The Leader Peptide of Yeast Atp6p Is Required for Efficient Interaction with the Atp9p Ring of the Mitochondrial ATPase*

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Atp6p (subunit 6) of the Saccharomyces cerevisiae mitochondrial ATPase is synthesized with an N-terminal 10-amino acid presequence that is cleaved during assembly of the complex. This study has examined the role of the Atp6p presequence in the function and assembly of the ATPase complex. Two mutants were constructed in which the codons for amino acids 2–9 or 2–10 of the Atp6p precursor were deleted from the mitochondrial ATP6 gene. The concentration of Atp6p and ATPase complex was approximately 2 times less in the mutants. The lower concentration of ATPase complex in the leaderless mutants correlated with less Atp6p complexed with the Atp9p ring of the F0 sector and with accumulation of an Atp6p-Atp8p complex that aggregated into polymers destined for eventual proteolytic elimination. We propose that the presequence either targets Atp6p to the Atp9p or signals insertion of the Atp6p precursor into a microcompartment of the membrane for more efficient interaction with the Atp9p ring. Despite the ATPase deficiency, growth of the leaderless atp6 mutants on respiratory substrates and the efficiency of oxidative phosphorylation were similar to that of wild type, indicating that the mutations did not affect the proton permeability of mitochondria.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—The genotypes and sources of the S. cerevisiae strains used in this study are listed in Table 1. The compositions of the media used to grow yeast have been described previously (14).

Construction of a Yeast Mutant Expressing Leaderless Atp6p—The wild type ATP6 locus from nucleotide position −316 upstream of the initiator codon to nucleotide position +275 downstream of the stop codon was PCR-amplified using strain FY1679 mtDNA3 (sequenced by Fourny et al. (15)) as a template and primers 5′-CGggatccCAGTGGGGAAGGAGTGA-3′ and 5′-CGggatccGCGGACCCAYAGGAGG and 5′-CGggatccCATGGGGAAGGAGTGA-3′. The PCR product was cut at its ends with BamHI and internally in the ATP6 coding sequence with EcoRI. The resulting two BamHI-EcoRI fragments were cloned into pUC19 to give pSDC8 and pSDC19. The plasmid pSCDs, containing the 5′ region of ATP6, was mutagenized with primers COUP1 and COUP2 or SANSCOUP1 and SANSCOUP2 (Table 2), using the QuikChange kit from Stratagene, to give plasmids pRK12 and pRK13, respectively. The fragments with the mutated ATP6 sequences were removed from pRK12 and pRK13 with BamHI and EcoRI and cloned into pJM2 to obtain plasmids pRK14 and pRK15.

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3 The abbreviations used are: mtDNA, mitochondrial DNA; MOPS, 4-morpholinepropanesulfonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; ρ0′, genome, wild type mitochondrial DNA; ρ0′ mutant, respiratory-deficient mutant lacking mitochondrial DNA; ρ−, mutant, respiratory-deficient mutant with a partially deleted mitochondrial genome.
respectively. pJM2 contains COX2 as a genetic marker for mitochondrial transformation (12). The plasmid pSDC19 was cut with EcoRI and SapI and cloned into pRK14 and pRK15. The resulting plasmids pRK16 and pRK17 contain the ATP6 coding sequence with a deletion of codons 2–9 and 2–10 of the ATP6, respectively.

pRK16 and pRK17 were co-transformed with the LEU2-bearing plasmid pFL46 into strain DFS160/ρ0 by microprojectile bombardment using a biolistic PDS-100/He particle delivery system (Bio-Rad) as described previously (16). Mitochondrial transformants were identified among the leucine-independent clones by their ability to produce respiring clones when crossed to the nonrespiring strain NB40-3C, containing a deletion in the mitochondrial COX2 gene (16). The mitochondrial synthetic ρ- transformants were crossed to MR10. The latter strain is isogenic with MR6 except that it contains the mtDNA of FY1679 (15) with ATP6 replaced with ARG8m (13). Haploid cells with the nuclear genome of MR10 and ρ- recombinant mtDNA in which ARG8m was replaced with the mutant ATP6 genes of the synthetic ρ- strains, were identified by crosses to SDS30 (13), a ρ- strain containing only ATP6 (Fig. 1).

