Mitochondrial intermediate peptidase and the yeast frataxin homolog together maintain mitochondrial iron homeostasis in Saccharomyces cerevisiae

Steven S. Branda, Zhi-yong Yang*, Anne Chew and Grazia Isaya§

Department of Pediatric and Adolescent Medicine, Mayo Clinic and Foundation, 200 First Street SW, Rochester, MN 55905, USA

Received January 22, 1999; Revised and Accepted March 15, 1999

Friedreich’s ataxia (FRDA) is a neurodegenerative disease typically caused by a deficiency of frataxin, a mitochondrial protein of unknown function. In Saccharomyces cerevisiae, lack of the yeast frataxin homolog (YFH1 gene, Yfh1p polypeptide) results in mitochondrial iron accumulation, suggesting that frataxin is required for mitochondrial iron homeostasis and that FRDA results from oxidative damage secondary to mitochondrial iron overload. This hypothesis implies that the effects of frataxin deficiency could be influenced by other proteins involved in mitochondrial iron usage. We show that Yfh1p interacts functionally with yeast mitochondrial intermediate peptidase (OCT1 gene, YMIP polypeptide), a metalloprotease required for maturation of ferrochelatase and other iron-utilizing proteins. YMIP is activated by ferrous iron in vitro and loss of YMIP activity leads to mitochondrial iron depletion, suggesting that YMIP is part of a feedback loop in which iron stimulates maturation of YMIP substrates and this in turn promotes mitochondrial iron uptake. Accordingly, YMIP is active and promotes mitochondrial iron accumulation in a mutant lacking Yfh1p (yfh1Δ), while genetic inactivation of YMIP in this mutant (yfh1Δoct1Δ) leads to a 2-fold reduction in mitochondrial iron levels. Moreover, overexpression of Yfh1p restores mitochondrial iron homeostasis and YMIP activity in a conditional oct1Δ mutant, but does not affect iron levels in a mutant completely lacking YMIP (oct1Δ). Thus, we propose that Yfh1p maintains mitochondrial iron homeostasis both directly, by promoting iron export, and indirectly, by regulating iron levels and therefore YMIP activity, which promotes mitochondrial iron uptake. This suggests that human MIP may contribute to the functional effects of frataxin deficiency and the clinical manifestations of FRDA.

INTRODUCTION

Friedreich’s ataxia (FRDA) is an autosomal recessive disease characterized by the association of progressive gait and limb ataxia, cardiomyopathy and diabetes (1). The FRDA locus (FRDA) has been identified and shown to encode a new mitochondrial protein, designated frataxin (2–6). In most FRDA patients, a GAA trinucleotide repeat expansion in the first intron of FRDA interferes with transcription and results in reduced levels of frataxin (2,5,7–9). Although the functional consequences of frataxin deficiency are not yet clear, insights have been gained through the study of yeast mutants lacking the yeast frataxin homolog (YFH1 gene, Yfh1p polypeptide) (3). Yfh1p-deficient yeast show mitochondrial iron accumulation, hypersensitivity to oxidative stress and loss of mitochondrial DNA (mtDNA) integrity (3,4,10,11). These features suggest that Yfh1p is required for mitochondrial iron homeostasis and that the primary effect of Yfh1p deficiency is mitochondrial iron overload, which increases production of hydroxyl radicals and thereby leads to oxidative damage of mtDNA and mitochondrial enzymes (3,10,12). The fact that iron deposits and multiple iron–sulfur enzyme deficiencies have been found in the myocardium of FRDA patients (13,14) strongly suggests that the function of frataxin is similar to that of Yfh1p and that the degenerative lesions of FRDA result from oxidative damage in the heart and possibly all other tissues affected in this disease (15).

Expansion-positive FRDA patients show considerable variability in age of onset and severity of symptoms, however, and 30–70% of this variance has been attributed to as yet unknown factors that modify the effects of frataxin deficiency (16–20). It is conceivable that such modifiers include mitochondrial proteins that interact with frataxin and that genetic variants of these proteins influence the expression of frataxin deficiency. For instance, recent studies have shown that two mitochondrial proteins, Saccharomyces cerevisiae Ssq1p (21) and rat mitochondrial processing peptidase (MPP) (6), are required for the maturation of Yfh1p and frataxin, respectively, and it seems plausible that human homologs of Ssq1p and MPP may play a role in the clinical variability of FRDA. In this study, we report another interaction with potential implications for FRDA, involving Yfh1p and yeast mitochondrial intermediate peptidase.

*Present address: Cardiovascular Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA
*To whom correspondence should be addressed: Tel: +1 507 266 0110; Fax: +1 507 284 1399; Email: isaya@mayo.edu
(OCT1 gene, YMIP polypeptide). [The open reading frame YKL134C, encoding the yeast mitochondrial intermediate peptidase, has recently been renamed OCT1 (YMIP polypeptide). OCT1 was previously referred to as MIP1 (22), but this name was first assigned to open reading frame YOR330C.] YMIP is a metal-dependent leader peptidase which catalyzes the second of two processing steps required for the maturation of a number of nuclear encoded mitochondrial proteins (22,23). These proteins are translated in the cytoplasm as larger precursors carrying the motif Rx(L)xF/L/I/xS/T/G/xxxx(↓) at the C-terminus of their leader peptide and are imported into the mitochondrial matrix and initially cleaved by MPP two peptide bonds from the R residue in the motif (24,25). This yields a processing intermediate with a characteristic N-terminal octapeptide, which is specifically removed by YMIP to yield the mature protein (26,27). YMIP homologs have been characterized in lower eukaryotes as well as mammals (28–30) and the mature protein (26,27). YMIP homologs have been characterized in lower eukaryotes as well as mammals (28–30) and the mature protein (26,27). YMIP homologs have been characterized in lower eukaryotes as well as mammals (28–30) and the mature protein (26,27). YMIP homologs have been characterized in lower eukaryotes as well as mammals (28–30) and the mature protein (26,27). YMIP homologs have been characterized in lower eukaryotes as well as mammals (28–30) and the mature protein (26,27).

### RESULTS

**High copy YFH1 suppresses oct1° but not oct1Δ yeast**

In previous studies, we found that substitution of a single amino acid within the putative catalytic domain of YMIP (Gly→Leu at position 578; G578L) yielded a temperature-sensitive mutant (oct1°; see Table 1 for strain genotypes) which expresses the YMIP(G578L) protein but is unable to efficiently process YMIP substrates and maintain respiratory function at the restrictive temperature (37°C) (23,31). In the present study, we identified proteins that interact with YMIP by screening for multicopy suppressors of oct1° (i.e. yeast genes that, when overexpressed, suppress the conditional phenotype of oct1°). A fragment of chromosome IV containing the YFH1 gene and two additional open reading frames was isolated twice based on its ability to partially restore growth of oct1° on YPEG (1% yeast extract, 2% peptone, 2% ethanol and 3% glycerol) plates at 37°C (data not shown) and deletion analysis revealed that a multicopy vector carrying only YFH1 (YE-YFH1) was sufficient for suppression (Fig. 1A, lane 4; the suppressed mutant is referred to as oct1°[YFH1] throughout). On the other hand, YFH1 on a centromeric vector did not suppress oct1° (data not shown).

