Genetic and Biochemical Basis for Viability of Yeast Lacking Mitochondrial Genomes

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ABSTRACT

Yme1p, an ATP-dependent protease localized in the mitochondrial inner membrane, is required for the growth of yeast lacking an intact mitochondrial genome. Specific dominant mutations in the genes encoding the α- and γ-subunits of the mitochondrial F_1F_0-ATPase suppress the slow-growth phenotype of yeast that simultaneously lack Yme1p and mitochondrial DNA. F_1F_0-ATPase activity is reduced in yeast lacking Yme1p and is restored in *yme1* strains bearing suppressing mutations in F_1-ATPase structural genes. Mitochondria isolated from *yme1* yeast generated a membrane potential upon the addition of succinate, but unlike mitochondria isolated either from wild-type yeast or from yeast bearing *yme1* and a suppressing mutation, were unable to generate a membrane potential upon the addition of ATP. Nuclear-encoded F_0 subunits accumulate in *yme1* yeast lacking mitochondrial DNA; however, deletion of genes encoding those subunits did not suppress the requirement of *yme1* yeast for intact mitochondrial DNA. In contrast, deletion of *INH1*, which encodes an inhibitor of the F_1F_0-ATPase, partially suppressed the growth defect of *yme1* yeast lacking mitochondrial DNA. We conclude that Yme1p is in part responsible for assuring sufficient F_1F_0-ATPase activity to generate a membrane potential in mitochondria lacking mitochondrial DNA and propose that Yme1p accomplishes this by catalyzing the turnover of protein inhibitors of the F_1F_0-ATPase.

MOST eukaryotic cells require a functional, intact mitochondrial chromosome for viability and are termed "petite negative." An exception is *Saccharomyces cerevisiae*, a petite-positive budding yeast that can grow on fermentable carbon sources if mitochondrial DNA (mtDNA) is partially deleted (ρ^-) or even completely absent (ρ°). Mutation of several different nuclear genes of *S. cerevisiae* creates petite-negative strains, yeast that are unable to grow or that grow very slowly on fermentable media in the absence of mtDNA. *S. cerevisiae* that simultaneously lack a functional mitochondrial ATP/ADP translocator and an intact mitochondrial genome are inviable (Kovacova *et al.* 1968), presumably because there is no ATP in the matrix of mitochondria and thus no electrical potential across the inner mitochondrial membrane. A membrane potential is necessary for the import of proteins into mitochondria (Gasser *et al.* 1982; Schleyer *et al.* 1982), itself an essential process in eukaryotic cells (Baker and Schatz 1991).

Mutational inactivation of the α-, β-, γ-, or δ-subunits of the F_1 portion of the mitochondrial ATP synthase also creates petite-negative strains of *S. cerevisiae* (Weber *et al.* 1995; Giraud and Velours 1997; Chen and Clark-Walker 1999; Kominsky and Thorsness 2000). The absence of the δ-subunit prevents assembly of the F_1 catalytic portion of mitochondrial ATP synthase and, when coupled with deletions or loss of mtDNA, interferes with the generation of an electrical potential across the inner mitochondrial membrane (Giraud and Velours 1997). In the absence of mtDNA, a membrane potential cannot be created by either the action of the electron transport chain or the pumping of protons by the F_1F_0-ATPase. Consequently, an intact mitochondrial ATP synthase is needed to assure sufficient flux of ATP and ADP through the ATP/ADP translocator. This exchange of ATP (−4 electrical charge) for ADP (−3 electrical charge) is necessary to establish a membrane potential to support mitochondrial protein import (Giraud and Velours 1997).

Yme1p is an inner mitochondrial membrane protein with a putative ATP and metal-dependent protease activity (Pearce and Sherman 1995; Leonhard *et al.* 1996; Weber *et al.* 1996). *S. cerevisiae* cells that lack Yme1p display several phenotypes indicative of impaired mitochondrial function (Thorsness *et al.* 1993). Of particular interest for this study is the *yme1* petite-negative phenotype; *yme1* strains grow very slowly when coupled with deletions in, or the complete loss of, mtDNA. Three genes have been identified that, when mutated, are able to suppress this phenotype. *ynt1-1 (RPT3)*, which encodes a subunit of the 26S protease, suppresses all of the *yme1* phenotypes (Campbell *et al.* 1994). Specific dominant mutations in two genes encoding ATP synthase F_1 subunits, *ATP1-75* and *ATP3-1*, suppress the *yme1* petite-negative phenotype (Weber *et al.* 1995; Kominsky and Thorsness 2000). Heterologous expression of *YME1* in the intrinsically petite-negative yeast *Schizosaccharomyces pombe* allows this yeast to grow in the ab-

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TABLE 1

Yeast strains

Strain	Genotype[a]	Source
DKY40	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 atp4::URA3 [ρ$^+$, TRP1]*	This study
DKY44	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 atp7::URA3 [ρ$^+$, TRP1]*	This study
DKY48	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 atp4::URA3 [ρ$^+$, TRP1]*	This study
DKY50	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 atp7::URA3 [ρ$^+$, TRP1]*	This study
PTY44	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 [ρ$^+$, TRP1]*	Thorsness and Fox (1993)
PTY52	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 [ρ$^+$, TRP1]*	Thorsness *et al.* (1993)
PTY93	*MAT***a** *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP1-75 [ρ$^+$, TRP1]*	Weber *et al.* (1995)
PTY109	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 ATP3-1 [ρ$^+$, TRP1]*	Weber *et al.* (1995)
PTY190	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 inh1Δ::kanMX6 [ρ$^+$, TRP1]*	This study
PTY191	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 tim11Δ::kanMX6 [ρ$^+$, TRP1]*	This study
PTY192	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 inh1Δ::kanMX61 tim11Δ::kanMX6 [ρ$^+$, TRP1]*	This study
PTY193	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 inh1Δ::kanMX6 [ρ$^+$, TRP1]*	This study
PTY194	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 tim11Δ::kanMX6 [ρ$^+$, TRP1]*	This study
PTY195	*MAT*α *ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 inh1Δ::kanMX61 tim11Δ::kanMX6 [ρ$^+$, TRP1]*	This study

[a] The mitochondrial genotype is bracketed.

sence of mtDNA (Kominsky and Thorsness 2000). On the basis of these observations, we propose that Yme1p plays a role in the regulation of ATP synthase. The studies presented here more closely examine the biochemical and genetic basis for the *yme1* petite-negative phenotype.

MATERIALS AND METHODS

Strains: The *Escherichia coli* strains used for preparation and manipulation of DNA were DH5α [F$^-$ *end, hsdR17* (r_k^- m_k^+), *supE44*, *thi-1*, λ*recA*, *gyrA96,relA1*, Δ(*argF-lacZYA*) *U169*, ϕ*80*, *lacZ*ΔM15] and XL1 Blue [*recA1*, *endA1*, *gyrA96*, *thi-1*, *rsdR17*, *supE44*, *relA1*, *lac*, *(F′ proAB*, *lacI^{q}ZDM15*, Tn*10 (tet′)*)]. The genotypes of *S. cerevisiae* strains used in this study are listed in Table 1. Standard genetic techniques were used to construct and analyze the various yeast strains (Sherman *et al.* 1986).

