ATP6 Homoplasmic Mutations Inhibit and Destabilize the Human F₁F₀-ATP Synthase without Preventing Enzyme Assembly and Oligomerization*§

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The molecular pathogenic mechanism of the human mitochondrial diseases neurogenic ataxia and retinitis pigmentosa and maternally inherited Leigh syndrome was determined in cultured human cells harboring homoplasmic T8993G/T8993C point mutations in the mitochondrial ATP6 gene, which encodes subunit 6 of the F₁F₀-ATP synthase. Immunoprecipitation and blue native electrophoresis showed that F₁F₀-ATP synthase assembles correctly in homoplasmic mutant mitochondria. The mutants exhibited a tendency to have an increased sensitivity to subsaturating amounts of oligomycin; this provided further evidence for complete assembly and tight coupling between the F₁ and F₀ sectors. Furthermore, human ATP synthase dimers and higher homo-oligomers were observed for the first time, and it was demonstrated that the mutant enzymes retain enough structural integrity to oligomerize. A reproducible increase in the proportion of oligomeric-to-monomeric enzyme was found for the T8993G mutant suggesting that F₁F₀ oligomerization is regulated in vivo and that it can be modified in pathological conditions. Despite correct assembly, the T8993G mutation produced a 60% inhibition in ATP synthesis turnover. In vitro denaturing conditions showed F₁F₀ instability conferred by the mutations, although this instability did not produce enzyme disassembly in the conditions used for determination of ATP synthesis. Taken together, the data show that the primary molecular pathogenic mechanism of these deleterious human mitochondrial mutations is functional inhibition in a correctly assembled ATP synthase. Structural instability may play a role in the progression of the disease under potentially denaturing conditions, as discussed.

The F₁F₀-ATP synthase is a ubiquitous enzyme that works as a rotary motor, harnessing the electrochemical proton gradients to carry out ATP synthesis from ADP and Pᵢ. It is composed of a membrane-embedded proton-translocating sector (F₀), coupled to a soluble sector (F₁) that contains catalytic sites for ATP synthesis/hydrolysis. Several of the most deleterious human mitochondrial diseases, such as the maternally inherited Leigh syndrome (MILS) (1, 2), neurogenetic ataxia and retinitis pigmentosa (NARP) (3), and some cases of Leber hereditary optic neuropathy (4), are caused by point mutations in the mitochondrial ATP6 gene that encodes subunit 6 of the ATP synthase F₀ sector.

The same single point mutation can produce either MILS or NARP, depending on the mtDNA mutation load (1, 5, 6). The mtDNA mutations most frequently associated with NARP or MILS are T8993G, T8993C, T9176G, and T9176C, which replace the conserved leucine residues at positions 156 or 217 of subunit 6 by arginine or proline, respectively (6, 7).

NARP/MILS mutations inhibit ATP synthesis (5–77%) and hydrolysis (0–14%) in isolated mitochondria from patient cells (8–12). A similar inhibition is observed when these mutations are modeled in Escherichia coli (13–15). The substitution of Leu-156 by arginine (mutation T8993G) is more deleterious to ATP synthesis than its substitution by proline (mutation T8993C) (60 versus 25% inhibition in human mitochondria) (6, 8) and therefore leads to more severe NARP/MILS manifestations (16–19). In addition, mitochondria bearing the T8993G mutation are hyperpolarized (9, 20) and have an increased production of reactive oxygen species (20). These functional defects could be caused either by impairment of enzyme assembly or by proton conduction blockade in a completely assembled enzyme. In both scenarios, there would be fewer functional F₀ sectors, and thus ATP synthesis inhibition would occur.

