Iron use for haeme synthesis is under control of the yeast frataxin homologue (Yfh1)

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The YFH1 gene is the yeast homologue of the human FRDA gene, which encodes the frataxin protein. Saccharomyces cerevisiae cells lacking the YFH1 gene showed very low cytochrome content. In Δyfh1 strains, the level of ferrochelatase (Hem15p) was very low, as a result of transcriptional repression of HEM15. However, the low amount of Hem15p was not the cause of haeme deficiency in Δyfh1 cells. Ferrochelatase, a mitochondrial protein, able to mediate insertion of iron or zinc into the porphyrin precursor, made primarily the zinc protoporphyrin product. Zinc protoporphyrin instead of haeme accumulated during growth of Δyfh1 mutant cells and, furthermore, preferential formation of zinc protoporphyrin was observed in real time. The method for these studies involved direct presentation of porphyrin to mitochondria and to ferrochelatase of permeabilized cells with intact architecture, thereby specifically testing the iron delivery portion of the haeme biosynthetic pathway. The studies showed that Δyfh1 mutant cells are defective in iron use by ferrochelatase. Mössbauer spectroscopic analysis showed that iron was present as amorphous nano-particles of ferric phosphate in Δyfh1 mitochondria, which could explain the unavailability of iron for haeme synthesis. A high frequency of suppressor mutations was observed, and the phenotype of such mutants was characterized by restoration of haeme synthesis in the absence of Yfh1p. Suppressor strains showed a normal cytochrome content, normal respiration, but remained defective in Fe–S proteins and still accumulated iron into mitochondria although to a lesser extent. Yfh1p and Hem15p were shown to interact in vitro by Biacore studies. Our results suggest that Yfh1 mediates iron use by ferrochelatase.

INTRODUCTION

The YFH1 gene is the yeast homologue of the human FRDA gene, which encodes the frataxin protein. Mutations of FRDA associated with decreased frataxin expression are responsible for Friedreich’s ataxia, the most common autosomal-recessive neurodegenerative disease of Caucasians (1,2). Both genes code for mitochondrial proteins that are involved in iron homeostasis and cellular respiration (3–8), but their precise roles are unknown. Cardiac tissues from patients with Friedreich’s ataxia exhibit iron deposition, deficiencies in many iron–sulphur cluster enzymes and reduced mitochondrial DNA (8,9). In addition, fibroblasts from these patients show hypersensitivity to oxidative stress that can be rescued by treatment with iron chelators (10). A link to haeme biosynthesis has not been uncovered, and blood, bone marrow and red cell development appear to be normal in patient with frataxin deficits. Frataxin is downregulated during erythroid development, suggesting that this protein is not involved in the high-volume iron trafficking that accompanies red cell production in the bone marrow (11). However, activities of haeme enzymes in other tissues of Friedreich’s ataxia patients have not been assessed, leaving open the possibility that tissue-specific haeme deficiencies may exist.

Yeast cells lacking Yfh1p mirror many of the phenotypes observed in disease tissues from patients with Friedreich’s ataxia. These cells have defective respiration (3,5,12–14), unstable mitochondrial DNA and hypersensitivity to oxidative stress (15). The Δyfh1 mutant cells have a severe respiratory defect and accumulate iron in their mitochondria (16). The mechanism by which frataxin prevents iron accumulation is not known.

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stressed (3–5). The assembly of Fe–S centres is impaired and cytochrome concentrations are low (4,8). Iron uptake is much greater than in wild-type cells, with most of the iron being found in the mitochondria (3,12). The involvement of Yfh1p in the assembly of Fe–S centres has been described in several studies (8,15–18), but no work has been devoted yet to investigate the possible specific involvement of Yfh1p in the synthesis of haeme, a major iron cofactor synthesized into the mitochondria. An impediment to understanding the function of Yfh1p or frataxin has been the complex nature of the cellular phenotypes resulting from depletion or loss of function. Here we reexamine the role of Yfh1p in iron homeostasis with special emphasis on haeme synthesis. We describe a switch from haeme synthesis to zinc protoporphyrin synthesis that occurs in absence of Yfh1p. A highly sensitive fluorimetric method is used to demonstrate this switch. Previous studies have not noted effects of Yfh1p on haeme formation in yeast, and this may be due to the high frequency of suppressor mutations that mask this phenotype. Here we describe the characteristics of such suppressor mutants, and the effects on haeme formation in the absence of Yfh1p.