Media: *E. coli* strains containing plasmids were grown in Luria-Bertani medium (10 g bactotryptone, 10 g NaCl, 5 g yeast extract/liter; Maniatis *et al.* 1982) supplemented with 125 μg/ml of ampicillin. Yeast strains were grown in complete glucose medium (YPD) containing 2% glucose, 2% bacto peptone, and 1% yeast extract; complete ethanol-glycerol medium (YPEG) containing 3% glycerol, 3% ethanol, 2% bacto peptone, 1% yeast extract; or minimal glucose medium (SD) containing 2% glucose, 6.7 g/liter yeast nitrogen base without amino acids (Difco), supplemented with the appropriate nutrients. Nutrients were uracil at 40 mg/liter, adenine at 40 mg/liter, tryptophan at 40 mg/liter, lysine at 60 mg/liter, and leucine at 100 mg/liter. For agar plates, Bactoagar was added at 20 g/liter. Where indicated, ethidium bromide was added at 25 μg/ml (Weber *et al.* 1995) and geneticin was added at a concentration of 300 μg/ml.

Nucleic acid techniques: All manipulations of DNA were performed using standard techniques (Sambrook *et al.* 1989). Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Plasmid DNA was prepared by boiling lysis (Maniatis *et al.* 1982).

Creation of *ATP4*, *ATP7*, *INH1*, and *TIM11* null alleles: The *ATP4* and *ATP7* null mutants were created via one-step gene replacement using constructs, a gift of Jean Velours. *atp4*Δ yeast cells were made using the plasmid pSU3-5 (Paul *et al.* 1989). pSU3-5 was digested with *Eco*RI and *Hin*dIII and the resulting 1.79-kb fragment containing the *atp4*Δ gene disrupted by *URA3* was used to transform PTY44. The resulting strain, DKY40, was tested for its ability to respire and for the presence of the *atp4*Δ mutation using both polymerase chain reaction (PCR) and Western blot analysis. *atp7*Δ yeast cells were made using the *ATP7* null plasmid construct, as described (Norais *et al.* 1991). The plasmid was digested using *Bam*HI and *Hin*dIII and the 3.4-kb fragment containing the *atp7*Δ gene interrupted with *URA3* was used to transform PTY44. The resulting strain, DKY44, was tested for its ability to respire, and the *atp7*Δ mutation was verified using both PCR and Western blot analysis.

Null alleles of *INH1* and *TIM11* were created by homologous gene replacement using DNA fragments generated by PCR *in vitro* as described (Longtine *et al.* 1998). Plasmid pFA-13MyckanMX6 was used as a template for PCR. Oligonucleotides used in the PCR reaction to generate DNA for the disruption of *INH1* were: 5′-CAC GCA TTA CTA CAG CAC ACT TTT ATA CAG TTC CAC AAT ACG GAT CCC CGG GTT AAT TAA-3′ (forward primer) and 5′-CTT CTG CGG AAA CGC ATG ATT ATT TGG TCA TCG AGT CAA TGA ATT CGA GCT CGT TTA AAC-3′ (reverse primer). Oligonucleotides used in the PCR reaction to generate DNA for the disruption of *TIM11* were: 5′-AGG AAG TAT TAT ATC GGA ACA TAA CGT ATA TAG GAA CTA GCT GAG TGA GTC GGA TCC CCG GGT TAA TTAA-3′ (forward primer) and 5′-CAT CTA GCG AAC GAG AAT CCA TCA TAA CTT CGT CAT TCA GTG CGA GCT AAG AAT TCG AGC TCG TTT AAAC-3′ (reverse primer). PCR-generated DNAs were used to transform the yeast strain PTY44. Transformants resistant to geneticin (Sigma Chemical, St. Louis) were putative null alleles of *INH1* or *TIM11* and were verified by PCR.

Isolation of mitochondria and immunodetection of mitochondrial proteins: Mitochondrial isolation was performed essentially as described (Daum *et al.* 1982). Cells were grown in 1 liter of the indicated media for 2 days. For the isolation of ρ° mitochondria, one-half of the 1-liter culture was washed in sterile water, resuspended in 1 liter of synthetic media with ethidium bromide (25 μg/ml), and grown for an additional 2 days. This 2-day time frame typically resulted in >90% of the cells becoming cytoplasmic petites, either ρ$^-$ or ρ°, without

significant accumulation of extragenic suppressors. Yeast cells were collected, treated with zymolyase to create spheroplasts, and broken with a dounce homogenizer. Mitochondria were collected by differential centrifugation and further purified by running the crude mitochondrial fraction through a 20% percoll-density gradient (Yaffe 1991). Mitochondrial yield was determined with the Coomassie protein assay (Pierce, Rockford, IL). Protein fractions were resolved on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Bio-Rad, Richmond, CA) as described previously (Hanekamp and Thorsness 1996). Atp4p and Atp7p were detected using antisera that were a gift from Jean Velours. Atp1p and Atp2p were detected using antisera that were a gift from David Mueller (Lai-Zhang and Mueller 2000). Arg8p was detected using antiserum that was a gift from Thomas Fox (Steele *et al.* 1996). Signals were detected using the enhanced chemiluminescence detection method (Amersham, Buckinghamshire, UK).

Determination of mitochondrial F_1F_0-ATPase activity and mitochondrial membrane potential: ATPase activities were determined using isolated mitochondria essentially as described (Tzagoloff 1979). Studies were performed in parallel, with and without 2 μg/ml oligomycin. Each reaction was performed in triplicate. Five micrograms of mitochondria were incubated at 37° for 12 min. The ATPase activities of ρ° mitochondria (Figure 2B) were determined using material prepared from cells treated with ethidium bromide in batch cultures as described above.

The effect of succinate or ATP addition upon the inner mitochondrial membrane potential was monitored by two different methods. Changes in the membrane potential in response to added succinate (Figure 5) were assayed by examining the potential dependent uptake of the fluorescent dye 3,3′-dipropylthiocarbocyanine iodide (Molecular Probes, Eugene, OR; Yaffe 1991). Fluorescence was monitored using an SLM4800S spectrofluoremeter operating in steady-state mode. Samples were excited at 620 nm and emission was measured at 670 nm. Each reaction was performed using 150 μg of mitochondria in a final volume of 2 ml. At the indicated times, succinate was added to a final concentration of 5 mm, or the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone was added to a final concentration of 0.2 μm. Changes in membrane potential in response to added ATP (Figure 6) were assayed by monitoring the potential dependent quenching of the fluorescent dye rhodamine-123 (Molecular Probes; Giraud and Velours 1997). Fluorescence was monitored using a FluoroMax-2 spectrofluoremeter operating in steady-state mode. Samples were excited at 498 nm and emission was measured at 530 nm. Each reaction was performed using 200 μg of mitochondria in a final volume of 2.5 ml. At the indicated time, ATP was added to a final concentration of 1 mm. A total of 50 ng of valinomycin was subsequently added as indicated. Membrane potential measurements for *yme1* mitochondria were made using ρ° mitochondria prepared from a batch culture treated with ethidium bromide. Membrane potential measurements for wild-type and *yme1 ATP1-75* mitochondria were made using ρ° mitochondria prepared from clonal cell cultures derived from "pure" ρ° strains. All experiments were performed in triplicate for each species of mitochondria.

