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Yeast and Human Frataxin Are Processed to Mature Form in Two Sequential Steps by the Mitochondrial Processing Peptidase*

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Frataxin is a nuclear-encoded mitochondrial protein which is deficient in Friedreich's ataxia, a hereditary neurodegenerative disease. Yeast mutants lacking the yeast frataxin homologue (Yfh1p) show iron accumulation in mitochondria and increased sensitivity to oxidative stress, suggesting that frataxin plays a critical role in mitochondrial iron homeostasis and free radical toxicity. Both Yfh1p and frataxin are synthesized as larger precursor molecules that, upon import into mitochondria, are subject to two proteolytic cleavages, yielding an intermediate and a mature size form. A recent study found that recombinant rat mitochondrial processing peptidase (MPP) cleaves the mouse frataxin precursor to the intermediate but not the mature form (Koutnikova, H., Campuzano, V., and Koenig, M. (1998) *Hum. Mol. Gen.* 7, 1485–1489), suggesting that a different peptidase might be required for production of mature size frataxin. However, in the present study we show that MPP is solely responsible for maturation of yeast and human frataxin. MPP first cleaves the precursor to intermediate form and subsequently converts the intermediate to mature size protein. In this way, MPP could influence frataxin function and indirectly affect mitochondrial iron homeostasis.

Recent studies have shown that the yeast frataxin homologue (*YFH1*, gene; Yfh1p, polypeptide) is a nuclear-encoded mitochondrial protein (1–4) and that its deficiency results in mitochondrial iron overload (1, 2, 5), which in turn leads to increased production of free radicals and loss of mitochondrial function (1). Similarly, iron deposits (6), multiple mitochondrial enzyme deficiencies (7), and hypersensitivity to oxidative stress (8) have been reported in studies on Friedreich's ataxia (FRDA),[1] a recessively inherited neurodegenerative disease caused by a deficiency of human frataxin (9, 10). Thus, it is believed that frataxin plays a critical role in mitochondrial iron homeostasis and free radical toxicity and that this function is conserved between yeast and mammals (7, 11). Not surprisingly, Yfh1p and mammalian frataxin share similar pathways of mitochondrial import and processing. The Yfh1p precursor (pYfh1p) is imported by isolated yeast mitochondria and processed to an intermediate (iYfh1p) and a mature size (mYfh1p) form (12). Production of mYfh1p is impaired in mitochondria isolated from yeast with mutations in the mitochondrial Hsp70 homologue Ssq1p, and similar to Yfh1p-deficient yeast (*yfh1*Δ) (1, 2, 5), *ssq1* mutants accumulate large amounts of mitochondrial iron (12), indicating that production of mYfh1p is required for mitochondrial iron homeostasis. The mouse frataxin precursor is also cleaved twice, and missense mutations corresponding to those found in FRDA patients dramatically reduce the efficiency of the second cleavage (13), further demonstrating the importance of proteolytic processing for frataxin function. Mitochondrial processing peptidase (MPP; EC 3.4.24.64) (14) was shown to catalyze conversion of the mouse frataxin precursor to intermediate form, but the peptidase responsible for formation of mature frataxin was not identified (13). Additionally, it has not yet been established whether the intermediate forms of Yfh1p and frataxin represent productive intermediates, in that they are actually processed to the mature form. In this study, we analyze proteolytic processing of Yfh1p and human frataxin and demonstrate that both proteins are processed to the mature form in two sequential steps by MPP.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Media—The strains used in this study are all isogenic derivatives of strain YPH501 (see Table I). Construction of *oct1*Δ, *yfh1*Δ, and isogenic ρ^0 strains was described previously (15, 16). For complementation of *yfh1*Δ by Yfh1p-*myc*, a polymerase chain reaction fragment encoding the Yfh1p C terminus fused in-frame to the 9E10 c-*myc* epitope was synthesized using a sense oligonucleotide complementary to the *YFH1* coding sequence upstream of a unique *Acc*I site and an antisense oligonucleotide specifying the 3′-end of the *YFH1* coding sequence, the 9E10 c-*myc* epitope coding sequence, a stop codon, 22 base pairs of the *YFH1* 3′-flanking DNA, and a *Bam*HI site. This polymerase chain reaction product was substituted for the 3′-region of the *YFH1* gene by digestion with *Acc*I and *Bam*HI, yielding a *YFH1-myc* fusion construct. A centromeric *TRP1*-based YCplac22-*YFH1-myc* plasmid was then used to transform the *yfh1*Δ[*YFH1*] strain (16) and replace the *URA3*-based YCp50-*YFH1* plasmid, which was eliminated by counterselection with 5-fluoroorotic acid, yielding the *yfh1*Δ[*YFH1-myc*] strain.

Mitochondrial Fractionation—The *yfh1*Δ[*YFH1-myc*] strain was grown in SSGD (6.7% bacto-yeast nitrogen base without amino acids, 0.3% yeast extract, 2% galactose, and 0.05% dextrose, supplemented with amino acids and other growth requirements) at 30 °C to an A_{600} of ~2, spheroplasts were prepared and homogenized, and the nuclear (1,000 × *g* pellet), heavy (3,000 × *g* pellet), and light (17,000 × *g* pellet) mitochondrial fractions were separated by differential centrifugation.

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[1] The abbreviations used are: FRDA, Friedreich's ataxia; MPP, mitochondrial processing peptidase: PAGE, polyacrylamide gel electrophoresis; IMP, inner membrane peptidase; MIP, mitochondrial intermediate peptidase; Cyt b_2, cytochrome b_2.

