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The Metalloprotease Encoded by *ATP23* Has a Dual Function in Processing and Assembly of Subunit 6 of Mitochondrial ATPase

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In the present study we have identified a new metalloprotease encoded by the nuclear *ATP23* gene of *Saccharomyces cerevisiae* that is essential for expression of mitochondrial ATPase (F_1-F_O complex). Mutations in *ATP23* cause the accumulation of the precursor form of subunit 6 and prevent assembly of F_O. Atp23p is associated with the mitochondrial inner membrane and is conserved from yeast to humans. A mutant harboring proteolytically inactive Atp23p accumulates the subunit 6 precursor but is nonetheless able to assemble a functional ATPase complex. These results indicate that removal of the subunit 6 presequence is not an essential event for ATPase biogenesis and that Atp23p, in addition to its processing activity, must provide another important function in F_O assembly. The product of the yeast *ATP10* gene was previously shown to interact with subunit 6 and to be required for its association with the subunit 9 ring. In this study one extra copy of *ATP23* was found to be an effective suppressor of an *atp10* null mutant, suggesting an overlap in the functions of Atp23p and Atp10p. Atp23p may, therefore, also be a chaperone, which in conjunction with Atp10p mediates the association of subunit 6 with the subunit 9 ring.

INTRODUCTION

The proton-translocating ATPase (F_1-F_O) of mitochondria catalyzes a vectorial transfer of protons to the matrix compartment when it functions as an ATP synthase (Boyer, 1997; Senior *et al.,* 2002). It also promotes a transfer of protons from the matrix to the intermembrane space when ATP is hydrolyzed (Boyer, 1997; Senior *et al.,* 2002). F_O is a hydrophobic protein consisting of upward of nine different subunits in fungal and mammalian mitochondria (Velours and Arselin, 2000; Ackerman and Tzagoloff, 2005). Proton transfer occurs at an interface between subunit 6 (subunit a) and subunit 9 (subunit c), the latter being present in 10–11 copies forming a ring structure that rotates with respect to the single subunit 6 (Nakamoto *et al.,* 1999).

Three subunits of the ATPase complex of yeast mitochondria are encoded by mitochondrial DNA (mtDNA) (Hensgens *et al.,* 1979; Macino and Tzagoloff, 1979, 1980; Macreadie *et al.,* 1983). They are the already-mentioned subunits 6 and 9 of F_O, and subunit 8, another component of F_O, the function of which is still unclear at present. The remaining subunits of F_O as well as the five subunits of F_1 ATPase are products of nuclear genes that are imported into different compartments of the organelle where they assemble with their mitochondrial partners to form the holoenzyme. In addition to the structural and catalytic subunits of the F_1-F_O complex, the nuclear genome also codes for proteins that are not part of the complex but are essential for its assembly. Three such factors have been shown to be necessary for oligomerization of the F_1 ATPase (Ackerman and Tzagoloff, 1990a; Lefebvre-Legendre *et al.,* 2001). Other factors have been implicated in expression of subunits 6 and 9 of F_O (Ackerman and Tzagoloff, 1990b; Payne *et al.,* 1991; Helfenbein *et al.,* 2003; Ellis *et al.,* 2004).

Subunit 6 is synthesized with an N-terminal extension that is proteolytically removed after insertion/assembly of the precursor (Michon *et al.,* 1988). Until now, the enzyme responsible for the maturation of the subunit 6 precursor has not been identified. As part of an effort to catalogue and functionally characterize nuclear gene products involved in assembly of the respiratory pathway, we have screened respiratory-deficient mutants of *Saccharomyces cerevisiae* for defects in the ATPase complex. In the present communication we report that the ATPase subunit 6 precursor is processed to the mature protein by the metallopeptidase encoded by the nuclear gene *ATP23* (reading frame YNR020C on chromosome XIV). Our results also indicate that the efficiency of processing of the precursor depends on the presence of Atp10p, a mitochondrial inner membrane protein previously shown to be required for biogenesis F_O (Ackerman and Tzagoloff, 1990b). In addition to their roles in processing of subunit 6, Atp23p and Atp10p are also essential for assembly of this subunit into a functional F_O.

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Abbreviations used: ρ^o mutant, respiratory-deficient mutant lacking mitochondrial DNA; ρ^- mutant, respiratory-deficient mutant with a partially deleted mitochondrial genome; *pet* mutant, respiratory-deficient mutant of yeast with a mutation in a nuclear gene; DOC, potassium deoxycholate; PMSF, phenylmethylsulfonyl fluoride; SMP, submitochondrial particles.

MATERIALS AND METHODS

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 elsewhere (Myers *et al.,* 1985).

Table 1. Genotypes and sources of yeast strains

Strain	Genotype	Source
D273-10B/A21	MATα *met6*	Tzagoloff *et al.* (1976)
CB11	MATa *ade1*	ten Berge *et al.* (1974)
W303	MATa/α *ade2-1/ade2-1 his3-1,15/his3-1,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*	Dr. Rodney Rothstein[a]
W303-1A	MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	Dr. Rodney Rothstein[a]
W303-1B	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	Dr. Rodney Rothstein[a]
aW303ΔPET494	MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet494::HIS3*	This study
aW303ΔATP23,PET494	MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp23::HIS3 pet494::HIS3*	W303ΔATP23 × aW303ΔPET494
E884	MATα *met6 atp23-1*	Tzagoloff and Dieckmann (1990)
aE884/UL1	MATa *leu2-3,112 ura3-1 atp23-1*	E884 × W303-1A
W303ΔATP23	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp23::HIS3*	This study
aW303ΔATP23	MATa *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp23::HIS3*	This study
W303ΔATP10	MATα *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 atp10::LEU2*	Ackerman and Tzagoloff (1990b)

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Preparation of Yeast Mitochondria and ATPase Assays

Mitochondria were prepared by the method of Faye *et al.* (1974) except that Zymolyase 20,000 instead of Glusulase was used to convert cells to spheroplasts. For the localization of Atp23p, mitochondria were obtained by the method of Glick (1985). ATPase activity was assayed by measuring release of P_i from ATP (King, 1932) at 37°C in the presence or absence of oligomycin.

