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Copper redox chemistry of plant frataxins

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ABSTRACT

The presence of a conserved cysteine residue in the C-terminal amino acid sequences of plant frataxins differentiates these frataxins from those of other kingdoms and may be key in frataxin assembly and function. We report a full study on the ability of *Arabidopsis* (AtFH) and *Zea mays* (ZmFH-1 and ZmFH-2) frataxins to assemble into disulfide-bridged dimers by copper-driven oxidation and to revert to monomers by chemical reduction. We monitored the redox assembly-disassembly process by electrospray ionization mass spectrometry, electrophoresis, UV–Vis spectroscopy, and fluorescence measurements. We conclude that plant frataxins AtFH, ZmFH-1 and ZmFH-2 are oxidized by Cu^{2+} and exhibit redox cysteine monomer – cystine dimer interexchange. Interestingly, the tendency to interconvert is not the same for each protein. Through yeast phenotypic rescue experiments, we show that plant frataxins are important for plant survival under conditions of excess copper, indicating that these proteins might be involved in copper metabolism.

1. Introduction

Frataxin is a mitochondrial protein whose deficiency in humans is the primary cause of Friedreich's ataxia, a cardio and neurodegenerative disease characterized by oxidative stress and iron accumulation in mitochondria [1–5].

Iron is necessary for mitochondria to synthesize different iron-containing biomolecules, especially iron-sulfur clusters [6]. As in other cellular parts, mitochondria must store metal ions in available and non-toxic forms since some free metal ions, such as those of copper and iron, can rapidly generate hydroxyl radicals, the most damaging and destructive reactive oxygen species [7]. To avoid the presence of free metal ions, mitochondria have mechanisms for metal homeostasis, controlling their uptake, storage and release. The protein frataxin potentially fulfills some of these functions. Although some evidence relates frataxin to iron metabolism [1–5], the molecular functions of frataxin remain unclear.

The most studied frataxins correspond to bacterial, yeast and human frataxins. Findings reported for these proteins are mutually inconsistent, which preclude a unified picture of iron chemistry in frataxin. On the other hand, even less is known regarding frataxin's chemistry with metal ions other than iron [8].

A common feature of all frataxins studied is their assembly. In some cases, assembly seems to be a consequence of iron incorporation into the protein. The assembly of yeast frataxin, for instance, seems to be driven by iron oxidation and accumulation by iron core formation [2]. In addition, iron core degradation results in protein disassembly [9]. Human frataxin assembly has been proposed as a method for detoxifying redox-active iron [10]. However, in the case of human frataxin iron does not seem to be the main factor for assembly [11]. Despite some controversy in the literature, the assembly is clearly a physiological property of frataxin. This protein could use different molecular forms to accomplish its function [12].

Since most frataxin studies have been limited to bacterial, yeast and human frataxins, plant frataxins present an especially interesting perspective for studying frataxin function. Furthermore, a study with metal ions different from iron is necessary to untangle frataxin's metal coordination chemistry. In this sense, there is some evidence that frataxin might be involved in copper metabolism. *S. cerevisiae* frataxin null mutants are more sensitive to Cu than wild type cells [13]. Friedreich's ataxia patients show altered Cu distribution in the dentate nucleus of the central nervous system [14], and in the fly model of the disease

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there is a generalized increase in copper content [15]. Moreover, in frataxin knockdown flies, treatment with copper chelators ameliorated some of the symptoms without altering the iron accumulation phenotype, implying a direct role of Cu in the pathophysiology of the disease.

It has been only very recently that Ha-Duong et al. reported a study on copper affinity by yeast frataxin, concluding that copper affinity for yeast frataxin is even higher than that of iron [16]. Therefore, further studies are needed to give an answer to the increasing interest to study the interaction between copper and frataxins.

In this paper, we contribute a glimpse into frataxin protein chemistry through studies on plant frataxins. In particular, we focus on the copper chemistry of frataxins belonging to *Arabidopsis* and *Zea mays*. In contrast to other known frataxins, plant frataxins contain one conserved cysteine (Cys) in their C-terminal amino acid sequences [17]. Cys residues can undergo several reversible modifications such as oxidation to form disulfide bridges, sulfenic acid, glutathionylations or alkylations. Therefore, the presence of a Cys residue in plant frataxins differentiates them from those of other kingdoms and may be vital in plant frataxin assembly and function.

