Mutations of the mitochondrially encoded ATPase 6 gene modeled in the ATP synthase of *Escherichia coli*

Isla Ogilvie[1], Roderick A. Capaldi*

Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA

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Abstract Defects of respiratory chain protein complexes and the ATP synthase are becoming increasingly implicated in human disease. Recently, mutations in the ATPase 6 gene have been shown to cause several different neurological disorders. The product of this gene is homologous to the *a* subunit of the ATP synthase of *Escherichia coli*. Here, mutations equivalent to those described in humans have been introduced into the *a* subunit of *E. coli* by site-directed mutagenesis, and the effects of these mutations on the ATPase activity, ATP synthesis and ability of the enzyme to pump protons studied in detail. The effects of the mutations varied considerably. The mutation L262P (9185 T-C equivalent) caused a 70% loss of ATP synthesis activity, reduced DCCD sensitivity, and lowered proton pumping activity. The L207P (8993 T-C equivalent) reduced ATP synthesis by 50%, affected DCCD sensitivity, while proton pumping was only marginally affected when measured by the standard AMCA quenching assay. The other mutations studied affected the functioning of the ATP synthase much less. The results confirm that modeling of these point mutations in the *E. coli* enzyme is a useful approach to determining how alterations in the ATPase 6 gene affect enzyme function and, therefore, how a pathogenic effect can be exerted.

Key words: ATP synthase; ATPase 6 gene mutation; Proton pumping defect

1. Introduction

An F_1F_0 type ATP synthase is found in the inner mitochondrial membrane, the inner membrane of bacteria, and the thylakoid membrane of chloroplasts, where it functions to convert the free energy of the proton motive force into the chemical energy source ATP. This large enzyme complex is composed of two major parts, the F_1 part which contains the catalytic sites for ATP hydrolysis and synthesis, and the F_0 part which contains the proton channel. The simplest enzyme form is that of bacteria, which is composed of only eight different subunits. In contrast, the mitochondrial (mt) ATP synthase contains 16 or 17 different subunits (reviewed in [1–3]).

Two subunits of the mt ATP synthase, subunits 6 and 6AL, are encoded on mtDNA [4]. Mutations in mtDNA, including ones in the ATP synthase genes, have been implicated in several human diseases [4]. The most common mutation of ATPase 6, the T8993G mutation, has been found in several studies [5–7] to cause variously NARP, Leigh's syndrome and FSBN, each a serious metabolic, mostly neurological disease of childhood. Other mutations of subunit 6 have been reported [8–14] and implicated in the above diseases, as well as in LHON [15], but the evidence that the specific mutation described is responsible for the observed phenotype is not always easily obtained. One issue is that many of these interesting mutations are heteroplasmic; that is, they occur only in a percentage of the many copies of mtDNA per cell. The significance of this heteroplasmy is related to the so-called threshold effect of mitochondrial diseases[4,16]. It is possible to deplete some cells of components of oxidative phosphorylation without observable effect on cellular functioning. For example, inhibition of up to 70% of the cytochrome *c* oxidase activity of muscle cells by inhibitors, or through a heteroplasmic mutation, shows little or no effect on the overall rate of oxidative phosphorylation [17]. Threshold levels of each enzyme are likely to be different for different tissues, in different developmental stages, and in age-related ways. Therefore, heteroplasmic mutations of mtDNA with a partial reduction of enzyme assembly or functioning may be deleterious in some, but not all cell types.

Examination of genotype-phenotype relationships for mutations of the ATPase 6 gene is additionally complicated by the difficulty in measuring structural and functional parameters of the ATP synthase in the limited amounts of tissue or in cell lines derived from patients. Straightforward approaches to measuring the amount of enzyme assembled are still not available, and measurements of ATP synthesis are difficult and rarely performed. One way that has been used to evaluate the functional effects of identified ATPase 6 mutants is to make similar amino acid changes in the equivalent subunit of the ATP synthase from *Escherichia coli*, subunit *a* [18]. By this approach, Hartzog and Cain [18] confirmed the pathogenicity of the T8993G mutation. They showed that this change of a Leu to Arg affected stability, blocked proton translocation, and abolished ATP synthesis. Since this earlier study, several new mutations of the human ATPase 6 gene have been reported. Here, we have examined these new mutations when generated in the *E. coli* enzyme.