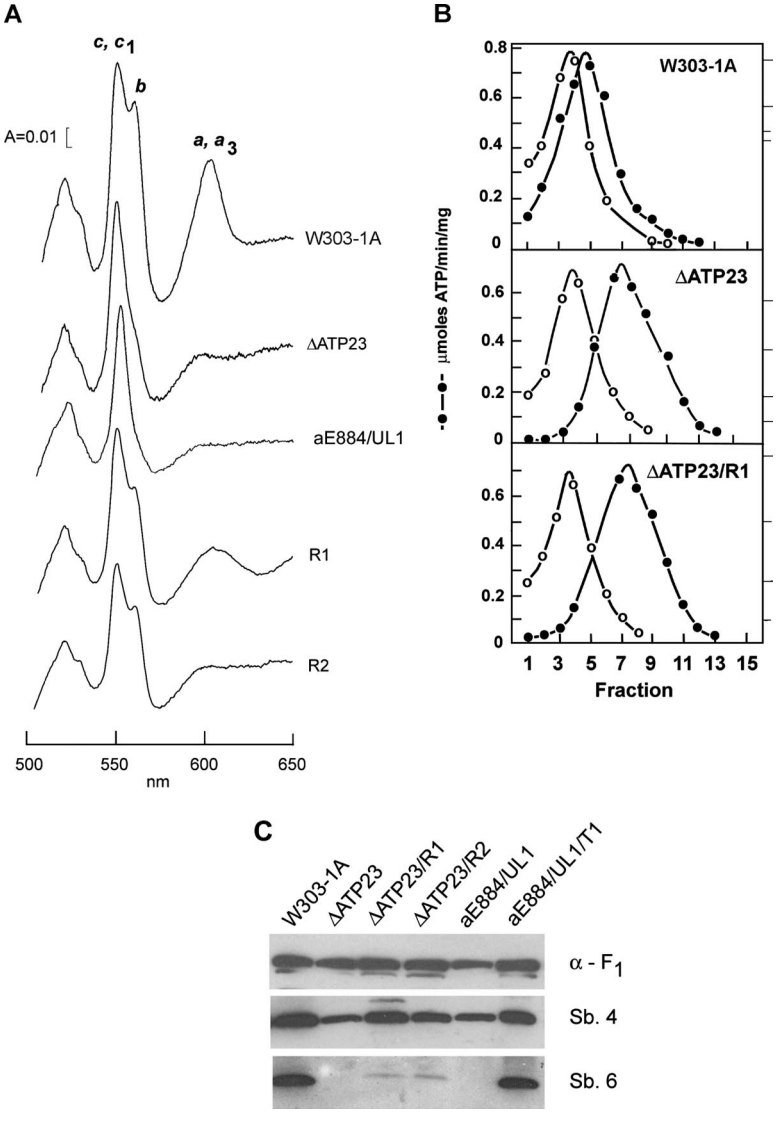

Figure 1. Phenotype of *atp23* mutants. (A) Spectra of mitochondrial cytochromes in wild type, *atp23* mutants, and revertants. Mitochondria of the wild-type strain W303-1A, the *atp23* mutants aE884/UL1 and W303ΔATP23 (ΔATP23), and two independent revertants of W303ΔATP23 (R1, R2) were extracted with potassium deoxycholate at a protein concentration of 5 mg/ml as described previously (Tzagoloff *et al.*, 1975). Difference spectra of the extracts oxidized with potassium ferricyanide and reduced with sodium dithionite were recorded at room temperature. The α-absorption bands corresponding to cytochromes *a*, a_3, and *b* and cytochromes *c* and c_1 are indicated. The percentages of $\rho^{o/-}$ mutants were 85% for W303ΔATP23, and 20% for the revertants. (B) Sedimentation of mitochondrial ATPase. Mitochondrial of the wild-type strain W303-1A, of the *atp23* null mutant aW303ΔATP23, and of revertant W303ΔATP23/R1 were suspended at a protein concentration of 10 mg/ml in 2 mM ATP, 1 mM EDTA, and 20 mM Tris-Cl, pH 7.5. The suspension was adjusted to a final concentration of 0.4% with a 10% solution of Triton X-100 (Tzagoloff and Meagher, 1971) and centrifuged at 250,000 × g_{av} for 15 min. The clarified extracts (0.5 ml) were mixed with 45 μg of β-galactosidase and applied on top of 5 ml of 7–20% linear sucrose gradients containing 2 mM ATP, 20 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 0.1% Triton X-100. After centrifugation at 65,000 rpm in a Beckman SW65 rotor for 3.5 h, 16 equal fractions were collected and assayed for ATPase and β-galactosidase (Wallenfels, 1962). (C) Western analysis of mitochondrial F_O and F_1 subunits. Mitochondria (40 μg protein) from the same strains as in panel A as well as the *atp23* point mutant harboring the wild-type *ATP23* gene (aE884/UL1/T1) were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with polyclonal antibodies to the α subunit of F_1 and subunits 4 and 6 of F_O. After a second reaction with peroxidase-conjugated anti-rabbit IgG, the antibody-antigen complexes were visualized with the SuperSignal chemiluminescent substrate kit (Pierce, Rockford, IL).

Cloning and Sequencing of ATP23

ATP23 was cloned by transformation of the respiratory-deficient mutant aE884/UL1 (MATa *leu2-3,11, ura3-1 atp23-1*) with a yeast genomic library consisting of partial Sau3A fragments of nuclear DNA cloned in YEp24 (Botstein and Davis, 1982). This plasmid library was kindly provided by Dr. Marian Carlson (Department of Genetics and Development, Columbia University). Transformation of aE884/UL1 (10^8 cells) with 10 μg of library DNA yielded a single uracil-independent and respiratory-competent clone (aE884/UL/T1). The plasmid pG200/T1 conferring respiratory competence to the mutant was amplified in *Escherichia coli* RR1 and was used to subclone the gene. The sequences at the junctions of the 8.2-kb insert in pG200/T1 were sequenced and matched to the region of chromosome IX between nucleotides 666087 and 674355.

Disruption of ATP23

The following strategy was used to delete most of the *ATP23* coding sequence. The 2.3-kb EcoRI-HindIII fragment containing the *ATP23* reading frame and flanking sequences was transferred to pUC18. The resultant plasmid (pG200/ST5) was used as a template for PCR amplification of the entire plasmid and insert except for the internal 608 nucleotides coding for residues 58–260 of *ATP23*. The bidirectional primers used for the amplification were 5′-ggcgcggatccgtctcc-accactcaa and 5′-ggcgcggatccgatacgagaccgtttg. The resultant product was digested with BamHI and ligated to the yeast *HIS3* gene on a 1-kb BamHI fragment yielding pG200/ST7. The deleted *atp23::HIS3* allele, isolated from pG200/ST7 as a 2.8-kb linear XbaI-XmnI fragment, was substituted for the wild-type gene by homologous recombination (Rothstein, 1983).

Construction of the E→Q Mutant and of a Hybrid Gene Expressing Atp23p with a C-terminal Hemagglutinin Tag

ATP23 with a E168Q mutation was made by amplification of two separate fragments. The first containing 177 nucleotides of 5′ sequence plus 507 nucleotides of coding sequence with two nucleotide changes to create a unique MfeI site and the glutamine codon, was amplified with primers 5′-ggcggatccgggccaaatattgaactag and 5′-ggccaattgat-gcgaaagcgtatcctc and was digested with a combination of BamHI and MfeI. The remainder of the gene starting with the glutamine codon and containing 155 nucleotides of 3′ sequence was amplified with primers 5′-ggccaattgattcattatt-tcgatgatct and 5′-ggcaagcttgacattctaaggcatcc. This product was digested with MfeI and HindIII. The two PCR fragments were ligated to YEp352 and to YIp352 (Hill *et al.*, 1986) linearized with BamHI and HindIII to yield pG200/ST14 and pG200/ST15, respectively.

