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 mutations in the genes encoding the α- and γ-subunits of the mitochondrial F₁F₀-ATPase suppress the slow-growth phenotype of yeast that simultaneously lack Yme1p and mitochondrial DNA. F₁F₀-ATPase activity is reduced in yeast lacking Yme1p and is restored in yme1 strains bearing suppressing mutations in F₁-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 F₀ 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 F₁F₀-ATPase, partially suppressed the growth defect of yme1 yeast lacking mitochondrial DNA. We conclude that Yme1p is in part responsible for assuring sufficient F₁F₀-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 F₁F₀-ATPase.

M ost 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 (ρ⁰). 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 translocase 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 F₁ 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 F₁ 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 F₁F₀-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 integral 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 or 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 20S protease, suppresses all of the yme1 phenotypes (Campbell et al. 1994). Specific dominant mutations in two genes encoding ATP synthase F₁ 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-
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− edn, hsdR17 (rK− m−), supE44, thi-1, λ recA, gyrA96, relA1, Δ(argF-lacZYA) U169, φ 80, lacZΔM15) and XL1 Blue (recA1, endA1, gyrA96, thi-1, relA1, supE44, relA1, lac, (F’ proAB, lacZDMD15, Tn10 (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, 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, histidine 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. ATPΔ yeast cells were made using the plasmid pSU3-5 (Paul et al. 1989). pSU3-5 was digested with EcoRI and HindIII and the resulting 1.79-kb fragment containing the ATPΔ 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 ATPΔ mutation using both polymerase chain reaction (PCR) and Western blot analysis. ATPΔ yeast cells were made using the ATP7 null plasmid construct, as described (Noras et al. 1991). The plasmid was digested using BamHI and HindIII and the 3.4-kb fragment containing the ATPΔ gene interrupted by URA3 was used to transform PTY44. The resulting strain, DKY44, was tested for its ability to respire, and the ATPΔ 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 pFA13Myc-kanMX6 was used as a template for PCR. Oligonucleotides used in the PCR reaction to generate DNA for the disruption of INH1 were: 5’-CAG GGA TTA CTA GAG CAT CAC ACT TTG GCC TAG TAT CAC ATG AAC CAA TTC (forward primer) and 5’-CTT CAA CGC AAT GAA CGG ATG TTT TGC GTT GAC TTG TGA AGG TCT (reverse primer). Oligonucleotides used in the PCR reaction to generate DNA for the disruption of TIM11 were: 5’-GTG AGG AAG TAT TAT ATC GGA ACA TAA GGT GCA ATA TAG GAA CTA GGT GAG TGA TGA GGG GTT CCG GGT TAA TTG AAC-3’ (forward primer) and 5’-CTT CAA CGC AAT GAA CGG ATG TTT TGC GTT GAC TTG TGA AGG TCT (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 ρ0 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 ρ0, without

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Source</th>
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<tbody>
<tr>
<td>DKY40 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 atp4::URA3 [p+, TRP1]</td>
<td>This study</td>
</tr>
<tr>
<td>DKY44 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 atp7::URA3 [p+, TRP1]</td>
<td>This study</td>
</tr>
<tr>
<td>DKY48 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::URA3 atp4::URA3 [p+, TRP1]</td>
<td>This study</td>
</tr>
<tr>
<td>DKY50 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::URA3 atp7::URA3 [p+, TRP1]</td>
<td>This study</td>
</tr>
<tr>
<td>PTY52 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::URA3 [p+, TRP1]</td>
<td>Thorsness et al. (1993)</td>
</tr>
<tr>
<td>PTY190 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 inh1Δ::kanMX6 [p+, TRP1]</td>
<td>This study</td>
</tr>
<tr>
<td>PTY191 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 tim11Δ::kanMX6 [p+, TRP1]</td>
<td>This study</td>
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<tr>
<td>PTY192 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 inh1Δ::kanMX6 [p+, TRP1]</td>
<td>This study</td>
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<tr>
<td>PTY193 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::URA3 tim11Δ::kanMX6 [p+, TRP1]</td>
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<tr>
<td>PTY194 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::URA3 tim11Δ::kanMX6 [p+, TRP1]</td>
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<tr>
<td>PTY195 MATa ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1Δ::URA3 inh1Δ::kanMX6 [p+, TRP1]</td>
<td>This study</td>
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* The mitochondrial genotype is bracketed.
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 (Hankew 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_F_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°C 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'-dipropylthiobacocyanine iodide (Molecular Probes, Eugene, OR; Yaffe 1991). Fluorescence was monitored using an SLM 4800S spectrofluorometer 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 mitochondrial protein 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 3-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 spectrofluorometer 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**

