Frataxin and mitochondrial carrier proteins, Mrs3p and Mrs4p, cooperate in providing iron for heme synthesis

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Running title: Frataxin and Mitochondrial Carrier Proteins

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Frataxin is a conserved mitochondrial protein implicated in cellular iron metabolism. Deletion of the yeast frataxin homolog (YFH1) was combined with deletions of MRS3 and MRS4, mitochondrial carrier proteins implicated in iron homeostasis. As previously reported, the Δyfh1 mutant accumulated iron in mitochondria, whereas the triple mutant (ΔΔΔ) did not. When wild-type, Δmrs3/4, Δyfh1 and ΔΔΔ strains were incubated anaerobically, all strains were devoid of heme and protected from iron and oxygen toxicity. The cultures were then shifted to air for a short time (4-5 hours) or a longer time (15 hours), and the evolving mutant phenotypes were analyzed (heme dependent growth, total heme, cytochromes, heme proteins, and iron levels). A picture emerges from these data of defective heme formation in the mutants, with a markedly more severe defect in the ΔΔΔ than in the individual Δmrs3/4 or Δyfh1 mutants (a “synthetic” defect in the genetic sense). The defect(s) in heme formation could be traced to lack of iron. Using a real-time assay of heme biosynthesis, porphyrin precursor and iron were presented to permeabilized cells and the appearance and disappearance of fluorescent porphyrins were followed. The Mrs3/4p carriers were required for rapid iron transport into mitochondria for heme synthesis, whereas, there was also evidence for an alternative slower system. A different role for Yfh1p was observed under conditions of low mitochondrial iron and aerobic growth (revealed in the ΔΔΔ), acting to protect bioavailable iron within mitochondria and to facilitate its use for heme synthesis.

INTRODUCTION

Frataxin is a mitochondrial protein, deficiency of which has been implicated in the human disease Friedreich’s ataxia (1). This is a progressive disease in which specialized tissues (neurons of dorsal root ganglia, cardiac muscle cells, pancreatic beta cells) undergo mitochondrial degeneration and cell death (2). A link to iron metabolism has been established, originally by the characterization of a yeast strain deleted for the yeast frataxin homolog (YFH1) (3). In this yeast mutant, iron accumulates in mitochondria as toxic inorganic precipitates (4); at the same time, iron proteins, including iron-sulfur and heme proteins, are decreased or inactive (4,5). The iron accumulations in mitochondria generate damaging oxidative intermediates. Many of the phenotypes of the frataxin deletion mutant appear to be secondary consequences of oxidative damage to proteins, lipids, and DNA (6,7). Critical to understanding the functions of frataxin will be the ability to distinguish primary consequences of lack of frataxin from secondary toxic events.

Frataxin has a highly conserved sequence and structure. Human (8), bacterial (9), and yeast (10) forms have been structurally characterized and exhibit similar folds. The structures are characterized by a platform of six or seven beta sheets and two alpha helices in a separate plane, with a hydrophobic core and a negatively charged groove between the alpha helices. The biochemical functions of frataxin have remained poorly defined, in part because of difficulties in
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distinguishing primary and secondary phenotypes of frataxin deficient mutants. Iron-sulfur cluster formation (11), aconitase repair (12), heme synthesis (4), antioxidant function (5,13), and iron storage (14) have been proposed as functions of frataxin. Protein interactions within mitochondria are likely to play an important role in frataxin functions, and interactions with Isa1p (15), aconitase (12), and ferrochelatase (4,16) have been described. Isa1p is a scaffold protein which functions as an intermediate in Fe-S cluster synthesis (17,18), and a role of frataxin in this process has been supported by biochemical studies (11,19,20). Genetic data also support this function of frataxin, in that mutations in various genes involved in Fe-S cluster synthesis combine with frataxin mutations to produce synthetic lethality (21,22). Ferrochelatase is the mitochondrial enzyme involved in iron insertion into porphyrin to make heme. Consistent with a role in heme synthesis, a high-affinity interaction of frataxin and ferrochelatase has been described (4,10,20).

Mrs3p and Mrs4p are homologous proteins located in the mitochondrial inner membrane. These proteins belong to the mitochondrial carrier family and have been implicated in iron metabolism (23). Some data suggest that these carriers are able to transport iron into mitochondria (24), although the mutant phenotypes resulting from deletions of both genes (∆mrs3/4) are mild. Complex regulatory changes occurring in the ∆mrs3/4 mutant strains make it difficult to ascertain which effects are primary and which are secondary (25). Knockouts of the major mitochondrial carriers involved in delivery of iron for heme synthesis would be expected to cause heme deficiency, and yet this has not been observed in the ∆mrs3/4 mutant. Cytochrome levels are often good indicators of heme synthesis, and these are low or abnormal in mutants involved in heme biosynthesis (26). Cytochromes in the ∆mrs3/4 mutant were reportedly normal (23).

Here we describe a novel genetic and biochemical interaction implicating frataxin and Mrs3/4p in heme synthesis. In some cases, the various yeast strains were grown in the absence of oxygen and shifted to air, thereby providing a uniform starting point for experiments without heme and without iron toxicity. The roles of the Mrs3/4p carriers and frataxin in iron handling for heme synthesis could then be distinguished by biochemical assays. By using these types of experiments, the Mrs3/4p carriers were implicated in iron delivery into mitochondria whereas Yfh1p was implicated in making iron bioavailable within mitochondria.

**EXPERIMENTAL PROCEDURES**

**Strains and genotypes** 53-76 (MATalpha ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 his3-Δ200 leu2-Δ1 cyh2) was derived from strain YPH500 by growth in the presence of cycloheximide and selection for resistant clones. Similarly 53-75, named wild-type in the text, (MATa ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 his3-Δ200 leu2-Δ1 cyh2 ∆mrs3::URA3) was made by introducing deletions for MRS4 (using a G418 cassette) and MRS3 (using URA3) into strain 53-75 (27). Correctness of the genomic insertions was verified by PCR. 59-32, called Δyfh1 shuffle strain, (MATa ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 his3-Δ200 leu2-Δ1 cyh2 ∆yfh1::TRP1 [pRS318-LEU2-CYH2-YFH1]) was constructed from 53-75 as described. In this strain, the Δyfh1 mutation is covered by the CEN and CYH2 containing plasmid pRS318 with the YFH1 HindIII genomic fragment (28). The plasmid was removed by counter selection on media containing 10 mg/ml of cycloheximide, which is toxic in the presence of the CYH2 allele. The mutant following removal of the covering plasmid was called simply Δyfh1. 82-43 (MATa ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 his3-Δ200 cyh2 Δyfh1::TRP1 Δmrs4::KAN Δmrs3::URA3 [pRS318-YFH1-LEU2]) was a shuffle strain derived from 59-32 in which deletions for MRS4 (using a G418 cassette) and MRS3 (using URA3) have been introduced. The pRS318 plasmid was removed by treatment with cycloheximide, and the mutant was referred to as ΔΔΔ or Δmrs3/4/Δyfh1.

**Genetic Procedures** Crosses were performed by patching strains of opposite mating types and picking zygotes with a micromanipulator after four hours. Crossing of the shuffle strains with the 53-76 parental strain was performed in this manner, and the YFH1 plasmid was removed from the diploid by counterselection on cycloheximide.
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before sporulation. Experiments with Δyfh1 or ΔΔΔ mutants were initiated with the shuffle strains, and the covering plasmid was removed by counter selection. Complete removal of the covering plasmid was verified for each experiment by testing for leucine auxotrophy, since the YFH1 bearing pRS318 plasmid also carried a LEU2 marker gene. For most experiments, counter selection was performed anaerobically by streaking shuffle strains on agar plates consisting of YPAD with 0.2% Tween 80, 40 µg/ml ergosterol and 10 mg/ml cycloheximide in an anaerobic chamber.

