Mutational Studies with Atp12p, a Protein Required for Assembly of the Mitochondrial F1-ATPase in Yeast

IDENTIFICATION OF DOMAINS IMPORTANT FOR Atp12p FUNCTION AND OLIGOMERIZATION*

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The Atp12p protein of Saccharomyces cerevisiae is required for assembly of the F1 moiety of the mitochondrial ATP synthase. The current work has used mutant forms of Atp12p in an effort to learn about amino acids and/or domains that are important for the action of the protein. In one set of studies, the mutant atp12 genes were cloned and sequenced from 13 independent isolates of chemically mutagenized yeast. Of the 10 different mutant alleles that were identified, 9 (8 nonsense and 1 frameshift) lead to the early termination of the protein. A single missense mutation that substitutes lysine for Glu-289 was identified in two of the atp12 strains. Analysis of several Atp12p variants, each with different substitutions at Glu-289, showed that the functional activity of Atp12p is compromised when non-acidic residues are introduced at position 289 in the sequence. In other work, deletion analysis led to the assignment of two domains in Atp12p; the functional domain of the protein was mapped to the sequence between Gln-181 and Val-306, and a structural domain (Asp-307 through Gln-325) was identified that confers Atp12p the ability to oligomerize with other proteins in mitochondria.

The ATP synthase of mitochondrial, chloroplast, and bacterial membranes is a key enzyme involved in energy production (1–3). The enzyme is composed of a catalytic moiety F1, attached peripherally to an integral membrane component F0. The subunit composition of F0 varies among species, while F1 has been highly conserved in evolution. In most organisms studied, F1 contains five different subunits in the stoichiometric ratio: α3β2γδε (1–3). X-ray diffraction data for the F1 have been obtained with the mitochondrial enzyme from both bovine (4) and rat liver (5, 6). The a and β subunits alternate in a hexagonal array (4, 5) surrounding the amino and carboxyl termini of the γ subunit (4). The δ and ε subunits are not visible in the current crystal structures. These subunits likely reside at the base of the enzyme since they are required for binding F0 to F1 (7, 8).

In Saccharomyces cerevisiae the F1 subunits are encoded by nuclear genes (9–13) and, with the exception of ε (11), are synthesized as precursors containing an amino-terminal mitochondrial targeting sequence that is cleaved during import (14). The reactions of sorting the F1 subunits into mitochondria and the subsequent folding reactions within the organelle are mediated by heat-shock proteins (Hsps)1 that serve as “molecular chaperones” in the cell (15). With respect to the biogenesis of mitochondrial proteins (such as the F1), the combined activities of the cytoplasmic and mitochondrial Hsp70-Hsp40 chaperone pairs are proposed to mediate the translocation of unfolded proteins into mitochondria; folding the polypeptide chain in the matrix is then facilitated by the Hsp60 and Hsp10 proteins (for review, see Refs. 15 and 16). In the specific case of F1 biogenesis, the final steps in the enzyme formation require two proteins, Atp11p and Atp12p, neither of which has significant sequence homology with other proteins in the data banks (17–19). Yeast mutants that are deficient for either Atp11p or Atp12p accumulate the F1α and β subunits in large protein aggregates instead of forming the enzyme oligomer (17). In contrast to Hsp60-deficient strains, which show aggregation of both the mature and precursor forms of the F1β subunit (20), only the mature form of the F1 subunits is observed in atp11 and atp12 mutants (17). For this reason, Atp11p and Atp12p are suggested to act at a point in the F1 assembly pathway that is downstream from the Hsp60 step.

In considering the type of action elicited by Atp11p and Atp12p during F1 assembly, it is informative to compare the phenotypes of atp11 and atp12 strains with those of yeast that have null mutations in the individual F1 structural subunit genes. For instance, yeast that are deficient for the α subunit harbor the β subunits as aggregated proteins; likewise, the α subunit aggregates in the β subunit null strains (17). As mentioned above, aggregation of the F1α and β subunits is the signature phenotype of atp11 and atp12 strains. In contrast, the α and β subunits remain soluble in mitochondria of γ (13), δ (12), or ε (11) null mutants, despite the fact that the absence of any of these subunits blocks F1 assembly. Moreover, in γ subunit-deficient yeast, the α and β subunits sediment in linear sucrose gradients to the position where αβ dimers would be expected (13). Thus, it appears that aggregation of the F1α and β subunits prevails only under conditions in which αβ dimerization is not possible, such as in the α or β subunit null strains, or in yeast that lack a protein (i.e. Atp11p or Atp12p) whose function may be to mediate αβ oligomerization. The proposal that Atp11p and Atp12p serve as chaperones during F1 biogenesis is supported by the fact that Atp11p and Atp12p are present in mitochondria at levels that are several orders of magnitude lower than the amount of F1α and β subunit protein (21) which is in accord with the fact that there is only a small pool of unassembled F1 proteins in the steady state (22).

1 The abbreviations used are: HSP, heat-shock protein(s); PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; MBP, maltose binding protein; PMSF, phenylmethylsulfonyl fluoride.

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The present paper reports on features of the Atp12p protein that are important for its action as an F₁ subunit. The primary translation product of the ATP12 gene is a 37-kDa precursor protein, which is cleaved to generate the mature polypeptide (33 kDa) following import into mitochondria (18). In previous work Atp12p was shown to be a soluble protein of the mitochondrial matrix that sediments in linear sucrose gradients as an oligomer of 70–80 kDa (18). The current work presents evidence that the Atp12p oligomer observed in mito-

**TABLE I**

| Strain        | Genotype | Sources |
|---------------|----------|---------|
| D273–10B/A1   | a met6   | Ref. 23 |
| W303–1A      | a ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1–1 | Ref. 18 |
| aW303ATP12   | a ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1–1 atp12–2:LEU2 | Ref. 18 |
| C264         | a met6 atp12–1 | Ref. 17 |
| E394         | a met6 atp12–1 | Ref. 17 |
| E54          | a met6 atp12–2 | Ref. 18 |
| E905         | a met6 atp12–3 | Ref. 18 |
| N540         | a met6 atp12–4 | Ref. 18 |
| E822         | a met6 atp12–5 | Ref. 18 |
| E823         | a met6 atp12–5 | Ref. 18 |
| Z40          | a met6 atp12–6 | Ref. 18 |
| P366         | a met6 atp12–7 | Ref. 18 |
| aW150        | a ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1–1 atp12–8 | This study |
| aW111        | a ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1–1 atp12–9 | This study |
| N242         | a met6 atp12–10 | This study |

