Defining the impact on yeast ATP synthase of two pathogenic human mitochondrial DNA mutations, T9185C and T9191C.

Anna Magdalena Kabala[1,2], Jean-Paul Lasserre[2], Sharon H. Ackerman[3], Jean-Paul di Rago[2], Roza Kucharczyk[1]*

[1]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
[2]Institut de Biochimie et Génétique Cellulaires, CNRS UMR5095, Université Bordeaux Segalen, 1 Rue Camille Saint-Saëns, Bordeaux 33077 cedex, France.
[3]Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan, USA
*To whom correspondence should be addressed. Email: roza@ibb.waw.pl, Phone: +48 225921217, Fax: +48 226584636

Abstract

Mutations in the human mitochondrial ATP6 gene encoding ATP synthase subunit *a*/6 (referred to as Atp6p in yeast) are at the base of neurodegenerative disorders like Neuropathy Ataxia Retinitis Pigmentosa (NARP), Leigh syndrome (LS), Charcot-Marie-Tooth (CMT), and ataxia telangiectasia. In previous studies, using the yeast *Saccharomyces cerevisiae* as a model we were able to better define how several of these mutations impact the ATP synthase. Here we report the construction of yeast models of two other ATP6 pathogenic mutations, T9185C and T9191C. The first one was reported as conferring a mild, sometimes reversible, CMT clinical phenotype; the second one has been described in a patient presenting with severe LS. We found that an equivalent of the T9185C mutation partially impaired the functioning of yeast ATP synthase, with only a 30% deficit in mitochondrial ATP production. An equivalent of the mutation T9191C had much more severe effects, with a nearly complete block in yeast Atp6p assembly and an >95% drop in the rate of ATP synthesis. These findings provide a molecular basis for the relative severities of the diseases induced by T9185C and T9191C.

Key words: ATP synthase, *ATP6*, mitochondria, energetics, disease, mtDNA mutation.

Introduction

A quite large number of point mutations (sixteen) has been found in the mitochondrial *ATP6* gene in patients presenting with various neurodegenerative disorders, Neurogenic Ataxia and Retinitis Pigmentosa (NARP), Leigh syndrome (LS), Leber's Hereditary Optic Neuropathy (LHON), Charcot-Marie-Tooth (CMT) or ataxia telangiectasia [1-8]. The *ATP6* gene encodes ATP synthase subunit *a*, which is referred to as Atp6p in yeast. The ATP synthase (also called complex V) synthesizes ATP from ADP and inorganic phosphate using the energy of the electrochemical proton gradient established by the mitochondrial electron transport chain (complexes I-IV) [9]. Atp6p is a key subunit of the F_O proton-translocating domain of the ATP synthase. Proton movements mediated by Atp6p lead to the rotation of a transmembrane ring of Atp9p subunits (referred to as subunit *c* in humans) which ends up in conformational changes at the level of the catalytic sites in the F_1 extra-membrane domain of the enzyme that favor the synthesis ATP and its release into the mitochondrial matrix [10, 11]. We previously constructed yeast models of the pathogenic ATP6 mutations T8993G [12], T8993C [13], T9176G [14], T9176C [15] and T8851C [16]. The effects of these mutations on yeast ATP synthase correlated well with those observed in humans, which reflects the high level of evolutionary conservation within the regions of Atp6p affected by these mutations.

Two other pathogenic mutations at the focus of the present study were described at positions 9185 (T9185C) and 9191 (T9191C) of ATP6 [17]. The first one changes a leucine into proline at position 220 near the carboxyl terminus of the protein. It was found in thirty-four patients from eight independent families suffering from LS, NARP, CMT or spinocerebellar ataxia syndromes [3, 17-21]. In all cases the disease was maternally inherited, with a relatively mild, sometimes reversible, clinical phenotype and occurred at a minimum of 85% heteroplasmy. Mitochondria from patients's cells (muscle or skin fibroblasts) showed normal complexes I-IV activities [3, 21] and only a slightly reduced ATPase activity [18, 20]. The second mutation,

T9191C, was found in a patient presenting with very severe LS [17]. It changes a leucine to proline at position 222 of the human homolog of yeast Atp6p. This mutation causes a substantial (50%) reduction in mitochondrial ATPase activity and a lower respiration rate (60% *vs*. control) [17]. We report here yeast models of the mutations T9185C and T9191C that help to better define how they impact the ATP synthase.

