Mrs3p, Mrs4p, and Frataxin Provide Iron for Fe-S Cluster Synthesis in Mitochondria*

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Yeast Mrs3p and Mrs4p are evolutionarily conserved mitochondrial carrier proteins that transport iron into mitochondria under some conditions. Yeast frataxin (Yfh1p), the homolog of the human protein implicated in Friedreich ataxia, is involved in iron homeostasis. However, its precise functions are controversial. Anaerobically grown triple mutant cells (Δmrs3/4/Δyfh1) displayed a severe growth defect corrected by in vivo iron supplementation. Because anaerobically grown cells do not synthesize heme, and they do not experience oxidative stress, this growth defect was most likely due to Fe-S cluster deficiency. Fe-S cluster formation was assessed in anaerobically grown cells shifted to air for a brief period. In isolated mitochondria, Fe-S clusters were detected on newly imported yeast ferredoxin precursor and on endogenous aconitase by means of [35S]cysteine labeling and native gel separation. New cluster formation was dependent on iron addition to mitochondria, and the iron concentration dependence was shifted dramatically upward in the Δmrs3/4 mutant, indicating a role of Mrs3/4p in iron transport. The frataxin mutant strain lacked protein import capacity because of low mitochondrial membrane potential, although this was partially restored by growth in the presence of high iron. Under these conditions, a kinetic defect in new Fe-S cluster formation was still noted. Import of frataxin into frataxin-minus isolated mitochondria promptly corrected the Fe-S cluster assembly defect without further iron addition. These findings show that Mrs3/4p transports iron into mitochondria, whereas frataxin makes iron already within mitochondria available for Fe-S cluster synthesis.

The inherited human neurodegenerative disease Friedreich ataxia has been attributed to deficiency of the conserved mitochondrial protein frataxin (1). The disease is characterized by cell death of cardiac muscle cells, dorsal root ganglia, and pancreatic beta cells (2). The loss of function phenotypes have been intensively studied in various model organisms. A connection to iron metabolism was established by the observation of iron accumulation in mitochondria of a yeast strain deleted for yeast frataxin homolog (YFH1) (3). Low levels of Fe-S cluster (4) and heme proteins (5) have also been associated with lack of frataxin. However, oxidative damage (6) and secondary genetic mutations (5) induced by accumulated iron in the Yfh1-minus cells have made it problematic to understand the primary functions of frataxin.

Yeast frataxin (7) shares common sequence and structural features with its human (8) and bacterial counterparts (9). The overall structure of frataxin has a unique fold characterized by a platform of six or seven β-sheets and two α-helices situated above the plane. Negatively charged surfaces on the protein have been found to interact with iron in vitro, although the in vivo relevance of this binding remains to be demonstrated (7). Besides iron, frataxin was recently found to physically interact with Isu1p (10), the scaffold protein for Fe-S cluster synthesis. Interactions have also been described for frataxin with ferrochelatase (5), aconitase (11), and succinate dehydrogenase (12). Frataxin has been found to form self-assembling multimers in vitro in certain buffers (13), although the in vivo relevance of this phenomenon remains unproven (14). The diversity of the interacting partner proteins provides support for numerous hypotheses regarding frataxin function, suggesting that it may be involved in Fe-S cluster synthesis (15–17), heme biosynthesis (5), aconitase repair (11), respiratory regulation (12, 18), iron detoxification (19), iron storage (20), and oxidative stress protection (3).

Mrs3p and Mrs4p are homologous proteins found in the mitochondrial inner membrane (21). Homologs have been identified in other species, including humans (22), zebrafish (23), and Cryptococcus neoformans (24). Disruption of zebrafish Mrs3/4p ortholog (Mfrn) resulted in hypochromic anemia and erythroid maturation arrest, consistent with a critical role in iron delivery into mitochondria for heme synthesis (25, 26). A role of Mrs3/4p in iron delivery for heme synthesis in mitochondria has been shown in yeast, although additional routes of mitochondrial iron entry must exist as evidenced by the mildness of loss of function growth phenotypes. An unusual phenotype was created by combining deletions of Mrs3/4 transporters and deletion of frataxin. In this triple mutant, iron accumulation in mitochondria associated with frataxin deficiency was prevented, but the defect in heme synthesis was exacerbated (26). Here we present assays for parsing the complex phenotypes related to the function of the transporters and

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frataxin in Fe-S cluster synthesis. The assays show that Mrs3p/4p transport iron into mitochondria for Fe-S cluster synthesis, whereas frataxin facilitates use of mitochondrial iron for Fe-S synthesis.

MATERIALS AND METHODS

Strains and Genotypes—A congenic set of four strains (wild type, Δmrs3Δ4, Δyfh1Δ, and ΔΔΔ) was constructed in the YPH499 background as described (26). The triple mutant, including Δmrs3::URA3, Δmrs4:: KanMX(4), and Δyfh1::TRP1, was called ΔΔΔ. The Δyfh1Δ and ΔΔΔ strains were covered by a CEN plasmid containing a genomic copy of YFH1 and CYH2. This plasmid was removed by counterselection with cycloheximide under anaerobic conditions prior to initiating experiments.

Media Composition—Rich YPAD medium contained 1% yeast extract, 2% peptone, 2% D-glucose, and 0.01% adenine. YPAD contained 2% raffinose instead of glucose. Defined YPAR contained 6.7 g/liter yeast nitrogen base without amino acids, 2% glucose, 0.8 g/liter amino acid supplements, and 1.5% Difco agar as a solidifying agent for agar plates. Iron-medium contained 150 mM NaCl, 10% glycerol, 1 mM sodium citrate, 1 mM HCl, pH 7.5, and 0.1 M sorbitol to adjust the total volume to 50 or 100 ml. The medium in sealed glass flasks. Cells were grown anaerobically in sealed flasks containing glucose as a carbon source. Cultures were grown in air for an additional 4 h at 30 °C with shaking and then harvested. Harvested cells were permeabilized and frozen with liquid nitrogen vapors as described (26). Mitochondria were isolated from permeabilized cells by douncing and differential centrifugation (28).

