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MtDNA and Nuclear Mutations Affecting Oxidative Phosphorylation: Correlating Severity of Clinical Defect with Extent of Bioenergetic Compromise

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Rates of ATP synthesis were studied in cultured skin fibroblasts treated with digitonin. In fibroblasts from patients with complex I deficiency, complex IV and complex V deficiency rates of ATP synthesis were decreased below the levels found in controls. In mitochondria isolated from cultured lymphoblasts, ATP synthesis was also decreased by 35–50% in cases of Leigh's disease due to complex I, complex IV, or complex V deficiency. Calculating the effect of the mutations in the various complexes on the overall efficiency of oxidative phosphorylation, we show that the mtDNA 8993 mutation which affects the activity of the F_1F_0 ATPase (complex V) has the strongest effect.

KEY WORDS: ATP synthesis; mtDNA; complex I; complex IV; complex V.

INTRODUCTION

Disorders which affect the functioning of the mitochondrial respiratory chain in man produce a wide variety of symptoms (Wallace, 1993). Progressive syndromes caused by specific mutations in mitochondrial tRNA species encoded on mitochondrial DNA include myoclonus epilepsy with ragged red fibers (MERRF) (Shoffner *et al.*, 1990), mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto *et al.*, 1990), and mitochondrial myopathy and cardiomyopathy (MCM) (Zeviani *et al.*, 1991). On the other hand, autosomal recessive inheritance of respiratory chain disorders in childhood produce either fatal infantile lactic acidosis (FILA) (DiMauro *et al.*, 1988; Moreadith *et al.*, 1984) or a progressive neurological deterioration known as Leigh's disease (Robinson *et al.*, 1992, 1987; Van Coster *et al.*, 1991). Defects in complex I or IV of the respiratory chain and disorders of the pyruvate dehydrogenase complex can produce both of these conditions. In a similar fashion, multiple different mutations in mitochondrial DNA affecting various subunits of complex I, complex III, or complex IV can all produce the identical syndrome of Lebers hereditary optic neuropathy (LHON) (Wallace, 1992).

Although the extent of compromise of oxidative phophorylation in many of the above diseases is not known, some estimates have been made using cultured cell systems. For instance, Majander *et al.* (1991) showed decreases in oxidative phosphorylation in lymphoblast mitochondria from patients with LHON with either homoplasmic 3460 or 11778 mutations. Estimates of skin fibroblast mitochondria oxidative phosphorylation from patients with FILA or Leigh's disease showed severe impairment for the former and a mild impairment for the latter condition (Robinson *et al.*, 1986).

One mtDNA disorder, the 8993 mtDNA mutation, can cause a variety of symptoms such that the degree of heteroplasmy of the mutation dictates the severity of the disease phenotype (Holt *et al.*, 1990). Percentages heteroplasmy of the 8993 mutation above

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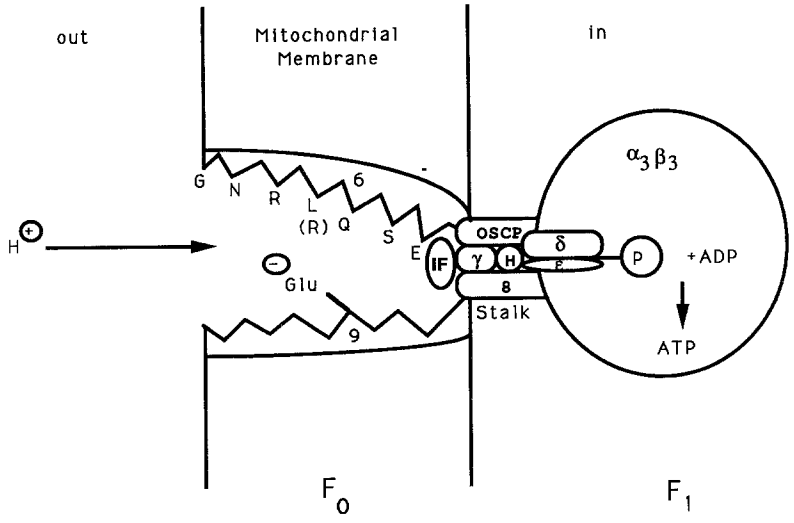