Western analysis showed that oct1°[YFH1] (Fig. 1B, lane 4) contained an ~23 kDa protein corresponding to the mature form of Yfh1p (21) at levels that were much higher than those detected in wild-type cells (wt) (lane 1) and apparently similar to those detected in a wild-type strain carrying YE-YFH1 (wt[YFH1]) (lane 2); however, densitometric analysis of under-exposed fluorograms revealed that oct1°[YFH1] contained

---

**Table 1. Saccharomyces cerevisiae strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH501</td>
<td>MATαURA3-52URA3-52 LYS2-801 &lt;br&gt; ade2-101 &lt;br&gt; OCT1::LEU2</td>
<td>(22)</td>
</tr>
<tr>
<td>Y6041</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>(22)</td>
</tr>
<tr>
<td>oct1Δ</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>(22)</td>
</tr>
<tr>
<td>wt</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>(23,31)</td>
</tr>
<tr>
<td>oct1°</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>(23,31)</td>
</tr>
<tr>
<td>oct1°[YFH1]</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>yfh1Δ</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>yfh1Δ[YFH1]</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>yfh1Δ[oct1Δ]</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>Isogenic g6</td>
<td>MATαURA3-52 LYS2-801 &lt;br&gt; ade2-101-&lt;br&gt; OCT1::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>KL14-4B</td>
<td>MATαauxotroph [p9]</td>
<td>A. Tzagoloff</td>
</tr>
<tr>
<td>CB11</td>
<td>MATαadel1 [p9]</td>
<td>(51)</td>
</tr>
<tr>
<td>M7-40-4D</td>
<td>MATαadel1 [mit (cob1)]</td>
<td>(51)</td>
</tr>
<tr>
<td>M9-3-6C</td>
<td>MATαadel1 [mit (ox2)]</td>
<td>(51)</td>
</tr>
<tr>
<td>M10-15-4D</td>
<td>MATαadel1 [mit (ox3)]</td>
<td>(51)</td>
</tr>
</tbody>
</table>
Yfh1p at levels 2-fold higher than those in wt[YFH1] (data not shown). Importantly, the YE-YFH1 plasmid did not suppress a disruption mutant completely lacking YMIP (oct1Δ): transformation of an oct1Δ/OCT1 diploid with YE-YFH1 prior to sporulation and tetrad dissection (i.e. prior to loss of mtDNA integrity) yielded oct1Δ[YFH1] haploids that overproduced Yfh1p but could not grow on YPEG (data not shown). Thus, the suppression achieved through overexpression of Yfh1p required the presence of YMIP (G578L) and in fact al the levels of this protein in oct1Δ[YFH1] (lane 4) were even higher than those of YMIP in wt and wt[YFH1] (lanes 1 and 2).

To determine whether overexpression of Yfh1p improved YMIP(G578L) activity at the restrictive temperature, we examined by western analysis the processing of three representative YMIP substrates: the enzyme ferrochelatase (HEM15 gene, Hem15p polypeptide), subunit IV of cytochrome c oxidase (COX4 gene, CoxIV polypeptide) and the Rieske iron–sulfur protein of the cytochrome bc1 complex (RIP1 gene, Fe/S polypeptide) (Fig. 1B). We found that oct1Δ[YFH1] (lane 4) contained the intermediate (i) and mature (m) forms of Hem15p in amounts that were comparable with those detected in wt and wt[YFH1] (Fig. 1A, lanes 1 and 2) and actually produced more mCoxIV and accumulated less iCoxIV than did the wild-type strains (Fig. 1A, compare lane 4 with lanes 1 and 2), indicating that these YMIP substrates were processed quite efficiently in oct1Δ[YFH1]. In contrast, overexpression of Yfh1p did not restore processing of iFe/S, as oct1Δ[YFH1] accumulated iFe/S and contained no detectable mFe/S (Fig. 1A, lane 4); trace amounts of mFe/S may have been produced, however, given that oct1Δ[YFH1] was able to grow on non-fermentable carbon sources (Fig. 1A, lane 4). Yfh1p overexpression did not influence the levels or processing of a protein cleaved to the mature form by MPP, the β subunit of F1-ATPase (ATP2 gene, F1β polypeptide) (26, 32; Fig. 1B). Moreover, YE-YFH1 did not suppress a conditional MPP mutant (mif2 ts) (33) or three conditional Fe/S mutants (JPJ1-r101T, JPJ1-r102L and JPJ1-r157S) (34; data not shown), indicating that YFH1 is not a general suppressor of strains with defects in mitochondrial processing or respiratory function, respectively. On the other hand, YE-YFH1 partially suppressed another temperature-sensitive mutant (H565R) (31) which, similar to oct1Δ, carries a point mutation in the catalytic domain of YMIP (data not shown). These observations support the conclusion that YFH1 suppressed oct1Δ through a positive and specific effect on YMIP.

Yfh1p overexpression rescues YMIP(G578L) activity in oct1Δ
Further characterization of the mechanism by which YE-YFH1 suppressed oct1Δ required a comparative analysis of oct1Δ and oct1Δ[YFH1] at the restrictive temperature and, because this could not be accomplished on YPEG plates (Fig. 1A), we