We have studied the influence of this Cys residue on the assembly of three recombinant plant frataxins, one from *Arabidopsis thaliana* (AtFH) and two from *Zea mays* (ZmFH-1 and ZmFH-2). The physiological role of AtFH is well known since frataxin-deficient plants were characterized [18]. AtFH is related, among other functions, to iron and heme metabolism, ROS (reactive oxygen species) protection and NO signaling [19]. As we have recently reported, *Zea mays* is the first organism reported to contain two frataxin isoforms [17]. Interestingly, ZmFH-1 and ZmFH-2 have different inclinations to form dimers under aerobic conditions [17].

Because metal ions, in particular Cu^{2+}, act in redox cysteine-cystine conversion in other proteins [20–21], we assess the plant frataxins' copper-driven oxidation leading to dimerization as well as the chemical reduction returning the monomers. We have monitored the redox assembly-disassembly process by electrospray ionization mass spectrometry (ESI-MS), native PAGE (polyacrylamide gel electrophoresis), UV–Vis (ultraviolet-visible) spectroscopy and fluorescence titration. We conclude that Cu^{2+} oxidizes plant frataxins AtFH, ZmFH-1 and ZmFH-2 and that these proteins exhibit redox cysteine monomer – cystine dimer exchange, although their tendencies to interconvert are not the same. In addition, functional complementation assays performed in *S. cerevisiae* showed that plant frataxins can rescue yeast frataxin-null mutants from excess copper toxicity, indicating a physiological and conserved role for the protein in copper metabolism.

This is the first study on plant frataxins' metal chemistry. We hope to present a basic picture to help complete the puzzle of this enigmatic protein.

2. Results and discussion

As already described [17], recombinant ZmFH-1, ZmFH-2 and AtFH plant frataxins were synthesized as monomers, and kept away from O_2 to avoid conformational changes [17]. The proteins were treated with a slight excess of Cu^{2+} (see Experimental) to evaluate their tendencies to form disulfide-bridged dimers by oxidation of their cysteine residues. The mixtures of protein and Cu^{2+} were analyzed by ESI-MS (Fig. 1).

After addition of Cu^{2+} to ZmFH-2, a major dimeric species of 28,830 Da appears together with a small fraction of the unreacted monomer with a MW of 14,416 Da (Fig. 1B). No monomeric Cu-ZmFH-2 complexes were detected but dimeric Cu-$(ZmFH\text{-}2)_2$, Cu_2-$(ZmFH\text{-}2)_2$ and Cu_3-$(ZmFH\text{-}2)_2$ species were the main species detected (Fig. 1B). Interestingly, the addition of a chemical reductant such as DTT (dithiothreitol) makes the copper dimer signals practically disappear while the apo-monomer results in a major peak with some unresolved peaks probably due to small amounts of Cu-species.

The behavior of ZmFH-1 towards Cu^{2+} is different to that of ZmFH-2. After incubating ZmFH-1 with Cu^{2+}, the mass peaks in the ESI-MS

