Characterization of ATP11 and Detection of the Encoded Protein in Mitochondria of Saccharomyces cerevisiae*

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In Saccharomyces cerevisiae, expression of functional F1-ATPase requires two proteins encoded by the ATP11 and ATP12 genes. Mutations in either gene block some crucial late step in assembly of F1, causing the α and β subunits to accumulate in mitochondria as inactive aggregates (Ackerman, S. H., and Tzagoloff, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 87, 4986–4990). In the present study we have cloned and determined the sequence of ATP11. The encoded product is protein of 37 kDa with no obvious homology to any known protein.

In vitro import assays of ATP11 precursor and immunoechemical evidence indicate that the protein is located in mitochondria. A fusion was made between ATP11 and a short sequence coding for 78 amino acids with the biotination signal of bacterial transcarboxylase. The protein expressed from this construct complements atp11 mutants, indicating that the addition of the extra 78 amino acids at the carboxy terminus of the ATP11 protein does not compromise its function. The hybrid protein is detected in mitochondria with antibodies and with peroxidase-conjugated avidin. Biotinlated ATP11 protein can be partially purified by affinity chromatography on monomeric or tetrameric avidin coupled to Sepharose. A fraction eluted from the avidin column and enriched for the biotinated ATP11 protein also contains the α and β subunits of F1-ATPase.

F1 is an important catalytic component of mitochondrial, chloroplast, and bacterial energy-transducing ATPases (1, 2). F1-ATPases are hetero-oligomers composed of five different subunit polypeptides. The two major subunits, referred to as α and β, are each present in three copies per oligomer (3). The other subunits are present in single copy. At present very little is known about the requirements and pathway for assembling this rather complex structure. Is there an obligatory order in which the subunits interact with one another? Is the final quaternary structure achieved through the intervention of other nonstructural proteins, or is it a spontaneous process guided solely by protein-protein recognition determinants on the surfaces of the folded subunits? These and other related questions remain unanswered.

We have recently reported two genetically distinct groups of Saccharomyces cerevisiae mutants whose inability to grow on nonfermentable carbon sources was ascribed to a block in F1 assembly (4). These mutants define two nuclear genes, ATP11 and ATP12, with important roles in the expression of a functional mitochondrial ATPase. Even though mitochondria of atp11 and atp12 mutants are generally deficient in ATPase activity, they have nearly normal concentrations of mature size α and β subunits. Instead of being part of the F1-F0 complex, however, the F1 subunits are present as aggregated proteins readily separable from the membrane fraction. Based on their phenotype, atp11 and atp12 mutants were proposed to be arrested in ATPase assembly at some step after import and cleavage of the F1 subunit precursors.

To facilitate further studies of the ATP11 and ATP12 proteins and to learn more about their functions, we have characterized their respective genes. In an earlier paper we reported the sequence of ATP12 and some properties of the encoded product (5). In the present studies we demonstrate that ATP11, like ATP12, is synthesized as a larger precursor and is cleaved during its import into mitochondria. The cloned ATP11 gene has enabled us to construct two different gene fusions. One fusion was used to prepare antibodies against the ATP11 protein. The second fusion has permitted the protein to be tagged in vivo with biotin (6). The biotinated ATP11 protein is functional based on its ability to restore respiratory competence to atp11 mutants. The possible association of the ATP11 protein with the α and β subunits of F1 has been assessed by partial purification of the biotinated derivative on avidin affinity columns.

MATERIALS AND METHODS

Yeast Strains and Growth Media—The genotypes and sources of the mutant and wild-type yeast strains used in the present study are listed in Table I. Mutants assigned to complementation group G13 were isolated by mutagenesis of the wild-type haploid strain S. cerevisiae D273-10B/A1 as described previously (9). Escherichia coli RR1 (proA,leuB, lacY, galK, xyl-5, mit-1, ara-14, rpsL, supE, hsdS, X) was used as a host for all the recombinant plasmid constructs. Yeast was routinely cultivated in the following media: YPD (2% glucose, 2% peptone, 1% yeast extract), YPGal (2% galactose, 2% peptone, 1% yeast extract), YEFG (3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract), WO (2% glucose, 0.67% yeast nitrogen base without amino acids (Difco)). Amino acids and other growth requirements were added where needed at a final concentration of 20 μg/ml. The solid media had identical compositions except for the addition of 2% agar.