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**MATERIALS AND METHODS**

**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. Chemically induced mutations were obtained as described (24). Yeast was grown in the following media: YPD (2% glucose, 2% peptone, 1% yeast extract), YPGal (2% galactose, 2% peptone, 1% yeast extract), YEPG (3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract), WO (2% acetate), and for constructing shuttle vector, YEp352, which was then ligated to the 2-μ vector, YEp352 (25), after the vector was opened with EcoRI, made blunt-ended with Klenow, and then digested with SstI. Atp12(2VA31/p) is coded for in pG57/V239. For this plasmid construction, a 1.0-kb SstI-HindII fragment of DNA that was prepared from pG57/ST4 and ligated to the 2-μ vector, YE352 (25), was then ligated to the designated position in the Atp12p primary structure (18). The details of the plasmid constructions are provided in Footnote 3. All of the plas-

**DNA Sequencing**—The oligonucleotide primers used for sequencing and for constructing atp12 deletion/truncation mutants are listed in Table II. Genomic DNA was purified from yeast and served as the template for PCR amplification of the atp12 gene using the primers 1, 2, and 9. The 1048-bp PCR product was digested with BamHI and SstI and ligated at these restriction sites in the yeast/E. coli shuttle vector, YE352 (25). The atp12 gene carried in the plasmids was sequenced by the dyeoxy method (26) with Sequenase (U. S. Biochemical Corp.). Both strands of two separate PCR products were sequenced for each atp12 mutant.

**Yeast Plasmid Constructions**—The plasmids used in this study are described in Table III. Atp12p is numbered from 1 to 325, where residue 1 is the initiator methionine in the primary translation product (18). In cases where sequences were removed from the 3′ end of the ATTP12 gene, the plasmids and the encoded products are named according to where the mutant protein is truncated. This is indicated by the single-letter code and number (18) of the last retained amino acid. For mutant proteins that have deletions from the amino terminus, the plasmids and the encoded products are designated by the Δ symbol, followed by the span of ATTP12 codons removed. The number of amino acids that are deleted from the carboxyl or the mature amino terminus of the mutant Atp12p proteins is indicated in Table III. In naming the plasmids coding for mutant Atp12p proteins harboring missense mutations, the single-letter code is used to indicate the amino acid substitution at the designated position in the Atp12p primary structure (18).
I, blunt-ending with Klenow, and digesting with 

\[ \text{Bam} \text{I} \text{- Sau} \text{I} \text{vectors purchased from CLONTECH. For these constructions, a 1-kb} \]

...Plasmids whose construction utilized PCR (p657/E289D, p657/E289A, pG57/E289D, pG57/ST21, pG57/E289K plasmids. To make pG57/E289D, the 1–124 bp of pG57/E289A were used as a template for the PCR reaction. The PCR products were ligated to BamHI digested YEp352, and the resulting plasmid was sequenced to verify that there were no coding frame changes. The two PCR products were ligated to 

**Preparation of Yeast Mitochondria—**Yeast were grown aerobically in liquid YPGal at the temperature indicated in the experiment to early

**Table II**

| Primer | Sequence (5’ → 3’) | ATP12 gene location | Restriction sites |
|--------|--------------------|---------------------|------------------|
| 1      | GCCGCCACGCTTCAACAACTTTC | +36 to +15 | BamHI |
| 2      | GCCGCCACGCTTCAACAACTTTC | +300 to +317 |  |
| 3      | GCCGCCACGCTTCAACAACTTTC | +524 to +541 |  |
| 4      | GCCGCCACGCTTCAACAACTTTC | +739 to +755 |  |
| 5      | GCCGCCACGCTTCAACAACTTTC | +164 to +181 |  |
| 6      | GCCGCCACGCTTCAACAACTTTC | +548 to +565 |  |
| 7      | GCCGCCACGCTTCAACAACTTTC | +554 to +571 |  |
| 8      | GCCGCCACGCTTCAACAACTTTC | +770 to +787 |  |
| 9      | GCCGCCACGCTTCAACAACTTTC | +990 to +1012 |  |
| 10     | GCCGCCACGCTTCAACAACTTTC | +82 to +99 |  |
| 11     | GCCGCCACGCTTCAACAACTTTC | +975 to +995 |  |
| 12     | GCCGCCACGCTTCAACAACTTTC | +1045 to +1045 |  |
| 13     | GCCGCCACGCTTCAACAACTTTC | +258 to +276 |  |
| 14     | GCCGCCACGCTTCAACAACTTTC | +730 to +755 |  |
| 15     | GCCGCCACGCTTCAACAACTTTC | +745 to +762 |  |
| 16     | GCCGCCACGCTTCAACAACTTTC | +755 to +771 |  |
| 17     | GCCGCCACGCTTCAACAACTTTC | +910 to +927 |  |
| 18     | GCCGCCACGCTTCAACAACTTTC | +927 to +944 |  |
| 19     | GCCGCCACGCTTCAACAACTTTC | +944 to +961 |  |
| 20     | GCCGCCACGCTTCAACAACTTTC | +961 to +978 |  |

**Table II**

**Oligonucleotide primers for PCR and sequencing**

| Plasmid | Encoded product | Number of amino acids deleted | Carbonyl terminus | Mature amino terminus |
|---------|-----------------|-------------------------------|-------------------|---------------------|
| pG57/ST4 | Wild type Atp12p | 0 |  |  |
| pG57/V306 | Atp12(V306)p | 19 |  |  |
| pG57/V283 | Atp12(V283)p | 42 |  |  |
| pG57/P299 | Atp12(P299)p | 86 |  |  |
| pG57/1–81 | Atp12(1–81)p | 51 |  |  |
| pG57/1–124 | Atp12(1–124)p | 94 |  |  |
| pG57/1–180 | Atp12(1–180)p | 150 |  |  |
| pG57/1–224 | Atp12(1–224)p | 194 |  |  |
| pG57/E289K | Atp12(E289K)p | 194 |  |  |
| pG57/E289A | Atp12(E289A)p | 194 |  |  |
| pG57/E289D | Atp12(E289D)p | 194 |  |  |
| pG57/E289Q | Atp12(E289Q)p | 194 |  |  |
| pG57/ST21 | YHistag-Atp12p | 194 |  |  |
| pG57/ST22 | Gal4p-AD-Histag-Atp12p | 194 |  |  |
| pG57/ST23 | Gal4p-AD-Histag-Atp12p | 194 |  |  |

**Table III**

**Atp12 yeast plasmids and encoded proteins**

With the exception of pG57/ST4 (Ref. 18), the present study is the source for all of the plasmids. The multi-copy (2μ) vector, YEpl532 (25), was used for all plasmid constructions.