Topology models indicate that NARP/MILS mutations cluster in the most conserved region of human subunit 6 (6, 7, 21), which is involved in proton flux through F₀ (22, 23). The core of this conserved region is an essential arginine residue (Arg-159 in the human enzyme) that undergoes a sequential interaction with the essential carboxyl in each c subunit during enzyme rotation and proton translocation (24). Thus, NARP/MILS mutations could hinder proton translocation by charge redis-

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§ The abbreviations used are: MILS, maternally inherited Leigh syndrome; BN-PAGE, blue native-PAGE; IF 1, F₁-ATPase inhibitor protein; NARP, neurogenic ataxia and retinitis pigmentosa; OSCP, oligomycin sensitivity conferring protein; RFLP, restriction fragment length polymorphism; SMP, submitochondrial particles; BisTris, 2-[bis(2-hydroxyethyl) amino]-2-(hydroxymethyl)propane-1,3-diol.
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tributions or subtle structural changes in the interface of subunit 6 and the barrel of c subunits (7, 9).

On the other hand, the transmembrane location of NARP/MILS mutations may induce subunit mis-folding and interfere with the assembly of the complete $F_{1}F_{0}$. In this scenario soluble $F_{1}$ and incomplete $F_{1}F_{0}$ complexes would be found in mitochondria, as reported for mtDNA-less Rho$^{0}$ cells lacking ATP$6$ and ATP$8$ (9, 25). Incomplete $F_{1}F_{0}$ complexes have been found in postmortem heart tissue from MILS patients (26), in NARP/MILS cybrids (27), and in skeletal muscle biopsies from these patients (25). In contrast, a normal content of $F_{1}F_{0}$ subunits, including subunit 6, was found in the enzyme isolated from patient cultured fibroblasts (9). To establish the molecular mechanism through which NARP/MILS mutations induce disease, it is crucial to determine unambiguously whether these point mutations impair $F_{1}F_{0}$ assembly.

Most of the previous structural studies have been done with heteroplasmic cells, where wild type and mutant ATP$6$ genes are co-expressed. To avoid wild type complementation in ATP synthase assembly or function, we used homoplasmic T8993G and T8993C cultured human cells to assess conclusively the effect of these mutations on the assembly of the ATP synthase. A novel immunoprecipitation procedure (28) was instrumental to isolate the whole $F_{1}F_{0}$ complex from human mitochondria. In parallel, blue native electrophoresis (BN-PAGE) (29) was used to estimate the amounts of native $F_{1}F_{0}$ complexes in wild type and mutant mitochondria.

The eukaryotic $F_{1}F_{0}$ ATP synthase forms homodimers (30–32) and larger oligomers (33) that contribute to mitochondrial type and mutant mitochondria. To establish the molecular mechanism through which NARP/MILS mutations induce disease, it is crucial to determine unambiguously whether these point mutations impair $F_{1}F_{0}$ assembly.

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The eukaryotic $F_{1}F_{0}$ ATP synthase forms homodimers (30–32) and larger oligomers (33) that contribute to mitochondrial cristae morphology (34). To date, this oligomerization has not been studied for the human enzyme. Here we demonstrate that ATP$6$ mutations do not impair dimerization and homo-oligomerization of the human $F_{1}F_{0}$. Functional and instability defects produced in the whole $F_{1}F_{0}$ by these mutations are assessed and discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—T8993G and T8993C patient skin fibroblasts were kindly provided by Professor Brian H. Robinson (Hospital for Sick Children, Toronto, Canada). Control human MRC5 fibroblasts were purchased from In Vitro S.A. Homoplasmic T8993G and T8993C cybrids (produced by the fusion of enucleated patient skin fibroblasts with Rho$^{0}$ 143B/TK osteosarcoma cells) and control Rho$^{0}$ 143B/TK osteosarcoma cells were derived from cell cultures at the laboratory of Prof. Rosalba Carrozzo, as described in Ref. 20. Cell culture was carried out in high glucose media, as described before (9), but in the presence of antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 μg/ml amphotericin B) from Invitrogen.

**Antibodies**—Monoclonal antibodies, 12F4AD8, anti-α, anti-OSCP, and anti-IF$_{1}$, were from Mitosciences and were used according to the manufacturer.

**DNA Isolation, PCR, and RFLP**—Total DNA was isolated from 1 × 10$^{6}$ cells. A segment of the ATP$6$ gene was amplified by PCR; products were digested with the endonuclease Mspl (Promega) and separated in PAGE for RFLP analysis.