Fig. 1. The topology of the F_1F_0 ATPase complex in the mitochondrial membrane in diagramatic form. The F_1 segment of the ATPase is composed of $\alpha, \beta, \epsilon, \delta$, and γ subunits and is responsible for the binding sites of ADP, P_i, and the synthesis of ATP. The stalk portion of F_1 consists of δ and ϵ subunits, together with the oligomycin sensitivity conferring factor (OSCF), heat shock protein (H), and inhibitory factor IF_1 (IF). The transmembrane segment consists of units of ATPase 6 and 9 with ATPase 8 connecting both stalk and membrane-spanning subunits.

90% result in Leigh's disease and lactic acidemia; between 80% and 89% a later-onset disease with mental impairment, ataxia, and retinitis pigmentosa is evident; and between 60% and 80% the only symptom is a late-onset retinitis pigmentosa (Holt *et al.*, 1990; Tatuch *et al.*, 1992, 1993). Below 60% the subjects with 8993 mutation are asymptomatic. Using the 8993 mtDNA mutation as a prime example of how oxidative phosphorylation is impaired, we will consider the effects of various nuclear and mtDNA mutations on the rate of ATP synthesis.

MECHANISM OF INHIBITION OF ATPASE IN THE 8993 MTDNA MUTATION

The T → G mutation at 8993 mtDNA occurs in a fairly conserved region of the ATPase 6 gene coding sequence and results in a change of a conserved leucine to an arginine residue (Holt *et al.*, 1990). Polymorphisms in the ATPase 6 gene which result in an amino acid change are not uncommon, at least nine having been documented in relatively small studies of the human population (Marzuki *et al.*, 1991). A T → C mutation at 8993 mtDNA has recently been documented in a child with Leigh's disease (De Vris *et al.*, 1993). This base change results in a change of a leucine to a proline, thus bringing the known amino acid changes in ATPase 6 to 11.

The ATPase 6 gene product is part of the F_0 assembly of the F_1F_0 ATPase. The F_0 segment of the ATPase represents the membrane-spanning portion of the complex V ATPase of the mitochondrial oxidative phosphorylation system (Boyer, 1989). Together with the nuclear encoded subunit ATPase 9 and the mtDNA encoded subunit ATPase 8, it constitutes a channel-like proton acceptor complex which is designed to capture the energy constituted in the pH gradient and membrane potential to drive ATP synthesis (Fig. 1). The F_1 segment of the complex, which consists of $\alpha, \beta, \delta, \gamma$, and ϵ subunits together with the oligomycin sensitivity conferring factor (OSCF), a heat shock protein, and an inhibitor protein (IF_1), contains the adenine nucleotide binding sites and is responsible for the synthesis of ATP (Yamada and Huzel, 1993).

Studies in *E. coli* and yeast mitochondria which have the same basic arrangement of ATPase subunits have shown that the fourth transmembrane amphipathic helix of ATPase 6 and the second transmembrane helix of ATPase 9 are absolutely essential to the working of the F_0 proton channel and capture

