitor oligomycin. As long as the rate of proton translocation by the respiratory chain does not exceed the impaired proton-translocating capacity of F_0 due to this mutation, the ATP/2e$^-$ ratio will remain normal. However, when this capacity is exceeded, the ATP/2e$^-$ ratio declines, and some inhibition of state 3 respiration also sets in due to respiratory control (cf. [13] and above). Our observation is in good agreement with the finding by Hartzog and Cain [21], who reported inhibition of proton translocation by membrane-reconstituted F_1F_0 from E. coli carrying the analogous mutation. It also agrees with previous reports on mitochondria carrying a similar level of heteroplasmy of this mutation, which showed lowered rates of ATP synthesis, ATP hydrolysis [22], and phosphorylating respiration [13], as well as a decreased ATP/2e$^-$ ratio [12,13,22].

In complete contrast, the ile192thr LHON mutation has the same type of effect as a low concentration of an uncoupling

Table 1
Efficiency of oxidative phosphorylation (ATP/2e$^-$ ratio) in mitochondria from cybrid cells and lymphoblasts carrying the nt 9101 mutation

Cell line	Rate of electron transfer(nmol 2e$^-$/min·mg)	ATP/2e$-$
Cybrid cell mitochondria (NADH → cyt.c)		
nt 9101 [11]	56 ± 6.8	1.08 ± 0.16
Control [11]	58 ± 8.8	1.59 ± 0.26
Lymphoblast mitochondria (TMPD → O_2)		
nt 9101 [7]	93 ± 31	0.34 ± 0.15
Control [7]	110 ± 23	0.69 ± 0.19

Values are means ± SD; number of separate experiments in parenthesis.
Cybrid cell mitochondria were oxidising α-ketoglutarate by ferricyanide [5]; lymphoblast mitochondria respired with 0.2 mM TMPD (N,N,N',N'-tetramethylene-p-phenylenediamine) plus ascorbate.

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protonophore, where a limited proton leak conductance is created across the membrane. The lowered ATP/2e⁻ ratio is now due to the escape of protons across the membrane through this leak. Such an effect may be overcome by increas-
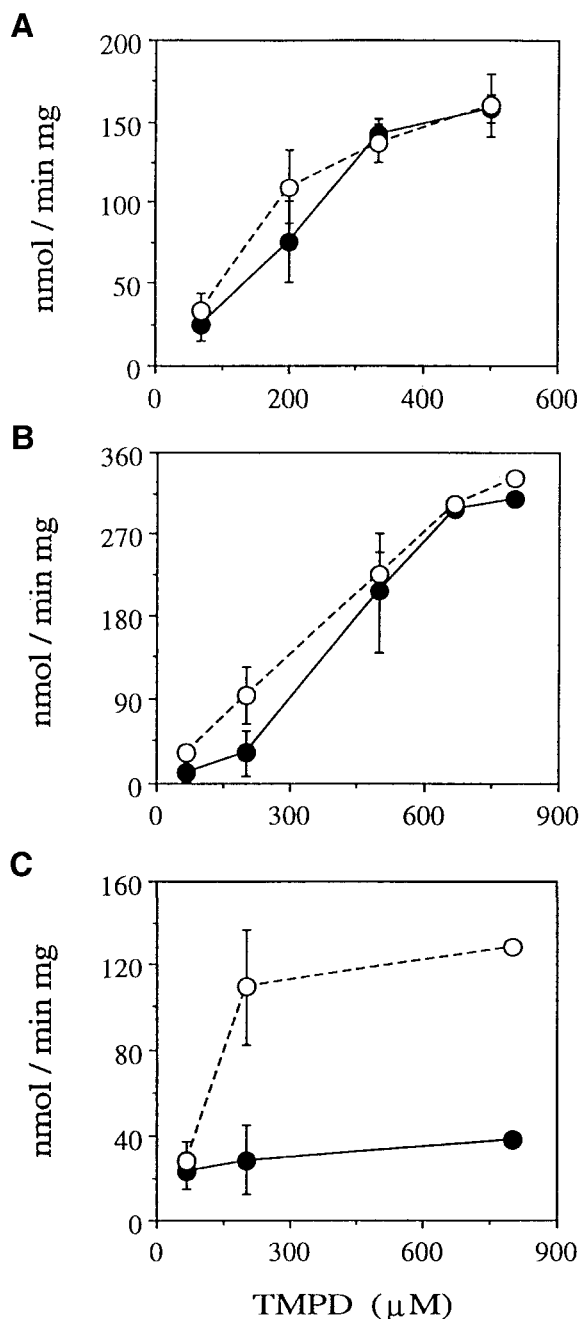
Fig. 2. The ATP/2e⁻ ratio as a function of respiratory rate. ●, Controls; ○, nt 9101 mutant; △, nt 8993 mutant. Conditions as in Fig. 1.