These values refer to the number of amino acids that are deleted from the amino terminus of the mature protein, which is the form of Atp12p that is generated when the mitochondrial leader sequence is removed.

The numbers refer to the Atp12p codons that were removed; in each case the remaining portion of the ATP12 gene is ligated to the Atp11p mitochondrial leader sequence (ATP11 codons 1–39, Ref. 21).

Fusion protein formed between the DNA binding domain of Gal4p and Histag-Atp12p.

Fusion protein formed between the activation domain of Gal4p and Histag-Atp12p.

Stationary phase. The method of Faye et al. (28) was used to prepare mitochondria with the exception that Zymolyase, instead of Glusulase, was used to digest the cell wall. Phenylmethylsulfonyl fluoride (PMSF) was added to 10 μg/ml final concentration during the cell-breaking step to minimize proteolysis.

**Solubilization of Atp12p from Mitochondria—**Two different methods, both of which give comparable results, were used to prepare mitochondrial extracts containing solubilized Atp12p. For some experiments, mitochondria were suspended at 10 mg/ml in 10 mM Tris-HCl, pH 8.0, and sodium deoxycholate was added to 1 mg/ml to permeabilize the membranes. Following a 15-min incubation at 0 °C, the suspension was centrifuged for 30 min at 4 °C, 50,000 rpm in a Beckman 70Ti rotor. Alternatively, mitochondria were suspended at 7–8 mg/ml in 0.4 ml of the buffer specified in the experiment and exposed to four 10-s bursts, with cooling in between, of sonic irradiation at 40% output (Branson...
Purification of Histag-Atp12p from E. coli Expression Systems—Two different plasmids were employed for the overproduction of Histag-Atp12p in bacteria. In one case, PCR was used to create the bacterial plasmid, pHISATP12, which encodes the mature form of Atp12p (with the mitochondrial leader sequence (18)) that carries a (6x)histidine sequence in Histag-Atp12p.

The plasmid pHISATP12 was created by subcloning a 1-kb Sau3A/HindIII fragment encoding the sequence for Histag-Atp12p from the plasmid pGS7/ST2 (Table III) into the pMAL-C2 vector that was prepared as an E. coli cells carrying the plasmid pMBPHISATP12 were grown in a 0.5-liter LB/ampicillin culture at 30 °C to mid-log phase. Following a 4-h induction with 0.5 mM isopropylthiogalactoside, the cells were harvested, sonicated, and centrifuged at 9,000 rpm in a Sorvall SA600 rotor. The crude extract (containing the recombinant fusion protein) was diluted five times with column buffer (10 mM Na2HPO4, pH 7.0, 500 mM NaCl, 0.25% Tween 20, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM EGTA). Following three cycles of freezing and thawing, the cell suspension was sonicated, brought to 0.5 M NaCl, and centrifuged at 9,000 rpm in a Sorvall SA600 rotor. The crude extract was then centrifuged for 3 min in a microcentrifuge at room temperature. The supernatant was collected, and the Ni-NTA beads were washed five times for 5 min with 0.14 M buffer (detergent-free) that contained 10 mM imidazole in 95% pure form. The Coomassie-stained gel in Fig. 2 shows a sample of the purified MBP-Histag-Atp12p protein (lane 2) from the pooled column fractions.

Affinity Precipitation of Histag-Atp12p from Mitochondrial Extracts—Mitochondria were suspended in 50 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 1 mM PMSF, and soluble extracts were prepared by sonic irradiation (see above). A 50-μl slurry of Ni-NTA resin was pre-equilibrated with buffer M (10 mM Tris-HCl, pH 8.0, 5 mM imidazole, 140 mM NaCl, 1% Triton X-100, 1 mM PMSF) and added to 0.14 M of sonic supernatant containing solubilized mitochondrial proteins. The suspension was mixed end-over-end for 30 min at room temperature and then centrifuged for 3 min at a microcentrifuge at room temperature. The supernatant was collected, and the Ni-NTA beads were washed five times for 5 min with 0.14 M of buffer M, and finally suspended in SDS-gel loading buffer in preparation for Western analysis.

Sedimentation Analysis of Atp12p—Mitochondria were suspended in 20 mM Tris-HCl, pH 8.0, and soluble extracts were prepared by sonic irradiation (see above) at 4 °C. Molecular weight markers (hemoglobin and myokinase, or hemoglobin and lipoamide dehydrogenase) were added to the solubilized protein samples, and the mixtures were centrifuged through 7–20% linear sucrose gradients under the conditions of sedimentation described (18). Previously described methods (18, 21) were used to define the positions of myokinase, hemoglobin, and lipoamide dehydrogenase peaks in the gradients. The gradient fractions were assayed by Western analysis for Atp12p.

Chemical Cross-linking—For cross-linking with amine-reactive bifunctional reagents, purified recombinant Histag-Atp12p was incubated at 0.4 mg/ml in 20 mM Na2HPO4, pH 7.5, 150 mM NaCl with either 5 mM disulfosuccinimidyl tartarate, 5 mM ethylene glycolbis(sulfosuccinimidylsuccinate), or 5 mM dithiobis(sulfosuccinimidylpropionate) at 22 °C for 30 min. The modification reactions were quenched by the addition of Tris-HCl, pH 8.0, to 50 mM concentration and denatured in SDS-gel loading buffer in preparation for electrophoresis. For experiments that employed glutaraldehyde, the purified Histag-Atp12p protein was incubated in 10 mM Tris-HCl, pH 7.5, at 0.4 mg/ml with 0.05, 0.1, or 0.5% glutaraldehyde at 22 °C for 10 min, at which time SDS-gel loading buffer was added, and the samples were loaded on SDS-polyacrylamide gels. In control experiments, 0.5 mg/ml hemoglobin was

![FIG. 1. SDS-polyacrylamide gel analysis of Histag-Atp12p purified from E. coli.](image1)

The figure shows a Coomassie-stained 12% SDS-polyacrylamide gel that was used to analyze recombinant MBP-Histag-Atp12p fusion protein that was purified from E. coli according to the procedure described under “Materials and Methods.” Lane 1 shows 2 μg of the Histag-Atp12p that was eluted from the Ni-NTA column. The migration of molecular mass standards is shown in lane 2. The size of the marker proteins is indicated in kilodaltons in the right-hand margin.

