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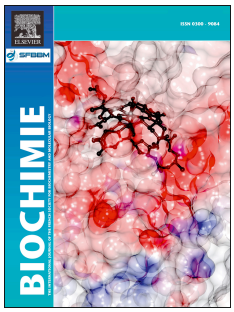

Ferrochelatase activity of plant frataxin

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Abstract

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Ferrochelatase activity of plant frataxin

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Abstract

Frataxin plays a key role in cellular iron homeostasis of different organisms. It is engaged in several activities at the Fe-S cluster assembly machinery and it is also involved in heme biosynthesis. In plants, two genes encoding ferrochelatases (*FC1* and *FC2*) catalyze the incorporation of iron into protoporphyrin IX in the last stage of heme synthesis in chloroplasts. Despite ferrochelatases are absent from other cell compartments, a remaining ferrochelatase activity has been observed in plant mitochondria. Here we analyze the possibility that frataxin acts as the iron donor to protoporphyrin IX for the synthesis of heme groups in plant mitochondria. Our findings show that frataxin catalyzes the formation of heme *in vitro* when it is incubated with iron and protoporphyrin IX. When frataxin is combined with AtNFS1 and AtISD11 the ferrochelatse activity is increased. These results suggest that frataxin could be the iron donor in the final step of heme synthesis in plant mitochondria, and constitutes an important advance in the elucidation of the mechanisms of heme synthesis in plants.

***Keywords*:** frataxin, ferrochelatase, heme, mitochondria

1. Introduction

Frataxin is a mitochondrial protein coded by the nuclear genome, and its deficiency was initially described in patients with Friedreich ataxia (FRDA), an autosomal recessive neuro- and cardio-degenerative disorder that represents the most common inherited ataxia in humans [1]. It was reported that frataxin plays an essential role in mitochondria biogenesis and is required for cellular iron homeostasis regulation in different organisms, iron-sulfur cluster assembly, heme metabolism, oxidative phosphorylation, and oxidative stress [2-11]. It has been reported that frataxin controls the iron used for heme synthesis, mediating the delivery to ferrochelatase in mammals [12, 13]. Furthermore, it was also reported that frataxin is essential for heme biosynthesis in plants [14].

Heme groups participate in several biological processes, including oxygen metabolism, oxygen transfer, electron transfer, and secondary metabolism, as a cofactor of different cellular hemoproteins located in various organelles [15]. In plants, the final step of the classical heme biosynthesis pathway is catalyzed by two ferrochelatase isoforms (FC1 and FC2), which insert the ferrous iron (Fe(II)) into protoporphyrin IX [15].

Different localization experiments such as the import of radiolabelled FC isoforms or the detection of FC coupled to fluorescent proteins showed that both plant FC are located in plastids. [16-18], however, it was suggested that FC1 could also be located in mitochondria [19, 20]. This mitochondrial localization is controversial since it was demonstrated that mitochondria could accept a variety of chloroplast proteins when in vitro import assays were performed [18, 20, 21]. In this way, it is possible that other proteins contribute to the ferrochelatase activity observed in plant mitochondria [18, 22, 23].

Considering that one of the proposed functions for frataxin is to deliver iron to ferrochelatase in the final step of heme synthesis in mammalian mitochondria [13] and that frataxin also interacts with other proteins such as a cysteine desulfurase (AtNFS1) and the small mitochondrial protein AtISD11, we further characterized the possible role of *Arabidopsis* frataxin (AtFH) (alone and in the presence of the other Fe-S biosynthetic proteins) as a potential mitochondrial iron donor. Thus, we evaluated the possibility that AtFH acts as an iron donor to protoporphyrin IX for the synthesis of heme groups in plant mitochondria.

2. Materials and Methods

2.1 Cloning, expression, and purification of AtFH, AtNFS1, and AtISD11

AtFH was induced and purified as described previously [24]. Gene synthesis was performed for AtNFS1 and AtISD11, with codon optimization for the expression in *E. coli* cells (Genscript). AtNFS1 CDS sequence was cloned into a pET32a plasmid containing a TEV protease recognition site from amino acid 420 to 426 and a His_{6x}-tag from amino acid 427 to 432, flanked by NdeI and XhoI restriction sites (pAtNFS1 plasmid). AtISD11 CDS sequence was cloned into a pRSFDuet-1 plasmid (named pAtISD11m), also containing a TEV recognition site (amino acids 67 to 72) and a C-terminal His_{6x}-tag.