**Isolation of Human Mitochondria**—Mitochondria were isolated from 180 to 200 × 10$^{6}$ cells by differential centrifugation, as described in Ref. 9. Submitochondrial particles (EDTA-SMP) were prepared by sonicating isolated mitochondria as in Ref. 8.

$F_{1}F_{0}$ immunoprecipitation was carried out from 3 mg of human mitochondria solubilized with n-dodecyl-β-D-maltoside (Sigma), as described previously (28). Where indicated, prior to solubilization, human mitochondrial membranes were obtained by subjecting mitochondria to an osmotic shock, as described previously (9).

**BN Electrophoresis, Second Dimension SDS-PAGE, and Western Blot**—BN-PAGE was as described previously (29). The solubilization buffer consisted of 0.75 M ε-aminocaproic acid, 50 mM BisTris-HCl, pH 7. Digitonin or n-dodecyl-β-D-maltoside (Sigma) was used for mitochondrial protein extraction. Titration curves were carried out to establish the appropriate detergent amounts to extract intact $F_{1}F_{0}$ complexes in its monomeric and oligomeric forms. ATPase activity was developed in native gels as described previously (34). Single native lanes were submitted to second dimension SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed with antibodies as described previously (35).

**ATP Synthesis and Hydrolysis Assays**—Spectrophotometric measurements of ATP synthesis rates coupled to NADP$^{+}$ reduction were carried out at 340 nm with 30 μg of isolated mitochondrial membrane as described (36), including 100 μM diadenosine pentaphosphate to inhibit adenylate kinase activity. Alternatively (see Fig. 5A), the phosphorylation of ADP by inorganic phosphate ($^{32}$P) was used to determine ATP synthesis (8).

ATP hydrolysis rates were measured spectrophotometrically with mitochondrial membranes obtained by osmotic shock, using an ATP-regenerating system, as described before (9). Where indicated, prior to measurements, mitochondrial membranes were resuspended in “activation conditions” consisting of 125 mM KCl, 2 mM EDTA, 30 mM Tris-SO$_{4}$ pH 8.0, and protease inhibitors (0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and incubated at the indicated times at 40°C to release the inhibitory protein (IF$_{1}$). Control “nonactivation conditions” were 0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4 and protease inhibitors (as above). To verify IF$_{1}$ release, membranes were pelleted by centrifugation at 15,000 × g, and supernatants were assayed by Western blot for the presence of IF$_{1}$. ATP hydrolysis was carried out by SMP was measured by the colorimetric determination of P$_{i}$ released from ATP, as reported (8).

**Other Methods**—Protein was measured by a modified Lowry method (see Ref. 37). SDS-PAGE was according to Laemmli (38). Densitometry analyses were carried out with Alpha-DigiDoc computer software coupled to a Kodak DC290 digital camera.

**RESULTS**

**T8993G Mutation Loads**—We studied two human cell cultures harboring the T8993G mutation, namely skin fibroblasts and cybrids, with their respective controls. The T8993G mutant mtDNA load was determined in each cell culture by RFLP with the endonuclease Mspl on a 764-bp ATP$6$ gene fragment. The wild type restriction pattern was absent from mutant samples (<1% of total DNA, by densi-
tometry analysis), regardless of the amount of genomic DNA used for amplification (supplemental Fig. 1). Therefore, we confirmed that the cells used in this study are homoplasmic for the T8993G mutation.

Homoplasmic T8993G Mutants Show Decreased ATP Synthesis—Oligomycin-sensitive ATP synthesis rates of homoplasmic T8993G mitochondria decreased to 40% of their respective controls (Fig. 1, A and B). This further confirms the homoplasmicity of the studied samples, because only the homoplasmic T8993G mutation results in 60% inhibition of ATP synthesis rates (9). Small amounts of wild type mtDNA complement substantially the ATP synthase activity; accordingly, mitochondria with 91% mutant load have only a 25% ATP synthase inhibition (9). Osteosarcoma mitochondria had 30% higher synthesis rates than those obtained from fibroblasts, probably because of a better yield of coupled mitochondrial membranes. However, the ATP synthesis rates of the four cell types showed a similar inhibition when incubated with saturating concentrations of oligomycin (Fig. 1, A and B).