Transformation of Yeast—The atp11 mutant strain C15U3(aura3-1, atp11-1) was grown in 50 ml of YPGal to a density of 10⁸ cells/ml. Approximately 5 × 10⁸ cells were transformed with a yeast genomic library (5 μg of DNA) by the procedure of Seges (10). The recombinant plasmid library consisting of partial Sau3A fragments of wild-type yeast nuclear DNA (5–15 kb in length) ligated to the BamHI

1 The abbreviations used are: kb, kilobase pair(s); SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; MOPS, 4-morpholinopropanesulfonic acid.

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purified by electrophoresis on 1% agarose and ligated to the BamHI ATP11 gene. The synthetic primer with the XbaI site shown above is developed with 10, 20, and 50 mM NaPO4, pH 7.5. The protein eluted with a combination of BamHI and XbaI. The digested fragment was the plasmid was ligated to a 270-base pair XbaI-BamHI fragment and 3'-flanking sequences was used as a template for the polymerase the native gene was modified by eliminating the termination codon and XbaI sites in the multiple cloning region of the yeast/E. coli shuttle vector YEp352 (17). After linearization with XbaI and PstI, the plasmid was ligated to a 270-base pair XbaI-BamHI fragment coding for the biotinization sequence of Propionibacterium shermanii transcarboxylase. This fragment, obtained from the plasmid pCY66 and coding for the carboxyl-terminal 78 amino acids of the transcarboxylase, has been shown to be a substrate for in vivo biotination in yeast (6). The Eagon of the bacterial sequence resulted in an in-frame fusion of the entire ATP11 gene to the biotination signal sequence (at the XbaI site). The ligation of the downstream BamHI to the PsI of the vector caused both sites to be destroyed.

Miscellaneous Procedures—Standard procedures were employed for restriction endonuclease analysis of DNA, purification and ligation of DNA fragments, transformations of and recovery of plasmid DNA from E. coli, and nick translation of DNA (18). The conditions of Myers et al. (19) were used for Southern hybridizations. DNA was sequenced by method of Maxam and Gilbert (20). For Western blot analysis, proteins were separated on 12% polyacrylamide gels run in the electrophoresis system of Laemmli (21) with the separation buffer adjusted to pH 8. The running buffer contained 0.05 M Tris, 0.38 M glycine, and 0.1% SDS. After transfer to nitrocellulose the Western blots were reacted with antibodies against various mitochondrial proteins. In most analyses the γ-globulin fraction was purified by chromatography of antisera on DE52 cellulose. The blot was then reacted with 125I-protein A and was washed by the protocol of Schmidt et al. (22). Protein concentrations were determined by the method of Lowry et al. (23).

RESULTS

Properties of atp11 Mutants—The phenotype of atp11 and atp12 strains is identical and has already been reported (4). Briefly, the mutants are deficient in F1 ATPase activity both in the mitochondrial and postmitochondrial supernatant fractions. The absence of enzyme activity correlates with a defect in assembly of the α and β subunits of F1 into the normal oligomer. Mature size subunits are detected in mitochondria, but instead of being associated with the hydrophobic Fo unit of the inner membrane they are present as large aggregates that can be quantitatively separated from the membranes by centrifugation of sonically disrupted mitochondria on isopycnic sucrose gradients (4).

Mitochondrial ATPase mutants can be distinguished by their genetic properties depending on whether the mutations affect the Fo or F1 component. As a rule mutations in genes coding for Fo subunits cause a marked instability in mitochondrial DNA as a result of which such mutants convert to secondary derivatives at a high frequency (24-26). In mutants in F1 also express the absence of ATPase but have no significant effect on the stability of mitochondrial DNA (27, 28). It is of interest that both atp11 and atp12 strains have genetic properties consistent with lesions in F1. Like most other pet mutants (nuclear mutants of yeast defective in mitochondrial respiration) they produce 1-5% ρ0 derivatives.

Cloning and Sequencing of ATP11—The ATP11 gene was cloned by complementation of aC15/U3 with a yeast genomic library. This mutant was transformed with a recombinant plasmid library consisting of 5-15 kb of partial Sau3A fragments of yeast genomic DNA ligated to the BamHI site of

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### TABLE I

| Strain       | Genotype                    | Sources                          |
|--------------|-----------------------------|----------------------------------|
| D273-10B/A1  | a+p+,met6                   | Ref. 7                           |
| CB11         | a+p+,ade1                   | Ref. 8                           |
| W303-1A      | a+p+,ade2-1,his3-1,leu3-3,112,trp1-1,ura3-1 | Ref. 4 |
| XS1077-2D    | a+p+,pha2,met2,pet2,ade12-1,lys2,ade2,gal7 | C15 × W303-1A |
| C15          | a+p+,met6,atp11-1           |                                  |
| aC15/U3      | a+p+,ure3-1,atp11-1         |                                  |
| C15/U1       | a+p+,ure3-1,atp11-1         |                                  |
| W3033deltaT1 | a+p+,ade2-1,his3-1,leu3-3,112,trp1-1,ura3-1,atp11-1,His3 | This study |