Preparation of Yeast Mitochondria and Respiratory and ATPase Assays—Mitochondria were prepared by the method of Faye et al. (17) except that Zymolyase 20,000 (MP Biochemicals, Inc., Aurora, OH) instead of Glusulase was used to convert cells to spheroplasts. ATPase activity
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was assayed by measuring the release of inorganic phosphate from ATP (18) at 37 °C in the presence or absence of oligomycin. Respiration, membrane potential, and ATP synthesis measurements were performed on isolated mitochondria prepared by the method described by Guérin et al. (19). Oxygen consumption rates were measured with a Clark electrode in respiration buffer containing 0.65M mannitol, 0.36 mM EGTA, 5 mM Tris-phosphate, 10 mM Tris-maleate, pH 6.8, as described previously (20). To measure rates of ATP synthesis, mitochondria (0.3 mg/ml) were placed in a 2-ml thermostatically controlled chamber at 28 °C in respiration buffer. The reaction was started by the addition of 4 mM NADH and 1 mM ADP and stopped by the addition of oligomycin (3 μM/ml) and a 0.15 mg/ml mitochondrial proteins, 10 μl of ethanol (EtOH), 6 μg/ml oligomycin (oligo), 0.2 mM potassium cyanide (KCN), 50 μM ADP, 1 μM ATP, and 3 μM CCCP.

In Vivo Labeling Mitochondrial Gene Products—Cells were grown overnight in YPGal and inoculated into 10 ml of medium containing 2% galactose, 0.67% yeast nitrogen base without amino acids and the appropriate auxotrophic requirements. Cells equivalent to an A600 of 0.5 were harvested at a growth density of 1–2 A600. After centrifugation and washing with 40 mM potassium phosphate, pH 6.0, containing 2% galactose, the cells were suspended in 500 μl of the same buffer, and 15 μl of freshly prepared aqueous solution of cycloheximide (7.5 mg/ml) was added. The cells were incubated at 24 °C for 5 min prior to the addition of 50 μCi of [35S]methionine (100 Ci/mmol; GE Healthcare). The reaction was terminated after 30 min with 500 μl of 20 mM methionine and 75 μl of 1.8 M NaOH, 1 mM β-mercaptoethanol, and 0.01 M phenylmethylsulfonyl fluoride. An equal volume of 50% trichloroacetic acid was added, and the mixture was centrifuged in a microcentrifuge for 5 min at 14,000 rpm. The precipitated proteins were washed once with 0.5 M Tris (free base) and two times with water and were suspended in 45 μl of sample buffer (22).

RESULTS

Genetic Stability of Mutants Expressing Atp6p without the N-terminal Leader Peptide—Two strains of yeast expressing leaderless Atp6p differing only by one extra amino acid at the N terminus were constructed by the procedure described under “Experimental Procedures” and schematically illustrated in Fig. 1. The atp6Δ2-9a and atp6Δ2-10a alleles with deletions of codons 2–9 and 2–10, respectively, were introduced by microprojectile bombardment into DFS160, a kar1 mutant with an arg8 auxotrophic marker and lacking mtDNA (12). The mutant genes of the resultant synthetic p–p– clones were recombined into the full-length mitochondrial genome by crosses to MR10, a respiratory defective mutant in which a mitochondrial variant of ARG8 was substituted for most of the ATP6 coding sequence (Fig. 1A) (13). Recombinant clones with atp6Δ2-9a and
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**TABLE 3**

Mitochondrial respiratory and ATP synthesis activities of mitochondria from wild type and atp6 mutants

Mitochondria were isolated from wild type strain MR6 and atp6 leaderless mutants RKY46 and RKY48-1 grown for 5-6 generations in rich galactose medium at 28 °C. The additions were 0.15 mg/ml proteins, 4 mM NADH, 150 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 respiratory activities were assayed in triplicate with the S.D. values indicated.

| Strain | Respiration rates | ATP synthesis |
|--------|-------------------|---------------|
|        | +NADH | +NADH + ADP | +NADH + CCCP | Asc/TMPD + CCCP | RC* | −Oligo | +Oligo | Inhibition |
| MR6    | 298 ± 66 | 624 ± 50 | 975 ± 1 | 2300 ± 12 | 2.1 | 655 | 22.4 | 97.7 |
| RKY46  | 165 ± 12 | 443 ± 26 | 965 ± 28 | 1484 ± 3 | 2.8 | 446 | 36 | 92.0 |
| RKY48-1| 166 ± 28 | 422 ± 28 | 797 ± 15 | 1337 ± 26 | 2.5 | 459 | 54 | 88.3 |

* Respiratory control.