Immunoprecipitation of the Human F1F0 Complex from Homoplasmic Control and T8993G (L156R) Mitochondria—Aggeler et al. (28) described a one-step isolation approach to immuno-capture the whole, functional, oligomycin-sensitive F1F0 complex from small amounts of solubilized human mitochondria with a monoclonal antibody (12F4AD8). Because the antibody recognizes the native F1 portion,4 enzyme assembly can be tested with this procedure, i.e. in the properly assembled F1F0 complex, F0 subunits will co-immunoprecipitate with the F1 portion. By SDS-PAGE of immunoprecipitated samples and band densitometry analyses, it was possible to estimate the ratio of F0 to F1 subunits. Subunit 6 was present at similar intensity ratios in the immunoprecipitated F1F0 complexes from control and mutant mitochondria (Fig. 2, A and B). Thus, assembly of the human F1F0-ATP synthase was not prevented by the L156R mutation, neither in fibroblasts (Fig. 2A) nor in cybrids (Fig. 2B). In both cell types, the protein yields of immunoprecipitated F1F0 were similar between control and mutant mitochondria.

These results are consistent with those previously obtained with the human ATP synthase isolated by affinity chromatography from heteroplasmic (91%) T8993G mitochondria (9). As in that study, the F1F0 subunits resolved here were α, β, γ, b, OSCP, d, and subunit 6; however, small subunits of F1 (ε, IF1) and F0 (ε, f, g, F6, A6L, and c) were not resolved.

For the gel shown in Fig. 2B, mitochondrial membranes were obtained by osmotic shock prior to solubilization and immunoprecipitation. This improved the purity of the enzyme, and it was useful to analyze the amount of F1 subunits released from mitochondria by osmotic shock as markers of ATP synthase disassembly. The content of subunits α, OSCP, and IF1 was similar in the supernatants of shocked control and mutant mitochondria, as shown by Western blot (supplemental Fig. 2). Furthermore, no differences in subunit content or intensity ratios were found between F1F0 immunoprecipitated from whole or osmotic-shocked mitochondria (not shown). Taken together, these results confirm that the mutant subunit 6 incorporates properly into the fully assembled human F1F0-ATP synthase.

Native Electrophoresis of Control, T8993G, and T8993C Mitochondria—We used blue native-PAGE to compare the amounts of native monomeric and oligomeric F1F0 extracted from control and mutant mitochondria, as an alternative method to check for assembly defects caused by subunit 6 mutations. To compare the effects of leucine 156 substitutions by arginine or proline, we included T8993G (L156R) and T8993C (L156P) human fibroblast mitochondria.

Fig. 3, A and B, shows the bands corresponding to the native oxidative-phosphorylation complexes I, III, IV, and V, obtained from dodecyl maltoside mitochondrial extracts. The band containing F1F0 (complex V) was assigned by in-gel development of ATPase activity and by the subunit pattern found in second dimension SDS-PAGE (not shown). The complete monomeric F1F0 was present in similar amounts in control and mutant mitochondria (both L156R and L156P) as confirmed by densitometry ratios of complex V (ATP synthase) and complex III (dimeric bc1) (Fig. 3, A and B).

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4 J. J. García, unpublished observations.
To detect small amounts of incomplete \( F_{1}F_{0} \) or soluble \( F_{1} \) as markers of enzyme disassembly, individual BN-PAGE lanes were submitted to second dimension SDS-PAGE and anti-\( \alpha \) Western blotting. In control (Fig. 3C) as well as in L156R (Fig. 3D) mitochondria, \( \alpha \) subunits were detected only in two positions of the blot, corresponding to the BN-PAGE migration of monomeric \( F_{1}F_{0} \) and \( F_{1} \), respectively. Equivalent results were obtained for L156P mutants (not shown). These experiments demonstrated that small quantities of \( F_{1} \) are detectable in human mitochondrial extracts, but these are not increased in mutant samples as compared with controls. Taken together the data confirm that L156R and L156P NARP/MILS mutations do not impair monomeric \( F_{1}F_{0} \) assembly.