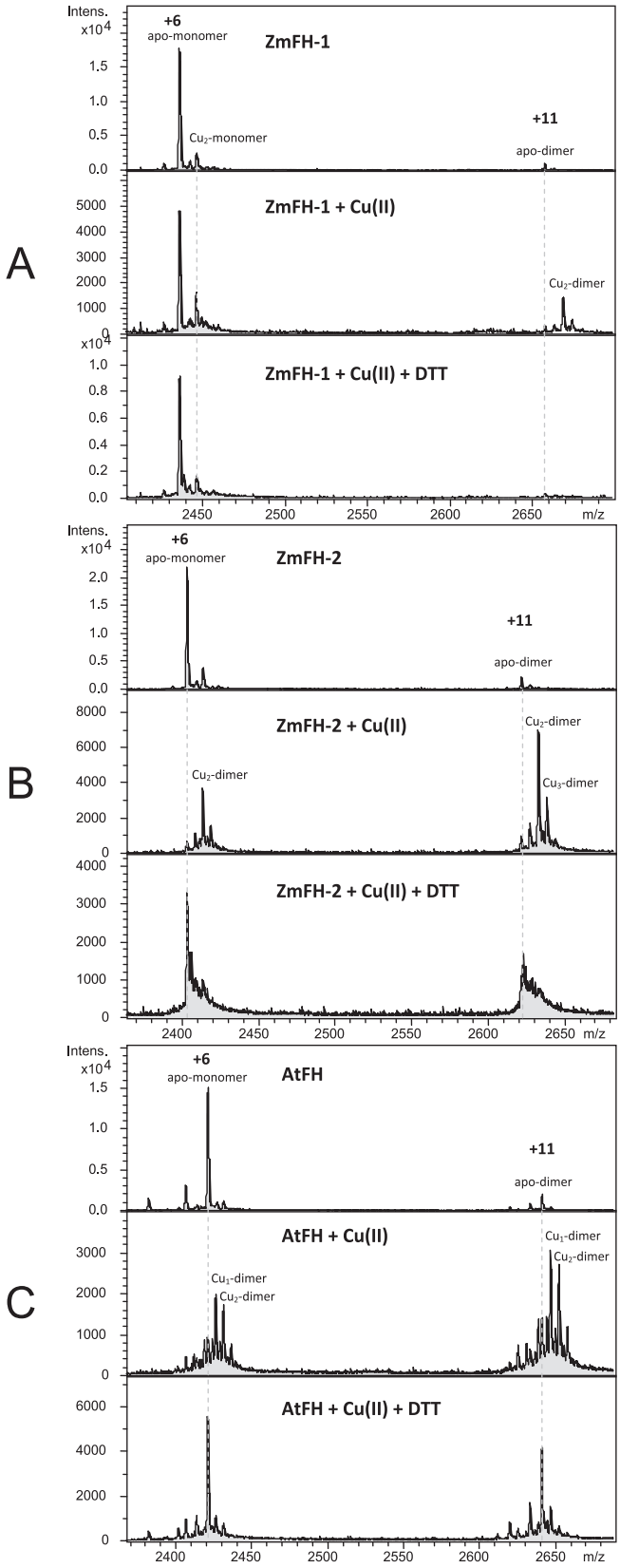

Fig. 1. ESI-MS spectra of plant frataxins. Mass spectra of the ZmFH-1 (A), ZmFH-2 (B) and AtFH (C) plant frataxins before and after addition of Cu^{2+}, and after subsequent addition of DTT. All the spectra show the +6 charge state for the monomeric form while the corresponding +11 charge state for the dimeric species. Notice that when the dimeric forms are the main species, the Cu-dimer species can be also observed at the +12 charge state (around 2420 *m/z*), which coincides and overlaps with the minor Cu-monomer species.

spectrum (Fig. 1A) corresponding to the ZmFH-1-dimer are clearly minor in comparison with those found for ZmFH-2 (Fig. 1B). Addition of DTT to the mixture of ZmFH-1 and Cu^{2+} resulted in a single peak corresponding to the monomer form (Fig. 1A). In contrast, addition of Cu^{2+} to AtFH produces several forms of AtFH-dimer. Copper-loaded dimers Cu-$(AtFH)_2$ and Cu_2-$(AtFH)_2$ render the predominant signals, although a minor apo-dimeric species is also detectable (Fig. 1C). Since the addition of DTT results in the appearance of apo-monomeric and apo-dimeric signals of similar intensity, we can conclude that the conversion from dimers to monomers is not complete in AtFH (Fig. 1C).

These findings suggest that the three plant frataxins studied, ZmFH-2, ZmFH-1 and AtFH, form disulfide-bridged dimers through cysteine oxidation by Cu^{2+} although with different extension. Whereas the monomer of ZmFH-1 seems to resist oxidation to form the dimer, the dimer of AtFH resists reduction to form the monomer. ZmFH-2 exhibits an intermediate behavior.

To confirm the existence of a redox reaction between the plant frataxins and Cu^{2+}, we carried out a spectrophotometric study on the mixtures of the proteins and Cu^{2+}, using BCS (bathocuproinedisulfonic acid disodium salt) as a Cu^{+} sensor. The $[Cu^{I}(BCS)_2]$ complex would form as a result of the reduction of Cu^{2+} by frataxin and could be detected by monitoring its UV–Vis absorbance at 480 nm (ε^{480} = 14,000 M^{-1}).

