YFH1-mediated iron homeostasis is independent of mitochondrial respiration

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Abstract The human gene frataxin and its yeast homolog *YFH1* affect mitochondrial function. Deficits in frataxin result in Friedreich ataxia, while deletion of *YFH1* results in respiratory incompetence. We determined that as long as respiratory incompetent yeast express Yfh1p they do not accumulate excessive mitochondrial iron. Deletion of *YFH1* in respiratory incompetent yeast results in mitochondrial iron accumulation, while the reintroduction of Yfh1p results in mitochondrial iron export. Further, overexpression of Yfh1p has no effect on oxygen consumption in wild-type yeast grown in either fermentative or respiratory carbon sources. We conclude that the effect of Yfh1p on mitochondrial iron metabolism is independent of respiratory activity.

Key words: Mitochondrial respiration; Iron; Yeast; *YFH1*; Frataxin; Friedreich ataxia

1. Introduction

In the budding yeast *Saccharomyces cerevisiae*, *YFH1* is a nuclear gene that encodes a mitochondrial protein [1]. Frataxin is the human ortholog of *YFH1*, which when defective results in Friedreich ataxia, a disease affecting nerves and the heart [2]. Deficits in *YFH1* lead to the accumulation of iron within mitochondria [3,4]. It has been suggested that excess mitochondrial iron reacts with H_2O_2, generated through respiratory activity, leading to the formation of toxic oxygen radicals [1]. These radicals damage mitochondrial DNA resulting in respiratory incompetent cells [5]. Conditions that limit cytosolic iron concentration maintain the respiratory activity of Δ*yfh1* strains. It is unclear, however, how Yfh1p affects mitochondrial iron export, as Yfh1p is not a membrane-bound protein and can not be an iron transporter. A recent report has suggested that frataxin is a regulator of respiratory activity. This conclusion was based on analyzing the effect of overexpression of frataxin in cultured mammalian 3T3-preadipocytes [6]. When induced to differentiate into adipocytes, cells transfected with a retroviral construct overexpressing frataxin showed an increase of O_2 consumption, ATP levels and mitochondrial membrane potential. Based on these observations it was suggested that frataxin was a master regulator of respiration, although the affected step in metabolism is unknown.

In this communication we examined the role of respiration on iron metabolism in Δ*yfh1* yeast strain. First, we demonstrate that respiratory incompetent yeast, which have deletions of the mitochondrial genome, do not show excessive iron accumulation if they have a functional Yfh1p. Second, we demonstrate that deletion of *YFH1* in respiratory incompetent yeast results in mitochondrial iron accumulation, which is relieved upon reintroduction of Yfh1p. These studies indicate that the effect of Yfh1p on mitochondrial iron accumulation is independent of respiratory activity.

2. Materials and methods

2.1. Strains and growth media

DY150 (*MATa*, *ura3-52, leu2-3,112, trp1-1, his3-11, ade2-1, can1-100*(oc)) and DY1457 (*MATα*, *ura3-52, leu2-3,112, trp1-1, his3-11, ade6, can1-100*(oc)) were derived from the W303 strain of *S. cerevisiae*. The METYFH1 strain was generated by crossing the Δ*yfh1* (*MATa*, *ura3-52, leu2-3,112, trp1-1, ade2-1, can1-100*(oc), *yfh1::HIS3*) strain with DY1457 as described previously [7]. The resulting diploid was transformed with a plasmid containing a *MET3* promoter-regulated HA-tagged *YFH1* construct (pMET3YFH1), as described previously [4]. The transformed diploid was sporulated and dissected on complete minimal media lacking methionine and uracil. A haploid Δ*yfh1* strain, with respiratory activity maintained by the pMET3YFH1 plasmid, was identified and is designated METYFH1 (Δ*yfh1*, pMET3YFH1). The respiratory competence of METYFH1 was maintained by growing the cells in the medium lacking methionine and uracil. To generate a rho^0 version of the METYFH1 strain, cells were treated with ethidium bromide at 20 μg/ml to select for the loss of mitochondrial genome. All growth media used in this study were described previously [7].