**TABLE 4**

ATPase activities of mitochondria from wild type and atp6 mutants

Mitochondria were prepared from the respiratory competent haploid strains MR6 and the atp6 leaderless mutants RKY48-1, RKY48-2, RKY46, and RKY47. A303AATP23/ST15 has a copy of the E168Q mutant atp3 gene inserted at the ara31 locus in nuclear DNA. Samples of the cultures were used to determine the percentage of ρ− cells. ATPase activity was measured at 37 °C. The values reported are averages of duplicate assays with the S.D. values indicated.

| Strain     | α* | ATPase | Inhibition |
|------------|----|--------|------------|
|            |    | Without oligomycin | With oligomycin |
|            |    | μmol/min/mg | μmol/min/mg |
| Experiment 1 |    |            |            |
| MR6        | >99 | 5.90 ± 0.010 | 0.75 ± 0.005 | 87 |
| RKY48-1    | >99 | 5.26 ± 0.008 | 1.65 ± 0.005 | 69 |
| RKY48-2    | >99 | 5.19 ± 0.016 | 1.52 ± 0.002 | 71 |
| RKY46      | >99 | 5.02 ± 0.000 | 1.37 ± 0.002 | 73 |
| RKY47      | >99 | 5.17 ± 0.01  | 1.38 ± 0.002 | 73 |
| Experiment 2 |    |            |            |
| W303-1A    | >99 | 4.43 ± 0.40  | 0.77 ± 0.15  | 83 |
| W303ΔATP23 | >99 | 2.62 ± 0.30  | 2.21 ± 0.20  | 16 |
| aW303ΔATP23/ST15 | >99 | 4.25 ± 0.07 | 0.32 ± 0.05 | 92 |

* The percentage of α* in the atp23 null mutant ranges from 20 to 30%.

atp6Δ2-10a were identified by the acquisition of arginine dependence and were further verified to have a ρ+ genome with the mutant genes by sequence analysis (not shown).

In view of earlier findings that mutations in ATPase genes promote an instability of the mitochondrial genome resulting in the production of ρ− clones, two independent recombinants with the atp6Δ2-9a allele (RKY46, RKY47) and the atp6Δ2-10a allele (RKY48-1 and RKY48-2) were checked for the percentage of ρ− mutants in cultures grown under nonselective conditions in rich glucose medium. The four mutants were found to contain less than 5% secondary ρ− clones, indicating that the absence of the Atp6p leader did not adversely affect the stability of mtDNA.

Respiratory Growth and Activities of Leaderless Mutants—To ascertain if deletion of the Atp6p leader peptide affects assembly of this subunit into a functional F1, RKY46 and RKY48-1 were tested for growth on the nonfermentable carbon sources glycerol/ethanol, and isolated mitochondria were assayed for several activities related to respiration and oxidative phosphorylation. Both mutants grew nearly identically to wild type on rich glycerol/ethanol, indicating that the absence of the leader peptide did not compromise the respiratory capacity (Fig. 2A). This is supported by the visible spectra of mitochondrial cristomes in RKY48-1 or RKY46, which were similar to those of the wild type MR6 (Fig. 2B). Measurements of NADH oxidation in the mutants in the presence of the uncoupler CCCP were not significantly different from wild type (Table 3), although state 3 respiration was reduced by ~30%. The cytochrome oxidase activity was also 40% lower in the mutants (Table 3).

ATPase and Phosphorylative Activities Are Partially Reduced in the Leaderless Mutants—The growth properties of the mutants indicated that the rate of ATP synthesis was not a limiting factor in utilization of the nonfermentable carbon sources. This, however, did not exclude the possibility of a partial perturbation of the ATPase, since the phenotype of S. cerevisiae does not correlate strictly with the level of ATPase, and some reduction of the enzyme (up to 80%) is tolerated without appreciably affecting growth of this organism on respiratory substrates (28).