In Vitro Transcription and Translation of Mitochondrial Precursor Proteins—pSP64T-Yah1, containing the full-length Yah1p, and pSP64T-Yfh1, containing the full-length Yfh1p open reading frame, were constructed by PCR amplification and subcloning with restriction enzyme sites for NdeI and Xhol. Site-directed mutations were introduced into pSP64T-Yah1 to change Fe-S cluster liganding cysteines to alanines (C104A and C107A). The mutated plasmid was called pSP64T-CysM. Circular pSP64T plasmids (30 μg for each) were then linearized with BamHI, recovered by ethanol precipitation, and resuspended in nuclease-free water to a final concentration of 1 μg/μl. In vitro transcription (Ribomax SP6, Promega) was then used to produce mRNA. Each reaction contained a final concentration 0.1 μg/μl of linearized DNA and was incubated at 37 °C for 3.5 h. Transcribed RNA was purified (RNA clean-up kit, Qiagen) and quantitated on a formaldehyde gel. The proteins were translated from the in vitro transcribed mRNA using an in vitro translation system (rabbit reticulocyte system, Promega) in the presence of [35S]cysteine following the manufacturer’s directions. 35S-Labeled proteins were analyzed by SDS-PAGE to confirm their molecular weight and purity.

Bacterial Expression and Purification of Full-length Yah1p—The open reading frame for Yah1p was amplified and inserted into pET21b between NdeI and Xhol restriction enzyme sites, and the plasmid was transformed into BL21 cells. Protein expression was induced in 20 ml of culture volume at 37 °C, by addition of 1 mM isopropyl-β-D-galactopyranoside to LB ampicillin medium and, 15 min later, addition of 100 μg/ml rifampicin. After 2 h and 45 min, cells were harvested, washed, and sonicated in buffer consisting of 50 mM Tris–HCl, pH 8.0, 1 mM PMSF. Inclusion bodies were recovered by centrifugation at 15,000 × g. Following several cycles of sonication and centrifugation, the inclusion bodies were solubilized in 500 μl of 8 M urea, 50 mM Tris–HCl, pH 8.0, 1 mM PMSF. The solubilized inclusion bodies were centrifuged at 24°C for 100,000 × g for 40 min. The supernatant analyzed by SDS gel showed a single major protein band and was ~95% pure.

Import of Mitochondrial Precursor Proteins into Permeabilized Cells or Isolated Mitochondria—Permeabilized cells or isolated mitochondria were resuspended in 0.5× compensating buffer (20 mM Heps-KOH, pH 7.5, 0.6 M sorbitol, 40 mM KOAc, 10 mM Mg(OAc)2, 5 mM methionine, 1 mM diithiothreitol, 4 mM ATP, 1 mM GTP, 0.1 mg/ml BSA, 5 mM, NADH). HS-BSA buffer (20 mM Heps-KOH, pH 7.5, 0.1 mg/ml BSA, 0.6 M sorbitol) was added to adjust the total volume to 50 or 100 μl. 5 μl of radioactive protein probe or 1 μl of (2 μg/μl) urea-denatured full-length Yah1p was added for each import reaction. [35S]Cysteine, unlabeled cysteine, valinomycin (5 μM from a 0.5 mM stock made in ethanol), and iron were added in some experiments as indicated in the figure legends. The import assay was incubated at the indicated temperature for 10 or 15 min. In some experiments, trypsin was used to digest unimported precursors. In those cases, 50 μl of 0.4 mg/ml trypsin in HS-BSA buffer was added and incubated on ice for 35 min. After trypsin digestion, 100 μl of 5 mg/ml soybean trypsin inhibitor and 2 μl of 200 mM PMSF were added and incubated on ice for 5 min to neutralize trypsin. Ice-cold HS-BSA buffer for mitochondria or hyperosmotic HS-BSA buffer for perme-

2 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; DISC3(5), 3,3’-dipyridyliadiocarbocyanine iodide; WT, wild type.
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**Results**

Iron-dependent Growth under Anaerobic Conditions—Iron-dependent growth of the wild type parent was observed under anaerobic conditions, with growth and colony size increasing with iron supplementation (Fig. 1). The mutant lacking MRS3/4 was more severely retarded by iron chelation than the wild type, but colony size recovered and resembled the wild-type growth rate with addition of chelator plus 20 μM iron. The frataxin deletion strain (Δyfh1) exhibited no discernable iron phenotype compared with wild type under anaerobic growth conditions. By contrast, the triple mutant (Δmrs3/4Δyfh1 or ΔΔΔ) exhibited dramatic iron-dependent growth. For this strain, there was almost no growth under low iron conditions, and colony size only began to approach the wild type on addition of 350 μM iron. The iron concentration of 350 μM exceeds the capacity of the 1 mM chelator (ferrozine, which functions at 3:1 stoichiometry), and so iron is available over and above the contents of standard defined media.

In *Saccharomyces cerevisiae*, heme is not made under anaerobic conditions, because enzymatic steps in the porphyrin biosynthetic pathway utilize oxygen as an obligatory cofactor. Under anaerobic growth conditions, oxidative stress should be minimal, although some cellular oxidants may still be formed as intermediary metabolites or toxic by-products. Oxidative stress from iron accumulation and Fenton chemistry should not occur under such conditions. By contrast, Fe-S cluster synthesis proceeds even under strictly anaerobic conditions. Thus, the slow anaerobic growth such as was observed for the triple mutant might indicate a problem with Fe-S cluster formation.

