

The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins

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Received April 26, 2002; Revised June 20, 2002; Accepted June 21, 2002

The mitochondrial matrix protein frataxin is depleted in patients with Friedreich's ataxia, the most common autosomal recessive ataxia. While frataxin is important for intracellular iron homeostasis, its exact cellular role is unknown. Deletion of the yeast frataxin homolog *YFH1* yields mutants ($\Delta yfh1$) that, depending on the genetic background, display various degrees of phenotypic defects. This renders it difficult to distinguish primary (early) from secondary (late) consequences of Yfh1p deficiency. We have constructed a yeast strain (Gal-YFH1) that carries the *YFH1* gene under the control of a galactose-regulated promoter. Yfh1p-deficient Gal-YFH1 cells are far less sensitive to oxidative stress than $\Delta yfh1$ mutants, maintain mitochondrial DNA, and synthesize heme at wild-type rates. Yfh1p depletion causes a strong reduction in the assembly of mitochondrial Fe/S proteins both *in vivo* and in detergent extracts of mitochondria. Impaired Fe/S protein biogenesis explains the respiratory deficiency of Gal-YFH1 cells. Furthermore, Yfh1p-depleted Gal-YFH1 cells show decreased maturation of cytosolic Fe/S proteins and accumulation of mitochondrial iron. This latter phenotype is common for defects in cytosolic Fe/S protein assembly. Together, our data demonstrate a specific role of frataxin in the biosynthesis of cellular Fe/S proteins and exclude most of the previously suggested functions. Friedreich's ataxia may therefore represent a disorder caused by defects in Fe/S protein maturation.

INTRODUCTION

Friedreich's ataxia (FRDA) is the most common inherited ataxia and is transmitted in an autosomal recessive fashion (for recent reviews see 1–4). The disease is characterized by degeneration of the large sensory neurons and spinocerebellar tracts. Characteristic symptoms include ataxia of the limbs, areflexia, muscle weakness and skeletal deformities, cardiomyopathy, sensory loss, and increased incidence of diabetes mellitus. FRDA is caused by severely reduced levels of frataxin, a mitochondrial matrix protein (5,6). Deletion of the frataxin gene in mice leads to early embryonic lethality, demonstrating an important role of the protein during embryonic development (7). Histological and biochemical analyses of tissues from FRDA patients and conditional mouse mutants have shown that frataxin defects result in the accumulation of iron deposits within mitochondria (8,9).

Homologs of frataxin have been identified in most eukaryotes, from yeasts to humans. Distant homologs are found in many proteobacteria, but the protein has so far not been detected in other bacterial lineages and is absent in archaea (10–14). Its three-dimensional structure does not resemble any known

protein- or cofactor-binding motif (15–17); however, frataxin shares weak sequence homology with bacterial proteins involved in tellurite resistance (18). The most intensely studied member of the frataxin family is the yeast frataxin homolog Yfh1p. Deletion of the *YFH1* gene (yielding $\Delta yfh1$ cells) leads to growth defects on non-fermentable carbon sources, a tendency to lose the mitochondrial DNA (mtDNA), sensitivity to oxidative agents, and iron accumulation within mitochondria (10–12). Depending on the strain background, the severity of these phenotypes differs quite substantially, thus rendering conclusions on the function of frataxin rather difficult.

Several different functions have been proposed for mitochondrial frataxin. Most of these proposals are centered around the influence of frataxin on cellular iron homeostasis. Genome-wide analysis of gene expression in yeasts has shown that frataxin deficiency results in enhanced expression of genes that are under the control of the iron-sensing transcription factors *AFT1/AFT2*, including genes involved in iron uptake (19). Since the *AFT* regulatory system is induced by low cytosolic iron concentration; (20–22), the deletion of frataxin appears to result in a redistribution of iron from the yeast cytosol to mitochondria. Mitochondrial iron accumulation was explained by a function of

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frataxin in the efflux of iron from mitochondria (23), since induction of *YFH1* in Yfh1p-depleted cells led to the efflux of accumulated iron from mitochondria. While this study clearly shows the reversibility of the mitochondrial iron accumulation, the precise role of frataxin in iron export remains open. Two reports propose a function of frataxin as a mitochondrial iron storage protein (24,25). Recombinant yeast and human frataxin were found to assemble into oligomers reminiscent of the cytosolic iron storage protein ferritin. The oligomer but not the monomer was reported to bind iron. However, three-dimensional structures of frataxin from several species did not provide any clues as to the higher-order structure and did not identify any specific iron-binding sites (15–17).

Further, a role of frataxin in the assembly and/or maintenance of Fe/S proteins was proposed on the basis of decreased activities of mitochondrial Fe/S proteins in yeast and mouse mutants, and in FRDA patients (9,18,26). However, the effects were usually small (about 2-fold) or even fully absent in some tissues of affected patients. This raised the question of whether these defects arose from damage by the oxidative stress prevailing in frataxin-deficient cells (10,11), or were due to defects in the biogenesis of the Fe/S proteins. While an *in vitro* study was taken to propose a function in biogenesis (27), continuous oxidative damage of Fe/S clusters in human FRDA cells was suggested to be the result of hampered superoxide dismutase signaling (28). Further suggestions for frataxin function include a general role as a key regulator of respiration, a function in mtDNA maintenance, and a role in cellular antioxidant defense (29,30).

From previous analyses, it is difficult to assess whether the phenotypes associated with frataxin deficiency are directly linked to the protein's cellular role or whether these defects represent late, secondary consequences. In addition, use of deletion mutants generally carries the possibility that the cells will develop strategies suppressing the original phenotype. In this work, we have circumvented these problems and created a yeast mutant (termed Gal-YFH1) harboring a regulatable *YFH1* gene. Similar mutants have proven useful for the functional analysis of numerous cellular pathways. The Gal-YFH1 strain allowed the depletion of the *YFH1* gene product, thus minimizing the chances of the cells developing secondary phenotypes or acquiring the ability to suppress the original defects. By directly measuring the maturation of cellular Fe/S proteins *in vivo* and *in vitro*, we show that Yfh1p plays a specific role in this mitochondrial process. Consequently, the impairment of the maturation of cellular Fe/S proteins appears to be the primary consequence of frataxin deficiency from which other phenotypic features are derived.