To express Atp23p tagged with hemagglutinin (HA; Atp23p-HA), *ATP23* in pG200/T1 was amplified with the primers 5′-acacacctagagctcacaattcaa and 5′-ggcgagctcaagcgtagtctgggacgtcgtatgggtatctgtaaatctcatcaaacgg. The product, consisting of 308 nucleotides of 5′ sequence and the entire *ATP23* gene fused in frame at its 3′ end to a short sequence coding for the HA tag, was digested with SacI and cloned in YEp352 and YIp352 (Hill *et al.*, 1986), yielding pG200/ST12 and pG200/ST13, respectively.

Miscellaneous Procedures

Standard methods were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from *E. coli* (Sambrook *et al.*, 1989). Proteins were separated on SDS-PAGE in the buffer system of Laemmli (1970). Protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

Phenotype of atp23 Mutants

E884 and its derivative aE884/UL1 are respiratory-deficient mutants previously assigned to complementation group G200 of a nuclear *pet* mutant collection (Tzagoloff and Dieckmann, 1990). The respiratory-deficient growth phenotype of the two mutants is complemented by a ρ^o tester confirming the presence of a recessive mutation in nuclear DNA. When grown under nonselective conditions on rich glucose medium, the mutant produces 70–80% $\rho^{-/o}$ derivatives, indicating that the mutation has a secondary effect on the stability of mtDNA. This is also reflected in the visible spectrum of mitochondria, which shows a deficiency of cytochromes *a*, a_3, and *b* (Figure 1A). The pleiotropic reduction of respiratory chain components is commonly found in mutants with a defective ATPase in which either the synthesis of F_1 or F_O is impaired (Paul *et al.*, 1989; Arselin *et al.*, 1996; Helfenbein *et al.*, 2003; Ellis *et al.*, 2004).

Table 2. Respiratory and ATPase activities of mitochondria from wild-type and *atp22* mutants

Strain	% ρ^+	ATPase (μmol/min/mg) −Oligomcyin	ATPase (μmol/min/mg) +Oligomycin	% Inh
Experiment 1				
W303-1B	>99	5.28 ± 0.03	1.15 ± 0.03	77
D273-10B/A1	>99	5.65 ± 0.02	1.38 ± 0.03	76
E884	35	1.80 ± 0.05	1.82 ± 0.08	0
aE884/UL1	20	2.36 ± 0.01	2.41 ± 0.05	0
W303ΔATP23	15	2.17 ± 0.07	2.22 ± 0.02	0
W303ΔATP23/R1	76	2.26 ± 0.04	1.93 ± 0.01	15
W303ΔATP23/R2	80	2.51 ± 0.05	1.96 ± 0.06	22
W303ΔATP23 + ATP23(E→Q) (i)	66	4.47 ± 0.34	0.33 ± 0.06	92.6
W303ΔATP23 + ATP23(E→Q) (e)	76	6.96 ± 0.43	1.39 ± 0.07	80
Experiment 2				
W303-1A	>99	4.70	0.42	91
W303ΔATP10	37	1.90	1.78	6
W303ΔATP10 + ATP23 (i)	45	2.63	1.80	32
W303ΔATP10 + ATP23 (e)	47	1.80	1.05	42

Mitochondria were prepared from cells grown in YPGal. Samples of the cultures were used to test for the percentage of $\rho^{-/o}$ cells. ATPase activity was measured at 37°C. The values reported in experiment 1 are averages of duplicate assays with the ranges indicated.

Analyses of F_1 and F_O subunits and measurements of ATPase activity in isolated mitochondria indicated that the phenotype of aE884/UL1 was likely to stem from a defect in F_O. The mutant was found to have essentially no immunologically detectable subunit 6 (Figure 1C), a hallmark of mutants with defective F_O (Paul *et al.*, 1989, 2000). The decrease in subunit 4, which does not turnover in F_O mutants as rapidly as subunit 6, was less pronounced. This was also true of the α subunit of F_1, which can assemble with its four partner subunits to form the active oligomer even in the absence of F_O (Tzagoloff, 1969; Schatz, 1968). The presence of F_1 was confirmed by assays of ATPase activity. Although the wild-type enzyme was more than 70% inhibited by oligomycin, the ATPase activity of mitochondria from E884 or aE884/UL1 was completely insensitive to the inhibitor even though the cultures consisted of 35 and 20% ρ^+ cells, respectively (Table 2). Sedimentation of mitochondrial extracts in sucrose gradients also indicated that the ATPase in the mutant had properties similar to those of the F_1 oligomer (Figure 1B).

Cloning and Disruption of ATP23

A plasmid (pG200/T1) capable of rescuing the respiratory defect of aE884/UL1 was obtained by transformation of the mutant with a genomic plasmid library constructed in the *URA3* shuttle plasmid YEp24. The nuclear DNA insert of pG200/T1 was used to localize the gene in a 2.3-kb EcoRI-BglII fragment (Figure 2A) with YNR020C of chromosome XIV as the only complete reading frame. This gene, henceforth referred to as *ATP23*, was determined to have a single G-to-A transition at nucleotide 756 of the gene in aE884/UL1. The mutation creates a premature TGA stop, resulting in a protein lacking the C-terminal 16 residues.

A partially deleted allele of *ATP23*, constructed by the strategy depicted in Figure 2B, was introduced into the