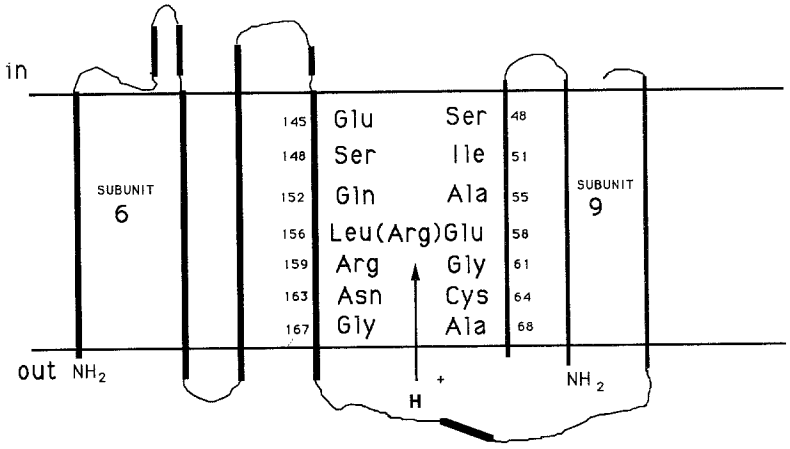

Fig. 2. The arrangement of amphipathic helices in the ATPase 6 and 9 proteins in the mitochondrial membrane. The scheme is that predicted by Cox *et al.* (1986) for the arrangement of five amphipathic helices in the mitochondrial membrane. The fourth amphipathic helix is crucial to the mechanism of oxidative phosphorylation containing the Leu^{156} and Arg^{159} residues. Leu^{156} becomes Arg^{156} in the 8993 mutation. The ATPase 9 protein is shown with its second transmembrane helix opposite the fourth transmembrane helix of ATPase 6.

mechanism (Cox *et al.*, 1986). In all organisms an acidic residue in the ATP 9 protein halfway up the channel formed by the helices of ATPase 6 and 9 is responsible for proton capture (Fillingame, 1992). Opposite this residue (glutamate in eukaryotes, aspartate in *E. coli*) on ATPase 6 are conserved leucine and arginine residues separated by two amino acids (Cox *et al.*, 1986). Changing this leucine residue to an arginine as occurs in the 8993 mtDNA mutation causes the appearance of an extra positively charged residue in the channel (Fig. 2). We have hypothesized that this interferes with the protonation of the glutamate on ATPase 9 (Holt *et al.*, 1990). Changing the equivalent leucine to an arginine in *E. coli* completely abolishes ATPase activity and ATP synthesis (Hartzog and Cain, 1993).

The arginine residue R^{159} of the human sequence is conserved throughout all species in the equivalent position of the fourth amphipathic helix at the ATP 6 protein. Mutation to a lysine abolishes proton transport in *E. coli*, implicating a major role for this residue (Howitt and Cox, 1992). However, revertants with amino acid substitutions in the first amphipathic helix of ATP 6 in *E. coli* are able to pump protons and make ATP, so the system for proton transport in F_0 is a complex one involving interaction between several of the helices of ATP 6 and the ATP 9 or "c" protein embedded in the energy-transducing membrane (Pati and Brusilow, 1991).

IMPAIRMENT OF ATP SYNTHESIS AND CONTROL OF FLUX IN OXIDATIVE PHOSPHORYLATION

How then does the impairment of the F_1F_0 ATPase affect the workings of the ATPase complex and the process of oxidative phosphorylation as a whole? We measured the activity of oligomycin-sensitive ATPase activity in mitochondria of lymphoblasts from two patients with > 95% mutant mtDNA 8993 and found it to be 35% of mean control activity and 42% of the lowest control activity (Tatuch and Robinson, 1993). The immediate implications of this measurement are that the complex still functions, albeit at a slower rate, despite the fact the R^{156} substitutes for L^{156} in the ATPase 6 subunits. The rate of ATP synthesis in digitonin-treated fibroblasts (Fig. 3) is reduced to between 45–49% of the rates in control fibroblasts with NAD-linked substrates, succinate/rotenone, and ascorbate/TMPD. The rate of ATP synthesis in isolated lymphoblast mitochondria was reduced to 67% with NAD-linked substrates, 58% with succinate/rotenone, and 54% with ascorbate/TMPD (Fig. 4). In both the fibroblast system and the lymphoblast mitochondria the pattern of impairment of ATP synthesis closely resembled that obtained with patient fibroblasts and lymphoblasts with cytochrome oxidase deficiency and Leigh's disease (Robinson *et al.*, 1986; Tatuch *et al.*, 1993;

