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Defining the pathogenesis of human mtDNA mutations using a yeast model: The case of T8851C☆

Roza Kucharczyk[a,c], Marie-France Giraud[a], Daniel Brèthes[a], Monica Wysocka-Kapcinska[c], Nahia Ezkurdia[a,1], Bénédicte Salin[a], Jean Velours[a], Nadine Camougrand[a], Francis Haraux[b], Jean-Paul di Rago[a,*]

[a] Institut de Biochimie et Génétique Cellulaires, CNRS UMR5095, Université Bordeaux Segalen, 1 Rue Camille SaintSaëns, Bordeaux 33077 cedex, France
[b] CNRS-UMR 8221, iBiTec-S, CEA Saclay, F 91191 Gif-sur-Yvette, France
[c] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

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ABSTRACT

More and more mutations are found in the mitochondrial DNA of various patients but ascertaining their pathogenesis is often difficult. Due to the conservation of mitochondrial function from yeast to humans, the unique ability of yeast to survive without production of ATP by oxidative phosphorylation, and the amenability of the yeast mitochondrial genome to site-directed mutagenesis, yeast is an excellent model for investigating the consequences of specific human mtDNA mutations. Here we report the construction of a yeast model of a point mutation (T8851C) in the mitochondrially-encoded subunit *a*/6 of the ATP synthase that has been associated with bilateral striatal lesions, a group of rare human neurological disorders characterized by symmetric degeneration of the corpus striatum. The biochemical consequences of this mutation are unknown. The T8851C yeast displayed a very slow growth phenotype on non-fermentable carbon sources, both at 28 °C (the optimal temperature for yeast growth) and at 36 °C. Mitochondria from T8851C yeast grown in galactose at 28 °C showed a 60% deficit in ATP production. When grown at 36 °C the rate of ATP synthesis was below 5% that of the wild-type, indicating that heat renders the mutation much more deleterious. At both growth temperatures, the mutant F_1F_o complex was correctly assembled but had only very weak ATPase activity (about 10% that of the control), both in mitochondria and after purification. These findings indicate that a block in the proton-translocating domain of the ATP synthase is the primary cause of the neurological disorder in the patients carrying the T8851C mutation.

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1. Introduction

Most of the cell's energy, in the form of ATP, is produced in humans by an F_1F_o-type ATP synthase (or complex V) located in the inner mitochondrial membrane (Saraste, 1999). This enzyme synthesizes ATP from ADP and inorganic phosphate using the energy of the electrochemical proton gradient established by the mitochondrial electron transport chain. Pronounced defects in the ATP synthase can cause various disorders, especially in the highly energy-demanding neuromuscular system (Schon et al., 2001; Houstek et al., 2006; Kucharczyk et al., 2009c). One of the genes most frequently implicated in such disorders is the mitochondrial *ATP6* gene encoding ATP synthase subunit *a* (referred to as Atp6p or subunit 6 in yeast). This is a key subunit of the F_o proton-translocating domain of the ATP synthase. Proton movements mediated by subunit *a* lead to the rotation of a transmembrane ring of subunit *c*, which results in conformational changes of the catalytic sites in the F_1 extra-membrane domain of the enzyme that favor ATP synthesis and its release into the mitochondrial matrix. (Fillingame et al., 2003).

The best known and studied of the pathogenic *ATP6* mutations are T8993G, T8993C, T9176G and T9176C that have been found in patients presenting with NARP (neuropathy ataxia retinitis pigmentosa) or MILS (Maternally-Inherited Leigh's Syndrome). Unequivocal genetic evidence for the pathogenesis of these mutations has been reported and all impair the ATP synthase to some extent (Kucharczyk et al., 2009c). The unique ability of yeast to survive without production of ATP by oxidative phosphorylation and the amenability of the yeast mitochondrial genome to site-directed

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* Corresponding author. Tel.: +33 5 56 99 90 43; fax: +33 5 56 99 90 51.
E-mail address: jp.dirago@ibgc.cnrs.fr (J.-P. di Rago).

[1] Present address: Liver Diseases Laboratory, Liver Unit, Department of Internal Medicine, Institut de Recerca Hospital Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain.