Assays of RKY46 and RKY48-1 mitochondria indicated a small but measurable decrease in total ATPase activity as well as a doubling of the oligomycin-insensitive activity (Table 4). The latter finding suggests that some of the hydrolytic activity measured in the mutant mitochondria is contributed by F1, ATPase that is not functionally coupled to F0. This was also supported by the detection of F1 in the blue native gel of the mutant mitochondria (Fig. 3B) and the sedimentation of the β-subunit of F1 as a broad peak composed of F1-F0 (Fig. 3C).

The rate of ATP synthesis was 30% lower in the mutant mitochondria, consistent with the reduced amount of F1-F0 (Table 3). In contrast, the respiratory control or the ratio of state 3 (+ ADP) to state 4 respiration, a measure of phosphorylation efficiency was, if anything, slightly higher in the mutant than in wild type. This indicates that the ATPase assembled from the leaderless Atp6p is as efficient in coupling proton translocation to electron transport as the enzyme formed from the precursor.

NADH- and ATP-dependent proton translocation of mitochondria was tested using rhodamine 123 fluorescence...
quenching to measure changes in electrical potential. The results of these experiments confirmed that the kinetics with which the membrane potential was established upon the addition of substrate were similar in wild type and mutant mitochondria (Fig. 1C). This was also true for the transition of state 4 to state 3 respiration after the addition of ADP. The reestablishment of the state 4 potential following exhaustion of ADP, however, occurred more slowly in the mutants, probably because of a lower rate of ATP synthesis (Table 3). The addition of ATP to KCN-inhibited mitochondria induced a rapid increase in membrane potential in both wild type and mutant mitochondria. The dissipation of the potential by oligomycin, a measure of proton leakage, was slightly slower in the mutants, perhaps because of their lesser ATPase content.

Less Atp6p Is Assembled into the ATPase Complex in the Mutants — The partial loss of oligomycin sensitivity and the lower rate of ATP synthesis pointed to a partial deficit of F0 in the leaderless Atp6p mutants. This was confirmed by the 2-fold lower steady-state concentration of Atp6p (Fig. 3A) and the presence of F1 ATPase and the unassembled Atp9p ring in mutant mitochondria (Fig. 3B). All of the Atp6p in both wild type and the leaderless mutant co-sedimented with the F1 subunit in regions corresponding to F1-F0 (Fig. 3C). Atp9p forms a ring consisting of 10 subunits, even when assembly of F0 is blocked by mutations in other subunits of F0, such as Atp6p. This is demonstrated by the atp6 null mutant MR10 (13) which is able to assemble F1 and the Atp9p ring, although F0 and F1-F0 are not detectable (Fig. 3B).

Turnover of Atp6p in Wild Type and the Leaderless Mutant — The reduced amount of Atp6p in the leaderless mutant could be caused by decreased synthesis, faster degradation, or less efficient assembly of the leaderless subunit into the ATPase complex. An effect of the mutation on translation was excluded by

FIGURE 3. Immunodetection of ATPase constituents. A, total mitochondrial proteins from the wild type strain MR6 and the two atp6 mutants were separated on a 12% polyacrylamide gel (22), transferred to nitrocellulose, and reacted with rabbit polyclonal antibodies against Atp6p and the F1 ATPase. The blot was treated with peroxidase-coupled anti-rabbit IgG, and antibody-antigen complexes were visualized with the Super Signal Kit (Pierce). B, mitochondrial extracts of wild type strain MR6, the atp6 leaderless mutants RKY46 and RKY48-1, and the atp6 deletion strain MR10 were prepared with 2 g of digitonin/g of protein and subjected to blue native PAGE. The gels were incubated with ATP-Mg2⁺ and Pb2⁺ to reveal ATPase activity. The protein complexes were transferred to polyvinylidene difluoride membranes and probed with antibodies against Atp9p. C, wild type (MR6) and mutant (RKY48-1) mitochondria at 10 mg/ml were extracted with 0.5% dodecyl maltoside. The extracts were layered on top of 4.6 ml of a 7–25% linear sucrose gradient and were centrifuged at 65,000 rpm in a Beckman SW65Ti rotor for 3.5 h. Thirteen equal fractions were collected, and samples were separated by SDS-PAGE on a 12% polyacrylamide gel (22). The proteins were transferred to nitrocellulose and reacted sequentially with a rabbit polyclonal antibody against Atp6p and the F1 subunit of F1. The blot was reacted with anti-rabbit IgG and visualized as in A.