Results

Cytochrome deficiency in strains lacking Yfh1p

Yeast cells lacking the YFHI gene grew slowly (or not at all, depending on the strain) on non-fermentable carbon sources, consistent with a defect in mitochondrial function. The colonies appeared depigmented and measurement of total cellular haeme revealed global haeme deficiency in the mutant cells (0.01 nmol haeme/mg dry weight) compared with the wild-type (0.2 nmol haeme/mg dry weight). Low-temperature spectra of Δyfh1 mutant whole cells revealed a virtual absence of b, c and (a + a3) cytochrome signals (Fig. 1). In contrast, a signal from zinc protoporphyrin was observed in the mutant cells, and this signal was further enhanced by zinc supplementation of the growth media. This observation is significant because biosynthesis of Fe-PPIX (haeme) and Zn-PPIX both require porphyrin precursor and ferrochelatase activity, differing only in the final metal insertion step. The prevalence of Zn-PPIX synthesis rather than haeme synthesis in Δyfh1 cells did not result from increased zinc accumulation in Δyfh1 cells compared with wild-type cells. Actually, the total zinc content of the cells was lower in Δyfh1 cells than in wild-type cells (402 μg zinc per g Δyfh1 cell paste and 457 μg zinc per g wild-type cell paste). A detailed study of zinc metabolism in Δyfh1 cells will be published elsewhere.

A general problem that has impeded characterization of Yfh1p function is the variability and instability of the phenotypes of the deletion strains. In part this is due to a tendency to lose functional mtDNA, thereby becoming rho minus or rho zero. A mutant phenotype characterized by increased frequency of secondary nuclear mutations has also been associated with Yfh1p loss of function (19). Therefore, in order to distinguish primary effects from secondary changes ensuing from lack of YFHI, we created a strain in which the sole copy of YFHI was placed under the control of a galactose inducible promoter. In this strain, regulated expression of Yfh1p allowed correlation of phenotypes with different Yfh1p expression levels. If this strain was grown in raffinose, a non-inducing carbon source, for 24 h, Yfh1p was undetectable by immunoblotting of isolated mitochondria, and the cells exhibited phenotypes similar to the Δyfh1 strain. However, under these conditions, rho minus conversion or secondary genetic changes were not observed (not shown). As was observed for the deletion strain, cytochromes were undetectable in the low temperature spectra, whereas Zn-PPIX was clearly discerned as a 580 nm absorbance peak (Fig. 2). Cytochrome c was an abundant haeme protein of the intermembrane space was undetectable by blotting in these cells. When the cells were exposed to galactose, the promoter was rapidly induced and Yfh1p expression reached a maximum within 2 h. Cytochromes in general and cytochrome c in particular were recovered, although the time course of recovery seemed delayed with respect to the recovery of Yfh1p levels. Yfh1p was completely restored at the 2 h time point, whereas cytochromes recovery lagged behind (Fig. 2). The critical Yfh1p function involved in haeme formation presumably occurs during this time interval.

Loss of function of Yfh1 affects the final step of haeme synthesis

The final step in haeme biosynthesis involves iron insertion into PPiX and is catalysed by ferrochelatase, the product of the HEM15 gene. The Hem15 protein, localized to the mitochondrial inner membrane, was markedly decreased in Δyfh1 mitochondria (Fig. 3A). HEM15 mRNA was also lower in the Δyfh1 mutant than in wild-type cells (Fig. 3B), as also reported by others (20). Other key enzymes of the haeme pathway were unchanged in yfh1 mutant cells: the amounts of Hem1p...
(δ-aminolevulinate synthase) and Hem13p (coproporphyrinogen oxidase) were the same as in the wild-type (Fig. 3A), and the activity of Hem14p (protoporphyrinogen oxidase) was normal (see below). These data suggested that the defect in haeme synthesis in yfh1 mutant cells might be due to low level of ferrochelatase protein. We therefore sought to correct the Hem15p deficiency by using a multicopy plasmid to increase expression of the gene in the Δyfh1 strain. Surprisingly, the Δyfh1 cells transformed with YEp351-HEM15 grew even more slowly than the untransformed cells, forming tiny colonies on agar plates one week after transformation (not shown). The total haeme and cytochrome contents of the transformed mutant cells remained much lower than the wild-type cells (not shown). Thus, the cytochrome defect of Δyfh1 cells does not result from a lack of Hem15p. Moreover, while cytochromes were almost undetectable in Δyfh1 cells, a peak of Zn-PPIX was clearly apparent in these cells (see below). Zinc is an alternative substrate of ferrochelatase (21). Thus, although Hem15p was expressed at very low levels in the Δyfh1 mutant, the protein was still produced and probably functional in the mutant (see below).