![FIG. 2. SDS-polyacrylamide gel analysis of the MBP-Histag-Atp12p fusion protein purified from E. coli.](image2)

The figure shows a Coomassie-stained 12% SDS-polyacrylamide gel that was used to analyze the recombinant MBP-Histag-Atp12p fusion protein that was purified from E. coli according to the procedure described under "Materials and Methods." The migration of molecular mass standards is shown in lane 1; the size of the marker proteins is indicated in kilodaltons in the left-hand margin. Lane 2 shows 2 μg of the MBP-Histag-Atp12p fusion protein that was eluted from the Amylose column.
Mutational Analysis of Atp12p

also Ref. 18) shows the presence of the mutant form of Atp12p in mitochondrial samples prepared from the atp12 strains, C264, E822, E695, N242, and P366. Such proteins are deleted for up to 86 amino acids from the carboxyl terminus of Atp12p. Mutant Atp12p proteins that are predicted to have 172 or more amino acids removed from the carboxyl terminus were not detected in Western blots of total mitochondrial protein.

Respiratory Properties of Yeast That Produce Genetically Engineered Forms of Atp12p with Deletions of Sequences from the Carboxyl or the Amino Terminus—The atp12 nonsense alleles (see above) encode truncated forms of Atp12p, which are inactive when produced in single copy from the chromosome. To investigate the possibility that carboxyl sequences could be dispensable with if the level of truncated Atp12p is raised in the cell, a series of multi-copy plasmids were constructed with atp12 genes that have deletions of sequences from the 3’ end of the gene (Table III). These plasmids direct the synthesis of the mutant proteins, Atp12/P239p, Atp12/V283p, and Atp12-V306p, which are missing 86, 42, and 19 amino acids, respectively, from the carboxyl terminus (Table III). The position of the last retained amino acid in these mutant proteins is indicated with an arrow in the Atp12p sequence shown in Fig. 3.

The properties of the plasmid-borne Atp12p mutant proteins were evaluated in the genetic background of a respiratory-deficient yeast strain that harbors a disruption at the Atp12p locus (aW303ΔATP12 (18)) (Table V). None of the mutant proteins that have sequences deleted from the carboxyl terminus conferred to the host strain the ability to grow within 48 h using ethanol-glycerol (EG), a non-fermentable carbon source (Table V). Low levels of respiratory activity were observed only for the strain that produces Atp12(V306p), which eventually grows on EG plates after 3–4 days at 30 °C. Western analysis established that the plasmid-produced Atp12(V306p), Atp12(V283p), and Atp12(P239p) proteins were present in mitochondria isolated from the respective yeast transformants at 30–80% of the wild type level (Fig. 4 and Table V). Similar results were obtained when mitochondrial extracts (solubilization with 0.1% sodium deoxycholate), rather than total mitochondria, were analyzed by Western blots (data not shown). With respect to the Western analysis it is important to note that while the antigen used to raise the polyclonal Atp12p antiserum in previous experiments (18) full length, mature Atp12p protein were prepared as described (18) and used at a dilution of 1:100.

RESULTS

Characterization of the atp12 Mutants Obtained by Chemical Mutagenesis—Thirteen independent yeast isolates, with mutations in the ATP12 gene, were obtained by chemical mutagenesis with nitrosoguanidine and ethyl methane sulfonate (24). These strains are respiratory-deficient due to a defect in the 

Yeast Two-hybrid Screen—The yeast two-hybrid screen (29) employed yeast vectors (pACT2 and pAS2-1) and a host strain CG-1945 (ura3-52 his3-200 yep2-01 ade2-101 trpl-1901 leu2-3, 112 gal4-524 gal80-538 cly2 LYS2-GAL1UAS-GAL1PACT-HIS3 URA3-GAL1PACT-CYC1PACT-lacZ) that were supplied in the MATCHMAKER Two-hybrid System 2 purchased from CLONTECH. The construction of Atp12p plasmids with pACT2 and pAS2-1 (pG57/ST22, pG57/ST23, Table III) is described in Footnote 3. Expression from the GAL1PACT-CYC1PACT-lacZ reporter gene was determined using 5-bromo-4-chloro-3-indolyl β-D-galactosidase as a chromogenic substrate for 

Mitochondrial ATPase Activity—Mitochondrial ATPase activity was determined using 5-bromo-4-chloro-3-indolyl β-D-galactosidase as a chromogenic substrate for β-galactosidase in the colony-lift assay described in the CLONTECH manual.

Footnote 3. Expression from the GAL1PACT-CYC1PACT-lacZ reporter gene was determined using 5-bromo-4-chloro-3-indolyl β-D-galactosidase as a chromogenic substrate for β-galactosidase in the colony-lift assay described in the CLONTECH manual.

Mutational Analysis of Atp12p

| atp12 mutation | Nucleotide | Codon change | Amino acid | Amino acids deleted from carboxyl terminus |
|----------------|------------|--------------|------------|------------------------------------------|
| E54            | 154        | CAG → TAG    | 52         | 274                                      |
| Z43            | 165        | TGG → TGA    | 55         | 271                                      |
| C90            | 305        | TGG → TGA    | 103        | 223                                      |
| aW111          | 415        | CAA → TAA    | 139        | 187                                      |
| N540           | 460        | CAG → TAG    | 154        | 172                                      |
| aW150          | 720        | TGG → TGA    | 240        | 86                                       |
| F366           | 720        | TGG → TGA    | 240        | 86                                       |
| N242           | 749        | TTA → TAA    | 250        | 76                                       |
| E695           | 799        |             | 267        | 48                                       |
| E822           | 865        | GAA → AAA    | 289        |                                          |
| E823           | 865        | GAA → AAA    | 289        |                                          |
| E394           | 890        | TGG → TAG    | 297        | 29                                       |
| C264           | 299        | TGG → TAG    | 297        | 29                                       |

* a These designations indicate the names of the mutant strains.
* b Sequence numbering according to Ref. 18.
* c Number of amino acids deleted assumes truncation at the site of the nonsense mutation.
* d Addition of an "A" nucleotide at position 799 in the ATP12 coding sequence causes a frameshift at amino acid 267, which results in the early termination of the protein at amino acid 277.
* e Missense mutation that changes Glu-289 to Lys.