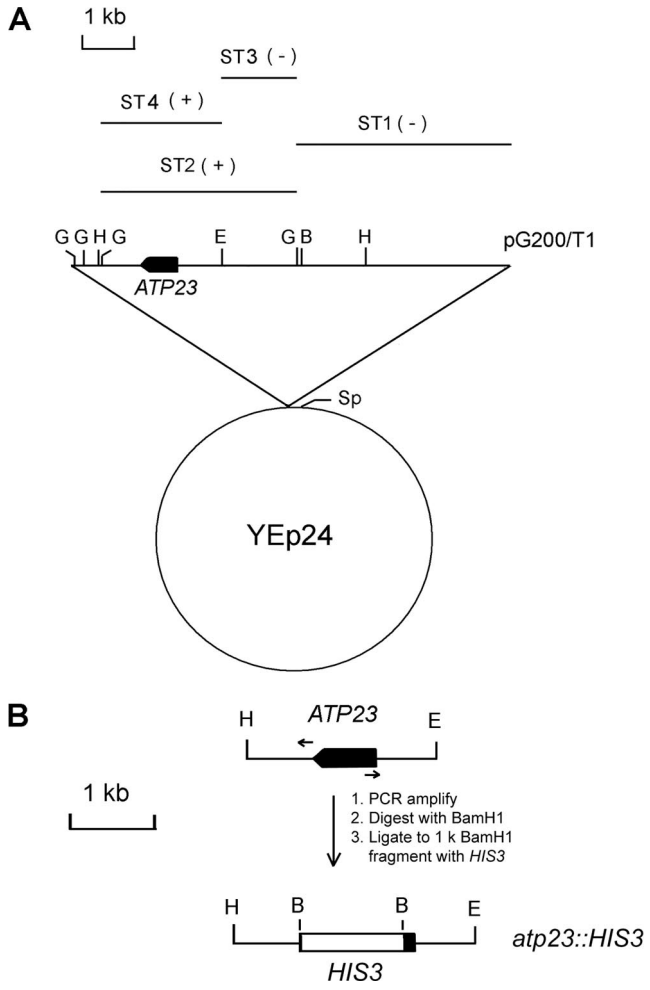

Figure 2. Cloning and disruption of *ATP23*. (A) Restriction maps of pG200/T1 and of subclones. The locations of the restriction sites for EcoRI (E), HindIII (H), BamHI (B), and BglII (G) are marked on the nuclear DNA insert in pG200/T1. The SphI (Sp) site in the vector is indicated for orientation purposes. The regions of the pG200/T1 nuclear DNA insert subcloned in YEp352 are depicted by the solid bars. The plus and minus signs indicate complementation or lack thereof, respectively, of the *atp23* mutant aE884/UL1. The location and direction of transcription of *ATP23* are indicated by the solid arrow in the pG200/T1 insert. (B) Construction of a partially deleted *atp23* allele. The details of the construction are described in *Materials and Methods*.

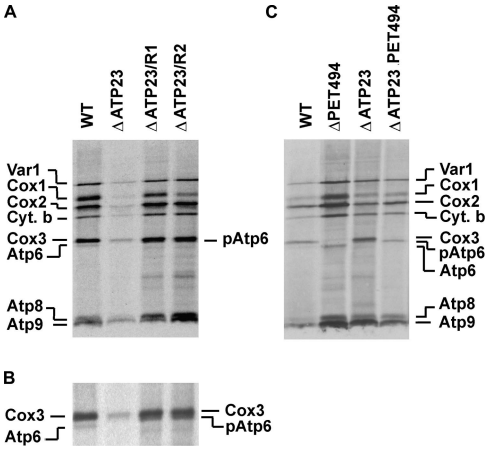

Figure 3. Aberrant form of subunit 6 in the *atp23* null mutant. (A) Mitochondrial translation products in wild type, the *atp23* null mutant, and revertants. Cells were grown in rich 2% galactose medium (YPGal), labeled with [^{35}S]methionine for 30 min and separated on a 17.5% polyacrylamide gel as described previously. Proteins were transferred electrophoretically to nitrocellulose and exposed to Kodak XAR film overnight (Eastman Kodak, Rochester, NY). The labeled mitochondrial translation products identified in the margin are ribosomal protein Var1, subunits 1 (Cox1), subunit 2 (Cox2), subunit 3 (Cox3) of cytochrome oxidase, cytochrome *b* (Cyt. b), and subunits 6 (Atp6), subunit 8 (Atp8), and subunit 9 (Atp9) of the ATPase. The aberrant form of ATPase subunit 6 (pAtp6) seen in the null mutant and revertants migrates slightly below Cox3. The lesser labeling of the in vivo synthesized proteins in the mutant is due to the high percentage of $\rho^{o/-}$ cells in the culture (82%). The two revertants, in which labeling of the mitochondrial products are comparable to wild type, had only 20% $\rho^{o/-}$ cells in the cultures. (B) The region of Cox3p and Atp6/pAtp6 was expanded to better visualize the difference in migration of the normal and aberrant subunit 6. (C) Mitochondrial translation products in single *atp23*, *pet494* and *atp23/pet494* double mutants. Whole cells were labeled with [^{35}S]methionine, separated on a 17.5% polyacrylamide gel, and exposed to x-ray film as in panel A. The *pet494* mutation almost completely blocks translation of Cox3p allowing a better display of the difference in migration of Atp6p in the *atp23* mutant.

respiratory haploid strains W303-1A and W303-1B. The null mutants (W303ΔATP23 and aW303ΔATP23) were respiratory deficient, did not complement E884, and displayed a biochemical phenotype similar to that of the point mutant. The *atp23* null mutants had severely depressed levels of cytochromes *a*, a_3, and *b* (Figure 1A) and were grossly deficient in subunit 6 but not in subunit 4 or the α-subunit of F_1 (Figure 1C). The ATPase activity of the null mutant, like that of E884, was also insensitive to oligomycin (Table 2).

Null Mutants in ATP23 Express an Aberrant Form of Subunit 6

Even though there is no immunologically detectable subunit 6 in the *atp23* null mutant, it is able to synthesize a novel form of this protein. Pulse-labeling of whole cells with [^{35}S]methionine in the presence of cycloheximide disclosed the presence of a novel mitochondrial translation product with a migration that is slightly retarded relative to mature subunit 6 (Figure 3, A and B). Because of its proximity to subunit 3 (Cox3p) of cytochrome oxidase in SDS-PAGE, the in vivo translation assays were also done with the *atp23* mutant carrying a second mutation in *PET494*, which codes for a *COX3*-specific translation factor (Costanzo and Fox, 1986). The results obtained with the double mutant lacking Cox3p confirmed the slower migration of subunit 6 (Figure 3C).

Atp23p Is a Metalloprotease That Processes the Subunit 6 Precursor of the Yeast ATPase

The primary translation product predicted by DNA sequence of *ATP23* has a mass of 32.2 kDa. This is consistent with the apparent molecular mass of 30 kDa estimated by SDS-PAGE (see below). Atp23p is present in diverse fungi, animals, and plants (Figure 4). All members of this protein family have a HEXXH motif characteristic of metalloproteases (Jongeneel *et al.*, 1989). The two conserved histidine and the glutamic acid residues of this motif are essential for protease activity (Becker and Roth, 1992). The importance of this sequence for the function of Atp23p was assessed by substitution of the glutamic acid by glutamine. The mutant gene was introduced into an *atp23* null background either on a multicopy plasmid (W303ΔATP23/ST14) or by insertion of the gene in an integra-