2. Materials and methods

2.1. Construction of mutants in the uncB gene

A 2658 bp fragment of the *unc* operon containing the *uncB, E, F, H* and part of *uncA* genes was isolated from pRA100 [19] using the restriction enzymes *Hin*dIII and *Sfi*I. The fragment was inserted into the *Hin*dIII-*Sma*I sites of M13mp18 by first blunting the *Sfi*I end. Five mutations were introduced into the *uncB* gene by site-directed mutagenesis [20] using the oligonucleotides shown in Table 1. Site-directed mutagenesis was carried out using *E. coli* strain CJ236,

*Corresponding author. Fax: (1) (541) 346-4854.
E-mail: rcapaldi@oregon.uoregon.edu

[1] Present address: Montreal Neurological Institute, 3801 rue Université, Montreal, Que. H3A 2B4, Canada.

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Table 1
List of oligonucleotides used to create mutants

Human muta-tion	*E. coli* mutation	Oligonucleotide
8851 T-C	4457 TT-CG	CAC TGG GCG TAC GTA TCC TGA TTC
8993 T-C	4605 T-C	CCA GTT TCA CCC GGT TTG CG
9101 T-C	4685 CA-AC	GTG GTG GTC AAC GTG GAT CCT G
9176 T-C	4762 T-C	CCT CAT GGT TCC GAC GAT CGT C
9185 T-C	4770 GT-CC	CTG ATG ATC CCC TAT CTG TCG

and all subcloning was carried out in XL1 blue cells. The presence of the mutations for 9176 T-C was confirmed by sequencing single-stranded DNA from the M13mp18 plasmid using the following oligonucleotide as a primer: CAC TGG GTG TAC GTA TCC TGA TTC. The other mutations were shown to be present due to the change in restriction patterns they conferred. The 8851 T-C mutation resulted in the gain of a *Sna*BI site at position 4458 of the *unc* operon; the 8993 T-C mutation produced the gain of an *Nci*I site at position 4605; the 9101 T-C mutation, the loss of a *Tsp*45I site at 4680; and the 9185 T-C mutation, the loss of a *Pvu*I site at position 4769. The mutated portions of the *uncB* gene were excised from M13mp18 using *Dra*III (sites at positions 4403, 5895 and 10995). The 1582 bp fragment containing the mutant and the additional 5610 bp fragment of the *unc* operon were inserted back into the pRA100 plasmid. The *Dra*III sites each consist of a different sequence and, therefore, orientate themselves correctly on ligation. The presence of the 8851 T-C, 8993 T-C, 9101 T-C, and 9185 T-C mutations was checked using restriction digests as for the M13mp18, and the resulting plasmids were designated pIO 13, pIO 8, pIO 9 and pIO 10 respectively. Correct insertion of the 1582 bp fragment for the 9176 T-C (pIO 11) mutation was done by inserting it into pIO 8 and looking for the loss of the *Nci*I site introduced at position 4605. Once constructed, the plasmids were transformed into the AN888 strain where the F_1F_0 is expressed. AN1460 was used as a control strain. AN888 cells containing each of the plasmids pIO 8–13 and AN1460 were grown in 2 l of LB broth overnight. The cells were then harvested and an inner membrane fraction isolated as described in Aggeler et al. [21].

2.2. ATPase assays and DCCD sensitivity

ATPase activities of the mutant inner membranes were measured using the method of Lötscher et al. [22] and the DCCD sensitivity of these activities was assessed as described before [21].

2.3. ATP hydrolysis-driven proton pumping and ATP synthase assays

ATP hydrolysis-driven proton pumping was measured using the method described by Aggeler et al. [21] where changes in pH due to proton pumping across the membrane are assayed with 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching.