**Media composition**

Rich YPAD medium contained 1% yeast extract, 2% peptone, 2% D-glucose, and 0.01% adenine. YPAR contained 2% raffinose instead of glucose. Defined medium contained 6.7 g/l yeast nitrogen base without amino acids, 2% glucose, 0.8g/l amino acid supplements and 1.5% Difco agar as a solidifying agent for agar plates. Iron buffered plates consisted of modified defined media to which pH buffer (50 mM MES pH 6.1) and iron chelator (1 mM ferrozine) were added. Different amounts of ferrous iron (0, 20, 50, 350 µM) were added back to establish windows of available free iron as described (29). For some experiments the following additions were included: 0.2% Tween 80, 40 µg/ml of ergosterol, 40 µg/ml methionine and 20 µM or 50 µM hemin. Hemin stocks were made by dissolving powder in a small amount of 1 M KOH and diluting into a larger volume of buffer (0.2 M TrisCl pH 8.0) and then adjusting the volume and pH to 7.8 with concentrated HCl to give a final concentration of 10 mM. For anaerobic growth, agar plates were placed in an anaerobic chamber with a BBL GasPak (Becton Dickinson). For anaerobic growth of liquid cultures, argon gas (Airgas) was bubbled through the media in sealed glass flasks. Cells were grown at 30 °C as stationary cultures (anaerobic) or with circular agitation (aerobic).

**Ferric reductase** The assay was performed as described (30).

**Iron levels** Samples of permeabilized cells (800 mg equivalent protein) or mitochondria (80 mg equivalent protein) were placed in microcentrifuge tubes. Following treatment with iron-free nitric acid, these were subjected to metal analysis by ICP-MS (laboratory of Dr. Robert Poppenga, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, PA 19348).

**Catalase** Potassium phosphate buffer (1 ml, 30 mM pH 7.0) was added to a quartz cuvette. H2O2 (10µl, 1.3M) was then added to give a 13 mM final concentration. The reaction was started by addition of 10 µl lysate from 30 X 10^6 cells and absorbance at 240 nm was measured for 2 minutes. The rate of decrease, reflecting catalase activity, was converted to activity by the extinction coefficient 4.36 X 10^4 cm^2/mol (31).

**Aconitase** Activity was measured in permeabilized cells (10 µl) in assay buffer (50 mM TrisCl pH 7.5, 0.5% Tween 80). The assay follows conversion of isocitrate to cis-aconitate over time measured by absorbance at 240 nm (32).

**Ferrochelatase activity in mitochondrial lysate (from added iron)** Protoporphyrin IX (PPIX, 200 ng) was added to mitochondrial lysates (prepared in air) in 2 ml assay buffer (0.1M TrisCl pH 7.5, 0.5% Tween 80). The lysate was rendered anaerobic by bubbling with argon for 5 minutes in a cuvette with a rubber septum. A baseline fluorescence tracing was obtained with excitation 410 nm and emission 632 nm. The assay was initiated by injection of 10 µM of ferrous iron (final concentration) from a 200 mM stock of ferrous ammonium sulfate in an anaerobic stoppered vial. A time trace was collected, and the initial decrease in slope of the PPIX fluorescence represented the ferrochelatase activity in the lysate (33,34).

**Cell permeabilization** Cells were grown anaerobically in sealed flasks containing YPAD supplemented with 0.2% Tween 80 and 20 mg/ml of ergosterol bubbled with argon. After 48 hours, the cultures were washed in water and resuspended in an equal volume of YPAR at O.D.₀₀₀ of about 1. Cultures were grown in air for an additional 4 hours at 30 C with shaking and then harvested. After washing with water, cells were treated with 0.1 M Tris pH 9.4, SO₄²⁻, 100 mM dithiothreitol for 10 minutes at 30 C. Spheroplasts were formed by digestion with Zymolase (1 mg/g wet weight, Seikagaku) in 10 ml spheroplasting buffer (1.2 M sorbitol, 20 mM KH₂PO₄ pH 7.5) for 1 hour at 30 C. Spheroplasts were pelleted by centrifugation (1,500 x g), washed with buffer at room temperature and...
allowed to metabolically recover in 20 ml of recovery solution (0.35 volumes of 2 M sorbitol per 0.75 volumes of YPAD medium) by incubating at 30°C with gentle shaking for 20 minutes. Recovered spheroplasts were then pelleted by centrifugation (1,500 X g) at room temperature, and gently resuspended in permeabilization buffer (20 mM Hepes KOH pH 6.8, 400 mM sorbitol, 150 mM potassium acetate, 2 mM manganese acetate). The spheroplast suspensions were aliquoted into microcentrifuge tubes and suspended over liquid nitrogen vapors to allow slow freezing (35,36).

**Heme synthesis in permeabilized cells (from endogenous iron)** An aliquot of permeabilized cells (100 µl of 0.5 g/ml wet weight) was thawed and very gently added to assay buffer consisting of 50 mM TrisCl pH 7.5, 0.6 M sorbitol in a water jacketed fluorescence cuvette at 30°C. Protoporphyrinogen, a non-fluorescent heme precursor (37), was added at 50 nM or 250 nM final concentration. Time traces were collected to monitor the appearance of PPIX (excitation 410 nm, emission 632 nm) and the appearance of zinc protoporphyrin (excitation 410 nm, emission 587 nm).

**Heme synthesis in permeabilized cells (from exogenous iron)** Ferrous ammonium sulfate (10 µM) was prepared by dissolving in water and bubbling with argon in a septum-sealed vial. Iron was removed with a syringe and injected into the assay cuvette to give a final concentration of 10 µM. NADH (5 mM final concentration) was added at the same time as the iron and preincubated with the permeabilized cells for 2 minutes at 30°C before each assay. Time traces (180 seconds) at 632 nm or repeated emission scans from 550 nm to 650 nm (each requiring 100 sec) were collected. Protoporphyrinogen oxidase activity was measured fluorimetrically (38) by treating the permeabilized cells with the iron and zinc chelator bipyridyl (10 mM added from a 1 M stock in ethanol) and dithiothreitol (5 mM) prior to addition of the protoporphyrinogen.

**Heme synthesis in permeabilized cells measured by radiolabeling with ⁵⁵Fe** An aliquot of permeabilized cells (100 µl) was centrifuged in a microcentrifuge tube at 1500 x g at 4°C for 10 minutes. Cells were resuspended in assay buffer (0.1 M Tris-Cl, 0.6 M sorbitol, pH 7.5) and the volume was adjusted to 83 µl. NADH (5 mM) and ferrous ascorbate (10 µM) were added and the reaction was incubated at 30°C for 2 minutes. Protoporphyrinogen (0.5 nmoles) was added and the reaction was incubated at 30°C for an additional 3 minutes. The reaction was stopped by immersing the tube in ice water, and then 300 µl HCl (0.2M) was added. The tube was vortexed for 30 seconds. Methyl ethyl ketone (500µl) was added and the tube was vortexed for 30 seconds. The sample was centrifuged (20,000 x g) for 5 minutes and 200 µl of the supernatant fraction was removed and subjected to scintillation counting.

**RESULTS**

**Synthetic slow-growth phenotype from combining Δmrs3, Δmrs4 and Δyfh1** The pleiotropic Δyfh1 phenotype, characterized by accumulations of toxic iron in mitochondria and deficiencies of iron proteins, has been difficult to tease apart in terms of cause and effect. Deletions of MRS3 and MRS4 in the Δyfh1 context (ΔΔΔ) have been reported to revert the mitochondrial iron accumulation phenotype (23). Thus, in this mutant strain, deficiencies resulting from toxic mitochondrial iron overload should be ameliorated whereas, by contrast, phenotypes resulting from interruption of iron trafficking or iron utilization should be exacerbated.

A haploid parental strain was transformed with constructs to delete MRS4 and then to delete MRS3. The single and double mutants grew normally in the rich or defined medium used for selection of the knockout transformants, and...
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secondary genetic changes were not observed. By contrast, the Δyfh1 strain was slow growing and genetically unstable in air. More rapidly growing suppressor mutations appeared with high frequency, and in many of the suppressor mutants, a severe global heme deficiency, associated with the Δyfh1 mutation, was reverted (4). In a previous report describing the triple mutant (Δmrs3/4/Δyfh1; ΔΔΔ) a Δyfh1 strain with normal cytochromes was used as the starting point, and therefore phenotypes of this strain or its derivatives due to heme deficiency may have been obscured by the presence of suppressor mutations (23). To minimize such problems from suppressors, shuffle strains were created in which the Δyfh1 deletion was “covered” by a plasmid carrying a wild-type YFH1 genomic allele. Three crosses were performed between the parental strain and the Δyfh1 shuffle, the Δmrs3/4 mutant, or the ΔΔΔ shuffle strain, respectively. The covering YFH1 plasmids were removed from the diploid shuffle strains by counterselection with cycloheximide, exposing the Δyfh1 allele. The diploids, which appeared uniform in their growth properties, were then sporulated, and tetrads were dissected. Tetrad clones were photographed after five days, and genotypes were determined by PCR with appropriate primers. The backcross of Δyfh1 yielded two larger wild-type and two smaller Δyfh1 tetrad colonies (Fig. 1A). The backcross of the Δmrs3/4 strain yielded tetrads of all expected genotypic combinations (MRS3 and MRS4 are unlinked), and colony sizes were indistinguishable from wild-type in all cases (Fig. 1B). In the cross involving Δmrs3/4 and Δyfh1, tiny colonies were observed (Fig. 1C). In all cases these were confirmed to carry three mutations: Δmrs3, Δmrs4 and Δyfh1.