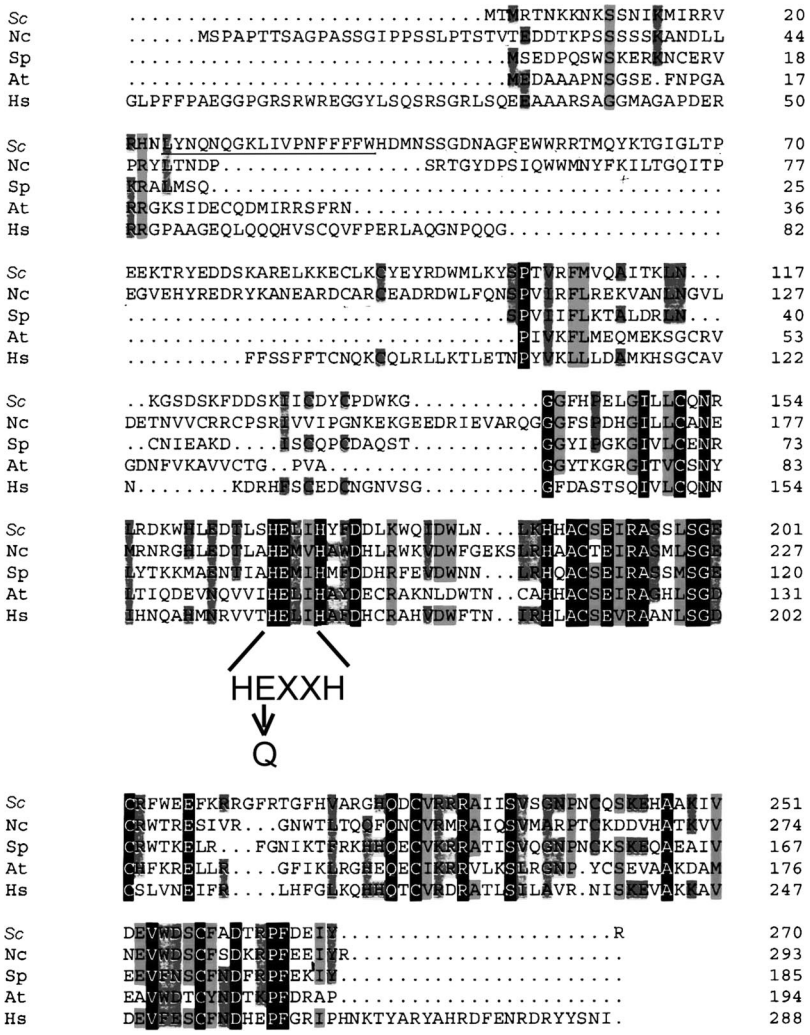

Figure 4. Alignment of yeast Atp23p with fungal, animal and plant homologues. The sequences of the *S. cerevisiae* (*Sc*), *Neurospora crassa* (*Nc*), *Schizosaccharomyces pombe* (*Sp*), *Aradopsis thaliana* (*At*), and *Homo sapiens* (*Hs*) Atp23p were aligned with the ClustalW program (Chenna *et al.*, 2003). Residues conserved in all five sequences are boxed in dark gray, and those conserved in some but not all five homologues are boxed with the lighter shade of gray. The HEXXH motif highlighted in large letters shows the glutamic acid residue mutated to a glutamine. The putative transmembrane domain near the N-terminal end of the *S. cerevisiae* sequence is underlined.

tive plasmid at the *ura3* locus of nuclear DNA (W303ΔATP23/ST15). In both cases, the gene expressing the E168Q mutant protein restored wild-type growth of the *atp23* null strain on glycerol/ethanol (Figure 5A).[1]

To ascertain if cells with the E168Q mutation in Atp23p have a normal or aberrant form of subunit 6, mitochondrial translation products were labeled with [^{35}S]methionine in vivo. These assays indicated that despite its ability to rescue growth on glycerol/ethanol, the *ATP23* mutant gene did not restore expression of normal subunit 6 (Figure 5B). The absence of mature subunit 6 in transformants with the E168Q mutation suggested that Atp23p is a protease responsible for removing the N-terminal 10 residues from the subunit 6 precursor. The ability of the E168Q protein to restore respiration and oligomycin-sensitive ATPase activity (Table 2) indicates that the subunit 6 precursor is capable of assembling into a functional F_O. The requirement of wild-type Atp23p or the E168Q mutant protein for respiratory sufficiency implies that in addition to its proteolytic activity Atp23p has another function related to F_O assembly.

Mutation(s) in Mitochondrial DNA Suppress the atp23 Null Mutation

The *atp23* mutant gives rise to spontaneous revertants capable of slow growth on glycerol/ethanol (Figure 5C). Two such revertants (W303ΔATP23/R1 and R2) had spectra intermediate between that of wild type and the mutant, with partial restoration of cytochromes *a*, a_3, and *b* (Figure 1A). The ATPase activity of mutant mitochondria was completely insensitive to oligomcyin, whereas the activity measured in the two revertants was partially inhibited (15 and 22%) by oligomcyin (Table 2). The fact that most of ATPase in the revertants remained insensitive to oligomcyin is consistent with the sedimentation properties of the ATPase in the mutant and the R1 revertant (Figure 1B). Unlike the F_1-F_O complex of wild-type mitochondria, the position of the AT-

[1] While these studies were in progress, we learned that Osman *et al.* (2007) obtained similar data.

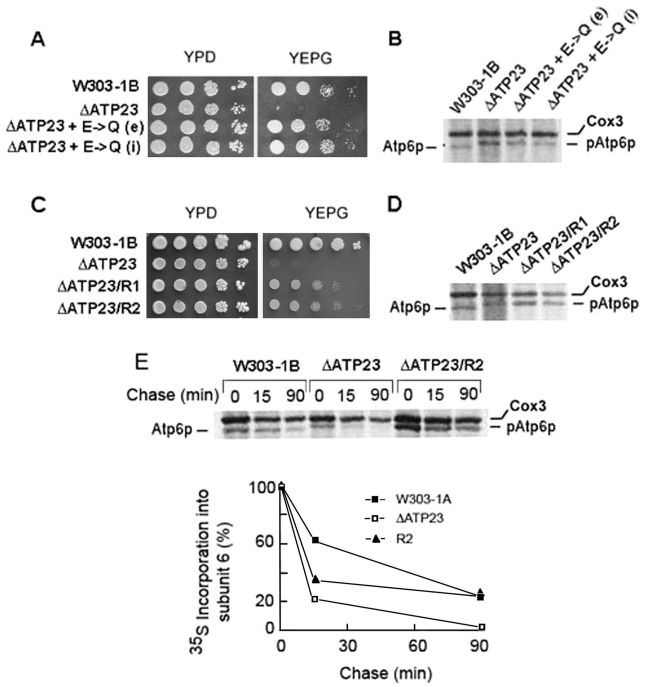

Figure 5. Complementation of the *atp23* mutant with the E168Q mutant *ATP23* gene. (A) Growth phenotype of the *atp23* null mutant transformed with mutant *ATP23*. The parental wild-type W303-1B, the *atp23* null mutant W303ΔATP23 (ΔATP23), and the null mutant expressing the E168Q mutation either from the chromosomally integrated gene [ΔATP23 + E→Q (i)] or from the gene on a multicopy plasmid [ΔATP23 + E→Q (e)] were grown in liquid YPD. Serial dilutions of the cultures were spotted on rich glucose (YPD) and rich ethanol/glycerol (YEPG) plates and incubated at 30°C for 2 d. (B) The strains used in panel A were labeled with [^{35}S]methionine in the presence of cycloheximide. Total cellular proteins were separated on a 12.5% polyacrylamide gel containing 4 M urea and 25% glycerol and transferred to nitrocellulose, and the blot was exposed to an x-ray film overnight as described in the legend to Figure 3A. The positions of mature (Atp6) and novel form of subunit 6 (pAtp6) are indicated by the arrows. (C) Growth of *atp23* revertants on glycerol/ethanol. The parental wild-type W303-1B, the *atp23* null mutant W303ΔATP23 (ΔATP23), and two independent revertants of the null mutant (ΔATP23/R1 and R2) were grown in liquid YPD. Serial dilutions of the cultures were spotted on rich glucose (YPD) and rich ethanol/glycerol (YEPG) plates and incubated at 30°C for 2 d. (D) The wild type and mutant strains shown in panel C were labeled, and the radioactive translation products were visualized as in panel B. (E) The top part of the panel shows the turnover of subunit 6 in wild type and in *atp23* mutants. The parental wild-type W303-1B, the *atp23* null mutant W303ΔATP23 (ΔATP23), and a revertant of the null mutant (W303ΔATP23/R2) were labeled in vivo for 20 min with [^{35}S]methionine in the presence of cycloheximide. Puromycin and excess unlabeled methionine were added, and samples were taken after the indicated times of chase at 30°C. Proteins were separated on a 12.5% gel containing 4 M urea and 25% glycerol. The radioactivity associated with Atp6p was quantified with a PhosphorImager. The results normalized to the values obtained at time zero of the chase are shown in the bottom part of panel E.

