Molecular Biology of the Cell
Vol. 7, 307–317, February 1996

Biochemical and Functional Analysis of the *YME1* Gene Product, an ATP and Zinc-dependent Mitochondrial Protease from *S. cerevisiae*

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Submitted July 31, 1995; Accepted November 6, 1995
Monitoring Editor: Thomas D. Fox

Inactivation of *YME1* in yeast causes several distinct phenotypes: an increased rate of DNA escape from mitochondria, temperature-sensitive growth on nonfermentable carbon sources, extremely slow growth when mitochondrial DNA is completely absent from the cell, and altered morphology of the mitochondrial compartment. The protein encoded by *YME1*, Yme1p, contains two highly conserved sequence elements, one implicated in the binding and hydrolysis of ATP, and the second characteristic of active site residues found in neutral, zinc-dependent proteases. Both the putative ATPase and zinc-dependent protease elements are necessary for the function of Yme1p as genes having mutations in critical residues of either of these motifs are unable to suppress any of the phenotypes exhibited by *yme1* deletion strains. Yme1p co-fractionates with proteins associated with the mitochondrial inner membrane, is tightly associated with this membrane, and is oriented with the bulk of the protein facing the matrix. Unassembled subunit II of cytochrome oxidase is stabilized in *yme1* yeast strains. The data support a model in which Yme1p is an ATP and zinc-dependent protease associated with the matrix side of the inner mitochondrial membrane. Subunit II of cytochrome oxidase, when not assembled into a higher order complex, is a likely substrate of Yme1p.

INTRODUCTION

Mutations in the nuclear gene *YME1* result in several phenotypes indicative of a loss of mitochondrial function (Thorsness *et al.*, 1993). These include an increased rate of escape of DNA from mitochondria and its subsequent capture by the nucleus, an inability to grow on nonfermentable carbon sources at 37°, and extremely slow growth when cells contain both a *yme1* nuclear mutation and large deletions of the mitochondrial genome. In addition, cells harboring a mutation in *YME1* contain punctate and grossly swollen mitochondria instead of the normal reticulated network of mitochondrial compartments (Campbell *et al.*, 1994). *YME1* encodes a protein, Yme1p, that is localized to mitochondria, has a molecular mass of 82 kDa, and contains two conserved sequence elements; the first is implicated in the binding and hydrolysis of ATP, and the second is homologous to the active site residues of neutral, zinc-dependent protease (Thorsness *et al.*, 1993; Campbell *et al.*, 1994). Yme1p is closely related to the *Escherichia coli* protein FtsH, which was identified in a screen for cell division mutants (Tomoyasu *et al.*, 1993b). Both Yme1p and FtsH contain a putative ATPase domain and the zinc-dependent protease element. FtsH is an essential inner membrane protein that has been shown to have both ATPase and zinc-dependent proteolytic activities (Tomoyasu *et al.*, 1993a, 1995).

The putative nucleotide binding domain in Yme1p is contained within a block of approximately 200 amino acids that is strongly conserved in a large and growing family of homologous ATPases known as the AAA-protein family. Two genes recently isolated from yeast, *YTA10/AFG3* (Guelin *et al.*, 1994; Schnall *et al.*, 1994) and *YTA12/RCA1* (Schnall *et al.*, 1994; Tzagoloff *et al.*, 1994) encode proteins that are very homologous to Yme1p and include the sequence elements characteristic of the ATPase and zinc-dependent protease domains. Yta10p has been implicated in the degrada-

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Table 1. Yeast strains

Strain	Genotype	Source
PTY44	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 [ρ⁺, TRP1]*	(Thorsness and Fox, 1993)
PTY52	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 [ρ⁺, TRP1]*	(Thorsness *et al.*, 1993)
JM43	*MATα his4-580 trp1-289 leu2-3, 112 ura3-52 [ρ⁺]*	(McEwen *et al.*, 1986)
JM43Δyme1	*MATα his4-580 trp1-289 leu2-3, 112 ura3-52 yme1-Δ1::URA3 [ρ⁺]*	This study
GDIV	*MATα his4-580 trp1-289 leu2-3, 112 ura3-52 cox4-Δ1::LEU2 [ρ⁺]*	(McEwen et al., 1986)
GDIVΔyme1	*MATα his4-580 trp1-289 leu2-3, 112 ura3-52 cox4-Δ1::LEU2 yme1-Δ1::URA3 [ρ⁺]*	This study
W303-1A	*MATa ade2-1 his3-11,15 leu2-3, 112 ura3-1 trp1-1 [ρ⁺]*	(Ackerman and Tzagoloff, 1990)
W303Δyme1	*MATa ade2-1 his3-11,15 leu2-3, 112 ura3-1 trp1-1 yme1-Δ1::URA3 [ρ⁺]*	This study
W303Δatp11	*MATa ade2-1 his3-11,15 leu2-3, 112 ura3-1 trp1-1 atp11::LEU2 [ρ⁺]*	(Ackerman and Tzagoloff, 1990)
W303Δatp11,yme1	*MATa ade2-1 his3-11,15 leu2-3, 112 ura3-1 trp1-1 atp11::LEU2 yme1-Δ1::URA3 [ρ⁺]*	This study
W303Δatp12	*MATa ade2-1 his3-11,15 leu2-3, 112 ura3-1 trp1-1 atp12::LEU2 [ρ⁺]*	(Ackerman and Tzagoloff, 1990)
W303Δatp12,yme1	*MATa ade2-1 his3-11,15 leu2-3, 112 ura3-1 trp1-1 atp12::LEU2 yme1-Δ1::URA3 [ρ⁺]*	This study

tion of polypeptides in the mitochondrial inner membrane (Pajic *et al.*, 1994). Rca1p is involved in the assembly of respiratory chain and ATP synthase complexes (Tzagoloff *et al.*, 1994). In addition, Yme1p is homologous to the yeast proteins Sec18p and Pas1p. Sec18p is required for membrane fusion events in the secretory pathway (Eakle *et al.*, 1988; Wilson *et al.*, 1989) and Pas1p is involved in peroxisome biogenesis (Erdmann *et al.*, 1991). Finally, Yme1p is homologous to Ynt1p, which was isolated as a bypass suppressor of a *YME1* deletion (Campbell *et al.*, 1994). An altered form of Ynt1p, which is homologous to a subunit of the 26S proteosome, can partially compensate for the absence of Yme1p. It has been proposed that subunits of the 26S proteosome to which Ynt1p shows homology may be involved in substrate selection for associated protease activities (Rechsteiner *et al.*, 1993).