Congenic strains were grown in 96-well plates containing rich YPAD media, and growth was followed by frequent turbidity measurements using a plate reader. Wild-type was indistinguishable from Δmrs3/4 (short lag phase and 2 hour doubling time in logarithmic phase). The Δyfh1 strain grew more slowly (much longer lag time and 5 hour doubling time in logarithmic phase). The ΔΔΔ strain was the most severely impaired (longest lag phase and 8 hour doubling time in logarithmic phase) (Fig. 2A). The small colony size of the triple mutant tetrad clone was thus not due to delayed germination but rather to genuine slow growth. In the final hours of the experiment (hours 30-40), growth of the triple mutant appeared to accelerate (Fig. 2A), perhaps due to outgrowth of suppressor mutant clones. These suppressors of the ΔΔΔ slow growth were also apparent as larger colonies surrounded by pinpoints in serial dilutions of the triple mutant grown on solid agar plates (Fig. 2B, ΔΔΔ).

Effects of oxygen exposure, heme, and iron availability on the mutant growth phenotypes

A variety of growth conditions were tested to further characterize the cause of the severe slow growth of the ΔΔΔ mutant. Interestingly, all strains, including the triple mutant, grew at similar rates and formed similar sized colonies when grown anaerobically in rich YPAD media with Tween 80 and ergosterol (Fig. 3A). The explanation for this observation is unknown at this time, but could be due to less iron toxicity, different iron requirements of anaerobically grown cells, or activation of alternative and functionally redundant iron utilization systems under anaerobiosis. In any case, this surprising observation was useful, because it enabled us to initiate experiments with healthy, uniformly grown cells of different genotypes.

Wild-type, Δmrs3/4, Δyfh1 and ΔΔΔ strains were grown anaerobically for 48 hours in liquid media allowing uniform growth, and serial dilutions were then inoculated on rich medium agar anaerobically (Fig. 3A) or defined medium agar plates in air (Fig. 3B-F). After 3 days, photographs were taken. After switching to aerobic conditions, the wild-type grew best, followed by Δmrs3/4, Δyfh1 and ΔΔΔ respectively. The red color of the wild-type strain is due to accumulation of adenine precursor, which occurs as a consequence of the ade2-101 mutation. The decreased pigmentation apparent in the Δyfh1 and ΔΔΔ spots may be due to slower growth and/or decreased respiratory activity in these strains (42).

The serial dilutions were also tested on defined medium with 20 µM hemin. On the supplemented defined media plates, growth of all strains (including wild-type) improved and the difference between wild-type and Δmrs3/4 was entirely abrogated (Fig. 3D). An interpretation of these results is that the shift from anaerobic to aerobic conditions created an increased requirement for heme synthesis, necessary to
provide cofactors for respiratory complexes that are synthesized only under aerobic conditions (43). Heme deficiency was reflected by a partial hemopathy of the wild-type strain following shift from no air to air. Although growth compromise in the Δyfh1 mutant was more severe than in the wild-type or Δmrs3/4, correction by hemin addition was slight. For the ΔΔΔ, growth impairment was very severe (1 spot appearing in serial dilutions, Fig. 3B), and significant correction was observed with 20 µM hemin added to the medium (3 spots appearing in serial dilutions, Fig. 3D). Increasing the amount of hemin to 50 µM did not lead to enhancement of growth by the mutant strains (compare Fig. 3E to Fig. 3D). Tween 80 (source of unsaturated fatty acids) and ergosterol (source of sterols) were tested for their effects on these phenotypes, because these biosynthetic pathways utilize heme proteins. Methionine was added because of involvement of siroheme in methionine synthesis. Significant growth enhancement was observed, similar to effects of heme addition alone (Fig. 3C). Addition of heme, Tween 80, ergosterol and methionine was similar to addition heme alone, with the exception of the Δyfh1 mutant, which showed slightly improved growth under these conditions (Fig. 3F).

Iron availability was manipulated by addition of the iron chelator, ferrozine, to defined medium. Ferrozine and MES buffer at pH 6.1 were added to defined media, and ferrous iron was added back, establishing an iron buffer with different windows of available free iron (29). The panel of strains was grown anaerobically and plated in air on agar plates with these different iron concentrations. The growth compromise of the Δmrs3/4 mutant compared with wild-type was apparent only in iron limited conditions. The Δmrs3/4 strain grew more slowly than wild-type in ferrozine 1 mM (Fig. 4A, E) or ferrozine 1 mM with 20 µM ferrous iron (Fig. 4B, F), but not in ferrozine 1 mM with 50 µM (Fig. 4C, G) or 350 µM (Fig. 4D, H) ferrous iron. This phenotype has been described by others (23,25). Interestingly, the Δyfh1 and triple ΔΔΔ mutants also showed compromised growth on iron limited plates that was partially alleviated by iron supplementation (compare Δyfh1 or ΔΔΔ in 4A versus 4B, C, D). After more prolonged incubations in air (16 hours) prior to patching on the chelated agar plates (Fig. 4E-H), the retardation of Δmrs3/4 under iron limitation was less severe. By contrast, for the Δyfh1 strain and particularly for the ΔΔΔ strain, growth compromise was more severe with longer air exposure. The growth promoting effects of iron were still apparent, however, especially for the ΔΔΔ strain (compare Fig. 4E and H). Following still more prolonged air exposure (greater than 24 hours), Δyfh1 and ΔΔΔ strains were severely compromised, and iron enhancement of growth was not observed (not shown). Supplementation with hemin, Tween 80, ergosterol, and methionine dramatically improved growth of all mutants (Fig. 4 I-L). These agar plate experiments suggest that heme deficiency plays a role in limiting growth of these mutants (Δmrs3/4, Δyfh1, and ΔΔΔ), and that oxygen exposure and iron availability strongly modulate these phenotypes.

**Ferrochelatase protein and activity levels**

A set of congenic strains including wild-type, Δmrs3/4, Δyfh1 and ΔΔΔ were grown anaerobically for 48 hours. During this period, growth of all strains was uniform. The cultures were then shifted to air for four hours with raffinose as the carbon source. At this point, heme deficiency of mutants compared with the wild-type was already apparent, and was quite severe in the ΔΔΔ (see below, Fig. 7). Heme synthesis involves ferrochelatase activity, acting on porphyrin and ferrous iron in the mitochondrial inner membrane. In seeking to understand the basis of the heme deficient phenotype, we examined ferrochelatase protein and activity. We and others previously observed a marked decrease in ferrochelatase protein levels in Δyfh1 strains grown in air (4,44,45). This decrease was even more marked in the ΔΔΔ mutant grown in air (not shown). However, anaerobically grown Δyfh1 and ΔΔΔ cells exhibited ferrochelatase levels indistinguishable from wild-type (data not shown), and following shift to air for 4 hours, levels were very slightly decreased in these mutants compared with wild-type (Fig. 5A). Ferrochelatase protein was not altered in Δmrs3/4 mutants under any conditions. The basis for the air dependent decrease in ferrochelatase protein in Δyfh1 and ΔΔΔ cells has not been determined. The levels of Mir1p, a phosphate carrier protein of the mitochondrial carrier protein family, were not...
altered. Yfh1p was absent in the deleted strains and unaltered in abundance in the Δmrs3/4 strain compared with wild-type.

