

# Low iron concentration and aconitase deficiency in a yeast frataxin homologue deficient strain

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**Abstract** Deletion of the yeast frataxin homologue, *YFH1*, elicits accumulation of iron in mitochondria and mitochondrial defects. We report here that in the presence of an iron chelator in the culture medium, the concentration of iron in mitochondria is the same in wild-type and *YFH1* deletant strains. Under these conditions, the activity of the respiratory complexes is restored. However, the activity of the mitochondrial aconitase, a 4Fe-4S cluster-containing protein, remains low. The frataxin family bears homology to a bacterial protein family which confers resistance to tellurium, a metal closely related to sulfur. Yfh1p might control the synthesis of iron-sulfur clusters in mitochondria.

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**Key words:** Friedreich's ataxia; Mitochondrion; Aconitase; Iron; Tellurium

## 1. Introduction

Iron is an essential metal in mitochondria, it is present in the heme of cytochromes and in iron-sulfur cluster-containing proteins. However, it is a highly reactive chemical which generates toxic free radicals via the Fenton reaction and its concentration must be tightly controlled. Several mitochondrial proteins, including Atm1p, an ATP-binding cassette transporter located in the inner membrane [1], Ssq1p [2], a Hsp70p-like chaperone, Oct1p, a metalloprotease involved in the maturation of mitochondrial precursors [3], and Yfh1p [4–7], the yeast frataxin homologue, have been shown to control iron homeostasis. Deletion of the yeast frataxin homologue gene (*YFH1*) ( $\Delta YFH1$ ) elicits a 10–15-fold increase in mitochondrial iron accumulation [4,5]. Iron makes deposits within the mitochondrial matrix [2] and cannot be extruded from mitochondria [8]. Moreover, loss of the *YFH1* gene is associated with permanent activation of the high-affinity iron transport system in the plasma membrane [4], a process which might contribute further to mitochondrial iron accumulation. Iron accumulation is associated with mitochondrial dysfunction, the severity of which is strain dependent [4–6]. In some strains, only mitochondrial respiration is decreased while in others, respiratory deficiency is associated with instability or total loss of mitochondrial DNA. Deficiency in aconitase has also been reported [9].

Elucidation of the function of Yfh1p will have a great medical impact. Mutations in the frataxin gene are responsible for Friedreich's ataxia, a recessive neurodegenerative disease often associated with cardiomyopathy with an estimated incidence of 1 in 30 000 Caucasians ([10]). The localization of human frataxin to mitochondria [11] and the presence of iron deposits in myocardial tissues of Friedreich's patients [12] suggest that yeast and human frataxins may have a similar function.

In the current paper, we have re-investigated the activity of the respiratory complexes and aconitase under culture conditions which allow us to obtain the same low iron concentration in wild-type and  $\Delta YFH1$  mitochondria.

## 2. Material and methods

### 2.1. Strains and media

The *Saccharomyces cerevisiae* strains were W303-1B (*MAT $\alpha$  ade2-1 leu2-3, 112, his3 ura3-1 trp1-1*) and its isogenic deletant derivative, W303-1B $\Delta YFH1$  ( $\Delta YFH1$ ), in which the *YFH1* gene has been replaced by a kanamycin resistance cassette [5]. Cells were grown in raffinose synthetic medium (2% raffinose, 0.67% yeast nitrogen base (Difco), 0.5% ammonium sulfate, a mixture of amino acids and the required auxotrophic supplements). FeSO<sub>4</sub> was solubilized in 0.1 N HCl and immediately added to the culture medium at the indicated concentration.

### 2.2. Preparation of mitochondria and measurement of respiratory activities

Mitochondria were prepared after spheroplast lysis as previously described [5]. Oligomycin sensitive ATPase, antimycin A sensitive NADH cytochrome *c* reductase and cytochrome *c* oxidase were measured as previously described [5]. Aconitase activity was measured by a standard procedure using isocitrate as the substrate [13]. Succinate dehydrogenase activity was determined by measuring oxygen consumption with a Clark-type electrode and using succinate and phenazine methosulfate in the presence of antimycin A.