Yeast Two-hybrid Screen—The yeast two-hybrid screen (29) employed yeast vectors (pACT2 and pAS2-1) and a host strain CG-1945 (ura3-52 his3-200 yep2-01 ade2-101 trpl-1901 leu2-3, 112 gal4-524 gal80-538 cly2 LYS2-GAL1UAS-GAL1PACT-HIS3 URA3-GAL1PACT-CYC1PACT-lacZ) that were supplied in the MATCHMAKER Two-hybrid System 2 purchased from CLONTECH. The construction of Atp12p plasmids with pACT2 and pAS2-1 (pG57/ST22, pG57/ST23, Table III) is described in Footnote 3. Expression from the GAL1PACT-CYC1PACT-lacZ reporter gene was determined using 5-bromo-4-chloro-3-indolyl β-D-galactosidase as a chromogenic substrate for β-galactosidase in the colony-lift assay described in the CLONTECH manual.

Miscellaneous Procedures—Standard techniques were used for restriction endonuclease analysis of DNA, purification and ligation of DNA fragments, and transformations of and recovery of plasmid DNA from E. coli (31). Yeast transformation was performed as described (32). The method of Laemmli (33) was used for SDS-polyacrylamide gel electrophoresis. Western blotting followed the procedure of Schmidt et al. (34). Antibodies against the full-length, mature Atp12p protein were prepared as described (18) and used at a dilution of 1:100.

RESULTS

Characterization of the atp12 Mutants Obtained by Chemical Mutagenesis—Thirteen independent yeast isolates, with mutations in the ATP12 gene, were obtained by chemical mutagenesis with nitrosoguanidine and ethyl methane sulfonate (24). These strains are respiratory-deficient due to a defect in the F1-ATPase assembly pathway and fail to utilize non-fermentable carbon sources for growth (17). The biochemical properties of some of the mutant atp12 strains were reported previously (18). In the current work, the mutant genes were cloned and sequenced from each of the atp12 strains to determine the type of genetic lesions that alter Atp12p function (see under “Materials and Methods”). This analysis disclosed a single missense allele (carried in strains E822 and E929) and nine mutant alleles (eight frameshifts and one frameshift) that cause early termination of the Atp12p protein (Table IV). The genetic information in the latter strains should lead to the synthesis of Atp12p with deletions of 29, 48, 76, 86, 172, 187, 203, 271, or 274 amino acid residues from the carboxyl terminus (Table IV). The positions of the chemically induced mutations are indicated in the Atp12p sequence shown in Fig. 3, using arrows that are flagged with the name of the mutant strain (highlighted in the black boxes). Western analysis (data not shown, see
The mean values from the analysis of two separate Western blots are reported; a representative blot that shows data for cells grown at 30 °C is given. Values obtained for the protein band intensities were corrected to account for the size of the mutant proteins relative to full-length Atp12p. The amount of Atp12p protein was determined using an AMBIS 4000 imaging system to scan Western blots of total mitochondrial protein. The yeast were scored for growth after 48 h following replica plating from glucose to ethanol/glycerol media. Yeast were grown at 30 °C unless indicated otherwise.

### Table V: Respiratory phenotype of yeast that produce truncated forms of Atp12p

| Plasmid-borne protein | Amount of Atp12p | Mitochondrial ATPase activity |
|-----------------------|------------------|--------------------------------|
|                       |                  | Minus oligomycin<sup>a</sup> | Plus oligomycin<sup>a</sup> | Oligomycin-sensitive activity |
|                       |                  | µmol Pi/min/mg | µmol Pi/min/mg | % control level |
| wtAtp12p              | ++               | 5.60 (5.35) min 0.63 (0.90) | 4.71 ± 0.13 | 100 |
| wtAtp12p, 23 °C       | ++               | 5.48 (6.33) min 0.76 (0.76) | 5.14 ± 0.10 | 100 |
| Atp12V306p            | 80               | 0.31 (0.20) min 0.20 (0.20) | 0.10 ± 0.01 | 5 |
| Atp12V283p            | 50               | 0.24 (0.12) min 0.14 (0.13) | 0.30 ± 0.01 | 5 |
| Atp12P239p            | <=5              | 0.01 (0.00) min 0.01 (0.00) | 0.01 ± 0.01 | 5 |
| Atp12(Δ-181)p         | <=5              | 4.24 (4.39) min 0.83 (0.47) | 3.27 ± 0.26 | 78 |
| Atp12(Δ-124)p         | <=18             | 0.89 (0.95) min 0.09 (0.26) | 0.35 ± 0.05 | 14 |
| Atp12(Δ-124)p, 23 °C  | <=20             | 3.57 (3.29) min 0.38 (0.46) | 3.01 ± 0.18 | 59 |
| Atp12(Δ-180)p         | <=5              | 0.38 (0.12) min 0.03 (0.00) | 0.24 ± 0.12 | 5 |
| Atp12(A2-124)p        | None             | 3.50 (3.14) min 0.29 (0.16) | 3.10 ± 0.12 | 60 |

<sup>a</sup> Atp12p was produced from a multi-copy plasmid.

<sup>b</sup> Yeast were scored for growth after 48 h following replica plating from glucose to ethanol/glycerol media. ++, good growth; +, moderate growth; −, no growth.

<sup>c</sup> The amount of Atp12p protein was determined using an AMBIS 4000 imaging system to scan Western blots of total mitochondrial protein. The values obtained for the protein band intensities were corrected to account for the size of the mutant proteins relative to full-length Atp12p. The mean values from the analysis of two separate Western blots are reported; a representative blot that shows data for cells grown at 30 °C is given in Fig. 4.

<sup>d</sup> Results from two separate mitochondrial preparations are shown; one set of values obtained ± oligomycin is given in parentheses.

<sup>e</sup> The mean values are reported.