Ferrochelatase activity was measured by a fluorimetric assay (33,34) in mitochondrial lysates to which iron and PPIX were added. Mitochondria equivalent to 80 µg of protein were isolated from permeabilized cells following anaerobic growth and 4 hours in air. Mitochondrial lysates were then mixed with 200 ng PPIX and rendered anaerobic by bubbling argon gas through the sample in a stoppered cuvette. The assay was started by injecting 10 mM ferrous iron and observing the decrease of PPIX fluorescence over time. Activities for wild-type, Δyfh1 and ΔΔΔ strains were comparable, although Δmrs3/4 exhibited more activity than the others (Fig. 5B). The decrease of PPIX fluorescence represented conversion of more than 60 ng of fluorescent PPIX to non-fluorescent heme by ferrochelatase in each of the samples. Thus, the heme deficiency of the mutants including the ΔΔΔ strain cannot be explained by lack of ferrochelatase protein or ferrochelatase activity. In fact, the amount of ferrochelatase activity present in 100 µl of permeabilized cells was estimated to be 10 times the activity measured here (since 10 µl of cells were equivalent to approximately 80 µg of mitochondria as assessed by comparison of Hem14p activities, data not shown). This amount of ferrochelatase activity should be able to rapidly convert 600 ng of PPIX to heme. Thus, the failure to convert PPIX to heme in the permeabilized cells of the ΔΔΔ genotype (see below) was not due to deficiency of ferrochelatase, which was present in tremendous excess, but rather to lack of iron availability.

Iron concentrations and heme proteins
Iron content of whole cells and mitochondria were determined for each of the congenic strains (wild-type, Δmrs3/4, Δyfh1 and ΔΔΔ) by ICP-MS. In whole cells, there was roughly a 2-fold increase in iron content in all the mutants compared with wild-type. The mitochondria, by contrast, showed increased iron levels in the Δyfh1 and slightly increased levels in Δmrs3/4 mitochondria, but virtually no change in the ΔΔΔ (Fig. 6A). Thus, the cellular iron content of the ΔΔΔ mutant was increased although the mitochondrial iron content was not increased. This characteristic of the triple mutant has been reported by others (23,25). None of the mutants, including the ΔΔΔ, exhibited lowered iron concentrations which could account for deficient heme synthesis, although a key unknown is the form of the iron in vivo which might restrict its bioavailability.

Ferric reductase and catalase activities were measured. Ferric reductase is highly regulated by iron and copper concentrations, but is also dependent on the availability of heme and flavin cofactors (46,47). In this experiment, ferric reductase activity was measured in the mutants following shift to air and growth for 4 hours. The activity was negligible in the wild-type and induced above this background in all three mutants, particularly Δyfh1. However, the picture was different after inducing reductase expression by growth in the presence of iron chelator. Under those conditions, activity in the wild-type was induced 70-fold, activity in Δmrs3/4 was induced 3-fold, but activities in Δyfh1 and ΔΔΔ were decreased. The meaning of these results is complicated, because of the opposite effects of iron limited induction of transcription and cofactor limitation; however, lack of heme in the Δyfh1 and ΔΔΔ strains could account for the relative deficiency of reductase in those strains. Catalase is a heme containing tetramer localized to peroxisomes. Catalase activity was measured in the panel of strains after growing for 4 hours in air. Activity was indistinguishable from wild-type in the Δmrs3/4 mutant but was significantly depressed in the Δyfh1 mutant and even more so in the ΔΔΔ mutant, again consistent with heme deficiency in those strains. (Fig. 6C)

Heme biosynthesis in permeabilized cells from endogenous iron stores
Heme biosynthesis was evaluated more directly, making use of a permeabilized cell system that allows direct presentation of porphyrin to mitochondria (4). As before, strains were grown anaerobically, shifted to air in rich raffinose media for four hours. The yeast cells were digested with zymolyase, allowed to recover, and slowly frozen in manganese acetate buffer over liquid nitrogen vapors in order to permeabilize the plasma membrane while leaving organelles intact (35). Control experiments to evaluate the quality of the permeabilization were performed. In
permeabilized wild-type cells, mitochondrial matrix enzymes such as aconitase were not detected when cells were assayed in isotonic buffer, but aconitase was detected following hypotonic shock to rupture the mitochondria (Fig. 7A). On the other hand, the cytoplasmic enzyme, 3-phosphoglycerate kinase, was quantitatively recovered by brief exposure of the permeabilized cells to hypertonic buffer (1.2 M sorbitol) as described (35).

Permeabilized cells from wild-type, Δmrs3/4, Δyfh1 and ΔΔΔ were presented with protoporphyrinogen (PPO, 50 nM) a non-fluorescent precursor of protoporphyrin IX (PPIX). In the presence of the iron and zinc chelator bipyridyl, all strains showed comparable time dependent increases in fluorescence (excitation 410 nm, emission 632 nm), consistent with in situ formation of PPIX catalyzed by the enzyme protoporphyrinogen oxidase (encoded by the HEM14 gene) (Fig. 7B). This shows that this step of heme biosynthesis is unaffected in the mutants, and that in all the strains, equivalent amounts of PPIX substrate are available for the final step of heme biosynthesis.

In the physiologic pathway of heme biosynthesis, the fluorescent PPIX produced by Hem14p is delivered to Hem15p (ferrochelatase) in the mitochondrial inner membrane; iron is also presented to ferrochelatase, which converts porphyrin to heme, a non-fluorescent compound. Thus iron delivery to ferrochelatase for heme synthesis correlates with decrease of the fluorescent signal due to PPIX formation. In wild-type, Δyfh1, and Δmrs3/4 permeabilized cells (without chelator treatment) PPO did not elicit any fluorescent signal, indicating adequate iron delivery. By contrast, in the absence of chelator, in the permeabilized cells from the ΔΔΔ mutant, there was a sharp upward sloping fluorescent signal, indicating rapid formation of PPIX and failure to convert it to heme (Fig. 7C). The interruption in heme synthesis was not absolute, as shown by the lesser slope of PPIX formation in the absence of chelator versus the slope in the presence of chelator (compare ΔΔΔ in 7B and 7C); however, the process was significantly impaired in the triple mutant. Zinc protoporphyrin (excitation 410 nm, emission 587 nm), an alternative end product of ferrochelatase formed from zinc and PPIX substrates, was not produced in the presence of chelator (not shown) and was minimally detected in the absence of chelator in all strains (Fig. 7D) under these conditions.

In summary, the data show that formation of heme from protoporphyrin was specifically compromised in the ΔΔΔ mutant. This was a synthetic phenotype in the genetic meaning of the term; the biochemical defect was elicited by a combination of mutations (ΔΔΔ), while it was not apparent in the individual mutants (Δyfh1 or Δmrs3/4). However, the individual mutants were, in fact, not normal in their iron handling for heme synthesis. Their respective biochemical defects could be detected and distinguished in the more detailed studies that follow.

**Total heme and cytochromes**

The panel of mutants was grown anaerobically to uniform density and then allowed to grow in air for an additional 5 hours or 15 hours in rich raffinose media. Cells were harvested and total heme levels were measured using the pyridine hemochromogen method (40). After 5 hours growth in air, heme levels were slightly decreased in Δmrs3/4, more so in Δyfh1 and severely depressed in ΔΔΔ (more than 5 fold decreased compared with wild-type) (Table I). This rank order of deficiencies was maintained after 15 hours growth in air. However, the heme deficiency of the Δyfh1 mutant in particular was much more exaggerated at the later time point than at the earlier time point (Table I). Because heme was absent in all the strains under the anaerobic conditions at the outset of the experiment, we were able to calculate rates for heme formation during the first 5 hours in air and the subsequent 10 hours in air (Fig. 8). The calculated rate for wild-type heme synthesis was initially 5.1 nmoles/h/g wet weight and declined to 4.4, perhaps reflecting an initial increased need to synthesize respiratory complexes during the burst of mitochondrial biogenesis that occurs after shifting to aerobic growth. The slightly decreased level of total heme formed per hour did not change much during the two time periods for the Δmrs3/4 strain. The initial rate of heme formation in the Δyfh1 cells, 2.7 nmoles/h/g wet weight, declined dramatically with time in air to 0.3 nmoles/h/g wet weight during the second time period. In the triple mutant, even at the initial time period, heme formation was severely compromised and the
measured rate was 1.0 nmoles/h/g. During the second period, this decreased further to 0.5 nmoles/h/g. Thus, time in air following anaerobic growth was a strong modifier of the total cellular heme and the rate of its biosynthesis in the different mutants.

