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Catalytic Activities of Mitochondrial ATP Synthase in Patients with Mitochondrial DNA T8993G Mutation in the ATPase 6 Gene Encoding Subunit *a**

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Alessandra Baracca‡, Silvia Barogi§, Valerio Carelli¶, Giorgio Lenaz‡, and Giancarlo Solaini§‖

From the §Scuola Superiore di Studi Universitari e di Perfezionamento S. Anna, Settore di Medicina, Via Giosuè Carducci 40, 56127 Pisa, Italy, the ‡Dipartimento di Biochimica, Università di Bologna, 48026 Bologna, Italy, and the ¶Department of Ophthalmology, University of Southern California School of Medicine, Los Angeles, California 90033

We investigated the biochemical phenotype of the mtDNA T8993G point mutation in the ATPase 6 gene, associated with neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP), in three patients from two unrelated families. All three carried >80% mutant genome in platelets and were manifesting clinically various degrees of the NARP phenotype. Coupled submitochondrial particles prepared from platelets capable of succinate-sustained ATP synthesis were studied using very sensitive and rapid luminometric and fluorescence methods. A sharp decrease (>95%) in the succinate-sustained ATP synthesis rate of the particles was found, but both the ATP hydrolysis rate and ATP-driven proton translocation (when the protons flow from the matrix to the cytosol) were minimally affected. The T8993G mutation changes the highly conserved residue Leu^{156} to Arg in the ATPase 6 subunit (subunit *a*). This subunit, together with subunit *c*, is thought to cooperatively catalyze proton translocation and rotate, one with respect to the other, during the catalytic cycle of the F_1F_0 complex. Our results suggest that the T8993G mutation induces a structural defect in human F_1F_0-ATPase that causes a severe impairment of ATP synthesis. This is possibly due to a defect in either the vectorial proton transport from the cytosol to the mitochondrial matrix or the coupling of proton flow through F_0 to ATP synthesis in F_1. Whatever mechanism is involved, this leads to impaired ATP synthesis. On the other hand, ATP hydrolysis that involves proton flow from the matrix to the cytosol is essentially unaffected.

Both the neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP)[1] syndrome and the maternally inherited Leigh disease have been associated with the mtDNA T8993G point mutation in the ATPase 6 subunit gene (subunit *a*) of the mitochondrial ATP synthase (F_1F_0-ATPase), being the variable load of the mutant mtDNA (heteroplasmy) associated with the different clinical expression (1–3).

The ATP synthase is the key enzyme for ATP production in mammalian cells. It catalyzes ADP phosphorylation using a proton electrochemical gradient generated by the electron transport chain (4–7). It is a ubiquitous, evolutionary conserved enzyme composed by two main sectors: F_1, a soluble catalytic sector comprising five different subunits that is bound through two stalks to F_0 (8, 9), and the membrane sector that contains up to 10 different subunits (4, 10). Among these are the evolutionary conserved ATPase 6 and the *N*,*N*′-dicycloexylcarbodiimide-binding protein (proteolipid), equivalent to subunits *a* and *c* of the *Escherichia coli* enzyme, respectively (10).

A large body of evidence supports the notion that F-ATPases of all species consist of similar structural motives and that reversible ATP synthesis coupled to a proton flux across F_0 is mediated by conformational changes transmitted from the membrane sector to the catalytic sector of the F_0F_1 complex (4, 11–13). Recent experimental evidence (14–16) supports a mechanical coupling between the F_0 and F_1 sectors based on the rotation of a central rotor, consisting of the smaller subunit(s), with respect to a hexagonal ring made up of the main polypeptides containing the catalytic sites. Energy transduction in this model would occur through a rotation within the F_0 sector powered by proton flow, which promotes ATP synthesis and release. Evidence also suggests that the structural rearrangements concerned with this machinery include subunit *c* of both bacteria and mitochondria (13, 16). Mutant analyses and chemical modification indicate the existence of intermolecular interactions between the transmembrane helices of subunits *c* and *a*. These interactions are thought to be involved in proton translocation since several residues of the *E. coli* enzyme, including Arg^{210} (equivalent to Arg^{159} of the mitochondrial enzyme) and others within the most highly conserved regions of subunits *a* (residues 190–220) and *c* (Asp^{61}, equivalent to Glu^{58} of human subunit *c*), have been shown to be essential for this function (17–20).

