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Research paper

Nitric oxide prevents Aft1 activation and metabolic remodeling in frataxin-deficient yeast

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ABSTRACT

Yeast frataxin homolog (Yfh1) is the orthologue of human frataxin, a mitochondrial protein whose deficiency causes Friedreich Ataxia. Yfh1 deficiency activates Aft1, a transcription factor governing iron homeostasis in yeast cells. Although the mechanisms causing this activation are not completely understood, it is assumed that it may be caused by iron-sulfur deficiency. However, several evidences indicate that activation of Aft1 occurs in the absence of iron-sulfur deficiency. Besides, Yfh1 deficiency also leads to metabolic remodeling (mainly consisting in a shift from respiratory to fermentative metabolism) and to induction of Yhb1, a nitric oxide (NO) detoxifying enzyme. In this work, we have used conditional Yfh1 mutant yeast strains to investigate the relationship between NO, Aft1 activation and metabolic remodeling. We have observed that NO prevents Aft1 activation caused by Yfh1 deficiency. This phenomenon is not observed when Aft1 is activated by iron scarcity or impaired iron-sulfur biogenesis. In addition, analyzing key metabolic proteins by a targeted proteomics approach, we have observed that NO prevents the metabolic remodeling caused by Yfh1 deficiency. We conclude that Aft1 activation in Yfh1-deficient yeasts is not caused by iron-sulfur deficiency or iron scarcity. Our hypothesis is that Yfh1 deficiency leads to the presence of anomalous iron species that can compromise iron bioavailability and activate a signaling cascade that results in Aft1 activation and metabolic remodeling.

1. Introduction

Frataxin is a mitochondrial protein highly conserved throughout evolution, with orthologous counterparts in almost all organisms, including mammals, bacteria, fungi and plants [1]. The budding yeast *Saccharomyces cerevisiae* contains an orthologous protein named Yfh1 (for Yeast Frataxin Homolog 1) and therefore has been widely used to explore frataxin function [13]. It is also well stablished that frataxin deficiency causes Friedreich Ataxia in humans [16,6].

Several functions have been proposed for frataxins. Most of them are related to iron metabolism, because frataxin deficiency has been found to cause misregulation of this process in several organisms. In this context, the biosynthesis of iron-sulfur clusters is the function that has attracted more support because frataxins have been found interacting with several mitochondrial proteins involved in this process. Among these proteins we can find Nfs1, a cysteine desulfurase which supplies sulfur, and Isu1, a protein that interacts with Nfs1 and acts as an scaffold were nascent iron-sulfur clusters are assembled. The current model of iron-sulfur biogenesis suggests that frataxins would regulate cysteine desulfurase activity and therefore would stimulate iron-sulfur biogenesis [28]. Nevertheless, the analysis of the effects of frataxin deficiency in several models indicates that frataxin is not essential for iron-sulfur biogenesis in vivo and suggests that frataxin may have additional functions [35]. In this regard, frataxin has also been related with heme biosynthesis [32], iron storage and/or detoxification [29], and modulation of iron regulatory protein-1 activation [9]. Frataxin may be also interacting with components of the OXPHOS system [12] and may confer oxidative stress protection [3].

In yeast, frataxin/Yfh1 deficiency induces the expression of several proteins involved in iron uptake. This phenomenon results in iron overload and is caused by activation of the iron regulator Aft1 [14]. Nevertheless, the mechanisms leading to activation of Aft1 in Yfh1 deficient cells have not been investigated in detail. Aft1 is regulated by an iron-sulfur cluster, which under iron-sufficient conditions stabilizes a protein complex that retains Aft1 inactive in the cytosol. Iron scarcity or impaired iron-sulfur biogenesis prevent the formation of such cluster and activate Aft1, resulting in increased iron uptake [23]. In Yfh1 deficient cells, it has been assumed that Aft1 activation would be caused by the loss of such iron-sulfur cluster. However, previous research from our group using conditional Yfh1 mutants provided two observations which challenged this hypothesis: i) activation of Aft1 could be observed earlier than iron-sulfur loss [22]; ii) loss of iron-sulfur containing

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proteins in Yfh1 deficient yeasts required the presence of Cth2, an Aft1 target that binds to mRNAs from iron-containing proteins and promotes its degradation [21]. Therefore, we hypothesize that in Yfh1 deficient yeast, Aft1 may be activated by a mechanism different than iron-sulfur cluster deficiency.