FIG. 3. **Description of Atp12p mutants.** The protein sequence for the “mature” form of Atp12p is shown in single-letter code. The names of the atp12 strains, which were obtained by chemical mutagenesis, are highlighted in the black boxes; the arrow indicates the amino acid residue that is mutated in each of the strains. The mutation in strain E695 shifts the frame at Asp-267. Strains E822 and E823 produce Atp12p with a Glu-289 → Lys missense mutation. In the remaining atp12 strains, the denoted amino acid is mutated to a stop codon. The white boxes bear the names of the plasmid-borne Atp12p deletion mutants. **Arrows** are used to indicate the first Atp12p amino acid in the Δ1–81, Δ1–124, Δ1–180 Atp12p proteins resulting in the addition of sequence between the mitochondrial leader peptide (of Atp11p) and the mature Atp12p polypeptide fragments, any or all of these proteins might have amino acids coded for in the linker sequence preceding the Atp12p amino acid that is indicated in the figure (see Footnote 3).
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on EG plates. Moreover, there was 60% of the control level of oligomycin-sensitive ATPase activity measured in mitochondria isolated from these transformants, following their growth at 23 °C in galactose media (Table V). The effect of temperature on the oligomycin-sensitive ATPase activity measured in mitochondrial extracts of deoxycholate-treated mitochondria. This observation is in accord with the report that the ability of yeast to utilize non-fermentable substrates for growth correlates with the presence of at least 15% mitochondrial ATPase activity (35). Yeast transformants harboring the atp12 mutations E289A or E289Q show normal growth on EG plates but exhibit 60–70% reduction in the level of mitochondrial oligomycin-sensitive ATPase activity (Table VI). In the case of the mutation E289D, wild type levels of mitochondrial ATPase activity are observed whether the mutant protein is produced from a multi-copy plasmid (Table VI) or from a single copy plasmid (data not shown). Since all four mutant proteins are detected in mitochondria at near wild type levels, the data suggest that the reduced activity of Atp12p harboring either the E289K, E289A, or E289Q mutations is not due to a reduction in the amount of the mutant proteins but is likely to be a consequence of having a non-acidic residue at position 289 in the sequence.

Sedimentation Analysis of Plasmid-borne Atp12p Variants—The molecular mass of mitochondrial Atp12p was estimated from its sedimentation properties in linear sucrose gradients to be in the range of 70–80 kDa (18). The molecular mass of the mature protein, as estimated from its migration in SDS gels relative to the precursor (18), is 33 kDa. These results suggest that the protein observed in sucrose gradients is oligomeric. Sedimentation analysis was used to determine if the ability of Atp12p to form higher ordered oligomers is lost when sequences are deleted from the amino or carboxyl terminus or as a result of mutations at Glu-289. Soluble mitochondrial extracts were prepared from the yeast transformants that overproduce mutant forms of Atp12p and centrifuged through linear sucrose gradients in the presence of molecular weight standards. Only the deletion mutants of Atp12p, whose presence was detected in Western blots of mitochondrial samples (see Table V), were analyzed in these studies, i.e. Atp12p, Atp12p, Atp12p, and Atp12p. The sizes predicted for monomers of mutant forms of the protein are given in Table VII. The three proteins that are missing carboxyl sequences (Atp12p, Atp12p, and Atp12p) co-sediment with myokinase (Mₚ = 21,000) in sucrose gradients (Fig. 6). This result suggests that these three mutant proteins are present as monomers in the mitochondrial matrix. On the basis of sequence, the size of the Atp12p mono- mer is comparable to that of Atp12p (Table VII), yet the sedimentation profiles for these two proteins are significantly different (Fig. 6). The apparent larger size noted for the amino-terminal deletion mutant suggests that Atp12p forms an oligomer in vivo (Table VII). With respect to the mutant proteins that are substituted at Glu-289, the sedimentation behavior of all four proteins (Fig. 6) was shown to be comparable with that of wild type mitochondrial Atp12p (18). These results indicate that the E289K, E289D, E289A, and E289Q mutations have no effect on Atp12p oligomerization.

Analysis of the Oligomeric State of Atp12p—Efforts were made to determine if mitochondrial Atp12p is a homo- or hetero-oligomer. As part of this work, Histag-forms of Atp12p synthesized in E. coli were used to evaluate the size of Atp12p in the absence of other mitochondrial proteins. Control experiments had shown that the presence of the (6x)histidine tag sequence does not alter the functional properties of Atp12p. Such experiments employed an atp12 null mutant transformed with a yeast plasmid (pG57/ST21, Table III) that encodes a protein chimera in which the Atp11p leader sequence is used to sort the tagged Atp12p protein (called YHistag·Atp12p for Yeast Histag·Atp12p) to mitochondria. The (6x)histidine sequence is retained on YHistag·Atp12p following cleavage of the heterologous leader peptide as indicated by the fact that YHistag·Atp12p (but not the non-tagged form of the protein) is selectively precipitated from mitochondrial extracts with NiNTA beads (procedure described under “Materials and Methods,” data not shown). Notably, the atp12 null mutation is complemented by the plasmid-borne YHistag·Atp12p as indicated by the ability of the transformed strain to grow on ethanol-glycerol plates at the wild type rate and to exhibit wild type levels of oligomycin-sensitive mitochondrial ATPase activity.
The data from one of the experiments are shown in Fig. 6. The mean values from the analysis of two separate Western blots are reported; one of the blots is shown in Fig. 5. 

Results from two separate mitochondrial preparations are shown; one set of values obtained ± oligomycin is given in parentheses. The mean values are reported.

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**Mutational Analysis of Atp12p**

Yeast were grown at 30 °C.

| Plasmid-borne protein<sup>a</sup> | Growth on ethanol/glycerol<sup>b</sup> | Amount of Atp12p<sup>c</sup> | ATPase activity<sup>d</sup> | Oligomycin-sensitive activity<sup>d</sup> |
|----------------------------------|-------------------------------------|-----------------------------|--------------------------|----------------------------------|
|                                  |                                     |                             | Minus oligomycin<sup>e</sup> | Plus oligomycin<sup>e</sup> | % control level | μmol Pi/min/mg | % control level | μmol Pi/min/mg |
| Wild type Atp12p                 | ++                                  | 100                         | 5.60 (5.35)               | 0.63 (0.09)                  | 4.71 ± 0.13   | 100            | 4.71 ± 0.13   | 100 |
| Atp12/E289Kp                     | −                                   | 84                          | 0.69 (0.55)               | 0.18 (0.00)                  | 0.53 ± 0.02   | 11             | 0.53 ± 0.02   | 11  |
| Atp12/E289Dp                     | ++                                  | 82                          | 5.50 (4.74)               | 0.56 (0.13)                  | 4.78 ± 0.17   | 100            | 4.78 ± 0.17   | 100 |
| Atp12/E289A<sup>e</sup>p         | ++                                  | 85                          | 1.82 (1.78)               | 0.16 (0.00)                  | 1.72 ± 0.06   | 37             | 1.72 ± 0.06   | 37  |
| Atp12/E289Q<sup>e</sup>p         | ++                                  | 100                         | 1.50 (1.22)               | 0.21 (0.00)                  | 1.26 ± 0.04   | 27             | 1.26 ± 0.04   | 27  |

<sup>a</sup> Atp12p was produced from a multi-copy plasmid.