Cytochromes were evaluated by examining low temperature spectra (Fig. 9). Consistent with the known oxygen requirements for critical enzymatic and regulatory steps in heme biosynthesis, cytochromes were not detected in anaerobically grown cells for any of the strains (48). For the wild-type, following shift to air, cytochromes appeared, and cytochrome c₆, b₅, c₂, b₆, and cytochrome oxidase peaks could be discerned (Fig. 9, peaks 1-5). Interestingly a ZnPP peak was visible in the 5 h tracings and disappeared in the 15 h tracings. All other cytochrome peaks became more prominent at 15 h. In the Δmrs3/4 mutant, cytochrome peaks were apparent at 5 and 15 hours, and these were even greater in height than in the wild-type. ZnPP peaks were also present and persisted in the 15 h time point, unlike in the wild-type (Fig. 9, peak 6). In the Δyfh1 mutant, cytochrome peaks were present at 5 h and slightly diminished compared with wild-type. A prominent ZnPP alpha peak was also present (Fig. 9, peak 6) and a ZnPP beta peak was apparent at 15 hours (Fig. 9, peak 7). In the ΔΔΔ mutant, a marked decrease in cytochromes was noted at the early 5 h time point, and this defect was more severe than in the other mutants. With more time in air, the pattern was virtually unchanged, consistent with total heme measurements indicating negligible new heme synthesis during this period (Table 1). ZnPP did not accumulate under these conditions.

Heme synthesis in permeabilized cells, effects of exogenous iron and time in air

Cells were grown in glucose based defined medium, shifted to air in raffinose based defined medium and permeabilized after growing for an additional 4 h or 15 h aerobically. Heme synthesis was tested by adding PPO following brief exposure to NADH (to maximally energize the mitochondria) and 10 μM ferrous iron. Note that these experiments differed from those shown in Fig. 7 in that the cells were grown in defined medium, a higher concentration of PPO was used (10 fold more), and the effects of exogenous iron were tested here. Initial rates of PPIX were measured (Fig. 10) with or without added iron and data for longer time courses showing the appearance and disappearance of fluorescent porphyrin products (PPIX and ZnPP) were also collected (Fig. 11). The initial positive slope of PPIX formation from added PPO in the wild-type was abrogated by addition of iron (Fig. 10A), and the tracings for the Δyfh1 mutant were similar in this regard (Fig. 10C). However, Δmrs3/4 and ΔΔΔ curves showed very little response to added iron (Fig. 10B and D, black diamonds versus open circles). Closer inspection revealed that the curves diverged later in the time courses (at 140 seconds in Fig. 10B and at 110 seconds in Fig. 10D), indicating that exogenous iron was gaining access to ferrochelatase in these mutants albeit at a slower rate than in the wild-type. The initial slope indicating PPIX formation with or without iron was much steeper in the ΔΔΔ than in the other strains, consistent with the more severe phenotype of this mutant.

In the 15 hour experiment, the positive slope of PPIX formation in the wild-type was abolished by iron addition as in the 4 hour experiment (Fig. 10E). For the Δmrs3/4 and ΔΔΔ mutants, the effect of adding iron was much diminished compared with the wild-type (Fig. 10F and H). However the effects of added iron in these mutants (indicated by diverging black and gray curves) were more marked at 15 hours than at 4 hours, perhaps indicating increased activity of a backup iron transport system different than the one mediated by Msr3/4p. The curves for the Δyfh1 mutant were altered significantly in the 15 hour time point (Fig. 10G). Large amounts of ZnPP not reflected in these curves (which only show PPIX formation) continued to be made by the Δyfh1 mutant following administration of NADH and iron (Fig. 11A’-D’). An identical experiment was
performed with the strains following 15 hours growth in air (Fig. 11E-H) and again following the administration of NADH and iron (Fig. 11E'-H'). In most cases, PPIX was entirely converted to heme or ZnPP by the end of the time course. However, the rates of porphyrin formation and utilization were very different for the various mutants as were the responses to added iron. Data showing these scans were plotted on three dimensional displays with emission wave length on the X-axis, time in seconds on the Y-axis and fluorescence intensity in the Z-axis. If the PPIX signal was increasing compared with the previous tracing, the line was shown in red, whereas if the PPIX signal was less than the previous tracing the line was shown in blue.

For wild-type, PPIX formation rapidly increased to a maximum (Fig. 11A, 11E; 2 red tracings) and rapidly declined. In later scans at the 4 h point, some ZnPP was made (hump seen with emission at 587 nm consisting of blue lines in Fig. 11A). This finding fits perfectly with the cytochrome scans (Fig. 9) showing the presence of ZnPP in wild-type cells grown under similar conditions without added porphyrins. Addition of iron to the permeabilized wild-type cells blunted or abrogated the fluorescent signals indicating use of exogenous iron and conversion of porphyrin to heme (11A', E').

For the \( \Delta mrs3/4 \) mutant, the height of the PPIX signal was greater than in the wild-type, and the rates of the appearance and disappearance of the PPIX signal were delayed (Fig. 11B, 11F). ZnPP was formed slowly in both 4 and 15 hour experiments (again consistent with the cytochrome spectra). The rise in PPIX indicating delayed availability of iron for heme was more severe at the 4 h point than at the 15 h point. Significantly, addition of exogenous iron resulted in the disappearance of all ZnPP and conversion of PPIX to heme, although the time course for this effect was delayed compared with the wild-type (Fig. 11B' and F').

For the \( \Delta yfh1 \) mutant, a large amount of ZnPP was formed, particularly at the 15 hour time point. At the 4 hour time point, the initial rate of PPIX formation was similar comparing wild-type and \( \Delta yfh1 \) (Figs. 10A and 10C), but the rising PPIX signal was prolonged during the longer time course (Fig. 11A compared with 11C, 3 red tracings compared with 5 red tracings). The defect was more severe with increasing time in air (compare the \( \Delta yfh1 \) scans at 4 hours in Fig. 11C showing 5 red tracings, with the \( \Delta yfh1 \) scans at 15 hours in Fig. 11G showing 9 red tracings). The addition of exogenous iron was able to almost entirely bypass the iron delivery problem for heme synthesis at the 4 hour time point (compare the wild-type scans in Fig. 11A’ with the scans for the \( \Delta yfh1 \) mutant in Fig. 11C’). This was no longer the case at 15 hours, at which point exogenous iron could no longer bypass the defect (compare the wild-type scans in Fig. 11E’ with the scans for the \( \Delta yfh1 \) mutant in Fig. 11G’).

In the triple mutant, the defect in iron use for heme synthesis was most severe. This was indicated by the increased height of the PPIX peak (Fig. 11D and H). A kinetic defect was also apparent, indicated by the delay in the appearance of the maximum PPIX signal (5 red curves or 4 red curves in Fig. 11D or Fig. 11H). Addition of iron did not bypass the defect, although the mutant phenotypes (increased height of PPIX peaks and delayed appearance of the maximum) were mitigated by iron addition (Fig. 11D’ and H’). At the end of the time course, all PPIX was consumed and mostly converted to heme.

An entirely different method was also used to measure new heme synthesis. The 15 hour permeabilized cells (i.e. mutants grown anaerobically, shifted to air for 15 hours and permeabilized) were incubated with NADH and \(^{55}\text{Fe}\) ferrous ascorbate. PPO was added, and after incubation of 3 minutes at 30°C, heme was extracted. Newly formed heme was detected in the wild-type (1209 cpm). A decreased amount of newly formed heme was detected in the \( \Delta mrs3/4 \) (499 cpm) and \( \Delta yfh1 \) (568 cpm) mutants. A more severely decreased amount of newly formed heme was extracted from the \( \Delta\Delta\Delta \) mutant (203 cpm). These results show, by a completely independent assay, that the \( \Delta mrs3/4 \) and \( \Delta yfh1 \) strains are defective in delivery of exogenous iron for heme synthesis and that the \( \Delta\Delta\Delta \) defect is more severe.