In this work, we provide further evidences that Aft1 is not activated by iron-sulfur deficiency in Yfh1-deficient yeasts. These evidences have been obtained upon the analysis of the relationship between frataxin and yeast flavohemoglobin (Yhb1). We had previously found this protein induced in a proteomic and transcriptomic analysis of Yfh1-deficient yeast [21]. This protein attracted our attention, as Yhb1 had been found interacting with Yfh1, although the physiological relevance of such interaction was not explored [12]. Yhb1 is a flavoprotein with a globin-like domain as well as NAD and FAD binding sites. It has NO reductase activity and therefore it is believed to act in NO detoxification. Yhb1 is localized in the mitochondria in anoxic conditions, while a dual cytoplasmatic/mitochondrial localization is observed in aerobic conditions [7]. It has also been recently shown to be involved in sulfur detoxification [10]. In mammals, neuroglobin it is structurally related to the globin domain and could conserve some of its functions [2]. After focusing on the analysis of the relationship between frataxin, Yhb1 and NO in yeast, we have found that Aft1 activation in Yfh1-deficient yeast is prevented by NO, further supporting that Aft1 activation upon Yfh1 deficiency occurs through a mechanism not dependent on iron-sulfur deficiency.

2. Results

2.1. Yhb1 is induced upon Yfh1 depletion

In order to explore in more detail the relationship between Yhb1 and Yfh1, we first confirmed the previously reported induction of Yhb1 in Yfh1-deficient cells [21]. We used a conditional Yfh1 mutant in which *YFH1* expression is under the control of a *tet* promoter (*tetO$_7$YFH1* strain). In this strain *YFH1* can be repressed by doxycycline addition to the growth media. In addition, this strain is able to grow in non-fermentable carbon sources such as glycerol, avoiding the repressing effect of glucose on several mitochondrial activities [22]. To confirm induction of Yhb1, this protein was GFP-tagged in a *tetO$_7$YFH1* strain and its content analyzed by anti-GFP western blot. Fig. 1A indicates that a clear induction of Yhb1-GFP could be observed 6 h after doxycycline addition. We also analyzed cellular localization by fluorescence microscopy. We could observe that Yhb1-GFP was localized both in the cytoplasm and mitochondria, as previously reported by other authors. Microscopic images confirmed the increase in protein content upon Yfh1 depletion, but we did not observe any change in the dual localization of Yhb1-GFP (Fig. 1B). We finally analyzed the presence of the *holo* form of Yhb1 in both control and frataxin deficient cells. Yhb1 contains a heme group and a FAD binding site. Both groups can be detected using non-denaturing (native) polyacrylamide gels and a Chemidoc XRS imaging system. Flavins can be directly detected in gels due to its fluorescent properties. Heme can be detected after transfer to PVDF membranes due to its peroxidase activity. Thus, cell extracts from control and doxycycline treated *tetO$_7$YFH1* cells were separated in native polyacrylamide gels and either imaged to detect the fluorescence from protein-bound flavins or transferred to PVDF membranes to detect heme. Extracts from *Δyhb1* mutants were also loaded and were used to identify the flavin and heme bands corresponding to Yhb1. As shown in Fig. 1C, the heme signal corresponding to Yhb1 is strongly increased upon Yfh1 depletion. This result confirms the induction of Yhb1 and indicates that the protein is present in the *holo* form. Of note, using this analytical approach, the heme from Yhb1 appears as the most intense band, supporting previous data about the abundance of this protein. Yhb1-bound FAD could also be detected in both control and doxycycline treated *tetO$_7$YFH1* cells. However, the low signal-to-noise ratio from this flavin signal did not allow reliable quantitative analysis of this band.