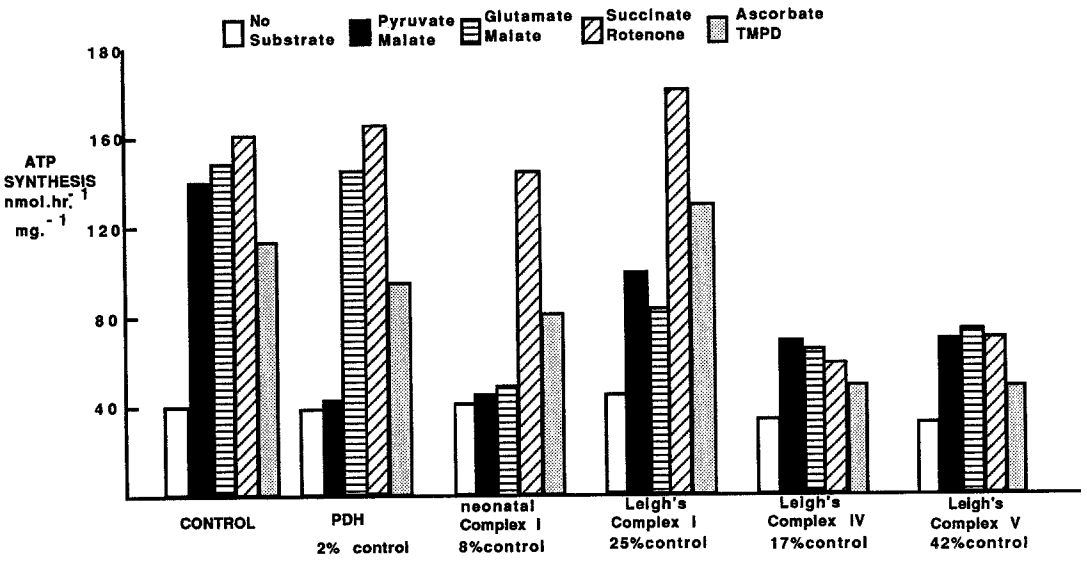

Fig. 3. ATP synthesis in digitonin-treated cultured skin fibroblasts of patients with lacticacidemia. ATP synthesis was measured as described by Robinson *et al.* (1986) in digitonin-treated fibroblasts with either no substrate; with 5 mM pyruvate, 1 mM L-malate; 5 mM glutamate, 5 mM L-malate; 8 mM succinate, 1 μm rotenone; or 1 mM ascorbate, 0.1 mM TMPD. Cell lines used were control; PDH-E_1 deficiency (2% normal activity); complex I deficiency (8% normal activity) with fatal neonatal lacticacidosis; complex I deficiency (25%) with Leigh's disease; complex IV deficiency (17%) with Leigh's disease; and complex V deficiency in a patient > 95% heteroplasmic for the 8993 mtDNA mutation.