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YEp24 (11). A single respiratory-competent and uracil-independent clone (aC15/U3/T1) was obtained by transformation of approximately \(5 \times 10^6\) cells with 5 \(\mu\)g of the plasmid library. The acquired uracil prototrophy and respiratory competence of aC15/U3/T1 cosegregated indicating both phenotypes to be dependent on the presence of an autonomously replicating plasmid. Plasmid pG13/T1, isolated from clone aC15/U3/T1, was ascertained by restriction mapping to have a nuclear DNA insert of 10.5 kb (Fig. 1).

The gene was localized by transferring different regions of the pG13/T1 insert to the E. coli/yeast shuttle vector YEp382 (17), and the resultant plasmids were tested for their ability to complement an atpl1 mutant. The results of the transformations with these constructs indicated the gene to be integral to a 3-kb BamHI fragment defined by pG13/ST3 (Fig. 1). The complementing region was further narrowed down to a 1.6-kb BamHI-KpnI fragment (pG13/ST4) by removal of 1.4 kb of DNA from pG13/ST3. This plasmid restored oligomycin-sensitive ATPase in mitochondria and conferred growth on the nonfermentable substrate glycerol.

The 1.6-kb BamHI-KpnI fragment cloned in pG13/ST4 was sequenced by the method of Maxam and Gilbert (20) by 5’ end labeling of the restriction sites shown in Fig. 2. All the sites were crossed from neighboring sites, and both strands were sequenced. The sequence of this region disclosed only a single full-length reading frame that was identified as ATP11 based on the subcloning results and the properties of a mutant showing the locations of the BamHI (B), KpnI (K), and SphI (S) sites is presented above the circular map of YEp24. The bars in the upper part of the figure correspond to DNA fragments transferred from pG13/T1 to the yeast/E. coli shuttle vector YEp352. Complementation of the atpl1 mutation in aC15/U3 is indicated by the plus signs. The ATP11 gene is denoted by the solid arrow pointing in the direction of transcription. The SphI site of YEp24 provides the orientation of the insert in pG13/T1.

![Restriction maps of pG13/T1 and subcloning of ATP11.](image)

The restriction sites used for 5’ end labeling and the approximate distances sequenced are indicated by the arrows. The symbols used to denote the restriction sites are: KpnI (○), PvuII (■), TaqI (▲), HinfI (△) BamHI (□), and DelI (◇). The locations and direction of transcription ATP11, DAL82, and of PHA2 are shown above the restriction map by the open bars and solid arrows, respectively. bp, base pairs.

The sequence of the BamHI-KpnI fragment has enabled us to map ATP11 relative to two other genes. Analysis of the sequence revealed a second open reading frame initiated from an ATG 146 nucleotide downstream of ATP11. Only the region of the gene coding for the amino-terminal 92 amino acids is present in the pG13/ST4 insert. This partial sequence showed significant homology to the bifunctional E. coli chorismate mutase/dehydratase encoded by pheA (30) (Fig. 4). Deletion of the sequence coding for the amino-terminal part of this protein confers a requirement of phenylalanine but not tyrosine or tryptophan for growth. The primary sequence homology and the phenylalanine auxotrophy suggest that this gene corresponds to yeast prephenate dehydratase encoded by PHA2 (31, 32). As discussed later this assignment is substantiated by genetic complementation tests. The second partial sequence included in the BamHI-KpnI fragment is the first six codons of the DAL82 gene coding for a protein required for induction of the allantoin degradation pathway (33). DAL82 is transcribed from the opposite strand and begins 166 nucleotides upstream of the ATP11 ATG. Even though the sequence of the entire 1.6-kb BamHI-KpnI fragment has been determined in connection with the characterization of DAL82 (33), neither ATP11 nor the PHA2 gene was reported because of several missing nucleotides in the reported sequence.