The light mitochondrial fraction was resuspended at 2 mg protein/ml in either isotonic (0.6 M mannitol, 20 mM HEPES-KOH, pH 7.4, 1 mg/ml bovine serum albumin) or hypotonic (20 mM HEPES-KOH, pH 7.4, 1 mg/ml bovine serum albumin) buffer and incubated for 25 min at 4 °C with gentle vortexing for 30 s every 5 min, essentially as described (17). When indicated, this treatment was carried out in the presence of 100 μg/ml proteinase K, with or without 1% Triton X-100. Proteinase K treatment was stopped by addition of 100 mM phenylmethylsulfonyl fluoride. Mitochondria were then resuspended in 20 mM HEPES-KOH, pH 7.4, 100 mM KCl and subjected to five cycles of freezing and thawing. Finally, the disrupted organelles were separated into soluble (matrix) and insoluble (membrane) fractions by centrifugation at 165,000 $\times$ *g*. Fractions were precipitated with 10% trichloroacetic acid (15), protein concentration was determined by ultraviolet absorption (18), and aliquots were analyzed by SDS/PAGE, Western blotting, and chemiluminescence. Yfh1p was detected using a monoclonal antibody against the 9E10 c-*myc* epitope or a polyclonal antibody (16); frataxin was detected using a polyclonal antibody against a GST-human frataxin fusion protein.[2] Antisera against mitochondrial Hsp60 and cytochrome b_2 (Cyt b_2) were gifts from other investigators.

Mitochondrial Import and Processing Assays—[^{35}S]Methionine-labeled precursors were synthesized *in vitro* by coupled transcription-translation (Promega). Previously described procedures were used for isolation of yeast (19) and rat liver (20) mitochondria. Translation mixture (6 μl) containing ^{35}S-labeled precursor was incubated with mitochondria (total protein, 80 μg) in import buffer (0.6 M mannitol, 20 mM HEPES-KOH, pH 7.4, 1 mM ATP, 1 mM $MgCl_2$, 40 mM KCl, 5 mM methionine, 3 mg/ml bovine serum albumin, 20 mM phosphocreatine, and 200 μg/ml phosphocreatine kinase) for 20 min at 27 °C (15). Upon import, reactions were either separated into mitochondrial pellet and post-mitochondrial supernatant by centrifugation at 14,000 $\times$ *g* for 5 min at 4 °C or first treated with proteinase K (250 μg/ml for 30 min at 0 °C) or trypsin (400 μg/ml for 5 min at 4 °C) and then separated into pellet and supernatant in the presence of protease inhibitors. To dissipate the inner membrane potential, mitochondria were incubated with 30 μM carbonyl cyanide *m*-chlorophenyl-hydrazone for 5 min at 0 °C prior to import. Crude matrix fractions were prepared by sonication of mitochondria followed by centrifugation at 165,000 $\times$ *g* for 30 min (15). Recombinant yeast MPP was prepared essentially as described by Geli (21); as determined by SDS/PAGE and Coomassie blue staining, the final enzyme preparation contained only αMPP and βMPP subunits (>99% purity).[3] One unit of recombinant MPP was arbitrarily defined as the amount of enzyme that converts 95% of the yeast F_1-ATPase subunit β precursor (p$F_1\beta$) contained in 5 μl of translation mixture to the mature form in 5 min at 27 °C in a total reaction volume of 50 μl of 10 mM HEPES-KOH (pH 7.4), 1 mM dithiothreitol, 1 mM $MnCl_2$ (HDM buffer). Import and processing reactions were directly analyzed by SDS/PAGE and fluorography. For analysis of Yfh1p and human frataxin processing, we used *T* = 12.5% separating gels (total length, 12.5 cm) overlaid with *T* = 4% stacking gels (*T* denotes the total concentration of acrylamide and bisacrylamide), from 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 75 min (pYfh1p) or 30 min (frataxin) after the samples had reached the bottom of the separating gel.

RESULTS AND DISCUSSION

To analyze mitochondrial import and processing of Yfh1p, the *YFH1* coding sequence was cloned into an *in vitro* expression vector, and radiolabeled pYfh1p was synthesized by coupled transcription-translation. SDS/PAGE analysis of the translation mixture revealed a major band with an apparent molecular mass of ~28 kDa, much larger than the predicted size of pYfh1p (~19.5 kDa) (Fig. 1*A*, *lane 1*; *lanes 8* and *MW* show the mobility of pYfh1p relative to those of standard proteins). A difference of almost 10 kDa between the predicted molecular mass and the electrophoretic mobility of pYfh1p was reported previously (12), and similar discrepancies were noted for human (10) and mouse (13) frataxin as well. Such differences are observed regardless of whether Yfh1p and frataxin are produced in intact cells or *in vitro* translation assays (Ref. 13 and this study), and therefore it seems unlikely that they result from post-translational modifications. A more likely explanation is that the extremely hydrophilic nature of frataxin causes it to bind less SDS as compared with standard proteins of the same mass and that this results in lower electrophoretic