Figure 1. Complementation of oct1Δ by overexpression of Yfh1p. (A) High copy YFH1 restores oct1Δ growth on non-fermentable carbon sources at the restrictive temperature. Yeast strains (see Table 1 for genotypes) were grown on YPEG plates at 30°C for 6 days and then streaked onto fresh YPEG plates at low cell density and grown at 37°C for 10 days. (B) Yfh1p overexpression restores processing of two YMIP substrates in oct1Δ[YFH1]. Total yeast cell extracts were prepared from strains that could grow on YPEG plates at 37°C and analyzed by western blotting. i, intermediate form of YMIP substrates; m, mature form of YMIP substrates. (C) Yfh1p overexpression correlates with decreased expression and improved function of YMIP(G578L). Yeast grown on YPEG plates at the permissive temperature were resuspended in liquid YPEG to an OD600 of ~0.2 and grown at 30°C to an OD600 of ~1.5. Cultures were then shifted to 37°C and the cells harvested after 14 h and analyzed by western blotting. (D) YE-YFH1 is maintained at very high copy number in oct1Δ[YFH1] but not wt[YFH1]. Total RNA was isolated from the cultures described above and analyzed by northern blotting. Each lane contains ~30 µg total RNA and essentially identical levels of the two yeast tRNAs, as determined by ethidium bromide staining of the agarose gel before blotting (data not shown). The yeast RNA polymerase II subunit 2 (RPO22) transcript was used as an internal loading control. The autoradiographs shown represent successive hybridizations of different radiolabeled DNA probes to the same membrane.
sought milder growth conditions that would enable oct1ts to remain viable on YPEG at 37°C. Thus, wild-type and oct1ts strains carrying YE-YFHI or YEp24 vector were grown to an OD_{600} of 1.5 (~36 h) in liquid YPEG at 30°C, at which point the cultures were shifted to 37°C for 14 h before the cells were harvested for western and northern analyses (Fig. 1C and D, respectively). We found that oct1ts did remain viable under these conditions, as it continued to grow at 37°C at a rate similar to that observed for oct1ts[YFHI] (data not shown). Western analysis showed that, relative to wild-type (Fig. 1C, lane 5), oct1ts[YFHI] produced very large amounts of Yh1p (Fig. 1C, lane 8) and northern analysis confirmed that this correlated with very high levels of the YFHI (Fig. 1D, lane 8) and URA3 (Fig. 1D, lane 12) transcripts, which are both transcribed from the YE-YFHI plasmid. On the other hand, the levels of Yh1p and YFHI transcript in wt[YFHI] (Fig. 1C and D, lane 6) were higher than normal (Fig. 1C and D, lane 5) but never approached those detected in oct1ts[YFHI] (Fig. 1C and D, lane 8). In fact, the amounts of URA3 transcript detected in these strains indicated that whereas oct1ts retained a very high number of copies of the YE-YFHI plasmid (Fig. 1D, lane 12) but freely lost the YEp24 vector (Fig. 1D, lane 11), wt instead lost YE-YFHI even more rapidly than YEp24 (Fig. 1D, lanes 10 and 9). Together with the fact that Yh1p expression was not increased in oct1ts as compared with wt (Fig. 1C and D, lanes 7 and 5), these results indicate that oct1ts[YFHI] produced levels of Yh1p sufficient for suppression only through maintenance of a very high number of copies of the YE-YFHI plasmid.

It seemed possible that Yh1p might suppress oct1ts by increasing the levels of YMIP(G578L), which could partially compensate for loss of proteolytic activity at the restrictive temperature. However, western analysis revealed that oct1ts[YFHI] actually contained YMIP(G578L) at levels that were significantly lower than those detected in oct1ts (Fig. 1C, lanes 8 and 7). Nevertheless, the levels of YMIP(G578L) in oct1ts[YFHI] (Fig. 1C, lane 8) were higher than those of YMIP in wt (Fig. 1C, lane 5) and wt[YFHI] (Fig. 1C, lane 6), a trend we had also observed in cells grown on YPEG plates (see Fig. 1B, lanes 4, 1 and 2). In fact, YMIP and the OCT1 transcript were nearly undetectable in wt and wt[YFHI] (Fig. 1C and D, lanes 5 and 6), indicating that only catalytic amounts of enzyme were required under the conditions used in this experiment. To assess whether Yh1p overexpression affected the stability of YMIP(G578L), radiolabeled YMIP(G578L) was synthesized in vivo at either 30 or 37°C and its levels monitored by immunoprecipitation during a 30 h chase at the restrictive temperature. This analysis showed that the turnover rate of YMIP(G578L) was similar in oct1ts and oct1ts[YFHI] (data not shown), indicating that the stability of the mutant enzyme was not affected by overexpression of Yh1p. Together with the fact that the levels of YMIP and YMIP(G578L) (Fig. 1C) paralleled those of the OCT1 and G578Loc1 transcripts (Fig. 1D), respectively, these data indicate that oct1ts overexpressed YMIP(G578L) to compensate for its decreased activity and that Yh1p overexpression reduced this requirement, probably by improving the function of YMIP(G578L). Indeed, overexpression of Yh1p correlated with a significant improvement in the processing of at least one YMIP substrate, Hem15p (Fig. 1C). Although oct1ts[YFHI] produced less mHem15p and accumulated more iHem15p than did wt and wt[YFHI] (Fig. 1C, compare lane 8 with lanes 5 and 6), the ratio of mHem15p to iHem15p was ~10-fold higher in oct1ts[YFHI] than in oct1ts (Fig. 1C, compare lanes 8 and 7). Additionally, oct1ts[YFHI] contained 4-fold more total Hem15p protein but comparable amounts of the HEM15 transcript, relative to oct1ts (Fig. 1C and D, compare lanes 8 and 7), while no such effect on Hem15p levels was observed in an octΔ strain overexpressing Yh1p (data not shown). These data indicate that high levels of Yh1p rescued YMIP(G578L) activity and also indirectly improved the import and/or stability of its substrates.

### Loss of YMIP activity leads to mitochondrial iron depletion

Considering that both Yh1p and YMIP reside in the mitochondrial matrix (unpublished data; 22), it seemed possible that overexpressed Yh1p might directly interact with YMIP(G578L) and/or its substrates to improve processing in oct1ts[YFHI]. However, no such physical interaction was detected by western analysis of Yh1p, YMIP(G578L), CoxIV, and Fe/S immunoprecipitates isolated from oct1ts[YFHI] mitochondrial matrix fractions (data not shown). Similarly, we detected no co-immunoprecipitation of Yh1p with wild-type or mutant YMIP from solubilized wild-type or oct1ts[YFHI] mitochondria, respectively, regardless of whether the mitochondria were treated with membrane-permeant crosslinking agents prior to solubilization (data not shown). Furthermore, Yh1p did not co-immunoprecipitate with radiolabeled CoxIV that had been imported into isolated wild-type mitochondria, regardless of whether import was slowed by low temperature or the mitochondrial treated with crosslinkers prior to solubilization (data not shown). Thus, we have found no evidence of physical interaction between Yh1p and YMIP or its substrates, suggesting that Yh1p overexpression improves YMIP(G578L) activity in oct1ts through indirect means.