An unresolved question was why Hem15 levels were low in the Δyfh1 strain. HEM15 transcription was found to be regulated in an iron-dependent manner, without dependence on Akt1p or Akt2p, the iron regulatory transcription factors (22,23). As shown in Figure 3B, iron addition to the growth media was correlated with a 2–3-fold increase in transcript abundance whether or not Akt1 or Akt2 was present. In the Δyfh1 strain the transcript was virtually undetectable. The molecular mediators of this iron dependent regulation remain to be determined. It is generally admitted that Akt-dependent genes are upregulated in Δyfh1 cells because cytosolic iron is low in these cells, most of the iron being sequestered in the mitochondrial compartment (14). In the case of HEM15, an Akt-independent gene, downregulation of transcription was probably again mediated by alterations in cytoplasmic iron levels, but the regulatory pathway appears to be a novel one.

Iron unavailability for haeme synthesis in Δyfh1 cells

Studies of in vitro haeme synthesis are often difficult to interpret, since ferrochelatase has a high affinity for ferrous iron, and in vivo iron availability to ferrochelatase probably depends on crucial factors related to iron compartmentalization or availability of an electron donor. Therefore, addition of exogenous ferrous ions and protoporphyrin IX (PPIX) to isolated mitochondria will result in haeme synthesis in vitro, even if no haeme synthesis occurred in vivo.

The level of ferrochelatase protein in Δyfh1 cells was lower than in wild-type cells, although the residual protein level varied depending on the yeast genetic background from roughly 10% (X498-1A) to 25% (S150-2BΔyfh1) of normal. This residual ferrochelatase protein was active as shown by the presence of Zn-PPIX in the mutants. Furthermore, the residual ferrochelatase in Δyfh1 mutants was able to mediate haeme formation (as measured by the pyridine haemochromogen method) when iron as ferrous ascorbate (or

**Figure 2.** Low-temperature spectra of whole cells of the strain GAL-Yfh1 various times after induction of YFH1 transcription. Cells of the GAL-Yfh1 strain were grown for 24h on YPR, diluted 20-fold into fresh YPR medium and grown for another 16h. At this time (t = 0), part of the cells were harvested and galactose (2%) was added to the culture. Cell aliquots were harvested 2 and 5h after galactose addition. Low-temperature spectra of whole cells were recorded (left panel). Yfh1p and Cyc1p levels were estimated by blotting of the mitochondrial proteins (right panel). The figure shows one experiment out of three independent experiments done.

**Figure 3.** Blotting of proteins of the haeme biosynthesis pathway in various isogenic yeast strains (A), and iron-dependent and Akt-independent transcription of HEM15 (B). The strains used were all isogenic to S150-2B. (A). Whole cell extracts (Hem1, Hem13) or mitochondrial extracts (Hem15) were prepared after overnight growth of the cells on YPD + 15 μg/ml hemin. Proteins were separated by SDS-PAGE before being blotted and revealed with the specific Hem1, Hem13 and Hem15 antibodies. (B). Total RNA was probed with HEM15 and ACT1 by northern blotting. Strains were grown in minimum media without iron or supplemented with 50 μM Fe(III)-citrate.
ferric citrate + NADH) and PPIX were added to isolated mitochondrial membranes. By contrast, haeme in Δyfh1 cells (X498-1A or S150-2B) was virtually undetectable, leading us to conclude that an additional defect in iron or porphyrin availability to ferrochelatase must exist in these cells. We then developed an in vitro assay to measure endogenous iron availability to ferrochelatase, using permeabilized whole cells (Fig. 4A) or intact mitochondria (Fig. 4B). We used protoporphyrinogen instead of PPIX as the substrate of reaction, and no exogenous metals, so that only endogenous iron or zinc could be incorporated into PPIX to form haeme or Zn-PPIX. Protoporphyrinogen is the substrate of protoporphyrinogen oxidase, an inter-membrane space enzyme, which converts protoporphyrinogen into PPIX. Protoporphyrinogen and haeme are both non-fluorescent, while PPIX and Zn-PPIX are both highly fluorescent. The use of protoporphyrinogen as substrate allowed monitoring with high sensitivity the rate of PPIX and Zn-PPIX synthesis. In the presence of a metal chelator such as EDTA or 8-hydroxyquinoline, haeme and Zn-PPIX formation from PPIX was inhibited, and we measured the rate of PPIX formation from protoporphyrinogen, i.e. protoporphyrinogen oxidase activity. This activity was comparable in both wild-type and yfh1 mutant cells (Fig. 4A, left panel), showing that the porphyrin substrate for ferrochelatase was not the limiting factor for haeme synthesis. When no chelator was added, part of the PPIX synthesized could be used by ferrochelatase to form haeme or Zn-PPIX with endogenous metals. The rate of haeme synthesis can then be calculated as:

\[ \text{rate of haeme synthesis} = \frac{\text{PPIX} + \text{Zn-PPIX}}{\text{total PPIX}} \]

where PPIX is the PPIX formed from protoporphyrinogen, and Zn-PPIX is the Zn-PPIX formed from PPIX. Protoporphyrinogen instead of PPIX as the substrate of porphyrinogen oxidase decreases the availability to ferrochelatase, using permeabilized whole cells grown on YPR. PYF1 transcription was either repressed (t=0; squares) or induced for 2 h (t=2 h; triangles) or for 5 h (t=5 h; circles) by galactose, as for Figure 2. Mitochondria were isolated from the cells incubated at 100 μM protoporphyrinogen to the cell suspension (OD 10). Formation of PPIX (λ_max 410 nm, λ_em 632 nm) and of Zn-PPIX (λ_max 420 nm, λ_em 587 nm) was followed fluorometrically. (a) Porphyrinogen oxidase activity; PPIX synthesis was monitored after chelation of all endogenous metals (iron and zinc) by adding 10 mM 8-hydroxyquinoline to the permeabilized cell suspension. (b) Synthesis of PPIX in the presence of endogenous metals. (c) Synthesis of Zn-PPIX in the presence of endogenous metals. (B) Cells of the strain GAL-Yfh1 were grown on YPR. YFH1 transcription was either repressed (t=0; squares) or induced for 2 h (t=2 h; triangles) or for 5 h (t=5 h; circles) by galactose, as for Figure 2. Mitochondria were isolated from the cells and incubated at 100 μM protoporphyrinogen to the permeabilized cell suspension. The reaction was initiated by adding 2 μM protoporphyrinogen to the cell suspension (OD 10). Formation of PPIX (λ_max 410 nm, λ_em 632 nm) and of Zn-PPIX (λ_max 420 nm, λ_em 587 nm) was followed simultaneously (upper panels and bottom left panel). Haeme formation (bottom right panel) was calculated as total PPIX (measured in the presence of EDTA) minus (Zn-PPIX + PPIX) (measured without EDTA). The figure shows one experiment out of three independent experiments done.
the synthesis of haeme (Fig. 4B) and of cytochromes (Fig. 2) was maximum only 5 h after induction.

Iron precipitation in an inorganic form in Δyfh1 mitochondria

The observation that iron was unavailable for haeme synthesis in Δyfh1 cells raised a question of why this should be so. The physical state of the iron was examined in mutant mitochondria using Mössbauer spectroscopy. Figure 5 shows Mössbauer spectra of mitochondria purified from a wild-type (YPH499) recorded at 4.3 K in a small perpendicular field of 20 mT. No Mössbauer signal was visible, indicating an iron concentration therein of lower than 300 μM and a lack of iron accumulation. In contrast, the mitochondria of the isogenic Δyfh1 mutant (X498-1A) displayed a well-resolved quadrupole doublet at 4.3 K (spectrum not shown) exhibiting an isomer shift δ = 0.53(4) mm s⁻¹, a quadrupole splitting ΔEQ = 0.63(1) mm s⁻¹ and a line width Γ = 0.57(1) mm s⁻¹ (numbers in brackets correspond to calculated error of last digit). No ferrous iron was observed. These Mössbauer parameters are typical of a high-spin ferric iron bound to oxygen/nitrogen in an octahedral arrangement. Very similar parameters were found for various bacterioferritins at this temperature (24−27).

Mitochondria of a Δyfh1 suppressor strain isolated from X498-1A (sup4+, see Fig. 5) exhibited a doublet with almost the same Mössbauer parameters (δ = 0.52 mm s⁻¹, ΔEQ = 0.67 mm s⁻¹, Γ = 0.53 mm s⁻¹; not shown). However, the degree of ferric iron accumulation per gram mitochondria was approximately one-quarter of that found in the original Δyfh1 strain.