RESULTS

F_1-ATPase activity is compromised in *yme1* cells: *yme1* yeast grow very slowly in the absence of mtDNA. This phenotype is easily scored by culturing cells in the presence of ethidium bromide, which causes the quantitative loss of mtDNA from cells (Slonimski *et al.* 1968; Fox *et al.* 1991). We identified dominant mutations in two F_1 subunits, *ATP1-75* and *ATP3-1*, that suppress this petite-negative phenotype of *yme1* strains (Figure 1; Weber *et al.* 1995; Kominsky and Thorsness 2000). In light of this observation, we examined F_1F_0-ATPase activity in mitochondria isolated from wild-type, *yme1*, and *yme1* strains bearing suppressors of the petite-negative phenotype. Mitochondria from strains that contained an intact mitochondrial genome (ρ^+) and from strains that lacked mtDNA (ρ°) were assayed in the presence and absence of oligomycin, an inhibitor of coupled F_1F_0-ATPase activity. As shown in Figure 2A, the mitochondrial ATPase activity in *yme1* ρ^+ cells is 15% lower than that in wild type. In contrast, the suppressed *yme1 ATP1-75* and *yme1 ATP3-1* strains displayed a marked increase in the level of mitochondrial ATPase activity, ~20% higher than that in wild type. Additionally, the total mitochondrial ATPase activity in the suppressed strains was less sensitive to oligomycin. The uncoupled

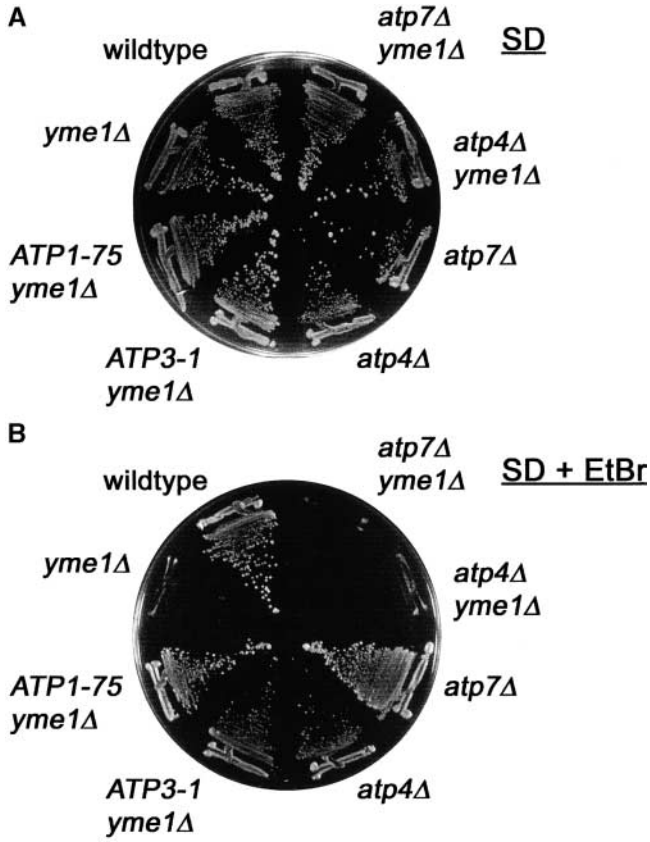


Figure 1.—Suppression of the *yme1* petite-negative phenotype. The indicated strains were streaked onto a synthetic glucose plate either lacking (A) or containing (B) 25 μg/ml ethidium bromide and were incubated for 5 days at 30°. Growth of yeast in the presence of ethidium bromide induces the quantitative loss of mtDNA (Slonimski *et al.* 1968; Fox *et al.* 1991). Strains were the following: *yme1*Δ, PTY52; *ATP1-75 yme1*Δ, PTY93; *ATP3-1 yme1*Δ, PTY109; *atp4*Δ, DKY40; *atp7*Δ, DKY44; *atp4*Δ *yme1*Δ, DKY48; *atp7*Δ *yme1*Δ, DKY50; and wild type, PTY44.

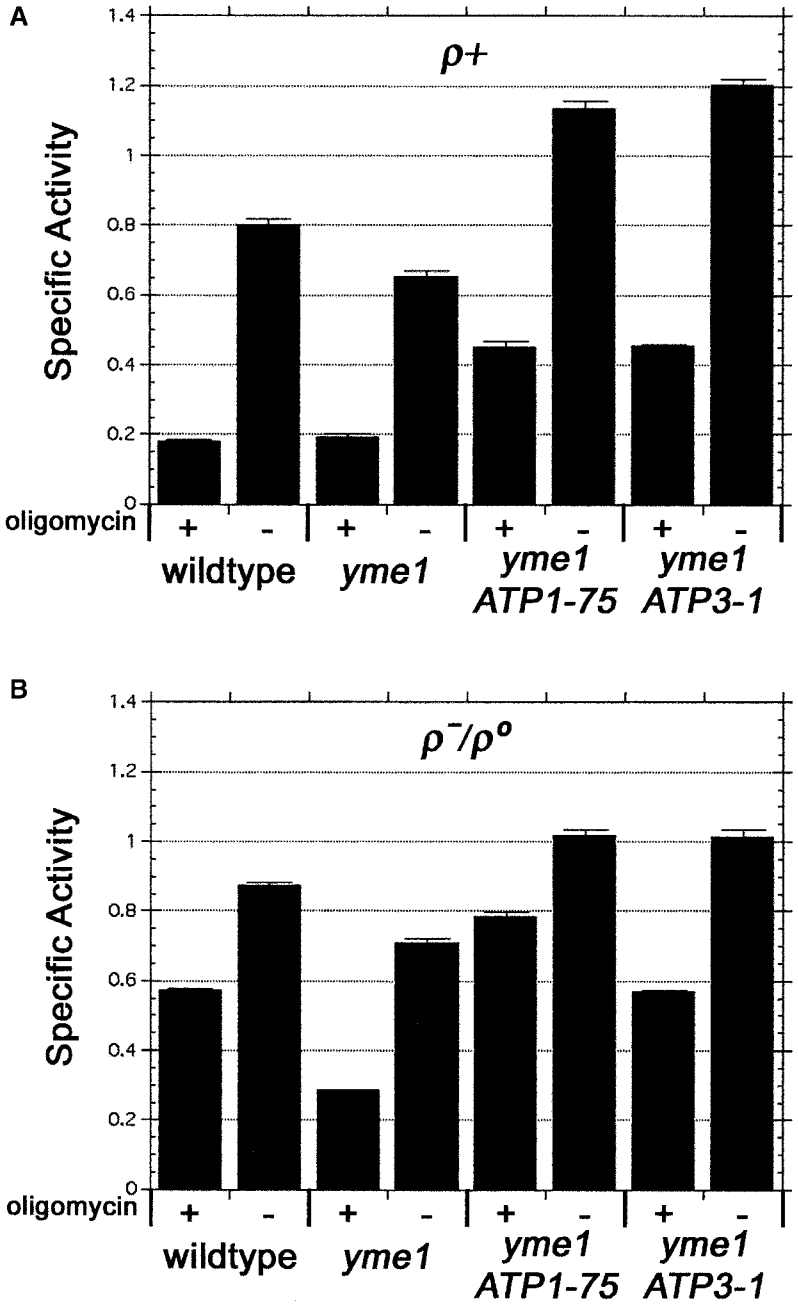
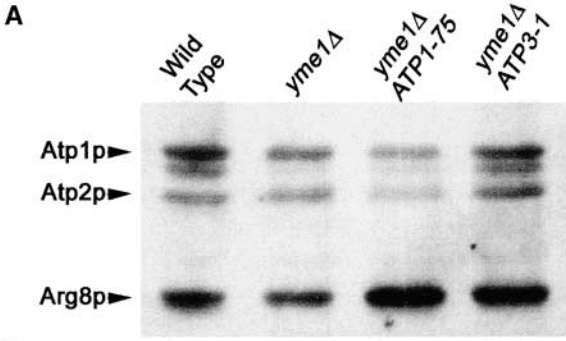