Mitochondrial \( F_{1}F_{0} \) can further assemble into dimers (30, 32) and higher oligomers (33) that control cristae morphology. Therefore, to explore \( F_{1}F_{0} \) oligomerization in the L156R/L156P human mutants, mitochondrial digitonin extracts were subjected to BN-PAGE, followed by second dimension SDS-PAGE and \( \alpha \) subunit immunodetection. The \( \alpha \) subunits were found at the positions corresponding to the migration of \( F_{1}F_{0} \) supercomplexes (V) (31) and monomeric \( F_{1}F_{0} \) in BN-PAGE (Fig. 4, A–E). The identity of the supercomplexes as ATP synthase homo-oligomers was corroborated by parallel analyses of bovine heart mitochondria (not shown). From the several oligomeric forms of the human ATP synthase detected, the most abundant had an apparent mass of 1400 kDa and migrated between the monomeric complex I (1000 kDa) and the supercomplex composed by I + III, (1500 kDa), thus corresponding to dimeric \( F_{1}F_{0} \). This dimer dissociated progressively as the digitonin concentration was raised in parallel to an enrichment of monomeric \( F_{1} \) and \( F_{1}F_{0} \) (Fig. 4, A–E). On the other hand, larger \( F_{1}F_{0} \) oligomers showed a lower mobility than the I + III, supercomplex (see far left side of Fig. 4, A–C) and therefore are likely composed by ATP synthase trimers and/or tetramers. However, formation of ATP synthase hetero-oligomers, i.e., associations with other mitochondrial proteins such as adenine nucleotide and \( P_{i} \) carriers as in the ATP synthasome (39), is also possible. These larger ATP synthase oligomers had a better resistance to dissociate with increasing digitonin concentrations than the dimer. In osteosarcoma mitochondrial extracts, these oligomers seem absent (Fig. 4, D and E); however, further BN-PAGE and two-dimensional PAGE analyses are required to determine whether these apparent differences simply reflect a lower electrophoretic resolution of the osteosarcoma mitochondrial extracts. Alternatively, these differences could reflect changes in protein expression and/or lipid content between control and osteosarcoma mitochondrial preparations. To avoid errors in the densitometric estimations derived from variations in electrophoretic resolution, the densities of all ATP synthase oligomers observed were added together in a single composed density. This is indicated in Fig. 4, A–E as “\( V_{n} \).”

The ATP synthase oligomers were found in all human mitochondrial preparations, regardless of the presence of an \( ATP_{6} \) mutation (Fig. 4, A–E). Because the proportion of oligomeric enzyme is strongly dependent on the amount of detergent used for extraction, we carefully titrated detergent-to-protein ratios in control and mutant mito-
chondria. This procedure was instrumental for comparing by densitometry the proportion of oligomeric (V) and monomeric (V) F1F0 between ATP6 mutants and their controls. The oligomer-to-monomer ratios (V/V) are plotted in Fig. 4, F and G, for fibroblasts and cybrids, respectively. The results show that the L156R/L156P mutations do not impair dimerization or higher order oligomerization of the human F1F0-ATP synthase. Rather, a tendency to increase F1F0 oligomerization was observed in mitochondria harboring the L156R homoplasmic mutation (Fig. 4, F and G). Mitochondria solubilized from different preparations at 1.5 g of digitonin/mg protein had an average oligomer-to-monomer ratio of 0.62 ± 0.11 for control and 1.47 ± 0.43 for L156R cybrids (n = 5, representative experiments are shown in Fig. 4 and supplemental Fig. 3).