A Γ-value of 0.57 mm s⁻¹ indicates a line-width broadening which can be associated with relaxation or superparamagnetic phenomena. Indeed, further broadening of the Mössbauer lines (δ = 0.53(4) mm s⁻¹, ΔEQ = 0.64(1) mm s⁻¹, Γ = 0.73(1) mm s⁻¹) occurred at 1.9 K (Fig. 5). Moreover, the formation of a second unstructured component (42% of absorption area) was observed. In contrast to what was found in bacterioferritins, no indication for a distinct magnetic hyperfine field or a narrow ranged field distribution was visible. This and the features of a high field spectrum (7 T, not shown) are consistent neither with a superparamagnetic transition as observed in bacterioferritins, nor with a magnetic transition of antiferromagnetically μ-oxo-coupled systems. The featureless broadening is best explained by a broad distribution of individual hyperfine fields originating from many magnetically non-equivalent ferric ions. Thus, our data are consistent with the presence of small and very amorphous nano-particles of iron in Δyfh1 mitochondria. Various attempts to visualize these particles on PAGE failed. The material remained in the wells of the gel, as seen by Fridovich staining (not shown). There were only very small amounts, if any, of protein associated with these particles (0.1 μg protein/μg iron), which could represent non-specific adsorption. Phosphate and iron determination resulted in a Fe/P ratio of 1/2.9 (8). We conclude that iron was essentially present in Δyfh1 mitochondria as nano-particles of ferric phosphate. In fact, an EXAFS analysis (not shown) supported the structural model of ferric phosphate as the main iron compound of Δyfh1 mitochondria. A complete analysis of

Some authors described normal cytochrome production in Δyfh1 cells (28). According to this observation, the cytochrome and respiratory defects reported by others for Δyfh1 mutants (3) could depend on a particular genetic background, on the growth conditions, or could result from rho minus conversion of the cells. Our results, however, do not support this hypothesis. We constructed a Δyfh1 shuffle strain where the yfh1 deletion was covered by a shuffle plasmid bearing a wild-type copy of YFH1. Cells of this strain formed isolated, depigmented colonies when plated on YPD + cycloheximide, but did not grow on YPG + cycloheximide plates (not shown). When Δyfh1 cells from a YPD plate were inoculated in liquid medium with glycerol as the carbon source, growth was delayed by a lag of 1–3 days (not shown). The cells harvested after 5 days on glycerol medium showed a normal cytochrome spectrum, unlike cells grown on raffinose as the carbon source, which were completely depigmented (Fig. 6). Cells from the glycerol culture did not recover the original phenotype of Δyfh1 (lack of cytochrome) when re-inoculated on a raf-based medium (Fig. 6). This result indicates that some inheritable change(s) occurred to the cells during their growth on glycerol. Indeed, Δyfh1 cells accumulated suppressor mutations with high frequency, which was easily observed on plate. When a mat of Δyfh1 cells was plated onto a YPG plate, numerous colonies grew on a background of non-growing (or poorly growing) cells (Fig. 7). The same observation was
made with Δyfh1 cells from different genetic backgrounds, including YPH499, S150-2B, CM3260 and W303 (not shown). We analysed several suppressor colonies of Δyfh1 from various genetic backgrounds. Most of the time, the suppressor strains exhibited the same phenotype as presented in Fig. 8. Cells recovered a normal cytochrome content (Fig. 8A) and normal activity of haeme-containing enzymes such as catalase (not shown). The activity of enzymes containing an iron–sulphur cluster remained low (Fig. 8B). Ferrochelatase and cytochrome c levels were increased compared to the original Δyfh1 mutant (Fig. 8C). Respiratory activity was similar to that of the wild-type (Fig. 8D). Cell iron accumulation decreased compared with the original Δyfh1 mutant but was still higher than in the wild-type (Fig. 8E), and iron still accumulated in the mitochondria, although to a lesser extent than in the original Δyfh1 mutant (not shown). Resistance of the cells to oxidative stress was increased (Fig. 8F). The suppressor phenotype of glycerol-growing cells resulted from nuclear mutation(s).

We crossed Δyfh1 cells showing the suppressor phenotype with an original Δyfh1 mutant of the same background with the opposite mating type. Features of the diploid were intermediate between the suppressor strain and the non-suppressed deletion strain, indicating semi-dominance of the suppressor mutation (Fig. 8C). Following sporulation and tetrad dissection, the suppressor phenotype was recovered in the tetrads, showing a 2:2 segregation of the suppressor characteristics (Fig. 8C). Such nuclear (semi-dominant) suppressor mutations occurred with high frequency in Δyfh1 cells submitted to the selection pressure of a non-fermentable carbon source or oxidative stress. The high rate of new suppressor mutations has prevented us from identifying the suppressor gene by complementation. A genomic library was constructed from a Δyfh1 suppressor strain and used to transform an original Δyfh1 strain. Transformants were selected on a copper-rich medium allowing growth of suppressors but not of original Δyfh1 cells. All the colonies analysed were new suppressor strains (not shown).