<sup>b</sup> Yeast were scored for growth after 48 h following replica-plating from glucose to ethanol/glycerol media. ++, good growth; +, moderate growth; −, no growth.

<sup>c</sup> The amount of Atp12p protein was determined using an AMBIS 4000 imaging system to scan Western blots of total protein in mitochondrial extracts. The mean values from the analysis of two separate Western blots are reported; one of the blots is shown in Fig. 5.

<sup>d</sup> Results from two separate mitochondrial preparations are shown; one set of values obtained ± oligomycin is given in parentheses.

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**TABLE VII**

**Oligomeric state of variant forms of Atp12p**

| Plasmid-borne protein<sup>a</sup> | Monomeric size<sup>b</sup> | Form of Atp12p<sup>c</sup> |
|----------------------------------|-----------------------------|----------------------------|
| Atp12/E289Kp                     | 30.9                        | Monomer                    |
| Atp12/E289Dp                     | 28.2                        | Monomer                    |
| Atp12/E289A<sup>e</sup>p         | 33.1                        | Oligomer                   |
| Atp12/E289Q<sup>e</sup>p         | 33.1                        | Oligomer                   |
| Atp12/E289K<sup>e</sup>p         | 33.1                        | Oligomer                   |

<sup>a</sup> Atp12p was produced from a multi-copy plasmid.

<sup>b</sup> These values are for “mature” Atp12p, which is produced following cleavage of the mitochondrial sorting signal.

<sup>c</sup> Evidence for the monomeric or oligomeric state of Atp12p is based on sedimentation analysis that was performed with two separate mitochondrial samples. The data from one of the experiments are shown in Fig. 6.

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Histag-Atp12p was overproduced in *E. coli* from the plasmid pHISATP12 and purified to homogeneity (see Fig. 1). The sedimentation behavior of Histag-Atp12p (mass = 35.7 kDa) in 7–20% sucrose gradients (Fig. 7) is significantly different from that of Atp12p that is lacking the last 19 amino acids, which migrates as a monomer (see sedimentation profile for Atp12/E289Kp (mass = 39.0 kDa) in Fig. 6). The size difference (~5 kDa) between Histag-Atp12p and Atp12/E289Kp is too small to account for the different migration of the two proteins in this experiment; similar work performed with the native and biotin-tagged forms of Atp11p indicated comparable sedimentation profiles for the proteins despite a difference of ~9 kDa in their size (21). The sedimentation profile of bacterially produced Histag-Atp12p is much more similar to that of the wild type mitochondrial protein (18) and to the mitochondrial Atp12p variants substituted at Glu-289 (Fig. 6), which sediment in sucrose gradients as oligomers. This analysis suggested that the native form of Atp12p is a homo-oligomer. However, the results from additional experiments conflict with this proposal. Studies with purified bacterial Histag-Atp12p that were designed to visualize a covalently linked Atp12p dimer gave negative results. SDS gels run with purified Histag-Atp12p in the absence of 2-mercaptoethanol did not show evidence of disulfide-linked dimers; such linkages were predicted as possible since there are three cysteines in the Atp12p polypeptide chain. In other work, three amine-reactive bifunctional chemical reagents (disulfosuccinimidyl tartarate, ethylene glycolbis(sulfosuccinimidylglycinolate), and glutaraldehyde), were used to cross-link purified recombinant Histag-Atp12p as described under “Materials and Methods”; there was no evidence in SDS gels for cross-linked Histag-Atp12p dimers generated by any of the chemical modification reactions (data not shown). Notably, under the same conditions cross-linking of hemoglobin by glutaraldehyde was observed suggesting that the experiment was properly designed to identify the presence of oligomers. Additional evidence against the formation of Atp12p homo-oligomers was obtained using the yeast two-hybrid screen (29), in which the two plasmids used in the assay both carried the gene for Histag-Atp12p. No activation of the *lacZ* reporter gene was observed suggesting that Atp12p monomers do not interact under the conditions of this assay. Although the negative results with the two-hybrid screen is not final proof against Atp12p homo-oligomerization, it is in agreement with the results from the cross-linking experiments that were performed with a battery of chemical modifiers.

The possibility was considered that the apparent migration of recombinant Histag-Atp12p as a homodimer in sucrose gradients (Fig. 7) was due to an artifact imposed by the purification procedure, which included extraction from inclusion bodies under denaturing conditions followed by renaturation, and efforts were made to overproduce the protein in bacteria in soluble form. To this end, a recombinant fusion protein formed between maltose binding protein (MBP) and Histag-Atp12p was found to remain soluble following bacterial cell lysis (see under “Materials and Methods”). Purified MBP-Histag-Atp12p...
Fig. 6. Western blots showing the sedimentation properties of mutant forms of Atp12p. For sedimentation analysis of Atp12p variants, mitochondria were isolated from a W303ΔATP12 transformants that produce the mutant proteins indicated in the figure and sonicated as described under “Materials and Methods.” The solubilized mitochondrial protein samples (200 μl) were each mixed with hemoglobin (M_r = 64,500) and myokinase (M_r = 21,000), and loaded on 4.8-ml 7–20% sucrose gradients. After centrifuging the samples at 42,000 rpm for 16.3 h in an SW55Ti rotor at 4 °C, 16 fractions were collected from the bottom of the tube. The position of Atp12p protein in the gradients is indicated in the Western blots shown in the figure. The numbers above each blot indicate fractions of the gradient; sucrose density increases going from right (high numbers) to left (low numbers). Spectrophotometric and enzymatic assays, respectively, were used to define the peak positions in the gradients for hemoglobin (Hb, filled arrowhead) and myokinase (MK, open arrowhead) as described (21). Western analysis followed the procedures described in the legend to Fig. 4.