### 2.3. Measurement of iron concentration

Non-heme, non-Fe-S mitochondrial iron was measured using bathophenanthroline sulfonate (BPS) in the presence of dithionite as described [14].

## 3. Results

### 3.1. Identical low iron concentrations can be obtained in wild-type and $\Delta YFH1$ mitochondria

When yeast cells were cultivated in a glucose rich medium which had a high iron content, a 10–15-fold increase in the mitochondrial iron concentration was observed in a  $\Delta YFH1$  mutant as compared to a wild-type strain [4,5]. In parallel, cellular respiratory activity was less than 10% of the wild-type [5]. However, under these conditions, the decreased cellular respiration results from the combination of respiration repression by glucose and iron toxicity. By comparison, raffinose

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**Abbreviations:** BPS, bathophenanthroline sulfonate; ORF, open reading frame

Table 1  
Iron concentration in mitochondria of wild-type and  $\Delta YFH1$  strains

Culture medium	Iron in mitochondria (nmol/mg protein)	
	Wild-type	$\Delta YFH1$
Raffinose <sup>a</sup>	4.5 ± 0.5	9.7 ± 2.0
Raffinose+10 $\mu$ M iron <sup>b</sup>	10.6	133
Raffinose+10 $\mu$ M BPS <sup>c</sup>	5.0	3.3
Raffinose+5 $\mu$ M BPS <sup>d</sup>	4.7	8.0

<sup>a</sup>Iron concentration resulting from several independent cultures on standard raffinose medium.

<sup>b</sup>Addition of 10  $\mu$ M iron to the standard medium.

<sup>c</sup>Addition of 10  $\mu$ M BPS to the standard medium.

<sup>d</sup>Addition of 5  $\mu$ M BPS to the standard medium.

synthetic medium offers an excellent respiratory carbon source and a lower iron concentration (approximately 1  $\mu$ M). When  $\Delta YFH1$  cells were shifted from glucose rich to raffinose synthetic medium, the iron concentration in  $\Delta YFH1$  mitochondria progressively decreased to reach approximately twice that of the wild-type strain after 9 h of culture (data not shown).

When 10  $\mu$ M ferrous iron was added to the culture medium, the mitochondrial iron content was more than 10 times higher in the  $\Delta YFH1$  mutant than in the wild-type strain (Table 1). However, when most of the iron present in the culture medium was trapped by an iron chelator such as BPS, the concentration of iron in mitochondria was not significantly different in the wild-type and  $\Delta YFH1$  strains. Iron accumulation in mitochondria can thus be prevented by limiting the iron availability in the culture medium. It can thus be expected that under these conditions, iron does not produce oxidative damage to  $\Delta YFH1$  mitochondria.

### 3.2. Restoration of mitochondrial respiratory activities in the $\Delta YFH1$ mutant

The activity of the respiratory complexes was measured in cells grown in raffinose synthetic medium with various iron concentrations. When the iron concentration was higher in  $\Delta YFH1$  than in wild-type mitochondria, the activity of cytochrome oxidase and, to a lesser extent, that of oligomycin sensitive ATPase and succinate dehydrogenase were significantly decreased (Tables 2 and 3). The activity of antimycin A sensitive NADH cytochrome *c* reductase, which measures the electron flux through complex III, was not significantly decreased (Table 2). When the concentration of iron was identical in  $\Delta YFH1$  and wild-type mitochondria (Tables 2 and 3, +10  $\mu$ M BPS), the activity of the respiratory complexes was fully (or almost fully) restored. These data show that the deletion of the *YFH1* gene does not impair the respiratory function per se. A decrease in the respiratory activities corre-

lates with an increased mitochondrial iron content and is probably the result of oxidative damage.