The biochemical effect of the T8993G mutation has not been clarified yet, although it was suggested to be an impairment of the F_1F_0-ATPase complex possibly due to a proton channel defect. However, it remains unclear whether ATP hydrolysis is affected by the mutation. Vazquez-Memije *et al.* (21) and Tatuch *et al.* (2) showed that the ATP hydrolysis rate of both skin fibroblast and muscle mitochondria from patients harboring >95% abnormal mtDNA did not change significantly with respect to controls. Tatuch and Robinson (22), using mitochondria isolated from lymphoblastoid cell lines with high percentage mutant mtDNA, subsequently found the rate of ATP synthesis reduced by 33–46%, whereas the ATPase activity was 42% reduced compared with controls. Similar results were reported by others (23). These findings were considered indirect evidence of impaired proton channel function (F_0) in NARP patients. However, Houstek *et al.* (24) found the rate of mito-

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‖ To whom correspondence should be addressed. Tel.: 39-050-883320; Fax: 39-050-883215; E-mail: gsolaini@sssup.it.

[1] The abbreviations used are: NARP, neurogenic muscle weakness, ataxia, and retinitis pigmentosa; ACMA, 9-amino-6-chloro-2-methoxyacridine; SMP, submitochondrial particle; bp, base pair(s); PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

chondrial ATP production in 99% mutant fibroblasts 2-fold lower than in normal fibroblasts and proposed that the mutation could induce structural instability of the enzyme complex. Finally, Trounce *et al.* (25) examined the respiration rate of mitochondria isolated from both patient-derived cell lines and cybrids with the T8993G mutation and found both decreased ADP-stimulated respiration rates and ADP/oxygen ratios suggestive of a proton channel and ADP phosphorylation defect in the F_1F_0-ATPase. However, the proton transport activity of the mutated enzyme has never been measured directly.

To address this point and to clarify whether the ATP hydrolysis rate of the Leu[156]-to-Arg mutated mitochondrial F_1F_0-ATPase subunit *a* was impaired, we analyzed the catalysis and proton translocating properties of the enzyme in platelet-derived submitochondrial particles from three patients, belonging to two unrelated families, harboring at least 80% mutant mtDNA. An ATP-driven proton transport activity similar to that of the enzyme from controls was observed. We analyze this finding in relation to the results of ATP synthesis and hydrolysis rates and discuss the implications for the current model of proton translocation through F_0 and its coupling to the conformational changes in F_1 leading to ATP synthesis.

EXPERIMENTAL PROCEDURES

Materials—ATP, ADP, oligomycin A, valinomycin, Hepes, Tris, trichloroacetic acid, and ACMA were obtained from Sigma. 1243-102 ATP monitoring reagent, a mixture of luciferin and luciferase, was a product of BioOrbit (Turku, Finland).

Samples Investigated—We investigated three previously reported Italian patients, from two unrelated Italian families, carrying high percentages of the T8993G mutation. Patients 1 and 2 are two sisters previously reported by Puddu *et al*. (26), and patient 3 belongs to a second unrelated family (proband of family F) reported by Uziel *et al.* (27). All three patients presented a disease clinically compatible with the original description of NARP syndrome (1). We also investigated 12 controls randomly chosen from the general population. Informed consent was obtained in all cases.

Isolation of Platelet Mitochondria and Submitochondrial Particles Preparation—Human platelets were isolated and purified from 100 ml of venous blood under standardized conditions as previously reported (28). To isolate mitochondria, platelets were suspended in a hypotonic medium (10 mM Tris-Cl, pH 7.6); 4 min later, the suspension was centrifuged at 1500 × *g* for 10 min; and finally, the supernatant was centrifuged at 10,000 × *g* for 20 min to precipitate mitochondria. This procedure was repeated twice. The mitochondria were suspended at 4–8 mg/ml in 0.25 M sucrose and 2 mM EDTA, pH 8. Coupled submitochondrial particles (SMPs) were prepared essentially according to Baracca *et al*. (28) by exposing mitochondria to sonic oscillation on a Labsonic U Braun sonicator for 20 s at the minimum output. The particles were suspended in 0.25 M sucrose to give a protein concentration of 6–8 mg/ml and immediately assayed for ATP-driven proton-pumping activity and for ATP hydrolysis and synthesis.