To further characterize Yme1p we have carried out a biochemical and functional analysis of this protein. Here, we report the location of Yme1p in the mitochondrial compartment as well as the solubility properties and orientation of this protein in the membrane. Furthermore, we define the importance of both the putative ATPase and zinc-dependent protease elements in the biological function of Yme1p, via site-directed mutagenesis. Finally, we identify a putative substrate of Yme1p, subunit II of cytochrome oxidase. Similar experimental results describing the location of Yme1p in mitochondria and stabilization of subunit II of cytochrome oxidase have recently been reported by others (Nakai *et al.*, 1995; Pearce and Sherman, 1995).

MATERIALS AND METHODS

Strains and Genetic Methods

The *E. coli* strain used for preparation and manipulation of DNA was DH5α [F^- *end hsdR17* (r_k^- m_k^+) *supE44 thi-1 λrecA gyrA96 relA1 Δ(argF-lacZYA) U169 ϕ80 lacZ*ΔM15] and the strain used for transformation after second strand synthesis during site specific mutagenesis was BMH 71–18 mut S *[thi, supE, Δ(lac-proAB), [mutS::Tn10][F', proA+B+, lac I^qZΔM15]*. The yeast strains used in this study are described in Table 1.

Media

E. coli containing plasmids were grown in LB (10 g/l Bacto Tryptone, 10 g/l NaCl, and 5 g/l yeast extract) plus 125 μg/ml of ampicillin. Yeast strains were grown in complete glucose medium (YPD), complete ethanol and glycerol medium (YPEG), complete galactose medium (YPGAL), or minimal glucose medium (SD medium) supplemented with the indicated nutrients (Thorsness and Fox, 1993). Solid media was made by the addition of 15 g/l Bacto Agar. Media components were purchased from Difco (Detroit, MI). Ampicillin and nutrients were obtained from Sigma (St. Louis).

Mitochondrial Fractionation and Detection of Yme1p

Yeast strains were grown on YPEG media to mid log phase at 30°, harvested by centrifugation, and mitochondria were prepared as described (Daum *et al.*, 1982). Mitochondria were resuspended in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (TE) at a protein concentration of 5 mg/ml in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml leupeptin, sonicated on ice with a Branson Sonifier 250 for five 3-s pulses at an output setting of 80%, and sedimented for 60 min at 3500 × *g* at 4° in a Beckman Type 65 rotor (Fullerton, CA). The sonicated supernatant fraction was removed and the pellet containing the submitochondrial particles was resuspended vigorously in TE at a protein concentration of 10 mg/ml. The inner and outer mitochondrial membranes were then separated as described (Daum *et al.*, 1982). Briefly, the submitochondrial particles were layered on a 13 ml linear 30–50% sucrose gradient buffered with TE. Gradient centrifugation was carried out in a Beckman SW-28 rotor at 100,000 × *g* for 18 h and the fractions containing the inner and outer mitochondrial membranes were washed three times in TE and collected each time by centrifugation at 20,000 × *g*. Protein fractions were resolved on a 10% SDS-polyacrylamide gel (Laemmli, 1970), which was then equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 15 min, and electroblotted onto nitrocellulose (Bio-Rad, Richmond, CA) at 12 V for 30 min with a Trans Blot SD cell. Following transfer, the membrane was stained with 0.5% Ponceau S in 5% acetic acid, destained with Tris-buffered saline (TBS), (20 mM Tris, 137 mM NaCl, pH 7.6) and blocked with TBS

containing 5% dried milk for 60 min. The membrane was decorated with polyclonal antibodies directed against Yme1p for 2 h at a dilution of 1:2000 in TBS containing 1% dried milk, 0.1% Tween 20, and subsequently washed several times in the same solution lacking antisera. The membrane was then incubated with a second antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) at a dilution of 1:2000 for 60 min, washed, and immune complexes were identified by chemiluminesence (ECL; Amersham). The purity of the mitochondrial fractions was confirmed by identifying marker proteins with antibodies to the γ-subunit of the F1-ATPase (Atp3p, a gift from M. Douglas, Sigma Aldrich Corp., St. Louis, MO) and Omp45p (a gift from R. Jensen, Johns Hopkins University, Baltimore, MD).

To determine the degree of association of Yme1p with the mitochondrial inner membrane, submitochondrial particles at a final protein concentration of 1 mg/ml were extracted at 4° with one of the following: 1.5 M NaCl, 10 mM Tris (pH 7.5); 100 mM alkaline carbonate; or 0.5% Triton X-100, 10 mM Tris (pH 7.5). Extractions were done in the presence of protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The soluble and insoluble components were separated by centrifugation at 9600 × *g* for 15 min and analyzed by immunoblotting with antibodies directed against Yme1p as described above.

The orientation of Yme1p in the mitochondrial inner membrane was determined essentially as described by Burgess *et al.* (1994). Fifty micrograms of purified mitochondria from strain PTY44 were suspended in 0.6 M mannitol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH7.4) at 10 mg/ml. The outer membrane was disrupted by diluting the suspension of mitochondria with 9 vol of 20 mM HEPES-KOH (pH 7.4) and incubating at 0°C for 30 min. Mitoplasts and intact mitochondria were centrifuged at approximately 16,000 × *g* for 5 min at 4°C and resuspended in 10 mM Tris-HCl, pH 7.4. Intact mitochondria and mitoplasts were digested with proteinase K at a final concentration of 25 μg/ml for 30 min on ice. In parallel, mitoplasts were also digested with proteinase K in the presence of 1% deoxycholate. PMSF was added to all reactions at a final concentration of 1 mM. Digested mitochondria and mitoplasts as well as undigested controls were recovered by centrifugation at 16,000 × *g* for 5 min at 4°C, and subjected to SDS-PAGE. Proteins were analyzed by immunoblotting with antibodies directed against Yme1p, Yme2p, and Atp1p.