2.2. Analysis of mitochondrial function

The presence of mitochondrial genome was determined by DAPI, 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR, USA) stain. A mid-log phase culture was harvested and washed with water and 95% methanol. Cells were resuspended in 1 ml 95% methanol. DAPI was added at a final concentration of 4 μg/ml and cells were incubated at room temperature in the dark for 10 min. The nuclear and mitochondrial DNA stains were examined by fluorescence microscopy.

Oxygen consumption was assayed using a Yellow Springs oxygen electrode as described previously [8].

2.3. Iron uptake assay

Iron transport assays were performed as described [9]. In a 0.5 ml reaction, 5×10^6 cells were incubated with ^{59}Fe ($FeCl_3$, Perkin-Elmer, Norwalk, CT, USA) at 0.5 μM in LIM-EDTA buffer with 1 mM ascorbate. Cells were incubated at 30°C for 10 min, and then washed on filters with EDTA-containing buffer to remove unincorporated iron. The filter was air-dried and radioactivity was determined. The uptake activity was expressed as pmol of ^{59}Fe per min per 10^6 cells.

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2.4. ^{59}Fe pulse-chase and subcellular fractionations

Exponentially growing cultures were harvested and washed three times with cold LIM-EDTA. Cells (1×10^9) were incubated with ^{59}Fe (0.5 μM in the presence of 1 mM ascorbate in 100 ml LIM-EDTA) at 30°C for 10 min. Cells were harvested, washed three times with cold EDTA-containing buffer and then incubated in fresh culture media for 2 h. Cells were harvested, spheroplasts were prepared and subcellular organelles were recovered by centrifugation of a post-nuclear supernatant at $15\,000\times g$ for 30 min as described by Radisky et al. [4]. The membrane pellet was layered onto a 0–25% iodixanol gradient, which was centrifuged at $12\,000\times g$ for 2 h. The gradient was then fractionated into 16 fractions. Radioactivity of ^{59}Fe in each fraction was determined. The data were plotted as the radioactivity in each fraction expressed as a percentage of the total radioactivity in the post-nuclear supernatant. As shown in our previous studies, using this protocol, mitochondria are localized to fractions 3–9 as ascertained by Western blotting using antibody against porin [4,7].

3. Results

YFH1 is required to maintain mitochondrial iron homeostasis in rho^0 cells.

Our previous studies showed that excessive mitochondrial iron accumulation occurs in cells lacking Yfh1p, which eventually leads to a respiratory deficit [4]. The loss of respiratory activity is time dependent and in our previous experiments, mitochondrial iron metabolism was examined in cells that were respiratory competent. Those studies left two questions unanswered. Does iron accumulate in respiratory incompetent mitochondria? Can *YFH1* affect iron metabolism in respiratory incompetent cells? To examine these questions we generated rho^0 cells using ethidium bromide to delete the mitochondrial genome. A yeast strain was generated that had a deletion in *YFH1* but was respiratory competent due to the expression of a plasmid borne *YFH1* under the control of the *MET3* promoter. These cells were grown in the absence of methionine and were incubated with ethidium bromide to select for the loss of mitochondrial genome. Loss of the mitochondrial genome was verified in three ways: (1) rho^0 cells (designated METYFH1-rho^0) were unable to grow on respiratory substrates; (2) rho^0 cells showed the absence of mitochondrial DNA staining when stained with DAPI (Fig. 1A); (3) rho^0 cells showed a marked reduction in oxygen consumption (Fig. 1B). We also measured the effect of overexpression