mtDNA Analysis—Total DNA was extracted from a pellet of the same platelets used for the biochemical assays by the standard phenol/chloroform purification method. To detect the T8993G mutation, a convenient 551-bp segment of mtDNA was amplified by PCR using the pair of primers originally described by Holt *et al.* (1), Forward/8648–8665 and Reverse/9199–9180, according to the Cambridge sequence. The PCR conditions were as follows: one cycle of denaturation for 5 min at 94 °C; 29 cycles each consisting of denaturation for 80 s at 94 °C, annealing for 100 s at 56 °C, and extension for 120 s at 72 °C; and a final cycle of "superextension" for 5 min at 72 °C. This last cycle minimizes the possible formation of heteroduplexes between mutant and wild-type strands. The presence of the mutation was detected by restriction fragment length polymorphism analysis after digestion with the restriction endonuclease *Ava*I. The T8993G mutation introduces a new *Ava*I restriction site; consequently, the 551-bp amplified fragment will be cut in two 345- and 206-bp fragments. The digestion products were separated through a 3% NuSieve-containing 0.5% agarose gel and visualized by UV transillumination after ethidium bromide staining. The co-presence of all three fragments of 551, 345, and 206 bp indicated the heteroplasmy (coexistence of wild-type and mutant mtDNA). The mutation proportion was calculated as the ratio of the 345- and 206-bp fragments *versus* the 551-bp fragment evaluated as intensity of the bands in the gel photographs using the Molecular Analyst PC image analysis software for the Bio-Rad GS-670 densitometer.

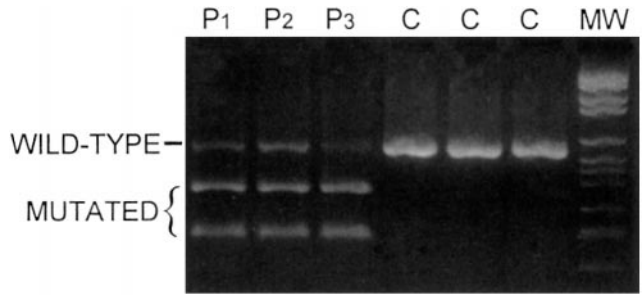

FIG. 1. **mtDNA analysis of platelets.** The *Ava*I digestion of PCR product shows, in the *first three lanes*, the presence of three fragments corresponding to the coexistence of wild-type (551 bp) and mutant (345 and 206 bp) mtDNAs in the patients investigated. Densitometric evaluation indicated the following relative percentages of heteroplasmy: *P1*, 12% wild-type and 88% mutant; *P2*, 20% wild-type and 80% mutant; *P3*, 7% wild-type and 93% mutant. The *Ava*I digestion indicates that the mutation is T8993G. The T8993C mutation occurring at the very same nucleotide position introduces a *Hpa*II restriction site (but not *Ava*I) that is thus the restriction enzyme discriminating between the two mutations (33). Mutant mtDNA was absent in the control samples.

Chemiluminescent Methods for Monitoring ATP Hydrolysis and Synthesis—The ATP synthesis rate was assayed by incubating 20–30 μg of submitochondrial particles in 25 μl of 0.25 M sucrose, 50 mM Hepes, 0.5 mM EDTA, 2 mM $MgSO_4$, 2 mM KH_2PO_4, and 0.2 mM ADP, pH 7.4. 20 mM succinate was added to start the reaction. Incubation was carried out for 10 min at 30 °C, and 5 μl of 50% trichloroacetic acid was added to stop the reaction. The mixture was centrifuged to remove precipitated protein, and the resulting extract was assayed for ATP by the luciferin/luciferase chemiluminescent method (29). Three assays were carried out for each sample.