**Aconitase Activity Deficiency under Anaerobic Conditions**—Aconitase, an abundant 4Fe-4S cluster protein, requiring an intact cluster for its activity, was studied as an indicator of the status of Fe-S cluster proteins in anaerobically grown cells. Wild type and mutants were grown under strict anaerobiosis, ruptured with glass beads under argon, and rapidly assayed for activity or processed in sample buffer for denaturing gel analysis. The objective of these procedures was to minimize oxidative damage to the protein, such as might occur during air exposure or during processing. Under these conditions, wild type and Δmrs3/4 cells showed similar aconitase enzyme activities. The Δyfh1 mutant exhibited significantly less activity (less than one-third of wild type) and the triple mutant had undetectable activity (Fig. 2B). In general there was good correlation of aconitase activities and protein levels, with Δyfh1 showing less protein and ΔΔΔ showing even less protein (Fig. 2A). In the latter case, this must represent inactive protein because aconitase activity was entirely undetectable in these cells (Fig. 2, A and B). For cultures grown anaerobically and then shifted to aerobic growth conditions for 4 h, aconitase activity was virtually

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**FIGURE 1. Anaerobic iron-dependent growth.** Congenic strains wild type (wt), Δmrs3/4, Δyfh1, and ΔΔΔ were grown in defined media with Tween and ergosterol under anaerobiosis established by bubbling argon through the growth medium. After 48 h, the cells were briefly removed from the flask and counted. Serial 1:5 dilutions starting with 5 × 10⁵ cells were spotted onto agar plates, and the plates were placed in an anaerobic chamber for 3 days before being photographed. The agar plates contained MES buffer, pH 6.1, standard defined medium supplemented with Tween and ergosterol, 1 mM ferrozine, and various amount of ferrous ammonium sulfate (none, 20, 50, and 350 μM).
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A. Yah1p

B. CysM

C. Aconitase activity

D. Anaerobic and aerobic aconitase activity

FIGURE 2. Anaerobic and aerobic aconitase activity. Strains wild type (wt), Δmrs3/4, Δyfh1, ΔΔΔ were grown anaerobically in YPAD/TE for 48 h. Cells were pelleted by a brief centrifugation and broken with glass beads under argon in a septum-sealed vial. The lysis buffer contained 50 mM Tris-Cl, pH 8, 1 mM sodium citrate, and 1.5% n-octyl-β-D-glucoside. An amount equivalent to 250 µg of total protein was tested for aconitase protein or Pgk1 protein by immunoblotting (A). Aconitase activity in the lysate was measured spectrophotometrically (B). Similar experiments were performed with cultures that were shifted to aerobic conditions in YP raffinose for 4 h or 18 h (D). In these cultures TE was omitted, but cell lysis and assay conditions were the same.

FIGURE 3. Import of radiolabeled Yah1p or Yah1p cysteine mutant precursors into mitochondria of permeabilized cells. Yeast strains were grown anaerobically and shifted to aerobic conditions for 10 min. A, radiolabeled Yah1p was imported into 20 µl of permeabilized cells (0.5 g/ml) for wild type (wt), Δmrs3/4, ΔΔΔ, and Δyfh1 (lanes 1–4, respectively). B, radiolabeled CysM mutant of Yah1p was imported into 20 µl (0.5 g/ml) of wild type-permeabilized cells in the absence (lane 1) or presence of valinomycin (val) (lane 2). Following the import reactions, trypsin digestion was performed as described under “Materials and Methods,” and the mitochondrial matrix fractions were separated on SDS gel or native gel. A, precursor; m, mature Yah1p; α, Yah1p apoprotein; h, Yah1p holoprotein.

Unchanged compared with anaerobic conditions (Fig. 2C). With longer air exposure (18 h), the pattern of aconitase activities was altered, perhaps because of regulatory adaptations and toxic effects related to oxidative stress (Fig. 2D).

Native Gel Assay of Fe-S Cluster Formation on Imported Yeast Ferredoxin, Yah1p—Ferredoxin (Yah1p), a nuclear encoded mitochondrial protein is imported into mitochondria, whereupon the targeting signal of the precursor form is proteolytically processed by matrix proteases, generating a mature form (30, 31). The mature Yah1p is then a substrate for addition of Fe-S clusters within mitochondria. Radiolabeled Yah1p precursor was imported into mitochondria, and Fe-S cluster synthesis was assessed by measuring formation of holoprotein on a native gel as described previously (30, 31). The precursor form of wild-type ferredoxin was synthesized in reticulocyte lysate in the presence of [35S]cysteine, labeling the polypeptide chain. To demonstrate that this method does indeed distinguish Fe-S cluster-loaded ferredoxin from apoprotein, a cysteine mutant (CysM) was constructed. In this mutant Yah1p, cluster-liganding cysteines (Cys-104 and Cys-108) were mutated to alanines, rendering it incapable of binding an Fe-S cluster. This cysteine mutant precursor was tested in parallel with wild-type Yah1p.

Following import of radiolabeled full-length or CysM forms of Yah1p into permeabilized cells for 10 min, the mitochondrial matrix fraction was isolated and separated by SDS or native gel electrophoresis. The appearance of a mature processed form resistant to externally added protease indicated that it had been imported into mitochondria (Fig. 3A, m, SDS). The import and processing of Yah1p did not occur in the presence of valinomycin, a potassium ionophore that disrupts the mitochondrial membrane potential (Fig. 3B, SDS, lane 2). On native gel, imported radiolabeled Yah1p migrated as holoprotein (h) or slower apoprotein (α) species (Fig. 3A), whereas the imported CysM generated only the apoprotein forms (Fig. 3B, lane 1).