### 3.3. Low aconitase activity in a $\Delta YFH1$ mutant

Rötig et al. [9] had previously reported that aconitase activity was very low in heart biopsies of Friedreich patients. Similar results were obtained using extracts from yeast cells grown in glucose rich medium [9]. As aconitase contains a 4Fe-4S cluster which is extremely labile [15], a low aconitase activity was interpreted as the result of oxidative degradation of the Fe-S cluster by the free radicals generated by iron [9]. We verified that when high iron concentrations were present in  $\Delta YFH1$  mitochondria, aconitase activity was low (Table 3). However, even when, after growth in the presence of BPS, no iron accumulation occurred in  $\Delta YFH1$  mitochondria, aconitase activity was still less than 50% of that of the wild-type strain (Table 3). This low activity, which cannot easily be explained by iron toxicity, might result from a defect in iron-sulfur cluster biogenesis.

### 3.4. Homology between frataxin homologs and a tellurium resistance protein family

Frataxin homologs have previously been shown to form a unique protein family which has been largely conserved from Gram-negative bacteria (Cyay) to humans [16], including yeasts and plants. Using the Cyay open reading frame (ORF) from *Escherichia coli* as the query, a search for sequence homology was carried out in the database of unfinished microbial genomes provided by the Institute for Genomic Research (Fig. 1). The *E. coli* Cyay ORF was not only aligned with the Cyay ORFs from other organisms but also with a *Clostridium acetobutylicum* ORF, which actually belongs to a protein family associated with tellurite resistance (Ter) (Fig. 1). The Ter family has members in *C. acetobutylicum*, *Bacillus subtilis*, *E. coli*, *Deinococcus radiodurans*, *Dictyostelium* and *Synechocystis* [17]. The Ter genes which encode proteins of unknown function and related sequences are organized in operons borne by plasmids or by the chromosome. Interestingly, when a reciprocal sequence homology search [18] was run against the *C. acetobutylicum* ORF, in addition to Ter ORFs, many Cyay and frataxin homologues were found, suggesting a phylogenetic relationship between Cyay/frataxin and Ter families (Fig. 1). Amino acid sequence homology is restricted to the N-terminal part of Ter ORFs and corresponds to the conserved core region of the Cyay/frataxin family. In both families, the conserved region is characterized by the presence of numerous glycine residues, suggesting a flexible domain, and by an invariant tryptophan residue. The latter is followed by an hydrophobic residue in the fra-

Table 2  
Activity of the respiratory complexes in wild-type and  $\Delta YFH1$  mitochondria

Culture medium <sup>a</sup>	Cytochrome oxidase (U/min/mg protein)		NADH cytochrome <i>c</i> reductase ( $\mu$ mol/min/mg protein)		Oligomycin sensitive ATPase ( $\mu$ mol/min/mg protein)	
	Wild-type	$\Delta YFH1$	Wild-type	$\Delta YFH1$	Wild-type	$\Delta YFH1$
Raffinose <sup>b</sup>	42	17	2.7	3.3	6.0	5.5
+10 $\mu$ M iron <sup>b</sup>	46	9	2.2	2.4	5.5	3.0
Raffinose <sup>c</sup>	59	12	2.5	2.0	4.5	3.5
+10 $\mu$ M BPS <sup>c</sup>	50	34	3.5	3.2	4.6	5.5

<sup>a</sup>The media and cultures are as in Table 1.

<sup>b</sup>The data have been obtained from cultures carried out simultaneously.

<sup>c</sup>The data have been obtained from cultures carried out simultaneously and independently of cultures b.