ATP hydrolysis was assayed as follows. Submitochondrial particles (10 μg) were incubated for 10 min at 30 °C in 25 μl of buffer containing 0.25 M sucrose, 50 mM Hepes, and 2 mM $MgCl_2$, pH 8, and 1 mM ATP was added to start the reaction. To stop the reaction, trichloroacetic acid was added, and non-hydrolyzed ATP was determined in a diluted sample of the reaction mixture by the luciferin/luciferase method as described above. Three assays were carried out for each sample.

Proton-pumping Activity—The proton-pumping activity coupled to the ATP hydrolysis of submitochondrial particles was determined from the quenching of ACMA fluorescence induced upon 0.8 mM ATP addition to the assay medium as described (30). Briefly, the assay medium contained (in a 1-ml final volume) 0.25 M sucrose, 10 mM Tricine, 50 mM KCl, 2.5 mM $MgCl_2$, pH 8, 1 μg of valinomycin, 0.5 μM ACMA, and 0.1 mg of submitochondrial particles. The reaction, performed under continuous mixing, was started by the addition of ATP. Two assays of each sample were carried out at 25 °C on a Jasco P450 spectrofluorometer with excitation and emission at 412 and 510 nm, respectively.

Other Methods—Protein concentration was measured using the method of Lowry *et al.* (31) in the presence of 1% deoxycholate.

Statistics—All data are presented as mean ± S.E. The significance of differences was evaluated by unpaired *t* tests and accepted when $p \leq 0.05$.

RESULTS

mtDNA Analysis—The results of mtDNA analysis are shown in Fig. 1. All three NARP patients presented high percentages of mutant mtDNA, *i.e.* 80% or more (range 80–93%), compatible with the heteroplasmy ratios previously reported (26, 27). We performed the restriction fragment length polymorphism analysis using the same PCR method commonly used for diagnostic purposes. An underestimation of the mutant mtDNA ratio could be due to heteroduplex formation, but this effect had been minimized by performing a final superextension cycle as previously reported (32). Moreover, standard curves prepared with PCR products obtained by Tatuch *et al.* (2) with the same primers we used demonstrated a negligible deviation due to heteroduplex artifacts. Because we are not doing a strict correlation between the mutation load and the biochemical results in the single samples, we can affirm that our biochemical

observations are related to a percentage, conservatively estimated, of at least 80% of T8993G mutant mtDNA in the same tissue sample investigated, the platelets. We also excluded the presence of the T8993G mutation in the platelet mtDNA of the controls.

ATP Synthase and ATPase Activities of Submitochondrial Particles from Platelets of NARP Patients—To assess the biochemical implications of the T8993G mutation, we measured rates of coupled ATP synthesis, ATP hydrolysis, and ATP-driven H^+ pumping. Since the available assays for ATP hydrolysis require large amounts of biological material and the amount of submitochondrial particles obtainable from patients' platelets is small, we used an assay procedure, based on the measurement of the luminescence emitted by the hydrolysis of ATP in the presence of luciferin and luciferase, that consumes as little as 10 μg of protein/assay. Owing to this method, which allowed us to assay ATP hydrolysis and ATP synthesis in very small samples of particles, we could also assay the ATP-driven proton-pumping activity of the particles from the same blood sample.

TABLE I
Mitochondrial activities of the F_1F_0-ATPase from platelets of patients carrying the Leu^{156} to Arg mutation in subunit α

The data are presented as means ± S.E.M.

Activity	Specific activity
ATP synthesis (nmol/min/mg)	
Controls[a]	2.93 ± 0.4
Patient 1	0.25
Patient 2	0.11
Patient 3	0.11
Patients' mean	0.16 ± 0.05[b]
ATP hydrolysis (nmol/min/mg)	
Controls	37 ± 4.5
Patient 1	30
Patient 2	21
Patient 3	29
Patients mean	27 ± 2.8
ATP-driven proton transport (Q/min/mg)[c]	
Controls	52 ± 6.5
Patient 1	69
Patient 2	55
Patient 3	76
Patients' mean	67 ± 6.4

[a] The number of all controls was 12.
[b] Significant difference compared with controls is indicated by $p \leq 0.01$.
[c] Q indicates the fluorescence quenching of ACMA induced upon addition of MgATP to platelet-coupled sumitochondrial particles.