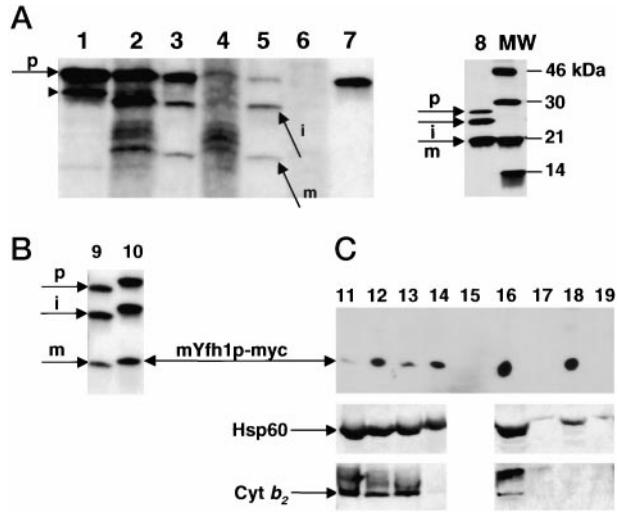

FIG. 1. **Yfh1p is imported by isolated mitochondria, and localizes to the mitochondrial matrix *in vivo*.** *A*, import of radiolabeled pYfh1p into isolated yeast mitochondria. *Lane 1*, Yfh1p translation mixture; *lane 2*, total import reaction; *lane 3*, mitochondrial pellet; *lane 4*, post-mitochondrial supernatant; *lanes 5* and *6*, as *lanes 3* and *4*, respectively, with proteinase K added to the import reaction prior to its separation into pellet and supernatant; *lane 7*, as *lane 3*, with carbonyl cyanide *m*-chlorophenyl-hydrazone added to mitochondria before addition of translation mixture; *lane 8*, as *lane 5*; *MW*, molecular weight markers. Samples were directly analyzed by 12.5% SDS/PAGE and fluorography, as described under "Experimental Procedures"; for *lanes 8* and *MW*, electrophoresis was stopped 75 min earlier. The letters *p*, *i*, and *m* denote the precursor, intermediate, and mature forms of Yfh1p, respectively. The *arrowhead* indicates a nonspecific product in the translation mixture. *B*, import and processing of pYfh1p-*myc* by isolated mitochondria. *Lanes 9* and *10* are as in *panel A*, *lane 5*, except that radiolabeled pYfh1p-*myc* was used in *lane 10*. *C*, localization of mYfh1p-*myc* to the mitochondrial matrix. Mitochondria were isolated from *yfh1*Δ[YC-*YFH1-myc*] yeast, and mitochondrial subfractions were analyzed by Western blotting. Anti-*myc* monoclonal antibody was used to detect Yfh1p-*myc*, and specific antisera were used to detect endogenous Hsp60 and Cyt b_2, which were used as matrix and intermembrane space markers, respectively. Yfh1p-*myc* was analyzed in one blot, and a second blot was used for analysis of both Hsp60 and Cyt b_2. 250 μg of total protein was loaded in *lanes 11–15*, and 90 μg was loaded in *lanes 16–19*. *Lane 11*, intact mitochondria; *lane 12*, intact mitochondria treated with proteinase K; *lane 13*, mitochondria subjected to hypotonic shock and reisolated by centrifugation; *lane 14*, mitochondria subjected to hypotonic shock in the presence of proteinase K and reisolated by centrifugation; *lane 15*, as *lane 14*, except that proteinase K treatment was performed in the presence of Triton X-100 (this particular analysis was not performed for Hsp60 and Cyt b_2). Note that Cyt b_2, a soluble intermembrane space protein, remained associated with the mitochondria after hypotonic shock (*lane 13*) but became fully accessible to proteinase K (*lane 14*), indicating that hypotonic treatment disrupted but did not completely remove the outer mitochondrial membrane. Note also that the levels of immunodetectable mYfh1p-*myc* were significantly increased after treatment with proteinase K (compare *lanes 11* and *13* with *lanes 12* and *14*); because this was not observed for the other proteins analyzed, it appears that clarification of the mitochondrial fraction by protease treatment somehow enhanced immunodetection of mYfh1p-*myc*. *Lanes 16* and *17*, intact mitochondria treated with proteinase K (as in *lane 12*) subjected to repeated cycles of freezing and thawing and separated into soluble (*lane 16*) and insoluble (*lane 17*) fractions by ultracentrifugation. *Lanes 18* and *19*, mitochondria subjected to hypotonic shock in the presence of proteinase K (as in *lane 14*), further subjected to repeated cycles of freezing and thawing, and separated into soluble (*lane 18*) and insoluble (*lane 19*) fractions. Attempts to improve the dot-like appearance of the mYfh1p-*myc* band in *lanes 16* and *18* were not successful due to a significant distortion of the protein samples during electrophoresis in 12.5% SDS/PAGE.

[2] P. Cavadini and F. Taroni, manuscript in preparation.
[3] J. Adamec, unpublished results.

TABLE I
S. cerevisiae strains

Strain	Genotype	Reference
YPH501	MAT**a**/α *ura3–52/ura3–52 lys2–801^{amber}/lys2–801^{amber} ade2–101^{ochre}/ade2–101^{ochre} trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*	15
Y6041 (wild type)	MAT**a** *ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*	15
*oct1*Δ	MATα *ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 oct1*Δ::*LEU2*	15
*yfh1*Δ	MATα *ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 yfh1*Δ::*HIS3*	16
*yfh1*Δ*[YFH1]*	MATα *ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 yfh1*Δ::*HIS3* + YC-*YFH1*	16
*yfh1*Δ*[YFH1-myc]*	MAT**a** *ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 yfh1*Δ::*HIS3* + YC-*YFH1-myc*	This study
Isogenic ρ^0	MAT**a** *ura3–52 lys2–801^{amber} ade2–101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 [rho^0]*	16

mobility. In agreement with this interpretation, we show in this study that N-terminally deleted variants of Yfh1p migrate in SDS/PAGE at rates slower than predicted from their molecular masses but proportional to the number of deleted amino acids (see below).

Incubation of radiolabeled pYfh1p with isolated yeast mitochondria yielded two major processing products with apparent molecular masses of ~27 kDa and ~21 kDa (designated intermediate (i), and mature (m), respectively) (Fig. 1*A*, *lane 2*; *lanes 8* and *MW* show the mobilities of iYfh1p and mYfh1p relative to those of standard proteins). Both iYfh1p and mYfh1p were associated with the mitochondrial pellet (*lane 3*) and were protected from externally added proteinase K (*lane 5*) but were degraded when protease treatment was performed in the presence of Triton X-100 (not shown). When the inner membrane potential was dissipated by addition of carbonyl cyanide *m*-chlorophenyl-hydrazone prior to import, the precursor was still associated with the mitochondrial pellet (*lane 7*) but was not protected from proteinase K (not shown), nor were the two processing products formed (*lane 7*). Thus, pYfh1p is specifically imported by isolated yeast mitochondria, and its translocation to a protease-protected compartment is associated with two proteolytic events.