Anaerobically grown cells were shifted to aerobic conditions for 4 h and permeabilized. Radiolabeled Yah1p precursor was then imported for 10 min. There was no detectable import in the Δyfh1 mutant (Fig. 3A, lane 4). The failure of import was not restricted to ferredoxin; preprotein for Put2p was likewise not imported, suggesting a general defect in mitochondrial protein import (data not shown). Approximately equal amounts of precursor were imported and processed in the other three strains (Fig. 3A, SDS, m).

Fe-S cluster forming activities on imported Yah1p were evaluated by native gel. The apoprotein signal in the Δmrs3/4 mutant was slightly increased compared with the wild type. In the triple mutant, the apoprotein was markedly increased, and holo-Yah1p was visibly decreased. As expected from the lack of import activity in the Δyfh1 cells, imported apoprotein or holo-
protein were not detected on native gel electrophoresis of these cells (Fig. 3A, native). Similar results were obtained in experiments with isolated mitochondria (not shown).

**Dependence of New Cluster Formation in Mitochondria on Iron from Outside Mitochondria, Role for Mrs3/4p Transporters—** The radiolabeled Yah1p precursor imported into mitochondria was present in very small absolute amounts (estimated at less than 1 ng per 5 μl in each reaction). Experiments designed to examine iron dependence of Fe-S cluster formation were unsuccessful. We reasoned that this might be due to the substoichiometric amounts of substrate and the presence of a small amount of contaminating or stored iron in the mitochondria. Therefore, we tested bacterially expressed urea-denatured precursor with a C-terminal His₆ tag, which could be imported into mitochondria in much larger quantities. Following the import reactions, the imported mature tagged protein (m-h₆) was detected by Western blotting and could be distinguished from the endogenous (e) protein (see Fig. 4A). Equivalent amounts of endogenous and imported Yah1-His₆ were detected by blotting in both wild-type and Δmrs3/4 mutant mitochondria following 10 min of import (Fig. 4A).

By contrast with the reticulocyte lysate synthesized precursors, formation of new Fe-S clusters on urea-denatured Yah1p precursors was strongly dependent on the addition of exogenous iron (Fig. 4B). In these latter assays, the precursor protein was unlabeled, and the import reaction into intact mitochondria was performed in the presence of radioactive [³⁵S]cysteine. Thus, newly formed Fe-S clusters were labeled with [³⁵S] and were detected as described previously (30, 31). Mitochondria isolated from cells grown anaerobically and shifted to air for 4 h were examined. Yah1-His₆ was imported into mitochondria in the presence of [³⁵S]cysteine and different amounts of iron (ferrous ascorbate). Imported Yah1p labeled on its Fe-S cluster appeared as a compact band migrating far down into the native gel (Fig. 4B, lane h). In the absence of precursor, this band was not detected (Fig. 4B, lane 15). In wild type mitochondria, the signal showed a strong dependence on added iron (Fig. 4B, compare lane 1 with no iron added and lane 2 with 1 μM iron added). The intensity of the signal remained the same with higher concentrations of added iron (Fig. 4B, lanes 2–7). Another signal was detected oriented near the top of the resolving portion of the gel and independent of precursor import (Fig. 4B, lane 15). Interestingly, this signal also increased in intensity with added iron (Fig. 4B, lanes 1–7). Further studies (data not shown) demonstrated that this signal represents aconitase, which becomes labeled on its Fe-S cluster. It was a surprise to detect labeling of aconitase in intact mitochondria, because new aconitase protein is not synthesized under these conditions. The observation implies the existence of an apoprotein pool of aconitase and repair machinery for inserting clusters into preformed apoproteins. The conditions and requirements for aconitase cluster labeling will be presented elsewhere.

In Δmrs3/4 mutant mitochondria, radiolabeled Fe-S clusters were detected on newly imported Yah1-His₆ and on pre-existing aconitase. Iron addition enhanced holoprotein formation on Yah1p, but the concentration dependence was shifted upwards. In the presence of 1 μM iron, only 5–10% of the wild-type level of holoferrredoxin was detected. In the presence of 20 μM added iron, the optimal concentration, 50–80% of the wild-type level was observed. Interestingly, iron dependence for labeling of endogenous aconitase with radioactive sulfur was also shifted upward in the Δmrs3/4 mutant (Fig. 4B, lanes 8–14). For comparison, reticulocyte lysate-synthesized ferreferredoxin, [³⁵S]cysteine labeled in the polypeptide chain, was imported into wild-type mitochondria (Fig. 4B, lane 16). Under these conditions, the aconitase band was not labeled, but both apo-(a) and holoferrredoxin (h) bands were visible (see Fig. 4B). These data suggest that mitochondrial iron import for Fe-S cluster formation is mediated by Mrs3p and Mrs4p, the high affinity mitochondrial transporters; the data also point to the existence of lower affinity iron transport system(s).