FIGURE 2.—ATPase activity in *yme1* yeast. Five micrograms of ρ^+ (A) or ρ° (B) mitochondria isolated from the indicated strains were assayed in triplicate. Data are means +/− standard error of the mean. Reactions were incubated without (−) or with (+) oligomycin (2 μg/ml) to determine the fraction of inhibited ATPase activity. ATPase specific activity is expressed as [micromoles of P_i per minute per microgram of protein (×1000)]. Strains were the following: *yme1*, PTY52; *yme1 ATP3-1*, PTY109; *yme1 ATP1-75*, PTY93; and wild type, PTY44.

ATPase activity, F_1-ATPase, was twofold greater in *yme1* yeast strains bearing the *ATP1-75* or *ATP3-1* mutations than in the unsuppressed *yme1* strain.

Because *yme1* ρ° yeast do not grow well enough to allow accumulation of ρ° cells for biochemical analysis, we devised a scheme to rapidly induce the loss of mtDNA from ρ^+ cultures of yeast by addition of 25 μg/ml ethidium bromide (MATERIALS AND METHODS). This treatment generated >90% cytoplasmic petites without significant accumulation of extragenic suppressors. We assayed mitochondrial ATPase activity and oligomycin-insensitive F_1-ATPase activity from wild-type, *yme1*, and suppressed *yme1* cytoplasmic petite strains prepared in this manner (Figure 2B). For all strains, total mitochondrial ATPase activity of ρ° cells was essentially unchanged from that of the corresponding ρ^+ cells. The proportion of oligomycin-insensitive F_1-ATPase activity, however, was significantly different in ρ^+ and ρ° cells. Typically, in a homogenous ρ° cell population, mitochondrial ATPase activity is uncoupled and thus oligomycin insensitive due to the absence of a complete F_0 complex. This is largely observed in wild-type ρ° cells prepared from batch ethidium bromide treatment, in which 66% of ATPase activity is oligomycin insensitive, compared to 15% in ρ^+ cells. The remaining oligomycin-sensitive ATPase activity in ρ° wild-type cells likely reflects an incomplete production of ρ° cells (up to 10% of cells were ρ^+ in the ethidium-bromide-treated cultures) and



B

Strain	Relative [Arg8p]	Normalized [Atp1p]	[Atp2p]
Wild Type	100	100	100
yme1Δ	64	88	96
yme1Δ ATP1-75	256	16	14
yme1Δ ATP3-1	232	54	100

FIGURE 3.—Quantitation of Atp1p and Atp2p in wild-type and *yme1* yeast. (A) Immunodetection of mitochondrial proteins. Approximately 15 μg of mitochondria from the indicated strains was resolved on a denaturing 7.5% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with antibodies against the α- and β-subunits of F_1F_0-ATPase (Atp1p and Atp2p, respectively) and Arg8p, a protein found in the mitochondrial matrix. (B) Relative concentration of Atp1p and Atp2p in wild-type and *yme1* yeast. The blot in A was digitized and relative signals quantitated using Molecular Analyst software from Bio-Rad. To control for differences in sample concentration, the relative amount of Atp1p and Atp2p in each lane was normalized to the amount of Arg8p. The wild-type concentrations of Arg8p, Atp1p, and Atp2p were set to 100. Strains: wild type, PTY44; *yme1*, PTY52; *yme1 ATP1-75*, PTY93; and *yme1 ATP3-1*, PTY109.

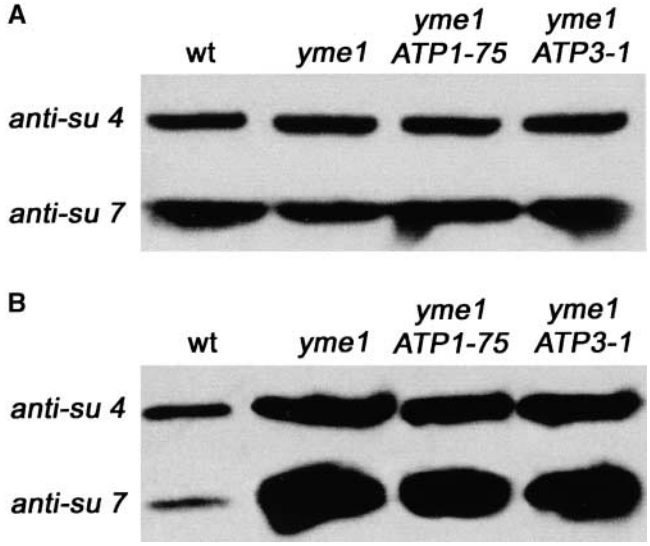


FIGURE 4.—Accumulation of F_0 subunits in *yme1* yeast. Fifteen micrograms of mitochondria from the indicated strains were resolved on a denaturing 12% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with antibodies against Atp4p (anti-su 4) and Atp7p (anti-su 7). (A) ρ^+ mitochondria. (B) ρ° mitochondria. Strains: wt, PTY44; *yme1*, PTY52; *yme1 ATP3-1*, PTY109; and *yme1 ATP1-75*, PTY93.

the presence of residual mitochondrially encoded proteins maintained during outgrowth of the ethidium-bromide-treated cultures. In contrast to wild type, only 40% of ATPase activity in *yme1* ρ° mitochondria is oligomycin insensitive. This suggests that a significant proportion of the F_1 complex is still coupled to F_0 subunits in *yme1* yeast. Mitochondrial ATPase activities in *yme1* ρ° cells bearing suppressing mutations in *ATP1* and *ATP3* are, as in ρ^+ strains, significantly higher than those in wild-type or *yme1* yeast. The majority of ATPase activity in these suppressed *yme1* strains is oligomycin-insensitive F_1-ATPase activity, as was true for wild type.