RESULTS

The yeast frataxin homolog Yfh1p is required for respiratory activity of yeast

We constructed a conditional *Saccharomyces cerevisiae* mutant termed Gal-YFH1 in which the 5'-upstream sequence of the *YFH1* gene is replaced by the galactose-regulated *GALI-10* promoter using a PCR-mediated DNA replacement procedure (see Materials and Methods). Promoter exchange resulted in

the moderate (3-fold) overexpression of Yfh1p when Gal-YFH1 cells were grown in the presence of galactose (Fig. 1A). Upon cultivation in the absence of galactose, *YFH1* expression declined to levels hardly detectable by immunostaining analysis. Yfh1p-depleted Gal-YFH1 cells exhibited wild-type growth on glucose-containing rich media (Fig. 1B). On minimal media supplemented with glucose, cells gave rise to small colonies, whereas hardly any growth was detectable in the presence of glycerol, a non-fermentable carbon source. Thus, the Yfh1p-depleted cells show a behavior typical of a *petite* phenotype, which is indicative for respiratory deficiency in yeast (31). As judged by DNA staining with DAPI, the cells did not lose the mtDNA, even after extended growth under depleting conditions (not shown). In addition, when the levels of Yfh1p were elevated by shift to minimal medium with galactose, wild-type growth of Gal-YFH1 cells was restored, demonstrating the reversible character of the Yfh1p deficiency (not shown). This feature distinguished these cells from some of the deletion mutant strains $\Delta yfh1$ (11,12) and suggested that the loss of mtDNA is an indirect consequence of frataxin deletion. Interestingly, growth of Gal-YFH1 cells on glucose-containing minimal media could be partially improved by supplementation with glutamate and methionine (not shown). This auxotrophic behavior is reminiscent of mutants impaired in amino acid biosynthetic pathways which involve enzymes with Fe/S clusters (see below).

To further demonstrate the specificity of our Gal-YFH1 mutant, we transformed these cells with plasmids containing the yeast (not shown) and human frataxin genes (Fig. 1C). Expression of these genes fully complemented the growth defect of Gal-YFH1 cells on non-fermentable carbon sources. Upon expression of site-directed mutants of the human gene (amino acid exchanges G130V and W158A), an intermediate growth rate was observed. The G130V mutation in human frataxin causes a mild form of FRDA, while that of W158A results in a more severe form of the disease in human patients (see 32). In yeast, neither mutation caused a complete loss of frataxin function. In summary, our conditional mutant strain Gal-YFH1 appears to be suitable for studies of frataxin function, as it allows the rapid depletion of the Yfh1p protein concentration and the study of mutated frataxin proteins.

Depletion of Yfh1p leads to iron accumulation and reduced enzyme activities of Fe/S proteins in mitochondria

In order to gain insight into the function of frataxin, we first studied the consequences of Yfh1p depletion on the mitochondrial iron content. Mitochondria were isolated from cells grown in galactose- or glucose-containing minimal media, and the concentration of 'free' (i.e. non-heme, non-Fe/S) iron was measured by the bathophenanthroline method (33). Similar to $\Delta yfh1$ cells (11), a 10-fold increase of the amount of iron was observed in mitochondria isolated from Yfh1p-depleted Gal-YFH1 cells as compared to wild-type organelles (Fig. 2A). Thus, in yeast the redistribution of iron from the cytosol to mitochondria appears to be an early consequence of the defect in frataxin function.

Next, we measured the enzyme activities of two mitochondrial Fe/S proteins. Upon depletion of Yfh1p, the activities of aconitase and succinate dehydrogenase were at least 5-fold

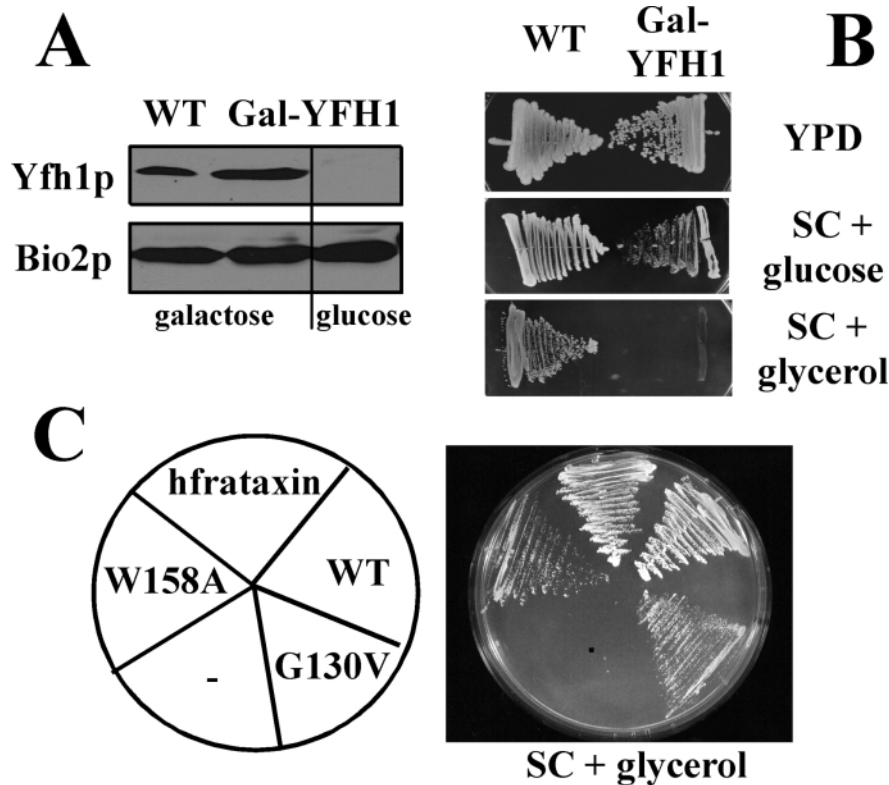


Figure 1. Characterization of a yeast strain for regulated expression of *YFH1*. (A) Immunostaining analysis for Yfh1p and Bio2p in mitochondria isolated from wild-type (WT) and Gal-YFH1 cells grown on synthetic minimal medium supplemented with galactose or glucose at 30°C. (B) Following depletion of Yfh1p by growth on solid SD medium at 30°C, wild-type and Gal-YFH1 cells were grown on rich medium supplemented with glucose (YPD), and synthetic minimal medium (SC) supplemented with glucose or glycerol. (C) Gal-YFH1 cells were transformed with plasmid p426GPD harboring wild-type human frataxin (hfrataxin), the corresponding mutants G130V and W158A or no DNA insert (-). These cells and wild-type cells were grown as described in (B).

reduced as compared to the wild-type situation (Fig. 2B). As shown by immunostaining analysis, the amounts of the corresponding polypeptide chains were not significantly altered under Yfh1p-depletion conditions (Fig. 2C). Furthermore, the activities of other enzymes, such as malate dehydrogenase, remained unchanged (Fig. 2B). Taken together, these findings indicate a specific decrease in the activity of mitochondrial Fe/S proteins upon depletion of Yfh1p. Since succinate dehydrogenase forms part of the mitochondrial electron transport chain, the results explain the loss of respiratory competence of Yfh1p-depleted Gal-YFH1 cells.

Reduced activities of mitochondrial Fe/S proteins are due to a defect in their maturation

The grossly reduced activities of mitochondrial Fe/S proteins may be caused either by a defect in the assembly of Fe/S proteins or by the oxidative damage of the Fe/S cluster holoproteins, which in turn may be a consequence of the increased iron concentration. To examine the reason for the reduced Fe/S protein activities in Yfh1p-depleted cells, we first analyzed the sensitivity of Gal-YFH1 cells to oxidative reagents upon Yfh1p depletion. As a reference, we used $\Delta yfh1$ cells that are highly sensitive to oxidative stress caused by external agents such as heavy metals and H_2O_2 (10,11).

