The T9176G mutation of human mtDNA gives a fully assembled but inactive ATP synthase when modeled in *Escherichia coli*

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Abstract A new mutation in human F_1F_0 ATPase6, T9176G, which changes Leu 217 to an Arg, has been described in two siblings with Leigh syndrome [Carrozzo et al. (2000) Neurology, in press]. This mutation was modeled in *Escherichia coli* by changing Leu 259 (the equivalent residue) to Arg and the properties of the altered ECF_1F_0 were compared to those of previously characterized ATPase6 mutants also modeled in the *E. coli* enzyme. The L259R change produced a fully assembled ECF_1F_0 which had no significant ATP hydrolysis, ATP synthesis or proton pumping functions. This is very different from previously described human ATPase6 mutations. The presence of Arg at position 259 in subunit *a* did not make membranes permeable to protons. We conclude that the mutation inhibits functioning by blocking the rotary motor action of the enzyme.

Key words: F_1F_0 ATP synthase; Mitochondrial; Subunit 6; T8993G; *Escherichia coli*

1. Introduction

An ATP synthase of the F_1F_0 type is present in the bacterial plasma membrane, chloroplast thylakoid membrane and mitochondrial cristal membrane and this enzyme is responsible for most of the ATP production in cells [1]. Not surprisingly, mutants that affect the functioning of the ATP synthase in humans are pathogenic. In mammals, including humans, two subunits of the enzyme complex, subunits 6 and A6L, are encoded on mitochondrial DNA (mtDNA) [2]. A number of pathogenic mutants in subunit 6 have been reported [3–8]. Here we have examined the functional consequences of a newly reported subunit 6 mutation, T9176G [9], by modeling this change of Arg for Leu in the *Escherichia coli* ATP synthase. The position of the mutation in human subunit 6 is residue 217, which is equivalent to position 259 in the *E. coli* *a* subunit. For reference we have compared the properties of the mutant *a*L259R with those of three other well described subunit 6 mutations. When modeled in *E. coli* these are L259P (equivalent nucleotide position T9176C in humans) [10], L207R (T8993G) [11] and L207P (T8993C) [10,12]. The T9176G mutation is different from the others. It is a fully assembled enzyme with essentially no ATPase activity and a very low ATP synthesis rate.

2. Materials and methods

2.1. Construction of mutants in the unc operon

Site-directed mutagenesis of a plasmid containing the *unc* operon [13] was performed using a two-step PCR method [14]. The first PCR reaction involves an upstream sense primer (GTACTGGGTCTGCCTGCACT) and one of the 3′ antisense mutagenic primers: L259R-TAGACGATCGTC**C**GAACCATGAAGA, L259P-TAGACGATCGTC**G**GAACCATGAAGA, L207R-AGTCGCAAACCG**C**GTGAAACTG. Nucleotides highlighted are mismatches to generate the desired mutation. These fragments were then used as the 5′ primers in a second PCR reaction together with a downstream antisense primer (TTTCGCGAGCTGTTGTTCAC). The product of the second PCR was digested with *Dra*III restriction endonuclease and the resulting 1581 bp fragment containing each mutation was subcloned into the pRA100 vector [13]. The construction of L207P was described earlier [10]. Sequence analysis of the purified plasmids was performed in order to confirm the presence of the mutated nucleotide. The plasmid containing the mutants was used to transform RA1 cells, which lack the *unc* operon [15]. Cells were harvested and an inner membrane fraction was isolated as described by Foster and Fillingame [16].

2.2. H^+ pumping measurements

100 μg of inner membranes were used to measure the flux of the protons across the membrane [17]. Changes in pH were monitored by 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching with an SLM 8000 fluorimeter at 480 nm using an excitation wavelength of 410 nm.

2.3. ATPase assay

20 μg of the inner membrane was used to measure the ATP hydrolysis ability of *E. coli* F_1F_0 ATPase (ECF_1F_0), following the method of Lotscher et al. [18]. Dicyclohexylcarbodiimide (DCCD) sensitivity was performed in parallel. Inhibition by 40 μM DCCD was monitored for 1 h at room temperature. To assay the ATPase activity of the F_1 part freed of the influence of F_0, the assay medium was supplemented with 0.5% *N,N*-dimethyldodecylamine-*N*-oxide (LDAO). The absorbance change at 340 nm was followed in a Beckman DU7 spectrophotometer.

2.4. ATP synthesis

50 μl of inner membrane, containing 50 μg of protein, was resuspended in 450 μl of 25 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 10% glycerol, 10 mM ADP, 10 mM K_2HPO_4. 2 mM NADH was used to start the synthesis of ATP; after 3 min of incubation at 37°C the reaction was stopped with 0.1 M trichloroacetic acid. The amount of ATP in the sample was determined using an ATP bioluminescent assay kit (Sigma chemical company). The light emitted, when firefly luciferase catalyzed the oxidation of D-luciferin, is proportional to the

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; LDAO, *N,N*-dimethyldodecylamine-*N*-oxide; DCCD, dicyclohexylcarbodiimide; ECF_1F_0, *Escherichia coli* F_1F_0 ATPase

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amount of ATP present in the sample and was followed in a luminometer (Turner TD-20 Luminometer).