The decreased F_1F_0-ATPase activity of *yme1* yeast may be the result of decreased levels of the F_1F_0-ATPase complex, increased inhibition of the F_1F_0-ATPase, or a combination of the two. Likewise, the increased F_1F_0-ATPase activity of the suppressed strains (*yme1 ATP1-75* and *yme1 ATP3-1*) may be the result of changes to the protein structure that increase activity, an increase in the accumulation of F_1F_0-ATPase protein, or a combination of the two. Consequently, the amount of F_1F_0-ATPase subunits α (Atp1p) and β (Atp2p) was determined using immunodetection of mitochondrial protein extracts bound to nitrocellulose (Figure 3). To compare the concentration of Atp1p and Atp2p found in each strain, the relative concentration of an unrelated mitochondrial protein, Arg8p, was determined and used to correct for differences in sample concentration (Figure 3B). In ρ^+ cells, *yme1* yeast cells have only a slight reduction in the amount of Atp1p and Atp2, indicating that the basis for the decreased ATPase activity in *yme1* mitochondria is due to inhibition of enzyme activity. In contrast, there was a sixfold decrease in the amount of Atp1p and Atp2p in *yme1Δ ATP1-75* yeast although these cells had 20% more F_1F_0-ATPase activity than wild type had (Figure 2A). Consequently, the turnover number of F_1F_0-ATPase with respect to ATP hydrolysis in the *yme1Δ ATP1-75* strain was increased >16-fold. Similarly, the *yme1Δ ATP3-1* strain exhibited a modest decrease in the relative concentration of Atp1p and consequently a modest increase in the ATPase turnover number, approximately threefold greater than that of wild-type F_1F_0-ATPase.

F_0 subunits accumulate in *yme1* ρ° cells: Previous work demonstrated that neither of the F_1 subunits, Atp3p and Atp1p, is turned over in a Yme1p-dependent manner (WEBER *et al.* 1995). Additionally, the presence of a higher-than-normal proportion of oligomycin-sensitive ATPase activity in *yme1* ρ° yeast suggested that the F_1 complex in those mitochondria might still interact with F_0 subunits. Therefore, we examined the fate of F_0 subunits in *yme1* cells. Immunodetection experiments were performed using polyclonal antibodies directed against

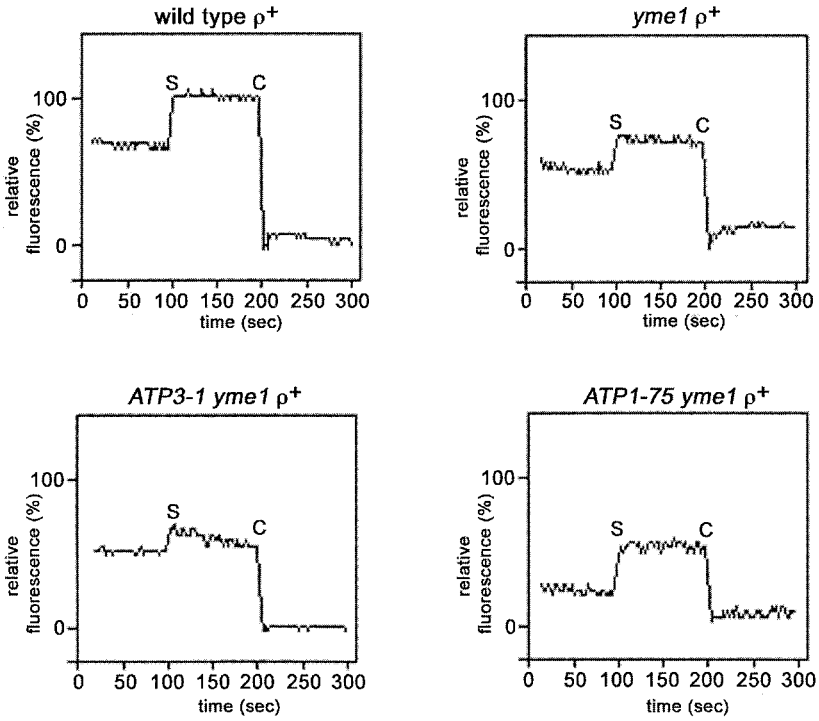


FIGURE 5.—Generation of an inner mitochondrial membrane potential by addition of succinate in ρ^+ yeast. ρ^+ mitochondria were isolated from wild-type, *yme1*, and suppressed *yme1 ATP3-1* and *yme1 ATP1-75* yeast. The potential dependent uptake of 3,3′-dipropylthiocarbocyanine iodide is expressed as percentage of relative fluorescence. The time point of addition of tris-succinate is indicated by S, and the time point of addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone is indicated by C. Strains: wild-type ρ^+, PTY44; *yme1* ρ^+, PTY52; *yme1 ATP3-1* ρ^+, PTY109; and *yme1 ATP1-75* ρ^+, PTY93.

Atp4p and Atp7p, two subunits of the F_0 complex. As shown in Figure 4A, there is no difference in the concentrations of these proteins in ρ^+ mitochondria isolated from wild-type, *yme1*, or suppressed *yme1* yeast. However, both Atp4p and Atp7p accumulate in *yme1* ρ° yeast as well as in the *yme1 ATP1-75* and *yme1 ATP3-1* mutants (Figure 4B). Other researchers have noted the Yme1p-dependent turnover of F_0 subunits 4, 5, 6, and 17 in *oxa1*Δ strains of yeast (LEMAIRE *et al.* 2000).

To determine whether the accumulation of Atp4p or Atp7p was the basis for the abnormally high proportion of oligomycin-sensitive ATPase activity of *yme1* ρ° yeast, we tested whether a null mutation in *ATP4* and/or *ATP7* suppressed the *yme1* petite-negative phenotype. Neither the *atp4*Δ *yme1* and the *atp7*Δ *yme1* double mutants (Figure 1) nor the *atp4*Δ *atp7*Δ *yme1* triple mutant (data not shown) grew in the absence of mtDNA; thus these mutations did not suppress the *yme1* ρ° lethality. The *atp4*Δ and *atp7*Δ mutants alone displayed no phenotype in the absence of mtDNA. It is possible that other F_0 subunits may be involved in *yme1* ρ° lethality, as four additional F_0 subunits are encoded in the nucleus. Alternatively, accumulation of F_0 subunits in *yme1* yeast may be a separate phenomenon from that of the petite-negative phenotype of these cells.

The inner mitochondrial membrane potential is diminished in *yme1* yeast: Inactivation of ATP synthase F_1 subunits coupled with the loss of mtDNA results in a decrease of the inner mitochondrial membrane potential (GIRAUD and VELOURS 1997). Because the petite-negative phenotype of *yme1* yeast can be rescued by mutations in two F_1 proteins, we examined the membrane potential in *yme1* cells. Changes in the membrane potential of mitochondria isolated from ρ^+ yeast in response to the addition of succinate were monitored by measuring the uptake of the fluorescent dye 3,3′-dipropylthiocarbocyanine iodide. These changes were recorded after the addition of a substrate, tris-succinate, and after the addition of an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone. Mitochondria prepared from the *yme1* ρ^+, *yme1 ATP3-1* ρ^+, and *yme1 ATP1-75* ρ^+ mutant strains all exhibit a reduction of membrane potential relative to wild type as judged by the relative change in fluorescence upon addition of the uncoupler (Figure 5).