**DISCUSSION**

The functions of frataxin have in large part been extrapolated from evaluation of loss-of-function phenotypes of mutants. However, many of these phenotypes are secondary to toxic effects of iron accumulation in mitochondria (49). Here we examined the role of frataxin in the \( \Delta\Delta\Delta \)
(Δmrs3/4/Δyfh1) mutant, which does not accumulate iron in mitochondria, allowing parsing of the phenotypes due to iron toxicity from those related to iron handling. The triplet mutant has been described previously, but emphasis was on the roles of Mrs3p and Mrs4p in intracellular iron distribution (23). We also observed effects on iron distribution in the triple mutant, but in addition, we observed striking effects on growth (worse in ΔΔΔ than in Δmrs3/4 or Δyfh1 individually). In addition, we observed a biochemical defect in generation of new heme, which was a “synthetic defect” (worse in ΔΔΔ than in Δyfh1 or Δmrs3/4) in the genetic sense that the combination of mutations interfered with redundant functions thereby uncovering critical functions of each one.

The final step in heme synthesis involves three components, porphyrin, ferrochelatase and iron, which must come together on the matrix side of the inner mitochondrial membrane (33). Ferrochelatase activity was measured using a fluorimetric assay, and in all the strains this was present in tremendous excess of what would be needed to synthesize wild-type levels of heme in these cells. The existence of excess ferrochelatase capacity has been described previously (26). Assays with permeabilized cells involved delivery of porphyrin precursor directly to mitochondria, and in these assays in situ synthesis of PPIX, the porphyrin heme precursor, was not impaired in any of the mutants. Thus, we could narrow down the problem in heme synthesis in the mutants to a problem with iron delivery or iron availability.

A key to the studies performed here was the use of anaerobic growth conditions to initiate experiments. Heme is not synthesized anaerobically (48), and iron toxicity largely does not occur under anaerobic conditions (50). Thus the use of anaerobically grown cells allowed us to find a uniform starting point (without heme and without iron toxicity) from which to evaluate the evolving mutant phenotypes after shifting to aerobic growth conditions. The large body of data presented here supports cooperating roles for Mrs3/4p and Yfh1p in heme formation. Deficiencies of total heme, cytochromes and heme proteins, heme synthesis from endogenous and exogenous iron were observed in the triple mutant and were more severe in the triple mutant than in the individual mutants.

In analyzing this large data set, it is useful to separate early and late effects, because there were major changes in some of the mutant phenotypes during increasing exposure to air. After shifting from anaerobic to aerobic conditions, the organism needs to synthesize cytochrome-containing respiratory complexes de novo, and this requires heme synthesis. Thus, in the wild-type we observed the gradual appearance and increase of cytochromes, while ZnPP formation was decreased with increasing time in air. Heme was absent under anaerobic growth conditions, and heme synthesis was activated upon shifting to aerobic growth. An intact high affinity mitochondrial iron uptake system was evident; in the permeabilized cell assay exogenous iron was rapidly made available to ferrochelatase inside the mitochondria allowing formation of new heme.

In the Δmrs3/4 mutant, heme levels were slightly decreased, but heme proteins and cytochromes appeared normal or slightly increased under the experimental conditions. Even the rate of appearance of cytochromes was not significantly altered compared with normal in the early or late periods in air. Total iron was increased, and mitochondrial iron was slightly increased although not in proportion to the total iron. Thus, the abnormalities to this point were very subtle. A striking finding in this mutant strain, however, was a block in delivery of iron for heme synthesis in the permeabilized cell assay. The slope indicating PPIX formation was not blunted at all following administration of exogenous iron addition (10 μM of ferrous ammonium sulfate), indicating that this iron did not immediately gain access to ferrochelatase. Over time, the iron did get in, and the PPIX was eventually converted to heme, although with a delayed time course. Comparing the 5 and 15 hour experiments, the delay was slightly less severe at the 15 hour time point. A simple explanation for this is that the Mrs3/4p carriers are responsible for iron transport into mitochondria for heme synthesis, and that this occurs with rapid kinetics, whereas an alternative slower system also exists. The relative contribution of the two systems may vary with growth conditions, with the accessory system being more active with increasing time in air. The Mrs3/4p system may also have higher affinity than the alternative system as has been suggested (24). The precise
intracellular substrate for transport which presumably is a ferrous iron chelate of some sort has not been determined.

In the Δyfh1 mutant, very marked changes in the phenotypes were noted with increasing air exposure. During the 4 hour time point, Δyfh1 already exhibited defects in heme synthesis. Cytochromes were only slightly diminished, but total heme levels were decreased by almost half. Significant ZnPP was being made as previously described (4), and this was supported by low temperature spectra and permeabilized cell experiments with added porphyrins. The formation of ZnPP indicates compromised iron availability to ferrochelatase, because zinc is a secondary substrate with much lower affinity for the enzyme. Exogenous iron (10 µM ferrous iron) largely bypassed the problem, and resulted in rapid and complete conversion of the porphyrin to heme, very similar to the wild-type. By the 15 hour time point, the situation changed dramatically, and heme synthesis in the Δyfh1 virtually ceased. The cytochromes no longer increased; the rate of heme synthesis dropped precipitously, and the permeabilized cell experiments showed extremely delayed rise and fall of fluorescent porphyrin peaks, again indicating lack of heme synthesis. Furthermore, addition of iron could no longer bypass the problem. An explanation for these findings may be that at the early time point, we are dealing with a lack of an iron chaperone, required to maintain iron in a bioavailable form for use by ferrochelatase. Thus, addition of significant amounts of ferrous iron, which is probably immediately bioavailable after getting into mitochondria may bypass the need for this function. At the later time point, toxic iron aggregates accumulating within mitochondria might produce oxidation of proteins, and also oxidation of added iron, as occurs during biomineralization (51). The oxidized iron would then be unavailable to ferrochelatase.

In the ΔΔΔ mutant, iron levels in mitochondria were normal, and so it was possible to discern a role of Yfh1p without toxic effects of iron accumulation. Global heme deficiency was much more severe than in the Δmrs3/4 or Δyfh1 strains, with 5-fold less total heme than wild-type at the 4 hour point and more than 7-fold less at the 15 hour point. This effect was consistent with observations of decreased cytochromes and heme proteins such as catalase in the triple mutant. The phenotype was apparent at the early time point and did not change radically with time in air, as was the case for Δyfh1. In the triple mutant, the rate of new heme formation was severely compromised (indicated by measurement of total heme formed/per hour in growing cells). This compromise also extended to heme formation from exogenously added iron, which showed delayed access to ferrochelatase and decreased availability in the permeabilized cell assay. In summary, the triple mutant phenotype was characterized by decreased iron availability for ferrochelatase due to a mitochondrial iron transport defect (ascribed to lack of Mrs3/4p) and an additional defect following iron transport (ascribed to lack of Yfh1p). This delay in iron utilization for heme beyond that ascribed to Mrs3/4p effects may reflect the iron chaperone or antioxidant function of Yfh1p. Yfh1p might protect bioavailable iron from oxidation, or deliver it to ferrochelatase, or both.

Yfh1p has recently been shown to physically interact with Isu1p (15), aconitase (12), ferrochelatase (4,10,20) and iron (10,52). Isu1p is a scaffold protein on which Fe-S clusters are assembled (18). Aconitase is an abundant Fe-S cluster containing enzyme of the Krebs cycle in the mitochondria (32). Ferrochelatase is the critical enzyme involved in iron insertion into porphyrin to form heme (26). The significance of these various protein-protein interactions are under active investigation. Yfh1p might be serving to protect iron against oxidation during iron utilization steps or might be involved in actual delivery of bioavailable metal to partner proteins (16). Alternatively Yfh1p might be serving an antioxidant function, and the protein interactions may be required. Finally, the protein interactions might represent formation of complexes, which have yet undiscovered structural roles (such as the recently discovered function of aconitase in nucleoid formation) (53).

A metallochaperone function of Yfh1p has been proposed, and might resemble the functions of copper chaperone proteins that bind copper and target it to different intracellular destinations by suitable protein-protein interactions (54,55). The phenotype of the mutants lacking copper chaperone proteins is instructive. These copper chaperone mutants show deficiency of particular
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copper proteins only in low copper environments (56). The chaperone functions become superfluous under copper replete conditions, because low-affinity or non-specific pathways for copper delivery exist that are able to bypass the chaperone requirement. Under conditions of our experiments, we observed a bypass of Yfh1p requirement for iron use for heme synthesis by addition of exogenous ferrous iron (4 h point, Fig. 11C’), which did not occur in the ΔΔΔ (Fig. 11D’) and was not explained by the mitochondrial iron transport block in Δmrs3/4 (Fig. 11B’). A likely explanation is that bioavailable iron was less in the ΔΔΔ, uncovering the need for Yfh1p chaperone function. Thus, frataxin might serve an iron chaperone function under conditions in which mitochondrial iron concentrations are low and heme requirements are high. Additional support for this hypothesis is provided by examination of the single Δyfh1 null mutant at the 4 hour time point. In this mutant, the initial rate of PPIX formation was similar to the wild-type (Fig. 10). However, delayed heme synthesis was observed as the time course progressed and presumably endogenous iron became limiting (Fig. 11C).