In Situ Disruption of ATP11---To confirm that the restoration of respiratory function in atpl1 mutants by pG13/ST4 is caused by complementation rather than extragenic suppression, the one-step gene substitution procedure (34) was used to create a mutant with a deletion in the gene. The chromosomal copy of ATP11 in the respiratory competent haploid strain W303-1A was replaced with a mutant allele deleted for all but the first 17 codons of the gene. The construction of the deletion allele is illustrated in Fig. 5. A 3.2-kb EcoRI fragment containing the entire reading frame of ATP11 and 5’- and 3’-flanking sequences was isolated from pG13/T1 and transferred to YEp352E (this plasmid lacks the multiple cloning sequence of YEp352 and has in its place a single EcoRI site). Digestion of the resultant plasmid with PvuII removed the ATP11 reading frame starting from codon 18 of the gene and an additional 690 base pairs of 3’-flanking sequence. The deletion included the amino-terminal coding region of the downstream open reading frame. The gapped
plasmid was ligated to a blunt-ended 1.8-kb fragment of DNA containing the yeast HIS3 gene (Fig. 5). The deleted allele, \( \Delta atp11:: HIS3 \), was recovered as a linear 3.1-kb EcoRI fragment and was used to transform W303-1A. Selection of transformants on minimal glucose medium supplemented with all the auxotrophic requirements of W303-1A except histidine yielded several respiratory deficient His\(^+\) clones. The His\(^+\) phenotype did not segregate, indicating stable integration of HIS3 into chromosomal DNA. The respiratory deficiency of one such clone (W303\(\Delta atp11\)) was checked by crosses to be complemented by a \( \rho^0 \) but not by an \( atp11 \) mutant.

The failure of W303\(\Delta atp11\) to be complemented by the \( atp11 \) tester was consistent with the substitution of the \( \Delta atp11:: HIS3 \) allele for the wild-type gene. This was supported by the results of the genomic Southern analysis shown in Fig. 5. Both the parental and mutant DNAs were digested with a combination of BglII and EcoRI and hybridized to the nick-translated 3.2-kb EcoRI fragment. As expected the probe hybridizes to the normal 3.2-kb EcoRI fragment lacking in the parental DNA the same probe hybridizes to four fragments. The two \( PvuII \) sites have been marked for reference.

### Fig. 3. Nucleotide sequence of \( ATP11 \) and flanking regions. The sequence presented includes the region between the BamHI and KpnI sites of the pG13/ST4 insert. Only the sequence corresponding to \( ATP11 \) has been translated and is shown above the DNA sequence. The partial sequences of the downstream PHA2 gene and of \( DAL52 \) (encoded in the opposite strand) have also been translated. The two \( PvuII \) sites have been marked for reference.

### Fig. 4. Homology of the \( PHA2 \) product to chorismate dehydratase. The translated open reading frame downstream of \( PHA2 \) and flanking regions. The sequence presented includes the region between the BamHI and KpnI sites of the pG13/ST4 insert. Only the sequence corresponding to \( ATP11 \) has been translated and is shown above the DNA sequence. The partial sequences of the downstream PHA2 gene and of \( DAL52 \) (encoded in the opposite strand) have also been translated. The two \( PvuII \) sites have been marked for reference.
shows the results of the Southern analysis of wild-type and mutant DNAs were digested with a combination of EcoRI and BglII, ant W303AATPll bearing insert (Fig. 5). To confirm that the respiratory defect of W303AATPll is a consequence of faulty F1 assembly we also measured the ATPase activity of isolated mitochondria and analyzed the physical properties of the F1 subunit. The deletion in W303AATPll encompassed both the ATP11 reading frame and a significant part of the downstream gene whose product was found to be homologous to E. coli chorismate mutase/prephenate dehydratase (30). W303AATPll grows very slowly on media supplemented with phenylalanine. The phenylalanine requirement was not complemented by XS1007-2A, that the partial removal of the downstream gene elicits a new reading frame and a significant part of the downstream gene whose product was found to be homologous to E. coli chorismate mutase/prephenate dehydratase (30). W303AATPll grows very slowly on media supplemented with the usual auxotrophic requirements of W303-1A, indicating that the partial removal of the downstream gene elicits a new growth requirement. Growth of the transformant on minimal medium with 1 mM valinomycin to collapse the proton gradient. The resistance of the ATP11 precursor to proteinase K digestion in the absence of Triton X-100 may be a result of its association with the outer membrane or may be related to a more protease-resistant conformation of the protein. Both precursors and mature proteins are completely sensitive to digestion by proteinase K if mitochondria are lysed with Triton X-100.