**F₁-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 F₁ 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 F₁F₀-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 F₁F₀-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
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 $\rho^+$ yeast do not grow well enough to allow accumulation of $\rho^-$ cells for biochemical analysis, we devised a scheme to rapidly induce the loss of mtDNA from $\rho^+$ cultures of yeast by addition of 25 $\mu$g/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 $\rho^-$ cells was essentially unchanged from that of the corresponding $\rho^+$ cells. The proportion of oligomycin-insensitive F$_1$-ATPase activity, however, was significantly different in $\rho^+$ and $\rho^-$ cells. Typically, in a homogenous $\rho^-$ 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 $\rho^-$ cells prepared from batch ethidium bromide treatment, in which 66\% of ATPase activity is oligomycin insensitive, compared to 15\% in $\rho^+$ cells. The remaining oligomycin-sensitive ATPase activity in $\rho^-$ wild-type cells likely reflects an incomplete production of $\rho^-$ cells (up to 10\% of cells were $\rho^+$ in the ethidium-bromide-treated cultures) and
Mitochondrial Membrane Potential in \( \rho^- \) Yeast

Figure 4.—Accumulation of F\( \text{O} \) subunits in \( \text{yme1} \) 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) \( \rho^+ \) mitochondria. (B) \( \rho^- \) mitochondria. Strains: wt, PTY44; yme1, PTY52; yme1 ATP3-1, PTY109; and yme1 ATP1-75, PTY93.

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<thead>
<tr>
<th>Strain</th>
<th>Relative</th>
<th>Normalized</th>
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<tr>
<td></td>
<td>[Arg8p]</td>
<td>[Atp1p]</td>
</tr>
<tr>
<td>Wild Type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>yme1( \Delta )</td>
<td>64</td>
<td>88</td>
</tr>
<tr>
<td>yme1( \Delta ) ATP1-75</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>yme1( \Delta ) ATP3-1</td>
<td>232</td>
<td>54</td>
</tr>
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The decreased F\( \text{F}_\text{\sigma} \)ATPase activity of \( \text{yme1} \) yeast may be the result of decreased levels of the F\( \text{F}_\text{\sigma} \)ATPase complex, increased inhibition of the F\( \text{F}_\text{\sigma} \)ATPase, or a combination of the two. Likewise, the increased F\( \text{F}_\text{\sigma} \)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\( \text{F}_\text{\sigma} \)ATPase protein, or a combination of the two. Consequently, the amount of F\( \text{F}_\text{\sigma} \)ATPase subunits \( \alpha \) (Atp1p) and \( \beta \) (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 \( \rho^+ \) cells, \( \text{yme1} \) yeast cells have only a slight reduction in the amount of Atp1p and Atp2p, indicating that the basis for the decreased ATPase activity in \( \text{yme1} \) mitochondria is due to inhibition of enzyme activity. In contrast, there was a sixfold decrease in the amount of Atp1p and Atp2p in \( \text{yme1} \) ATP1-75 yeast although these cells had 20% more F\( \text{F}_\text{\sigma} \)ATPase activity than wild type had (Figure 2A). Consequently, the turnover number of F\( \text{F}_\text{\sigma} \)ATPase with respect to ATP hydrolysis in the \( \text{yme1} \) ATP1-75 strain was increased >16-fold. Similarly, the \( \text{yme1} \) 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\( \text{F}_\text{\sigma} \)ATPase.