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mutagenesis (Bonnefoy and Fox, 2001) has allowed us to create yeast models of T8993G (Rak et al., 2007a), T8993C (Kucharczyk et al., 2009a), T9176G (Kucharczyk et al., 2009b) and T9176C (Kucharczyk et al., 2010). The ATP synthase defects observed in these yeast mutants correlate well with what is known about the consequences of these mutations in humans, which reflects a high level of evolutionary conservation within the regions of subunit *a* affected by these mutations.

Another *ATP6* mutation, T8851C, has been found in patients presenting with bilateral striatal lesions of childhood (BSLC), a group of rare neurological disorders characterized by symmetric degeneration of the corpus striatum (De Meirleir et al., 1995). This mutation leads to replacement of a relatively well-conserved tryptophan residue with arginine at position 109 of human subunit *a*. Nothing is known about its biochemical consequences. In this study, we show that in yeast the T8851C mutation severely affects the functioning of the ATP synthase and leads to several other secondary deficiencies that compromise the integrity of mitochondria.

2. Materials and methods

2.1. Construction of a yeast atp6-W136R mutant

Using the QuikChange XL Site-directed Mutagenesis Kit of Stratagene, we changed the tryptophan TGA codon at position 136 in the yeast *ATP6* gene into arginine AGA codon, with primers 5′-CTCTTTAAGTATTGTTATT**AGA**TTAGGTAATACTATTTTAGG and 5′-CCTAAAATAGTATTACCTAA**TCT**AATAACAATACTTAAAGAG (in bold the mutator codon). The mutation was introduced into a BamHI–EcoRI fragment of the *ATP6* locus cloned into pUC19 (plasmid pSDC8, see Zeng et al. (2007)). The mutated *ATP6* fragment was liberated and ligated with pJM2 (Steele et al., 1996) cut with BamHI and EcoRI, yielding plasmid pRK4. The 3′ part of the wild type *ATP6* locus was excised with SapI + EcoRI from pSDC9 (Rak et al., 2007a) and ligated between the same sites of pRK4, to give pRK10, thus reconstructing a complete *ATP6* gene containing the W136R mutation in pJM2. The ρ^0 strain DFS160 (all genotypes in Table 1) was co-transformed with pRK10 and the nuclear selectable *LEU2* plasmid pFL46 by microprojectile as described previously (Bonnefoy and Fox, 2001). Mitochondrial transformants were identified among the Leu^+ clones by their ability to produce respiring clones when mated with the non-respiring NB40-3C strain, which bears a deletion in the mitochondrial *COX2* gene. One mitochondrial transformant (synthetic ρ^- RKY36) was crossed to the *atp6::ARG8*^{m} deletion strain MR10 (Rak et al., 2007b) to produce cytoductants (called RKY39) harboring the MR10 nucleus in which the $ARG8^m$ ORF had been replaced with the *atp6*–W136R gene.

2.2. Purification and analysis of the kinetics of the yeast ATP synthase

To facilitate purification of the yeast ATP synthase, a hexa-histidine tag was added to one of its small components, subunit *i*, encoded by the nuclear ATP18 gene (Vaillier et al., 1999). The MR6 and RKY39 strains were transformed with a 3 kbp linear EcoR1–BamH1 fragment containing a 0.65 kbp sequence corresponding to the upstream region of ATP18, followed by the ATP18 coding sequence fused at the 3′ end to a sequence encoding the hexa-histidine tag, a lox-Kan^R-lox selection cassette and a 0.53 bp fragment analogous to the downstream region of the ATP18 gene. Transformants were selected on YPD + G418 (400 μg/ml) and analyzed by PCR (using whole cells) with the primers 5′-GGAACAATCAGCTGAGATGTG (forward) and 5′-CACGCATTACGAATATATTTCTATATAC (reverse) to verify the insertion of the EcoR1–BamH1 fragment at the ATP18 locus. Cells were transformed with the plasmid pSH47 to allow excision of the loxP-Kan^R-loxP cassette on galactose medium (Guldener et al., 1996). The plasmid pSH47 was then eliminated by growing the cells on 5-FOA medium 1.7% (w/v) yeast nitrogen base without nitrogen or amino acids, 2% (w/v) glucose, uracil (10 mg/l), proline (1 g/L), fluoroorotic acid (300 mg/l), supplemented with the required amino acids (20 mg/l)).