**Partial Restoration of the Low Mitochondrial Membrane Potential of Δyfh1 Cells by Growth in High Iron—** Membrane potential was measured for mitochondria isolated from the congenic strain set (wild type, Δmrs3/4, Δyfh1, ΔΔΔ) with the dye DiSC3(5). Fluorescence of this dye is quenched by concentration of the dye in mitochondria, which occurs in a membrane potential manner. Fluorescence is restored by uncouplers that interfere with membrane potential (29). Compared with the wild type, the

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**FIGURE 4. Iron dependence for de novo Fe-S cluster formation in isolated wild-type and Δmrs3/4 mutant mitochondria.** A, immunoblotting with anti-Yah1p antibody following import of Yah1p precursors for 10 min at 30 °C. Lane 1, wild type mitochondria, no precursor added; lanes 2 and 3, import of urea-denatured Yah1-His₆ into wild type and Δmrs3/4 mitochondria (200 μg), respectively; lane 4, import of radiolabeled reticulocyte lysate synthesized Yah1p into wild type mitochrondia. e, endogenous Yah1; p, unprocessed Yah1-His₆ precursor; m-H₆, matured processed Yah1-His₆. B, urea-denatured Yah1-His₆ precursor (2 μg) was imported into isolated mitochondria (200 μg) from wild-type or Δmrs3/4 mutant for 10 min at 30 °C in the presence of [³⁵S]cysteine and indicated concentrations of iron. Before the protein import, isolated mitochondria were washed with HS-BSA buffer containing 1 mM EDTA. Residual EDTA was then removed by additional washing with HS-BSA buffer. Lanes 1–7, wild-type (wt) mitochondria. Lanes 8–14, Δmrs3/4 mutant mitochondria. Lane 15, wild-type mitochondria, no precursor added. Lane 16, import of radiolabeled reticulocyte lysate-synthesized Yah1p into WT mitochondria, no added iron or [³⁵S]cysteine. Aco1, aconitase protein; h, Yah1 holoprotein; a, Yah1 apoprotein labeled on polypeptide.
membrane potentials for the Δmrs3/4 and ΔΔΔ were slightly decreased, whereas the Δyfh1 mutant exhibited an extremely low membrane potential, ~20-fold lower than the wild-type level (Fig. 5). These results explain the selective loss of precursor import capacity in the Δyfh1 mutant mitochondria.

In an effort to restore membrane potential in the mutant, the Δyfh1 strain was grown anaerobically, shifted to air for 4 h, permeabilized, and then returned to anaerobiosis for 90 min. At this point mitochondria were purified and evaluated. The deficient membrane potential was not improved by the anaerobic incubation, and the Δyfh1 membrane potential remained markedly less than the wild type handled in a similar manner (not shown). Surprisingly, growth in the presence of high concentrations of iron (100 μM ferrous ammonium sulfate, anaerobic growth shifted to air for 4 h) partially restored the membrane potential of Δyfh1 mitochondria. The other strains exhibited decreased overall membrane potential under these conditions, so the effect was to create an intermediate level of membrane potential in all the strains. The mechanisms are unknown by which growth in high iron partially restored membrane potential in Δyfh1 mitochondria (and decreased membrane potential in the other strains). However, these conditions restored import capacity to the Δyfh1 mutant mitochondria, making it possible to study Fe-S cluster formation on an imported apoprotein indicator protein.

Kinetic Defect in Fe-S Cluster Formation on Imported Yah1p in Frataxin-minus Mitochondria—The discovery of special conditions allowing restoration of membrane potential in the Δyfh1 mitochondria made possible new types of experiments. In one set of experiments, Fe-S cluster formation was examined on imported Yah1p (synthesized in reticulocyte lysate) for the panel of congenic strains (wild type, Δmrs3/4, Δyfh1, and ΔΔΔ). The strains were grown anaerobically in defined media supplemented with iron (glucose-based media with 100 μM ferrous ammonium sulfate and Tween/ergosterol mixture) and shifted to air for 4 h (raffinose-based media with 100 μM added iron), prior to isolation of mitochondria. The import with radiolabeled Yah1p was performed for 10 min and analyzed by SDS and native gels. The SDS gel showed import and processing of the Yah1p precursor in all four strains (Fig. 6A). When examined by native gel (Fig. 6B), the import reactions gave rise to holoprotein (h) and apoprotein (a) signals. Apoprotein signals were similar for all the strains, but holoprotein signals were markedly decreased for Δyfh1 and ΔΔΔ. The lack of proportionate increases in apoprotein signals in those reactions with decreased holoprotein was not clearly explained, but this result was reproducible. Perhaps the missing apoprotein species in those samples was aggregated or not well resolved on the native gels. This hypothesis is supported by time course experiments in which some but not all apoprotein can be chased into holoprotein. In the experiment presented here, holoprotein formation was equivalent for wild type and Δmrs3/4, similarly, holoprotein formation was equivalent for Δyfh1 and ΔΔΔ. The results therefore suggest that in vivo loading of cells with iron during growth effectively bypassed the need for Mrs3/4p function in iron delivery.
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was undertaken. Mitochondria were obtained by means of the special high iron growth conditions described above. In the first stage, full-length reticulocyte lysate-synthesized and radiolabeled Yfh1p was imported into mitochondria. Mitochondria were recovered by centrifugation, and a second import reaction was performed. In the second stage, urea-denatured Yah1p was imported in the presence of [35S]cysteine, with or without added iron (40 μM). Mitochondria were again recovered, and matrix fractions were analyzed for Yfh1p import, Yah1p import, and Fe-S cluster formation.