Site-directed Mutagenesis of Yme1p

Site directed mutagenesis of residues in the ATPase and zinc-dependent protease domains of Yme1p was carried out using the Altered Sites Mutagenesis System (Promega, Madison, WI). A 3.6-kb *Pvu*II restriction fragment containing the complete wild-type *YME1* gene was isolated from pPT50 (Thorsness *et al.*, 1993), and subcloned into the *Sma*I restriction site of pALTER-1. Plasmid DNA was recovered from DH5α, transformed into JM109, and phagemid DNA was prepared using the helper phage R408. Following mutant strand synthesis, double-stranded DNA was transformed into the *E. coli* strain BMH 71–18 mutS. Total plasmid DNA was isolated, transformed into the bacterial strain DH5α, and ampicillin-resistant colonies were identified. Plasmid DNA was prepared from several ampicillin-resistant colonies and sequenced using the Sanger dideoxy method (Sanger *et al.*, 1977) to confirm the presence of a mutation in either the ATPase domain or the zinc-dependent protease domain. A 4.0-kb *Pvu*II restriction fragment containing the mutagenized *YME1* gene was isolated from pALTER-1 and ligated into the yeast centromeric vector pRS315 (Sikorski and Hieter, 1989) that had been digested with *Pvu*II. These plasmids were named pA6 (lysine 327 of Yme1p changed to arginine), pZ3 (glutamate 541 of Yme1p changed to alanine), and pYME1, which directs expression of wild-type Yme1p. The resulting plasmid DNA was transformed into the yeast strain PTY52 and analyzed phenotypically. Construction of revertants of the site-directed mutant *YME1* genes carried by pA6 and pZ3 was as described above and the corresponding plasmids named pA6R and pZ3R. The mutant and wild-type *YME1* genes carried by pA6, pZ3, and pYME1 were excised by digestion with *Xho*I and *Sac*I. The 3-kb fragments bearing the *YME1* alleles were isolated and ligated to pRS314 vector DNA (Sikorski and Hieter, 1989) that had been digested with *Xho*I and *Sac*I. These plasmids were designated pRS314A6, pRS314Z3, and pRS314YME1. The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The degenerate oligonucleotide used for the construction of the ATPase mutants had the following sequence: +991, 5'-GCC AAC AAA GTT (C, A, or G)TA CCT GTA CC-3', +969. The degenerate oligonucleotide used for the construction of the zinc-dependent protease mutants had the following sequence: +2397, 5'-GGC ATG TCC AGC C(G, C, or A)C GTG GAA AGC-3', +2374. The oligonucleotides used to revert the ATPase and zinc-dependent protease mutants to wild type were +991, 5'-GCC AAC AAA GTT TTA CCT GTA CC-3', +969 and +2397, 5'-GGC ATG TCC AGC CTC GTG GAA AGC-3', +2374 respectively.

In Vivo Labeling of Mitochondrial Translation Products

The in vivo labeling of mitochondrial translation products was performed essentially as described by Nakai *et al.* (1994). Three milliliters of early stationary phase cells (Klett = 275) grown in YPD were harvested, washed once with distilled water, and resuspended in SFS media (2.9 mM $MgCl_2$, 7.3 mM KH_2PO_4, 8.6 mM NaCl, 3.6 mM $CaCl_2$, 18.0 mM NH_4Cl) containing 2% glucose. Cyclohexamide was added to a final concentration of 1 mg/ml and the cell suspension was incubated for 5 min at 30°. Mitochondrial translation products were radio-labeled in the presence of 50 μCi/ml [^{35}S]methionine (>1000 Ci/mmol, Amersham) for 30 min at 30°, washed once in SFS media, and chased in the presence of unlabeled methionine at a final concentration of 10 mM. One-half milliliter of cells was harvested at each time point and trichloroacetic acid was added to a final concentration of 10%. The cells were washed once with distilled water and resuspended in 150 μl water. Cell walls were disrupted by vortexing the cell suspension at maximum speed for 5 min with 300 μl glass beads. After centrifugation, the supernatants were decanted, and proteins were solublized by incubating the disrupted cells in the presence of 2% SDS for 30 min at room temperature. Following brief centrifugation, at 12000 × *g* to remove insoluble material, the supernatants were prepared for electrophoresis and resolved on a 10–20% Laemmli gradient gel (Laemmli, 1970).

Detection of Cox2p in Whole Cell Extracts

The yeast strains TF145 (a *cox2* deletion strain), GDIV-transformed with plasmid pRS314, and GDIVΔyme1, transformed with plasmids pRS314, pRS314YME1, pRS314A6, or pRS314Z3 were grown to stationary phase in 10 ml of minimal glucose media. The cells were collected and protein extracts were made as described (Pearce and Sherman, 1995). Briefly, cells were pelleted, washed with distilled water, and resuspended in 0.5 ml of distilled water. An equal volume of cold 0.4 M NaOH, 1.7% 2-mercaptoethanol was added and the cell suspension was then mixed and incubated on ice for 10 min. Twenty-five microliters of cold 100% trichloroacetic acid was added and the suspension was then mixed and incubated on ice for 10 min. The mixture was then spun in a microfuge for 10 min, and the supernatant was removed. The pellet was washed two times with cold acetone and allowed to dry. The pellet was brought up in 150 μl of loading buffer (4% SDS, 1 mM EDTA, 10% 2-mercaptoethanol, 20% glycerol, 0.15 M Tris-HCl, pH 6.8) and boiled for 5 min. Insoluble material was removed by spinning in a microfuge for 5 min, and the samples were then subjected to SDS-PAGE. Proteins were analyzed by immunoblotting with antibodies directed against Yme1p and Cox2p.

Nucleic Acid Techniques and DNA Sequencing

All manipulations of DNA were performed using standard techniques (Maniatis *et al.*, 1982). Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). DNA sequencing was carried out using plasmid DNA as template prepared by lysis by boiling (Maniatis *et al.*, 1982). Sequencing of double-stranded templates was performed using the nucleotide chain termination method (Sanger *et al.*, 1977) using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH).

RESULTS

Yme1p Is Associated with the Mitochondrial Inner Membrane

Yme1p is a mitochondrial protein (Figure 1A); (Thorsness *et al.*, 1993). To determine the location of Yme1p in the mitochondrial compartment, mitochondria were separated into a soluble fraction containing the components of the inner membrane space and matrix, and an insoluble membrane fraction. The insoluble membrane fraction was then placed over a 30–50% sucrose gradient, resolved into inner and outer membrane fractions, and subjected to immunoblotting using antisera to Yme1p (Thorsness *et al.*, 1993). As shown in Figure 1A, Yme1p co-fractionates with proteins associated with the membrane containing submitochondrial particle fraction (Figure 1A, lane 4), and upon further purification, the protein fraction containing the inner mitochondrial membrane (Figure 1A, lane 6). The Yme1p antisera cross-reacts with a band of slightly lower molecular weight present in mitochondria prepared from both wild-type and *yme1Δ* strains (Figure 1A, lanes 1 and 2). Degradation products of Yme1p can also be detected in mitochondria that have been subjected to freezing (Figure 1A, lanes 2, 4, and 6). Homogeneity of inner and outer mitochondrial membrane fractions was demonstrated by immunoblotting using antibodies to Omp45p, an outer membrane protein (Yaffe *et al.*, 1989) (Figure 1B), and Atp3p, a protein associated with the inner mitochondrial membrane (Paul *et al.*, 1996) (Figure 1C).