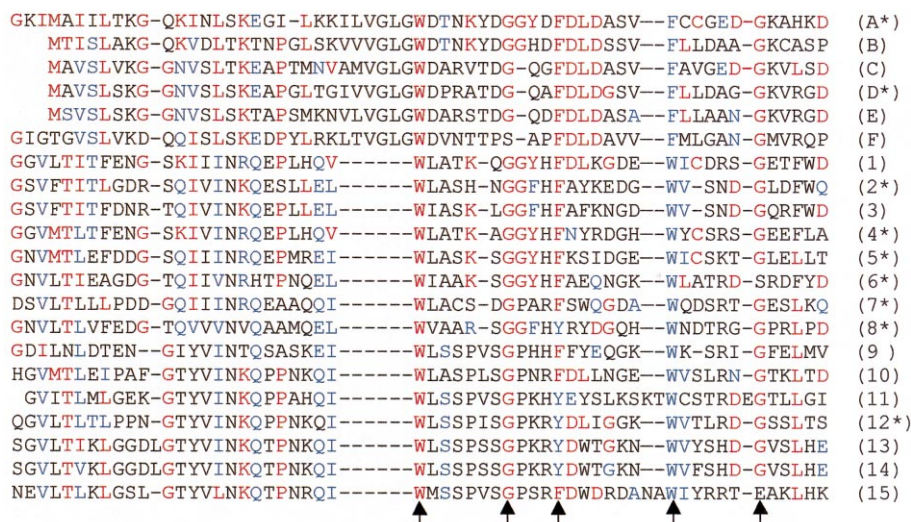


Fig. 1. Amino acid sequence homology between frataxin/Cyay and Ter protein families. Homologues of *E. coli* Cyay were searched for using the tBLASTn program on the NCBI server, in the database of unfinished microbial genomes (<http://www.ncbi.nlm.nih.gov/BLAST/unfinished-genome.html>). The sequence of *C. acetobutylicum* (AE001437) was used to run the Psi-BLAST program [18] with six iterations against the nr databases. Then, multiple amino acid alignment was performed using the Megalign program of DNASTAR and visual inspection of the sequences. Sequences A–F belong to the Ter family: A: *C. acetobutylicum* (AE001437); B: *B. subtilis*, YceD (dbj|BAA22251|); C: plasmid R478, TerE (gi|141975|); D: *D. radiodurans*; E: plasmid pMJ606, TerD (sp|P18781|); F: *Dictyostelium discoideum*, CABP1 (pir|B49752); 1: *E. coli*, Cyay (sp|P27838|); 2: *Actinobacillus actinomycetemcomitans*, Cyay; 3: *Haemophilus influenzae*, Cyay (gi|1573739); 4: *Yersinia pestis*, Cyay (sp|P46356|); 5: *Vibrio cholerae*, Cyay; 6: *Neisseria gonorrhoeae*, Cyay; 7: *Thiobacillus ferrooxidans*, Cyay; 8: *Bordetella pertussis*, Cyay; 9: *Rickettsia prowazekii*, Cyay (RP323); 10: *S. cerevisiae*, Yfh1 (YDL120w); 11: *Schizosaccharomyces pombe*, frataxin homologue (gi|3650374); 12: *Candida albicans*, frataxin homologue; 13: *Mus musculus*, frataxin (gi|2344992); 14: *Homo sapiens*, frataxin (gi|1237439); 15: *Arabidopsis thaliana*, frataxin homologue (gb|AAD14452|). The asterisk denotes unfinished microbial genomes. Red and blue letters indicate identical and conservative (I/V/L, T/S, F/Y, F/W, E/D/Q/N) residues, respectively, between the Ter and frataxin/Cyay families.

taxin/Cyay family and by an aspartate in the Ter family (Fig. 1). Tellurite resistance is not yet understood, however, tellurium and sulfur are closely related elements in the periodic table and it has been proposed that tellurite toxicity could result from the substitution of tellurium for sulfur in various biochemical reactions [19].