The submitochondrial particle preparations from both normal human and patients' platelets had similar cytochrome *c* oxidase (typically, it was 30 nmol/min/mg of protein), whereas the 5′-nucleotidase activity, used as a probe for contamination, was below the assay sensitivity (specific activity < 1 nmol/min/mg of protein for each sample). These findings indicate comparable and low contamination of the particles in control and mutant samples.

The succinate-sustained ATP synthase activity present in the submitochondrial particles from platelets of NARP patients is shown in Table I. The particles from all patients exhibited a greatly reduced ATP synthase activity ranging from 0.11 to 0.25 nmol/min/mg of protein with respect to the control mean of 2.93 nmol/min/mg of protein. Although, on the basis of the evaluated heteroplasmy of the patients' mitochondrial samples, nearly 20% of the F_1F_0 complexes are active, the ATP synthesis rate decreased by a factor of nearly 20 (on the mean basis, 5% residual synthesis activity). Our expected result was 20% residual activity or higher if the greater driving force for each individual normal F_1F_0 complex was taken into account, as according to Hatefi (6) and Matsuno-Yagi and Hatefi (34). However, the observed lower than expected ATP synthesis rate might be only apparent, for instance, given that the mutated

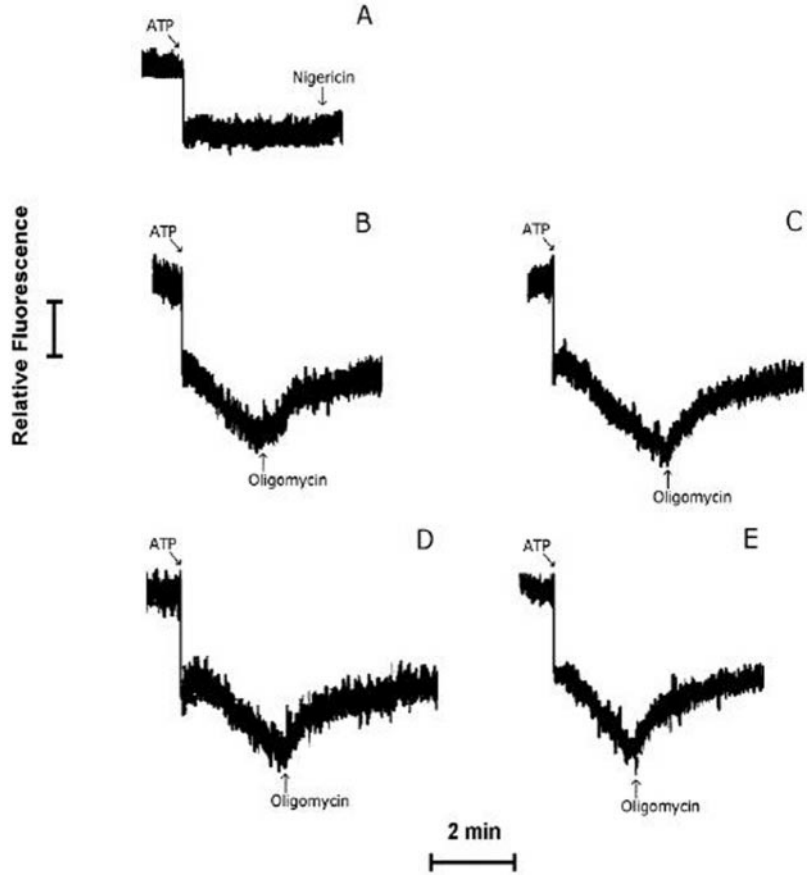

FIG. 2. **ATP-driven proton translocation of human submitochondrial particles containing normal and mutated F_1F_0-ATPases.** Proton gradient formation is indicated by quenching the fluorescence of ACMA, as described under "Experimental Procedures." *A*, SMPs preincubated with oligomycin showing fluorescence quenching upon addition of ATP (30); *B*, a typical trace of the control; *C–E*, traces of patients 1–3, respectively. 100 μg of SMP protein in each assay was used, and oligomycin was added at 0.2 μmol/mg of protein.