The solutions of the frataxin proteins were mixed with BCS, degassed and saturated with argon. Several equivalents of Cu^{2+} were added. Fig. 2 shows the UV–Vis spectra recorded 5 min after the addition of Cu^{2+} to each frataxin. The amounts of Cu^{+} generated by ZmFH-2 and AtFH are significantly larger than that of ZmFH-1. Based on the initial concentration of frataxins, it can be concluded that whereas ZmFH-2 and AtFH reduce Cu^{2+} in equimolar amounts, ZmFH-1 barely reaches a 10% of Cu^{2+} reduction. These values indicate that Cu^{2+} hardly oxidizes ZmFH-1. This frataxin, in contrast to ZmFH-2 and AtFH, is less prone to Cu^{2+} oxidation, in agreement with the previous ESI-MS results.

Additionally, spectra of fluorescence emission after excitation at 295 nm were collected after every addition of Cu^{2+}. The initial spectra exhibited maxima at 350 nm corresponding to the frataxin auto-fluorescence, which decreased continuously with Cu^{2+} additions (Fig. 3). The decrease in this fluorescence emission can be caused by either: (i) oxidation and dimerization of frataxin by Cu^{2+} or (ii) Cu^{2+} binding to frataxin, which would quench the fluorescence of the protein, or both processes simultaneously.

The Asp-Ala-His-Lys peptide (DAHK), a well-known Cu^{2+}-chelating ligand [23], was added in a two-fold excess (with respect to copper) to the final mixtures of frataxin and Cu^{2+} (3 equivalents/protein). DAHK binds free or weakly-bonded Cu^{2+}. Thus, fluorescence values obtained after addition of DAHK reveal the interactions between frataxin and Cu^{2+}. The fluorescence emission upon addition of DAHK differed

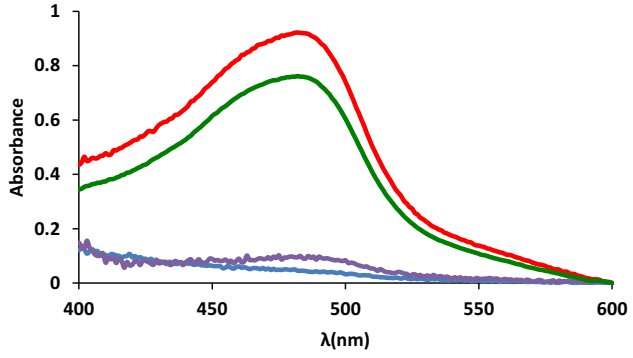

Fig. 2. UV–Vis spectra of [Cu(BCS)2] complexes generated after incubating plant frataxins with copper. UV–vis spectra of the mixtures of the ZmFH-1 (dotted line), ZmFH-2 (black solid line) and AtFH (dashed line) with BCS after 5 min of addition of Cu^{2+}. The grey solid line is the control: solution of Cu^{2+} with BCS in the absence of frataxins.

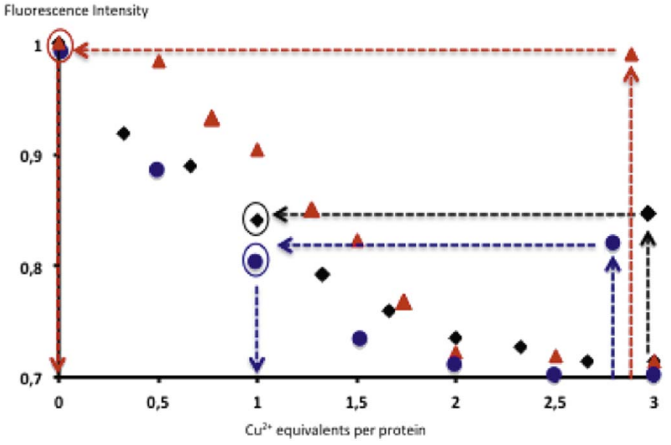

Fig. 3. Monitoring fluorescence of frataxins in the presence of copper. Fluorescence emission at 350 nm (excitation at 295 nm) upon addition of Cu^{2+} to the ZmFH-2 (black), ZmFH-1 (red) and AtFH (blue) plant frataxins. Dashed arrows indicate the fluorescence values obtained after addition of an excess of DAHK. For ZmFH-2 (black) and AtFH (blue), the fluorescence recovers values close to those obtained after the addition of 1 equivalent of Cu^{2+} per monomer of frataxin. For ZmFH-1 (red), the fluorescence goes back to the initial value (before addition of Cu^{2+}).