This pattern of processing sets pYfh1p apart from the vast majority of mitochondrial protein precursors, which are cleaved to the mature form in a single step by MPP (14). On the other hand, two-step processing has been reported for precursors targeted to the intermembrane space, which are cleaved sequentially by MPP and the inner membrane peptidase (IMP) (22), as well as for a subset of precursors targeted to the matrix or the inner membrane, which are processed by MPP and the mitochondrial intermediate peptidase (MIP; EC 3.4.24.59) (15, 20). In light of this, and considering that MPP was shown to catalyze conversion of the mouse frataxin precursor to intermediate form (13), we investigated whether IMP or MIP might be involved in mYfh1p production. Because the proteins cleaved by these peptidases are targeted to specific mitochondrial compartments, we sought to define the intramitochondrial localization of mYfh1p. Polyclonal antibodies against Yfh1p were not available at the time of these experiments, and therefore a sequence encoding the c-*myc* epitope (9E10) was fused to the *YFH1* coding sequence immediately upstream of the stop codon. A centromeric YC-*YFH1-myc* plasmid was then substituted for the YC-*YFH1* plasmid in strain *yfh1*Δ[*YFH1*] (Table I for strain genotypes), yielding *yfh1*Δ[*YFH1-myc*] derivatives that grew as well as the parental strain under a variety of conditions (not shown), indicating that the c-*myc* epitope does not affect Yfh1p function. Radiolabeled pYfh1p-*myc* was efficiently imported by isolated mitochondria and was cleaved to two products slightly larger than the iYfh1p and mYfh1p products generated upon import of untagged precursor (Fig. 1*B*, *lanes 9* and *10*), demonstrating that the c-*myc* epitope does not affect N-terminal processing of pYfh1p. Furthermore, it was previously reported that Yfh1p fused to five copies of the c-*myc* epitope localizes to mitochondria, as determined by immunofluorescence staining (3). These data clearly indicate that Yfh1p-*myc* is fully functional, and consequently its intramitochondrial localization must reflect that of the native protein. We therefore isolated mitochondria from the *yfh1*Δ[YC-*YFH1-myc*] strain and analyzed by Western blotting the Yfh1p-*myc* distribution in mitochondrial subfractions. In intact mitochondria we detected a single protein band (Fig. 1*C*, *lane 11*) that migrated identically to the mYfh1p-*myc* product formed upon import of radiolabeled pYfh1p-*myc* into isolated mitochondria (Fig. 1*B*, *lane 10*). Although the precursor form was not detected *in vivo*, overexposed blots did reveal low levels of iYfh1p-*myc* (not shown). The mYfh1p-*myc* product was associated with intact mitochondria (Fig. 1*C*, *lane 11*) as well as mitochondria subjected to hypotonic shock (to disrupt the outer mitochondrial membrane) (*lane 13*); furthermore, mYfh1p-*myc* was protected when mitochondria were subjected to proteinase K treatment (*lane 12*) or both hypotonic shock and proteinase K treatment (*lane 14*). In contrast, mYfh1p-*myc* was fully degraded when proteinase K was added to mitochondria in the presence of Triton X-100 (*lane 15*), indicating that protection of mYfh1p-*myc* from protease treatment requires an intact inner mitochondrial membrane. The mYfh1p-*myc* product was recovered in the soluble fraction derived from mitochondria that were subjected to hypotonic shock and then repeated cycles of freezing and thawing (to gently disrupt the inner membrane) (*lane 16*). This was also the case when the soluble fraction was derived from mitochondria that were subjected to both hypotonic shock and proteinase K treatment (to degrade any proteins external to the inner membrane) prior to freezing and thawing (*lane 18*). This fractionation pattern was similar to that of Hsp60, a soluble matrix protein (23), but different from that of Cyt b_2, a soluble intermembrane space protein (24) that was fully accessible to proteinase K after hypotonic shock (*lanes 14* and *18*). Furthermore, mYfh1p-*myc* partitioned differently from the Rieske iron-sulfur protein, an inner membrane protein (25) that was detected primarily in the membrane fractions (not shown). Thus, mYfh1p-*myc* behaved like a soluble mitochondrial matrix protein, and this result was confirmed for endogenous mYfh1p using a polyclonal antibody (not shown). Although localization to the matrix is consistent with the hydrophilic nature of Yfh1p (2), Campuzano *et al.* (10) showed by immunoelectronmicroscopy that human frataxin localizes at or near the inner mitochondrial membrane. Therefore, we cannot exclude the possibility that mYfh1p is loosely bound to the inner mitochondrial membrane in intact mitochondria and that this interaction is disrupted when mitochondria are fractionated.