F_1F_0 complexes could still fully exert the ATP hydrolysis activity. In fact, at variance with the ATP synthesis, the ATPase activity of the mutated particles was very close to that of controls (37.0 ± 4.5 nmol/min/mg of protein), without any statistically significant difference ($p > 0.3$) (Table I). This observation rules out the possibility that the content of F_1F_0-ATPase might be different in normal and mutated mitochondrial platelets, thus ruling out that a different content of the enzyme could account for the reduction of ATP synthase activity observed in the NARP patients. Finally, it should be mentioned that the oligomycin sensitivity of the different enzyme activities cannot be used as a reliable indicator of the F_1F_0-ATPase activity since it appears that the inhibitor affects the enzyme from controls and patients differently, as has very recently been suggested (35).

ATP-dependent Fluorescence Quenching—F_1F_0-ATPase-mediated proton-pumping activity in submitochondrial particles prepared from NARP patients' platelets was used as an indication of the enzyme proton channel function and of coupling between transport and catalysis. Acidification of inverted membrane vesicles was monitored by fluorescence of ACMA. Adding ATP to the suspension of SMPs, a time-dependent decrease in the fluorescence of the probe was observed until oligomycin was added (Fig. 2). To our surprise, particles derived from the three NARP patients had ATP-driven proton-pumping activity similar to that of controls, and proton transport was completely abolished when oligomycin was added to the particles. This indicates that the fluorescence quenching was in fact due to the ATP-driven proton translocation through the membrane and that the proton transport activity of the mutated enzyme is as sensitive to the inhibitor as the normal type. Although the quenching responses do not correlate linearly with the actual rate of H^+ pumping, the use of ACMA has been validated by several authors investigating different types of vesicles (30, 36, 37). Here, we supply evidence that by slowing the ATPase activity of control particles by lowering the ATP substrate concentration, both the ACMA fluorescence quenching rate and the steady-state fluorescence quenching extent were reduced (Fig. 3). Moreover, by inhibiting the enzyme with substoichiometric oligomycin, the ACMA quenching response was reduced (Fig. 3*D*). These observations indicate that ACMA fluorescence quenching measurements are a reliable means to reveal changes in the F_1F_0-ATPase proton transport activity of platelet submitochondrial particles. Therefore, we conclude that the mutated F_1F_0-ATPase is fully competent for ATP-driven proton transport.

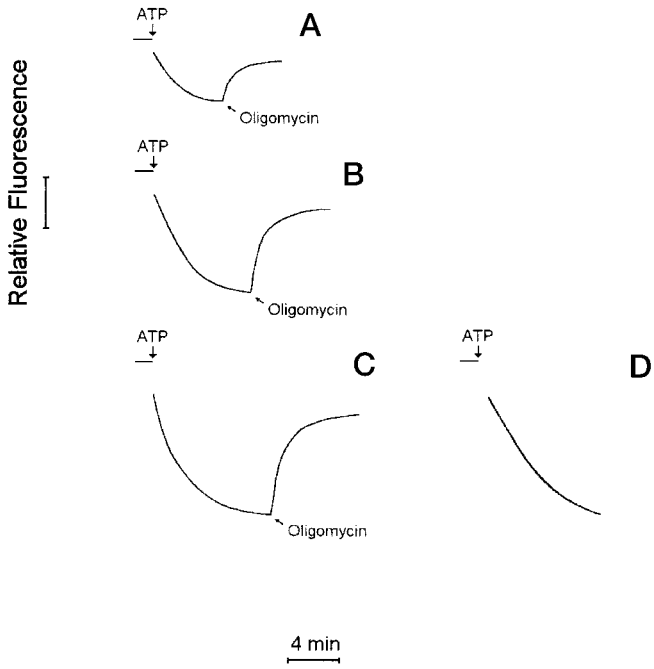

FIG. 3. **ATP-driven quenching of ACMA fluorescence by platelet submitochondrial particles.** The cuvette contained 0.2 mg of SMP protein in 1 ml of assay medium (0.25 M sucrose, 10 mM Tricine, 50 mM KCl, 2.5 mM $MgCl_2$, pH 8, 1 μg of valinomycin, and 0.5 μM ACMA (100 μg/ml in ethanol)). The reaction was started by the addition of increasing ATP concentrations, and the fluorescence emission was recorded at 510 nm after excitation at 412 nm. *A–C*, 0.2, 0.4, and 0.8 mM ATP, respectively, were added. *D*, the particles were preincubated with 0.2 nmol of oligomycin/mg of protein, and ATP was added at 0.8 mM (final concentration), as in *C*.