2.5. Other methods

Gel electrophoresis was preformed according to Laemmli [19] in a 15% gel. For Western blotting, gels were transferred to PVDF membranes (0.45 μM pore size, Millipore). Reactive bands were detected with ECL+ kit (Amersham Pharmacia Biotech) or with alkaline phosphatase methods (Bio-Rad). Anti-α, anti-γ and anti-*b* monoclonal antibody were prepared and characterized in this laboratory, anti-*a* polyclonal antibodies were a kind gift from Dr. S. Vik. Protein concentrations were determined with the BCA protein assay (Pierce chemical Co.).

3. Results

The rates of ATP synthesis, ATP hydrolysis and the DCCD sensitivity of the ATPase activities of the L259R mutant have been compared with the same measurements for L259P, L207R and L207P in Fig. 1A. It can be seen that the ATPase activity of the L259R mutant was very low and essentially the same as for an *unc*$^-$ strain with no F_1F_0 assembled. In comparison, the L259P and the two different mutants at position 207 each had ATPase activities of around 50% of the wild type. Note that the L259P and L207P mutants retain DCCD sensitivity, although at a lower level than the wild type, as reported previously [10], while the L207R mutant shows essentially no sensitivity to DCCD, as reported previously [11]. Fig. 1A also shows the activities of the four ATPase mutants after addition of LDAO to the membranes. This detergent disrupts the interaction between the F_1 and F_0 parts without releasing the F_1 from membranes [20]. The ATPase activity obtained in LDAO is therefore a measurement of the total amount of assembled F_1 on the membrane. As indicated in Fig. 1A, the amount of ECF_1 assembled in the L259R mutant was the same as in wild type (and in L259P and L207P mutants). Only in the mutant L207R was there significantly less ATPase assembled, and even then the effect was small. The ATP synthesis activity of the L259R mutant was very low, as was that of the L207R mutation. Both the L259P and L207P mutants retained some ATP synthesis function, i.e. 60% and 25%, respectively. As expected from the above results, the L259R mutant showed negligible ATP driven proton pumping activity when measured by the ACMA quenching assay (Fig. 2). However, NADH driven proton pumping was essentially normal, indicating that the mutation in subunit *a* has not made the membrane intrinsically permeable to protons.

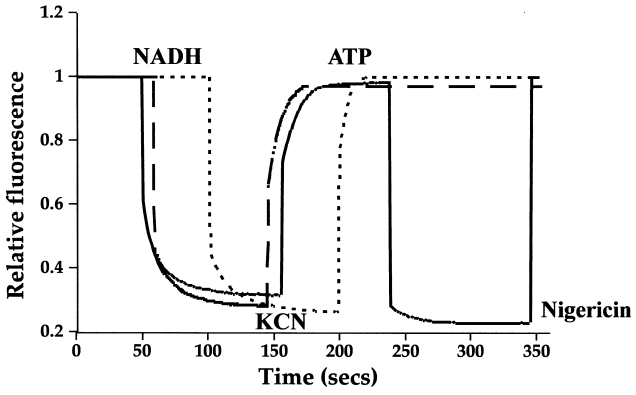

Fig. 2. NADH- and ATP-dependent ACMA quenching of membranes containing wild type (solid), L207R (dotted), and L259R (dashed) F_1F_0 ATP synthase. Membranes at 100 μg/ml in HMK buffer (10 mM HEPES pH 7.5, 5 mM $MgCl_2$, 100 mM KCl) were incubated with 1 μM ACMA, and 3.6 μM valinomycin. Assays were then supplemented with 2 mM NADH, 2 mM KCN, 2 mM ATP and 3.6 μM nigericin at the time points shown. Proton pumping in response to NADH demonstrates the integrity of the inner membrane vesicles, then this activity is eliminated by KCN. Proton pumping by F_1F_0 can then be demonstrated by addition of ATP at the time point shown.