In plate assays, Yfh1p depletion in Gal-YFH1 cells was not associated with an increased sensitivity to H_2O_2 , as cells were capable of growing at wild-type rates in the presence of H_2O_2 concentrations that were well above the threshold of toxicity for $\Delta yfh1$ cells (Fig. 3A). Upon more detailed examination of the doubling times in liquid cultures, Yfh1p-depleted Gal-YFH1 cells were found to be somewhat more susceptible to H_2O_2 than wild-type cells, but far less sensitive compared to $\Delta yfh1$ cells (Fig. 3B). The low residual sensitivity of Yfh1p-depleted Gal-YFH1 cells to H_2O_2 most likely reflects the oxidative stress caused by the mitochondrial iron deposits observed in these cells (see above). Clearly, the rapid depletion of Yfh1p was not immediately accompanied by the severe sensitivity to oxidative reagents that is observed in $\Delta yfh1$ cells, despite their similar high mitochondrial iron loads. Thus, the high sensitivity of $\Delta yfh1$ cells to oxidative reagents appears to be a late consequence of frataxin deletion. Taken together, these observations point towards a defect in the assembly of mitochondrial Fe/S proteins as the primary cause of the respiratory deficiency observed in the absence of Yfh1p.

To directly examine the assembly of a mitochondrial Fe/S protein, the *de novo* incorporation of radioactive ^{55}Fe into the mitochondrial biotin synthase (Bio2p) was investigated *in vivo*. The protein was overexpressed from the high copy (2 μ) plasmid p426GPD carrying *BIO2* under the control of a strong

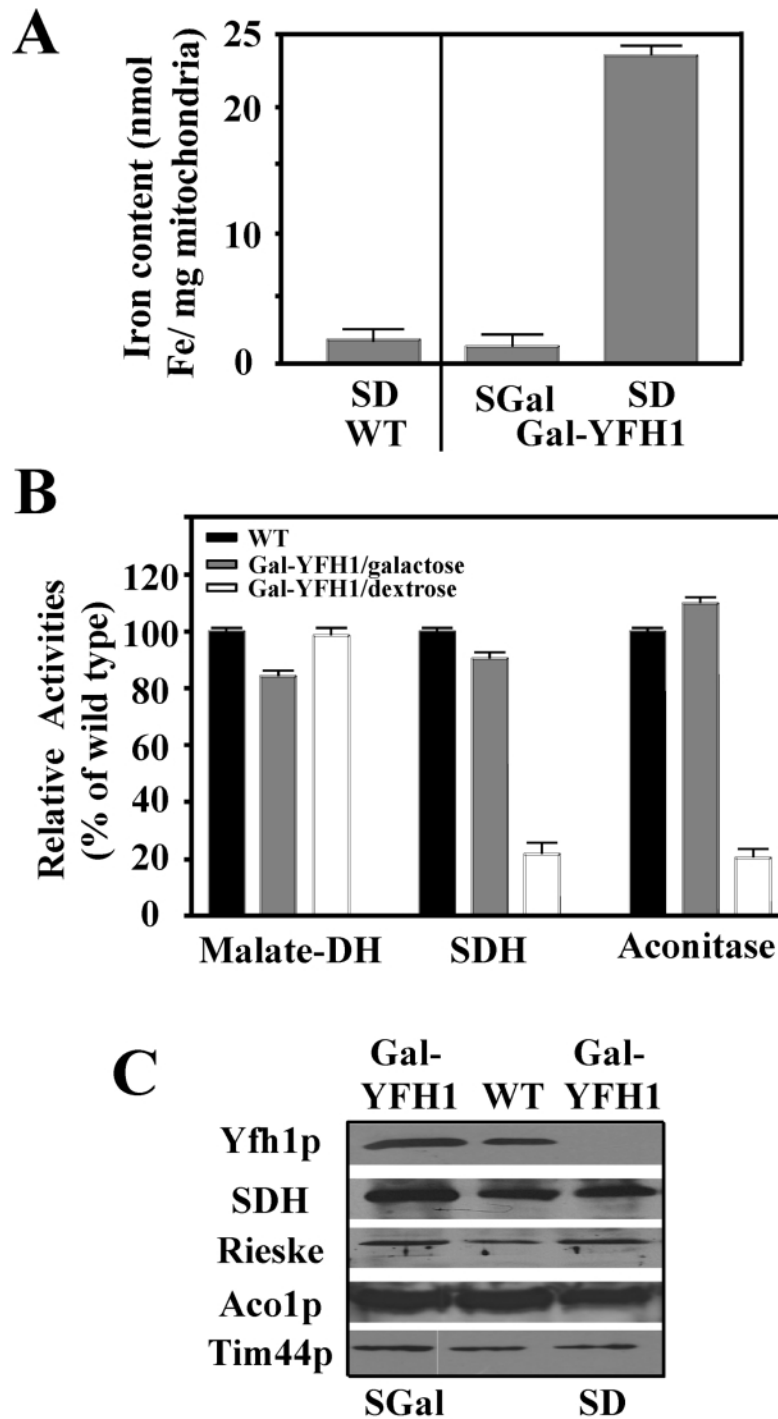


Figure 2. Depletion of Yfh1p induces the accumulation of iron within mitochondria and a specific defect in the activities of mitochondrial Fe/S proteins. Gal-YFH1 cells pre-incubated in SC medium supplemented with galactose or glucose and wild-type cells (WT) were grown in lactate medium for 16 h. Mitochondria were isolated and analyzed for: (A) non-heme, non-Fe/S ('free') iron content by the bathophenanthroline method (33); (B) the activity of malate dehydrogenase (Malate-DH), and the Fe/S proteins aconitase and succinate dehydrogenase (SDH); and (C) the levels of representative mitochondrial proteins by immunostaining. Error bars indicate the standard deviation of at least three independent measurements.

constitutive promoter. In wild-type or in Gal-YFH1 cells grown in the presence of galactose, a high amount of ^{55}Fe could be immunoprecipitated using Bio2p-specific antibodies (Fig. 4A), whereas only background signals ($<2\text{ pmol }^{55}\text{Fe/g cells}$) (34,35) were obtained using non-specific antisera or cells that

did not harbor overexpressed Bio2p (not shown). A strong reduction in the amount of ^{55}Fe associated with Bio2p was observed upon depletion of Yfh1p by growth of Gal-YFH1 cells in the presence of glucose, indicating a defect in the assembly of the Fe/S protein Bio2p (Fig. 4A). A similar