Materials and Methods

2.1. Construction of yeast atp6-S250P and atp6-L252P mutants. The strains used in the study are listed in Table 1. Using the QuikChange XL Site-directed Mutagenesis Kit of Stratagene, we changed the serine TCA codon at position 250 in the yeast *ATP6* gene into proline CCA codon, with primers 5' GTCTGGGCTATTTTAACAGCA**CCA**TATTTAAAAGATGCAGTATACTTACAT and 5' ATGTAAGTATACTGCATCTTTTAAATA**TGG**TGCTGTTAAAATAGCCCAGAC and the leucine TTA codon at position 252 into proline CCA codon, with primers 5' GTCTGGGCTATTTTAACAGCATCATAT**CCA**AAAGATGCAGTATACTTACAT and 5' ATGTAAGTATACTGCATCTTT**TGG**ATATGATGCTGTTAAAATAGCCCAGAC (in bold the mutator codon). The mutagenesis was performed on an EcoRI–BamHI fragment containing the last 38 codons of *ATP6* cloned in pUC19 (plasmid pSDC9) [12]. The mutated fragment was liberated by restriction with EcoRI and SapI and ligated with pSDC14 [12] cut with the same enzymes to reconstruct a whole *ATP6* gene with the S250P or L252P mutations. The resulting plasmids (pRK37 and pRK38, respectively) also contain the yeast mitochondrial *COX2* gene as a marker for mitochondrial transformation. The plasmids were introduced by co-transformation with the nuclear selectable *LEU2* plasmid Yep351 into the rho^0 strain DFS160 by microprojectile bombardment using a biolistic PDS-1000/He particle delivery system (Bio-Rad) as described [22]. Mitochondrial transformants (synthetic AKY13 and AKY14 respectively) were identified among the Leu+ nuclear transformants by their ability to produce respiring clones when mated to the nonrespiring NB40-3C strain bearing a deletion in the mitochondrial *COX2* gene. One AKY13 and AKY14 clone was crossed to the *atp6::ARG8m* deletion strain MR10 [23] for the production of clones (called AKY5 and RKY66) harboring the MR10 nucleus and where the *ARG8m* ORF [24] had been replaced by recombination with the mutated *atp6-S250P* or *atp6-L252P* genes. The AKY5 clone was

identified by its inability to grow in the absence of an external source of arginine and the ability to grow on respiratory medium. The RKY66 clone was identified by its inability to grow in the absence of an external source of arginine and the ability to grow on respiratory medium when crossed with the SDC30 strain bearing in the mitochondrial DNA the wild type copy of *ATP6* gene. Sequencing of the mutated *atp6* locus in AKY5 and RKY66 revealed no other changes than S250P or L252P, respectively.

2.2. Measurement of respiration and ATP synthesis/hydrolysis activities in whole mitochondria. For these assays, mitochondria were prepared by the enzymatic method of [25]. The rates of ATP synthesis were determined as described in [23]. 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 Heito (France) as described [26]. The different respiration states were measured after consecutive additions of 4 mM NADH for State 2, 150 µM ADP for State 3 and State 4, 4µM carbonyl cyanide m-chlorophenylhydrazone (CCCP) for uncoupled respiration and finally 12.5 mM ascorbate (Asc), 1.4 mM N,N,N,N,-tetramethyl-p-phenylenediamine (*TMPD*) for Complex IV respiration activity. The rates of ATP synthesis were determined in the same condition using 750 µM ADP. Aliquots were withdrawn from the oxygraph cuvette every 15 seconds and reaction was stopped by 3.5% (w/v) perchloric acid, 12.5 mM EDTA. Samples were then neutralized to pH 6.5 by addition of KOH, 0.3 M MOPS. ATP was quantified by luciferin/luciferase assay (ATPLite kit from Perkin Elmer) on a LKB bioluminometer. Participation of the F_1F_O-ATP synthase to ATP production was assessed by oligomycin addition (3 µg/ml). Variations in transmembrane potential ($\Delta\Psi$) were evaluated as in [27] by monitoring the quenching of rhodamine 123 fluorescence (0.5 µM) using a λ_{exc} of 485 nm and

a λ_{em} of 533 nm using a FLX Spectrofluorimeter (SAFAS, Monaco) under constant stirring. Transmembrane potential was generated by addition of ethanol [1% (v/v) final concentration]. ATP synthesis (state 3 of respiration) was initiated by addition of 50 μM ADP. When State 4 was reached, respiratory was inhibited by adding 0.3 mM KCN in order to measure the ΔΨ produced by the hydrolysis of the synthetized ATP. ΔΨ was collapsed by adding 4 μM CCCP. The specific ATPase activity at pH 8.4 of non-osmotically protected mitochondria was measured as described in [28].