Detection of Native ATP11 Protein with Antibodies and of Biotinylated ATP11 Protein with Peroxidase-conjugated Avidin—The mitochondrial localization of ATP11 protein was confirmed in two other ways. The first relied on the use of an

![Fig. 5. Disruption of ATP11. Partial restriction maps of the EcoRI fragment containing the wild-type and deleted allele Δatp1::HIS3 are shown in the upper part of the figure. The ΔATP11 gene is depicted by the solid bar and the 1.8-kb SmaI-HincII fragment used for the disruption by the dashed lines with the his3 gene shown as an open bar. The direction of transcription of ΔATP11 and HIS3 is indicated by the arrows. The locations of the EcoRI (E), PvuII (P), BglII (G) sites and of the PvuII-SmaI (P/S) and PvuII-HincII (P/H) junctions are indicated on the map. The lower part of the figure shows the locations of the Southern analysis of wild-type and mutant genomic DNA. Nuclear DNA was prepared from the His+ transformant W303AATPll, separated electrophoretically on a 1% agarose gel, and transferred to nitrocellulose. The blot was hybridized to the nick-translated 3-kb EcoRI fragment containing the entire ATP11 reading frame. The migration of known size standards is shown in the left margin.](image)

![Fig. 6. Mitochondrial import of in vitro synthesized ATP11 precursor. Each of three import reactions contained 20 μg of mitochondrial protein in 100 μl of buffer consisting of 3% (w/v) bovine serum albumin, 0.25 mM sucrose, 10 mM MOPS, pH 7.2, 70 mM KCl, 5 mM MgCl2, 2 mM NADH, and 1 mM ATP. Valinomycin (VALIN9) was added at a final concentration of 0.2 μM where indicated. The reactions were started by addition of 10 μl of a lysate mixture containing [35S]methionine. The transfer and concomitant processing of both radioactive precursors into a protease-protected compartment of mitochondria were confirmed by the results in Fig. 6. Incubation of labeled ATP11 and β subunit precursors with isolated mitochondria under previously described conditions led to the appearance of two new radioactive products, one of which corresponds to mature β subunit. The second major product displays properties expected of the mature ATP11 protein. It is approximately 4 kDa smaller than the precursor and has an apparent size of 32 kDa. Like the β subunit, the mature ATP11 protein is resistant to externally added proteinase K and is not detected when the transport assay is done in the presence of valinomycin to collapse the proton gradient. The resistance of the ATP11 precursor to proteinase K digestion in the absence of Triton X-100 may be a result of its association with the outer membrane or may be related to a more protease-resistant conformation of the protein. Both precursors and mature proteins are completely sensitive to digestion by proteinase K if mitochondria are lysed with Triton X-100.](image)
antibody prepared against a protein expressed from a hybrid trpE-ATP11 gene. This protein consisted of the carboxyl two-thirds of the ATP11 sequence starting from Asp112 fused to the amino-terminal half of component I of anthranilate synthetase (see "Materials and Methods"). As shown in Fig. 7, the antibody detects a 32-kDa protein in wild-type mitochondria. The identity of the 32-kDa protein as the ATP11 product is supported by its absence in W303ΔATP11 containing the Δatp11::HIS3 allele and its presence in substantially higher concentrations in an atp11 mutant transformed with ATP11 on a multicopy plasmid.

As an alternative approach, a sequence coding for a short polypeptide with the biotination site of bacterial transcarboxylase, was fused in frame to the last codon of ATP11. The hybrid gene in the multicopy shuttle vector YEp352 was able to complement the respiratory deficiency of C15/U1, indicating that the presence of the biotination signal at the carboxyl end of the ATP11 protein does not compromise its function. Mitochondria prepared from wild-type yeast and from a transformant expressing the biotinated ATP11 protein were fractionated to yield a soluble and a membrane fraction. Mitochondria and the soluble and insoluble protein fractions were separated by SDS-polyacrylamide gel electrophoresis and were transferred to nitrocellulose. Similar fractions were obtained from a strain with the disrupted ATP11 allele and from a transformant harboring normal ATP11 on a multicopy plasmid. The Western blot was first developed with peroxidase-conjugated avidin to visualize proteins with bound biotin and was then reacted with the antibody against ATP11 protein. The results of this experiment show that the antibody detects a protein some 8 kDa larger than wild-type ATP11 protein in mitochondria obtained from the transformant harboring the fusion gene (Fig. 8). This protein (BT-ATP11) is also detected with the peroxidase-conjugated probe, indicating the presence of covalently bound biotin. The apparent molecular weight of BT-ATP11 based on its properties on SDS-gel electrophoresis is 40,000 and is consistent with the expected size increase of ATP11 protein because of the extra 78 amino acids contributed by the biotination signal. Approximately 50% of the overproduced biotinated ATP11 protein is recovered in the soluble protein fraction after disruption of mitochondria by sonic irradiation (Fig. 8). A similar distribution was observed in a transformant harboring the wild-type ATP11 gene in a multicopy plasmid. In wild-type yeast, however, with a single chromosomal copy of ATP11, almost all of the ATP11 protein is released into the soluble fraction by sonic disruption of mitochondria. These results suggest that in overexpression strains approximately half of the ATP11 protein detected in mitochondria is probably present in an aggregated form because of improper folding after transport.