Further dissection of the complex functions of frataxin will require analysis of special environmental conditions and mutant alleles. For example, the frataxin of Candida albicans can be viewed as a “mutant” allele of the Saccharomyces cerevisiae frataxin. It is 47 percent identical at the amino acid level, and Candida frataxin expressed in S. cerevisiae will complement oxidative stress and iron accumulation phenotypes but will not correct the heme biosynthetic defects (13). Frataxin interaction with ferrochelatase has been mapped to a particular part of the frataxin molecule by structural studies (10), and thus iron chaperoning for heme synthesis might be mediated by this part of the molecule, while antioxidant functions might be mediated by separate frataxin domains.

The frataxin amino acid sequence and structure are highly conserved across evolution, and aspects of frataxin function are also conserved. A role of frataxin in Fe-S cluster formation in yeast and human mitochondria is supported by biochemical studies showing interaction of frataxin and Isu1p in yeast (15) and humans (20). Similarly, ferrochelatase is highly conserved, and the interaction of ferrochelatase and frataxin has been described in both yeast (4,10) and humans (16). Thus, there may be a role of frataxin in heme synthesis in humans, although heme protein deficiencies have not been observed in Friedreich’s ataxia patients. This may reflect species differences in frataxin functions. Alternatively, effects on heme attributable to mammalian frataxin may not have been observed because of organismal or cellular non-viability associated with heme altering or severe loss-of-function alleles. A role for frataxin in heme synthesis in mammals might also be apparent under special conditions such as during erythropoietic stress (57). Further work will be required to determine the precise roles of yeast or human frataxins in iron delivery for heme synthesis.

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LEGENDS TO FIGURES

Fig. 1. Demonstration of genetic interactions of $\Delta mrs3/4$ and $\Delta yfh1$. A. $\Delta yfh1 \times$ wild-type. The $\Delta yfh1$ shuffle strain, was crossed with a parental strain of opposite mating type, and the $YFH1$ containing plasmid was removed by counterselection on cycloheximide prior to sporulation. B. $\Delta mrs3/4 \times$ wild-type. The $\Delta mrs3/4$ strain, was backcrossed and sporulated. C. $\Delta mrs3/4/\Delta yfh1 \times$ wild-type. The $\Delta mrs3/4/\Delta yfh1$ shuffle strain was crossed with a parental strain of the opposite mating type, and the $YFH1$ containing plasmid was removed prior to sporulation. Panels A, B, C show tetrad colonies after five days incubation in air at 30 $^\circ$C, and Panels A’ B’ C’ show the corresponding genotypes. Only the cross including the three mutations (C) gave rise to tiny tetrad colonies and in every case (1c, 3a, 5b, 6c), these were confirmed to have the triple mutant genotype ($\Delta mrs3/4/\Delta yfh1$).

Fig. 2. Growth characteristics of mutants measured in YPAD media. A. Tetrad clones of the indicated genotypes were inoculated into rich glucose medium (YPAD) in microtiter wells at low density (O.D.600=.005). Cells were grown at 30 $^\circ$C in a water bath and density was monitored by periodic readings on a plate reader. Cell numbers were determined from a calibration curve. Doubling times in logarithmic phase were wild-type (2 hours), $\Delta mrs3/4$ (2 hours), $\Delta yfh1$ (5 hours), $\Delta\Delta\Delta$ (8 hours). B. Cells of the indicated genotypes were washed in water and serial dilutions (1:5) of 5 x 10^5 cells were inoculated onto YPAD agar plates and allowed to grow for 5 days. Heterogeneity of colony sizes was apparent in $\Delta yfh1$ and $\Delta\Delta\Delta$ dilutions.

Fig. 3. Anaerobic to aerobic shift elicits heme dependent growth phenotypes. Cells were grown anaerobically in YPAD with Tween and ergosterol in sealed flasks under argon gas for 48 hours. Serial dilutions (1:5) of 5 x 10^5 cells were plated on agar plates and grown at 30 $^\circ$C under various conditions. A. YPAD supplemented with Tween 80 and ergosterol, anaerobic growth. B. Defined medium, aerobic growth. C. Defined medium with Tween 80, ergosterol, and methionine, aerobic growth. D. Defined medium with 20 $\mu$M hemin, aerobic growth E. Defined medium with 50 $\mu$M hemin, aerobic growth. F. Defined medium with 50 $\mu$M hemin, Tween 80, ergosterol, and methionine, aerobic growth. Concentrations of additives were 0.2% Tween 80, 40 $\mu$g/ml ergosterol, and 40 $\mu$g/ml methionine.

Fig. 4. Anaerobic to aerobic shift elicits iron and heme dependent growth phenotypes. A-D. Cells were grown anaerobically as above and plated aerobically on defined medium agar plates with 50 mM MES buffer to maintain pH at 6.1, 1 mM ferrozine and various concentrations of iron. A. No additional ferrous iron. B. 20 $\mu$M. C. 50 $\mu$M. D. 350 $\mu$M. E-H. Anaerobically grown cells were transferred to YPAD liquid for 16 hours in air. Serial dilutions were then plated aerobically on agar plates containing 50 mM MES buffer, 1 mM ferrozine and various concentrations of ferrous iron. E. 0 added ferrous iron. F. 20 $\mu$M. G. 50 $\mu$M. H. 350 $\mu$M. I-L. Those same cells were plated aerobically on agar plates containing 50 mM MES buffer, 1 mM ferrozine, various concentrations of ferrous iron, 50 $\mu$M hemin, 0.2% Tween 80, 40 $\mu$g/ml ergosterol, and 40 $\mu$g/ml methionine. Abbreviated genotypes of the strains are indicated to the left of each row of serial dilutions, and a triangle to the right of the I-L blocks represents the increasing free iron concentrations in the different agar plates.

Fig. 5. Ferrochelatase protein levels and activities. A. Ferrochelatase protein levels. Mitochondria were isolated from permeabilized cells from cultures grown anaerobically and shifted to air in YPAR for 4 hours. Mitochondrial proteins were separated on a 14% SDS PAGE, transferred to nitrocellulose and incubated with antibodies to ferrochelatase protein (Hem15p), frataxin (Yfh1p) or the mitochondrial phosphate carrier protein (Mir1p). B. Continuous fluorimetric assay for ferrochelatase activity. Mitochondria (80 $\mu$g equivalent) from each strain of permeabilized cells were lysed on ice in 2 ml of buffer (50 mM TrisCl pH 7.5, 0.5% Tween 80), and exposed to air for 5 minutes. The lysate was placed in a septum stoppered fluorescence cuvette and bubbled with argon to render it anaerobic. PPIX substrate (200 ng) was injected and a baseline tracing was obtained. After 1 minute, 10 $\mu$M ferrous ammonium sulfate iron was injected from an anaerobic stock. Fluorescence was followed at 410 nm excitation and 632 nm emission under conditions identical to the permeabilized cell assays.
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Fig. 6. Iron contents and heme proteins. A. Iron contents of whole cells and mitochondria. Anaerobically grown cells (wild-type, ∆mrs3/4, ∆yfh1 and ∆ΔΔ) were shifted to air for 4 hours in raffinose based rich medium. Cellular and mitochondrial iron contents (100 µg protein equivalent) were measured by ICP-MS. B. Ferric reductase activity. Wild-type, ∆mrs3/4, ∆yfh1, ∆ΔΔ cells were grown anaerobically and shifted to air in YPAR for 4 hours. Cells were washed and incubated with 1 mM ferric ammonium sulfate and 1 mM BPS in 50 mM citrate buffer at pH 7.0 containing 13 mM H2O2. The decrease of absorbance at 240 nm was measured for the initial 2 minutes and catalase activity was calculated using the extinction coefficient 4.36 x 10^4 cm²/mol.