Pase in these strains relative to the β-galactosidase marker was similar to that previously reported for F_1 (Tzagoloff and Meagher, 1971). The subunit 6 precursor was also found to be more stable in the revertant than in the mutant. Almost none of the precursor was detected after 90 min of chase in the mutant (Figure 5E). In contrast ~25% was still present in both wild type and the revertant.

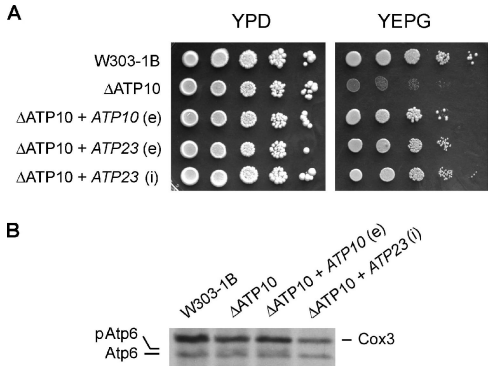

Figure 6. Suppression of the *atp10* null mutant by *ATP23*. (A) Growth of the *atp10* mutant and transformants on rich glycerol/ethanol. The wild-type strain W303-1A, the *atp10* null mutant W303ΔATP10 (ΔATP10), and the null mutant transformed either with *ATP10* in an episomal plasmid [(ΔATP10 + ATP10 (e)] or with *ATP23* on and episomal [ΔATP10 + ATP23(e)] or integrative plasmid [(ΔATP10 + ATP23(i)] were grown in liquid YPD. Serial dilutions were spotted on YPD and on YEPG medium and incubated at 30°C for 2 d. (B) The same strains (except for the *atp10* mutant with *ATP23* gene on an episomal plasmid) were labeled in vivo with [^{35}S]methionine in the presence of cycloheximide as described in the legend to Figure 3A. The position of mature subunit 6 (Atp6) is indicated by the arrow.

Only low amounts of the subunit 6 precursor and no mature protein were detected by Western analysis of the revertant mitochondria (Figure 1C). The subunit 6 precursor was also evident in the pattern of the mitochondrial translation products synthesized by the revertants (Figure 5D). Because suppression does not depend on cleavage of the precursor these results confirm that cleavage of the N-terminal 10 residues is not an essential step for expression of functional F_O in yeast.

The suppressors in two revertants were ascertained to have dominant mutations. Diploid cells issued from crosses of the revertants to the *atp23* mutant had a growth phenotype similar to that of the haploid revertant. This was not true of diploid cells obtained from crosses of ρ^o derivatives of the revertants to the *atp23* mutant, which indicated that the suppressors were in mtDNA. Attempts to localize the mutations in the mitochondrial genome by deletion mapping with a ρ^- library generated from the revertants or by direct sequencing *ATP6*, *ATP8*, and *ATP9* genes were unsuccessful. Introduction of the suppressor mutation in an *atp10* revertant reported previously (Paul *et al.*, 2000) also failed to rescue the *atp23* null mutant. Our inability to find mutations in any of the three mitochondrial ATPase genes suggests that the revertants are likely to have informational suppressors either in mitochondrially encoded tRNA or rRNA genes. No further attempts were made to identify the suppressor(s).

Suppression of atp10 Mutants by One or More Copies of ATP23

ATP10 codes for an inner membrane protein that was shown to target subunit 6 and to be necessary for the interaction of this F_O constituent with the subunit 9 ring (Tzagoloff *et al.*, 2004). Like other mutants blocked in F_O assembly, *atp10* mutants have severely reduced levels of subunit 6 (Paul *et al.*, 2000).

Transformation of *atp10* null mutant with an extra copy of *ATP23* integrated into nuclear DNA or with the gene in a