depending on the frataxin. The fluorescence emission of ZmFH-1 decreased as Cu^{2+} was added but returned to the initial value after DAHK addition (Fig. 3, red dashed arrow). The fluorescence emissions of ZmFH-2 and AtFH also decreased as Cu^{2+} was added. However, DAHK addition to ZmFH-2 and AtFH resulted in fluorescence emission values corresponding to those obtained after addition of one equivalent of Cu^{2+} per protein, not the initial values prior to Cu^{2+} additions (Fig. 3, black and blue dashed arrows).

The fluorescence measurement results match those obtained by ESI-MS and UV–Vis spectroscopy. The addition of Cu^{2+} to ZmFH-2 or AtFH produces a decrease in protein fluorescence due to their conversions, first into dimers and then into copper complexes. The addition of DAHK removes bound Cu^{2+} leading to frataxin copper-free dimeric forms. Thus the fluorescence values obtained after DAHK addition match those corresponding to the addition of the stoichiometric amount of Cu^{2+} required for oxidation/dimerization of ZmFH-2 or AtFH frataxins (Fig. 3, arrow and blue dashed lines). In contrast, ZmFH-1 fluorescence decreases mainly due to multiple copper ions binding to the monomer. After addition of DAHK, these Cu^{2+} ions are removed resulting in the initial frataxin copper-free monomer.

The described fluorescence experiment also illustrates that Cu^{2+} affinities of these frataxins are not higher, in either monomeric or dimeric forms, than that of DAHK ($2.6{\cdot}10^{14}$ M^{-1}) [23], since DAHK restores the fluorescence emission to the corresponding values of the copper-free forms.

In plant frataxins, dimerization takes place through the oxidation of their Cys residues. The three frataxins were labelled with 2-iodoacetamide, a common reagent used to covalently bind cysteine thiols, thus avoiding the formation of disulfide bonds. As hypothesized, the resulting labelled frataxins hardly produced dimers after addition of Cu^{2+}. Native PAGE (Fig. 4A) shows how the addition of Cu^{2+} to the iodoacetamide derivative of ZmFH-2 does not significantly modify its electrophoresis pattern. Likewise, its fluorescence does not significantly vary after step-by-step addition of Cu^{2+}. These results agree with the lack of formation of a disulfide-bridged dimer (Fig. 4B).

With the aim to deepening into the biological influence of plant frataxin on copper metabolism, a phenotype rescue experiment was performed in a frataxin knockout yeast strain to study the ability of ZmFH-1, ZnFH-2 and AtFH to restore the normal growth phenotype in high Cu conditions. Thus, wild type and Δyfh1 cells alone or complemented with each isoform were grown in complete YP-galactose medium supplemented with or without an excess of $CuSO_4$. As

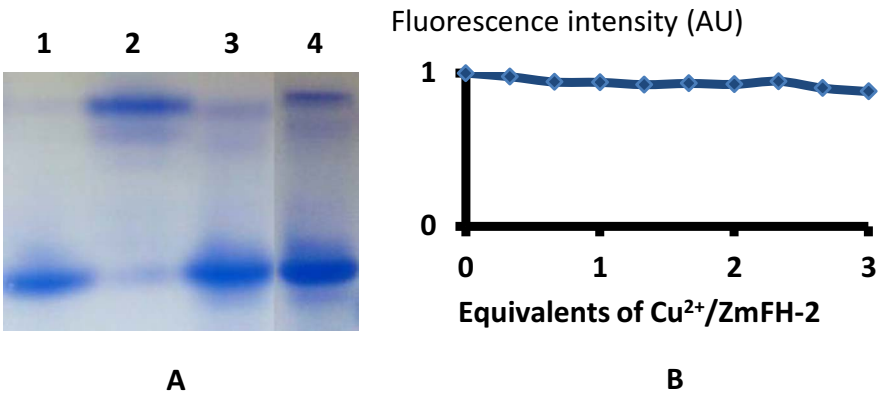

Fig. 4. Frataxins and labelled frataxins. A) Comparative native electrophoresis of ZmFH-2 before and once labelled with iodoacetamide. Lane 1 is ZmFH-2; Lane 2 is ZmFH-2 after addition of Cu^{2+}; Lane 3 is ZmFH-2 after labelling with iodoacetamide and Lane 4 is addition of Cu^{2+} to the labelled frataxin. The presence of some dimer in Lane 4 is noticeable probably due to the fact that iodoacetamide label was not complete. However, it is worth to notice the strong difference between lane 2 and lane 4, especially at the monomer level as well as the similarity between lane 3 and lane 4. B) Fluorescence emission at 350 nm (excitation at 295 nm) upon additions of Cu^{2+} to ZmFH-2 once labelled with iodoacetamide.