E. coli BL21 pLys cells harboring each plasmid were grown at 37°C in LB medium containing the corresponding antibiotics to an OD_{600} = 0.6. Protein production was induced by the addition of 1 mM IPTG and subsequent incubation for 4 h (AtNFS1) or 18 h (AtISD11) at 20°C. Cells were harvested and then resuspended in 20 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), disrupted by sonication and centrifuged at 10000 x *g* for 15 min at 4°C. AtNFS1 was obtained in the insoluble fraction and AtISD11 in the soluble fraction.

For the purification of AtNFS1, the pellet obtained was resuspended in 10 ml of 20 mM Tris-HCl, pH 7.5, 8 M urea and incubated with gentle shaking for 1 h at 25°C and then centrifuged at 10000 x *g* for 30 min at 4°C. The clarified *E. coli* extract was loaded onto a HiTrap chelating column (GE Healthcare, Sweden). The column was washed with 20 ml of 20 mM Tris-HCl, pH 7.5, 8 M urea, 20 mM imidazole and the recombinant protein was eluted by an imidazole gradient (20–500 mM) in 20 mM Tris-HCl, pH 7.5, containing 8 M urea. Purified fractions were diluted at 4°C with 20 mM Tris-HCl, pH 7.5, 0.5 mM PLP, 0.5 M NaCl, 20% (V/V) glycerol and concentrated with a 10 kDa centricon (Millipore) to sequentially eliminate imidazole and urea to refold the enzyme.

For the purification of AtISD11, the soluble fraction was centrifuged at 10000 x *g* for 30 min at 4°C. The clarified extract was loaded onto a HiTrap chelating column, and the protein was eluted in the same way as for AtNFS1. Purified fractions were diluted at 4°C with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl and 20% (V/V) glycerol and concentrated with a 3 kDa centricon (Millipore) to eliminate imidazole. After SDS–PAGE analysis, the purified proteins were stored at -80°C.

2.2 Assay of ferrochelatase activity

Ferrochelatase activity was assayed spectrophotometrically as reported [25] with modifications. AtFH (70 µM µg) or AtFH plus AtISD11 and AtNFS1 (about 70 µM each) were preincubated with 140 µM $(NH_4)_2Fe(SO4)_2$ for 10 min at 25°C in 100 mM Tris-HCl pH 8 and 1

mM DTT in a final volume of 100 µL [26]. The reaction was initiated with the addition of 10 µl of the above solution to 990 µl of the assay buffer (100 mM Tris-HCl pH 8, 0.5% (V/V) Tween 20, 1 mM DTT, 10 µM protoporphyrin IX) at 25°C. The molar extinction coefficients of protoporphyrin IX and hemin (both products from Sigma-Aldrich) were calculated using stock solutions in 0.1 N NaOH. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of heme per minute at 25°C. Fe(III) protoporphyrin was prepared as previously reported. Briefly, hemin was dissolved in 140 mM NaCl in 100 mM Tris-HCl, pH 9.8 (final concentration 1 mM). The solution was then adjusted to pH 7.5 with HCl and diluted in 50 mM phosphate buffer / 140 mM NaCl, pH 6.5 to obtain a 100 µM solution [27].

2.3 Thin layer chromatography (TLC)

Thin layer chromatography was performed using an aluminum-backed silica gel 60 F_{254} TLC and methanol as mobile phase. To compare the mobility of the compounds, we used standards of protoporphyrin IX and hemin in 0.1 N NaOH [28].

3. Results

We analyzed the absorption spectra of protoporphyrin IX and hemin. To discard any possible effect on the spectrum of the chlorine atom present in the hemin, a solution of Fe(III) protoporphyrin was prepared and the absorbance spectrum was also determined. The three compounds have a maximum absorbance at 398 nm. The results showed that the spectra of both hemin and Fe(III) protoporphyrin are similar, showing both an additional peak at 340 nm (Fig. 1A). So, we continue using hemin as a control in the subsequent analyzes. The molar extinction coefficients calculated were the following: protoporphyrin IX ε_{340} = 16700 M^{-1} cm^{-1}, protoporphyrin IX ε_{398} = 37000 M^{-1} cm^{-1}; hemin ε_{340} = 36700 M^{-1} cm^{-1} and hemin ε_{398} = 39700 M^{-1} cm^{-1}.