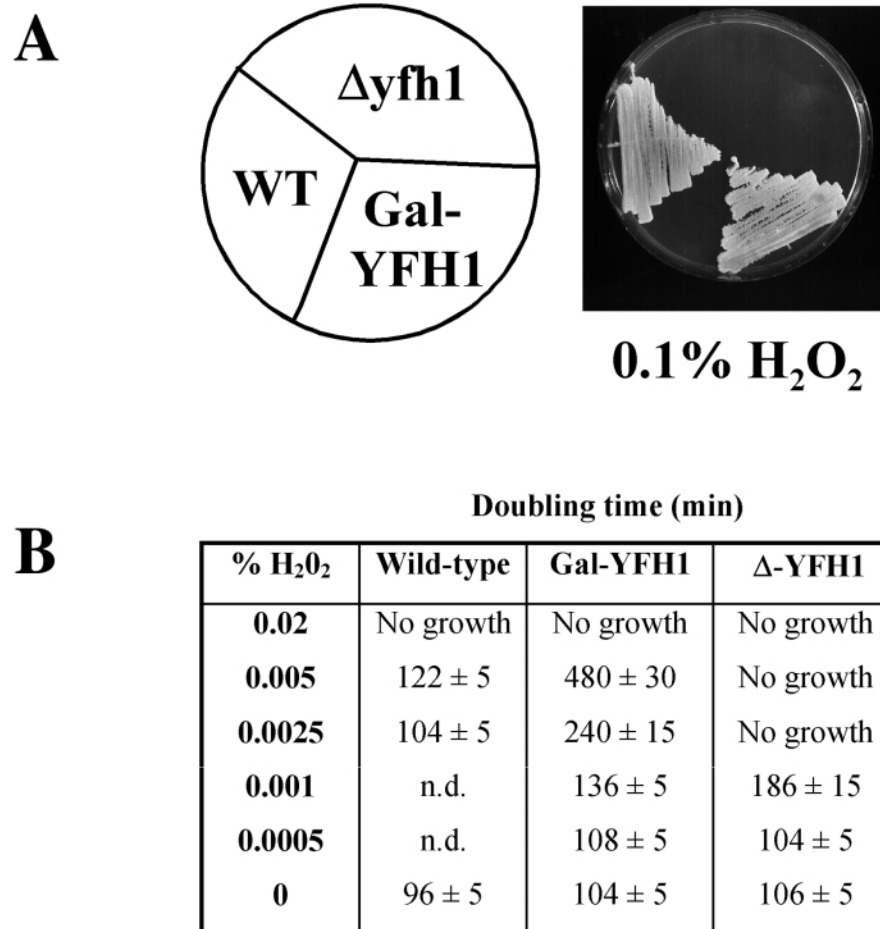


Figure 3. Depletion of Yfh1p in Gal-YFH1 cells does not cause severe sensitivity to oxidative reagents. (A) Wild-type, Yfh1p-depleted Gal-YFH1 and $\Delta yfh1$ cells were incubated for 2 days at 30°C on YPD agar plates containing 0.1% H₂O₂. (B) Doubling times of wild-type, Yfh1p-depleted Gal-YFH1 and $\Delta yfh1$ cells grown in liquid rich media (YPD) in the presence of the indicated concentrations of H₂O₂. Growth was recorded over a period of 12 h at 30°C. n.d., not determined.

severe decline in ⁵⁵Fe incorporation into Bio2p was observed in $\Delta yfh1$ cells.

To analyze the specificity of this defect in Fe/S protein assembly, we investigated the effect of Yfh1p depletion in Gal-YFH1 cells on the *in vivo* biosynthesis of another iron-containing component, namely heme. To this end, newly synthesized heme was determined from the incorporation of radioactive iron into protoporphyrin IX by extraction of radioactive heme into organic solvents (36). Depletion of Yfh1p did not significantly affect the biosynthesis of heme as compared to wild-type cells or Gal-YFH1 cells expressing *YFH1*, indicating that frataxin plays no decisive role in this process (Fig. 4B). Wild-type heme biosynthesis activity was obtained with $\Delta yfh1$ cells (not shown). In summary, loss of the enzyme activity of mitochondrial Fe/S proteins observed in the absence of Yfh1p seems to be due to a specific failure in the assembly of Fe/S proteins, and is not primarily caused by other more general consequences of the rather drastic alterations in cellular iron homeostasis upon Yfh1p deficiency.

In order to study the involvement of Yfh1p in Fe/S protein assembly in more detail, we employed an *in vitro* system that allowed us to follow Fe/S protein assembly in extracts of isolated mitochondria. In this assay, a detergent lysate was

prepared from mitochondria isolated from iron-starved cells overexpressing the mitochondrial Fe/S protein Bio2p. The extracts were incubated with radioactive ⁵⁵Fe under anaerobic conditions, and the incorporation of ⁵⁵Fe into mitochondrial Bio2p was determined from the amount of radioactivity that was co-immunoprecipitated from these extracts by antibodies against Bio2p. A detailed description of this assay is given elsewhere (37). The amount of ⁵⁵Fe incorporation into Bio2p reached wild-type levels with the use of mitochondria isolated from Gal-YFH1 cells bearing Yfh1p. Under Yfh1p-depleting conditions, a 7-fold reduction in Fe/S cluster assembly into Bio2p was observed (Fig. 5A). A similar decrease in Fe/S protein formation *in vitro* was detected in mitochondrial extracts from $\Delta yfh1$ cells. These results support the idea that Yfh1p plays a crucial role in the maturation of mitochondrial Fe/S proteins. The data obtained *in vitro* clearly exclude the possibility of defects in associated processes such as mitochondrial protein import, iron transport or iron homeostasis as the primary causes for the defect in Fe/S protein assembly. Moreover, since the mitochondria used in the *in vitro* assay were isolated from iron-starved cultures, their iron content was similar to that of wild-type organelles isolated from standard media (Fig. 5B). Hence, these data unambiguously exclude