2.3. Miscellaneous procedures. Determination of ρ^-/ρ^0 cells in yeast cultures, SDS–PAGE and BN–PAGE, western blotting, pulse labeling of mtDNA encoded proteins were performed as described in [23].

Results

3.1. Respiratory growth and genetic stability of yeast mutants atp6-S250P and atp6-L252P.

The leucine residues 220 and 222 of the human homolog of yeast Atp6p that are modified by the T9185C and T9191C mutations correspond respectively to serine 250 and leucine 252 of Atp6p [29]. The TCA and TTA codons specifying these residues were converted into proline CCA codon (see Materials and Methods). Yeast *atp6*-S250P clones grew well on non-fermentable carbon sources (like glycerol) whereas *atp6*-L252P ones failed to grow in these conditions, as shown by the drop tests in Fig.1. The few growing colonies in the *atp6*-L252P drops presumably arose from genetic suppressors restoring mitochondrial function.

Even though the mutant *atp6*-S250P had a normal respiratory growth this does not necessarily mean that mitochondrial ATP synthesis was not compromised. Indeed as previously shown through the analysis of numerous yeast ATP synthase mutants, the activity of this enzyme needs to be decreased by at least 80% to see an obvious respiratory growth defect, which indicates that ATP synthase is far from limiting for the proliferation of yeast cells producing ATP by oxidative phosphorylation [30, 31]. However, when the rate of mitochondrial ATP production is diminished cells become more sensitive to chemical inhibition of ATP synthase with oligomycin [31], a compound that is presumed to target the F_O because mutations in Atp9p and Atp6p can confer an increased resistance to it [32, 33]. Thus, the higher *in vivo* sensitivity to oligomycin of mutants in which ATP synthase is partially compromised is due to the fact that less of the drug is required to reach the 20% oxidative phosphorylation threshold under which the production of ATP becomes limiting for growth [31]. We therefore tested the *in vivo* sensitivity to oligomycin of the mutant *atp6*-S250P. As shown in Fig.1, in the presence of 0.25μg/ml oligomycin the *atp6*-S250P mutant stopped growing on glycerol whereas wild type yeast (*WT*, strain MR6) was unaffected. This finding indicated that the S250P change in Atp6p was not without any deleterious

consequence on the ATP synthase, which was confirmed by *in vitro* experiments on isolated mitochondria as described below. It is to be noted that the few revertants of the *atp6*-L252P mutant displayed a normal sensitivity to oligomycin, indicating that ATP synthase function was largely, if not totally, restored in these clones.

In yeast, defects in ATP synthase often increase the production of ρ^-/ρ^0 *petites* issued from large deletions in the mtDNA [34, 35]. The *atp6*-S250P mutant never produced more than 5% ρ^-/ρ^0 cells like the *WT*. The *atp6*-L252P mutant had a higher but still moderate propensity (36%) to produce *petites* showing that its severe respiratory growth deficiency was not due, at least solely, to a failure in mtDNA maintenance. Full respiratory competence was restored in *atp6*-L252P cells that contained a complete (ρ+) mitochondrial genome by crossing with SDC30, a synthetic ρ^- strain whose mitochondria contain only the wild type *ATP6* gene. This result proved that the L252P change in Atp6p was responsible for the observed respiratory growth phenotype of the *atp6*-L252 mutant.

3.2. Consequences of the atp6-S250P and atp6-L252P mutations on various activities related to respiration and oxidative phosphorylation.

3.2.1. Mitochondrial oxygen consumption. We first measured oxygen consumption in isolated mitochondria using NADH as an electron donor, at state 3 (i.e. in the presence of ADP, phosphorylating conditions), state 4 (i.e. without addition of ADP, basal respiration) and in the presence of the membrane potential uncoupler CCCP (i.e. conditions at which respiration is maximal). We also used ascorbate/TMPD to deliver electrons directly at the level of complex IV, the last complex of the electron transport chain. The *atp6*-S250P mutation had a minor impact in all tested conditions, with only a 10-15% decrease at state 3 with respect to *WT* (Table 2). Much more important oxygen consumption deficits (67-85%) were observed in *atp6*-L252P mitochondria. A respiratory defect, especially at the level of complex IV, is a

common property of yeast ATP synthase mutants [23, 36]. Not surprisingly, a pronounced decrease in the content of complex IV was observed also in the mutant *atp6*-L252P whereas the abundance of this complex was almost normal in the *atp6*-S250P mutant, as revealed by BN-PAGE analysis of mitochondrial protein digitonin-extracts (Fig. 2A).