Oligomycin Sensitivity in T8993G and T8993C Mutants—Based on studies on heteroplasmic T8993G cells, some groups (11, 40) propose that the pathogenic mechanism of NARP/MILS is a partial uncoupling of F0-proton translocation from F1-ATP synthesis. We evaluated functional coupling between F1 and F0 sectors in the homoplasmic T8993G cybrids by assaying oligomycin sensitivity during ATP synthesis and hydrolysis (Fig. 5, A and B). Oligomycin binds to F0, blocking the proton channel and thus inhibiting F1 catalysis in a well coupled enzyme. As shown in Fig. 5, A and B, the L156R mutant and control enzymes showed similar oligomycin sensitivities in both activities measured. However, the L156R mutant had a trend to show a higher oligomycin sensitivity that is more evident at oligomycin amounts around 0.02 μg/mL protein. This tendency is in consonance with the higher oligomycin sensitivity reported previously for ATP synthesis in fibroblasts with the same mutations (8). A similar trend was also observed with mitochondria isolated from human fibroblasts harboring the L156R/L156P mutations (not shown). These experiments provide further evidence of the tight coupling that exists between F1 and F0 sectors in the mutant enzyme preparations studied here.

F1F0 Destabilization in T8993G (L156R) Mutants—Because no F1F0 assembly defects were found in mitochondria from NARP/MILS mutants, we compared the functional and structural stability of the wild type and L156R mutant F1F0 by incubation in potentially destabilizing conditions. When F1F0 ATPase activity was measured in conditions of maximal turnover (“activation conditions,” i.e. in vitro conditions that release the inhibitory subunit IF1), the L156R mutants failed to increase their hydrolytic activities as the wild type does (Fig. 6). The time course of activation showed a classical bell-shaped curve for the wild type enzyme, corresponding to the balance between enzyme activation by IF1 release and enzyme inactivation that becomes evident after 90 min. In contrast the mutant did not show activation but only a tendency to inactivate along the whole time course. As shown in Fig. 6 (inset), the mutant and wild type whole mitochondria had a similar IF1 content (see α/IF1 ratios for lanes 1 and 2), and both mitochondrial membranes released a similar amount of IF1 to the supernatant after a 90-min incubation at 40 °C in activation conditions (lanes 5 and 6). Thus, the lack of activation in the mutant F1F0 is not a result of IF1 overexpression or failure to release IF1. We therefore looked for structural F1F0 alterations associated with the lack of mutant activation. Control and L156R whole mitochondrial membranes were activated during 90 min; afterward dodecyl maltoside extracts of “activated” and “nonactivated” membranes were obtained. These extracts were subjected to BN-PAGE, second dimension SDS-PAGE and immunode-
EDTA-SMP with saturating amounts of oligomycin (40 μM, around 80%) with saturating oligomycin. In contrast, for control mitochondria reached a similar maximal inhibition with a small increment in the spot corresponding to soluble F1 (Fig. 7D). Thus the monomeric form of the L156R mutant F1F0 is unstable under activation conditions, as compared with the wild type enzyme.

To look for evidence of F1 release from F0 under other destabilizing conditions, activated submitochondrial particles (EDTA-SMP) were prepared by sonication from control osteosarcoma and L156R cybrid mitochondria. The ATPase activities that remained in the particles were compared with the activity released to the supernatant. From the total ATPase activity obtained after sonication, 21 and 27% were found in the supernatant of control and L156R SMP after ultracentrifugation, respectively (not shown). In both cases the hydrolytic activity of the supernatants was 75–78% sensitive to the F1 inhibitor efrapeptin (75 μM/mg protein). Thus, release of F1 during SMP preparation is slightly higher in mutant mitochondria than in wild type.

As shown in Fig. 5B, the ATPase activities from mutant and control mitochondria reached a similar maximal inhibition (around 80%) with saturating oligomycin. In contrast, for EDTA-SMP with saturating amounts of oligomycin (40 μM/mg protein), ATPase activity was inhibited 72% in control, but only 58% in the L156R mutant (not shown), suggesting some enzyme uncoupling because of sonication that is more evident in the mutant.