Because previous studies (3,10,35) have demonstrated that loss of Yh1p leads to accumulation of ‘free’ mitochondrial iron [i.e. iron that is not bound to heme or iron–sulfur proteins (36); referred to as ‘mitochondrial iron’ throughout], we postulated that overexpression of Yh1p might affect mitochondrial iron levels in oct1ts. To test this possibility, wild-type and oct1ts strains bearing YE-YFHI or YEp24 were grown in liquid YPEG for ~100 h, shifted to 37°C for 14 h and their mitochondria isolated and analyzed for iron concentration (36). We found that relative to mitochondria isolated from wild-type strains, oct1ts mitochondria contained 40% less iron (P = 0.0845) and that octΔ mitochondria contained 60% less iron (P = 0.033) (Fig. 2), indicating that loss of YMIP activity was associated with loss of mitochondrial iron. In contrast, oct1ts[YFHI] mitochondria contained iron at levels that, while slightly lower than normal, were 40% higher than those measured in oct1ts (P = 0.2715) (Fig. 2). Although not statistically significant, the latter difference was reproduced in three independent experiments and appeared to specifically result from overexpression of Yh1p in oct1ts. Whether Yh1p overexpression also affects iron levels in wild-type cells could not be determined because by the time cells were harvested for isolation of mitochondria, wt[YFHI] had lost the YE-YFHI plasmid and no longer overexpressed Yh1p (data not shown). Importantly, Yh1p overexpression was not sufficient to pre-
Iron stimulates YMIP activity in vitro

Considering that Yfh1p prevented both depletion of mitochondrial iron and inactivation of YMIP(G578L), it seemed possible that iron might stimulate YMIP activity. To test this, crude mitochondrial matrix fractions derived from wt or oct1Δ were incubated with radiolabeled Yfh1p and yet contained no more mitochondrial iron than other oct1Δ strains (data not shown). Thus, overexpression of Yfh1p prevented loss of mitochondrial iron in oct1Δ in a way that depended upon the residual activity of YMIP(G578L).

Yfh1p and YMIP have opposing roles in iron homeostasis

To further examine the relationship between Yfh1p and YMIP, we disrupted the YFH1 and OCT1 genes individually and in combination and analyzed the resulting mutants in terms of growth phenotype and mitochondrial iron levels. A number of previous studies have demonstrated that disruption of YFH1 (yfh1Δ) can result in defective growth on fermentable and non-fermentable carbon sources, depending upon the genetic background of the parental strain (3,4,10,11). In our hands, yfh1Δ haploid derivatives of strain YPH501 were unable to grow on YPEG (Fig. 4A) or complement pΔ (KL14-4B and CB11, lacking mtDNA) or mit− (M7-40-4B, M9-3-6C and M10-15-4D, carrying single point mutations in mtDNA) strains (data not shown), which indicates loss of respiration and mtDNA integrity. Additionally, yfh1Δ grew poorly on YPD (1% yeast extract, 2% peptone, 2% dextrose), but remained viable even at 37°C (Fig. 4A). Transformation of a yfh1ΔΔYFH1 diploid with YFH1 on a centromeric plasmid (YC-YFH1) prior to sporulation yielded fully complemented haploid derivatives (designated yfh1ΔΔYFH1) that grew as well as the parental strain on both YPEG and YPD, whereas transformation of a yfh1Δ haploid with YC-YFH1 produced a yfh1ΔΔYFH1pΔ strain which failed to grow on YPEG but did grow better than yfh1Δ and as well as an isogenic pΔ strain on YPD (Fig. 4A). Therefore, in the YPH501 genetic background, Yfh1p was required not only for respiratory growth and mtDNA maintenance, but also for normal fermentative growth. In contrast, the growth phenotype of oct1Δ was similar to that of yfh1ΔΔYFH1pΔ and an isogenic pΔ strain (Fig. 4A), which is consistent with YMIP playing a role in respiratory but not fermentative growth. As might be predicted from these observations, a yfh1ΔΔoct1Δ double mutant showed a growth phenotype very similar to that of yfh1ΔΔ; note that although yfh1ΔΔoct1Δ did not grow as well as yfh1Δ on the particularYPD plate shown in Figure 4A, this difference was not detected in similar experiments and in liquid culture the mutants grew at the same rate (data not shown).

Previous studies have found that yfh1Δ mutants derived from different parental strains accumulate mitochondrial iron at levels ~10-fold higher than wild-type strains (3,10). In our hands, yfh1Δ mitochondria showed iron levels that varied in...
different mitochondrial preparations but were, on average, ~25-fold higher than those detected in wild-type mitochondria 
\( (P = 0.0001) \). In contrast, \( oct1^{Δ} \) and \( oct1^{Δ} \) mitochondria showed reduced levels of iron as compared with wild-type mitochondria (\( P = 0.0845 \) and \( P = 0.033 \), respectively) (Fig. 4B). It seemed possible that the mitochondrial iron depletion in \( oct1^{Δ} \) and \( oct1^{Δ} \) might result from loss of respiratory function and not directly from lack of YMIP activity. However, \( yfh1^{Δ}oct1^{Δ} \) mitochondria contained iron at levels that, while still much higher than wild-type (\( P = 0.0001 \)), were ~2-fold lower than those of \( yfh1^{Δ} \) mitochondria (\( P = 0.0013 \)) (Fig. 4B). Because both \( yfh1^{Δ} \) and \( yfh1^{Δ}oct1^{Δ} \) lack mtDNA but only the double mutant lacks YMIP, the difference in their mitochondrial iron levels must be a direct consequence of loss of YMIP activity in the latter strain. This point was further confirmed by the fact that transformation of \( yfh1^{Δ}oct1^{Δ} \) with \( OCT1 \) on a centromeric plasmid resulted in mitochondrial iron levels that were comparable with those measured in \( yfh1^{Δ} \) (data not shown). In summary, loss of \( Yfh1^{p} \) (\( yfh1^{Δ} \)) affected both respiratory and fermentative growth, presumably as a result of mitochondrial iron overload and oxidative damage of mitochondrial as well as other cellular components. On the other hand, loss of YMIP (\( oct1^{Δ} \)) primarily affected the maturation of its substrates, which in turn led to loss of respiratory function and reduced levels of mitochondrial iron. Finally, loss of YMIP in \( yfh1^{Δ}oct1^{Δ} \) did limit mitochondrial iron accumulation, but not to a degree sufficient to prevent the phenotypic manifestations associated with loss of \( Yfh1^{p} \).