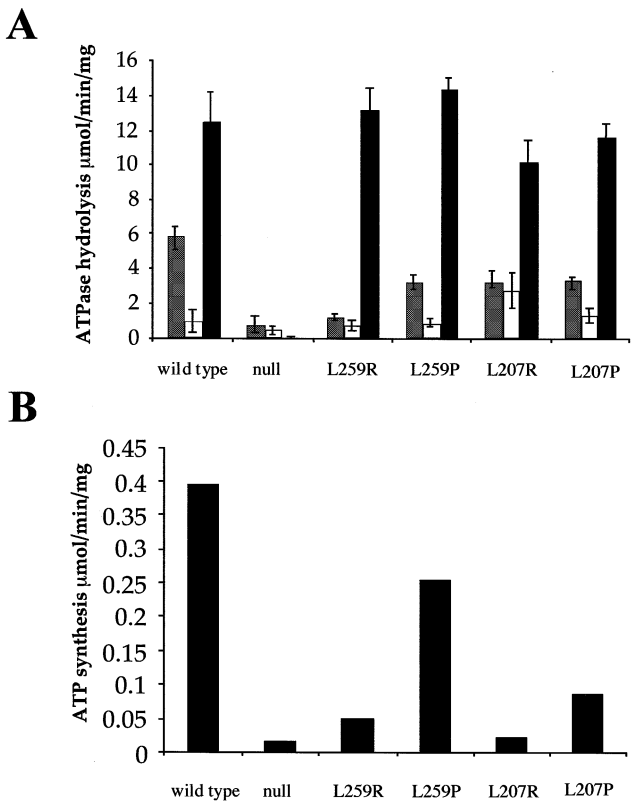

Fig. 1. ATPase and ATP synthase activities of wild type, null, L259R, L259P, L207R and L207P mutants. (A) ATPase activities of membranes were measured as described under Section 2. 20 μg of membranes were used in each case to measure activity (gray), DCCD sensitivity (white) and LDAO activation (black) of ATPase activity. Error bars indicate the maximum variation between four measurements. (B) ATP synthase activities of 50 μg samples of membranes were measured as described under Section 2. An average of three determinations is shown.

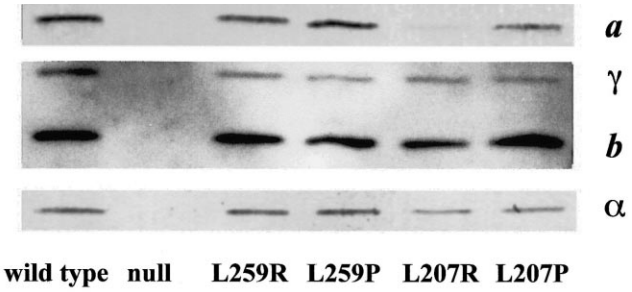

Fig. 3. Western blot of washed inner membranes probed with a polyclonal antibody against subunit *a* and monoclonal antibodies against subunits γ, *b*, and α. 20 μg of wild type, null, L259R, L259P, L207R, and L207P were loaded per lane.

Antibodies are available to all of the subunits of the ECF_1F_0. Western blotting with this set showed that the level of the enzyme in the L259R mutant (per mg of membrane protein) is similar to that of wild type, although this method of estimation is not as quantitative as obtained by the LDAO-stimulated ATPase activity measurement. More importantly, the blots in Fig. 3 show that the mutant L259R had a full complement of all subunits, as is the case for the L259P mutant. In the L207R mutant, assembly of the subunit *a* into the complex was poor while the levels of subunits *b*, α and γ were near normal.

4. Discussion

Mutations of mtDNA in the gene encoding ATPase6 are being reported with increasing frequency. The pathogenicity of these mutations depends on the amino acid altered, the percentage of enzyme molecules showing the mutation which is related to the heteroplasmy, and the tissue distribution of mutated DNA molecules [3]. Not surprisingly genotype–phenotype relationships are difficult to understand in such patients. The overall structure and functioning of the ATP synthase is well conserved in all forms of life. Subunit *a* of the ECF_1F_0 is 23% identical and has 44% homology with subunit 6 of the human enzyme. Most of the pathogenic mutations in humans are in amino acids conserved between the two subunit forms. This is the case for the recently described human mutant T9176G [9]. A recent study of a family harboring this mutation at different levels of heteroplasmy suggests that this particular mutation is as severe, or more severe, than other mutations described so far in subunit 6 [9]. The highly deleterious effect of substituting an Arg for a Leu at position 217 in humans is confirmed here by replacing the equivalent Leu (at position 259) with Arg in the *E. coli* enzyme. The mutant *a*L259R showed essentially no ATPase activity, which is different from all other subunit 6 mutations modeled in *E. coli*. ATP synthesis was also dramatically reduced and no measurable ATP driven proton pumping was obtained.

Most importantly the low levels of ATP hydrolysis and ATP synthesis do not result from a failure of the enzyme complex to assemble or from instability of the enzyme in the L259R mutant membranes. There are normal amounts of F_1 present which functions to give LDAO-stimulated ATPase activities equivalent to wild type. Moreover the levels of subunits including subunit *a* are normal. The L259R mutant clearly assembles much better than the L207R mutant when all experiments are run in parallel with both mutants.

The assembly of the F_1F_0 in human cells cannot be determined at present. We have monoclonal antibodies to subunits α, β, *d* and the inhibitor protein. Western blotting of fibroblast mitochondria from the patient with the T9176C mutation at close to homoplasmy with these antibodies shows normal levels of the four polypeptides (results not shown). This is not the case for Rho^o cells where ATPase6 is not synthesized and subunit *d* is diminished in amount [21]. Unfortunately an antibody against subunit 6 is not yet available with which to assess incorporation of this subunit.

In summary, the pathological mutation T9176G when modeled in ECF_1F_0 by replacing Leu 259 with an Arg produces a novel enzyme that has all of the subunits present, with an F_1 that is highly active when the interaction with F_0 is disrupted but yet shows no significant functioning in membranes. The mutation does not lead to uncoupling and is in every way equivalent to reacting F_1F_0 with the inhibitor DCCD. In recent models of functioning of the ATP synthase this would suggest that an Arg at position 259 blocks the rotation of the *c* subunit ring [22,23].

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