3.2.2. Mitochondrial ATP synthesis/hydrolysis. We analyzed further the influence of the *atp6*-S250P and *atp6*-L252P mutations by measuring the rate of ATP synthesis in isolated mitochondria, which was done in the presence of a large excess of external ADP to keep constant a minimal intra-mitochondrial concentration of ATP. An ~30% decrease in ATP synthesis rate was observed in *atp6*-S250P mitochondria while this activity was less than 10% that of *WT* (Table 2) in the *atp6*-L252P mutant. As the rates of oxygen consumption were reduced in similar proportions (see above), it can be inferred that the efficiency of oxidative phosphorylation (*i.e.* the number of ATP molecules formed per electron transferred to oxygen) was largely unaffected by both mutations.

We next measured the rate of ATP hydrolysis by non-osmotically protected mitochondria buffered at pH 8.4 and in the presence of saturating amounts of ATP, conditions under which this activity is maximal. Both mutants had an ATPase activity similar to that of the *WT* (Table 2). Of particular interest, oligomycin inhibited the ATPase activity by 85% in the *WT* and *atp6*-S250P samples, but only by 20% in the *atp6*-L252P mutant.

c) ATP-driven translocation of protons across the mitochondrial inner membrane – We next measured the proton-pumping activity coupled to F_1-mediated ATP hydrolysis in samples of whole mitochondria, using a fluorescent dye, Rhodamine 123, to monitor changes in the membrane potential ($\Delta\Psi$) (Figure 3). This dye accumulates inside the mitochondrial matrix, where its fluorescence is quenched, in response to an established $\Delta\Psi$ [27]. Before testing for ATP-driven proton translocation, the mitochondria were energized with ethanol in order to elicit release of the natural inhibitory peptide (IF1) that binds F_1F_O in the resting state

and prevents ATP hydrolysis [37]. The imposed ΔΨ was then collapsed with KCN and less than 2 minutes later ATP was added, i.e. well before rebinding of IF1 to F_1 could occur. The added ATP is counter-exchanged for ADP in the matrix compartment and is then hydrolyzed by the ATP synthase coupled to the pumping of protons out of the mitochondrial matrix through the F_O. Comparable levels of proton pumping coupled to ATP hydrolysis was manifested in the *WT* and *atp6*-S250P mitochondria by a large and sustained fluorescence quenching that was fully reversed upon addition of oligomycin. It is to be noted that the concentration of oligomycin used in these assays is in far excess of the one that is minimally required to inhibit all ATP synthase complexes in wild type mitochondria, which explains that the ATP-induced ΔΨ in mutant and wild type mitochondria showed the same sensitivity to oligomycin. In the growth tests of Figure 1 showing that the *atp6*-S250P mutant has an increased *in vivo* sensitivity to oligomycin, the drug was used at a suboptimal concentration not sufficient to inhibit the respiratory growth of wild type yeast. Mitochondria from the *atp6*-L252P mutant produced only a small change in fluorescence upon addition of ATP and this change was almost insensitive to oligomycin.

3.2.3. Assembly/stability of the ATP synthase in the atp6-S250P and atp6-L252P mutants. We finally investigated the influence of the *atp6*-S250P and *atp6*-L252P mutations on ATP synthase assembly/stability, by BN-PAGE analysis of mitochondrial proteins extracted with digitonin (Fig.2A). The BN gels were first stained with Coomassie brilliant blue. *WT* and *atp6*-S250P samples showed similar amounts of fully assembled ATP synthase dimers and monomers, whereas these complexes were barely detectable in *atp6*-L252P samples. The protein complexes were further analyzed in-gel via their ATPase activity. Two major ATPase signals corresponding to ATP synthase dimers and monomers were detected for both the WT and *atp6*-S250P mutant. Similar signals were seen also but with a much weaker intensity for the *atp6*-L252P mutant. Of particular interest this mutant displayed a strong lower-size

ATPase signal corresponding to free F_1. Finally, the protein complexes were transferred to a nitrocellulose membrane and decorated with antibodies against Atp6p, which further illustrated the failure of the *atp6*-L252P mutant to assemble correctly the ATP synthase.