The ability to generate a membrane potential in response to succinate is dependent upon electron transport, a feature absent in ρ° cells. Instead, the generation of membrane potential in ρ° mitochondria is created by the flux of ATP and ADP through the ATP/ADP translocator (GIRAUD and VELOURS 1997). Consequently, we examined the ability of wild-type and mutant yeast strains to generate a membrane potential using ATP by monitoring fluorescence of rhodamine 123 (Figure 6). Wild-type ρ^+ and ρ° mitochondria generated a membrane potential in response to added ATP, as indicated by the decrease in relative fluorescence (Figure 6A). The membrane potential was destroyed by the addition of the ionophore valinomycin, and the magnitude of the membrane potential can be judged by the relative change in fluorescence that occurred in response to addition of valinomycin. The greater mem-

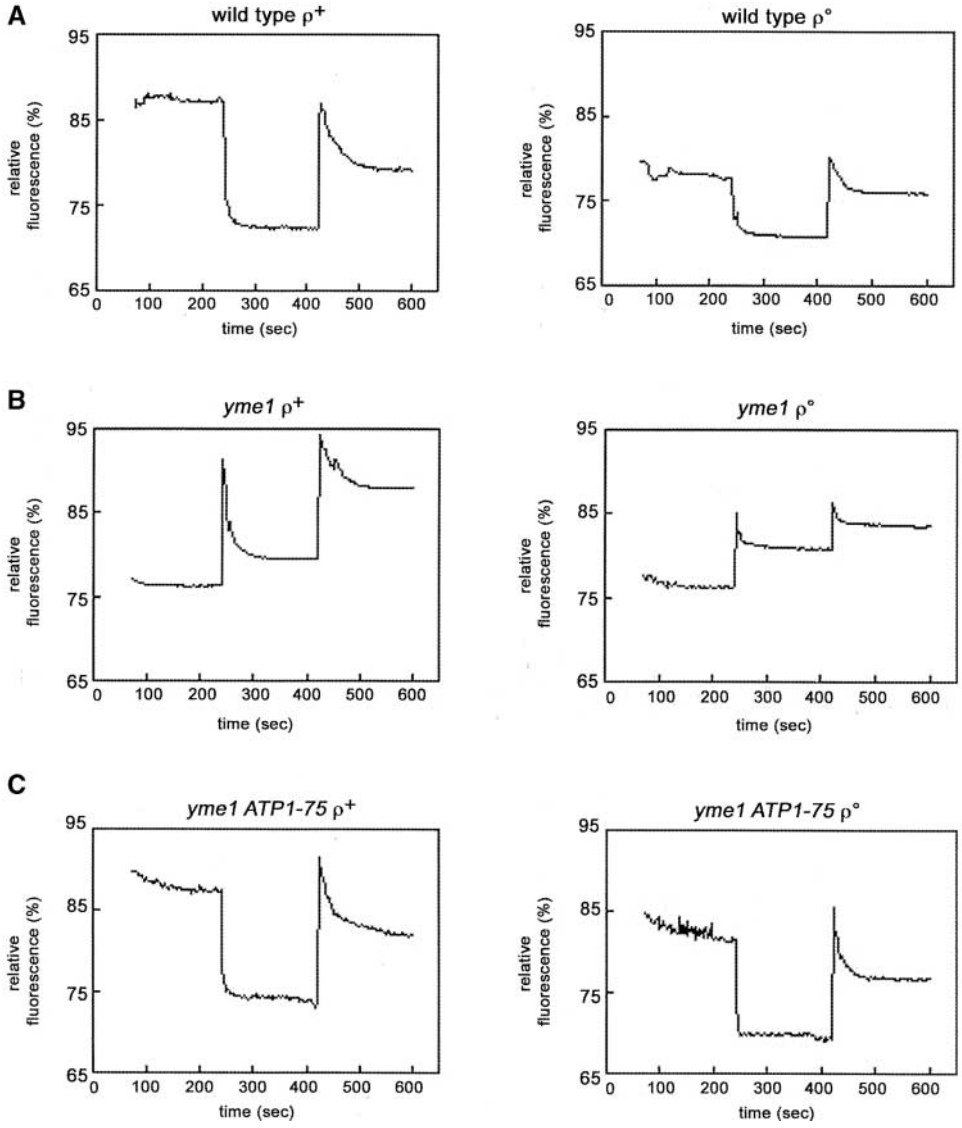


FIGURE 6.—Generation of an inner mitochondrial membrane potential in ρ^+ and ρ° yeast by addition of ATP. Mitochondria were isolated from wild-type, *yme1*, and *yme1 ATP-75* yeast. The ρ° mitochondria prepared from wild-type and *yme1 ATP1-75* strains are quantitatively ρ°. The ρ° mitochondria prepared from the *yme1* strain were generated from a batch culture of ρ^+ cells by treatment with ethidium bromide. The potential dependent quenching of rhodamine 123 fluorescence is expressed as percentage of relative fluorescence. ATP was added at ~240 sec, and the ionophore valinomycin was added at ~420 sec. Strains: wild-type ρ^+, PTY44; wild-type ρ°, PTY44ρ°; *yme1* ρ^+, PTY52; *yme1* ρ°, PTY52ρ°; *yme1 ATP1-75* ρ^+, PTY93; and *yme1 ATP1-75* ρ°, PTY93ρ°.

brane potential in ρ^+ as compared to that of ρ° mitochondria is probably a reflection of both the flux of ATP/ADP through the translocator and the ability to pump protons out of mitochondria upon ATP hydrolysis by the F_1F_0-ATPase. Strikingly, *yme1* mitochondria, whether ρ^+ or ρ°, did not generate a membrane potential in response to the addition of ATP, and the small potential present before the addition of ATP was actually destroyed (Figure 6B) by the addition of ATP. Mitochondria prepared from ρ^+ and ρ° *yme1* strains bearing a suppressing mutation in the α-subunit of ATP synthase (*yme1 ATP1-75* strains) once again generated a membrane potential in response to ATP (Figure 6C). The ρ° mitochondria from wild-type or *yme1 ATP1-75* yeast, whether prepared from clonal ρ° cultures (Figure 6C) or from ρ^+ strains treated with ethidium bromide (data not shown), generated a membrane potential in response to ATP. Hence, the petite-negative phenotype of *yme1* yeast is likely due to the inability of the mitochondria to generate a membrane potential in response to ATP.

Deletion of *INH1* partially suppresses the petite-negative phenotype of *yme1* ρ° cells: Mitochondrial ATPase activity in ρ° cells is necessary for the generation of a membrane potential in mitochondria (GIRAUD and VELOURS 1997). Since a *yme1* strain has low ATPase activity compared to that of wild-type strains and since suppressing mutations of the α- and γ-subunits of F_1-ATPase subunits lead to an increase in ATPase activity, it is possible that an inhibitor of F_1-ATPase accumulates in *yme1* strains. The accumulation of an F_1-ATPase inhibitor might contribute to the ρ° slow-growth phenotype of the *yme1* mutant. Several small peptides encoded in the nucleus of yeast inhibit F_1-ATPase activity. *INH1* encodes an intrinsic F_1F_0-ATPase inhibitor (ICHIKAWA *et al.* 1990; YOSHIDA *et al.* 1990). Inactivation of *INH1* shows no phenotype in otherwise wild-type yeast and has been proposed to inhibit the F_1F_0-ATPase when the F_1 and F_0 portions of the ATPase are uncoupled. Inactivation of *INH1* in a *yme1* background partially complemented the ρ° slow-growth phenotype (Figure 7). Two