The formation of heme was measured for 180 min by following the increase in absorbance at 340 nm in the presence of AtFH or AtFH+AtNFS1+AtISD11 (both pre-incubated in the presence of Fe(II) as described above). Under the established reaction conditions, a linear increase in the absorbance at 340 nm was observed, which indicates that the production of heme was constant at least until 3 h after the reaction started (Fig. 1B). The saturation plots for protoporphyrin IX are shown in Figure 1C. The specific activity of AtFH or AtFH+AtNFS1+AtISD11 were 3.08 ± 0.15 mU . mg^{-1} and 4.80 ± 0.32 mU . mg^{-1}, respectively. Thus, the complex formed by the three proteins showed about 60% more activity that the AtFH alone. Furthermore, we determined the $S_{0.5}$ values

for protoporphyrin IX. Both, AtFH or the AtFH+AtNFS1+AtISD11 complex displays similar $S_{0.5}$ values around 1.6 µM. These results indicate that although frataxin alone can catalyze the incorporation of iron into protoporphyrin IX, the presence of two other proteins such as AtNFS1 and AtISD11 increase the efficiency of the incorporation of iron to the porphyrin.

To evaluate if AtFH or the protein complex protect iron from oxidation, AtFH alone or in combination with AtNFS1 and AtISD11 were incubated at room temperature with Fe(II) for 10 min in a 2:1 molar ratio for Fe(II):AtFH. Figure 1D shows that AtFH and AtFH+AtNFS1+AtISD11 were able to maintain iron in solution, avoiding Fe(II) oxidation and precipitation as $Fe(OH)_3$, even after 30 min of oxygen exposure.

In order to confirm that the increase in absorbance observed in the kinetic analyses corresponded to an increase in the production of heme, we carried out an analysis of the reaction products by TLC. After verifying that the mobility of hemin is similar to that of Fe(III) protoporphyrin, we loaded onto the silica protoporphyrin IX and hemin as controls and the reaction mixture. The reaction was performed with saturating levels of protoporphyrin IX (10 µM) in the presence of AtFH+AtNFS1+AtISD11 pre-incubated with Fe(II) (Fig. 2). After the separation of the compounds, the formation of heme was observed (see Fig 2, lane 3), confirming the ferrochelatase activity of AtFH.

4. Discussion

Frataxin is an essential enzyme that plays a central role in both Fe-S and heme biosynthetic pathways. However, the connection between both pathways remains unclear, especially in photosynthetic organisms. Yeast cells lacking frataxin (*Δyfh*) are deficient in Fe-S cluster assembly and also in the use of iron by FC [12]. In addition, *Δyfh* cells also show low cytochrome content and reduced levels of other heme-containing proteins such as cytochrome c oxidase [29]. In this sense, it was described that YFH and two mitochondrial carrier proteins, MRS3 and MRS4 implicated in iron homeostasis, have a cooperative function in providing iron for heme and Fe-S synthesis [30, 31]. In humans, it has been reported that frataxin interacts with FC and mediates the delivery of iron in the final step of heme synthesis in mitochondria [13], however, it was demonstrated that plant FCs are located in chloroplasts and that there are no isoforms of FCs present in plant mitochondria [16-18]

We reported that AtFH, apart from its role in the biogenesis of Fe-S groups, also plays a role in the biosynthesis of heme groups [14]. *Arabidopsis* lines deficient in AtFH showed a decrease in heme content in leaves and flowers and in heme pathway transcripts such as *HEMA1* and *HEMA2*