Full-length frataxin radiolabeled in the polypeptide chain was generated by translation of specific mRNA in the presence of [35S]cysteine (Fig. 8A, p). The lower bands representing smaller Yfh1p species probably derived from internal initiations, because they were not imported. The mature frataxin (m-Yfh1) detected on this gel was present in equivalent amounts following import into wild-type and Δyfh1 mutant mitochondria (Fig. 8A, lanes 3 and 4 and 7 and 8, respectively). Imported Yah1p was not detected here because the polypeptide was not labeled. Only the Fe-S cluster was labeled following import of Yah1p, and this was sensitive to disassembly induced by the SDS. Instead, Yah1-His6 import was evaluated by immunoblotting of a duplicate SDS gel (Fig. 8B). The blot developed with antibody to Yah1p revealed three different species of this protein. The endogenous form migrated the most rapidly. The mature imported form migrated more slowly because of the His6 tag, and the precursor form migrated still more slowly because of the presence of the unprocessed leader sequence. The results show decrease of the endogenous form of Yah1p in the Δyfh1 mutant, consistent with a defect in cluster formation and turnover of the apoprotein during growth. The imported processed Yah1 holoprotein (m-h6) was present in wild-type and mutant import reactions, although a lesser amount was present in the Δyfh1 mitochondria. Aconitase protein was significantly decreased in the Δyfh1 mutant. Blotting with antibody against Ssc1p, the abundant Hsp70 chaperone, provided a loading control and was nearly equivalent for all reactions (Fig. 8B).

Strikingly, import of trace amounts of frataxin into wild-type mitochondria had little effect, but the same protein imported into frataxin-minus mitochondria restored Fe-S cluster formation on imported ferredoxin (h-Yah1) and endogenous aconitase (Aco1) (Fig. 8C). The level of Fe-S cluster labeling was close to the wild-type level for both endogenous aconitase and imported Yah1p labeling (Fig. 8C, compare lane 7 and lane 1). Note that these effects were not mediated by iron addition during the course of the experiment. Therefore, iron for this frataxin-mediated Fe-S cluster formation must be already present within the isolated mitochondria. Furthermore, although addition of 40 μM iron during the assay could not substitute for the lack of frataxin (Fig. 8C, lanes 5 and 6), iron added with frataxin present was able to enhance Fe-S cluster labeling on endogenous aconitase (compare lanes 7 and 8). Taken together, these results suggest that frataxin plays a direct function in Fe-S cluster synthesis. Other roles for frataxin are possible, but they are unlikely to account for the rapid frataxin-mediated restoration of new Fe-S formation in the time scale of this experiment.

The rate of Fe-S cluster formation on imported Yah1p was further examined in a kinetic experiment. In this experiment, radiolabeled precursor produced in reticulocyte lysate was imported into wild-type and Δyfh1 mitochondria (10 min at 18°C). At this point valinomycin was added to uncouple the mitochondrial membrane potential and to interrupt further precursor import. The mitochondria were then switched to 30°C for chase periods ranging from 5 to 40 min. During the chase, additional import did not occur, and the amount of processed mature protein was roughly equivalent for all time points (Fig. 7A). However, the same samples analyzed on native gel (Fig. 7B) showed time-dependent formation of holoprotein. In the wild type (Fig. 7B, lanes 1–4), the holoprotein signal progressively increased during the 40-min time course. In the Δyfh1 mitochondria (Fig. 7B, lanes 5–8), the holoprotein band was less intense at base line and after 10 or 20 min of chase. Following a 40-min chase period (Fig. 7B, lane 8), however, the holoprotein signal was markedly increased compared with base line and approached the intensity in the wild type. Thus, the defect in holo-Yah1p formation in the frataxin-minus strains was not absolute but rather a kinetic defect, with longer incubation times permitting additional holoprotein synthesis.

Frataxin Import Restores Fe-S Cluster-forming Activity to Frataxin-minus Mitochondria—To further examine the role of frataxin in Fe-S cluster formation, a two-stage import assay was undertaken. Mitochondria were obtained by means of the special high iron growth conditions described above. In the first stage, full-length reticulocyte lysate-synthesized and radiolabeled Yfh1p was imported into mitochondria. Mitochondria were recovered by centrifugation, and a second import reaction was performed. In the second stage, urea-denatured Yah1p was imported in the presence of [35S]cysteine, with or without added iron (40 μM). Mitochondria were again recovered, and matrix fractions were analyzed for Yfh1p import, Yah1p import, and Fe-S cluster formation.

Full-length frataxin radiolabeled in the polypeptide chain was generated by translation of specific mRNA in the presence of [35S]cysteine (Fig. 8A, p). The lower bands representing smaller Yfh1p species probably derived from internal initiations, because they were not imported. The mature frataxin (m-Yfh1) detected on this gel was present in equivalent amounts following import into wild-type and Δyfh1 mutant mitochondria (Fig. 8A, lanes 3 and 4 and 7 and 8, respectively). Imported Yah1p was not detected here because the polypeptide was not labeled. Only the Fe-S cluster was labeled following import of Yah1p, and this was sensitive to disassembly induced by the SDS. Instead, Yah1-His6 import was evaluated by immunoblotting of a duplicate SDS gel (Fig. 8B). The blot developed with antibody to Yah1p revealed three different species of this protein. The endogenous form migrated the most rapidly. The mature imported form migrated more slowly because of the His6 tag, and the precursor form migrated still more slowly because of the presence of the unprocessed leader sequence. The results show decrease of the endogenous form of Yah1p in the Δyfh1 mutant, consistent with a defect in cluster formation and turnover of the apoprotein during growth. The imported processed Yah1 holoprotein (m-h6) was present in wild-type and mutant import reactions, although a lesser amount was present in the Δyfh1 mitochondria. Aconitase protein was significantly decreased in the Δyfh1 mutant. Blotting with antibody against Ssc1p, the abundant Hsp70 chaperone, provided a loading control and was nearly equivalent for all reactions (Fig. 8B).