In any case, our results clearly indicate that mYfh1p is not localized to the intermembrane space and therefore exclude the possibility that IMP is involved in the maturation of Yfh1p. On the other hand, the fact that mYfh1p localizes to the matrix is consistent with the possibility that pYfh1p is processed in two sequential steps by two matrix-localized peptidases, MPP and MIP. Precursors cleaved by these two peptidases are characterized by a three-amino acid motif, R*X*↓(F/L/I)*XX*(S/T/

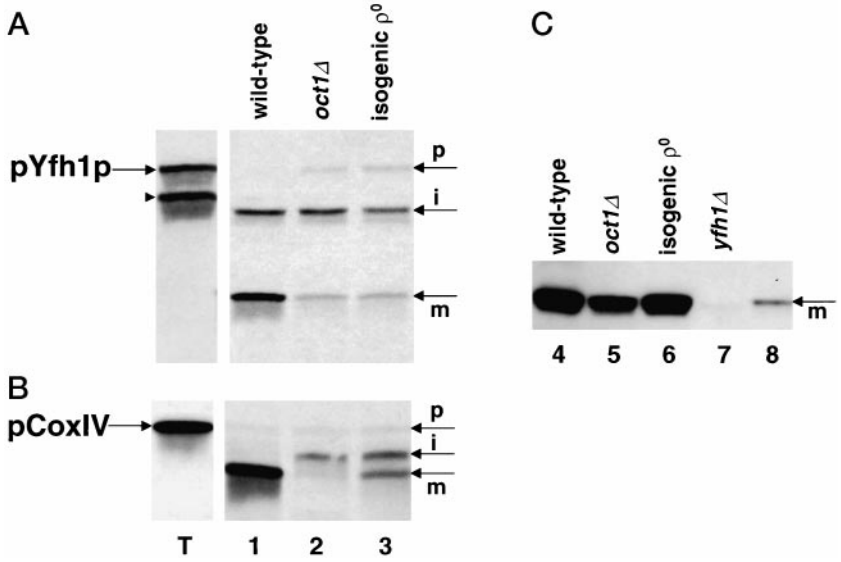

FIG. 2. **pYfh1p is processed to the mature form in yeast lacking MIP activity.** *A*, import and processing of radiolabeled pYfh1p by *oct1*Δ mitochondria. Mitochondria isolated from wild-type, *oct1*Δ, and isogenic ρ^0 yeast were incubated with Yfh1p translation mixture (*T*) for 20 min at 27 °C. Total import reactions were treated with proteinase K, and the mitochondrial pellet was reisolated by centrifugation and directly analyzed by SDS/PAGE and fluorography. The letters *p*, *i*, and *m* and the *arrowhead* are as in the legend of Fig. 1*A*. *B*, import and processing of a representative MIP substrate by *oct1*Δ mitochondria. Translation mixture containing radiolabeled CoxIV precursor (*T*) was incubated with isolated mitochondria, and processing was analyzed as described above. The letters *p*, *i*, and *m* denote pCoxIV, iCoxIV, and mCoxIV, respectively. *C*, detection of endogenous mYfh1p in *oct1*Δ yeast. Mitochondria were isolated from the strains indicated and aliquots analyzed by Western blotting using a polyclonal antibody against Yfh1p. *Lane 7*, mitochondria isolated from *yfh1*Δ yeast, which lacks endogenous Yfh1p due to disruption of the *YFH1* gene. *Lane 8*, radiolabeled mYfh1p produced upon import of pYfh1p by isolated mitochondria, as in Fig. 2*A*, *lane 1*.

G)*XXXX*↓, at the C terminus of their leader peptide (26–28). MPP initially cleaves these precursors two peptide bonds C-terminal to the Arg residue in the motif, yielding a processing intermediate with a typical N-terminal octapeptide, which is then specifically removed by MIP to yield the mature protein (29). The N-terminal region of neither pYfh1p nor the frataxin precursor contains this motif, however, suggesting that MIP is not involved in their maturation. In fact, radiolabeled pYfh1p was imported and processed to the mature form by mitochondria isolated from a knock-out mutant (*oct1*Δ) lacking yeast MIP (*OCT1*, gene; YMIP, polypeptide)[4] (Fig. 2*A*, *lane 2*). In contrast, *oct1*Δ mitochondria did not cleave the intermediate form of the cytochrome *c* oxidase subunit IV (iCoxIV) (Fig. 2*B*, *lane 2*), which is normally processed to the mature form by YMIP (15, 26). Interestingly, *oct1*Δ mitochondria produced less mature protein than did wild-type mitochondria (Fig. 2*A*, compare *lanes 1* and *2*), and in this respect behaved identically to mitochondria isolated from an isogenic ρ^0 strain (*lane 3*). Similarly, pCoxIV was inefficiently processed by *oct1*Δ and ρ^0 mitochondria (Fig. 2*B*, *lanes 2* and *3*), confirming that reduced production of mYfh1p by *oct1*Δ mitochondria is not indicative of a specific involvement of YMIP in pYfh1p processing. Rather, this effect probably results from loss of mitochondrial DNA in *oct1*Δ (26), a condition that is known to affect the efficiency of *in vitro* import assays (30). In agreement with these *in vitro* results, endogenous mYfh1p was detected by Western analysis of mitochondria isolated from *oct1*Δ yeast (Fig. 2*C*, *lane 5*), further demonstrating that YMIP is not directly involved in the maturation of Yfh1p.

Having excluded direct participation of IMP or MIP in Yfh1p processing, we tested the possibility that MPP might be solely responsible for production of both iYfh1p and mYfh1p. To determine whether typical MPP cleavage sites (26–28) are used in the processing of pYfh1p, we synthesized a series of N-terminally truncated versions of pYfh1p and used them as standards to map the N termini of iYfh1p and mYfh1p. In our SDS/PAGE system, iYfh1p ran slightly faster than a product translated from residue 21 of pYfh1p (designated M-iYfh1p) but slower than a product translated from residue 25 (Fig. 3). Thus, the first cleavage site must lie between residues 21 and 25, and indeed residues 19–22 (RYM ↓ I) match the consensus sequence R*X*(*X*/Y)↓(*X*/A/S), which is found at many MPP cleavage sites (26, 28) (Fig. 3). Similarly, mYfh1p ran between products translated from residues 52 and 56, and residues 50–53 (RFV ↓ E) also match the R*X*(*X*/Y) ↓(*X*/A/S) consensus sequence (Fig. 3). Moreover, the amino acids C-terminal to this putative MPP cleavage site include two serines and one threonine, which is consistent with observations that the mature N termini of mitochondrial proteins frequently contain small hydroxylated residues (26, 28).