(two genes coding for glutamyl-tRNA reductase proteins), *GSA1* and *GSA2* (two genes coding for glutamate-1-semialdehyde aminomutases), *HEMB1* and *HEMB2* (two porphobilinogen synthase genes) and *HEMF2* (coproporphyrinogen oxidase gene). Interestingly, *FC1* and *FC2* transcripts were increased (see below). In addition we found a decrease in catalase activity, an heme-containing enzyme, without affecting the levels of the three transcripts coding for catalases (CAT1, CAT2 and CAT3) or its protein content. Catalase activity was recovered only after the incubation of homogenates of cell cultures or mitochondrial suspensions with hemin, but no recovery of the activity was observed in the presence of the heme precursors aminolevulinic acid or protoporphyrin IX [14]. Those findings were in accordance with hemin rescue experiments performed in frataxin-deficient neuronal cells [32] and confirmed the deficiency at the heme cofactor level in AtFH deficient plants. However, it is also important to note that even in the presence of high levels of *FC1* and *FC2* transcripts, the addition of their substrate protoporphyrin IX was unable to restore catalase activity. The deficiency in heme content and also the recovery of normal enzymatic activity after the addition of hemin suggests that AtFH would have a significant role in heme production.

It has been suggested that in plants the synthesis of heme took place almost exclusively in plastids and exported to the cytosol and mitochondria [33-35]. However, about 30% of FC activity was found in mitochondria [18, 22, 23]. Since it has been reported that FC1 and FC2 are not imported into mitochondria in *Arabidopsis thaliana* [17], thus, there should be another source of FC activity.

The last common enzyme of heme and chlorophyll biosynthesis is protoporphyrinogen IX oxidase (PPO) which catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX by molecular oxygen. In plants, there exist two isoforms of PPO, the plastidic PPO1 and the mitochondrial PPO2 [36]. This is in agreement with the possibility of the existence of a final step of heme synthesis in mitochondria of plants.

We have shown that AtFH was able to bind and maintain Fe(II) in solution, as it was reported for the human homolog [37] preventing its oxidation and precipitation as $Fe(OH)_3$ when the cation is exposed to air. Furthermore both, AtFH or the complex AtFH+AtNFS1+AtISD11, "charged" with Fe(II) were able to deliver iron to protoporphyrin IX with the production of heme. We showed that the presence of AtNFS1 and AtISD11 increased the FC activity respect to AtFH alone. Both, the yeast and human homologs of NFS1 and ISD11 interact with frataxin [38], and frataxin has an activating allosteric effect on NFS1 catalytic activity [39, 40]. We have also shown that AtFH interacts with the plant NFS1 isoform, and that the presence of AtFH increases the cysteine desulfurase activity and the $S_{0.5}$ value for cysteine [41]. In this case, the ferrochelatase activity is performed by AtFH and AtNFS1 and AtISD11 seem to have a regulatory effect. Other report showed that YFH interacts with aconitase to deliver iron to the enzyme, converting the

oxidized [3Fe-4S] form into an active [4Fe-4S] protein, suggesting that frataxin could act as an iron chaperone for iron delivery [42]. Thus, the AtFH ferrochelatase activity might be another example of this iron chaperone function.

In summary, results presented here demonstrated that frataxin catalyzes the insertion of Fe(II) into protoporphyrin IX to form heme *in vitro*, becoming a member of the family of enzymes that add divalent metal cations to tetrapyrrole structures. This, as well as the localization of at least one isoform of PPO in plant mitochondria, and the lack of any FC isoform in this organelle suggests that AtFH could be responsible for the remaining ferrochelatase activity in plant mitochondria. Thus, plant frataxin could be involved in an alternative pathway to provide heme for different mitochondrial hemeproteins, catalyzing the last step of heme synthesis in this organelle.

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Figure Legends

Fig. 1. (A) Optical absorption spectra of protoporphyrin IX, hemin and Fe(III) protoporphyrin. About 10 µM of each compound was used for the determination of each spectrum. **(B)** Time course of the reaction catalyzed by AtFH (black squares) or AtFH+AtNFS1+AtISD11 (black circles). Production of heme was followed spectrophotometrically as described in the Materials and Methods section. **(C)** Kinetic analysis of *A. thaliana* frataxin's ferrochelatase activity alone (black circles), AtFH+AtNFS1+AtISD11 (black squares) and AtNFS1+AtISD11 (black diamonds). AtFH or AtFH+AtNFS1+AtISD11 were preincubated with iron (140 µM $(NH_4)_2Fe(SO_4)_2$) and the ferrochelatase activity was measured in the presence of different concentrations of protoporphyrin IX. **(D)** Incubation of Fe(II) alone (tube 1) or in the presence of AtFH (tube 2) or AtFH+ AtNFS1+AtISD11 (tube 3) in a 2:1 molar ratio for Fe(II):AtFH for 30 min exposed to air.