Thus, the presence of normal cytochrome concentration in some Δyfh1 strains (28) may result from suppressor mutations rather than from adaptation of the cells to particular growth conditions. Conversely, the lack of cytochromes in Δyfh1 was not a consequence of rho minus conversion of the cells, since induction of Yfh1 by galactose in the GAL-Yfh1 strain rapidly induced synthesis of all the cytochromes, with concomitant disappearance of Zn-PPIX, as shown above (Fig. 2). We conclude that Yfh1 is required for normal cytochrome synthesis in Δyfh1 cells, independent from the background and from the tendency of cells to loose mitDNA.

**Hem15p and Yfh1p interact in vitro**

Our biochemical experiments suggested that the final step of haeme biosynthesis required both Hem15p (ferrochelatase) and Yfh1p. We therefore looked for a direct interaction between ferrochelatase and Yfh1p using a real-time biomolecular interaction analyser, Biacore 2000, based on plasmon surface resonance measurements. Ferrochelatase (100 or 500 RU) was immobilized on research-grade CM5 sensor chips (Biacore) using a standard amine coupling procedure. Control experiments were run using bovine serum albumin (100 or 500 RU) and blank flow cells (activated carboxyl groups reacted with excess ethanolamine). A specific, high-affinity interaction between Yfh1p and ferrochelatase was measured when Yfh1p was used as the analyte (Fig. 9). Varying the concentration of Yfh1p from $7 \times 10^{-5}$ to $1.4 \times 10^{-6} \text{M}$ allowed us to estimate the dissociation constant ($4 \times 10^{-8} \text{M}$) by global fitting to a simple Langmuir model.
DISCUSSION

The loss-of-function of YFH1 results in striking maldistribution of cellular iron, with depletion of cytoplasmic iron and accumulation of mitochondrial iron. Here we show that mitochondrial iron in Δyfh1 cells, although present in large excess, is unavailable for haeme synthesis. Ferrochelatase, a mitochondrial protein, able to mediate insertion of iron or zinc into the porphyrin precursor, makes primarily the zinc protoporphyrin product. Zinc protoporphyrin instead of haeme accumulates during growth of Δyfh1 mutant cells and, furthermore, preferential formation of zinc protoporphyrin is observed in real time. The method for these studies involves direct presentation of porphyrin to mitochondria and to ferrochelatase of permeabilized cells with intact architecture, thereby specifically testing the iron delivery portion of the haeme biosynthetic pathway. The studies show that Δyfh1 mutant cells exhibit a defect in iron use by ferrochelatase. Multiple secondary effects and the complex pleiotropic phenotype of these cells has impeded definition of the function of frataxins. The defect in haeme synthesis in Yfh1p depleted cells, however, is readily reversed by reinduction of expression from a regulated promoter, and therefore the phenotype is not due to secondary effects such as mtDNA damage. The precise role of Yfh1p and frataxins in iron delivery for haeme synthesis remains to be defined. A role for Yfh1p in iron solubilization in mitochondria is possible and/or a role in directly handing of iron as described for metallation of copper proteins by copper chaperones (29). Ferrochelatase is able to use soluble ferric iron as a substrate for iron incorporation into PPIX, and iron is largely present as insoluble ferric particles in Δyfh1 mitochondria. This could explain why the large excess of iron in these mitochondria is not available for haeme synthesis, and maybe for other iron-requiring processes like Fe–S centre assembly. The involvement of Yfh1p might involve a role in maintaining solubility of iron in mitochondria. Our results show that Hem15p and Yfh1p physically interact in vitro. An in vivo interaction between these two proteins might mediate hand-off of iron for haeme formation. Adamec et al. (30) showed that self-assembled multimers of Yfh1p can sequester more than 3000 atoms of iron, and that iron can be released from the protein shell by a reducing agent. It is tempting to speculate that iron bound to such a Yfh1p intermediate may be the iron donor to ferrochelatase for haeme synthesis. Such a donor should exist as it is unlikely that there is ‘free’ iron in mitochondria, which is an environment particularly sensitive to radical reactions.

The role of Yfh1p in iron delivery for haeme synthesis recalls the recently described role of Yfh1p in iron delivery for Fe–S
cluster synthesis in mitochondria (8,15–18). The precise function of Yfh1p is likewise undefined in this process. A recent study by Puccio et al. (17) analyzed the sequence of events in tissues of transgenic mice following frataxin depletion. They found that the Fe–S assembly defect appeared prior to mitochondrial iron accumulation, suggesting a primary role for frataxin in iron utilization. Our work also indicates that Yfh1 is involved in mitochondrial iron use (for haeme synthesis as for Fe–S cluster synthesis) and for correct partitioning of iron between mitochondrial and cytoplasmic pools. Definition of the mechanisms of these effects will require additional work, and the identification of additional components of the mitochondrial iron transport system(s).