To confirm that pYfh1p is indeed processed in two steps by MPP, radiolabeled pYfh1p was incubated with recombinant yeast MPP, which was reconstituted from bacterially expressed and purified subunits using a procedure similar to that described previously by Geli (21). After 10 min of incubation (Fig. 4*A*, *lane 1*), most of the input pYfh1p was no longer detected, whereas iYfh1p was accumulated along with smaller amounts of mYfh1p; incubation for an additional 10 min (*lane 2*) resulted in increased production of mYfh1p with concomitant disappearance of iYfh1p. Given that most of the precursor was converted to iYfh1p during the first 10 min of incubation (*lane 1*), we conclude that the mYfh1p accumulated in the subsequent 10 min (*lane 2*) was produced by cleavage of iYfh1p. The fact that disappearance of the precursor band was not associated with a proportional increase in the intensity of the iYfh1p and mYfh1p bands (*lane 1*) can be explained by the loss of three of the four radiolabeled methionine residues present in the precursor sequence (codons 1, 16, and 21) upon processing to intermediate form (Fig. 3). On the other hand, iYfh1p and mYfh1p are each predicted to contain a single methionine

[4] 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* (15), but this name was first assigned to open reading frame YOR330C.

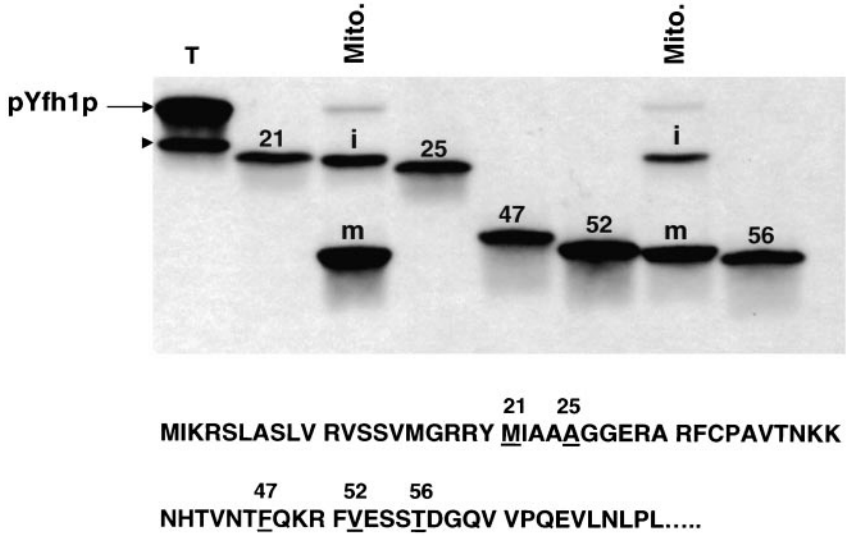

FIG. 3. **Potential MPP cleavage sites in the Yfh1p sequence.** A series of cDNAs encoding N-terminally truncated forms of Yfh1p were generated by polymerase chain reaction, and the corresponding polypeptides synthesized by coupled *in vitro* transcription-translation. *21* denotes a polypeptide translated from methionine 21 of the Yfh1p sequence (M-iYfh1p); *25*, *47*, *52*, and *56* denote polypeptides translated from methionine residues introduced at these positions of the Yfh1p sequence (the N-terminal portion of which is shown at the *bottom* of the figure). *T*, Yfh1p translation mixture; *Mito.*, mitochondrial pellets similar to that in Fig. 2*A*, *lane 1*. The letters *p*, *i*, and *m* and the *arrowhead* are as in the legend of Fig. 1*A*.