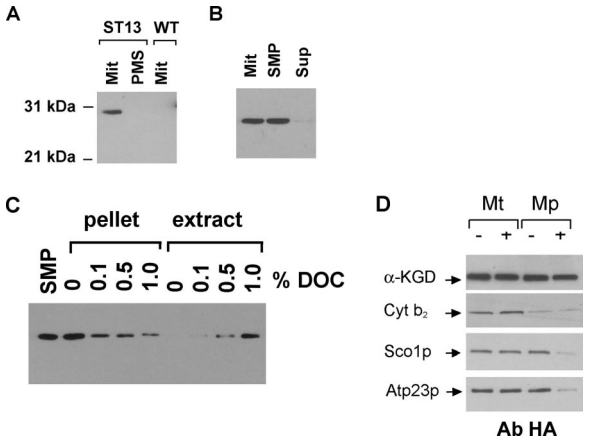

Figure 7. Localization of Atp23p in yeast mitochondria. (A) Mitochondria (Mit) and the postmitochondrial supernatant fraction (PMS) consisting mainly of cyotosolic proteins were prepared from the W303ΔATP23/ST13 (ST13), an *atp23* mutant with a chromosomally integrated copy of *ATP23* fused to a sequence coding for the HA epitope. Samples of mitochondria and PMS containing 40 μg protein were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with a monoclonal antibody against the HA tag. After a second reaction with anti-mouse IgG coupled to peroxidase, the antibody–antigen complexes were visualized with the SuperSignal chemiluminescent substrate kit (Pierce, Rockford, IL). (B) Mitochondria (Mit) of the transformant W303ΔATP23/ST13 expressing HA-tagged Atp23 was suspended at a protein concentration of 10 mg/ml in 0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 0.5 M EDTA (STE) and was disrupted by sonic irradiation for 3 s with a Branson Sonifier microtip. The suspension was centrifuged at 100,000 $\times$ g_{av} for 20 min. The supernatant (Sup) was collected, and the pellet (SMP) consisting of submitochondrial membrane vesicles was suspended in the starting volume of STE. Mitochondria (40 μg protein) and equivalent volumes of the supernatant and membrane pellet after sonic irradiation were separated on a 12% polyacrylamide gel and processed as in panel A. (C) Submitochondrial membranes (SMP) were suspended in STE at a protein concentration of 10 mg/ml and extracted with the indicated concentrations of potassium deoxycholate (DOC) in the presence of 1 M KCl. After centrifugation at 100,000 $\times$ g_{av} for 15 min, the extracts were collected and the pellets suspended in the starting volume of STE. Samples corresponding to 40 μg of the submitochondrial membranes were separated on a 12% polyacrylamide gel and processed as in A. The positions of mature subunit 6 (Atp6) is indicated by the arrow. (D) Mitochondria were prepared by the method of Glick (1985) from the W303ΔATP23/ST13 the *atp23* null mutant with a chromosomally integrated copy of the *ATP23* fusion gene expressing the protein with a C-terminal HA tag. The mitochondria were suspended in 0.6 M sorbitol, 20 mM HEPES, pH 7.4, at a protein 8 mg/ml in 0.6 M sorbitol, 20 mM HEPES, pH 7.5 (SH). Equal samples of mitochondria were diluted with 8 volumes of either 0.6 M sorbitol, 20 mM HEPES, pH 7.5, or 20 mM HEPES, pH 7.5, to cause lysis of mitochondria to mitoplasts. Proteinase K (prot K) was added to one-half of each sample to a final concentration of 100 μg/ml. After incubation for 60 min on ice, the reaction was quenched with 2 mM phenylmethylsulfonyl fluoride, and the mitochondria and mitoplasts were recovered by centrifugation at 100,000 $\times$ g_{av} for 10 min. The pellets were suspended in 0.6 M sorbitol, 20 mM HEPES, pH 7.5, and precipitated by addition of 0.1 volume of 50% trichloroacetic acid. The precipitated proteins were dissolved in Laemmli sample buffer and heated for 5 min at 90°C. Mitochondrial (Mt) and mitoplast (Mp) proteins (40 μg) were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with a mAb against the HA tag and with rabbit polyclonal antibodies against α-ketoglutarate dehydrogenase (α-KGD), Sco1p, and cytochrome b_2 (Cyt b_2). Antibody-antigen complexes were visualized as in panel A after a secondary reaction with either anti-mouse or anti-rabbit IgG.

high-copy plasmid conferred substantial growth on glycerol/ethanol (Figure 6A). Approximately 30% of subunit 6 in the atp10 null mutant was unprocessed (Figure 6B). In contrast only mature subunit 6 was detected in the *atp10* mutant harboring an extra copy of wild-type *ATP23*. Only weak suppression was observed when the *atp23* mutant was transformed with *ATP10* on a high-copy plasmid (data not shown). To determine if proteolytically inactive E→Q Atp23p also has suppressor activity, the mutant *ATP23* gene was introduced into the *atp10* mutant on an integrative or a high-copy episomal plasmid. Neither the high-copy or chromosomally integrated mutant gene restored respiration in the *atp10* null strain (data not shown).

Localization of Atp23p

The cellular localization of Atp23p was examined in W303ΔATP23/ST13, an *atp23* null mutant with a chromosomally integrated *ATP23* fusion gene expressing the protein with a C-terminal tag (Atp23p-HA). A monoclonal antibody against the HA tag detected a protein of ~30 kDa, consistent with the expected size of Atp23p (Figure 7A). Atp23p-HA was not present in the cytosolic fraction or in the comparable fractions of wild-type yeast (not shown). It was recovered in the membrane fraction (SMP) obtained from sonically disrupted mitochondria (Figure 7B) and was partially extracted from the membrane vesicles in the presence of salt and deoxycholate (Figure 7C).

Because the N terminus of subunit 6 faces the intermembrane space (Paumard *et al.*, 2000), processing of the precursor by Atp23p is likely to occur in this compartment. This location of Atp23p is consistent with the sensitivity of the HA-tagged protein to proteinase K in mitoplasts (Figure 7D). Treatment of mitoplasts but not mitochondria resulted in a decrease of Atp23p-HA to about the same extent as of Sco1p, an inner membrane protein facing the intermembrane space (Beers *et al.*, 2002). Cytochrome b_2, a soluble intermembrane constituent, was largely depleted in the mi-

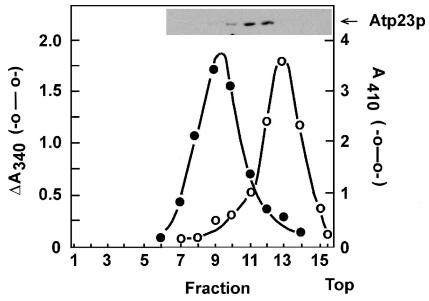

Figure 8. Sedimentation of Atp23p-HA in a sucrose gradient. Mitochondria prepared from W303ΔATP23/ST13 at a protein concentration of 10 mg/ml were adjusted to 1% potassium deoxycholate and 1 M KCl. After centrifugation at 105,000 $\times$ g_{av} for 15 min, the clear supernatant (0.25 ml) was diluted with an equal volume of 10 mM Tris-Cl, pH 7.5, containing 1.5 mg hemoglobin and 100 μg of bovine L-lactate dehydrogenase. The mixture was layered on top of 5 ml of a 7–25% linear sucrose gradient prepared in 10 mM Tris-Cl, pH 7.5, and 0.1% Triton X-100. The gradient was centrifuged in a Beckman SW65 rotor (Fullerton, CA) at 65,000 rpm for 5 h and fractionated into 15 equal-size fractions. The fractions were assayed for hemoglobin at 410 nm (○—○) and for lactate dehydrogenase by pyruvate-dependent oxidation of NADH at 340 nm (●—●). The distribution of Atp23p-HA was determined by Western analysis of the fractions with the mouse mAb against the hemagglutinin tag as in Figure 8. The size of Atp23p was estimated from the positions of the peak relative to those of the markers (Martin and Ames, 1961).

toplast fraction. In contrast, α-ketoglutarate dehydrogenase, a component of the soluble matrix α-ketoglutarate dehydrogenase complex, was protected against proteinase K in both mitochondria and mitoplasts, indicating that the inner membrane remained intact after lysis of mitochondria under hypotonic conditions (Figure 7D).

Sizing of Atp23p

The native size of Atp23p was assessed by sucrose gradient sedimentation. The HA-tagged protein was extracted from mitochondria of W303ΔATP23/ST13 with deoxycholate and centrifuged through a 7–25% sucrose gradient containing 0.1% Triton X-100. The peak of Atp23p-HA sedimented at a position midway between those of lactate dehydrogenase and hemoglobin, indicating an M_r of ~100,000 (Figure 8). This suggests that the native protein is homo-oligomeric or is part of a hetero-oligomeric complex.

DISCUSSION

Respiratory defective mutants of yeast have served as useful tools in identifying a large number of proteins that promote various events essential for assembly of the terminal respiratory pathway of mitochondria. The product of the *ATP23* gene reported here, like most of the other accessory proteins that target mitochondrially encoded subunits of the respiratory and ATPase complexes, acts on subunit 6, one of three subunits of F_O that are translated on mitochondrial ribosomes.