A biotinated protein of the same size and antigenic properties as BT-ATP11 was detected in the crude postmitochondrial supernatant fraction of transformants harboring the ATP11 fusion gene (data not shown). Because the size of BT-ATP11 in the postmitochondrial supernatant is identical to that found in mitochondria it probably corresponds to protein that leaked out of mitochondria during their isolation.

Purification of the Biotinated ATP11 Protein by Chromatography of an Avidin Affinity Column—The observation that biotinated ATP11 protein can be recovered in the soluble protein fraction of mitochondria made it possible to use an avidin affinity column for its partial purification. Both high affinity columns with tetrameric avidin coupled to Sepharose (37) and lower affinity columns containing monomeric avidin (38) have been used to purify biotin-containing proteins from relatively crude protein mixtures. The monomeric column has the advantage that biotin-containing proteins can be displaced under non-denaturing conditions with biotin (38). The release of proteins fixed onto tetrameric avidin requires extreme conditions of pH or the use of denaturing detergents.

The fractionation of the biotinated protein on both types of avidin columns is shown in Fig. 9. Biotinated ATP11 protein was assayed by Western analysis of the fractions using either antibody to ATP11 or peroxidase-conjugated avidin. Equivalent amounts of a mitochondrial extract from the transformant C15/U1/ST16 (lane 1) were passed over a monomeric and a tetrameric avidin column. Even though less than 50% of the protein applied to the monomeric column was retained (lane 2), a substantial portion eluted in the

![Fig. 7. Western blot analysis of ATP11 protein. Total mitochondrial protein (30 μg) from the respiratory competent strain D273-10B/A1, from the atp11 mutant W303ΔATP11, and from the transformant C15/U3/ST1 harboring the multicopy plasmid pG13/ST1 was separated on a 12% polyacrylamide gel and transferred to nitrocellulose paper. The blot was first treated with the antiserum against the trpE/ATP11 product followed by a second reaction with [125I]-protein A. The migrations of known size standards are indicated in the left margin.](image-url)

![Fig. 8. Expression of biotinated ATP11. Mitochondria were prepared from the respiratory-competent strain D273-10B and from C15/U1/ST16, an atp11 mutant transformed with the multicopy plasmid pG13/ST16 containing the ATP11 gene fused to the sequence coding for the biotination site of bacterial transcarboxylase. The mitochondria were disrupted by sonic irradiation and centrifuged at 106,000 × g, for 30 min. The membrane pellet was resuspended in the starting volume of 10 mM Tris-Cl, pH 7.5. Mitochondria (M), representing 40 μg of protein, and equivalent volumes of membrane pellet (P) and supernatant (S) were applied to 12% polyacrylamide gel. Mitochondria from the atp11 mutant W303ΔATP11 (ΔATP11) and from the same strain transformed with pG13/ST5 (ΔATP11/ST5) were also separated on the same gel. Plasmid pG13/ST5 is a multicopy vector containing the wild-type ATP11 gene. After electrophoresis, proteins were transferred to nitrocellulose and visualized with peroxidase-conjugated avidin (PCA) (36). The blot was then processed with antibody against the ATP11 protein (Anti-ATP11).](image-url)
Fig. 9. Fractionation of biotinylated ATP11 protein on avidin affinity columns. The monomeric column consisted of monomeric avidin coupled to Sepharose through an arm 12 atoms in length (Sigma). The tetrameric column was prepared by coupling native avidin to cyanogen bromide-activated Sepharose. Soluble protein extract at a protein concentration of 3 mg/ml was obtained from sonically disrupted mitochondria of C15/U1/S16 (see legend to Fig. 8). The extract was applied at a ratio of 1 mg of protein/0.1 ml of packed resin volume to either Sepharose coupled to monomeric or tetrameric avidin. The monomeric avidin column (MONO) was washed sequentially with 5 volumes of 0.01 M tetrameric avidin. The monomeric avidin column was packed in resin volume to either Sepharose coupled to monomeric or tetrameric avidin coupled to Sepharose through an arm 12 atoms in length. The mitochondrial extract and various column fractions (normalized to the volume of starting extract) were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. The smaller fraction released in the SDS wash (lane 3) was probably complexed to some residual tetrameric avidin present in the column. A much higher proportion of biotinylated ATP11 protein was adsorbed on the tetrameric column (lane 6). None of the complexed protein, however, was eluted with biotin (lane 7) although its recovery was quantitative in the hot SDS wash (lane 8). To obtain a better idea of the purification achieved on the monomeric column, the fraction eluted with biotin (lane 3) was separated by SDS-gel electrophoresis and the proteins visualized by silver staining. The gel revealed the most prominent band detected by the stain to be protein whose migration was identical to BT-ATP11 as described in the legend to Fig. 8. Lane 1, mitochondrial extract; lane 2, PBS eluate (this fraction contains proteins that are not adsorbed on the column); lane 3, biotin eluate; lane 4, glycine eluate; lane 5, SDS eluate; lane 6, PBS eluate; lane 7, biotin eluate; lane 8, hot SDS eluate.