The ability of the mutants to synthesize ATP was measured as follows: 150 mg of isolated inner membranes from each mutant was resuspended in 5 ml 50 mM MOPS (pH 7.0), 20 mM $MgCl_2$, 5 mM K_2PO_4, 1 mM ADP. A 500 μl aliquot was removed and 50 mM NADH added to the remainder to start the synthesis of ATP. After 5 min incubation at 37°C, the reaction was stopped with 1% TCA and the samples incubated at room temperature for 10 min. After dilution of the samples to less than 0.1% TCA concentration, the ATP content was assayed using the bioluminescent ATP assay (BioOrbit).

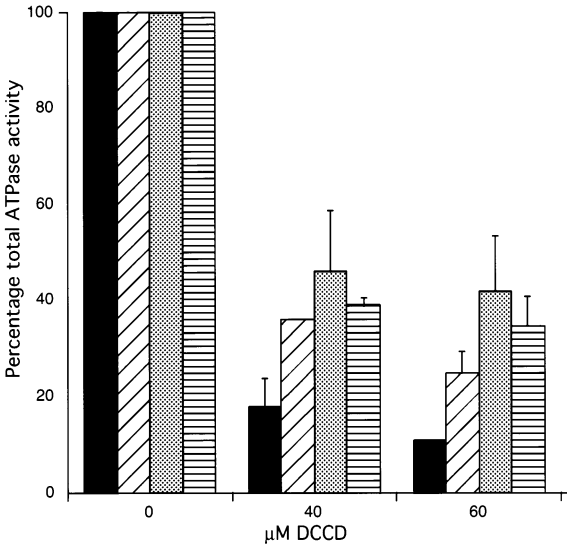

Fig. 1. DCCD sensitivity of ATP hydrolysis activity. Isolated inner membranes from wild-type (black), F158R (diagonal stripes), L262P (gray), and L207R (horizontal stripes) mutants were resuspended in 50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$ and 10% glycerol at a concentration of 0.1 mg protein/ml. Samples were then incubated with and without 40 μM and 60 μM DCCD for 1 h at 22°C. ATPase activities were measured as described by Lötscher et al. [22].

3. Results

A list of mutations that have been reported to date in the mitochondrially encoded ATPase subunit 6 gene is provided in Table 2 along with the disease(s) caused. Also, the equivalent mutations generated for the present study in the *E. coli* enzyme are given. Table 2 lists ATPase activity measured without and with LDAO present. With wild-type, the detergent releases F_1 from the constraints of the F_0 part [22]. Three other functional attributes of the ATP synthase were examined: DCCD sensitivity of the ATPase activity (Fig. 1), ATP-driven proton pumping (Fig. 2), and ATP synthesis (Fig. 3). The effects of the mutations varied widely.

4. Discussion

Two mutants, L207P and V262P, were significantly impaired in ATP synthesis and had reduced sensitivity to DCCD. In the case of the V262P mutation, there was a clear effect on proton pumping function as measured by the ACMA quenching assay. The L207P mutant showed no major effect on proton pumping, although ATP synthesis was reduced by 50%, cf. 70% for V262P. This may reflect the lack of sensitivity of the ACMA quenching assay at optimal ATP hydrol-

Table 2
Mutations of ATPase 6 gene, equivalent mutations in the *E. coli a* subunit, and effect on ATP hydrolysis

Human mutation	Amino acid change	Disease	Reference	Amino acid change in *E. coli*	ATPase activity basal	Level (fold) of stimulation of ATPase activity by LDAO
T8851C	W109R	FSBN	[8]	F158R	1.1	3.3
T8993C	L156P	NARP/Leigh's	[9,10]	L207P	1.1	4.3
T9101C	I192T	LHON	[15]	Q234T	0.7	5.9
T9176C	L217P	Leigh's	[11–13]	L259P	0.7	5.3
T9185C	L220P	Leigh's	[14]	V262P	1.2	2.9
Wild-type:					1.0	3.0