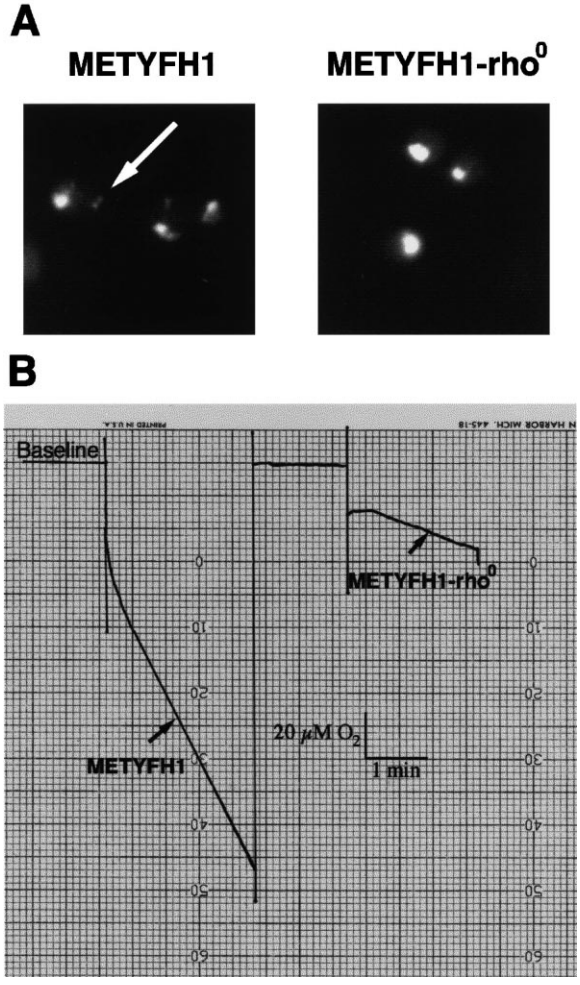

Fig. 1. Deletion of mitochondrial genome in METYFH1 strain by ethidium bromide. METYFH1 cells were incubated in ethidium bromide at 20 μg/ml in complete minimal media lacking both uracil and methionine for 48 h. The loss of mitochondrial genome was analyzed using DAPI (arrow points to a mitochondrial DNA staining in control cells) (A). Mitochondria respiration was determined by the rate of oxygen consumption in cells grown in glucose-containing media (B).

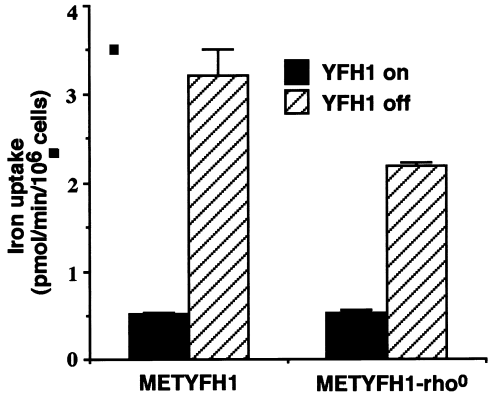

Fig. 2. Effect of YFH1 on high-affinity iron transport in METYFH1-rho^0 strain. The METYFH1-rho^0 strain was grown overnight in the presence or absence of 2 mM methionine. The rate of iron transport was determined using ^{59}Fe as described in Section 2.

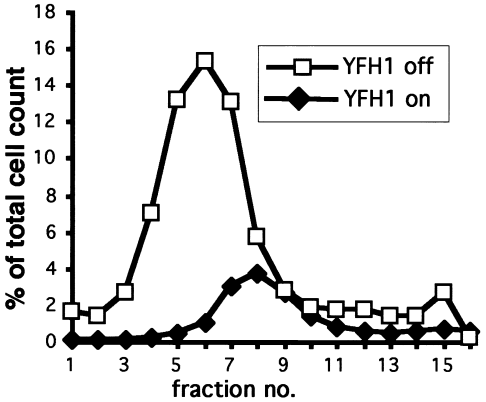

Fig. 3. Effects of *YFH1* on mitochondrial iron accumulation in METYFH1-rho^0 strain. The METYFH1-rho^0 strain was grown overnight in the presence ('YFH1 off') or absence ('YFH1 on') of 2 mM methionine. Mitochondrial iron was analyzed by ^{59}Fe labeling followed by subcellular fractionations on a 0–25% iodixanol gradient where the sedimentation of mitochondria-enriched fractions usually appears between fraction 3 and fraction 9.