Strikingly, import of trace amounts of frataxin into wild-type mitochondria had little effect, but the same protein imported into frataxin-minus mitochondria restored Fe-S cluster formation on imported ferredoxin (h-Yah1) and endogenous aconitase (Aco1) (Fig. 8C). The level of Fe-S cluster labeling was close to the wild-type level for both endogenous aconitase and imported Yah1p labeling (Fig. 8C, compare lane 7 and lane 1). Note that these effects were not mediated by iron addition during the course of the experiment. Therefore, iron for this frataxin-mediated Fe-S cluster formation must be already present within the isolated mitochondria. Furthermore, although addition of 40 μM iron during the assay could not substitute for the lack of frataxin (Fig. 8C, lanes 5 and 6), iron added with frataxin present was able to enhance Fe-S cluster labeling on endogenous aconitase (compare lanes 7 and 8). Taken together, these results suggest that frataxin plays a direct function in Fe-S cluster synthesis. Other roles for frataxin are possible, but they are unlikely to account for the rapid frataxin-mediated restoration of new Fe-S formation in the time scale of this experiment.

FIGURE 7. Defect in Δyfh1 is kinetic. Mitochondria from wild type (wt) or Δyfh1 were prepared from cells grown in high iron (100 μM). The radiolabeled Yah1 precursor prepared in reticulocyte lysate was imported into wild type or Δyfh1 mitochondria at 18°C for 10 min, and the import reactions were stopped by addition of valinomycin. The reactions were shifted to 30°C for varying periods, as indicated, to allow observation of time-dependent Fe-S cluster synthesis and loading on the imported Yah1p. Following import reactions, the mitochondria matrix fractions were separated on SDS (A) or native (B) gels. Lanes 1–4, wild-type mitochondria incubated 5, 10, 20, or 40 min following addition of valinomycin. Lanes 5–8, Δyfh1 mitochondria incubated for 5, 10, 20, or 40 min following addition of valinomycin. p, precursor; m, mature; a, Yah1 apoprotein; h, Yah1 holoprotein.

The rate of Fe-S cluster formation on imported Yah1p was further examined in a kinetic experiment. In this experiment, radiolabeled precursor produced in reticulocyte lysate was imported into wild-type and Δyfh1 mitochondria (10 min at 18°C). At this point valinomycin was added to uncouple the mitochondrial membrane potential and to interrupt further precursor import. The mitochondria were then switched to 30°C for chase periods ranging from 5 to 40 min. During the chase, additional import did not occur, and the amount of processed mature protein was roughly equivalent for all time points (Fig. 7A). However, the same samples analyzed on native gel (Fig. 7B) showed time-dependent formation of holoprotein. In the wild type (Fig. 7B, lanes 1–4), the holoprotein signal progressively increased during the 40-min time course. In the Δyfh1 mitochondria (Fig. 7B, lanes 5–8), the holoprotein band was less intense at base line and after 10 or 20 min of chase. Following a 40-min chase period (Fig. 7B, lane 8), however, the holoprotein signal was markedly increased compared with base line and approached the intensity in the wild type. Thus, the defect in holo-Yah1p formation in the frataxin-minus strains was not absolute but rather a kinetic defect, with longer incubation times permitting additional holoprotein synthesis.
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A two-stage assay was performed with 400 μg of protein import-competent mitochondria (as in Fig. 7). Before the two-stage assay, isolated mitochondria were washed with HS-BSA buffer containing 1 mM EDTA. Residual EDTA was then removed by additional washing with HS-BSA buffer. In the first stage, radiolabeled reticulocyte lysate-synthesized Yfh1p was imported into some samples, as indicated, at 18 °C for 10 min. In the second stage, urea-denatured Yah1-His6 was imported into all samples in the presence of [35S]cysteine. 40 μM ferrous ammonium sulfate was added to some samples, as indicated, at the beginning of the second stage of the assays. The samples were divided and analyzed by 14% denaturing SDS-PAGE and transferred to nitrocellulose. Samples 1–8 were loaded in the same order as A and B. Aconitase, Aco1, aconitase; h, Yah1 holoprotein for Yah1-His6 labeled in the cluster with [35S]sulfide (SDS-sensitive labeling); Yfh1, mature Yah1 protein labeled with [35S]cysteine in the polypeptide (SDS-resistant labeling). wt, wild type.

DISCUSSION

The process of Fe-S cluster formation is essential in eukaryotic cells, reflecting the existence of essential Fe-S cluster proteins such as those implicated in ribosome biogenesis (32, 33). Of course, iron must be required for Fe-S cluster formation. However, although many essential proteins have been implicated in the process of Fe-S cluster synthesis (34–36), none of these have been shown to provide iron (37). Iron delivery to mitochondria for use in Fe-S cluster formation (and heme synthesis) seems to be characterized by redundancy and alternative routes. Thus mutations in particular genes interfere with the process but do not abrogate it. Studies to elucidate this process therefore must rely on combinations of mutations, specialized growth conditions, and biochemical assays. Here we have characterized the roles of the mitochondrial carrier proteins Mrs3/4p and frataxin. We find that Mrs3/4p and frataxin act in the same pathway mediating iron utilization for Fe-S cluster synthesis. However, Mrs3/4p mediates iron delivery from outside to inside of mitochondria, whereas frataxin facilitates use of iron already present within mitochondria.

Mrs3p and Mrs4p are similar and functionally redundant mitochondrial carrier proteins implicated in iron homeostasis (21). Titration with different concentrations of ferrous iron in wild-type mitochondria showed maximum reconstitution of Fe-S cluster signal in the presence of 1 μM of added iron. By contrast, in the Δmrs3/4 mutant mitochondria a comparable level of synthesis was not achieved even with 40 μM added iron. The results demonstrate a rapid and high affinity Mrs3/4p-mediated iron transport system and also show the existence of a backup lower affinity system. Special growth conditions involving shift of cells from anaerobic to aerobic conditions may have facilitated detection of this activity because of increased iron demand for synthesis of respiratory complexes following the shift to air. These findings are consistent with biochemical studies using 55Fe labeling and immunoprecipitation of target Fe-S cluster proteins in wild-type and Δmrs3/4 mutant mitochondria (25).