ing the respiratory velocity, which may increase the rate of generation of the electrochemical proton gradient over that of the leak conductance. The normal maximal rate of ATP synthesis in this mutant agrees with the previously reported lack of marked changes in ATP hydrolysis activity [9]. A proton leak has been reported previously for a mutation in the E. coli a subunit [23], corresponding to $Gly^{167}Asp$ in the human subunit. Interestingly, the amino acids in position corresponding to 168 and 194 have been shown to interact in the E. coli enzyme [24], so that the Ile^{192} LHON site may be close to that of Gly^{167} in the structure. These findings suggest that substituting a hydrophobic side chain with a hydrophilic one in this domain of subunit a introduces a proton leak through F_0. Lewis et al. [25] proposed that the dielectric barrier across F_0 may be quite thin in the C-terminal domain of subunit a, relative to the thickness of the phospholipid bilayer.

The NARP mutation is expected to be much more destructive than the LHON mutation from the viewpoint of energy metabolism. In LHON the functional defect can be overcome by an increased respiratory turnover, whereas in NARP the metabolic defect will be exacerbated whenever there is a higher demand on the rate of ATP synthesis.

A lowered rate of oxidative ATP synthesis appears to be the common biochemical background of pathogenic importance in all LHON mutations studied thus far. This may result from inhibition of the respiratory chain, usually at complex I, but with an unaffected efficiency of oxidative phosphorylation [5]. Alternatively, as in the nt 9101 mutation described here, the same overall effect may be caused by a lowered efficiency of ATP synthesis, without inhibition of respiration. The net deficit is relatively small in all these LHON cases. Compensatory mechanisms were shown to minimise the metabolic consequences of LHON mutations in complex I [5]. Interestingly, the specific features of the nt 9101 mutation also allow such compensation, although by an entirely different mechanism.

The general view thus emerges that whilst the LHON mutations are generally homoplasmic, the resulting primary defect on the enzyme level can, in part, be metabolically compensated. This may be a key feature contributing to the high tissue specificity of this disease, and to its low incidence

Fig. 1. Relationship between rates of ATP synthesis and respiration in lymphoblastoid cell mitochondria. ○, Rates of O_2 consumption (nmol O/mg×min); ●, rates of ATP synthesis (nmol/mg×min). Isolated mitochondria were suspended in 0.25 M mannitol, 10 mM KCl, 0.2 mM EDTA, 1 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.2, with 0.5 mM ADP, 0.7 U/ml hexokinase (Boehringer-Mannheim), 20 mM glucose, 10 μM rotenone, 0.3 μg/ml antimycin, 5 mM ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Values are means of 3–7 experiments ± SD. A: Control; B: nt 9101 mutant; C: nt 8993 mutant. Epstein-Barr virus transformed lymphoblast cell lines [5] were from the LHON patient [9], from a NARP patient [12] and from 3 healthy control individuals. 80% of mtDNA is mutated in the nt 8993 cell line [12].
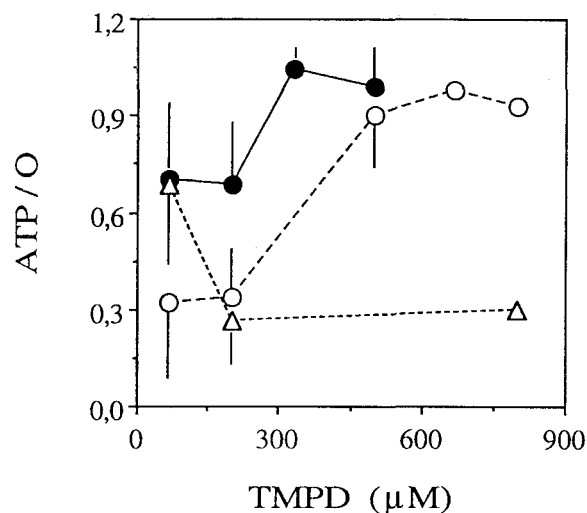
among individuals carrying the mutation. On the other hand, several other mtDNA mutations (e.g. the nt 8993 NARP mutation), lead to more severe metabolic defects which may be difficult to compensate for. In these cases the final clinical outcome, therefore, depends entirely on the degree of mtDNA heteroplasmy and its distribution in vital organs.

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