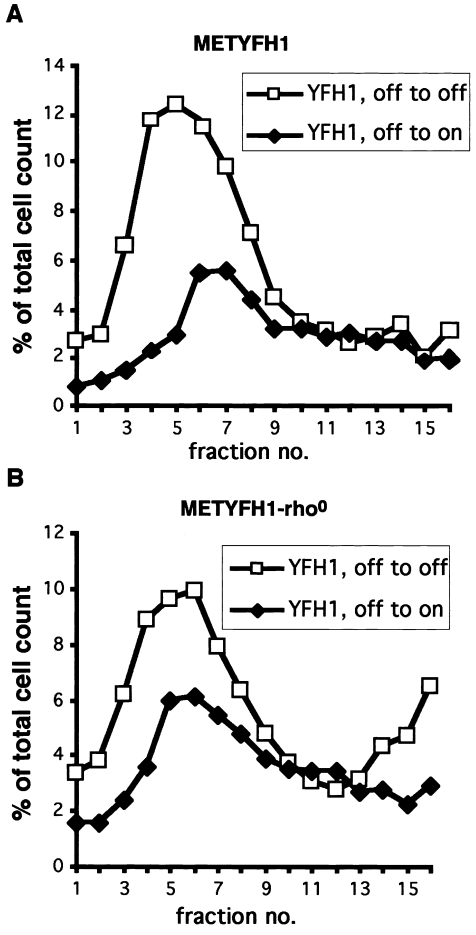

Fig. 4. Effects of *YFH1* on mitochondrial iron export in both rho$^+$ and rho^0 strains. Both METYFH1 (A) and METYFH1-rho^0 (B) cells grown in the presence of methionine were labeled with ^{59}Fe for 10 min and allowed to return to the same medium without ^{59}Fe for 2 h. At the end of the 2 h chase, half of each culture was washed and incubated in methionine-free medium ('off to on') for 12 h while the other half remained in the methionine medium ('off to off'). Cells were harvested and the distribution of ^{59}Fe was examined by subcellular fractionations on a 0–25% iodixanol gradient (B).

of Yfh1p on respiratory activity. Wild-type cells were transformed with plasmid-containing *YFH1* under the control of the *MET3* promoter. In the absence of methionine high levels of expression of Yfh1p occur [4]. Growth of cells in glycerol-ethanol increased the rate of oxygen consumption nine-fold over cells grown in glucose. Overexpression of Yfh1p in wild-type cells grown in either glucose or glycerol-ethanol had no affect on the rate of oxygen consumption (data not shown).

In cells that do not make Yfh1p, iron accumulates in the mitochondria at the expense of cytosolic iron [4]. Decreased cytosolic iron leads to increased transcription of the high-affinity iron transport system, comprised of Fet3p and Ftr1p, and increased cellular iron accumulation [1,4]. In respiratory competent cells, expression of Yfh1p from a *MET3*-regulated *YFH1* prevents mitochondrial iron accumulation and cytosolic iron depletion leading to low rates of iron transport [4]. Similarly, there is a normal rate of iron transport in METYFH1-rho^0 cells expressing Yfh1p. When METYFH1-rho^0 cells are incubated in the presence of methionine, which prevents transcription of *YFH1*, there is an increase in high-affinity iron transport activity (Fig. 2). One interpretation of this result is that the lack of Yfh1p leads to an increase in mitochondrial iron accumulation, which results in an induction of transcription of *FET3* and *FTR1*. To test this possibility we directly measured mitochondrial iron accumulation using $^{59}FeCl_3$. Mitochondria from METYFH1-rho^0 cells grown in the absence of methionine do not accumulate iron (Fig. 3). Mitochondrial iron accumulation, however, does occur when cells are grown in the presence of methionine, which results in the loss of Yfh1p. The amount of mitochondrial iron (fractions 3–9) accounts for 60% of total cell iron in cells that lack Yfh1p and 12% in cells that express Yfh1p. The amount of iron accumulated in mitochondria in METYFH1-rho^0 strains is similar to our previous report in METYFH1 strains [7]. This result demonstrates that simply being respiratory incompetent does not lead to mitochondrial iron accumulation.