Yme1p Is Tightly Associated with the Inner Membrane

To determine the degree of association of Yme1p with the mitochondrial inner membrane, sub-mitochondrial particles were treated with either 1.5 M NaCl, 100 mM alkaline carbonate, or 0.25% Triton X-100. As shown in Figure 2, Yme1p remained associated with the inner membrane fraction in the presence of 1.5 M NaCl and 100 mM alkaline carbonate. As predicted, Atp1p, a component of the F_1 portion of the ATP synthase and loosely associated with the inner membrane (Takeda *et al.*, 1986), was partially soluble in 1.5 M NaCl and largely soluble in 100 mM alkaline carbonate. Aac2p, an integral membrane protein (Lawson and Douglas, 1988), remained associated with the membrane in either salt wash and was partially soluble in 0.25% Triton X-100. Therefore, Yme1p behaves as an integral membrane protein. Unlike the related proteins FtsH (Tomoyasu *et al.*, 1993a), Yta10p (Pajic *et al.*, 1994), and Rca1p (Tzagoloff *et al.*, 1994), which contain two putative transmembrane domains each, Yme1p does not contain an obvious transmembrane domain (Thorsness *et al.*, 1993).

Orientation of Yme1p in the Mitochondrial Inner Membrane

Yme1p may play a role in either the degradation or maturation of mitochondrial proteins, utilizing the putative protease domain in the C-terminal half of the protein. A related protein, Yta10p, believed to be involved in the degradation of mitochondrial proteins, is oriented in the inner mitochondrial membrane with the C-terminal half containing the putative protease domain facing the matrix (Pajic *et al.*, 1994). We have similarly tested the orientation of Yme1p in the inner mitochondrial membrane. Because we had previously generated antibodies to the carboxyl terminal 105 amino acids of Yme1p (Thorsness *et al.*, 1993), it was possible to determine the orientation of the C-terminal domain. Mitoplasts, mitochondria that have had the

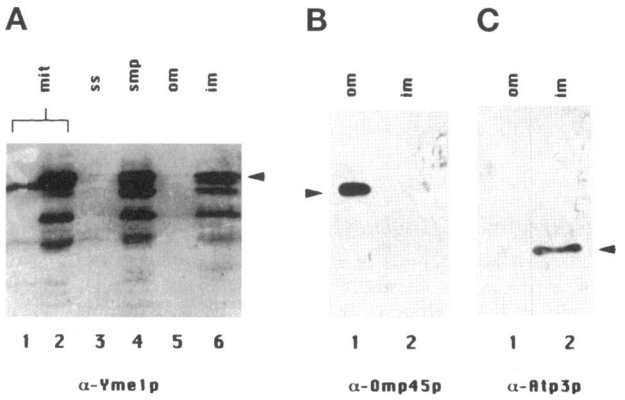

Figure 1. Localization of Yme1p in the mitochondrial compartment by immunoblotting. (A) Total mitochondrial protein was prepared as described from PTY52 (*yme1-Δ1::URA3*, lane 1) and the isogenic wild-type strain PTY44 (mit; lane 2). Mitochondria from PTY44 were further fractionated into the soluble sonicated supernatant (ss; lane 3) and insoluble submitochondrial particles (smp; lane 4). The insoluble submitochondrial particles were separated into two fractions containing the outer membranes (om; lane 5) and inner membranes (im; lane 6). Approximately 20 μg protein from each fraction was resolved in a 10% SDS polyacrylamide gel, blotted, and probed with polyclonal antibodies to Yme1p as described. (B and C) To assay the integrity of the protein fractions, outer membrane (lane 1) and inner membrane (lane 2) fractions were probed with polyclonal antibodies to an exclusively outer membrane protein, Omp45p (B), and an exclusively inner membrane protein, Atp3p (C). Arrowheads mark the positions of Yme1p, Omp45p, and Atp3, respectively.

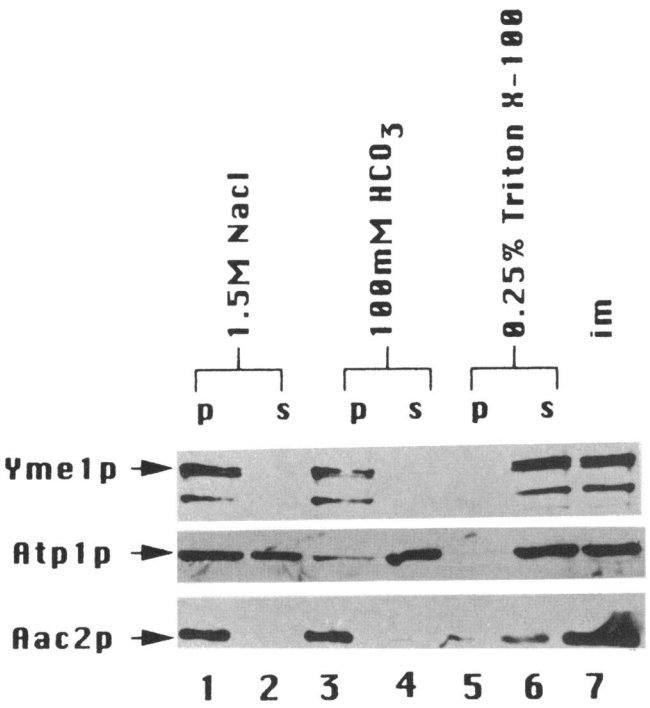

Figure 2. Yme1p is tightly associated with the mitochondrial inner membrane. One hundred micrograms of mitochondrial inner membrane protein fraction (im; lane 7) was extracted with either 1.5 M NaCl, 100 mM alkaline carbonate, or 0.25% Triton X-100 detergent as described. The soluble (s; lanes 2, 4, and 6) and insoluble fractions (p; lanes 1, 3, and 5) were resolved in a 10% Laemmli gel, blotted, and probed with antibodies against Yme1p, Atp1p (nonintegral inner membrane protein), and Aac2p (integral inner membrane protein).

outer membrane disrupted by osmotic shock, when treated with proteinase K did not allow the degradation of Yme1p (Figure 3). Similarly, the α subunit of the mitochondrial ATP synthase (Atp1p), which is localized to the matrix side of the inner mitochondrial membrane (Poyton and Mckemmie, 1979), was also protected from digestion with a protease added to isolated mitochondria or mitoplasts. The amount of both Yme1p and Atp1p were reduced in the mitoplast sample that had been treated with proteinase K, presumably due to disruption of the inner membrane in a fraction of the mitoplasts that subsequently allowed access of the protease to the matrix. Following solubilization of mitoplasts with 1% deoxycholate, both Yme1p and Atp1p were degraded in the presence of protease (Figure 3). Therefore, Yme1p is oriented such that the carboxyl terminus faces the matrix. As the apparent molecular weight of Yme1p did not change in mitoplasts treated with protease, we also conclude that no significant portion of the protein is located in the intermembrane space of mitochondria. Treatment of mitoplasts with proteinase K resulted in the digestion of Yme2p, an inner mitochondrial membrane protein (Hanekamp and Thorsness, 1995) that projects into the intermembrane space (Figure 3).