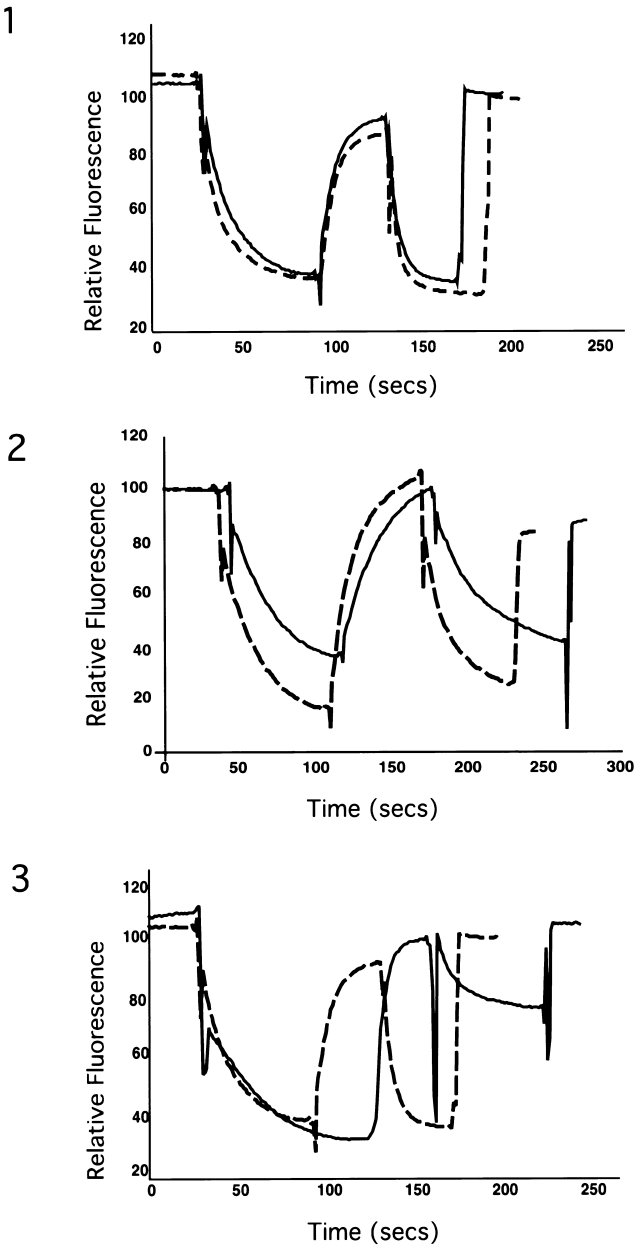

Fig. 2. Effect of *a* subunit mutants on proton pumping. Isolated inner membranes from the mutants and wild-type were resuspended in 10 mM HEPES, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$ at a concentration of 0.1 mg protein/ml. To this resuspension, 3.6 μM valinomycin was added as a K^+ ionophore followed by 1 μM ACMA. NADH (0.5 mM) was added, and the proton pumping by respiratory chain activity was thereby monitored. KCN (2 mM) was then added to block respiration and ATP (2 mM) added to measure ATP hydrolysis-driven proton translocation. Finally, nigericin at 3.6 μM was added to dissipate ion gradients. a: Wild-type (dashed line) and L259P (solid line); b: wild-type (dashed line) and L207P (solid line; c: wild-type (dashed line) and V262P (solid line).

ysis rates. The non-linearity of the ACMA quenching assay has been described elsewhere [23].

The above data for V262P and L207P are consistent with both mutations changing the structure of the *a* subunit, to alter coupling between catalytic sites and the proton channel, without fully destabilizing the binding of F_1 to F_0. In both cases, this must be without disruption and opening of the

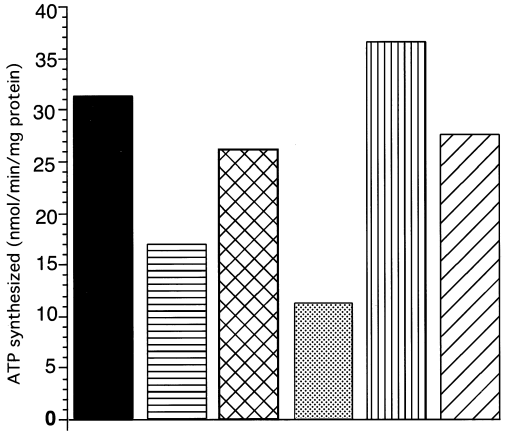

Fig. 3. ATP synthesis by *a* subunit mutants. Inner membranes from wild-type (black), L207P (horizontal stripes), Q234T (cross-hatches), V262P (gray), L259P (vertical stripes) and F258R (diagonal stripes) were treated as described in Section 2. ATP content was then assayed using the BioOrbit bioluminescent ATP assay.