Fig. 10. Purity of biotinylated ATP11 protein after chromatography on an avidin affinity column. Equivalent volumes of mitochondrial extract (lane 1), PBS wash (lane 2), and 14 volume equivalents of the 0.1% biotin eluate (lane 9) from the monomeric column (experiment of Fig. 9) were separated on a 12% polyacrylamide gel and stained with silver. The biotinylated ATP11 (BT-ATP11) protein is indicated by the dash. The migration of molecular weight standards are indicated in the left margin.

Fig. 11. Coelution of the ATP11 protein with F1, α and β subunits from tetrameric avidin. Mitochondrial extracts were prepared from the respiratory-competent strain D273-10B (D273) and the transformant C15/U1/S16 (S16). The extracts were applied to a tetrameric column equilibrated in PBS. The column was washed sequentially with PBS, 0.1% biotin in PBS, and 0.5% SDS under the conditions described in the legend to Fig. 9. The extracts and column fractions (normalized to the starting volume of extract) were separated on a 12% polyacrylamide gel and transferred to nitrocellulose paper. The Western blots were reacted with a mixture of antibodies against the α and β subunits of F1, and the ATP12 protein. Lanes 1, mitochondrial extract (15 μg of protein); lanes 2, PBS wash; lanes 3, biotin wash; lanes 4, hot SDS wash. The α and β subunits of F1, and ATP12 protein are identified in the left margin. The migration of molecular weight standards is shown in the right margin.

bacterial wash (lane 3). The smaller fraction released in the SDS wash (lane 5) was probably complexed to some residual tetrameric avidin present in the column. A much higher proportion of biotinylated ATP11 protein was adsorbed on the tetrameric column (lane 6). None of the complexed protein, however, was eluted with biotin (lane 7) although its recovery was quantitative in the hot SDS wash (lane 8). To obtain a better idea of the purification achieved on the monomeric column, the fraction eluted with biotin (lane 3) was separated by SDS-gel electrophoresis and the proteins visualized by silver staining. The gel revealed the most prominent band detected by the stain to be protein whose migration was identical to BT-ATP11 detected with peroxidase-conjugated avidin (Fig. 10). This protein was absent in the comparable fraction obtained by fractionation of a mitochondrial extract from a wild-type yeast strain although the other bands of higher molecular weight were present. BT-ATP11 was also a prominent band in the hot SDS wash of the tetrameric column although the pattern of proteins was considerably more complex (data not shown).

**Does the ATP11 Protein Interact with F1, α and β Subunits from Tetrameric Avidin?**—The fact that atp11 and atp12 mutants exhibit identical phenotypes (4) suggests that the encoded products may have a related function and therefore might be part of a single complex. The existence of an ATP11-ATP12 complex in mitochondria was tested in two ways. In previous studies, the ATP12 protein was determined to have a native molecular weight approximately twice that of the monomer as estimated by SDS-gel electrophoresis (5). The discrepancy in size could indicate that the native ATP12 protein is a homodimer of a hetero-oligomer complexed with some other protein. Because ATP11 seemed a reasonable candidate, molecular weight determinations were done on the ATP12 protein in W303ΔATP11, a strain unable to express ATP11. Within experimental error, no difference could be detected in the size of the native ATP12 protein in this mutant and in a wild-type strain. Avidin affinity chromatography also failed to provide evidence for the existence of an ATP11-ATP12 protein complex. Fractions obtained from the tetrameric avidin column were separated by SDS-gel electrophoresis and probed with antibody against the ATP12 protein and against the α and β subunits of F1. As shown in Fig. 11, no ATP12 protein could be detected in the hot SDS wash containing almost all of the biotinylated ATP11 protein applied to the column. Interestingly, a significant amount of both α and β subunits was detected in this fraction. An identical fractionation of a mitochondrial extract from wild-type yeast is also shown in Fig. 11. Although the SDS eluate from this column also had signals corresponding to the F1 subunits they were significantly weaker. The results of the fractionations indicate...
that ATP11 protein is unlikely to be complexed to ATP12 but could have some affinity either for F1 subunits or for the oligomeric enzyme itself.