Previously we demonstrated that expression of Yfh1p by a regulated promoter in cells with a Δyfh1 deletion resulted in the export of accumulated mitochondrial iron [7] (Fig. 4A). A similar result was obtained in cells that are respiratory incompetent. Induction of Yfh1p synthesis in METYFH1-rho^0 cells, whose mitochondria had previously accumulated iron due to lack of Yfh1p, resulted in export of mitochondrial iron (Fig. 4B). These results indicate that changes in mitochondrial iron transport depend on Yfh1p but are independent of respiratory activity.

4. Discussion

The function of Yfh1p/frataxin remains unknown. Loss of Yfh1p in yeast results in increased mitochondrial iron accumulation. In cells with a *YFH1* deletion, reduction in cytosolic iron levels prevents mitochondrial iron accumulation and preserves respiratory function. The function of Yfh1p, however, remains elusive, as is its effect on mitochondrial iron metabolism. A recent report suggested that frataxin, the mammalian homolog of Yfh1p, was a regulator of respiratory activity. This conclusion was based on an increase in respiratory activity when frataxin was overexpressed in line of cultured mammalian adipocyte-like cells. Our studies in yeast did not show any increased respiratory activity upon Yfh1p overexpression. Further, changes in mitochondrial iron metabolism resulting from a deficit in Yfh1p were independent of respiratory activity. Respiratory incompetent cells do not show mitochondrial iron accumulation as long as they have functional Yfh1p. In the absence of Yfh1p, respiratory incompetent cells will accumulate iron in mitochondria. This increased iron will exit the mitochondria upon the reintroduction of Yfh1p. These results indicate that mitochondrial iron metabolism, as affected by Yfh1p, is independent of respiratory activity. They also demonstrate that the energy required to either import iron into the mitochondria or export iron from mitochondria is not linked to respiration.

Frataxin is ortholog to *YFH1* as frataxin expressed in yeast can accomplish the critical function of Yfh1p, maintaining mitochondrial iron homeostasis and preserving mitochondrial respiratory activity. There is some question of leader sequence homology between yeast and mammalian proteins, as some studies indicate that the appropriate localization of frataxin in yeast requires a yeast leader sequence for proper mitochondrial localization [5,10], while other studies have not seen this

requirement [11]. All studies, however, have shown that beyond the leader sequence the bulk of the frataxin protein can complement Δ*yfh1* cells. If frataxin and *YFH1* are orthologs, then the results in yeast should be extrapolated to mammalian cells. How then can the results on mitochondrial respiration in frataxin-overexpressing mammalian cells be explained? One possibility is that the changes of oxidative phosphorylation in adipocytes are secondary to changes in iron metabolism. *YFH1* was initially identified as a high copy suppressor of a mutation in yeast that led to a growth defect on low iron medium [1]. The mutation was in a gene (YMR134W) that encodes an extra mitochondrial protein. Overexpression of *YFH1* complements this mutant by means of an increased rate of mitochondrial iron export, leading to increased cytosolic iron. An intriguing hypothesis is that increased iron export or some other mitochondrial product accelerates adipocyte differentiation. As changes in mitochondrial respiration and lipid accumulation are correlated with the stage of adipocyte differentiation, then anything which affects differentiation may lead to the observed changes in respiratory activity.

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