The cytochrome-deficient phenotype of Δyfh1 cells is often masked by nuclear mutation(s) that suppress the phenotype. The features of strains carrying such suppressor mutations are striking. Haeme synthesis is restored as indicated by complete recovery of cytochrome spectra and disappearance of Zn-PPIX. Haeme synthesis is restored, although iron accumulation and Fe–S cluster deficiency phenotypes are intermediate between the wild-type and deletion phenotypes. We frequently encountered such genetic changes with various Δyfh1 strains: strains were cytochrome-deficient when freshly isolated and acquired normal pigmentation after a few culture cycles on complete medium. Our finding that Δyfh1 cells accumulate suppressor mutation(s) fits well with a recent work (19), showing that the absence of Yfh1 in yeast leads to nuclear damage, increased chromosomal instability including recombination and mutation, and greater sensitivity to DNA-damaging agents. The frequency of these events may explain why the link of Yfh1p with cytochromes and haeme synthesis has not previously been described. The implication of the results is that the requirement of Yfh1p in haeme synthesis can be bypassed by nuclear gene mutations. Identifying the suppressor mutation(s) that allow(s) Δyfh1 cells to recover normal cytochrome synthesis and respiration is being undertaken. The unidentified suppressor mutation(s) might result in solubilizing part of the iron accumulated into Δyfh1 mitochondria, making it available for haeme synthesis, as suggested by our Mössbauer analysis. The low expression of frataxin in erythroid cells (11) and the lack of red cell phenotypes in the human frataxin deficiency disease Friedreich ataxia (11) likewise suggest that haeme synthesis can occur in the absence of frataxin in some settings.

Ferrochelatases from diverse species as mammals, Drosophila, Schizosaccharomyces pombe and some bacteria possess 2Fe–2S clusters that are critical for activity (reviewed in 31). In view of the role of frataxin in Fe–S cluster formation, an effect of frataxin deficiency on ferrochelatase function in these species might be inferred. By contrast, the ferrochelatase of S. cerevisiae lacks such Fe–S cluster and appears to be an outlier in this regard. One can speculate about the implications of this difference for the regulatory links between frataxin and ferrochelatase function. Perhaps, the effects of frataxin on Fe–S cluster assembly being insufficient to inactivate ferrochelatase in S. cerevisiae, alternative regulatory mechanisms evolved instead. At least two such mechanisms are observed here. First, a transcriptional mechanism is implied by the decreased Hem15 transcript levels observed in the absence of Yfh1. The mediators of this response are not Aft1 or Aft2, the previously identified iron regulatory proteins (22,23). Second a post-transcriptional effect of Yfh1 is required for active ferrochelatase function. This might involve a role of Yfh1 in delivery of the substrate iron for haeme formation. Alternative possibilities are that Yfh1 activates ferrochelatase activity by producing a folding or conformational change in the protein, or by contribution of a catalytic iron atom that serves a function analogous to the regulatory Fe–S cluster. The direction of these regulatory effects makes sense in terms of coupling haeme synthesis to the availability of the critical iron cofactor. In similar fashion, the first step in haeme biosynthesis, δ-aminolevulinate synthase in erythroid cells, is positively regulated by iron (32) and ferrochelatase in mammals is also regulated by iron via post-transcriptional mechanisms (33). Many questions remain: the molecular details of the regulatory effects of Yfh1 on ferrochelatase function remain to be defined. Also a question exists as to whether similar control mechanisms will be found in mammalian tissues.

MATERIALS AND METHODS

Strains, media and growth conditions

The strains of S. cerevisiae used in this study are described in Table 1. Unless otherwise stated, cells were grown at 30°C in complete YPD medium supplemented with 15 μg hemin/ml (YPD-hemin) for haeme-deficient strains. Other media used were YPG (yeast extract 1%, peptone 1%, glycerol 2%), YPR (yeast extract 1%, peptone 1%, raffinose 2%) and YPGal (yeast extract 1%, peptone 1%, galactose 2%). Cultures in iron-deficient medium were done in minimal YNB-glucose medium (yeast nitrogen base without copper and iron, Bio 101, Inc.) plus the required amino acids and 1 μM copper sulfate.