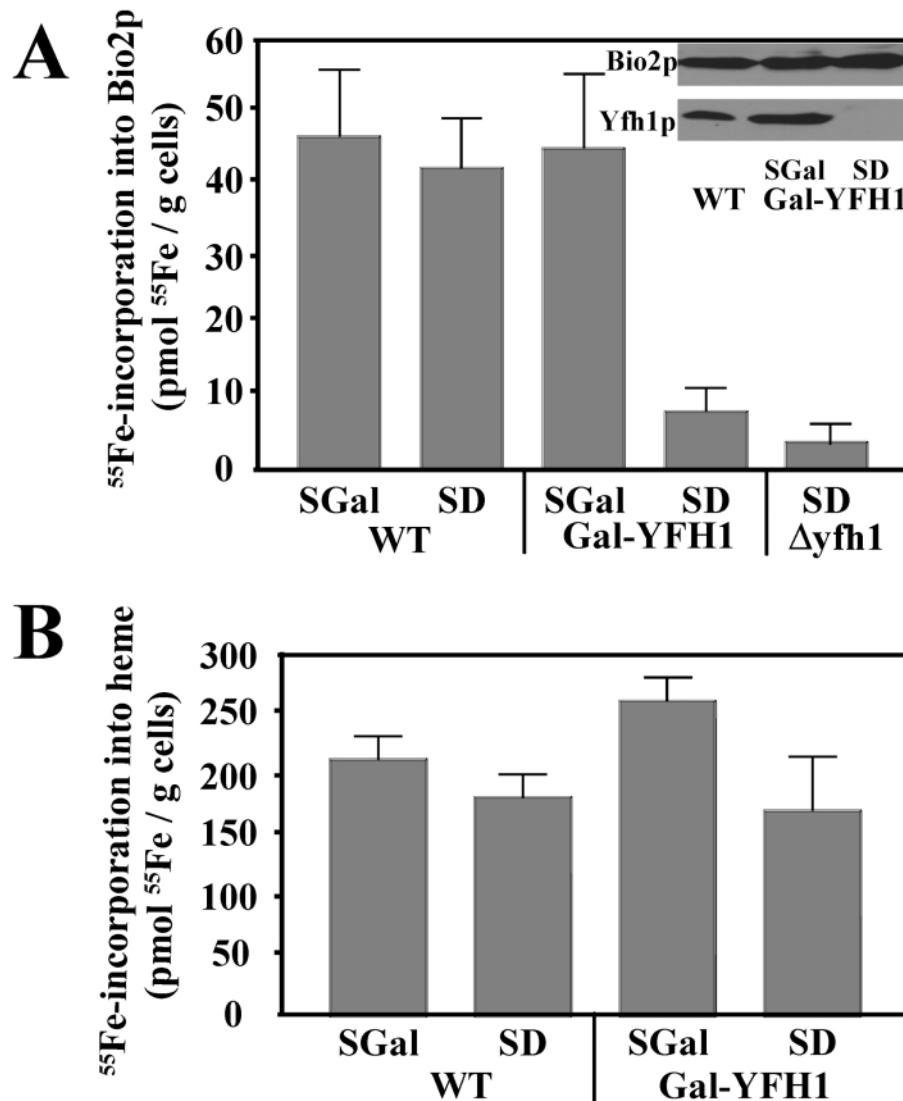


Figure 4. Yfh1p is required for the *de novo* maturation of mitochondrial Fe/S proteins *in vivo* but not for the synthesis of heme. Wild-type (WT), Yfh1p-depleted Gal-YFH1 and $\Delta yfh1$ cells expressing *BIO2* from the plasmid p426GPD were grown in 'iron-poor' minimal medium supplemented with either galactose (SGal) or glucose (SD) for 16 h. Cells were radiolabeled with ^{55}Fe for 90 min, and cell lysates were prepared. (A) Bio2p was immunoprecipitated from the lysates with anti-Bio2p antibodies and the amount of co-immunoprecipitated ^{55}Fe was quantified by liquid scintillation counting. The inset shows immunostaining of Yfh1p and Bio2p in mitochondrial preparations from the indicated cells. (B) Heme was extracted by butyl acetate, and the amount of extractable ^{55}Fe -labeled heme was estimated by liquid scintillation counting (36). Error bars indicate the standard deviation of at least three independent measurements.

deleterious effects of accumulated mitochondrial iron, such as oxidative damage of Fe/S holoproteins, as the primary cause of the observed Fe/S protein deficiency in mitochondria lacking Yfh1p.

Purified recombinant yeast and human frataxin have been shown to bind iron (24,25). Since the iron associated with the protein was reported to be firmly attached, it was suggested that Yfh1p might function as an iron storage protein. In order to test this hypothesis under *in vivo* conditions, the *YFH1* gene was overexpressed in wild-type cells using the yeast expression vectors p424GPD and pCM182, respectively. Cells were radiolabeled with ^{55}Fe , Yfh1p was immunoprecipitated from cell lysates, and the amount of ^{55}Fe associated with the immunoprecipitated material was quantified by liquid scintillation counting. Cell lysis and immunoprecipitation were

carried out under a variety of conditions, in order to screen for effects caused by chelators such as EDTA, detergents and reducing agents. All experiments were performed under aerobic and anaerobic conditions. However, under any condition tested, we did not detect any specific iron association with Yfh1p (not shown). The ability of the anti-Yfh1p antibody to immunoprecipitate Yfh1p was demonstrated using ^{35}S -radiolabeled cell extracts (not shown). We therefore conclude that either Yfh1p does not bind iron *in vivo*, or that the binding of iron to Yfh1p is too labile to be detectable with our assay. Furthermore, it is also possible that our antisera may fail to immunoprecipitate the iron-loaded form of Yfh1p. Taken together, our available data do not support a function of frataxin in mitochondrial iron storage, as such a role would require the stable binding of iron at high