**DISCUSSION**

The *ATP11* and *ATP12* genes of *S. cerevisiae* have been shown previously to be required for the assembly of the F1 subunits into a functional oligomer (4). The present studies were undertaken with several objectives in mind. The first was to characterize the *ATP11* gene and learn more about the properties of the protein. Second, it was important to establish whether ATP11 protein is located in mitochondria. Finally, we explored a recently described method for in *vivo* tagging proteins with biotin for purposes of their detection and purification (6).

The *ATP11* gene was cloned by transformation of an *atp11* mutant from complementation group G13 with a plasmid library of yeast nuclear DNA. The gene codes for a product of 39 kDa with a hydrophilic amino acid composition suggestive of a water-soluble protein. The amino-terminal 30–40 residues have been confirmed by *in vitro* import assays to constitute a mitochondrial import signal. The primary translation product expressed from an RNA transcribed from *ATP11* is translocated by an energy-dependent process into a protease-resistant compartment of yeast mitochondria. The mature protein is some 3–4 kDa smaller than the precursor. The transport-coupled processing of the ATP11 precursor constitutes strong evidence for its mitochondrial localization. This was confirmed by immunological assays of fractionated yeast. An antibody against a hybrid protein consisting of ATP11 fused to the amino-terminal half of the component I of anthranilate synthetase was found to react with a mitochondrial protein of 32 kDa present in wild-type yeast but not in a mutant with a deleted copy of *ATP11*. The protein detected by the antibody was identical to the processed ATP11 protein seen after its import into mitochondria.

The primary structure of the ATP11 product, deduced from the sequence of the gene, does not provide any obvious clues concerning the role of this protein in F1 assembly. The ATP11 protein is not homologous to any entries in either the GenBank or EMBL databases; nor does it have a domain that is recognized by programs designed to search for protein sequence motifs indicative of enzyme functions or substrate binding properties. The elucidation of how the ATP11 protein promotes F1 assembly will therefore require that it be available in a form sufficiently pure for functional studies. Our initial trials at purification have been hampered by the low abundance of the protein in mitochondria. As an alternative approach we constructed a fusion gene of *ATP11* and a sequence coding for the carboxyl-terminal biotination domain of *P. shermanii* transcarboxylase (5). The hybrid protein expressed from this gene has a carboxyl-terminal extension of 78 residues with a lysine situated 34 amino acids from the new carboxyl terminus that serves as the biotin acceptor (39). The ability of this gene to complement the respiratory defect of an *atp11* mutant indicates that the presence of the bacterial sequence does not abolish the normal function of the ATP11 protein. Furthermore, analysis of mitochondria from the transformant harboring a multicopy plasmid with the fusion gene disclosed the presence of a protein with the expected size which reacts with the anti-ATP11 antibody and with peroxidase-conjugated avidin. The detection of the hybrid protein with the avidin probe confirmed the *in vivo* biotination of the hybrid protein.

In addition to providing a convenient and sensitive means for assaying the ATP11 protein, the presence of covalently attached biotin makes it possible to use an avidin affinity column to remove most matrix proteins. Chromatography of a crude mitochondrial extract on a monoavidin column yields a fraction in which ATP11 protein is the major stained band detected with silver stain. Because the protein is displaced from the avidin column with biotin it should retain its native conformation. A more quantitative recovery of the ATP11 protein is obtained after affinity chromatography on Sepharose coupled to native tetrameric avidin. However, elution of the protein from this column requires harsher conditions such as SDS because of the higher affinity of tetrameric avidin for biotin. The native avidin column can be used to test for possible association of other proteins with the biotinylated component. The results of such analyses have failed to provide any evidence of an ATP11-ATP12 protein complex. They do, however, suggest that the ATP11 protein may interact with the α and/or β subunits of F1-ATPase.

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