FIGURE 4. Stability of newly synthesized Atp6p. A, mitochondria from the parental wild type MR6 and of the mutants RKY46 and RKY48-1 were labeled with [³⁵S]methionine for 30 min at 24 °C (29). Turnover of newly synthesized but unassembled Atp6p was measured by the addition of excess cold methionine and incubation for the indicated periods of time at 30 °C. Total mitochondrial proteins were separated on a 12.5% polyacrylamide gel prepared in the presence of 4 M urea and 25% glycerol. Proteins were transferred to nitrocellulose and exposed to x-ray film. The mitochondrially translated proteins identified in the margin are as follows: Var1p, a subunit of mitochondrial ribosomes; Cox1p, Cox2p, and Cox3p, subunits of cytochrome oxidase; cytochrome b, a subunit of the bc1 complex; Atp6p, Atp8p, and Atp9p, subunits of the ATPase. B, the bands corresponding to Cox1p and Atp6p in A were quantified with a PhosphorImager and normalized to the value obtained at zero time.
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FIGURE 5. Properties of Atp6p in the leaderless mutant. A, mitochondria were prepared from the parental wild type MR6 and the leaderless mutant RKY48-1 grown to early stationary phase in YPGal. The mitochondria (1 mg of protein) were labeled with $[^{35}S]$methionine for 20 min. The labeled mitochondria were treated with 1% dodecyl maltoside and clarified by centrifugation at 100,000 × g, for 20 min. The extracts were mixed with lactate dehydrogenase (132 kDa) and applied to 4.6 ml of 7–25% linear sucrose gradients prepared in the presence of 10 mM Tris-Cl, pH 7.5, 0.1% Triton X-100 and centrifuged for 8 h at 65,000 rpm in a Beckman SW65Ti rotor. The gradients were collected in 13 equal fractions, and samples were separated by SDS-PAGE on 12.5% polyacrylamide gel prepared in the presence of 4% urea and 25% glycerol (top) to resolve Atp6p and Cox3p and on a 17% gel (bottom) to separate Atp8p and Atp9p. Proteins were transferred to nitrocellulose and exposed to x-ray film. The radioactively labeled mitochondrial translation products are identified as in Fig. 3. The peak of lactate dehydrogenase in both gradients was between fractions 5 and 6. Although the increased resistance of Atp6p to digestion by externally added protease K in the mutant mitoplasts was observed reproducibly, the apparent increase in Cox1p stability in the mutant mitochondria treated with protease K was not. B, fractions 4 and 8 of the gradients shown in A were treated with preimmune serum or antiserum to Atp6p in the presence of protein A-Sepharose for 4 h at 4°C. Proteins adsorbed to the beads and samples of untreated fractions 4 and 8 (T) representing 30% of what was used for the immunoprecipitation were separated by SDS-PAGE on a 17% polyacrylamide gel. The lower of the two Atp6p bands is probably a proteolytic product.

in vivo labeling of mitochondrial translation products with $[^{35}S]$methionine in the presence of cycloheximide. Synthesis of Atp6p and of the other mitochondrial gene products was comparable in the mutant and wild type strains (Fig. 4A). Unexpectedly, Atp6p was more stable in the mutants (Fig. 4, A and B). After 90 min of chase, as much as 80–90% of Atp6p synthesized during a 30-min pulse was detected in the two mutants. In contrast, only 40% of the Atp6p synthesized in the wild type strain MR6 survived after 15 min of chase, and almost all of the protein was degraded after 90 min (Figs. 4, A and B).