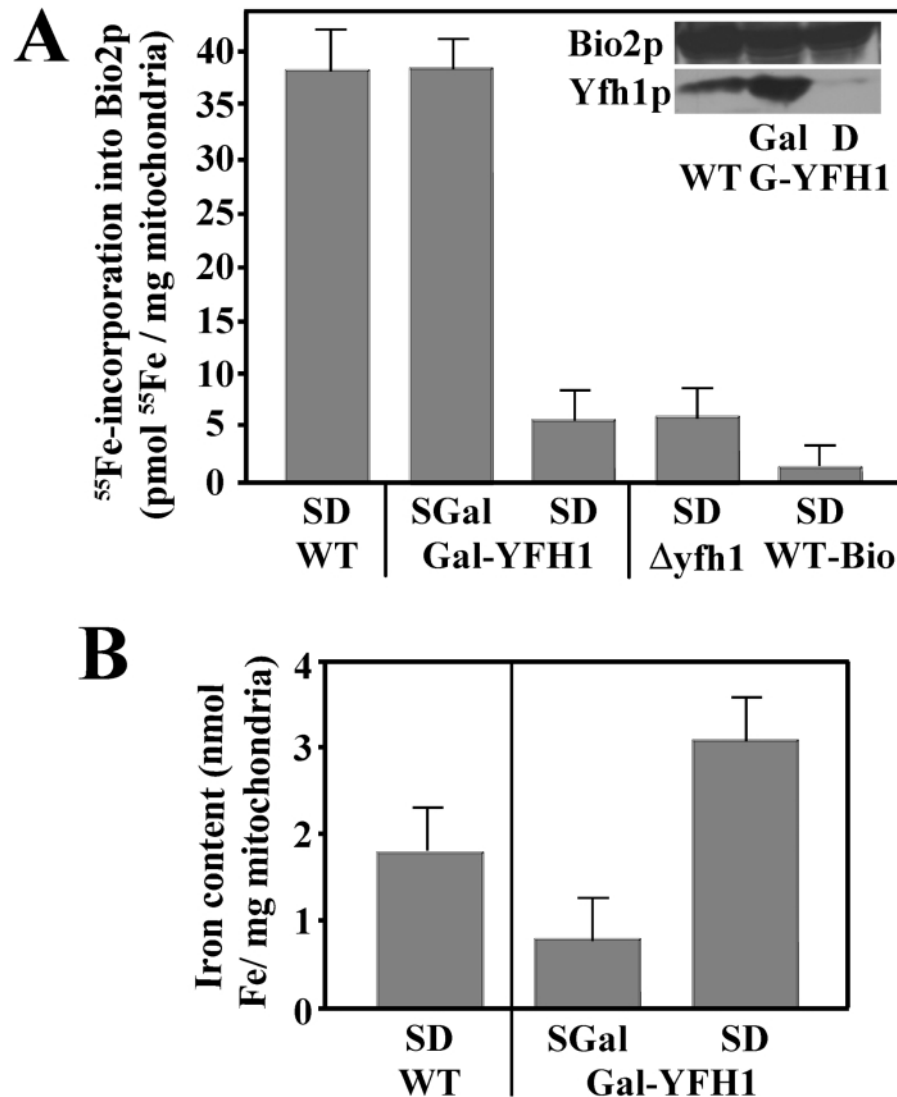


Figure 5. Yfh1p is crucial for the *de novo* maturation of mitochondrial Fe/S proteins *in vitro*. (A) Mitochondria were isolated from wild-type (WT), Yfh1p-depleted Gal-YFH1 cells and $\Delta yfh1$ cells after growth in iron-poor minimal medium supplemented with either galactose (SGal) or glucose (SD). Except for a wild-type control (WT-Bio2p), all cells contained a plasmid to overexpress *BIO2* (see Fig. 4A). Mitochondria were lysed in detergent and the extracts were radiolabeled with ^{55}Fe for 3 h under anaerobic conditions. The amount of ^{55}Fe incorporated into Bio2p was determined by immunoprecipitation as described in Figure 4. The inset shows immunostaining of Yfh1p and Bio2p in the mitochondrial extracts derived from wild-type and Gal-YFH1 cells. (B) The free iron contents of mitochondrial preparations from wild-type and Gal-YFH1 cells grown in iron-poor minimal medium in the presence of either glucose or galactose were determined as in Figure 2A. Error bars indicate the standard deviation of at least three independent measurements.

stoichiometry. However, they do not exclude a role of Yfh1p as an iron chaperone that may bind iron only transiently.

Yfh1p is involved in the maturation of cytosolic Fe/S cluster proteins

Several proteins of the Fe/S cluster (ISC) assembly machinery of the mitochondrial matrix, such as Nfs1p, Yah1p, Arh1p or Isa1p, are required for the maturation of Fe/S proteins in the cytosol (34,38–40). In order to test the involvement of Yfh1p in the assembly of Fe/S proteins in the cytosol, we followed the *de novo* incorporation of radioactive ^{55}Fe into the cytosolic Fe/S protein Leu1p and an HA-tagged version of Rli1p *in vivo* (34,35). The amount of ^{55}Fe incorporated into both reporter

proteins was reduced to 40% of wild-type levels upon depletion of Yfh1p in Gal-YFH1 cells (Fig. 6A and B). A similar decrease in Fe/S cluster assembly into Leu1p was observed with the use of $\Delta yfh1$ cells. In all cases, immunostaining analyses established that the polypeptide chains of Leu1p and Rli1p were present at wild-type levels under these conditions (Fig. 6A and B). These data suggest an involvement of Yfh1p in the assembly of cytosolic Fe/S proteins. In keeping with this notion, we noted that the slow growth of Gal-YFH1 cells on glucose-containing minimal media can be improved significantly by the addition of methionine and glutamate (not shown). The auxotrophy for methionine is best explained by an impairment in the function of the cytosolic Fe/S protein sulfite reductase (Met17p), and that of glutamate by the inactivation

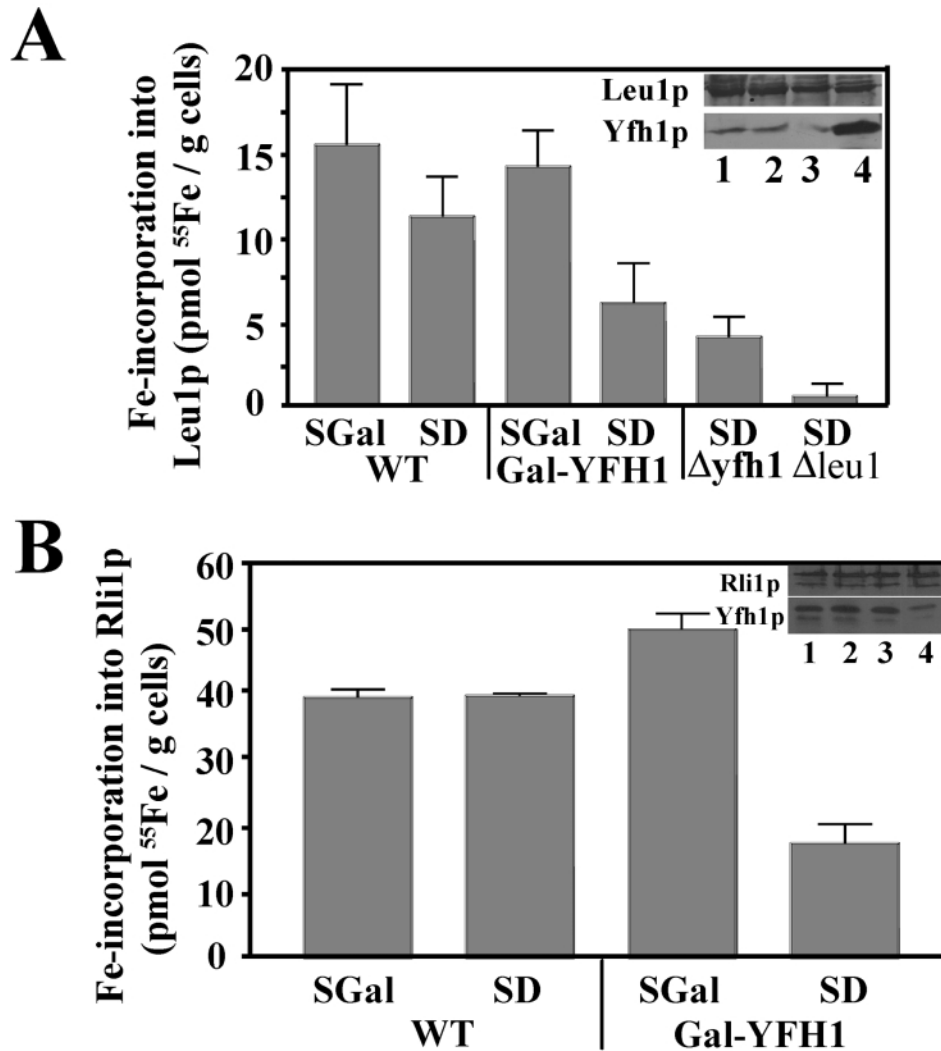


Figure 6. Depletion of Yfh1p results in decreased assembly of cytosolic Fe/S proteins. Wild-type (WT) cells, Yfh1p-depleted Gal-YFH1 cells and Δ yfh1 cells expressing an HA-tagged version of Rli1p from the plasmid p424GPD were grown in iron-poor minimal medium supplemented with either galactose (SGal) or glucose (SD) for 16 h. Cells were radiolabeled with ⁵⁵Fe for 4 h and cell lysates were prepared. The *de novo* Fe/S cluster assembly into (A) Leu1p and (B) Rli1p was analyzed by immunoprecipitation using antibodies against Leu1p and the HA epitope, respectively. Error bars indicate the standard deviation of at least three independent measurements. The insets show the immunostaining of Yfh1p, Leu1p and Rli1p-HA in the extracts investigated. (Part A: 1, wild-type/SD; 2, wild-type/SGal; 3, Gal-YFH1/SD; 4, Gal-YFH1/SGal. Part B: 1, wild-type/SGal; 2, wild-type/SD; 3, Gal-YFH1/SGal; 4, Gal-YFH1/SD).

of cytosolic glutamate synthase (Glt1p) and mitochondrial aconitase, both involved in glutamate production (41,42).