In these experiments with isolated intact mitochondria, iron added as a reduced iron salt (ferrous ascorbate) was rapidly and efficiently utilized for Fe-S cluster formation within mitochondria. However, this is unlikely to be the form of iron that is presented to mitochondria within living cells. The true biological substrates for Mrs3/4p transport specifically and for mitochondrial iron transport generally have not been identified. Recent structural data on the mitochondrial carrier protein family might provide a hint relevant to this question (38). The carrier protein family is a large family of proteins located in the mitochondrial inner membrane, allowing exchange of substrates between cytosol and mitochondrial matrix. In an analysis of predicted substrate-binding sites in the cytosolic conformation of family members, three classes were identified, keto acid carriers, amino acid carriers, and adenine nucleotide carriers. However, Mrs3/4p, although sharing many of the common motifs with other family members, exhibited unique predicted substrate-binding residues. The substrate binding pocket was predicted to transport a coordinated form of iron. Identifying this iron-coordinating molecule will be critical for further studies of Mrs3/4p iron transport.

A role of frataxin in iron use has been proposed, but as for Mrs3/4p, the existence of alternative mechanisms and pathways has confounded many studies. Iron can be used for Fe-S cluster formation (and heme synthesis) in the absence of frataxin in some settings. Several papers have observed that frataxin is not essential for Fe-S cluster synthesis, and even the
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A difficulty that had to be overcome in order test Fe-S cluster loading on imported indicator proteins was the loss of import capacity in the Δyfh1 mutant. This has been also noted by others (30). Import capacity was restored to the Δyfh1 mutant genetically by combination with Δmrs3 and Δmrs4 mutations; in this case, mitochondrial iron was lowered compared with the Δyfh1 alone (21, 26). By contrast import capacity was also restored by growth in the presence of iron (100 µM ferrous iron) for 4 h in air, and in this case, the mitochondria contained tremendously increased amounts of iron (about 20 times) compared with Δyfh1 grown in standard defined medium (not shown). The anaerobic growth and shift to air for 4 h may also have been important for producing these phenotypes. However, there appears to be a paradox: conditions that lowered mitochondrial iron (Δmrs3/4/yfh1) and conditions that raised mitochondrial iron (Δyfh1 grown in high iron) both restored membrane potential and import capacity to the Δyfh1 mutant mitochondria. The resolution of this paradox must await further study, but the data imply that all mitochondrial iron is not equal. Functional differences are likely to exist in mitochondrial iron pools. A beautiful demonstration of this fact has recently been published (40). In this paper, Culotta and co-workers (40) show that iron accumulating in Δyfh1 or Δmtm1 mutant mitochondria is differentially available for inactivation of Sod2p (only Δmtm1 iron is inserted). Thus iron in the mitochondria of Δyfh1 and Δmtm1 mutants is functionally distinct. Further work will be needed to explore the nature of these functional differences in mitochondrial iron pools.

The iron-loaded Δyfh1 mitochondria recovered import capacity, allowing characterization of the biochemical defect. As suggested by others, the defect in Fe-S cluster formation on imported ferredoxin was not absolute (30). Reticulocyte lysate-synthesized ferredoxin was imported efficiently but was converted to holoprotein inefficiently. The defect was kinetic, and longer incubations allowed the mutant to catch up with the wild type in new holoprotein synthesis. The key question as to whether the effects of frataxin on Fe-S cluster synthesis are direct or indirect was addressed by import of frataxin into frataxin-minus mitochondria. Following import, cluster formation was examined on imported Yah1p or endogenous aconitase. Dramatic restoration of cluster formation was observed during the 10–20 min of the assay. The effect was observed without adding iron during the assay, indicating that iron already present within mitochondria could be utilized for this process. An indirect effect or regulatory effect of frataxin, although not excluded, is unlikely during the brief time frame of the assay.

Other evidence of a role for frataxin in Fe-S cluster formation has been presented. The gene co-evolved with other Fe-S cluster assembly components, and they segregated together throughout evolution (41). Fe-S cluster deficiencies have been correlated with lack of frataxin or frataxin depletion in model systems (2). Fe-S cluster-deficient proteins have been described in impaired tissues of human disease patients with Friedreich ataxia (4). Genetic interaction of frataxin with Isu1p and other Fe-S cluster assembly components has been presented in the form of synthetic lethality of the combined mutations (14). Biochemical evidence of physical interaction of yeast frataxin with Isu1p, the Fe-S assembly scaffold protein has been provided, and frataxin has been shown to facilitate formation of the cluster on Isu1p (10). Interaction of the frataxin has been demonstrated in vitro (7, 20). However, uncertainty about whether frataxin is directly or indirectly involved in Fe-S cluster formation has persisted because of extensive data on the role of frataxin in oxidative stress protection and because of the known sensitivity of many Fe-S clusters to oxidative destruction. For example, human mitochondrial ferritin expression was able to correct growth phenotypes of a frataxin deletion strain in yeast (42), although this protein would not be expected to mediate Fe-S cluster formation, and the human protein would not be expected to interact with yeast Isu1p. Likewise manganese supplementation was able to rescue many of the phenotypes of the Δyfh1 mutant, including Fe-S cluster protein deficiencies (39). In view of the studies presented here, it appears that frataxin does indeed play a direct role in Fe-S formation and in mobilization of endogenous iron stores within mitochondria for Fe-S cluster synthesis. Antioxidant and other functions of the frataxin are also possible but probably do not come into play during the short time frames of the organelle import assays.

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