Fig. 2. Colorimetric detection by thin layer chromatography of the reaction products of AtFH+AtNFS1+AtISD11 preincubated with Fe(II) and protoporphyrin IX (lane 3). Lanes 1 and 2 show standard solutions of hemin and protoporphyrin IX, respectively.

Figure 1

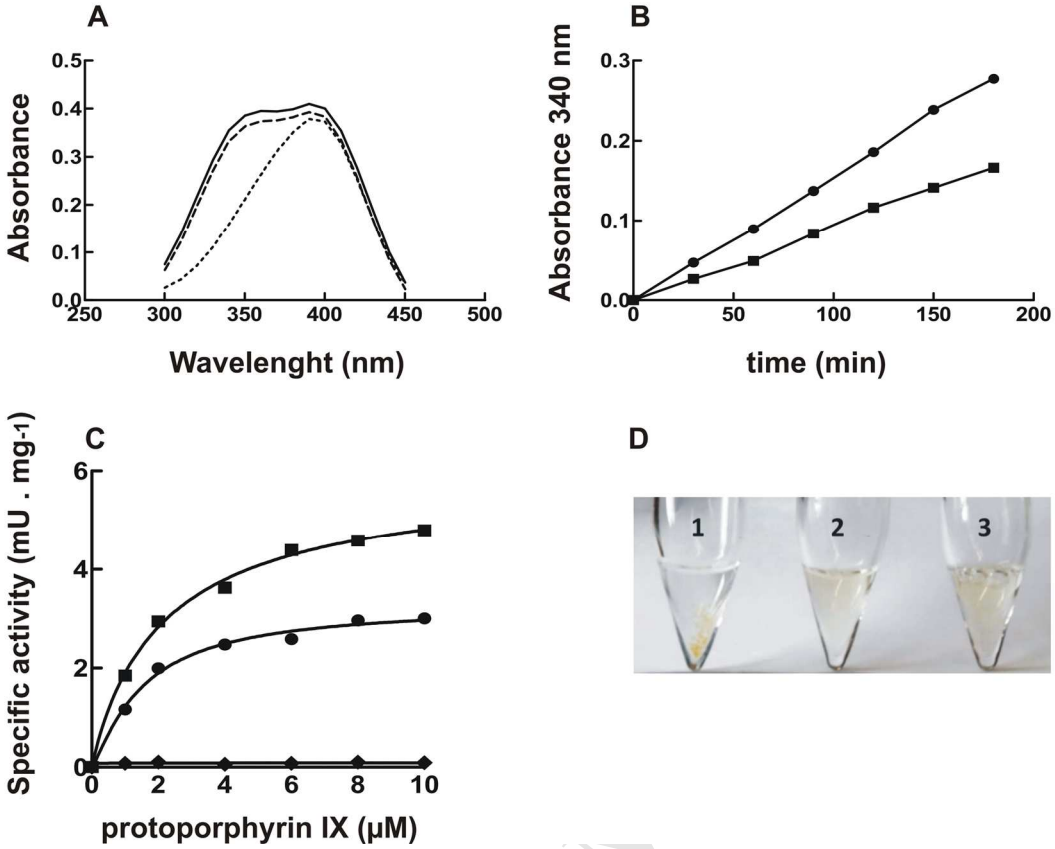
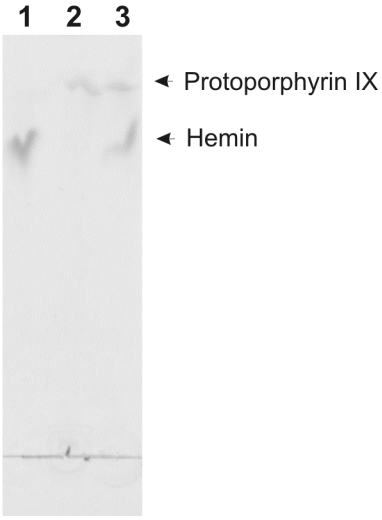


Figure 2



Highlights

- Frataxin acts as an iron donor to protophorphyrin IX
- AtNFS1 and AtISD11 increase the ferrochelatase activity of frataxin
- Frataxin could be the iron donor for heme synthesis in plant mitochondria