Fig. 7. Western blots showing the sedimentation properties of recombinant forms of Atp12p purified from bacteria. Histag-Atp12p and the fusion protein MBP-Histag-Atp12p were each purified from E. coli as described under “Materials and Methods” (see Figs. 1 and 2). The experiment with purified Histag Atp12p (loaded as 2 μg in 200 μl of Tris-HCl, pH 7.5) was performed as described in the legend to Fig. 6. The conditions for the sedimentation experiment with the MBP-Histag-Atp12p fusion protein (loaded as 2 μg in 200 μl of Tris-HCl, pH 7.5) were the same with the following exceptions. First, 10 mM maltose was included in the buffer to prevent aggregation of the maltose binding protein, which oligomerizes in the absence of maltose (36). Second, lipoamide dehydrogenase (100 kDa) was included with hemoglobin as a size standard in the gradient; lipoamide dehydrogenase activity was assayed as described (18). Finally, 15 fractions of identical volume were collected from the bottom of the tube. Western analysis to determine the position of the recombinant Atp12p proteins in the gradients was done as described in the legend to Fig. 4, and the results are illustrated as in Fig. 6.

(Fig. 2) (mass = 77 kDa) sediments in 7–20% sucrose gradients to a position between the hemoglobin (mass = 66 kDa) and lipoamide dehydrogenase (mass = 100 kDa) marker proteins (Fig. 7). This result indicates that the fusion protein migrates as a monomer. Thus, cumulatively, the results from cross-linking studies, the yeast two-hybrid screen, and sedimentation analysis of recombinant MBP-Histag-Atp12p suggest that pure, homogeneous Atp12p does not form homo-oligomers and that the oligomeric form of Atp12p observed in mitochondrial samples most likely originates from the interaction of Atp12p with one or more different gene products.

DISCUSSION

Sequence analysis of 13 independent isolates of yeast harboring chemically induced mutations in the ATP12 gene identified eight nonsense alleles, one frameshift mutation that leads to premature termination of the protein, and a Glu-289 → Lys substitution in the Atp12p primary structure (Table IV). The low frequency of missense mutations (1 in 10 alleles) is not a common feature of the pet mutant collection (24) from which the atp12 strains were obtained. A plausible reason why more missense mutations are not present among the atp12 strains is because the genetic screen that was used originally to select the strains (24) was based on the ability of cells to grow on non-fermentable carbons (ethanol-glycerol, EG). Thus, only the most severe mutations, which prevent Atp12p from assembling F_o in amounts sufficient to support growth on EG plates, were selected by the screen. Since most mutations found in the atp12 genes produce deletions, it would appear that Atp12p is relatively resilient to substitutions of individual residues. However, it was found that at least one position (residue 289) in the amino acid sequence may be of particular importance. Our study indicates that an acidic residue is required at this position for optimal Atp12p activity.

Identification of Domains in Atp12p—A schematic map of wild type Atp12p is presented in the upper part of Fig. 8. The mitochondrial targeting sequence (black-shaded region in the wild type Atp12p map) is estimated to be 30 amino acids long (Met-1 through Leu-30) on the basis of the relative migrations of the precursor and mature forms of Atp12p in SDS gels (18). Two domains were mapped in the mature protein using deletion mutants of Atp12p (for reference, Fig. 8 also includes schematic protein maps for the Atp12p deletion mutants and provides a summary of the characteristics of these proteins). The sequence between Gln-181 and Val-306 constitutes the functional domain of the protein (see hatched region in the wild type Atp12p protein map). The amino-terminal boundary of the functional domain in Atp12p is estimated to be 30 amino acids long (Met-1 through Leu-30) on the basis of the relative migrations of the precursor and mature forms of Atp12p in SDS gels (18). Two domains were mapped in the mature protein using deletion mutants of Atp12p (for reference, Fig. 8 also includes schematic protein maps for the Atp12p deletion mutants and provides a summary of the characteristics of these proteins). The sequence between Gln-181 and Val-306 constitutes the functional domain of the protein (see hatched region in the wild type Atp12p protein map). The amino-terminal boundary of the functional domain in Atp12p is estimated to be 30 amino acids long (Met-1 through Leu-30) on the basis of the relative migrations of the precursor and mature forms of Atp12p in SDS gels (18). Two domains were mapped in the mature protein using deletion mutants of Atp12p (for reference, Fig. 8 also includes schematic protein maps for the Atp12p deletion mutants and provides a summary of the characteristics of these proteins).
For details). The shown in the preparation of (6x)histidine-tagged Atp12p produced in homo- or hetero-oligomer provided conflicting results initially. The effective behavior of the protein (see above). Atp12(V306)p to form stable oligomers, likely explains the de- sequence, did not show evidence for interfering with the ability of ordered oligomer in mitochondria (18). Deletions made from the amino terminus and substitutions at Glu-289 in the sequence, did not show evidence for interfering with the ability of Atp12p to oligomerize (see data for Atp12(D1–124)p and the E289K, E289D, E289A, and E289Q proteins in Fig. 6). However, removing as few as 19 amino acids from the carboxyl terminus yields a mutant protein (Atp12(V306)p) that sediments like a monomer (Fig. 6). On this basis the carboxy-terminal sequence between Asp-307 and Gln-325 is designated as a discrete domain in Atp12p that is involved in its oligomerization. The loss of this domain, which reduces the ability of Atp12(V306)p to form stable oligomers, likely explains the defective behavior of the protein (see above).

Studies performed to determine if mitochondrial Atp12p is a homo- or hetero-oligomer provided conflicting results initially. A preparation of (6x)histidine-tagged Atp12p produced in E. coli was found to show sedimentation properties similar to the native mitochondrial protein (Fig. 7), which was interpreted to be representative of homo-oligomerization. However, such putative homo-oligomers were not visualized in cross-linking studies performed with the purified recombinant protein. There was also no evidence for intermolecular associations of Atp12p monomers using the yeast two-hybrid system. The ambiguity in the data was resolved by examining the sedimentation properties of a chimera (Fig. 2) between maltose binding protein and Histag-Atp12p. The MBP-Histag-Atp12p fusion protein purified from bacteria was shown to migrate as a monomer in linear sucrose gradients (Fig. 7). The results of this analysis, along with the chemical modification and genetic studies described above, argue against the formation of homo-oligomers of Atp12p. On this basis we suggest that the native form of Atp12p observed in mitochondrial samples is a hetero-oligomer.

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Mutational Studies with Atp12p, a Protein Required for Assembly of the Mitochondrial F$_1$-ATPase in Yeast: IDENTIFICATION OF DOMAINS IMPORTANT FOR Atp12p FUNCTION AND OLIGOMERIZATION

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