Iron uptake assays

Iron uptake was measured in microtitre plates. Cells were grown overnight at 30°C in YPD-hemin to the stationary phase. Cells were diluted 10-fold in the same medium and cultured for 6 h at 30°C. They were then washed once with 2% EDTA and three times with distilled water and suspended in 50 mM citrate.
Table 1. Genotype of the Saccharomyces cerevisiae strains used

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<tr>
<th>Strain name</th>
<th>Genotype</th>
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<td>S150-2B</td>
<td>MATa, his3-A1, leu2-3,112, trp1-289, ura3-52</td>
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<tr>
<td>S150-2Bp0</td>
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(tri-sodium) buffer (pH 6.5) containing 5% glucose to give about 2 mg protein/ml. The cell suspension was distributed into the wells of the micro-titration plate (50 μl cells/well) at 0 °C. Iron was added [as 55Fe(II)-ascorbate] to give a final concentration of 1–5 μM and the plate was incubated for 15–60 min at 30 °C. The cells were collected with a cell harvester (Brandel) and washed on the filter.

RNA isolation and northern analysis

RNA was extracted as described previously (34). Northern blotting and hybridization (at 42 °C in 50% [vol/vol] formaldehyde) were done essentially as described previously (35). The DNA fragment used as a probe for HEM15 was a 1.2 kb EcoRI–BamHI fragment and, for ACT1, a 1.2 kb BamHI–HindIII fragment.

Biacore experiments

Recombinant yeast ferrochelatase was overproduced in Escherichia coli (36) and purified as previously described (21). The specific activity of the recombinant protein (35 000 nmol/h/mg protein) was similar to that of the native enzyme. Recombinant mature Yfh1p (amino acids 52–174) was overproduced in E. coli as a His-tagged protein (37). Both proteins were extensively dialysed against HBS-buffer [10 mM HEPES, 150 mM NaCl and 0.005% (v/v) Tween 20, pH 7.4].

Mössbauer spectroscopy

In situ Mössbauer spectroscopy enables in principle the simultaneous identification of all major iron metabolites on a qualitative as well as on a quantitative level and of the kinetics of intracellular iron distribution without destruction of the cellular assembly (38,39). A prerequisite for in situ spectroscopy is an appropriate feeding of the cell cultures with 57Fe leading to 57Fe-iron concentrations high enough for detection by Mössbauer spectroscopy. 57Fe was purchased from Physicalische Messtechnik Lübeck. For preparation of Mössbauer samples cells were isotope-enriched with 57Fe by adding an aqueous 57Fe(III)–citrate solution (10 μM final) to the medium. As later demonstrated by Mössbauer spectroscopy, the intracellular isotopic enrichment with 57Fe was sufficiently high to obtain well-resolved spectra.

Samples of isolated mitochondria were transferred to cylindrical Mössbauer sample holders made of Delrin®, frozen and stored at 77 K until measurement. The Mössbauer spectrometer worked in conventional constant acceleration mode with sources of 0.9–1.85 GBq 57Co/Rh (Techsnabexport). The spectrometer was calibrated against a metallic 22Fe foil at room temperature yielding a standard line width of 0.25 mm/s. The Mössbauer cryostats were a helium bath cryostat (MD306, Oxford Instruments) or a superconducting magnet system with split coil geometry (Oxford Instruments). Isomer shift, quadrupole splitting, E0, and percentage of the total absorption area were obtained by least squares fits of Lorentzian lines to the experimental spectra.

Analytical methods

Protein concentrations were determined based on the Lowry method with the Bio-Rad D2 protein assay and the amount of iron was determined with the nitro-PAPS assay (40). Polyacrylamide gel electrophoresis (PAGE) was carried out by the Laemmli procedure (41). For SDS–PAGE the ready-to-use NuPAGE® electrophoresis system (Invitrogen) was used. Proteins were visualized by Coomassie brilliant blue staining. Iron loading of the protein could be detected on gels using the specific iron staining method of Fridovich (42). Phosphatase was determined employing literature methods (43,44).

Other

Protoplasts were lysed and fractionated to purify isolated mitochondria as previously described (45). Low temperature spectra (~191°C) of whole cells were prepared as described.
previously (46). Total haeme content of the cells was determined by the pyridine hemochromogen method (47). Zine content of the cells was measured by inductively coupled plasma atomic emission spectroscopy. Antibodies against Yfh1p, Hem15p and Hem13p were obtained as previously described (21,48,49). Ferrochelatase, zinc-chelatase and protoporphyrinogen oxidase activities were measured fluorometrically (21,50). Protoporphyrinogen was prepared from protoporphyrin by reduction with a sodium amalgam (48). When required, cells were made permeable by adding 20 μl of tolune–ethanol (4:1) to 1 ml of cell suspension (OD 10). Aconitase activity was measured spectrophotometrically on mitochondrial extracts of cells grown in WP with 2% raffinose (51).

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REFERENCES