In this study we present evidence that Atp23p is a mitochondrial protease that removes the 10-residue-long N-terminal prepeptide of the subunit 6 precursor (Michon *et al.*, 1988). The function of Atp23p was gleaned from the phenotype of a respiratory-deficient mutant previously assigned to complementation group G200 of our mutant collection (Tzagoloff and Dieckmann, 1990). In vivo labeling of the mitochondrial translation products in *atp23* point and null mutants and in partial revertants revealed the presence of a novel form of subunit 6, which migrates as a slightly larger protein than mature subunit 6. The retarded electrophoretic migration of subunit 6 was also observed in Western blots of total mitochondrial proteins in *atp23* revertants.

Atp23p is associated with the inner membrane in an orientation such that the C-terminus faces the intermembrane space. It is conserved among eukaryotic organisms from yeast to humans. It has an HEXXH motif previously shown to be part of the active sites of metalloproteases including zinc proteases (Jongeneel *et al.*, 1989; Becker and Roth, 1992). The two histidine and the glutamic residues of this motif participate in zinc binding and in the catalytic mechanism, respectively, and are essential for enzymatic activity. Substitution of the essential glutamic acid by glutamine prevents cleavage of the N-terminal prepeptide, confirming that Atp23p processes the subunit 6 precursor. Surprisingly, the E→Q mutant protein is able to restore normal growth of the *atp23* mutant on respiratory substrates, even though all the subunit 6 remained in the unprocessed form. The ability the subunit 6 precursor to assemble into a functional ATPase excludes removal of the 10 N-terminal residues as a necessary condition for the function of this F_O subunit.

The ATPase deficiency of *atp23* mutants implies that in addition to catalyzing processing of the subunit 6 precursor, Atp23p has still another function in assembly of the ATPase complex. Clues about the second function of Atp23p have emerged from the observation that *ATP23* is able to suppress the ATPase defect of *atp10* mutants. Atp10p was previously shown to form a complex with and to confer stability on newly synthesized but unassembled subunit 6 (Tzagoloff *et al.*, 2004). It was also inferred to be required for the association of subunit 6 with the subunit 9 ring (Tzagoloff *et al.*, 2004). This interaction may be a rate-limiting step in F_O assembly as Atp10p appears to minimize turnover of unassembled subunit 6 (Tzagoloff *et al.*, 2004). It is significant that ~5–15% of F_1-F_O ATPase is assembled in the *atp10* null mutant (Ackerman and Tzagoloff, 1990b). This probably represents the small fraction of subunit 6 that escapes degradation. The presence of an extra copy of *ATP23* is able to partially compensate for the absence of Atp10p suggesting that, when overexpressed, Atp23p helps to stabilize subunit 6. A possible explanation is that there is a cooperative interaction of Atp10p and Atp23p with the subunit 6 precursor. A physical interaction of the two proteins, if it occurs, is likely to be transient and unstable as the two proteins do not cosediment in sucrose gradients (data not shown). In the absence of either Atp10p or Atp23p the increased instability of the precursor would be expected to result in its more extensive turnover. The biochemical cooperativity of Atp23p and Atp10p is also indicated by the less efficient processing of subunit 6 precursor in the *atp10* mutant. Proteolytically inactive Atp23p can promote assembly of a functional ATPase complex, indicating that the subunit 6 precursor is functional. Unlike the wild-type Atp23p, the E→Q protein does not suppress the *atp10* mutant. This may be the result of a greater susceptibility of the subunit 6 precursor to degradation in the absence of Atp10p, even when there is excess mutant Atp23p present. These observations are integrated in the model shown in Figure 9.

The dual function of Atp23p is interesting in the light of evidence that subunit 6 is not synthesized as a precursor in

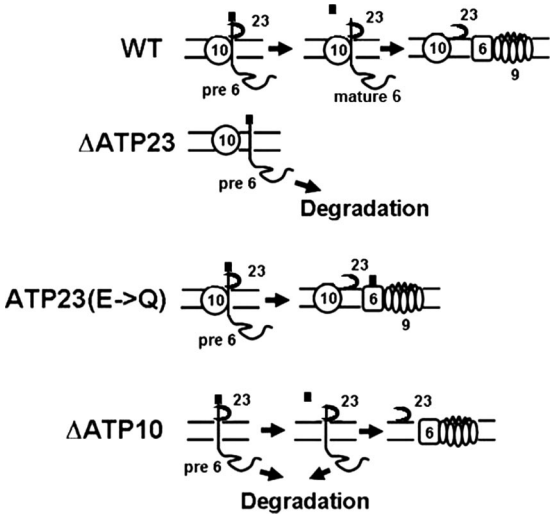

Figure 9. Postulated roles of Atp23p and Atp10p in assembly of subunit 6. The top panel shows processing of the subunit 6 precursor by Atp23p and its further interaction with the subunit 9 ring. Although processing of the subunit 6 precursor is shown to precede its interaction with the subunit 9 ring, the order could be reversed or the two events could occur concurrently. Degradation of the subunit 6 precursor as a result of the absence of Atp23p is illustrated in the second panel from the top. The third panel shows that in the presence of Atp23p with the E→Q mutation, processing of the precursor is prevented but the precursor is still able to interact with the subunit 9 ring. In the bottom panel the absence of Atp10 allows some of the precursor to be processed and assembled but most of subunit 6 is degraded. Although this model shows an interaction of Atp23p and Atp10p, there is no experimental evidence to support this at present.

many eukaryotes, including mammals, that have *ATP23* homologues. This raises the possibility that Atp23p may be required for assembly of the ATPase complex in these organisms as well. Atp23p may also act on other substrates that we are still not aware of.

Subunit 6 of the ATPase and subunit 2 (Cox2p) of cytochrome oxidase (COX) are the only mitochondrially encoded proteins of yeast synthesized as precursors. The amino terminal presequence of the Cox2p precursor (pCox2p) is cleaved by the IMP complex located in the intermembrane space of mitochondria (Jan *et al.*, 2000). Mutations affecting the proteolytic activity of the IMP complex block maturation of pCox2p and elicit a COX deficiency. A similar phenotype is observed in *cox20* mutants (Hell *et al.*, 2000). Cox20p is not a protease but forms a stable complex with pCox2p, a prerequisite for processing by the IMP complex (Hell *et al.*, 2000). Homologues of the yeast *COX20* gene have recently been reported in animals and other organisms that synthesize Cox2p without a presequence (Hell *et al.*, 1997). On the basis of these observations, Herrmann and Funes (2005) have inferred that the Cox20p acts both as a chaperone for proteolytic maturation of pCox2p by IMP and additionally promotes assembly of the mature subunit. This is also supported by observations that processing of pCox2p is not essential for its assembly and electron carrier function. Translocation of the N-terminal catalytic domain of pCox2p across the inner membrane depends on Oxa1p (He and Fox, 1997; Hell *et al*., 1997). The respiratory deficiency of a yeast mutant with a temperature-sensitive allele of *oxa1* has been shown to be suppressed by a mutation in *COX2* (Meyer *et al.*, 1997). The mutant with the suppressor mutation in Cox2p assembles functional COX even though it does not process pCox2p (Meyer *et al.*, 1997). The dual role of Cox20p in Cox2p biogenesis is in some ways similar to the results described here except that unlike Cox20p, which is not a protease, Atp23p functions both as the protease and assembly factor for subunit 6.

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