Fig. 7. Heme synthesis in permeabilized cells from endogenous iron (growth in rich medium). Wild-type, ∆mrs3/4, ∆yfh1, ∆ΔΔ cells were grown anaerobically and shifted to air in YPAR for 4 hours. Cells were permeabilized by freeze thaw in acetate buffer. A. Latency of aconitase activity. Aconitase activity of the wild-type permeabilized cells was measured in the presence or absence of 0.6 M sorbitol. Activity was apparent only after hypotonic shock generated by the absence of sorbitol. B. Protoporphyrinogen oxidase activity. Permeabilized cells (100 µl, 50 µg wet weight) were gently resuspended in 2 ml of assay buffer consisting of 50 mM TrisCl pH 7.5 and 0.6 M sorbitol to maintain intactness of the mitochondria. The metal chelator bipyridyl (10 mM) and dithiothreitol (5 mM) were added and cells were incubated for 3 minutes at room temperature and 1 minute at 30°C. Protoporphyrinogen (50 nM) was added and the rate of PPIX formation was followed fluorimetrically. The excitation was 410 nm and the emission was set at 632 nm, slit size of 4 nm and power of 600 V. C. Heme synthesis from endogenous iron. Same experiment as B. but without chelator. PPIX accumulation was observed only in the ∆ΔΔ cells, indicating deficient heme synthesis. D. Zinc protoporphyrin. Permeabilized cells were incubated as above in assay buffer only (no chelator) and protoporphyrinogen was added. Excitation was 410 nm and emission was 587 nm.

Fig. 8. Calculated rates of heme formation. Wild-type, ∆mrs3/4, ∆yfh1, ∆ΔΔ cells were grown anaerobically (under which conditions heme content was negligible). Cells were shifted to aerobic conditions in YPAR for an additional 5 hours or 15 hours before harvesting. Total cellular heme content was measured by the pyridine hemochromogen method (data shown in Table 1) for the two time points. The rates of heme production were calculated as nmoles/g wet weight/hour for the initial 5 hour period in air (black bars), and the subsequent 10 hour period in air (striped bars).

Fig. 9. Low temperature spectra. Low-temperature spectra of whole cells (wild-type, ∆mrs3/4, ∆yfh1, ∆ΔΔ) were recorded following anaerobic growth (t=0) or after 5 hours (t=5) or 15 hours (t=15) growth in YPAR under aerobic conditions. The labeled peaks are 1) beta band of cytochrome c; 2) beta band of cytochrome b; 3) alpha band of cytochrome c; 4) alpha band of cytochrome b; 5) cytochrome oxidase; 6) alpha band of zinc protoporphyrin; 7) beta band of zinc protoporphyrin.

Fig. 10. Heme synthesis in permeabilized cells from exogenous iron (growth in defined medium), initial rates of PPIX formation. Wild-type, ∆mrs3/4, ∆yfh1, ∆ΔΔ cells were grown anaerobically in defined medium containing glucose, 0.2% Tween 80 and 40 µg/ml ergosterol. The cultures were transferred to raffinose defined medium and grown aerobically for an additional 4 hours or 15 hours prior to permeabilization. An aliquot of permeabilized cells (100 µl, 50 µg wet weight) was gently resuspended in 2 ml of assay buffer in a fluorescence cuvette and preincubated at 30°C for 1 minute. Settings for the fluorimeter were excitation of 410 nm, emission of 632 nm, slit size of 4 nm, and power of 600 V. Protoporphyrinogen (250 nM) was added and the rate of PPIX formation was recorded for 180 seconds (black diamonds). In a parallel assay, cells were preincubated with 5 mM NADH and 10 µM ferrous iron for 2 minutes at 30°C prior to the addition of PPO substrate (open circles), and fluorescence was recorded for 180 seconds. Fluorescence intensity is shown on the vertical axis and time in seconds is shown on the horizontal axis. A. wild-type, 4 hours in air. B. ∆mrs3/4, 4 hours in air. C. ∆yfh1, 4 hours in air. D. ∆ΔΔ,
Frataxin and Mitochondrial Carrier Proteins

4 hours in air.  E. wild-type, 15 hours in air.  F. Δmrs3/4, 15 hours in air.  G. Δyfh1, 15 hours in air.  H. ΔΔΔ, 15 hours in air.

**Fig. 11. Serial emission scans in permeabilized cells following protoporphyrinogen administration, effects of time in air and exogenous iron.** Permeabilized cells were prepared, and experiments were performed exactly as for Fig. 10. Settings for the fluorimeter were as above, except that serial emission scans (550 to 650 nm) were recorded, with each scan taking 100 seconds. Data are presented as 3-dimensional plots with X-axis, Y-axis and Z-axis representing emission wavelength in nm, fluorescence intensity, and time in seconds, respectively. PPIX and ZnPP emitted fluorescence at characteristic wavelengths of 632 and 587 nm respectively, upon an excitation of 410 nm. If the PPIX signal increased compared with the previous tracing, the tracing was shown in red. If the PPIX signal decreased compared with the previous tracing, the tracing was shown in blue.  A-D. Cells shifted to air for 4 hours, no added iron.  A. wild-type, 4 hours in air, no added iron.  B. Δmrs3/4, 4 hours in air, no added iron.  C. Δyfh1, 4 hours in air, no added iron.  D. ΔΔΔ, 4 hours in air, no added iron.  A'-D'. Cells shifted to air for 4 hours, with exposure to exogenous iron.  A'. wild-type, 4 hours in air, with added iron.  B'. Δmrs3/4, 4 hours in air, with added iron.  C'. Δyfh1, 4 hours in air, with added iron.  D'. ΔΔΔ, 4 hours in air, with added iron.  E-H. Cells shifted to air for 15 hours, no added iron.  E. wild-type, 15 hours in air, no added iron.  F. Δmrs3/4, 15 hours in air, no added iron.  G. Δyfh1, 15 hours in air, no added iron.  H. ΔΔΔ, 15 hours in air, no added iron.  E'-H'. Cells shifted to air for 15 hours, with addition of exogenous iron.  E'. wild-type, 15 hours in air, with added iron.  F'. Δmrs3/4, 15 hours in air, with added iron.  G'. Δyfh1, 15 hours in air, with added iron.  H'. ΔΔΔ, 15 hours in air, with added iron.
Table I: Total Cellular Heme Levels

Wild-type, Δmrs3/4, Δyfh1, ΔΔΔ cells were grown anaerobically as described and shifted to air in YPAR for 5 hours or 15 hours before harvesting. Total heme content of the cells was determined by the pyridine hemochromogen method.

<table>
<thead>
<tr>
<th>Strains</th>
<th>5 h in air</th>
<th>15 h in air</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.7 ± 0.7 ²</td>
<td>69.8 ± 5.6</td>
</tr>
<tr>
<td>Δmrs3/4</td>
<td>19.8 ± 3.5</td>
<td>57.3 ± 3.5</td>
</tr>
<tr>
<td>Δyfh1</td>
<td>13.5 ± 3.5</td>
<td>16.7 ± 1.4</td>
</tr>
<tr>
<td>ΔΔΔ</td>
<td>5.2 ± 1.4</td>
<td>9.9 ± 1.7</td>
</tr>
</tbody>
</table>

1 nmoles/g wet weight
2 mean of 3 assays ± SE
Fig. 1
Fig. 2

A

Log$_2$ (cell #) vs. Time (h)

B

wt

Δmrs3/4

Δyfh1

ΔΔΔ
Fig. 3
Fig. 4

Air Exposure Before Plating

0 h 16 h 16 h + Supplements

[Fe]
Fig. 5

A

wt mrs3/4 yfh1 ΔΔΔ hem15

66
45
29
21

Hem15p

Mir1p

Yfh1p

B

Fluorescence (632nm)

0
0.06
0.12
0.18

0 50 100 150 200

Time (s)

- chelator

+ iron

40ng PPIX

wt

mrs3/4

Δ yfh1

ΔΔΔ
Fig. 6
**Fig. 7**
Fig. 8
Fig. 9
Fig. 10
Fig. 11
Frataxin and mitochondrial carrier proteins, Mrs3p and Mrs4p, cooperate in providing iron for heme synthesis
Yan Zhang, Elise R. Lyver, Simon A.B. Knight, Emmanuel Lesuisse and Andrew Dancis

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