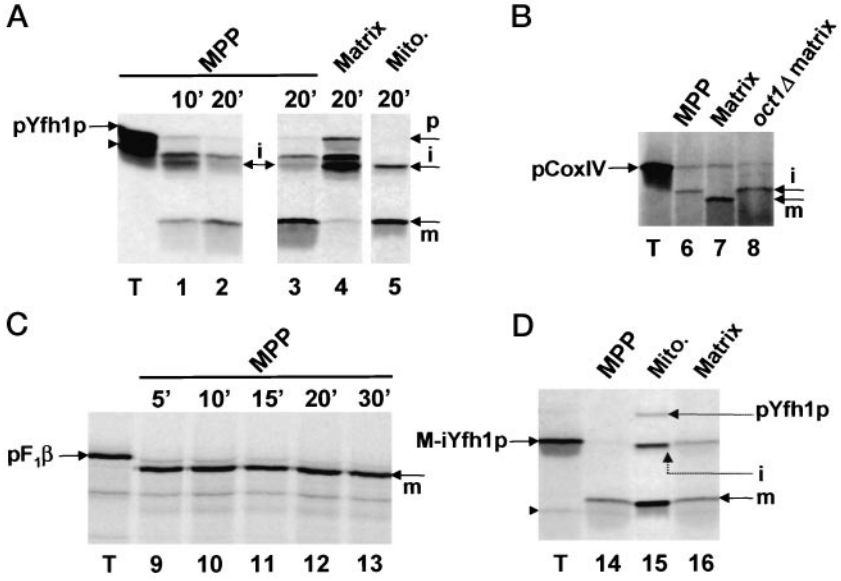

FIG. 4. **Two-step processing of pYfh1p by recombinant yeast MPP.** *A*, processing of pYfh1p. 5 μl of pYfh1p translation mixture (*T*) were incubated with 0.4 units of recombinant yeast MPP (*MPP*) in HDM buffer (total reaction volume, 20 μl), and 10-μl aliquots were withdrawn after 10 min (*lane 1*) and 20 min (*lane 2*) at 27 °C and directly analyzed by SDS/PAGE and fluorography. In two parallel reactions, pYfh1p was incubated with 0.2 units of MPP (*lane 3*) or matrix (total protein, 6 μg) derived from wild-type yeast mitochondria (Matrix; *lane 4*) for 20 min at 27 °C in HDM buffer (total reaction volume, 10 μl), and the processing reactions were analyzed as above. *Lane 5*, mitochondrial pellet similar to that in Fig. 2*A*, *lane 1*. The letters *p*, *i*, and *m* and the *arrowhead* are as in the legend of Fig. 1*A*. *B*, processing of pCoxIV. 5 μl of pCoxIV translation mixture (*T*) were incubated with 0.2 units of MPP (lane 6), wild-type matrix (total protein, 6 μg) (Matrix; *lane 7*), or matrix derived from *oct1*Δ mitochondria (total protein, 6 μg) (*oct1*Δ matrix; *lane 8*) in a total reaction volume of 10 μl, as described above. The letters *i* and *m* are as in the legend of Fig. 2*B*. *C*, processing of $pF_1\beta$. 5 μl of $pF_1\beta$ translation mixture (*T*) were incubated with 1 unit of MPP in a total reaction volume of 50 μl, and 10-μl aliquots were withdrawn at the indicated time points. *D*, processing of M-iYfh1p. 5 μl of M-iYfh1p translation mixture (*T*) were incubated with 0.2 units of MPP (*lane 14*) or wild-type matrix (Matrix; *lane 16*) in a total reaction volume of 10 μl, as described above. *Lane 15*, mitochondrial pellet similar to that in Fig. 2*A*, *lane 1*. The letters *i* and *m* are as in the legend of Fig. 1*A*; the *arrowhead* indicates a nonspecific product in the M-iYfh1p translation mixture.

residue (codon 109), and disappearance of the accumulated iYfh1p (*lane 1*) coincided with formation of an equal amount of mYfh1p (*lane 2*). Apparently identical products were generated whether pYfh1p was incubated with recombinant MPP (*lanes 1–3*), total mitochondrial matrix (*lane 4*), or isolated mitochondria (*lane 5*). It is important to note, however, that whereas mYfh1p was efficiently produced in isolated mitochondria (*lane 5*), only trace amounts of mYfh1p were produced by matrix (*lane 4*). Similarly, although 10-fold lower concentrations of MPP were sufficient for processing of pYfh1p to iYfh1p, higher enzyme levels and longer incubation times were required for conversion of iYfh1p to mYfh1p (not shown). The possibility that under these experimental conditions recombinant MPP might have cleaved iYfh1p nonspecifically seems unlikely for two reasons: first, under very similar conditions pCoxIV was processed to intermediate form only (Fig. 4*B*, *lane 6*); and second, $pF_1\beta$ was processed to the mature form after only 5 min of incubation with MPP (Fig. 4*C*, *lane 9*), but no further proteolysis occurred during an additional 25 min of incubation at 27 °C (*lanes 10–13*). Thus, a more likely explanation is that whereas MPP *per se* is sufficient to catalyze conversion of iYfh1p to mYfh1p, additional factors such as mitochondrial membrane integrity may affect the efficiency of this reaction. In fact, Knight *et al.* (12) showed previously that Ssq1p, a mitochondrial Hsp70 homologue, is required for formation of mYfh1p *in vivo*, suggesting that factors influencing the confor-