In SDS gels of total mitochondrial protein extracts, Atp6p was barely detected in the *atp6*-L252P mutant whereas the steady state levels of this protein were normal in the *atp6*-S250P mutant (Fig.2B). Pulse labeling of the proteins encoded by the mitochondrial genome revealed that Atp6p was synthesized efficiently in both mutants (Fig.4). It can be inferred that the nearly absence of Atp6p in the mutant *atp6*-L252P is caused by a high susceptibility of this protein to degradation. There is a visible difference in the migration of Atp6p in both mutants where this protein appears to be larger than in the *WT*. It seems unlikely that this effect is due to a block in the processing of the leader peptide of Atp6p, a stretch of 10 amino acids that is removed during assembly of the protein [38-41]. Indeed, if this were the case, both mutant proteins would have the same migration rate, which is not observed. Atp6p like other very hydrophobic proteins has aberrant electrophoretic properties; while it migrates as an 21 KDa protein it has a predicted molecular weight of about 30 KDa. The differences in the migration of Atp6p in the *atp6*-S250P and *atp6*-L252P mutants most likely result from the structural changes induced by the mutations themselves. That a single amino acid replacement may change the electrophoretic properties of a protein has been observed on many occasions (see Figure 3 in [42] for an example).

Discussion

We have investigated the consequences on yeast ATP synthase of two mutations of the human mitochondrial ATP6 gene, T9185C and T9191C, that were identified in patients suffering from neurological disorders [17]. Both mutations change a leucine residue into proline near the C-terminal end of the human homolog of yeast Atp6p, at positions 220 and 222 respectively. These leucine residues show moderate evolutionary conservation as manifested by the presence of different amino acids at corresponding positions in other species (like Ser, Val, Ile, and Gly at position 220; Ala, Met, Ser and Ile at position 222) (see [29] for amino acids alignments). In current folding models [29], these residues belong to the last α-helical transmembrane segment (helix V) of Atp6p. This segment would contact the Atp9p-ring and is presumed to play a key role in proton transport through the F_O [43]. Nearby the leucine 220 and 222 of the human homolog of yeast Atp6p are two residues within helix V, leucine 217 and tyrosine 221, that have possible crucial importance as indicated by their strict evolutionary conservation [29]. It is therefore not very surprising that replacing leucine 220 or 222 by an α-helix breaker residue like proline is detrimental to human health. Consistent with this, some of the most severe ATP6 pathogenic mutations were located at position 217 [44].

Mutations in yeast Atp6p equivalent to T9185C (*atp6*-S250P) and T9191C (*atp6*-L252P) lead to 30% and 90% drops in the rate of mitochondrial ATP synthesis respectively (Table 2). The *atp6*-S250P mutation had no visible influence on yeast ATP synthase assembly/stability (Fig.2A), indicating a partial functional impairment of the enzyme. There was no evidence of proton leakage across the mitochondrial inner membrane, and the efficiency of oxidative phosphorylation in *atp6*-S250P mitochondria in terms of ATP molecules synthesized per electron transferred to oxygen was almost normal. The main effect of this mutation is thus a partial functional impairment of F_O. Since the leucine 220 (serine

250 in yeast) is predicted to be vey close to the matrix side of the membrane, a proline in this position could possibly create some local structural modification resulting in a less efficient exit of protons from the F_O. Alternatively, the mutation might induce long-range effects impacting the entry of protons from the intermembrane space or their exchange with the *c*-ring near the middle of the membrane. Nearly identical defects, i.e. a 30% deficit in ATP production with no visible impact on assembly/stability of ATP synthase, were found in a yeast model of the T9176C mutation (amino acid position 217 in humans, 247 in yeast) that gives also relatively mild clinical phenotypes [31]. These findings reveal that a deficit in mitochondrial ATP production so modest as 30% is sufficient to impact human health.

The >95% drop in ATP synthesis in the *atp6*-L252P mutant is caused by defects in ATP synthase assembly. Only trace amounts of fully assembled F_1F_O complexes were detected by BN-PAGE analysis in this mutant (Fig.2A). The mutated Atp6p was synthesized efficiently (Fig.3) but failed to accumulate at the steady state (Fig.2B) indicating that it is rapidly eliminated from cells after synthesis [9, 45]. Yeast Atp6p is typically degraded when it cannot assemble. It is presumed to insert in a late step after assembly of the other ATP synthase subunits [23, 45]. When subjected to BN-PAGE analysis the Atp6p-less intermediate easily dissociates into several subcomplexes, among which free F_1 particles [23] (this study, Fig.2A). The rapid degradation of neo-synthesized Atp6p and the presence of substantial amounts of free F_1 in the *atp6*-L252P mutant are strong indications that the mutated protein is unable to be stably incorporated into ATP synthase. As the region of Atp6p affected by the L252P change is presumed to contact the *c*-ring, it is possible that the mutated protein cannot interact properly with the *c*-ring. Alternatively, the mutation may prevent insertion of Atp6p within the membrane or acquisition of a folded structure required to interact with the *c*-ring. In this respect, it is to be noted that the C-terminal region of Atp6p seems to be critical for interaction with Atp10p [46], an accessory protein that helps insertion of Atp6p into ATP