Newly Synthesized Atp6p Exists in Two Physically Distinct Forms in the Leaderless Mutant—The enhanced stability of newly synthesized leaderless Atp6p in the mutants suggested that its chemical context or environment may be different from that of processed Atp6p in wild type. This was confirmed by the physical properties of Atp6p in wild type and in the mutants. Mitochondrial products labeled in organello were extracted with dodecyl maltoside, size-fractionated on a sucrose gradient, and analyzed by SDS-PAGE. These conditions are the same as those used to solubilize the F$_1$–F$_0$ complex.

The results of this experiment indicated that most of the Atp6p co-sedimented with Atp9p in wild type but not in the mutant (Fig. 5A). Whereas Atp6p sedimented with an apparent mass of ~80 kDa in wild type, the distribution of Atp6p in the gradient of the mutant suggested a heterogeneous population of higher molecular mass. Similar results were obtained when digitonin instead of dodecyl maltoside was used for the extraction. In contrast, the sedimentation of Atp9p was very similar in wild type and the mutant. Another difference between the two strains was evident in the sedimentation behavior of Atp8p. The main peak of this subunit was distinct from that of Atp6p and Atp9p in wild type. In the mutant, Atp8p displayed a dual distribution with a fraction having sedimentation properties similar to wild type and another fraction that co-sedimented with Atp6p in the high molecular weight region of the gradient (Fig. 5A).

The two different forms of Atp6p were further characterized by immunoprecipitation of fractions 4 and 8 of each gradient with Atp6p antibody. The antigen-antibody complexes adsorbed to protein A were analyzed by SDS-PAGE. Two Atp6p bands were observed, the smaller of which may be a partial proteolytic product. The Atp6p antiserum also precipitated Atp9p from fraction 8 of both gradients, indicating that some of this subunit was complexes with the Atp9p ring. Because most of Atp6p in the mutant sedimented as high molecular weight aggregates, less of the subunit was recovered as a complex with Atp9p from fraction 8 of the mutant relative to wild type (Fig. 5B). As expected, more Atp6p was precipitated from fraction 4 of the mutant than wild type, and the presence of a strong Atp8p signal in the precipitate indicated that the faster sedimenting fraction of Atp8p in the mutant was complexes with Atp6p. This complex was also detected in the comparable fraction of wild type, although at much low abundance (Fig. 5B).

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A–D. With the exception of Var1, lysis of the outer membrane by hypotonic shock in the presence of proteinase K caused a substantial decrease of all of the labeled products in wild type, including Atp6p, of which less than 40% remained after the 45 min of digestion. A similar pattern of digestion occurred in the mutant mitoplasts, except that 82% of Atp6p remained after the digestion (Fig. 7, A and B). The more protected environment of Atp6p in the mutant was also evident when mitochondria, which had been converted to submitochondrial vesicles, were similarly treated with proteinase K (Fig. 7, C and D). The overall digestion of most of the labeled mitochondrial products was less in the submitochondrial particles than in mitoplasts. However, Atp6p again showed a greater resistance to proteinase K in the submitochondrial particles obtained from the mutant mitochondria. This was true regardless of whether proteinase K was added after disruption of mitochondria by ultrasound as shown in Fig. 7, C and D, or during the sonic treatment (not shown). The protease resistance of Atp6p in the inverted vesicles of the mutant provides strong evidence that the absence of the leader does not interfere with insertion of this ATPase subunit into the inner membrane.

DISCUSSION

Atp6p is known to interact with the Atp9p ring in F₀ to form the interface involved in translocation of protons during ATP synthesis or hydrolysis (30). In S. cerevisiae, this subunit is synthesized with a 10-amino acid-long N-terminal presequence, which was recently shown to be proteolytically removed by a metalloprotease encoded by ATP23 (11, 12). Inactivation of the proteolytic activity of Atp23p indicated that removal of the presequence is not an essential condition for assembly or function of the ATPase (11, 12). It remained unclear, however, if the presequence is needed for assembly of Atp6p with the other subunits of F₀.