**YMIP is active in \( yfh1^{Δ} \)**

The fact that mitochondrial iron levels in \( yfh1^{Δ} \) were reduced upon deletion of \( OCT1 \) suggests that YMIP promotes the iron accumulation associated with loss of \( Yfh1^{p} \). Therefore, YMIP should be active in cells lacking \( Yfh1^{p} \) and, indeed, pulse labeling experiments demonstrated that mCoxIV and mFe/S were produced in intact \( yfh1^{Δ} \) cells (Fig. 5), although the total amounts of CoxIV and Fe/S protein synthesized were drastically lower than those detected in wild-type strains (see below). Similarly, *in vitro* import assays showed that radio-labeled pCoxIV was imported and processed to the mature form by isolated \( yfh1^{Δ} \) mitochondria (data not shown). In agreement with these results, western analysis (Fig. 6A)
showed that \( \text{yfh1} \Delta \) (Fig. 6A, lane 4) contained YMIP at levels that were ~2-fold lower than wild-type (Fig. 6A, lane 1) but ~6-fold higher than those detected in an isogenic \( \rho^0 \) strain (Fig. 6A, lane 7). As northern analysis revealed a similar pattern in \( \text{OCT1} \) mRNA levels (Fig. 6B, compare lane 11 with lanes 8 and 14), it appears that relative to other strains lacking mtDNA, \( \text{yfh1} \Delta \) actually overexpressed YMIP. In contrast, \( \text{yfh1} \Delta \) and \( \text{yfh1} \Delta \text{oct1} \Delta \) contained very little CoxIV and almost no detectable Hem15p and Fe/S, but did contain three proteins processed to the mature form by MPP (\( \text{F}, \beta, \text{BMPP} \) and Hsp60) (26,37,38) at levels comparable with those detected in other \( \rho^0 \) strains (Fig. 6A and data not shown). Thus, while lack of mtDNA resulted in a global reduction in the levels of all mitochondrial proteins examined, Yfh1p deficiency led to an additional, more dramatic loss of YMIP substrates, but was also associated with an increase in YMIP expression. Northern analysis confirmed this pattern: strains lacking Yfh1p showed an additional loss of YMIP substrate transcripts but an ~6-fold increase in \( \text{OCT1} \) transcript (Fig. 6B). All strains contained similar amounts of the RNA polymerase II subunit 2 transcript (\( \text{RPO22} \)) (39), which served as an internal loading control (Fig. 6B).

Although the levels of YMIP substrate transcripts were decreased to similar degrees in strains lacking Yfh1p, the proteins themselves behaved somewhat differently. For instance, western analysis failed to detect Fe/S in \( \text{yfh1} \Delta \) and \( \text{yfh1} \Delta \text{oct1} \Delta \), but did reveal small amounts of CoxIV and Hem15p (Fig. 6A). To test whether lack of Yfh1p affects the stability of YMIP substrates, yeast were pulse labeled and the turnover rates of radiolabeled mFe/S and mCoxIV were monitored by immunoprecipitation during a 6 h chase. Over this period of time, mFe/S remained relatively stable in wild-type cells, but was degraded rapidly in an isogenic \( \rho^0 \) strain (\( t_{1/2} = 72 \text{ min} \)) and even more rapidly in \( \text{yfh1} \Delta \) (\( t_{1/2} = 36 \text{ min} \)) (data not shown), indicating that decreased protein stability contributed to the loss of Fe/S in \( \text{yfh1} \Delta \). Protein instability did not seem to play a role in the loss of mCoxIV in \( \text{yfh1} \Delta \), however, as this protein was equally stable in all three strains (data not shown). These results indicate that the near absence of YMIP substrates in \( \text{yfh1} \Delta \) strains was primarily due to a dramatic decrease in their expression. As YMIP substrates include a number of iron-utilizing proteins, such a decrease in their expression could serve to limit the demand for mitochondrial iron and thus prevent its further accumulation in \( \text{yfh1} \Delta \) strains. If this is the case, \( \text{yfh1} \Delta \) should show a similar decrease in the expression of iron-utilizing mitochondrial proteins other than YMIP substrates. Consistent with this possibility, cytochrome c\(_1\) (\( \text{CYT1} \) gene, Cyt c\(_1\) polypeptide), which is not a YMIP substrate but does contain a heme cofactor (40), was affected by loss of Yfh1p in much the same way as the YMIP substrates, in that neither Cyt c\(_1\) nor its transcript were detected in the \( \text{yfh1} \Delta \) or \( \text{yfh1} \Delta \text{oct1} \Delta \) mutants, whereas both were present in wild-type and, to a lesser degree, \( \rho^0 \) strains (Fig. 6).

The \( \text{FRDA} \) and \( \text{MIPEP} \) genes are similarly expressed in mouse tissues

As a first step in addressing a potential functional interaction between the human homologs of Yfh1p and YMIP, we compared the patterns of expression of the \( \text{FRDA} \) and MIP (\( \text{MIPEP} \)) genes in different mouse tissues by northern blotting (Fig. 7). We found that the relative amounts and tissue distribution of the \( \text{FRDA} \) transcript closely matched those of the \( \text{MIPEP} \) transcript, which is in agreement with the fact that the pattern of expression reported for the human \( \text{FRDA} \) gene (2) is very similar to the one we described previously for the human \( \text{MIPEP} \) gene (30). These data are consistent with the possibility that the interaction between Yfh1p and YMIP is conserved in mammalian cells.

DISCUSSION

In a screen designed to identify proteins that interact with YMIP, we isolated the \( YFH1 \) gene based on its ability to suppress the respiratory-deficient phenotype of a conditional \( \text{oct1} \Delta \) mutant. Complementation of \( \text{oct1} \Delta \) required overexpression of Yfh1p as well as the presence of YMIP(G578L) and correlated
Heart and skeletal muscle contain two forms of βFRDA related by addition of 100
we demonstrated that iCoxIV processing by YMIP was stimu-
partially restored upon overexpression of Yfh1p. Furthermore, activity and complements
loss that Yfh1p overexpression reconstitutes YMIP(G578L)
mental to YMIP(G578L) activity and that it is by limiting iron
mouse tissues (Clontech) was sequentially hybridized with 32 P-labeled probes
oct1ts metal-dependent peptidase (31), we hypothesized that over-
homeostasis (3,10,35), together with the fact that YMIP is a
YMIP(G578L) to a similar degree required 10-fold higher con-
the conclusion that loss of mitochondrial iron in
context of a crude mitochondrial matrix fraction supports
mechanism and whether iron is bound to the native enzyme
whether iron acts as the essential metal ion by binding to the
active site of YMIP or stimulates the enzyme by some other
mechanism and whether iron is bound to the native enzyme in vivo. However, the fact that Fe²⁺ stimulates YMIP activity in
the context of a crude mitochondrial matrix fraction supports
the conclusion that loss of mitochondrial iron in oct1Δ is detri-
mental to YMIP(G578L) activity and that it is by limiting iron loss that Yfh1p overexpression reconstitutes YMIP(G578L)
activity and complements oct1Δ.