#### 4. Discussion

We and others had previously reported that iron accumulates in mitochondria of  $\Delta YFH1$  mutants. This iron accumulation has been associated with various mitochondrial defects. A recent publication strongly suggests that in  $\Delta YFH1$  mu-

tants, iron cannot be extruded from mitochondria [8]. However, in the current report, we have shown that iron accumulation in  $\Delta YFH1$  mitochondria is not an ineluctable event. When a  $\Delta YFH1$  mutant is cultivated in the presence of an iron chelating agent that limits iron availability for the cell, the iron concentration in mitochondria is the same in wild-type and deletant strains. Under these conditions, activities of the respiratory complexes and oligomycin sensitive ATPase are restored. This means that the *YFH1* deletion per se is not deleterious to the mitochondrial function and suggests that the decreased respiratory activities are linked to oxidative damage caused by excess iron. However, even when in a  $\Delta YFH1$  mutant, the iron concentration in mitochondria was low, aconitase activity was still less than 50% of that in the wild-type strain. Aconitase is a 4Fe-4S cluster-containing enzyme whose iron-sulfur center is extremely labile [15]. Moreover, in vertebrate cells, incorporation of the iron-sulfur cluster into aconitase is reversible and this dissociation property plays a central role in iron homeostasis regulation [20]. As previously reported [9], our current data suggest that an excess iron elicits oxidative degradation of the iron-sulfur cluster of the aconitase. However, they also suggest that incorporation of the iron-sulfur cluster into aconitase might be impaired in a manner which is not linked to iron toxicity. Iron-sulfur cluster biogenesis in yeast mitochondria is still poorly understood, but there is growing evidence that homologues of the bacterial Nif operon play a central role in this process. Nfs1p [21] is the yeast homologue of the bacterial Nifs protein, a cysteine desulfurase using pyridoxal phosphate as a cofactor [22–25], which is involved in the delivery of sulfur to iron-sulfur clusters. Nfs1p is essential to yeast life, is conserved in humans [26] and has been shown to localize to the mitochon-

Table 3

Aconitase and succinate dehydrogenase activities in wild-type and  $\Delta YFH1$  mitochondria

Culture medium <sup>a</sup>	Succinate dehydrogenase (nmol O <sub>2</sub> /min/mg protein)		Aconitase (nmol/min/mg protein)	
	Wild-type	$\Delta YFH1$	Wild-type	$\Delta YFH1$
Raffinose <sup>b</sup>	110	65	283	66
+10 $\mu$ M iron <sup>b</sup>	100	60	341	47
Raffinose <sup>c</sup>	130	65	394	83
+10 $\mu$ M BPS <sup>c</sup>	130	110	400	195
Raffinose <sup>d</sup>	240	156	574	44
+5 $\mu$ M BPS <sup>d</sup>	229	257	507	184

<sup>a</sup>The media and cultures are as in Table 1.

<sup>b</sup>The data have been obtained from cultures carried out simultaneously.

<sup>c</sup>The data have been obtained from cultures carried out simultaneously and independently of cultures b and d.

<sup>d</sup>The data have been obtained from cultures carried out simultaneously and independently of cultures b and c.

drial matrix [27]. Like *YFH1* mutants, *nfs1* mutants accumulate iron in their mitochondria [28] and exhibit aconitase deficiency [21], even though succinate dehydrogenase, a respiratory complex which contains an iron-sulfur subunit, Sdh2p, is only moderately affected [21]. It has been proposed that in the *nfs1* mutants, iron-sulfur metabolism is sufficient to maintain succinate dehydrogenase in an active form while the more labile aconitase is deficient [21]. Thus, impaired incorporation of iron into iron-sulfur clusters could provide an explanation to iron accumulation and deposits in the mitochondrial matrix of  $\Delta YFH1$  strains.

In addition, we have found that the conserved core region of frataxin/Cyay proteins shares sequence similarity with the N-terminal region of small proteins conferring resistance to tellurium (Ter). Although the function of the Ter proteins is unknown, it is generally accepted that resistance to tellurite results from conversion to a less toxic form. However, tellurite is rare in the environment and it is very unlikely that the primary function of Ter proteins is linked to tellurite detoxification. Their large occurrence among bacteria suggests that they play an important and basic role in cellular life. Interestingly, tellurium is closely related to sulfur in the periodic table and has been proposed to interfere with sulfur metabolism [19]. Therefore, these data also support the hypothesis that Yfh1p might be involved, directly or indirectly, in the regulation of iron-sulfur cluster synthesis.

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