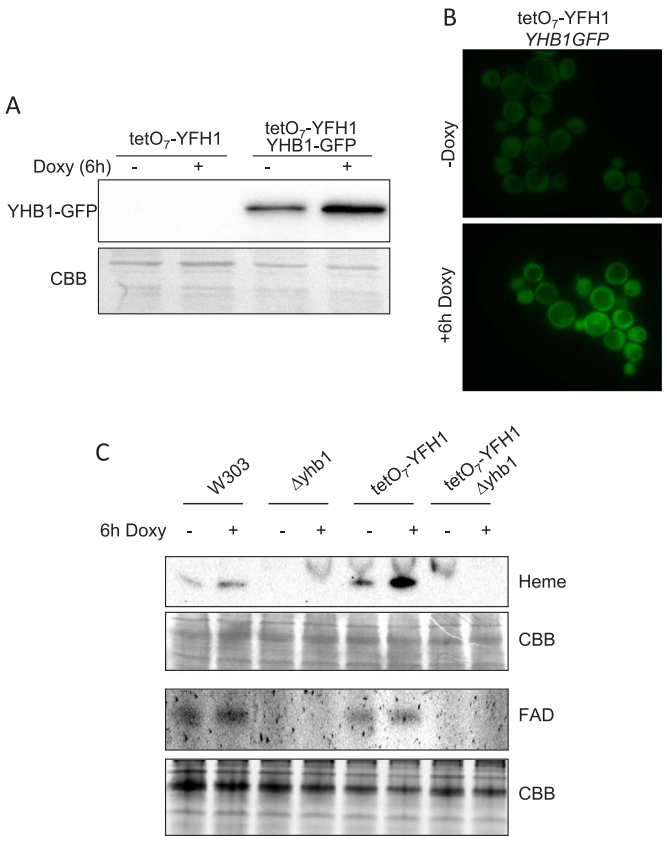

Fig. 1. Yhb1 is induced by Yfh1 deficiency. Yeast cells were grown in YPG and, where indicated, doxycycline was added to the culture media for 6 h in order to repress Yfh1 expression. A, whole cell extracts were analyzed by western blot using anti-GFP antibodies; B, *tetO$_7$YFH1 YHB1GFP* cells were analyzed by fluorescence microscopy. A punctuate pattern can be appreciated in both images, indicating the dual localization of Yhb1-GFP irrespective of the presence of doxycycline. C, whole cell extracts were loaded on native gels and the presence of the heme group and the flavin from Yhb1 were detected as described under experimental conditions. Protein load was verified by post-western Coomassie Briliant Blue (CBB) staining of the gels or PVDF membranes.

2.2. Increased nitroxidative stress in Δyfh1 cells

It has been proposed that Yhb1 would have a central role in regulating NO levels in yeast cells, as it has NO oxidase activity and is induced by nitroxidative stress. We hypothesized that induction of Yhb1 in Yfh1-deficient cells would be related to the presence of reactive oxygen or nitrogen species in Yfh1-deficient cells. Indeed, we had previously shown an increased O_2^- production in *Δyfh1* cells [13]. Thus we analyzed O_2^- and NO levels in *tetO$_7$YFH1* and *tetO$_7$YFH1Δyhb1* cells treated with doxycycline. Superoxide levels were measured using DHE, while NO levels were measured using DAF-FM DA and flow cytometry. As shown in Fig. 2A, loss of Yfh1 led to increased production of superoxide, while NO levels were increased in *Δyhb1* mutants (Fig. 2B), confirming the role of this protein in regulating NO levels. Interestingly, loss of Yfh1 promoted a slight but significant decrease in NO levels in both strains (Fig. 2B). This observation suggested us that O_2^- could be reacting with NO to form peroxynitrite, a highly reactive compound that can easily react with tyrosines to form nitrotyrosines. To confirm this hypothesis, we analyzed the presence of nitrotyrosines by western blot in *tetO$_7$YFH1* and *tetO7yfh1Δyhb1* cells and we observed a significant increase in the presence of this postraductional modification when cells where deficient in both Yfh1 and Yhb1 (Fig. 2C–D). These results confirm that Yfh1 loss leads to increased O_2^- production, which in Yhb1 deficient cells would react with NO to produce peroxynitrite.