YMIP is not only stimulated by iron, however, but also pos-
atively affects mitochondrial iron levels, as is evident from the
fact that oct1Δ and oct1Δ strains showed loss of mitochondrial iron. It is clear that loss of YMIP activity and not simply of the protein itself was responsible for mitochondrial iron depletion in these strains, as YMIP(G578L) was actually overexpressed in oct1Δ. Given that YMIP is required for the maturation of the major iron-utilizing protein (Hem15p) and several iron-containing proteins in mitochondria, it seems likely that YMIP activity increases the demand for mitochondrial iron and therefore stimulates uptake of iron by mitochondria. Identification of the protein partners of YMIP and their roles in mitochondrial iron movement will be required to fully elucidate this mechanism. The data available at this time, however, support the conclusion that YMIP is part of a positive feedback loop in which maturation of iron-utilizing proteins promotes mitochondrial iron uptake, which in turn stimulates YMIP activity. Thus, in the case of oct1Δ, partial inactivation of YMIP(G578L) at the restrictive temperature causes loss of mitochondrial iron, leading to further loss of YMIP(G578L) activity and eventually to loss of mtDNA integrity and irreversible loss of respiratory function. Overexpression of Yfh1p interrupts this cycle by preventing depletion of mitochondrial iron, thereby maintaining YMIP(G578L) in a partially active state and rescuing oct1Δ.

The fact that overexpression of Yfh1p increases mitochondrial iron levels in oct1Δ may seem difficult to reconcile with the role of Yfh1p in promoting mitochondrial iron efflux (35). However, considering that the levels of Yfh1p produced in oct1Δ[YFH1] greatly exceed physiologic levels (Fig. 1C), it seems plausible that under these conditions Yfh1p inhibits the process it normally promotes, perhaps through titration of another factor involved in iron movement. Were this to be the case, one might expect that overexpression of Yfh1p could be detrimental to wild-type cells and, in fact, we found that in contrast to oct1Δ, which retained a very large number of copies of the YE-YFH1 plasmid, wild-type strains rapidly lost this plasmid and never expressed Yfh1p at levels comparable with those in oct1Δ[YFH1]. Moreover, oct1Δ showed no increase in expression from its endogenous YFH1 allele, even though this mutant could clearly benefit from Yfh1p overexpression, indicating that Yfh1p expression is probably tightly regulated in yeast. It is important to note, however, that a modest degree of Yfh1p overexpression (<2-fold above basal levels) did not affect mitochondrial iron levels in the yfh1Δ[YFH1] strain, which stably maintained the YC-YFH1 plasmid at low copy number (Fig. 6B, lane 9, and data not shown). Therefore, it appears that rescue of oct1Δ by YE-YFH1 was a fortuitous case in which loss of mitochondrial iron, due to YMIP(G578L) inactivation, was blocked by artificially high and otherwise toxic levels of Yfh1p. Despite the fact that this positive effect may only be achieved in the genetic background of this or similar mutants, analysis of oct1Δ suppression by Yfh1p has been essential in uncovering a functional interaction between Yfh1p and YMIP that is relevant to mitochondrial iron homeostasis and might otherwise not have been identified, given the lack of direct physical interaction between Yfh1p and YMIP.

Our analysis indicates that YMIP plays a role in mitochondrial iron homeostasis and implies that, under physiological conditions, Yfh1p and perhaps other proteins involved in iron movement might influence YMIP activity through changes in mitochondrial iron levels. An increase in Yfh1p-mediated iron export, for example, could produce mitochondrial iron levels that are suboptimal for YMIP activity, inhibiting the maturation of Hem15p and other YMIP substrates and therefore leading to reduced uptake of iron. In fact, we have found that yeast that completely lack Yfh1p, and therefore accumulate extremely high levels of mitochondrial iron, show drastically reduced expression of YMIP substrates and Cyt c, a response which could serve to decrease mitochondrial iron uptake. On the other hand, unlike its substrates and all other mitochondrial proteins analyzed, YMIP itself was overexpressed in yfh1Δ rel-
ative to other \( \rho \) strains. Such an effect should contribute to mitochondrial iron accumulation in \( yfh1 \Delta \) and, indeed, \( yfh1 \Delta \) contains significantly more mitochondrial iron than does \( yfh1 \Delta oct1 \Delta \). Thus, loss of Yfh1p appears to elicit contradictory responses, with reduced expression of YMIP substrates and other iron-utilizing proteins predicted to decrease mitochondrial iron uptake and overexpression of YMIP leading to accumulation of mitochondrial iron. Further investigation will be required to determine whether these responses stem from a common proximal signal such as depletion of cytosolic iron, the signal for induction of the high affinity iron transport system (35). In any case, our data are consistent with a model in which YHlp maintains mitochondrial iron homeostasis both directly, by promoting iron export, and indirectly, by regulating the expression and activity of YMIP, which promotes mitochondrial iron uptake.

Iron deposits (13,14), multiple mitochondrial enzyme deficiencies (12) and hypersensitivity to oxidative stress (41) have been reported in studies of FRDA, a neurodegenerative disease caused by a deficiency of human frataxin (15). The striking similarity between these pathological findings and the alterations observed in \( yfh1 \Delta \) yeast (3,4,10,11) has led to the conclusion that frataxin is essential for mitochondrial iron homeostasis and that its function is conserved in eukaryotes (12,15). This view supports the use of yeast as a valid model to elucidate how frataxin deficiency leads to mitochondrial iron accumulation and to identify other factors influencing this phenotype. Indeed, genetic screens in yeast have recently enabled the identification of two mitochondrial proteins, MPP (6) and Ssq1p (21), that are required for proteolytic processing of frataxin and as such are predicted to affect frataxin levels in normal individuals as well as FRDA patients. Similarly, identification of \( YFH1 \) as a multicopy suppressor of the \( oct1 \Delta \) mutant has enabled us to show that YMIP is an iron-dependent peptidase which promotes mitochondrial iron uptake in both wild-type and \( yfh1 \Delta \) yeast. YMIP homologs have been identified in rat and human (28,30) and shown to be able to efficiently replace YMIP in yeast (29; unpublished data), demonstrating that the function of this peptidase is evolutionarily conserved. Furthermore, the relative amounts and tissue distribution of the \( FRDA \) gene transcript match those of the MIP gene (\( MIPEP \)) in human (2,30) and mouse (Fig. 7), suggesting that frataxin and human MIP (HMIP) may work together to maintain mitochondrial iron homeostasis. HMIP could therefore contribute to the mitochondrial iron imbalance believed to result from frataxin deficiency, while genetic or environmental factors that reduce HMIP activity could have a protective effect.