Site-directed Mutagenesis of Yme1p

Comparison of Yme1p sequence with protein databases reveals two hallmark sequence motifs (Thorsness *et al.* 1993; Campbell *et al.* 1994). Yme1p contains a highly conserved potential nucleotide binding domain spanning the region between residues 316 and 328 (G-V-L-L-T-G-P-P-G-T-G-K-T), a sequence element that is important for activity of homologous proteins (Ohana *et al.* 1993; Akiyama *et al.*, 1994). Alignment of the amino acid sequence of Yme1p with known neutral zinc-dependent proteases has led to the identification of sequences in Yme1p that are identical to those essential for zinc binding and catalytic activity (Vallee and Auld, 1990; Medina *et al.*, 1991). A short segment of Yme1p encompassing residues 540–546 (H-E-A-G-H-A-I) may supply two of the zinc ligands, histidine-540 and histidine-546. These conserved histidine residues are found in many metaloproteases including thermolysin, elastase of *Pseudomonas aeuruginosa*, and human amino peptidase N (Vallee and Auld, 1990). The glutamate residue corresponding to glutamate-541 of Yme1p and adjacent to the first coordinating histidine residue in these proteases is invariant, suggesting a role in enzymatic function.

To examine the importance of both the putative ATP binding domain and the zinc-dependent protease element in the biological function of Yme1p, the effects of mutations in these regions of the protein were analyzed. Degenerate oligonucleotides were used to convert the conserved lysine residue in the putative nu-

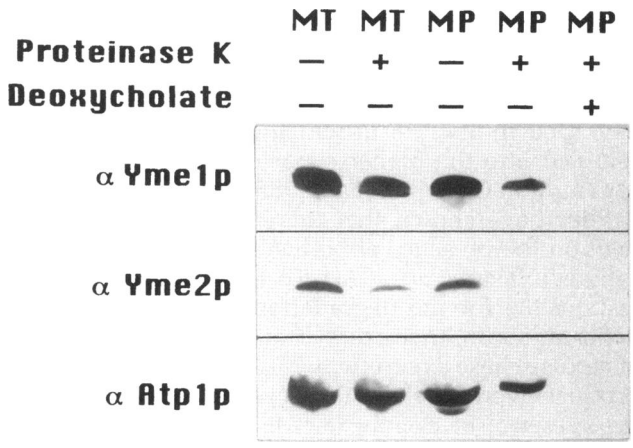

Figure 3. Yme1p is associated with the matrix side of the inner mitochondrial membrane. Mitochondria (MT) and mitoplasts (MP) were incubated with (+) and without (−) proteinase K, and either in the presence (+) or absence (−) of the detergent deoxycholate. Reaction mixtures were then subjected to SDS-PAGE, blotted, and probed with antibodies to Yme1p, Yme2p, and Atp1p.

cleotide binding element (residue 327) to either an arginine, threonine, or isoleucine, and mutations in the zinc-dependent protease element were introduced at the invariant glutamate (residue 541) to convert it to either a glycine, alanine, or valine. These introduced mutations were confirmed by DNA sequencing. The mutant derivatives of *YME1* were subcloned into a centromeric plasmid that was introduced into a strain harboring a null allele of *YME1*, and the resulting strains were scored for the four phenotypes associated with either the original *yme1–1* point mutant or a null allele of *YME1*. Expression of the mutant forms of Yme1p was confirmed by immunoblotting.

As shown in Figure 4, mutations that altered conserved residues in either the ATPase domain or the zinc-dependent protease domain abolished the function of the protein. All six point mutants displayed identical phenotypes when expressed in a *yme1* deletion background, although data for only two representative mutants are shown (lysine 327 changed to arginine and glutamate 541 changed to alanine). Strains expressing the *yme1* site-specific mutations, like the *yme1* deletion mutant strain, showed a high rate of DNA escape from mitochondria (Figure 4A), temperature-sensitive growth on fermentable carbon sources (Figure 4B), cold-sensitive growth on rich glucose media (Figure 4B), and an extremely slow growth when mitochondrial DNA was completely absent from the cell (Figure 4C). Furthermore, reversion of both the ATPase and zinc-dependent protease domain mutants to their wild-type forms by a second round of site-directed mutagenesis restored wild-type Yme1p function. This confirmed that the phenotypes observed in each of the site-directed mutants was due to a single amino acid change.