DISCUSSION

Platelets have been widely used in investigations of mitochondrial diseases and neurodegenerative disorders (38, 39). However, these studies were limited to mtDNA analysis and electron transfer activities, whereas no investigation has been performed on the protonophoric activity of the energy-conserving complexes. The latter is crucial in the pathophysiology of some disorders due to mtDNA mutations, in particular in NARP and maternally inherited Leigh disease phenotypes associated with the T8993G mutation. This mutation is now recognized as the most frequent mtDNA defect associated with Leigh disease (40), although its biochemical effect is still not fully understood.

The results reported in this work indicate that substitution of Leu^{156} with Arg of F_1F_0-ATPase subunit *a* causes a reduction of the ATP synthesis rate in platelet submitochondrial particles, whereas both ATPase and ATP-driven proton translocation through F_0 are not significantly affected. Thus, the ATP hydrolysis rate of the mutated enzyme, which is controversial in the literature (2, 21–23), appears slightly altered, with no statistically significant difference with respect to the control. The differences in data reported might be ascribed to the different biological systems assayed since Tatuch *et al.* (2) and Vazquez-Memije *et al.* (21) did not find any difference in ATPase activity between skin fibroblasts of patients (with >95% abnormal mtDNA) and controls, whereas Tatuch *et al.* (23) found reduced activity in lymphocyte mitochondria with >95% heteroplasmy, and Hartzog and Cain (41) found 50% decreased activity in mutated *E. coli* membranes. However, it remains to be seen why the mutated enzyme from different cells behaves differently. The enzyme may be differently expressed or regulated in different cells.

The second point addressed in this work concerns proton translocation. Our data clearly indicate that protons can be translocated by the mutated enzyme as efficiently as by the control, at least in the direction from the matrix (F_1-binding site) to the cytosolic side of the membrane. This observation was unexpected on the basis of reports in the literature (2, 22, 23, 41).

The T8993G mutation changes a highly conserved leucine 156 to an arginine in a transmembrane helix of subunit *a*. Modeling of F_0 subunits *a* and *c*, both thought to be involved in the proton channel (Fig. 4), shows that this mutation has the effect of placing a positive charge in the vicinity of Arg^{159}, a residue generally thought to play an essential role in both H^+ transport (13, 19, 42, 43) and the induction of the movement of subunits *c* relative to subunit *a* via protonation-deprotonation of the couple *a* Arg^{159}/*c* Glu^{58}.

The proposed topography of the transmembrane helices of subunits *a* (helix 4 (*h4*) and helix 5(*h5*)) and *c* (helix 2 (*h2*)) and amino acids considered to be involved in proton translocation through F_0, Arg^{159} (210 in *E. coli*), His^{168} (Glu^{219} in *E. coli*), and Glu^{194} (His^{245} in *E. coli*), are shown in Fig. 4. According to the

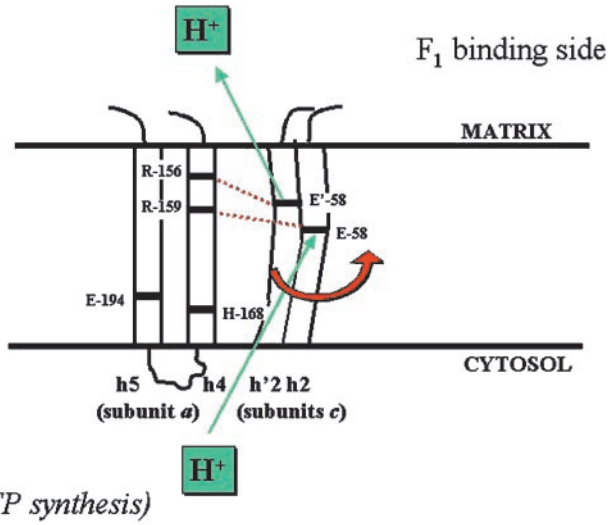

FIG. 4. **Schematic representation of proposed transmembrane helices of subunits *a* and *c* involved in proton translocation, as modified from Fig. 3 of Hatch *et al.* (42).** The amino acid residues discussed under "Discussion" are indicated. The *numbers* refer to the amino acid positions in the human subunits. The *green lines* represent the possible proton pathway; the *red broken lines* indicate the salt bridges referred to under "Discussion." *h*, helix.