Mitochondria from the wild type MR6 + ATP18$(His)_6$ and mutant MR39 + ATP18$(His)_6$ strains were prepared by the zymolyase method (Guerin et al., 1979). Submitochondrial particles were prepared according to (MacLennan and Asai, 1968) and centrifuged at 48,000 × *g* for 45 min at 4 °C. Pellets were suspended at a concentration of 10 mg of starting mitochondrial protein per ml of buffer “A” (150 mM potassium acetate, 10% (V/V) glycerol, 2 mM PMSF, 2 mM ε-ACA, 30 mM HEPES pH 7.4 and EDTA-free protease inhibitor cocktail) containing 0.75% (W/V) H_{12}-TAC (C_2H_5—C_6F_{12}—C_2H_4—S-poly-Tris-(hydroxymethyl)acrylamidomethane), as described in Talbot et al. (2009). After 30 min of incubation at 4 °C, the extract was clarified by centrifugation (30 min, 4 °C, 25,000 × *g*). The supernatant was diluted with two volumes of washing buffer “W” (50 mM NaCl, 10% (V/V) glycerol, 10 mM imidazole, 20 mM sodium phosphate pH 7.9) and mixed with Ni-NTA-agarose beads (Qiagen) such that there was 0.25 ml of slurry per 10 mg of starting mitochondrial protein. After an overnight incubation at 4 °C, beads were washed with 25 volumes of buffer “W” containing 0.1% (W/V) H_{12}-TAC. ATP synthase was eluted with buffer “E” (50 mM NaCl, 10% (V/V) glycerol, 250 mM imidazole, 0.1% (W/V) H_{12}-TAC, 20 mM sodium phosphate pH 7.9). Egg phosphatidylcholine (PC) purified according to Singleton et al. (1965) was suspended in 5 mM $MgCl_2$, 50 mM HEPES pH 7.5 to a concentration of 10 mg/ml and then sonicated three times for 5 min at 4 °C (75TS Annemasse sonicator, 120 V). Purified enzymes were mixed immediately after their elution from the Ni-NTA resin with PC, to a lipid/protein molar ratio of ∼250. Eluted proteins (0.4 mg), relipidated or not, were analyzed under native conditions (BN-PAGE) on a 3–13% linear gradient polyacrylamide gel (Schagger et al., 1994). The ATPase activity of the purified enzymes was evaluated by monitoring NADH oxidation at 340 nm through a coupled reaction with pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Tietz and Ochoa, 1958). The decrease in absorbance at 340 nm reflects ATPase activity. Activity assays were performed at 28 °C under magnetic stirring in 1.5 ml of activity buffer (20 units ml^{-1} of PK, 30 units ml^{-1} of LDH, 2 mM PEP, 0.2 mM NADH, 5 mM $MgCl_2$, 50 mM HEPES pH 7.5) using 15 μg of the enriched ATP synthase fraction. The molar extinction coefficient of NADH at 340 nm was 6220 M^{-1} cm^{-1} and the path length was 1 cm. Initial rates were measured for ATP concentrations from 11 μM to 2000 μM. Km_{app} and *V*max values were obtained by fitting data with a single site Michaelis–Menten kinetics using Graphpad Prism software.

2.3. Measurement of respiration and ATP synthesis/hydrolysis activities in whole mitochondria

For these assays, mitochondria were prepared by the enzymatic method of (Guerin et al., 1979). The rates of ATP synthesis were determined as described in (Rak et al., 2007a). For respiration ATP synthesis and transmembrane potential ($\Delta\Psi$) measurements, freshly prepared mitochondria were diluted to 0.15 mg/ml in the reaction medium thermostated at 28 °C and containing 10 mM Tris–maleate (pH 6.8), 0.65 M sorbitol, 0.3 mM EGTA, and 3 mM potassium phosphate. Oxygen consumption rates were measured using a Clarke electrode and a OXM204 oxymeter from Heto (France) as described (Rigoulet and Guerin, 1979). The different respiration states were measured after consecutive additions of 4 mM NADH for State 2, 150 μM ADP for State 3, 4 μM carbonyl cyanide

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