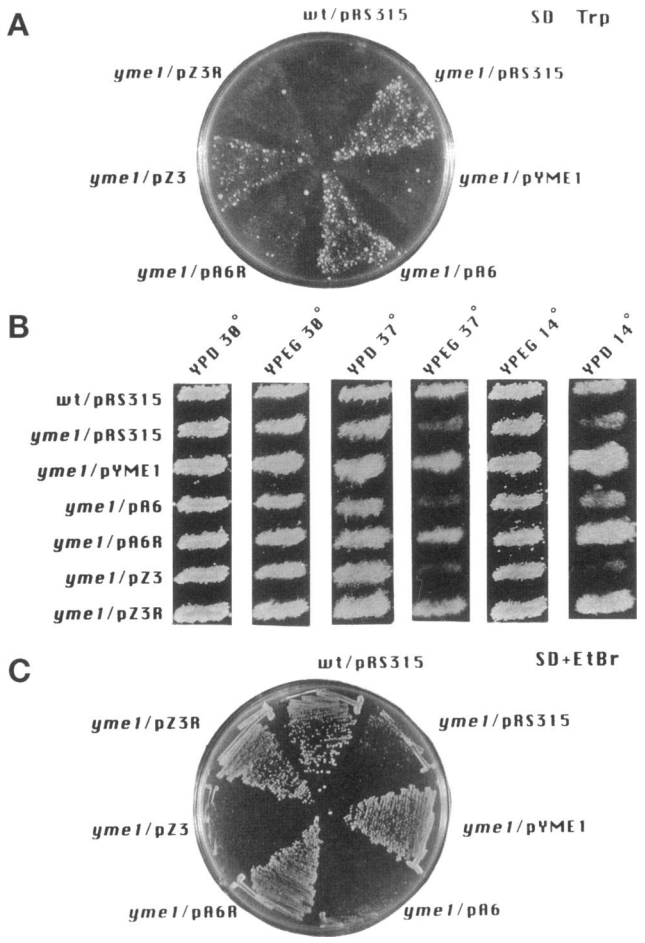

Figure 4. Phenotypic analysis of in vitro generated, site-directed *yme1* mutants. Site-directed mutations in *YME1* were generated as described. Plasmid pA6 carries a copy of *YME1* that has had a critical lysine of the ATPase motif (lysine 327) changed to an arginine. Plasmid pZ3 carries a copy of *YME1* that has had a critical glutamate in the zinc-dependent protease motif (glutamate 541) changed to an alanine. Plasmids pA6R and pZ3R contain the wild-type *YME1* allele generated by back mutation of plasmids pA6 and pZ3, respectively. The wild-type strain (wt) is PTY44 and the *yme1* mutant strain (*yme1*) is PTY52. (A) Each of the mutant plasmids (*yme1*/pA6 and *yme1*/pZ3) and in vitro–generated revertants (*yme1*/pA6R and *yme1*/pZ3R) carried by a *yme1* mutant strain were plated onto minimal media containing tryptophan. A wild-type strain harboring the plasmid without insert (wt/pRS315), a *yme1* mutant strain harboring a plasmid without insert (*yme1*/pRS315), or a *yme1* mutant strain containing a complementing plasmid (*yme1*/pYME1) were also plated as controls. The colonies were replica plated to media lacking tryptophan and after 3 days of incubation at 30°, they were scored for the appearance of Trp^+ papillae to assay the escape of DNA containing the *TRP1* gene from mitochondria (Thorsness and Fox, 1993). (B) To score for both the temperature-sensitive growth on nonfermentable carbon sources and the cold-sensitive growth on rich glucose media, each strain was grown on selective media and replica plated to YPD and YPEG plates that were incubated at 14°, 30°, or 37°. (C) Each of the seven strains was streaked onto a selective plate containing 25 μg/ml ethidium bromide and allowed to grow for 2 days at 30°. A single colony from each strain was then streaked to the same media and incubated for 3 days at 30°. Culturing yeast in the presence of ethidium bromide induces the rapid loss of mitochondrial DNA (Slonimski *et al.*, 1968).

Turnover of Mitochondrially Encoded Subunit II of Cytochrome C Oxidase

Subunit II of cytochrome *c* oxidase (Cox2p) is rapidly degraded in a strain in which this protein is not assembled into the higher order complex, such as in a *cox4* mutant (Dowhan *et al.*, 1985). Furthermore, recent studies have shown that this degradation is dependent on the presence of both ATP and divalent metal cations (Nakai *et al.*, 1994). To test whether Yme1p is responsible for the degradation of Cox2p in a *cox4*-deficient strain, the turnover of Cox2p was examined in a pulse chase experiment. Mitochondrial translation products were labeled in vivo with [^{35}S]methionine in the presence of cyclohexamide, and chased with an excess of unlabeled methionine for up to 4 h. In these experiments, Cox2p was stable in both the wild-type strain and the isogenic *yme1* deletion strain (Figure 5A). After a 60-min chase, greater than 70% of the original Cox2p remained in these strains. In contrast, in the *cox4*-deficient strain the level of Cox2p was

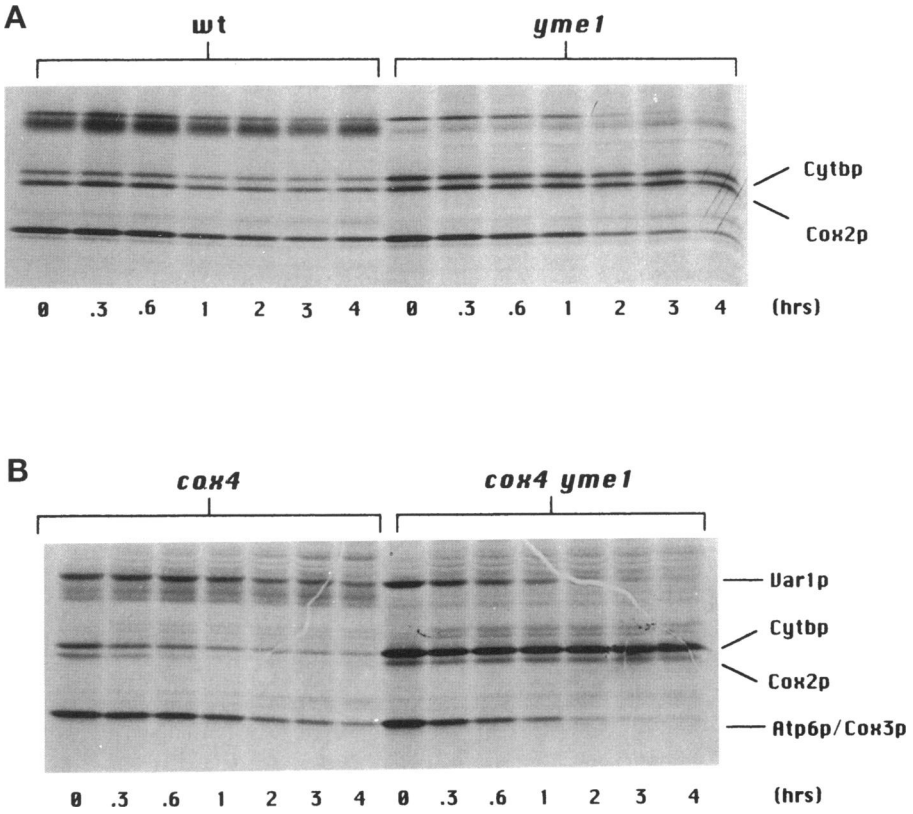

Figure 5. Pulse chase analysis of mitochondrial translation products. Mitochondrial translation products were labeled with [^{35}S]methionine in the presence of cyclohexamide. Following the removal of labeled methionine, cultures were sampled at the indicated times during a 4-h chase with unlabeled methionine. (A) Pulse chase analysis of the wild-type strain JM43 (wt) and the isogenic *yme1* deletion strain, JM43Δyme1 (*yme1*). (B) Pulse chase analysis of GDIV, a *cox4* null mutant strain isogenic to JM43 (*cox4*), and an isogenic strain, GDIVΔyme1, that contains a disruption of *YME1* (*cox4 yme1*).

reduced to approximately 8% of the original level after a 60-min chase (Figure 5B). Cox2p was stabilized in the *cox4 yme1* double mutant, such that the level of protein was greater than 90% of the original level after a 60-min chase (Figure 5B). The half-life of Cox2p in *cox4* strain was estimated to be 20 min, compared with 420 min in the *cox4 yme1* double mutant. In contrast to the changes seen in the level of Cox2p, the levels of Var1p and Atp6p remained fairly constant after 60 min in all of the strains.