synthase either directly or by protecting it against proteolytic degradation until it is assembled [47]. Nearly identical defects were found in a yeast model of the pathogenic mutation T9176G mutation, which changes the highly conserved leucine 217 (247 in yeast) into arginine [14]. This mutation results in very severe clinical phenotypes too similar to those of the patient with the T9191C mutation [44]. Since this mutation was found in only one patient, its pathogenesis remained uncertain. Our study provides strong evidence that this mutation was actually responsible for the clinical phenotypes displayed by this patient.

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Table 1. *Genotypes and sources of yeast strains*

Strain	Nuclear genotype	mtDNA	Source
DFS160	*MAT**a** leu2Δ ura3-52 ade2-101 arg8:: URA3 kar1-1*	ρ^o	[24]
NB40-3C	*MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII*	ρ^+ *cox2-62*	[24]
	arg8::hisG		
MR6	*MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG*	ρ^+	[23]
MR10	*MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG*	ρ^+ *atp6::ARG8*^{m}	[23]
SDC30	*MAT**a** leu2Δ ura3-52 ade2-101 arg8: URA3 kar1-1*	ρ^- *ATP6*	[23]
AKY13	*MATα leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1*	ρ^- *atp6-S250P*	This study
AKY14	*MATα leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1*	ρ^- *atp6-L252P*	This study
AKY5	*MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1*	ρ^+ *atp6 S250P*	This study
	CAN1 arg8::hisG		This study
RKY66	*MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1*	ρ^- *atp6-L252P*	This study
	CAN1 arg8::hisG		This study

Table 2. *Influence of the atp6-S250P and atp6-L252P mutations on yeast mitochondrial respiration, and ATP synthesis and hydrolysis activities*.
Mitochondria were isolated from wild type strain MR6 (*wt*) and mutants *atp6*-S250P and *atp6*-L252P grown for 5-6 generations in YPGALA medium (rich galactose) at 28°C. Reaction mixes for assays contained 0.15 mg/ml protein, 4 mM NADH, 150 (for respiration asays) or 750 (for ATP synthesis) µM ADP, 12.5 mM ascorbate (*Asc*), 1.4 mM N,N,N,N,-tetramethyl-p-phenylenediamine (*TMPD*), 4 µM CCCP, 3 µg/ml oligomycin (*oligo*). The two MR6 cultures contained 2-5% of $\rho^-/\rho°$ cells, while those of *atp6*-S250P and *atp6*-L252P contained 5% and 36% $\rho^-/\rho°$ cells. The values reported are averages of triplicate assays ± standard deviation. Respiratory and ATP synthesis activities were measured using freshly isolated, osmotically protected mitochondria buffered at pH 6.8. For the ATPase assays, mitochondria kept at –80°C were thawed and the reaction performed in absence of osmotic protection and at pH 8.4.

Strain	Respiration rates nmol $O.min^{-1}.mg^{-1}$				ATP synthesis rate nmol $Pi.min^{-1}.mg^{-1}$		ATPase activity µmol $Pi.min^{-1}.mg^{-1}$	
	NADH	NADH +ADP	NADH +CCCP	Asc/TMPD + CCCP	- oligo	+ oligo	- oligo	+oligo
MR6	470 ± 59	898 ± 124	1437 ± 316	2540 ± 434	1112 ± 185	52 ± 25	4.1 ± 1.6	0.48 ± 0.27
AKY5	420 ± 41	774 ± 61	1374 ± 129	2606 ± 185	779± 37	10 ± 5	4.5 ± 2.0	0.71 ± 0.37
RKY66	154 ± 1	180 ± 7	356 ± 57	559 ± 33	71 ± 1	29 ± 3	5.1 ± 0.3	4.1 ± 1.9

LEGENDS TO FIGURES

Fig.1. Respiratory growth of *atp6-S250P* and *atp6-L252P* mutants. Freshly grown cells of wild type yeast (MR6) and the *atp6* mutants were serially diluted and 5 µl of each dilution were spotted onto rich glucose, rich glycerol and rich glycerol + oligomycin. The plates were incubated at 28°C and photographed after the indicated number of days.