alternative mechanism proposed by Hatch *et al.* (42), during ATP synthesis, protons from the intermembrane space, through the involvement of Glu^{194} and His^{168}, would move to Glu^{58} of subunit *c*. This would destabilize the ionic interaction between that Glu^{58} residue and Arg^{159} of subunit *a*. Arg^{159} would then form a salt bridge with the next Glu^{58}, with the energy released driving the rotation of the ring of subunits *c* relative to subunit *a* (44), and a proton would be released from this residue to the matrix side of the membrane. This mechanism implies that the energy released by the H^+ moving down its concentration gradient drives the relative motion of the subunits as described above. One could speculate that the presence of Arg instead of Leu in position 156 of subunit *a* could impede the rotation of subunits *c* relative to subunit *a* due to a possible salt bridge between Arg^{156} of the mutated enzyme and Glu^{58} of that subunit *c* next to that interacting with Arg^{159}, therefore inhibiting the ATP synthase activity of the mutated enzyme. In contradistinction, the driving force for the reverse reaction is ATP hydrolysis on F_1 that moves the asymmetric rotor, of which the ring of subunits *c* is part (16). ATP-driven rotation of subunits *c* relative to subunit *a* might force H^+ to be released from the couple *a* Arg^{159}/*c* Glu^{58} to the cytosolic side of the membrane, whatever amino acid residue is present in position 156 of subunit *a*.

The experimental data may also be interpreted in light of another popular model for F_1F_0-ATPase proton transport coupled to subunit rotation (43), whereby the protons flow from the mitochondrial matrix side to the couple *a* Arg^{159}/*c* Glu^{58}, where the movement of subunits is induced, and then to the cytoplasm, no matter whether Leu or Arg is in position 156. The opposite flow of protons, from the cytoplasm to the matrix, appears most difficult to visualize in the mutated enzyme; the presence of the positively charged and bulky Arg instead of Leu in the pathway from the cytoplasm to the *a* Arg^{159}/*c* Glu^{58} couple might impede the H^+ flow, causing a block of subunit rotation and ATP synthesis inhibition.

The results reported in the present paper are consistent with the results of Cain and Simoni (19) in that they found that the mutations of the *E. coli* residue equivalent to human Leu^{156}, Leu^{207} to Cys or Tyr, resulted in partial loss of F_1F_0-ATP synthase activity, but failed to reduce ATP-driven proton-pumping activity. Similarly, in a very recent paper, Jiang and Fillingame (45) reported that changing Leu^{207} to Cys gave transformant strains that grew considerably slower than the wild type on succinate minimal medium, implying that oxidative phosphorylation was impaired.

Hartzog and Cain (41) reported that ATP synthesis and the ATP-driven proton flux through F_0 were abolished and that ~50% residual ATPase activity was still found in the membrane of an *E. coli* mutant when subunit *a* Leu^{207} was replaced with Arg, in contrast with the present findings, where ATP-driven H^+ pumping by the mutated mitochondrial enzyme was found to be hardly affected by the mutation. The above data suggest a possible difference between the importance of the homologous residues in *E. coli* and humans. It has to be considered that the essential residues Glu^{219} and His^{245} in *E. coli* are replaced by His^{168} and Glu^{203}, respectively, in humans. Moreover, possible subtle differences between the mammalian and bacterial complexes, for instance, in mechanisms present in the eukaryotic enzyme to control proton translocation through F_0, have to be considered; the mammalian F_1F_0 complex contains seven extra polypeptides located in the membrane domain that play unknown roles (46).

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Catalytic Activities of Mitochondrial ATP Synthase in Patients with Mitochondrial DNA T8993G Mutation in the ATPase 6 Gene Encoding Subunit *a*

Alessandra Baracca, Silvia Barogi, Valerio Carelli, Giorgio Lenaz and Giancarlo Solaini

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