The accumulation of unassembled Cox2p was examined in strains lacking Yme1p, or containing either wild-type Yme1p or one of the Yme1p point mutants that affect the ATP-binding motif or the zinc-protease motif (Figure 6). Utilizing antisera directed against Cox2p, it was observed that accumulation of unassembled Cox2p occurred in strains that either lacked Yme1p or expressed the point mutant forms of Yme1p that had been previously observed to be unable to complement a *yme1*Δ mutation (Figure 6, lanes 3, 5, and 6). Strains containing functional Yme1p, expressed either from the chromosomal locus (Figure 6, lane 2) or a plasmid (Figure 6, lane 4), did not accumulate Cox2p. Expression of the wild-type and mutant forms of Yme1p was confirmed by immunoblotting. Thus, the turnover of Cox2p that has not been assembled into a higher-order complex is dependent upon Yme1p having intact ATP-binding and zinc-protease motifs.

Degradation of Atp3p

Recently, we have shown that mutations in the gamma subunit of the mitochondrial ATP synthase, Atp3p, suppress the poor growth phenotype associated with *yme1* strains that lack mitochondrial DNA (Weber *et al.*, 1996). Based on this result, we examined whether Atp3p is also a substrate for Yme1p. We observed that, as for Cox2p, which is degraded in a mutant strain that is defective in assembly of the cytochrome oxidase complex, Atp3p is degraded in an *atp11* or *atp12* mutant strain (Ackerman and Tzagoloff, 1990; Bowman *et al.*, 1991) in which the F_1 portion of the ATP synthase is not assembled (Figure 7, lanes 3

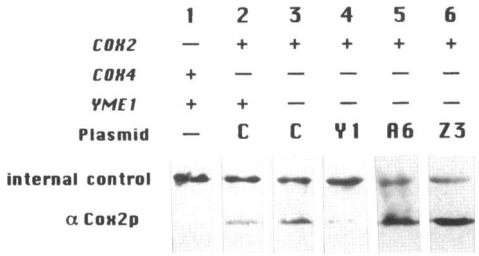

Figure 6. Accumulation of unassembled Cox2p in yeast expressing wild-type and mutant Yme1p. Whole cell protein extracts were subjected to SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with antisera directed against Cox2p. Lane 1, TF145 (a *cox2*Δ strain); lane 2, GDIV containing the vector control plasmid pRS314 (C); lane 3, GDIVΔyme1 containing the vector control plasmid pRS314 (C); lane 4, GDIVΔyme1 containing plasmid pRS314YME1 (Y1); lane 5, GDIVΔyme1 containing plasmid pRS314A6 (A6); and lane 6, GDIVΔyme1 containing plasmid pRS314Z3 (Z3). Genotypes of COX2, *COX4*, and *YME1* are indicated; "+" denotes a wild-type chromosomal allele and "−" denotes deletion of the chromosomal locus. The bands corresponding to Cox2p (αCox2p) and a cross-reacting band (internal control) are indicated.

and 5). However, we were unable to detect any stabilization of Atp3p in either an *atp11 yme1* or *atp12 yme1* double mutant (Figure 7, lanes 4 and 6).

DISCUSSION

Recently, several nuclear-encoded gene products have been identified that are involved in the turnover of mitochondrial proteins (Nunnari *et al.*, 1993; Pajic *et al.*, 1994). Our studies have focused on Yme1p, a putative ATP and zinc-dependent protease that is localized in mitochondria (Thorsness *et al.*, 1993). Mutations in *YME1* result in several growth defects as well as defects in mitochondrial morphology that are indicative of an impairment of mitochondrial function (Thorsness *et al.*, 1993). In this study, we have demonstrated that Yme1p is tightly associated with the inner mitochondrial membrane and is oriented in such a manner that when mitoplasts are treated with protease, Yme1p remains intact. In addition, we present data showing that mutations in conserved residues of both the ATPase and zinc-dependent protease sequence elements destroy the function of the protein, resulting in all of the phenotypes associated with the original *yme1–1* point mutant or the *yme1* null allele. Finally, we show that the degradation of mitochondrial encoded Cox2p is dependent on Yme1p. The association of Yme1p with the inner mitochondrial membrane (Nakai *et al.*, 1995) and the stabilization of Cox2p in yeast that do not assemble the cytochrome oxidase complex (Nakai *et al.*, 1995; Pearce and Sherman, 1995) has been noted by others.

Consistent with observations made by other groups working with homologous proteins, site-directed mutagenesis of highly conserved residues in both the consensus ATP binding and hydrolysis domain and the zinc-dependent protease domain destroys the function of Yme1p. It has recently been shown that Yta10p, which is highly homologous to Yme1p, Rca1p, and *E. coli* FtsH, and contains both of the conserved protein elements, is involved in the degradation of polypeptides in the mitochondrial inner membrane (Pajic *et al.*, 1994). Furthermore, the same investigators have shown that the proteolytic activity of Yta10p is dependent on ATP and divalent cations. We propose that Yme1p, like Yta10p, FtsH, and Rca1p, is an ATP and zinc-dependent protease. In support of this model for Yme1p function is the observation that in the absence of functional Yme1p, unassembled Cox2p is stabilized. It is likely that Yme1p is directly responsible for the degradation of Cox2p, however, it is possible that Yme1p activates another protease that is responsible for the turnover of Cox2p. The effect of the *yme1* mutation on Cox2p turnover was not apparent in strains that assembled Cox2p into a higher order structure, however, it may be that the small amounts of Cox2p that are unassembled and accumulate in the mutant strain are enough to cause several of the phenotypes observed. Yme1p may have additional substrates that, if not turned over, could contribute to the phenotypes observed in *yme1* strains.