Evidence presented here demonstrates that although a functional complex is formed in mutants expressing Atp6p without its normal N-terminal presequence, the process of F₀ assembly is some 2 times less efficient. This is supported by the presence of only half as much fully assembled complex in mutant mitochondria, the decreased oligomycin sensitivity of the ATPase, and detection in the mutant of F₁ and of the unassembled Atp9p ring. This ring structure is stable even when it is not part of the larger F₁ complex. Despite the ATPase deficit, the Atp6p leaderless mutants grow almost as well as wild type yeast on nonfermentable carbon sources, and there is minimal loss of phosphorylation efficiency. The lower rate of phosphorylation observed in the mutants is probably due to the lower rate of respiration rather than decreased coupling of the mitochondria.

To gain insights into the reasons for the less efficient assembly of the ATPase complex, the stability of Atp6p was examined in cells labeled in vivo in the presence of cycloheximide. Whereas virtually all of Atp6p was degraded during a 90-min chase in the wild type, the mutants retained 80–90% of the subunit, indicating that newly synthesized Atp6p is significantly more stable in the latter strains. The susceptibility of newly translated Atp6p to externally added protease in mitoplasts and inverted submitochondrial particles derived from mutant and wild type mitochondria was also
consistent with the protein being in a more protected environment in the mutant. Less than 20% of Atp6p was degraded by protease K in mitoplasts of the mutant, whereas more than 60% was degraded in wild type mitoplasts under the same conditions. A similar trend was seen in inverted vesicles obtained by sonic disruption of mutant and wild type mitochondria. The slower turnover of Atp6p in the mutant and the increased resistance to protease K in submitochondrial vesicles constitute strong evidence that the newly synthesized leaderless Atp6p is inserted into the inner membrane. Failure to do so would be expected to increase turnover of Atp6p and to make it more prone to digestion by exogenous proteinase in disrupted mitochondria.

Distinct from the results reported here for Atp6p, substitution of the normal mitochondrial COX2 gene by a variant coding for a leaderless Cox2p elicits a severe cytochrome oxidase deficiency (31). In this instance, the absence of the leader was found to greatly increase turnover of the protein. This and the fact that the cox2 mutant was rescued by a \( \rho^- \) suppressor genome in which the first 251 codons of the cytochrome b gene were fused to the mutant COX2 indicated that the leader of Cox2p is necessary for membrane insertion of the protein (31).

Sedimentation of detergent-solubilized mitochondria following in organello labeling of their endogenous gene products revealed that most of Atp6p in the mutant sedimented as heterogeneous high molecular weight aggregates. The sucrose gradients and immunoprecipitation also indicated that the polymeric fraction of Atp6p was complexed to Atp8p, part of which co-sedimented with Atp6p. In contrast, most of the Atp6p in the wild type displayed a symmetric distribution in the gradient with an estimated size of 80 kDa. Immunoprecipitation confirmed some of the newly translated Atp6p in wild type mitochondria to be associated with the Atp9p ring. Although complexes of Atp6p with Atp9p and of Atp6p with Atp8p were present in both mutant and wild type, only a small fraction of Atp6p was present in a complex with Atp8p in wild type, and approximately 2 times less of Atp9p was co-immunoprecipitated with Atp6p in the leaderless mutant.

The results of the sucrose gradient combined with the immunoprecipitation suggest that the association of Atp8p with \( F_0 \) or an \( F_0 \) intermediate normally occurs after formation of an Atp6p-Atp9p complex. This result is at variance with earlier conclusions that the interaction of Atp9p with Atp6p follows its interaction with Atp8p (32). We have no explanation for this discrepancy. Atp8p has been shown by cross-linking experiments to be in contact with Atp6p (33). This is consistent with our evidence that a large part of
Atp6p in the mutant is associated with Atp8p. The presence of only a small fraction of Atp6p in complex with Atp8p in wild type suggests that normally the interaction of the precursor with the Atp9p ring takes precedence over the reaction with Atp8p. In the mutant, the situation is reversed as the interaction of the leaderless Atp6p with Atp8p is favored. The results of the sucrose gradients also indicate that the fraction of Atp6p complexed to Atp8p has a tendency to aggregate into larger polymers. Although we prefer this interpretation, the possibility is not excluded that the pathway for subunit interactions is altered in the mutant, resulting in a less efficient assembly of the enzyme.