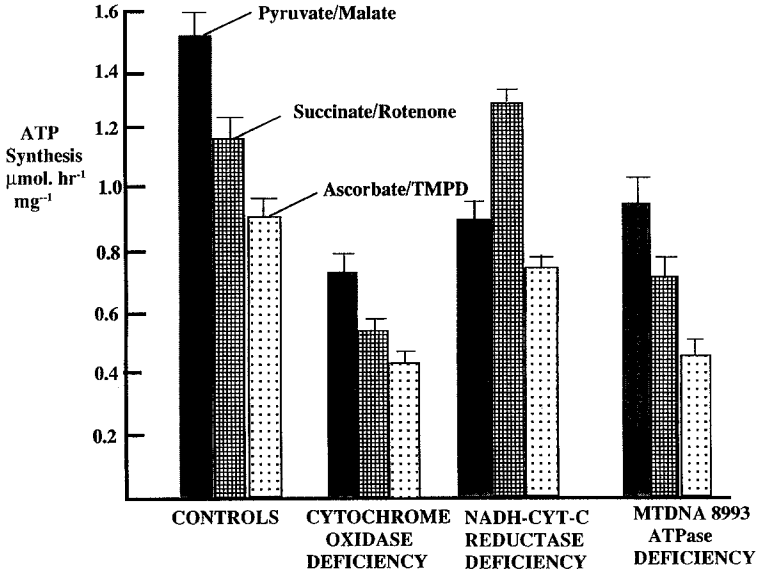

Fig. 4. The rate of ATP synthesis by isolated lymphoblast mitochondria from patients with the mtDNA 8993 mutation. ATP synthesis was measured as described by Tatuch and Robinson (1993) using either: (a) 5 mM pyruvate, 5 mL L-malate; (b) 5 mM succinate, 1 μM rotenone; or (c) 1 mM ascorbate, 0.1 mM TMPD as substrates as indicated above. Values are given in bar form as the mean ±S.E.M. for three controls: one patient with complex I deficiency, one patient with cytochrome oxidase deficiency, and three patients with > 95% mutant 8993 mtDNA ATPase deficiency. Each patient's lymphoblast mitochondria were tested on at least four separate occasions.

Tatuch and Robinson, 1993). Rates with NAD-linked substrates were lower for fatal neonatal lacticacidosis due to complex I deficiency and higher for complex I deficiency with Leigh's disease. Since Leigh's disease due to complex I deficiency seems to have in general a slower rate of progression than COX deficiency or 8993 deficiency (> 95%), it is perhaps not surprising that the capacity for ATP synthesis is slightly better (Moreadith *et al.*, 1984; Robinson *et al.*, 1992).

We have also shown conclusively that the ~ 35% depression of ATP synthesis with NAD linked substrates that applies in lymphoblast mitochondria is true for both low and high substrate concentrations (Tatuch *et al.*, 1993). Thus, the ATPase 6 mutation behaves much like a V_{max} type defect, with the deficit in ATPase operative at all values of the membrane potential and pH gradient. The metabolic control theory developed by Kacser and Burns (1979) and Heinrich and Rapoport (1974) has extensively been applied to the study of mitochondrial metabolism. The important elements of this theory are the derived control coefficients for each step in a pathway which measures the amount of control exertd by a particular enzyme step on the flux of a whole metabolic pathway. The most telling parameter, the control strength C_i, is given by

$$C_i = \frac{dJ/J}{de/e}$$

When J is the steady state of flux through the pathway and e is the concentration of enzyme, dJ is the fractional change in J brought about by the removal from the reaction of a fraction of e (de) tied up by an inhibitor (Kacser and Burns, 1979; Heinrich and Rapoport, 1974; Groen *et al.*, 1982). For a mutation that changes the V_{max} of an enzyme;

$$C_i = \frac{dJ/J}{dV_{max}/V_{max}}$$

since de/e in this case closely approximates to dV_{max}/V_{max}. Thus, for these mutations affecting oxidative phosphorylation in lymphoblast mitochondria, we calculate that the control strength of the ATPase 6 mutation is 0.568, 0.724 and 0.793 for NAD-linked substrates, succinate/rotenone, and ascorbate/TMPD, respectively. This compares with only 0.34 for cytochrome oxidase deficiency (17%) and 0.49 in the case of complex I deficiency (25%). The two control coefficients of these particular enzyme steps would in fact be somewhat lower than these values calculated here simply because we have used only one data point to calculate the control strength. However, it serves to illustrate the point that in severe cases of 8993 mtDNA mutation, for the reduction in activity (58%) of the ATPase, the resultant inhibition of the pathway (35%) at ATP synthesis has a significantly greater influence than the defects in complex I or complex IV. Recent observations on rat muscle mitochondria using oligomycin inhibition of the ATPase give a rather low control coefficienct for complex V of 0.07, 0.17 for complex IV, and 0.11 for complex I (Letellier *et al.*, 1993).

CONCLUSION

Thus, we see that rather minimal disturbances in energy metabolism in mitochondria can have major sequelae in patients with such disturbances. Complex V deficiency has a profound effect clinically despite the fact that oxidative phosphorylation may be compromised only 35%. Defects due to complex I deficiency and complex IV deficiency which produce very similar symptoms have a similar depression of oxidative phosphorylation capacity and also result in the same progressive neurological deterioration syndrome that we recognize as Leigh's disease.

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