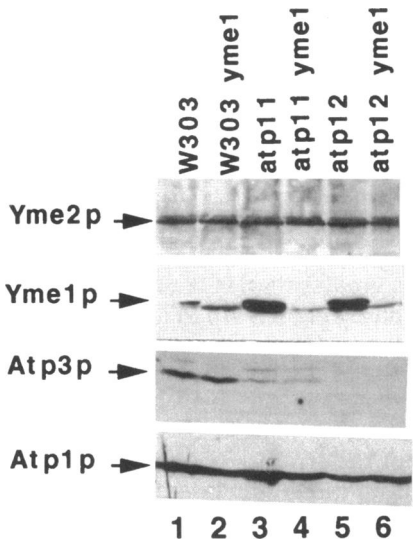

Figure 7. Immunoblot analysis of steady state levels of Atp3p. Yeast strains were grown up to late log phase in YPGAL, harvested, and whole cell protein extracts were prepared as described. Approximately 50 μg of total cell protein from each strain was resolved on a 10% Laemmli gel, blotted, and probed with antibodies to either Yme2p, Yme1p, Atp3p, or Atp1p. Lane 1, W303 (wild type); lane 2, W303 yme1 (*yme1* deletion strain); lane 3, atp11 (*atp11* deletion strain W303Δatp11); lane 4, atp11 yme1 (*atp11 yme1* double mutant strain W303Δatp11,yme1); lane 5, atp12 (*atp12* deletion strain W303Δatp12); and lane 6, atp12 yme1 (*atp12 yme1* double mutant W303Δatp12,yme1).

These results are suggestive of a more general model in which unassembled subunits of the ATP synthase or respiratory chain protein complexes are subject to degradation by a proteolytic system that is associated with the mitochondrial inner membrane. It is likely that Yme1p is a component of such a degradation pathway. Although Yme1p appears to be tightly associated with the inner mitochondrial membrane and behaves like an integral membrane protein, there is only a weak consensus for a single transmembrane domain located between residues 227 and 247. This is in contrast to Yta10p, Rca1p, and *E. coli* FtsH, all of which contain two putative transmembrane domains. One would predict that treatment of mitoplasts with protease would result in a truncated protein if Yme1p completely traversed the inner membrane and the amino terminus was exposed. However, Yme1p remains intact following treatment of mitoplasts with protease. It is likely that Yme1p is tightly associated with the inner membrane in a fashion that does not involve exposure of a significant portion of the protein to the intermembrane space.

Although we have identified one substrate for Yme1p, it is likely that other substrates exist. This is based on the observation made in our laboratory that each of the phenotypes associated with a *yme1* mutant strain can be suppressed individually. For example, we have identified *yme1* strains that are suppressed only for the temperature-sensitive growth phenotype observed on nonfermentable carbon sources (Kominsky and Thorsness, unpublished results), the cold sensitive growth phenotype observed on rich glucose media (Weber and Thorsness, unpublished results), and the poor growth phenotype associated with *yme1* yeast lacking mitochondrial DNA (Weber *et al.*, 1996). Probably the strongest argument in favor of a model in which Yme1p has multiple substrates is based on the observation that *yme1* strains lacking mitochondrial DNA grow very poorly and can be suppressed by mutations in Atp3p. Since Cox2p is mitochondrially encoded, this phenotype cannot be related to the accumulation of Cox2p. Along these lines, we tested the possibility that Atp3p is a substrate for Yme1p. Our analysis revealed that Atp3p was not stabilized in either the *atp11 yme1* or *atp12 yme1* double mutant strains and is therefore probably not a substrate for Yme1p. Yme1p may thus be acting on some other gene product and suppression of the slow growth phenotype by mutations in *ATP3* may be occurring via an indirect mechanism.

In light of the observation that Yme1p is likely an ATP and zinc-dependent protease, it is interesting to speculate about how the accumulation of unassembled subunits of the electron transport chain could result in the phenotypes observed in a *yme1* mutant strain. The accumulation of incompletely translated or unassembled subunits on the mitochondrial inner membrane may act as a signal for the turnover or autophagy of defective mitochondrial compartments by the vacuole. Autophagy of mitochondrial compartments by the vacuole may lead to the escape of mitochondrial DNA and subsequent migration to the nucleus. Autophagy of mitochondria by the vacuole in response to nutritional stress has been reported (Takeshige *et al.*, 1992). It is also possible that accumulation of unassembled protein subunits on the inner membrane compromises the integrity of this membrane, resulting in the escape of mitochondrial DNA through transient breaches in the membrane. The temperature-sensitive growth phenotype observed on nonfermentable carbon sources may also be due to the accumulation of subunits on the mitochondrial inner membrane. For example, the accumulation of incompletely translated or unassembled Cox2p may impair the function of cytochrome oxidase, particularly at elevated temperatures. The slow growth phenotype observed in *yme1* yeast lacking mitochondrial DNA may be due to a decrease in the number of functional mitochondrial compartments, thereby affecting the viability of the cell. By compromising the integrity of the mitochondrial inner membrane, the accumulation of protein subunits may have an indirect affect on the levels of ATP inside the matrix. Subsequent loss of the mitochondrial genome would result in the absence of a functional ATP synthase, which may be acting to compensate for altered ATP levels. It has been shown that ATP inside the matrix is necessary for viability of the cell (Eilers *et al.*, 1987). The poor growth phenotype on rich glucose media at 14° displayed by *yme1* mutant strains may also result from the accumulation of undigested Yme1p substrates. However, it is an affect that is likely to be complicated by the relative differences in growth rates and mitochondrial membrane compositions of yeast growing at sub-optimal temperatures on different media. In addition to displaying growth phenotypes, *yme1* mutant strains harbor mitochondrial compartments that are punctate and grossly swollen in appearance when compared with the reticulated network of mitochondrial compartments seen in wild-type strains of yeast (Campbell *et al.*, 1994). Again, the putative accumulation of protein subunits on the inner membrane in a *yme1* mutant may result in perturbations in the structure and topology of this membrane and thus affect the organization of the entire mitochondrial compartment.

In this report we have presented both a biochemical and functional analysis of Yme1p. Further biochemical analysis of Yme1p as well as genetic analysis will provide more insight into the exact role of Yme1p in the turnover of mitochondrial proteins. This information will lead to a better understanding of the role that protein turnover may play in regulating both the assembly of the complexes of the electron transport chain and mitochondrial function.

ACKNOWLEDGMENTS

We thank Robert Jensen, Michael Douglas, Sharon Ackerman, and Robert Poyton for providing us with antisera, yeast strains, and helpful discussions. This work was supported by a United States Public Health Service grant (GM47390) and a Junior Faculty Research Award from the American Cancer Society (JFRA-404) to P.E.T.

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