We next compared the stability of control and L156R mutant monomeric F1F0 by incubation of dodecyl maltoside mitochondrial extracts at 40 °C. In these experiments samples were heated directly in the solubilization buffer in which they were extracted and subsequently loaded onto blue native gels. A representative Coomassie-stained native gel is shown in Fig. 8, upper panel, and the lower panel shows the immunodetection of α subunits in an equivalent native gel. The L156R monomeric ATP synthase (complex V) was readily lost after a 2.5 min at 40 °C, yielding F1 and other disassembly intermediates. In contrast, most of the wild type enzyme resisted a 5-min incubation at 40 °C. It should be noted that the observed disassembly occurred only for complex V in these conditions; complexes I and III remained assembled at the incubation times shown. The F1F0 disassembly intermediate marked with an asterisk (Fig. 8, lower panel) is compatible with those described previously for TS993G mitochondria (25–27), which mainly contain F1 and c subunits. The detergent present in the samples (3 g of dodecyl maltoside/g of protein) probably accounts for the short times that are sufficient to observe enzyme disassembly in this experiment.

We also analyzed if the dimeric human F1F0 is destabilized by ATP6 mutations. Digitonin extracts that contained dimeric and monomeric F1F0 were obtained; aliquots were incubated at 40 °C for 90 min in the solubilization buffer used for extraction and compared in BN-PAGE and SDS-PAGE with their non-heated counterparts. After heating, the ratio of oligomeric-to monomeric F1F0 decreased from 0.64 to 0.33 for the control enzyme and from 1.89 to 0.34 for the L156R mutant (supplemental Fig. 3). This figure also shows that there is a higher IF1 content in the multimeric F1F0 complexes of control and mutant enzymes as compared with the IF1 amounts associated with monomeric F1F0 (supplemental Fig. 3, A and B). This result is in concordance with the role of IF1 in promoting dimerization of the whole F1F0 complex as found by IF1 reconstitution or its overexpression in bovine and rat mitochondria or submitochondrial particles (41). Supplemental Fig. 3 also shows that IF1 is released by heat from monomeric and oligomeric F1F0 in both control and mutant enzymes (compare A and B with C and D). Furthermore, no smearing of the α-signal was observed after heating, two-dimensional PAGE, and West-
properly assembled human F$_1$F$_0$-ATP synthase carrying subunit 6 mutations. Among the possible consequences of ATP$_6$ point mutations, impaired F$_1$F$_0$ assembly (26, 27), enzyme uncoupling (11, 40), and proton conduction blockade through F$_0$ (6, 7, 9) have all been proposed. To distinguish between them, concluding evidence on the effect of NARP/MILS mutations on F$_1$F$_0$ assembly was limited because the reported human F$_1$F$_0$ structural studies have been done mostly with heteroplasmic cells and provided apparently opposing results (9, 25–27). Here we demonstrate the proper assembly of the F$_1$F$_0$ complex in two T8993G homoplasmic human cell cultures, namely fibroblasts and cybrids, by immunoprecipitation (Fig. 2) and direct electrophoretic resolution of the native enzyme (Figs. 3 and 4). Furthermore, tight coupling between the F$_1$ and F$_0$ sectors in the L156R mutant enzyme was evidenced by the oligomycin sensitivity found in ATP synthesis and hydrolysis (Fig. 5). Because structural integrity is essential for oligomycin inhibition, this confirmed that the mutant F$_1$F$_0$ complex is correctly assembled, even in the presence of $>$99% T8993G mutated DNA copies.