Fig.2. ATP synthase and complex IV in the mutants *atp6-S250P* and *atp6-L252P*. A: BN-PAGE analysis of mitochondrial protein digitonin-extracts (50 µg). The proteins were, as indicated, stained in-gel by Coomassie brilliant blue and their ATPase activity, and by Western blot with antibodies against Atp6 and Cox2. Fully assembled, dimeric (V_2) and monomeric (V_1), F_1F_O-ATP synthase complexes accumulate normally in the atp6-250P mutant while only trace amounts are detected in the atp6-L252P mutant. This mutant accumulates large amounts of free F_1 particles. The anti-cox2 Western reveals that the atp6-L252P mutant has low contents in cytochrome oxidase (complex IV) while the levels of this enzyme in the atp6-S250P mutant are similar to those seen in the WT. B: SDS-PAGE of total mitochondrial proteins (20 µg). After migration the proteins were transferred to a nitrocellulose membrane and probed with antibodies against porin and Atp6p.

Fig.3. *ATP-driven energization of mitochondria*. Energization of the mitochondrial inner membrane in intact mitochondria from wild type, *atp6-S250P* and *atp6-L252P* mutants grown in rich galactose at 28°C was monitored by Rhodamine 123 (Rh-123) fluorescence quenching. The additions were 0.5 µg/ml Rhodamine 123, 0.15 mg/ml mitochondrial proteins (Mito), 10 µl of ethanol (EtOH), 0.2 mM potassium cyanide (KCN), 1 mM ATP, 6 µg/ml oligomycin (oligo) and 3 µM CCCP.

Fig. 4. *In vivo labeling of mitochondrial translation products*. Proteins encoded by mtDNA were labeled in whole cells from wild type (MR6) and strains bearing *atp6-S250P* and *atp6-L252P* mutations with [35S]-(methionine+cysteine) for 20 min in the presence of cycloheximide to inhibit cytosolic protein synthesis. After the labeling reactions, total protein extracts were prepared from the cells (0.2 OD at 650 nm) and separated by SDS-PAGE on a 16.5% polyacrylamide gel (left). For a better resolution of Cox3p and Atp6p, a 12% polyacrylamide gel containing 6 M Urea was used (right). The gels were dried and analyzed with a PhosphorImager.

Figure

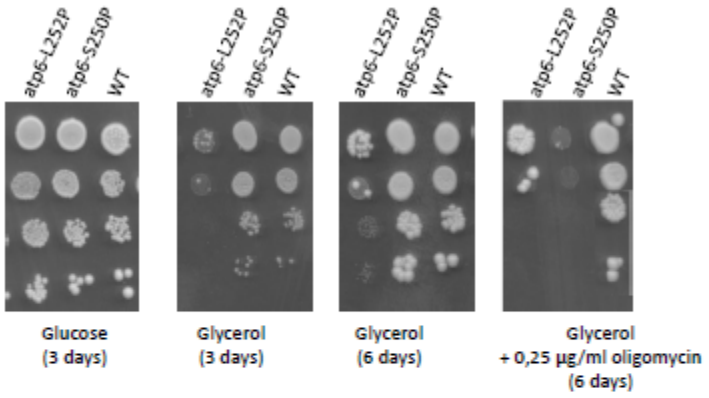
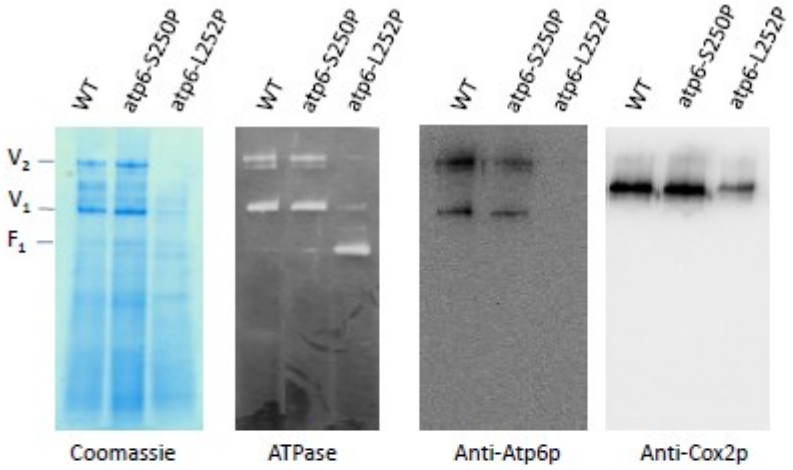
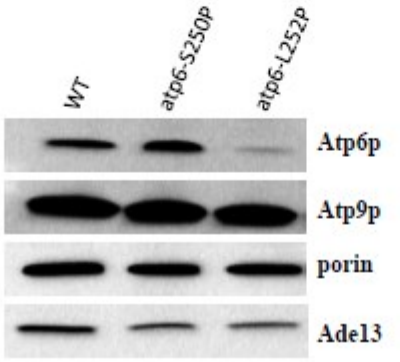