proton channel within the F_0 because electron transfer linked (NADH-driven) proton pumping was not altered measurably. At levels close to homoplasmy, the T9185C mutation has been reported to cause Leigh's disease [14], while the T8993C mutation has been reported to cause either NARP or Leigh's disease [9,10]. It is important to note that replacement of Leu by Pro in the T8993C mutation is less deleterious than the change of this Leu for Arg in the T8993G mutation. The L207R mutation in *E. coli* results in a poorly assembled and unstable enzyme [7,18]. At close to homoplasmy, the T8993G causes severe Leigh's disease [7–9].

Two other mutations, T9101C [15] (Q234T in *E. coli)* and T9176C [11–13] (L259P in *E. coli)*, affected functioning of the ATP synthase in a measurable way. In both, the membrane-bound ATP hydrolysis activity was significantly lower than in wild-type, but there was a high, essentially normal activated ATPase activity in the presence of LDAO, giving a more than fivefold activation by the detergent compared to threefold in wild-type or the V262P mutant. Neither the Q234T or L259P mutations affected proton pumping, DCCD sensitivity or ATP synthesis rates. It appears, therefore, that these mutations in the *a* subunit alter the extent to which the F_0 part controls the ATP hydrolysis reaction. This effect is often explained as a reversal of the inhibiting effect of the ε subunit on ATPase activity by binding of F_1 to F_0 [24,25]. Thus, the ATPase activity of ECF_1-ε is 10-fold higher than that of ECF_1+ε, but only threefold higher than that of ECF_1F_0. In fact, the ε subunit still exerts an effect on ATPase rates in the intact ATP synthase, as shown by both mutation studies of the ε subunit and proteolysis experiments [19,26,27]. Other mutations in the *a* subunit that alter the effect of the ε subunit on ATP hydrolysis have been reported recently [28].

How the T9101C and T9176C mutations cause LHON and Leigh's disease respectively is not made clear by the *E. coli* studies. The same is true of the study of the 8851 T-C mutation which converts a Trp at position 109 to an Arg in humans. In *E. coli*, the equivalent change of a Phe to Arg at position 158 has an altered DCCD sensitivity, but no other effects on the functioning of the ATP synthase. It could be that these last three described mutations are not the prime cause of the observed phenotype. However, this is unlikely

as the polymorphism is rare and, in each case, there are pedigree studies to support the conclusion that these mutations are pathogenic. One way that the T9101C, T9176C and T8851C mutations could each affect oxidative phosphorylation in humans is if these mutations alter the assembly and, therefore, steady-state levels of the ATP synthase. The mammalian enzyme has several more subunits than the bacterial ATP synthase [3,24,29], most of which are in the F_0 and/or stalk region. Altered interaction of the *a* subunit with these additional subunits could cause instability or reduced assembly of the complex. We have recently obtained monoclonal antibodies against subunits of the human ATP synthase, and can examine the assembly of the enzyme for the first time.

In summary, three known mutations of mtDNA in the ATPase subunit 6 gene (two described here and one described by Hartzog and Cain [18]) have now been shown to alter the functioning of the ATP synthase to an extent expected to affect oxidative phosphorylation in human cells. In the T8993C and T9176C mutants examined here, this is due to alterations in the coupling between the F_1 and the F_0 part, apparently in each case without making the membrane increasingly permeable to protons. For both mutations in humans, the pathogenicity will depend on tissue distribution, the extent of heteroplasmy, and the requirements for oxidative phosphorylation, i.e. threshold for normal functioning of the cell type.