**MATERIALS AND METHODS**

**Yeasts, strains and media**

The strains used in this study are shown in Table 1. Construction of \( oct1 \Delta \) and \( oct1 \Delta \) were described previously (22,23,31). For construction of \( yfh1 \Delta \) and \( yfh1 \Delta oct1 \Delta \), PCR-directed integration (42) was used to generate \( yfh1 \Delta HIS3/YFH1 \) and \( yfh1 \Delta HIS3/YFH1 \). The \( oct1 \Delta :LEU2/OCT1 \) heterozygous disruption strains, \( yfh1 \Delta HIS3 \) and \( yfh1 \Delta HIS3 \), \( oct1 \Delta :LEU2 \) derivatives were then isolated by sporulation and tetrat analysis and integration of the disrupted alleles at the correct loci confirmed by PCR amplification of total genomic DNA. For complementation of \( yfh1 \Delta \), a phage clone of a segment of yeast chromosome IV (ATCC 70458) was digested with \( S_{\text{stl}} \) to yield \( YFH1 \) plus 620 bp of 5' and 310 bp of 3'-flanking DNA. This fragment was cloned into a centromeric URA3-based vector (YCP50) to produce the construct YC-\( YFH1 \). This plasmid was then used to transform the heterozygous \( yfh1 \Delta :HIS3/YFH1 \) and \( yfh1 \Delta [YFH1] \) derivatives isolated by sporulation and tetrat analysis. The \( yfh1 \Delta [YFH1] \) plasmid was produced by direct transformation of \( yfh1 \Delta \) with YC-\( YFH1 \). An isogenic \( \rho \) strain was obtained by ethidium bromide treatment (43) of Y6041.

The following liquid and solid media were used in this study, supplemented with amino acids and other growth requirements as needed: YPD (1% yeast extract, 2% peptone, 2% dextrose); YPEG (as YPD except with 2% ethanol and 3% glycerol instead of dextrose); YPGal (as YPD except with 2% galactose and 0.05% dextrose instead of 2% dextrose); SD (6.7% bacto-yeast nitrogen base without amino acids, 2% dextrose).

**Multicopy suppressor screen**

The \( oct1 \Delta \) mutant was transformed with a yeast genomic library on a 2\( μ \)YEp24 vector (M. Carlson, Columbia University, New York, NY). Approximately 287 000 transformants were selected on SD plates at 23°C and then replica-plated onto YPEG plates and screened for their ability to grow at 37°C. A clone containing bp 245 718–250 349 of yeast chromosome IV (LP12) was isolated twice in the screen. Deletion derivatives of LP12 were constructed by restriction digestion and tested by transformation of \( oct1 \Delta \) and growth on YPEG at 37°C. Suppressor plasmid YE-\( YFH1 \) consists of an 800 bp genomic fragment containing the \( YFH1 \) coding sequence plus 200 bp of 5' and 150 bp of 3'-flanking DNA, cloned into the YEp24 vector.

**Antibodies**

A fusion protein consisting of bacterial glutathione S-transferase (GST) fused to Yfh1p was expressed in *Escherichia coli* and injected into one rabbit. Western analysis of yeast cell extracts demonstrated that a total immune serum from this animal recognized a protein of ~23 kDa. This band was not recognized a protein of ~23 kDa. This band was not recognized by a pre-immune serum (data not shown) and was absent in \( yfh1 \Delta \) strains (Fig. 6A, lanes 4 and 6). This is consistent with a recent report showing that radiolabeled YHlp is imported by isolated mitochondrial and processed to an intermediate product of ~27 kDa and a putative mature form of ~23 kDa (21). We have observed an identical pattern of processing upon incubation of radiolabeled YHlp with isolated yeast mitochondria or mitochondrial matrix fractions and have demonstrated that the putative mature form runs identically to the endogenous ~23 kDa protein detected by western analysis of total cell extracts (data not shown). These data indicate that our polyclonal antibody specifically recognizes the mature form of YHlp.

**GST fusion constructs** were also used to generate specific antisera against yeast Fe/S and CoxIV proteins. Polyclonal antisera against Hem1p, F. β. Hsp60 and βMPP, as well as a monoclonal antibody against Cyt c, were provided by other investigators (R. Labbe-Bois, Institut Jacques Monod, Paris, France; M. P. Yaffe, UCSD, San Diego, CA; A. L. Horwich,
Western analysis

In one set of experiments (Fig. 1A and B), yeast were streaked on YPEG plates and grown at 30°C for 6 days and then streaked onto fresh plates and grown at either 30 or 37°C for 10 days. In a second set of experiments (Fig. 1C), yeast were grown in 50 ml YPEG at 30°C until they reached an OD_{600} of 1.5 (~36 h) and then shifted to 37°C for 14 h. In a third set of experiments (Fig. 6A), yeast were grown in liquid YPGal at 30°C to an OD_{600} of 3 (~72 h). Cells were harvested by plate scraping or centrifugation, resuspended in water and extracted by precipitation with 10% trichloroacetic acid (TCA) (22). Protein concentrations were determined using the Bradford dye binding assay (Bio-Rad, Hercules, CA) and confirmed by Coomassie Blue staining of protein gels. Proteins were detected using specific primary antibodies, as described above, followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Amersham, Piscataway, NJ). Immunodetection of Yfh1p and most other proteins analyzed in this study required ~300 µg total protein/lane, whereas detection of YMIP and Hem15p required ~3-fold more total protein. Analysis of YMIP, CoxIV and F₁β used previously described SDS–PAGE conditions (23); analysis of Yfh1p used CoxIV SDS–PAGE conditions, while analysis of Fe/S, Cyt c₁ and βMFP used F₁β conditions. In most experiments, successive hybridizations of the same membrane were used to detect Yfh1p and CoxIV and F₁β and Fe/S. Analysis of Hem15p required T = 8.3% separating gels (12.5 cm total length) overloaded with T = 4% stacking gels (T denotes the total concentration of acrylamide and bisacrylamide), using a stock solution of 40:1.7 acrylamide:bisacrylamide; electrophoresis was started at 180 V, shifted to 240 V after the samples had completely entered the separating gel and continued for an additional 90 min after the samples had reached the bottom of the separating gel.

In vitro precursor protein processing

Previously described methodologies were used in these experiments. Briefly, yeast were grown in YPGal at 30°C to an OD_{600} of ~2.0 and their mitochondria isolated (49). Soluble (‘crude matrix’) fractions were prepared by sonication of mitochondria followed by centrifugation at 165 000 g for 30 min (22). The CoxIV precursor was radiolabeled in vitro using a coupled transcription–translation system (Promega, Madison, WI). In a typical processing reaction, crude matrix (6 µg total protein) was added to 10 mM HEPES–KOH (pH 7.4), 1 mM DTT containing varying concentrations of FeCl₂ (reagent grade, 99% pure; Alfa AESAR, Ward Mill, MA) in a final volume of 8 µl. After 5 min at 23°C, 2 µl translation mixture was added and the incubation continued at 27°C for 20 min. Processing reactions were directly analyzed by SDS–PAGE and fluorography (22).