DISCUSSION

In this paper, we report the detailed analysis of a mutant yeast strain that allowed the depletion of the frataxin homolog Yfh1p by regulated gene expression. This strain enabled us to discriminate early from late phenotypic consequences of the decrease of Yfh1p levels and, consequently, to define the cellular role of Yfh1p. According to our analyses, Yfh1p depletion induces a respiratory deficiency, strong specific defects in the maturation of mitochondrial and extramitochondrial Fe/S cluster-containing proteins, and the accumulation of iron within mitochondria. Based on this study and data provided by previous investigations, we propose a role in Fe/S

protein maturation as the primary cellular function of frataxin. Other observed phenotypes can be envisioned as a consequence of defective Fe/S protein biogenesis. The respiratory deficiency of Yfh1p-depleted cells may easily be understood as a failure in Fe/S protein assembly of the respiratory chain complexes that contain several Fe/S clusters. The question of whether mitochondrial iron accumulation or Fe/S protein assembly deficiency represents the primary phenotypic consequence appears at first glance more difficult to answer, since the two effects occur almost simultaneously upon Yfh1p depletion in yeast. However, defects in the assembly of cellular Fe/S proteins in yeast are generally associated with the accumulation of iron within mitochondria (43–45). As a result of the accompanying iron depletion in the cytosol, cells impaired in Fe/S protein biogenesis increase their iron uptake (33,39). These characteristics closely resemble the behavior of frataxin-deficient cells and fit with the increased iron uptake observed in

Yfh1p-depleted Gal-YFH1 cells (not shown). Iron accumulation in mitochondria is also detected when proteins with a specific function in the maturation of cytosolic Fe/S proteins are inactivated (34,35). Strikingly, Yfh1p-deficient cells show a significant reduction in the maturation of cytosolic Fe/S proteins such as Rli1p and Leu1p. Moreover, the cells are impaired in the biosynthesis of amino acids that involve cytosolic Fe/S proteins, indicating a general defect in Fe/S protein assembly in the cytosol. This defective assembly of cytosolic Fe/S proteins in Yfh1p-depleted cells may therefore explain the mitochondrial iron accumulation. Taken together, the phenotype of Yfh1p-deficient cells is strikingly similar to that of yeast mutants impaired in components of the mitochondrial ISC assembly machinery (43,44).

A function of frataxin in Fe/S protein maturation is strongly supported by the use of a novel *in vitro* approach to study the assembly of eukaryotic Fe/S proteins. A deficiency in Yfh1p in mitochondrial extracts was associated with low efficiency in the maturation of Fe/S proteins *in vitro*. Since the iron load of the mitochondrial preparations used in these experiments was generally close to wild-type levels, iron-induced oxidative stress can be excluded as a cause of the inhibition of mitochondrial Fe/S protein maturation in this assay. Along the same lines, iron-induced oxidative damage cannot be the cause of the Fe/S protein assembly defects observed *in vivo* upon Yfh1p depletion, since our Fe/S protein assembly assay is carried out with iron-starved cells. Furthermore, our *in vitro* studies exclude a role of frataxin in processes that are only indirectly associated with Fe/S protein maturation, such as mitochondrial protein iron or cysteine import, iron storage, or general iron homeostasis. Finally, a role of frataxin in general iron import into mitochondria is rendered unlikely from the wild-type heme biosynthesis activity of yeast cells depleted in Yfh1p. We therefore propose that frataxin represents a novel component of the mitochondrial ISC assembly machinery.

Our investigations exclude several other functions previously proposed for frataxin (reviewed in 2 and 3). Yfh1p-depleted cells do not lose mtDNA and show only a moderate sensitivity to oxidative reagents, clearly distinguishing these cells from *YFH1*-deletion strains. Apparently, these effects are late consequences of Yfh1p deletion and are not directly related to frataxin function. Therefore, a role of frataxin in mtDNA maintenance and protection from oxidative stress can be ruled out from our study. The mitochondrial iron accumulation in Yfh1p-deficient cells has been taken to suggest a role of frataxin in the export of iron from the organelles back to the cytosol (23). Raising the levels of Yfh1p in Yfh1p-depleted cells induced the efflux of accumulated iron from mitochondria to the cytosol. We believe that these experimental observations are best explained by the reversible restoration of Fe/S protein assembly rather than by a direct role of Yfh1p in the regulation of iron export. The latter function would not readily explain the defects in both mitochondrial and cytosolic Fe/S proteins.

Frataxin is conserved in eukaryotes, and human frataxin can functionally replace the yeast protein (12). It is therefore likely that all eukaryotic proteins perform an orthologous function in Fe/S cluster biogenesis. Hence, in addition to X-linked sideroblastic anemia and cerebellar ataxia (XLSA/A) caused by mutations in the human *Atm1p* ortholog *ABC7* (46), FRDA may represent a disorder caused by defects in Fe/S protein

maturation. Homologs of Yfh1p are also found in eubacteria, mostly in β - and γ -proteobacteria (14). Disruption of the *E. coli* *cyaY* gene is not associated with conspicuous defects in bacterial iron metabolism, and bacteria show no signs of oxidative stress (13). From these observations, it was concluded that bacterial frataxin may not serve as a model for the eukaryotic homologs and may have a function distinct from these proteins. However, recent findings concerning Fe/S protein maturation in prokaryotes and eukaryotes (reviewed in 44) are compatible with the idea that frataxin may also play a role in the corresponding bacterial pathway. First, Yfh1p seems to perform a dispensable role in Fe/S protein maturation in yeast, unlike several other components of the ISC assembly machinery. Therefore, it seems plausible that the bacterial protein may play an auxiliary role in Fe/S protein biogenesis, explaining why it is difficult to track down its functional participation in this process in bacteria. Furthermore, the possibility of bypassing frataxin function would account for the fact that many bacteria do not possess a frataxin homolog, although these organisms appear to use the classical ISC assembly machinery for Fe/S protein maturation (14). Second, the lack of iron accumulation upon frataxin deletion in bacteria is easily understood from the fundamental differences in the mechanism of iron sensing and the regulation of iron homeostasis between eukaryotic and prokaryotic systems (see for example, 47 and 48). Bacteria impaired in Fe/S protein assembly have not been reported to accumulate iron. Thus, in light of these recent insights, iron accumulation is not an expected response to defects in bacterial Fe/S protein biogenesis (13). Third, bacterial *cyaY* genes show the same phylogenetic distribution as the *hscA* and *hscB* genes, which are usually encoded within the *isc* operons (14,49). The encoded proteins play a chaperone function in Fe/S cluster biogenesis (50–53), and hence a role of frataxin in the same process was postulated (14). Our data provide the experimental evidence for these theoretical predictions.