Cybrids and osteosarcoma control cells share the same nuclear background, which allows focusing on the pathogenic effects of the mtDNA mutation. Nevertheless, we had equivalent results with fibroblasts obtained from patients, which have not been genetically manipulated. The homoplasmicity of the mutant cultures used in this work guaranteed the study of enzymes containing either the normal or the mutated subunit 6, but not a mixture of them. Additionally, because the ATP synthase dimerizes in mitochondria, mainly through F$_0$ interactions (30, 32), we studied for the first time the existence of F$_1$F$_0$ dimers and higher oligomers in human mitochondria (Fig. 4). Human F$_1$F$_0$ oligomerization was not prevented by the mutations studied here. This further confirms the lack of assembly defects in both of the most common NARP/MILS mutations (L156R and L156P) and corroborates that the small F$_0$ subunits (which were not resolved here by SDS-PAGE) are present in the mutant F$_1$F$_0$ complex. These likely include subunits e and g, which are essential for ATP synthase dimer formation in yeast (30, 34). A consistent increase in oligomerization that is more evident in cybrids was found in L156R mitochondria. This suggests the interesting possibility that enzyme oligomerization may be upregulated as a compensatory response to the mutation, because the oligomeric enzyme could be more stable and/or more efficient to carry out ATP synthesis.

Taken together, our data show that it is possible to obtain a fully assembled but catalytically inhibited F$_1$F$_0$-ATP synthase from homoplasmic NARP/MILS mitochondria. Therefore, the L156R/L156P mutations per se do not impair the assembly route of the human F$_1$F$_0$-ATP synthase. Our results are in apparent contrast with the large amounts of sub-assembled F$_1$F$_0$ complexes found previously with BN-PAGE of T8993G mutant samples (25–27). However, most of those studies did not address whether ATP$_6$ mutations directly impair enzyme assembly, because they used postmortem (26) or biopsy muscular tissues (25), which have sustained metabolic stress because of disease and tissue extraction. On the other hand, when assembly was addressed in different T8993G cybrid lines (27), sub-assembled complexes were found only in some lines, although they all harbored the mutation. We consider that our results can be reconciled with the previous reports by assuming that either tissue-specific factors or mitochondrial damage during disease or sample extraction/preparation results in F$_1$F$_0$ disassembly. Thus enzyme disassembly would not be solely caused by the T8993G mutation, rather, it would be the result of a number of possible destabilizing factors.

In this line, we also showed that nonphysiological conditions such as sonication or mild heating destabilized the mutant enzyme more than the wild type. Although this instability conferred by the L156R mutation is in agreement with previous findings in ex vivo tissues (25, 26), it does not lead to enzyme disassembly in homoplasmic T8993G human mitochondria carrying out ATP synthesis at 40% of the wild type rate. F$_1$F$_0$ disassembly, as observed in the mutant mitochondria, was forced by our in vitro conditions (sonication, heating in the presence of detergents, or high salt and pH).

Our data show that the primary pathogenic mechanism of the studied NARP/MILS mutations is a decreased ATP synthesis turnover in a fully assembled and tightly coupled F$_1$F$_0$ complex. Given that the wild type and mutant mitochondria had comparable amounts of assembled F$_1$F$_0$, the inhibition in enzyme turnover is likely the result of a homogeneous population of assembled mutant enzymes working at half their capacities as a consequence of proton flow blockade through F$_0$, as has been proposed (6, 7, 9). A similar (59%) inhibition was observed in maximal ATPase turnover conditions (Fig. 6A, closed versus open circles). The enzyme disassembly observed in these conditions (Fig. 7) does not account entirely for the decrease in F$_1$F$_0$ ATPase rates, because the activities measured in Fig. 6 retained oligomycin sensitivity. Thus, as suggested before (6, 7, 9), these ATP$_6$ mutations could slow rotation of the whole central rotor because of their location at the rotor/stator interface of F$_0$ between subunit 6 and the ring of c subunits where protons are translocated.

More studies are required to determine the role of enzyme instability in NARP/MILS progression. Enzyme disassembly
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may occur in vivo in metabolically demanding conditions. ATP$_{6}$ mutations hyperpolarize mitochondria (9, 20), and this ultimately leads to an increase in the production of reactive oxygen species (20). If these effects are extended into a chronic mitochondrial damage during long lasting illness, disassembly of mutant F$_{1}$F$_{0}$ may occur, as a side effect of the mutation.

Finally, by determining the mechanism of ATP$_{6}$ mutations in homoplasmic cells derived from NARP/MILS patients, this work provides key elements to develop novel gene therapy strategies to overcome the functional and destabilizing consequences of these mutations on the human ATP synthase.

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