Iron determination

Mitochondrial isolation buffer [MIB; 20 mM HEPES–KOH (pH 7.4), 600 mM mannitol] and metal determination buffer [MDB; 10 mM MOPS (pH 6.5), 1% Triton X-100] (36) were passed through a Chelex 100 column (Bio-Rad) in order to remove adventitious metals. Yeast were grown in 1 l of either YPEG (wt, oct₁Δ and oct₁Δ[YFH1]) or YPGal (wt, oct₁Δ, yfh1Δ and yfh1Δoct₁Δ) at 30°C until they reached an OD_{600} of ~1.5 or 3.0, respectively; YPEG cultures were then shifted to 37°C for an additional 14 h. Note that analysis of wild-type

Northern analysis

In one set of experiments (Fig. 1D), yeast were grown in liquid YPEG at 30°C to an OD_{600} of 1.5 (~36 h) and then shifted to 37°C for 14 h. In other experiments (Fig. 6B), yeast were grown in liquid YPGal at 30°C to an OD_{600} of 3 (~72 h). Total RNA was prepared by extraction with hot acid phenol, size fractionated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and then transferred to a Hybond-N nylon membrane (Amersham) (48). A commercial blot (Clontech, Palo Alto, CA) was used in Figure 7. ³²P-labeled DNA probes were synthesized by PCR amplification and random priming and all hybridizations were carried out under stringent conditions. The autoradiographs shown in Figures 1D, 6B and 7 were obtained by successive hybridizations of different probes to the same membrane. Prior to each new hybridization, the old probe was stripped from the membrane by pouring a boiling solution of 0.1% SDS directly onto the membrane and allowing it to cool to room temperature; the efficiency of this treatment was confirmed by autoradiography.

In vivo protein labeling and immunoprecipitation

Yeast were initially grown in YPD at 30°C to an OD_{600} of ~3.0 (~40 h), collected by centrifugation, resuspended in SD to an OD_{600} of ~0.5 and grown at 30°C for an additional 3 h. Cells were then collected and resuspended in SD + 0.5 mg/ml BSA to an OD_{600} of ~2.5 and grown at 30°C in the presence of Tran32P label (ICN, Costa Mesa, CA), at 125 µCi/ml cells (44). In pulse labeling experiments, aliquots (1 ml) were removed at different time points and immediately extracted using 10% TCA at 4°C. In pulse-chase experiments, cells were radiolabeled for 1 h at 30°C, at which point a 10x chase solution (50 mM methionine, 10 mM cysteine, 2% yeast extract) was added and growth at 30°C continued for 6 h with 1 ml aliquots removed and extracted at the times indicated (44). To measure YMIP(G578L) stability in oct₁Δ and oct₁Δ[YFH1], cells were radiolabeled at either 30 or 37°C and chased at 37°C for >30 h. Immunoprecipitations were performed essentially as described previously (45). For co-immunoprecipitation experiments, mitochondria isolated from wild-type and oct₁Δ[YFH1] yeast were treated with the homobifunctional crosslinkers DSS and DSP (Pierce, Rockford, IL) and then solubilized, essentially as described (46). Import of radiolabeled CoxIV precursor at low temperature, to accumulate translocation intermediates for co-immunoprecipitation, was as described (47).
strains indicated that mitochondrial iron levels did not vary with growth medium (data not shown). Cells were harvested by centrifugation and mitochondria were isolated as described (49). The mitochondrial pellet was then washed twice with metal-free MIB and resuspended in metal-free MDB to a final concentration of 1 μg/μl. Iron was detected using a ferrozine colorimetric assay (Diagnostic Chemicals, Oxford, CT) (50) and a Boehringer Mannheim (Indianapolis, IN) Hitachi 911 analyzer; all determinations were confirmed by atomic absorption, using a Perkin-Elmer (Foster City, CA) 4100ZL spectrometer equipped with a transverse heated graphite furnace.

Statistical analysis

The iron concentration (nmol iron/mg mitochondrial protein) of each mitochondrial preparation was measured in at least three independent determinations on at least two independent mitochondrial preparations of each yeast strain. The natural logarithms of the measurements from the different strains were compared via a mixed model analysis of variance. The logarithmic transformation was performed as a variance stabilizing measure; the mixed model made it possible to account for variability due to both the different preparations and the multiple determinations made from each preparation. After the analysis of variance model confirmed that differences existed among the iron concentrations of the various strains (P < 0.0001), pairwise comparisons were made using Fisher’s least significant difference method.

The variability of different preparations within strains and determinations within preparations were both statistically larger than 0 (P < 0.0001 in each case) in the analysis of variance model. However, the variance arising from different determinations within preparations was both statistically larger than 0 (P < 0.0001 in each case) in the analysis of variance model. Therefore, the variance arising from different determinations was estimated to be <1% of the strain-to-strain variability. The variability due to different preparations was also small (2% of the variability among strains), but more noticeable than the variance of determinations within preparations. Because the differences among the determinations made from the same preparations were so small, their values were averaged together to arrive at a mean iron concentration for each mitochondrial preparation for the purposes of presentation. The mean values reported in Figures 2 and 4B represent the means of the average iron concentration obtained from each preparation. The vertical bars indicate the range of mean concentrations measured in the preparations.

ABBREVIATIONS

CoxIV, cytochrome c oxidase subunit IV; Cyt c1, cytochrome c1; Fβ, Fe/ATPase β subunit; Fe/S, Rieske iron–sulfur protein of cytochrome bc1 complex; FRDA, Friedreich’s ataxia; FRDA, frataxin gene; Hem15p, ferrochelatase; i, intermediate form of protein; m, mature form of protein; MIPEP, mammalian MIP gene; MPP, mitochondrial processing peptidase; mtDNA, mitochondrial DNA; OCT1, yeast mitochondrial intermediate peptidase gene (MIP1 in previous publications); p, precursor form of protein; p9, lacking mtDNA; ts, temperature-sensitive; YFH1, yeast frataxin homolog gene; Yfh1p, YFH1 gene product; YMIP, OCT1 gene product.

ACKNOWLEDGEMENTS

We thank A.L. Horwich, R. Labbe-Bois, G. Schatz, B.L. Trumpower, M.P. Yaffe, M. Carlson, P. Novick, J.D. Friesen and F. Taroni for reagents, W. Sakati and S. Sterling for technical assistance, and W.A. Fenton, F. Kalousek and M. Robinson for discussions. Iron determinations were performed by the Clinical Chemistry Laboratory at the Yale New Haven Hospital and by D.S. Ross in the Department of Plant and Soil Science at the University of Vermont. Statistical analysis was performed by S. Pankratz in the Department of Biostatistics at the Mayo Clinic. This work was supported by a grant from the Muscular Dystrophy Association and grant AG15709 from the National Institutes of Health.

REFERENCES


Human Molecular Genetics, 1999, Vol. 8, No. 6 1109