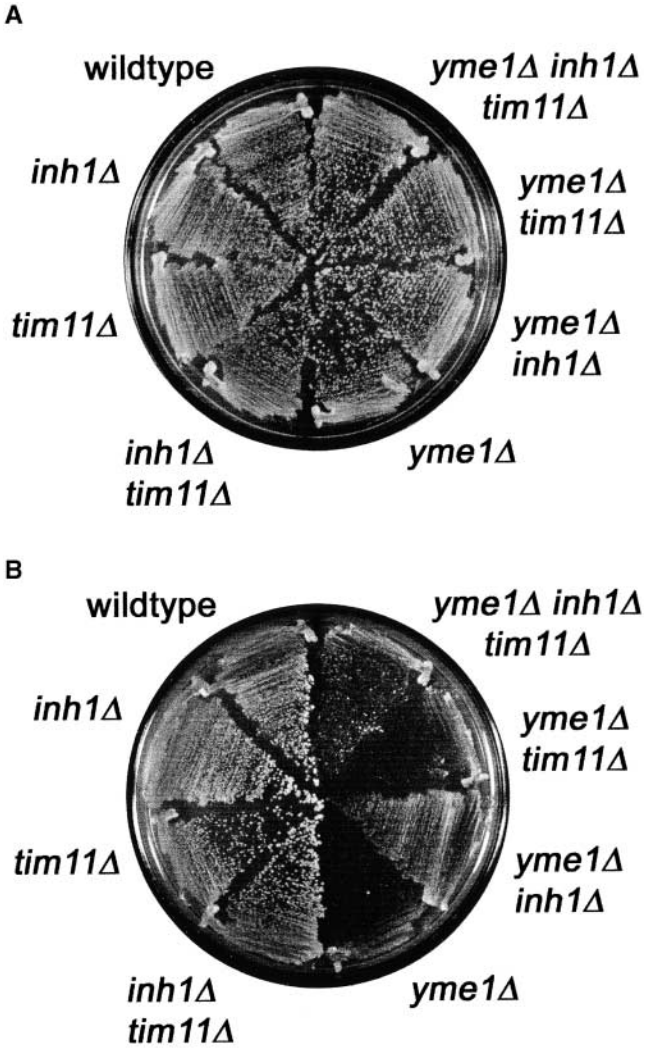


FIGURE 7.—Inactivation of an endogenous F_1F_0-ATPase inhibitor partially suppresses the *yme1* ρ° slow-growth phenotype. The indicated yeast strains were cultured on (A) synthetic glucose media (SD) or (B) synthetic glucose media that contained 25 μg/ml ethidium bromide (SD + EtBr) for 5 days at 30°, creating ρ° strains. Strains: wild type, PTY44; *inh1*Δ, PTY190; *tim11*Δ, PTY191; *inh1*Δ *tim11*Δ, PTY192; *yme1*Δ, PTY52; *yme1*Δ *inh1*Δ, PTY193; *yme1*Δ *tim11*Δ, PTY194; and *yme1*Δ *inh1*Δ *tim11*Δ, PTY195.

other nuclear genes, *TIM11* and *STF1*, also affect F_1F_0-ATPase activity. *TIM11* encodes a protein necessary for the assembly of F_1F_0-ATPase into dimers (ARNOLD *et al.* 1998), and *STF1* encodes a protein with sequence similarity to *INH1* (AKASHI *et al.* 1988). When we tested whether a *TIM11* deletion (Figure 7) or a *STF1* deletion (data not shown) rescued the *yme1* ρ° slow-growth phenotype, neither of these mutations, singly or in combination with each other or with *inh1*Δ, complemented the *yme1* ρ° slow-growth phenotype (Figure 7 and data not shown). Deletion of *INH1*, *TIM11*, or *STF1* did not create a slow-growth phenotype in otherwise wild-type ρ° strains (Figure 7 and data not shown).

DISCUSSION

Previous work has described a role for the F_1 portion of the mitochondrial ATP synthase in maintaining the viability of the yeast *S. cerevisiae* that lack mtDNA. Several mutations that lead to the loss of F_1-ATPase activity cause corresponding decreases in the viability of those mutant strains when they lack mtDNA (WEBER *et al.* 1995; GIRAUD and VELOURS 1997; CHEN and CLARK-WALKER 1999; KOMINSKY and THORSNESS 2000). GIRAUD and VELOURS (1997) proposed that yeast lacking mtDNA require exchange of adenine nucleotides through the inner membrane transporter to generate a membrane potential of adequate magnitude to support the import of proteins and that this exchange largely depends upon the activity of the F_1-ATPase. Import of ATP and its associated electrical charge of −4 into mitochondria is coupled to the export of ADP, which has an electrical charge of −3 (GASSER *et al.* 1982). Consequently, ATP hydrolysis in the mitochondrial matrix and a concomitant exchange of nucleotides across the inner membrane results in the generation of a membrane potential. Similarly, the exchange of ATP and ADP across the inner membrane and the hydrolysis of ATP by the F_1-ATPase are necessary for the generation of the mitochondrial membrane potential in human cells that lack mtDNA (BUCHET and GODINOT 1998; APPLEBY *et al.* 1999). The data presented here support this proposed role for the F_1-ATPase in yeast lacking mtDNA. The defect in *yme1* yeast that leads to an extreme slow-growth phenotype when yeast lack mtDNA (Figure1) is due to an inability to generate a potential across the inner mitochondrial membrane utilizing ATP (Figure 6), presumably as a result of decreased F_1-ATPase activity (Figure 2). Mutations that increase mitochondrial ATP synthase activity (Figure 2) and consequently increase the magnitude of the electrical potential across the inner mitochondrial membrane (Figures 5 and 6) suppress the *yme1* slow-growth phenotype (Figure 1). The dominant mutations that lead to suppression of the *yme1* slow-growth phenotype in ρ° cells map to residues in the α- and γ-subunits of the F_1-ATPase at the interface of the subunits (WEBER *et al.* 1995; KOMINSKY and THORSNESS 2000). These mutations increase the ability of the F_1F_0-ATPase to hydrolyze ATP (Figures 2 and 3), even in the presence of oligomycin or the endogenous peptide inhibitor Inh1p. One surprising result is the complete inability of *yme1* ρ^+ mitochondria to generate a membrane potential in response to ATP (Figure 6B). This may reflect a general defect of the *in organellar* regulation of the F_1F_0-ATPase in *yme1* yeast or even a general defect in the import of ATP into the mitochondrial matrix via the adenine nucleotide transporter. It seems likely that in *yme1* yeast the generation of a membrane potential is dependent upon at least a partially functioning electron transport chain.

Yme1p may be responsible for the proteolytic turnover of a regulator of F_1-ATPase activity, an activity that is particularly important in yeast lacking mtDNA. Inappropriate inhibition of the F_1-ATPase in ρ° cells would lead to impaired function of this complex and reduced electrical potential across the inner mitochondrial membrane. The suppressing mutations in *ATP1* and *ATP3* clearly increase the ATPase activity of F_1F_0-ATPase (Figures 2 and 3), potentially by decreasing the efficacy of an inhibitor. Yme1p controls the accumulation of the F_0 subunits Atp4p and Atp7p in yeast lacking mtDNA (Figure 4), but they are unlikely to be the hypothesized inhibitors of F_1-ATPase, as inactivation of either gene does not suppress the slow-growth phenotype of *yme1* yeast cells that lack mtDNA (Figure 1). However, the inactivation of the F_1F_0-ATPase inhibitor *INH1* partially complements the slow-growth phenotype of *yme1* ρ° strains (Figure 7), which supports a role for Yme1p in regulating F_1-ATPase by affecting the stability of an inhibitor.