Figure 1

Figure



A



B

Figure 2

Figure

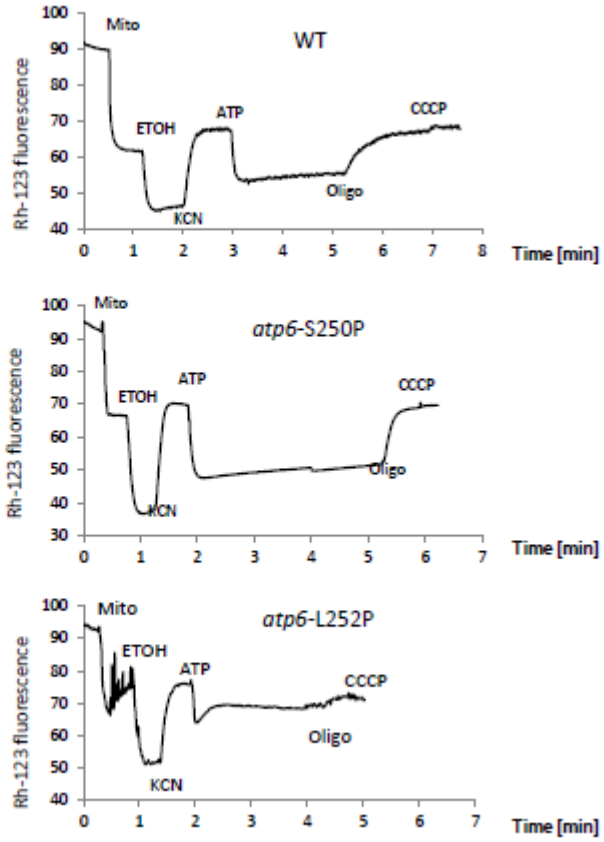


Figure 3

Figure

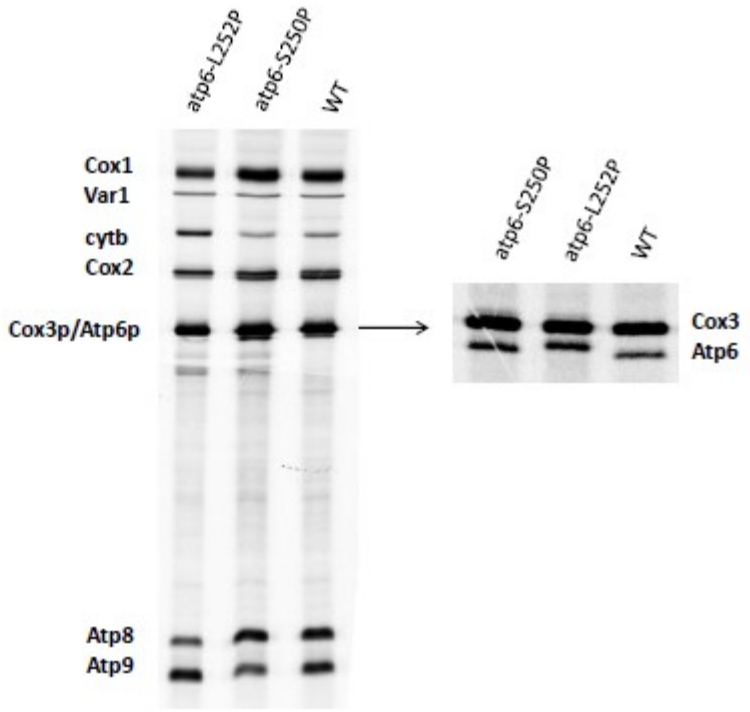


Figure 4