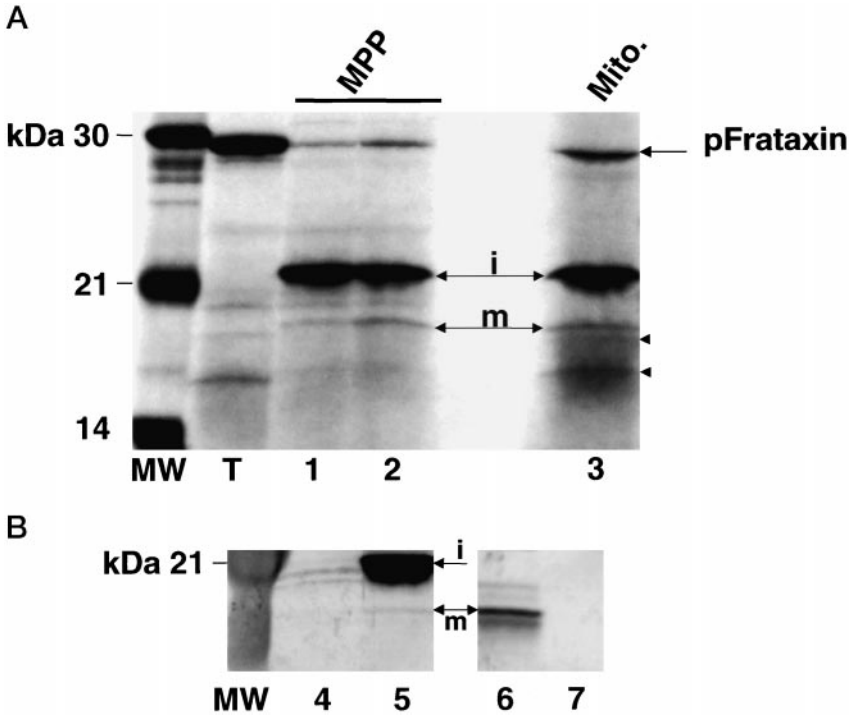

FIG. 5. **Processing of human frataxin by recombinant yeast MPP.** *A*, 6 μl of translation mixture (*T*) containing [^{35}S]methionine-labeled human frataxin precursor was incubated with 2 units of MPP in a total reaction volume of 20 μl, and a 10-μl aliquot was withdrawn from the processing reaction after 30 min at 27 °C (*lane 1*); one unit of MPP was added to the remainder of the reaction, and incubation continued for another 30 min (*lane 2*). *Lane 3*, mitochondrial pellet obtained by incubation of human frataxin precursor with freshly isolated rat liver mitochondria, followed by trypsin treatment. Processing and import reactions were directly analyzed by SDS/PAGE and fluorography. Note that the frataxin precursor used in this experiment contains an in-frame, C-terminal tag of 10 amino acids consisting of a methionine residue and the HA1 epitope. The letters *i* and *m* denote the intermediate and mature forms of frataxin, respectively. The products indicated by the *two arrowheads* in *lane 3* could be degradation products of frataxin, the significance of which remains to be established. *MW*, molecular weight markers. *B*, the following samples were analyzed by SDS/PAGE and Western blotting: *lane 4*, 6 μl of human frataxin precursor translation mixture; *lane 5*, processing reaction similar to that shown in Fig. 5*A*, *lane 1*, except that wild-type human frataxin precursor (*i.e.* lacking the C-terminal tag) was used; *lane 6*, 50 μg (total protein) of a human liver extract; *lane 7*, 1 unit of yeast MPP.

mation of iYfh1p may affect the rate of its conversion to mature form.

To further confirm that mYfh1p is produced from iYfh1p, we analyzed processing of the N-terminally truncated product translated from residue 21 of pYfh1p (M-iYfh1p), which is predicted to be one amino acid longer than iYfh1p (Fig. 3). We found that M-iYfh1p was processed to the mature form very efficiently by recombinant MPP (Fig. 4*D*, *lane 14*), and less efficiently by matrix (*lane 16*), a processing pattern similar to that observed for iYfh1p (Fig. 4*A*, *lanes 3* and *4*). The mature size product generated by cleavage of M-iYfh1p in these reactions (*lanes 14* and *16*) was indistinguishable from the mYfh1p produced upon import of pYfh1p into isolated yeast mitochondria (*lane 15*). This result provides further support to the conclusion that MPP first cleaves the Yfh1p precursor to the intermediate form and then converts this product to the mature form. Although two-step processing by MPP has been described for at least one other mitochondrial protein precursor (31), our observations do not agree with those of a previous study in which recombinant rat MPP appeared to process the precursor of mouse frataxin to the intermediate but not the mature form (13). One possible explanation for this discrepancy might be that rather than a purified enzyme, the previous study used crude extracts of bacterial cells that co-expressed both MPP subunits and perhaps a factor in these extracts inhibited the second cleavage. To test this possibility, we analyzed the processing of [^{35}S]methionine-labeled human frataxin precursor, the sequence of which is almost identical to that of mouse frataxin (4). Because the human frataxin sequence does not contain any methionine residues C-terminal to codon 76, we used a construct containing an in-frame C-terminal tag of 10 amino acids that includes a methionine residue (32). Upon incubation with recombinant yeast MPP, most of the input precursor was converted to the intermediate form (Fig. 5, *lane 1*); we also detected trace amounts of a smaller product with a mobility similar to that reported for mature frataxin (13) (*lane 1*). Addition of a fresh aliquot of MPP resulted in modestly increased conversion of the accumulated intermediate to the putative mature form (*lane 2*). This result was reproduced in three independent experiments, and a very similar pattern of processing was observed when the frataxin precursor was incubated with rat liver mitochondria (*lane 3*) or bacterially expressed recombinant rat MPP (not shown). To exclude the possibility that the C-terminal tag might interfere with cleavage of intermediate frataxin to the mature form, the wild-type frataxin precursor (*i.e.* lacking the C-terminal tag) was incubated with yeast MPP as described above, and processing reactions were analyzed by Western blotting. As was the case for the tagged precursor, we detected a major processing product corresponding to the intermediate form of frataxin and only trace amounts of the putative mature form (Fig. 5*B*, *lane 5*). The latter product migrated identically to mature frataxin, as detected in a variety of human tissue extracts (*lane 6* and not shown). Thus, under our experimental conditions, MPP did efficiently cleave the frataxin precursor to the intermediate form but could only partially process this intermediate to mature size protein. Given that the intermediate form of frataxin was processed very inefficiently even upon import into rat mitochondria (Fig. 5*A*, *lane 3*), it seems that similar to Yfh1p, frataxin is processed in two sequential steps by MPP and that species- and/or tissue-specific factors are involved in the second cleavage.

A number of recent studies indicate that Yfh1p and frataxin play conserved roles in mitochondrial iron homeostasis and free radical toxicity (1–8), supporting a model in which frataxin deficiency results in oxidative damage, which in turn leads to the degenerative lesions of FRDA (11). Moreover, the clinical variability observed in FRDA patients suggests that additional pathogenetic factors, such as mitochondrial proteins that interact with frataxin, may influence the phenotypic expression of frataxin deficiency (11). MPP was previously identified as the peptidase responsible for one of two cleavages required for the biogenesis of mouse frataxin (13), and we have demonstrated that MPP is solely responsible for two-step processing of yeast and human frataxin. Knight *et al.* (12) also identified Ssq1p, a mitochondrial Hsp70 homologue, as an additional factor required for cleavage of iYfh1p to mature form. Thus, it is tempting to speculate that this pattern of processing has a regulatory function and that genetic or environmental factors that influence the affinity of MPP for the frataxin intermediate might play a role in iron homeostasis and the clinical manifestations of FRDA.

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