A few previous reports have pointed out defects in Fe/S protein activities in frataxin-deficient cells, but so far no *in vivo* measurements of the assembly of Fe/S proteins have been performed. Hence, it remained unclear whether reduced Fe/S protein activities had emerged from damage of Fe/S clusters or from impaired biogenesis. In yeast, a *YFH1* deletion mutant harbored 5–10-fold lower activities of mitochondrial aconitase as compared to wild-type cells, whereas succinate dehydrogenase was reduced only 2-fold (18). Upon cultivation in low iron-containing media, only a 2-fold reduction of aconitase and hardly any decrease in succinate dehydrogenase activities were observed. This suggested that the majority of these defects in $\Delta yfh1$ cells were due to oxidative damage of the Fe/S clusters and were apparently caused by the iron overload in these cells (18). In comparison, we show here that Gal-YFH1 cells are strongly impaired in Fe/S protein maturation, even in the absence of excess iron, clearly demonstrating that Fe/S assembly deficiency is the primary event. Similarly, human endomyocardial biopsy tissues taken from FRDA patients showed a slight (2–3-fold) reduction in aconitase activities relative to healthy controls, but hardly any alterations of complexes II and III of the respiratory chain harboring Fe/S proteins (26). In other patient tissues, aconitase activity was not decreased at all. The best evidence for a function of frataxin in

Fe/S protein biogenesis so far comes from mutant mice carrying a targeted frataxin gene inactivation in muscle or neuronal tissues (9). These mice display a progressive deficiency in aconitase and complexes I to III of the respiratory chain, with hardly any defects in 2-week-old mice, yet 4-fold reduced activities after 7 weeks. Interestingly, this defect is followed by iron accumulation in mitochondria of affected tissues. Evidently, mutant mice differ in this feature from our yeast mutant cells, in which mitochondrial iron is elevated in parallel with the impairment in Fe/S protein maturation (see above). Therefore, this finding supports our conclusion that the mitochondrial iron overload in yeast is a consequence of the failure in Fe/S protein maturation. It is clear from previous data that the usually small effects make it difficult to conclude with certainty that a defect in Fe/S cluster biogenesis is the primary reason for hampered Fe/S protein activities. Our study circumvents these disadvantages by the possibility of rapidly decreasing the amount of frataxin and by the direct measurement of the *de novo* biosynthesis of Fe/S proteins.

Our investigation has established a function of frataxin in the biogenesis of cellular Fe/S proteins. The elucidation of the exact step of the functional involvement of frataxin is the next challenge in the analysis of this interesting protein. The combination of *in vivo* studies with our newly developed *in vitro* system should provide a gateway to tackle this problem.

MATERIALS AND METHODS

Yeast strains and cell growth

The following strains of *Saccharomyces cerevisiae* were used: W303-1A (*MATa, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112*), which served as wildtype, $\Delta yfh1$ (W303-1A, *yfh1:LEU2*) (11), and Gal-YFH1. In the last strain, the promoter of *YFH1* was exchanged for a galactose-inducible promoter by PCR-mediated DNA replacement (Lafontaine and Tollervey, <http://mips.gsf.de/proj/yeast/CYGD/db/index.html>). In brief, PCR fragments carrying the *HIS3* gene and the *GAL1-10* promoter region of vector pTL26 flanked by region -228 to -178, and -18 to +30 of the *YFH1* gene were used to transform strain W303-1A. Initial transformants were selected for histidine prototrophy. Integration at the correct chromosomal locus was verified by PCR amplification on DNA isolated from single colonies using primers flanking the site of integration (54). In order to deplete the amount of Yfh1p to physiologically critical levels, Gal-YFH1 cells were routinely pre-incubated for 4 days on glucose-containing solid minimal media in the absence of galactose prior to the analyses. Cells were grown in rich (YP) or minimal (SC) media containing the required carbon sources (55), or in lactate media (56).

⁵⁵Fe incorporation into Fe/S cluster apoproteins *in vivo* and *in vitro*

In vivo labeling of yeast cells with radioactive iron (⁵⁵FeCl₃) and measurement of ⁵⁵Fe incorporation into mitochondrial or cytosolic Fe/S proteins by immunoprecipitation and liquid scintillation counting was carried out as described previously (34). The following reporter proteins were used: mitochondrial

biotin synthase (Bio2p), a hemagglutinin (HA)-tagged version of cytosolic Rli1p (35), both overexpressed from the 2 μ plasmids p426GPD or p424GPD, which carry the strong constitutive promoter of the glyceraldehydes-3-phosphate dehydrogenase gene *TDH3* (57), and cytosolic isopropylmalate isomerase (Leu1p). For analyzing iron binding of Yfh1p *in vivo*, *YFH1* was amplified by PCR and inserted into the *Bam*HI and *Cla*I restriction sites of vectors p424GPD or pCM182, which carry a doxycycline-regulatable tet-O₂-promoter (58). W303-1A cells overexpressing *YFH1* from these plasmids were radiolabeled with 10 μ Ci ⁵⁵FeCl₃ and 1 mM ascorbate for 2 h at 30°C as described previously (34). Cells were collected and lysed mechanically by vortexing with glass beads, and Yfh1p was immunoprecipitated from the clarified lysate by Yfh1p-specific antibodies under both aerobic and anaerobic conditions (59). Radioactivity associated with immunoprecipitated material was quantified by liquid scintillation counting.

The determination of Fe/S cluster formation *in vitro* was carried out with mitochondria isolated from corresponding yeast strains overexpressing Bio2p from plasmid p426GPD or p424GPD, respectively. Cells were grown at 30°C in glucose- (SD) or galactose-containing (SGal) minimal media lacking added iron chloride (55). At an optical density of 1–2 OD₆₀₀, cells were harvested and mitochondria were isolated as described (56). In an anaerobic chamber, 100 μ g mitochondria were resuspended in 250 μ l buffer A (20 mM HEPES, pH 7.4, 50 mM KCl, 1 mM MgSO₄, 0.6 M sorbitol) (36) supplemented with 1 mM sodium ascorbate, 1 mM dithiothreitol, 1 mM NADH, 10 μ M pyridoxal phosphate, and 0.2 mM cysteine. Organelles were lysed by Triton X-100 (0.1% w/v final concentration) and incubated with 5 μ Ci ⁵⁵FeCl₃ for 3 h at 25°C under anaerobic conditions. Radiolabeling was terminated by addition of 2 mM EDTA, membrane debris was removed by centrifugation (10 min, 15 000 g), and Bio2p was immunoprecipitated from the clarified reaction mixture as described. Radioactivity associated with immunoprecipitated Bio2p was quantified by liquid scintillation counting.

Miscellaneous methods

The following published methods were used: manipulation of DNA and PCR (60); transformation of yeast cells (61); isolation of yeast mitochondria (56); preparation of whole cell lysates by mechanical cell disruption with glass beads (62); immunostaining and immunoprecipitation (59); and enzyme activities of malate dehydrogenase, aconitase (63) and succinate dehydrogenase (64,65). The standard error of the determination of enzyme activities was between 5% and 15%. The iron content of mitochondria was determined as described previously (33).

ACKNOWLEDGEMENTS

We thank Dr D. Tollervey for kindly providing plasmid pTL26 and Dr F. Foury for the yeast strain $\Delta yfh1$. Our work was supported by grants of the Sonderforschungsbereich 286, Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, the Hungarian Fund OKTA, and the Fritz-Thyssen-Stiftung.

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