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References

[1] Weber, J. and Senior, A.E. (1997) Biochim. Biophys. Acta 1319, 19–58.
[2] Boyer, P.D. (1997) Annu. Rev. Biochem. 66, 717–749.
[3] Fillingame, R.H. (1996) Curr. Opin. Struct. Biol. 6, 491–498.
[4] Wallace, D.C. (1992) Annu. Rev. Biochem. 61, 1175–1212.
[5] Holt, I.J., Harding, A.E., Petty, R.K.H. and Morgan-Hughes, J.A. (1990) Am. J. Hum. Genet. 46, 428–433.
[6] Tatuch, Y., Christodoulou, J., Fergenbaum, A., Clarke, J.T.R., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R. and Robinson, B.H. (1992) Am. J. Hum. Genet. 50, 852–858.
[7] Houstek, J., Klement, P., Hermanska, J., Houstkova, H., Hansikova, H., van der Bogert, C. and Zeman, J. (1995) Biochim. Biophys. Acta 1271, 349–357.
[8] De Meirleir, L., Seneca, S., Lissens, W., Schoentjes, E. and Desprechins, B. (1995) Pediatr. Neurol. 13, 242–246.
[9] Fujii, T., Haltori, H., Higuchi, Y., Tsuji, M. and Mitsuyoshi, I. (1998) Pediatr. Neurol. 18, 275–277.
[10] Santorelli, F.M., Mak, S.C., Vazquez-Meinije, E., Shanske, S., Kranz-Eble, P., Jain, K.D., Bluestone, D.L., De Vivo, D.C. and DiMauro, S. (1996) Pediatr. Res. 39, 914–917.
[11] Makino, M., Horai, S., Goto, Y. and Nonaka, I. (1998) Neuromusc. Disord. 8, 149–151.
[12] Thyagarajan, D., Shanske, S., Vazquez-Meinije, M., De Vivo, D.C. and DiMauro, S. (1995) Ann. Neurol. 38, 468–472.
[13] Campos, Y., Martin, M.A., Rubio, J.C., Solana, L.G., Garcia-Benayas, C., Terradas, J.L. and Arenas, J. (1997) Neurology 49, 595–597.
[14] Robinson, B.H. (unpublished).
[15] Majander, A., Lamminen, T., Juvonen, V., Aura, P., Nikoskelainen, E., Savontaus, M.-L. and Wikstrom, N. (1997) FEBS Lett. 412, 351–354.
[16] Grossman, L.I. and Schoubridge, E.A. (1996) BioEssays 18, 983–991.
[17] Davey, G.P., Peuchen, S. and Clark, J.B. (1998) J. Biol. Chem. 273, 12753–12757.
[18] Hartzog, P.E. and Cain, B.D. (1993) J. Biol. Chem. 268, 12250–12252.
[19] Aggeler, R., Chicas-Cruz, K., Cai, S.X., Keana, J.F.W. and Capaldi, R.A. (1992) Biochemistry 31, 2956–2961.
[20] Kunkel, T.A., Roberts, J.E. and Zakour, M.A. (1987) Methods Enzymol. 154, 367–382.
[21] Aggeler, R., Weinreich, F. and Capaldi, R.A. (1995) Biochim. Biophys. Acta 1230, 62–68.
[22] Lötscher, H.R., deJong, C. and Capaldi, R.A. (1984) Biochemistry 23, 4134–4140.
[23] Zhang, Y. and Fillingame, R.H. (1998) J. Biol. Chem. 270, 24609–24614.
[24] Fillingame, R.H. (1997) J. Exp. Biol. 200, 217–224.
[25] Deckers-Hebestreit, G. and Altendorf, K. (1996) Annu. Rev. Microbiol. 50, 79182.
[26] Tang, C. and Capaldi, R.A. (1996) J. Biol. Chem. 271, 3018–3024.
[27] Mendel-Hartvig, J. and Capaldi, R.A. (1991) Biochemistry 30, 1278–1284.
[28] Gardner, J.L. and Cain, B.D. (1999) Arch. Biochem. Biophys. 361, 302–308.
[29] Walker, J.E. and Collinson, I.R. (1999) FEBS Lett. 346, 39–43.