The increased stability of the Atp6p in the mutant may be a direct result of its association with Atp8p, which might mask protease-susceptible sites or an indirect effect due to the further polymerization of the Atp6p-Atp8p complex. It is important to point out, however, that the enhanced stability of Atp6p in the mutant applies only to the newly translated subunit under conditions when it is not incorporated into the ATPase complex. In growing cells, the Atp6p that is not recruited for ATPase assembly becomes a target for degradation and clearing from the membrane.

Although the above discussion helps to account for the stability of newly translated Atp6p and points to a plausible step at which assembly of the ATPase complex may be compromised in the mutant, it does not explain the slower assembly of Atp6p with the Atp9p ring. We propose two different but related mechanisms in which the leader peptide may help to increase the efficiency of F0 assembly (Fig. 8). In the first scenario, the Atp6p leader may function to guide the precursor to a physically distinct subcompartment of the inner membrane, where assembly of the mitochondrially translated subunits of the complex is initiated. Alternatively, the presequence may guide Atp6p to Atp9p through protein recognition, thereby assuring a kinetically favored interaction of the two. In either mechanism, the presequence acts to target Atp6p to the Atp9p ring.

Finally, the absence of a growth phenotype in the leaderless atp6 mutant does not signify that the leader does not play an important role in the overall health of the cell.

Growth of the leaderless mutants in this study was measured under optimal conditions that do not exist in the natural habitat of this yeast, where the 2-fold decrease of ATPase may be a serious disadvantage under some circumstances.

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REFERENCES
1. Boyer, P. D. (1997) Annu. Rev. Biochem. 66, 717–749
2. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) Biochim. Biophys. Acta 1553, 188–211
3. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
4. Ackerman, S. H., and Tzagoloff, A. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 13339–13344
5. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
6. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) Biochim. Biophys. Acta 1553, 188–211
7. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
8. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
9. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
10. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
11. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
12. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
13. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
14. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
15. Velours, J., and Arselin, G. (2000) J. Bioenerg. Biomembr. 32, 383–390
Rak, M., Tetaud, E., Godard, F., Sagot, I., Salin, B., Duvezin-Caubet, S., Slonimski, P. P., Rytka, J., and di Rago, J. P. (2007) J. Biol. Chem. 282, 10853–10864
14. Myers, A. M., Pape, K. L., and Tzagoloff, A. (1985) EMBO J. 4, 2087–2092
15. Foury, F., Roganti, T., Lecrenier, N., and Purnelle, B. (1998) FEBS Lett. 440, 325–331
16. Bonnefoy, N., and Fox, T. D. (2001) Methods Cell Biol. 65, 381–396
17. Faye, G., Kujawa, C., and Fukuhara, H. (1974) J. Mol. Biol. 88, 185–203
18. King, E. J. (1932) Biochem. J. 26, 292–297
19. Guérin, B., Labbe, P., and Somlo, M. (1979) Methods Enzymol. 55, 149–159
20. Rigoulet, M., and Guérin, B. (1979) FEBS Lett. 102, 18–22
21. Emaus, R. K., Grunwald, R., and Lemasters, J. J. (1986) Biochim. Biophys. Acta 850, 436–448
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Schägger, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223–231
25. Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D., Brôthes, D., di Rago, J.-P., and Velours, J. (2002) EMBO J. 21, 221–230
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
27. Tzagoloff, A., Akai, A., and Needleman, R. B. (1975) J. Biol. Chem. 250, 8228–8235
28. Mukhopadhyay, A., Uh, M., and Mueller, D. M. (1994) FEBS Lett. 343, 160–164
29. Hell, K., Tzagoloff, A., Neupert, W., and Stuart, R. A. (2000) J. Biol. Chem. 275, 4571–4578
30. Fillingame, R. H. (2000) Nat. Struct. Biol. 7, 1002–1004
31. Torello, A. T., Overholtzer, M. H., Cameron, V. L., Bonnefoy, N., and Fox, T. D. (1997) Genetics 145, 903–910
32. Hadikusumo, R. G., Meltzer, S., Choo, W. M., Bernadette Jean-Francois, M. J., Linnane, A. W., and Marzuki, S. (1988) Biochim. Biophys. Acta 933, 212–222
33. Stephens, A. N., Khan, M. A., Roucou, X., Nagley, P., and Devenish, R. J. (2003) J. Biol. Chem. 278, 17867–17875