F\( \text{O} \) subunits accumulate in \( \text{yme1} \) \( \rho^- \) cells: Previous work demonstrated that neither of the F\( \text{I} \) 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 \( \text{yme1} \) \( \rho^- \) yeast suggested that the F\( \text{I} \) complex in those mitochondria might still interact with F\( \text{O} \) subunits. Therefore, we examined the fate of F\( \text{O} \) subunits in \( \text{yme1} \) cells. Immunodetection experiments were performed using polyclonal antibodies directed against
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 p$^+$ mitochondria isolated from wild-type, yme1, or suppressed yme1 yeast. However, both Atp4p and Atp7p accumulate in yme1 p$^-$ yeast as well as in the yme1 ATP3-1 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 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 p$^-$ 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 p$^-$ 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 p$^-$ 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 p$^+$ 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 p$^+$, yme1 ATP3-1 p$^+$, and yme1 ATP1-75 p$^+$ 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 p$^-$ cells. Instead, the generation of membrane potential in p$^-$ 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 p$^+$ and p$^-$ 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-

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**Figure 5.**—Generation of an inner mitochondrial membrane potential by addition of succinate in p$^+$ yeast. p$^-$ 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 p$^+$, PTY44; yme1 p$^+$, PTY52; yme1 ATP3-1 p$^+$, PTY109; and yme1 ATP1-75 p$^+$, PTY93.
brane potential in ρ+ as compared to that of ρ0 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 F1F0-ATPase. Strikingly, yme1 mitochondria, whether ρ+ or ρ0, 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 ρ0 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 ρ0 mitochondria from wild-type or yme1 ATP1-75 yeast, whether prepared from clonal ρ0 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 ρ0 cells: Mitochondrial ATPase activity in ρ0 cells is necessary for the generation of a membrane potential in mitochondria (Giraudo 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 F1-ATPase subunits lead to an increase in ATPase activity, it is possible that an inhibitor of F1-ATPase accumulates in yme1 strains. The accumulation of an F1-ATPase inhibitor might contribute to the ρ0 slow-growth phenotype of the yme1 mutant. Several small peptides encoded in the nucleus of yeast inhibit F1-ATPase activity. INH1 encodes an intrinsic F1F0-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 F1F0-ATPase when the F1 and F0 portions of the ATPase are uncoupled. Inactivation of INH1 in a yme1 background partially complemented the ρ0 slow-growth phenotype (Figure 7). Two
other nuclear genes, TIM11 and STF1, also affect F$_1$F$_0$-ATPase activity. TIM11 encodes a protein necessary for the assembly of F$_1$F$_0$-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 $\rho^+$ slow-growth phenotype, neither of these mutations, singly or in combination with each other or with inh1 $\Delta$, complemented the yme1 $\rho^+$ 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 $\rho^+$ 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$_1$F$_0$-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; Appley 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 (Figure 1) 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 $\rho^+$ cells map to residues in the $\alpha$- and $\gamma$-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$_1$F$_0$-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* $\rho^+$ mitochondria to generate a membrane potential in response to ATP (Figure 6B). This may reflect a general defect of the in organelar regulation of the F$_1$F$_0$-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 $\rho^0$ 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$_1$F$_0$-ATPase (Figures 2 and 3), potentially by decreasing the efficacy of an inhibitor. Yme1p controls the accumulation of the F$_1$ 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$_1$F$_0$-ATPase inhibitor INH1 partially complements the slow-growth phenotype of yme1 $\rho^0$ strains (Figure 7), which supports a role for Yme1p in regulating F$_1$F$_0$-ATPase by affecting the stability of an inhibitor.

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