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LITERATURE CITED

Akashi, A., Y. Yoshida, H. Nakagoshi, K. Kuroki, T. Hashimoto *et al.*, 1988 Molecular cloning and expression of a gene for a factor which stabilizes formation of inhibitor-mitochondrial ATPase complex from *Saccharomyces cerevisiae*. J. Biochem. **104:** 526–530.

Appleby, R. D., W. K. Porteous, G. Hughes, A. M. James, D. Shannon *et al.*, 1999 Quantitation and origin of the mitochondrial membrane potential in human cells lacking mitochondrial DNA. Eur. J. Biochem. **262:** 108–116.

Arnold, I., K. Pfeiffer, W. Neupert, R. A. Stuart and H. Schagger, 1998 Yeast mitochondrial F1F0-ATP synthase exists as a dimer: identification of three dimer-specific subunits. EMBO J. **17:** 7170–7178.

Baker, K. P., and G. Schatz, 1991 Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature **349:** 205–208.

Buchet, K., and C. Godinot, 1998 Functional F1-ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted rho° cells. J. Biol. Chem. **273:** 22983–22989.

Campbell, C. L., N. Tanaka, K. H. White and P. E. Thorsness, 1994 Mitochondrial morphological and functional defects in yeast caused by *yme1* are suppressed by mutation of a 26S protease subunit homologue. Mol. Biol. Cell **5:** 899–905.

Chen, X. J., and G. D. Clark-Walker, 1999 Alpha and beta subunits of F1-ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **262:** 898–908.

Daum, G., P. C. Böhni and G. Schatz, 1982 Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. J. Biol. Chem. **257:** 13028–13035.

Fox, T. D., L. S. Folley, J. J. Mulero, T. W. McMullin, P. E. Thorsness *et al.*, 1991 Analysis and manipulation of yeast mitochondrial genes. Methods Enzymol. **194:** 149–165.

Gasser, S. M., G. Daum and G. Schatz, 1982 Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. J. Biol. Chem. **257:** 13034–13041.

Giraud, M. F., and J. Velours, 1997 The absence of the mitochondrial ATP synthase delta subunit promotes a slow growth phenotype of rho$^-$ yeast cells by a lack of assembly of the catalytic sector F1. Eur. J. Biochem. **245:** 813–818.

Hanekamp, T., and P. E. Thorsness, 1996 Inactivation of *YME2/RNA12*, which encodes an integral inner mitochondrial membrane protein, causes increased escape of DNA from mitochondria to the nucleus in *Saccharomyces cerevisisae*. Mol. Cell. Biol. **16:** 2764–2771.

Ichikawa, N., Y. Yoshida, T. Hashimoto, N. Ogasawara, H. Yoshikawa *et al.*, 1990 Activation of ATP hydrolysis by an uncoupler in mutant mitochondria lacking an intrinsic ATPase inhibitor in yeast. J. Biol. Chem. **265:** 6274–6278.

Kominsky, D. J., and P. E. Thorsness, 2000 Expression of the *Saccharomyces cerevisiae* gene *YME1* in the petite-negative yeast *Schizosaccharomyces pombe* converts it to a petite-positive yeast. Genetics **154:** 147–154.

Kovacova, V., J. Irmlerova and L. Kovac, 1968 Oxidative phosphorylation in yeast, IV: combination of a nuclear mutation affecting oxidative phosphorylation with cytoplasmic mutation to respiratory deficiency. Biochim. Biophys. Acta **162:** 157–163.

Lai-Zhang, J., and D. M. Mueller, 2000 Complementation of deletion mutants in the genes encoding the F1-ATPase by expression of the corresponding bovine subunits in yeast *S. cerevisiae*. Eur. J. Biochem. **267:** 2409–2418.

Lemaire, C., P. Hamel, J. Velours and G. Dujardin, 2000 Absence of the mitochondrial AAA protease Yme1p restores F0-ATPase subunit accumulation in an *oxa1* deletion mutant of *Saccharomyces cerevisiae*. J. Biol. Chem. **275:** 23471–23475.

Leonhard, K., J. M. Herrmann, R. A. Stuart, G. Mannhaupt, W. Neupert *et al.*, 1996 AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. EMBO J. **15:** 4218–4229.

Longtine, M. S., A. McKenzie, 3rd, D. J. Demarini, N. G. Shah, A. Wach *et al.*, 1998 Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast **14:** 953–961.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1982 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Norais, N., D. Prome and J. Velours, 1991 ATP synthase of yeast mitochondria: characterization of subunit d and sequence analysis of the structural gene *ATP7*. J. Biol. Chem. **266:** 16541–16549.

Paul, M., J. Velours, G. Arselin de Chateaubodeau, M. Aigle and B. Guerin, 1989 The role of subunit 4, a nuclear-encoded protein of the F0 sector of yeast mitochondrial ATP synthase, in the assembly of the whole complex. Eur. J. Biochem. **185:** 163–171.

Pearce, D. A., and F. Sherman, 1995 Degradation of cytochrome oxidase subunits in mutants of yeast lacking cytochrome c and suppression of the degradation by mutation of *yme1*. J. Biol. Chem. **270:** 20879–20882.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schleyer, M., B. Schmidt and W. Neupert, 1982 Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. Eur. J. Biochem. **125:** 109–116.

Sherman, F., G. R. Fink and J. B. Hicks, 1986 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Slonimski, P. P., G. Perrodin and J. H. Croft, 1968 Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal "petites." Biochem. Biophys. Res. Commun. **30:** 232–239.

Steele, D. F., C. A. Butler and T. D. Fox, 1996 Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc. Natl. Acad. Sci. USA **93:** 5253–5257.

Thorsness, P. E., and T. D. Fox, 1993 Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. Genetics **134:** 21–28.

Thorsness, P. E., K. H. White and T. D. Fox, 1993 Inactivation of

YME1, a member of the *ftsH-SEC18–PAS1-CDC48* family of putative ATPase-encoding genes, causes increased escape of DNA from mitochondria in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:** 5418–5426.

Tzagoloff, A., 1979 Oligomycin-sensitive ATPase of *Saccharomyces cerevisiae*. Methods Enzymol. **55:** 351–358.

Weber, E. R., R. S. Rooks, K. S. Shafer, J. W. Chase and P. E. Thorsness, 1995 Mutations in the mitochondrial ATP synthase gamma subunit suppress a slow-growth phenotype of *yme1* yeast lacking mitochondrial DNA. Genetics **140:** 435–442.

Weber, E. R., T. Hanekamp and P. E. Thorsness, 1996 Biochemical and functional analysis of the *YME1* gene product, an ATP and zinc-dependent mitochondrial protease from *S. cerevisiae*. Mol. Biol. Cell **7:** 307–317.

Yaffe, M. P., 1991 Analysis of mitochondrial function and assembly. Methods Enzymol. **194:** 627–643.

Yoshida, Y., T. Sato, T. Hashimoto, N. Ichikawa, S. Nakai *et al.*, 1990 Isolation of a gene for a regulatory 15-kDa subunit of mitochondrial F1F0-ATPase and construction of mutant yeast lacking the protein. Eur. J. Biochem. **192:** 49–53.

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