described previously, the null mutant showed a lower growth and a higher sensitivity to Cu than wild type cells. In contrast, complemented yeast cells showed an increased resistance to Cu with respect to the knockout yeast strain (Fig. 5). Cells expressing *ZmFH-1* and *ZmFH-2* grew until the fifth dilution after the metal treatment. Cells expressing *AtFH* also grew until the fifth dilution but less vigorously. Thus ZmFH isoforms are more effective than AtFH in restoring the phenotype. As plant frataxins are able to complement a yeast null strain, frataxin seems to be involved in Cu metabolism, a function possibly conserved through evolution.

3. Conclusions

This paper addresses the copper chemistry of plant frataxins for the first time. ZmFH-2, ZmFH-1 and AtFH exhibit a redox monomer–dimer interchange, although the inclination to interconversion is different for each protein. Cu^{2+} easily oxidizes and transforms ZmFH-2 into its dimer that is readily chemically reduced back to the monomer. ZmFH-1 is disinclined to oxidize and mainly exists in its monomeric form. AtFH resists dimer reduction to give back the monomer.

These remarkable differences in redox chemistry of plant frataxins, despite their similar amino acid sequences, may be tentatively explained in terms of the accessibility of the Cys residue in every protein. The free thiol group of ZmFH-1 may be buried in the interior of the protein and is not exposed to oxidants as it occurs for other proteins [22]. In contrast, the Cys residues of ZmFH-2 and AtFH are accessible and, thus, easily oxidized to form disulfide-bridged dimers. These dimers can be reversibly reduced to their respective monomers to different degrees. This kind of redox turnover has not yet been reported for a frataxin protein and outlines a new plausible scheme for frataxins relevant for understanding frataxin function in plants. Moreover, the involvement of frataxins in general copper metabolism is a result that deserves further investigation.

4. Experimental procedures

4.1. Synthesis of recombinant frataxins

AtFH, ZmFH-1 and ZmFH-2 were synthesized in 2 L Erlenmeyer flasks inoculated with an overnight pre-culture of *E. coli* BL21 DE3 cells transformed with the corresponding pGEX 4-T1 plasmid constructions. Cultures at $OD_{600\ nm}$ = 0.8 were induced with isopropyl β-d-thiogalactopyranoside (IPTG) 0.5 mM and grown for 3 h at 37 °C. Then, cells were harvested by centrifugation and resuspended in ice-cold phosphate saline buffer (PBS). Cell lysis was carried out by sonication in the presence of 0.5% β-mercaptoethanol in order to avoid protein oxidation. All of the following steps were performed using argon saturated buffers. After sonication and centrifugation, GST fusion proteins were isolated from supernatant by Glutathione Sepharose 4B (Amersham Pharmacia) batch affinity chromatography. Then, fusion constructs were excised by thrombin cleavage. Finally proteins were purified through FPLC, in a gel filtration Superdex 75 column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 7.0, as previously reported [17].

4.2. Non-denaturing polyacrylamide gel electrophoresis (PAGE)

Native PAGE was carried out in Mini-PROTEAN Tetra cell (Bio-Rad) using 14% acrylamide/bisacrylamide gels. Reagent preparation and stock solutions were done according to Ornstein-Davis Protocols (Bio-Rad Manual Instruction). 7 μg of protein samples were loaded per well. Gels were stained with Coomasie Brilliant Blue (R250).

4.3. Copper samples

ZmFH-2, ZmFH-1, AtFH (45 μM) were incubated in 20 mM Tris-HCl, pH 7.0 for 30 min with 2 equivalents of Cu^{2+} (as $CuCl_2{\cdot}2H_2O$) in nitrogen atmosphere. The mixture was then used for ESI-MS. The

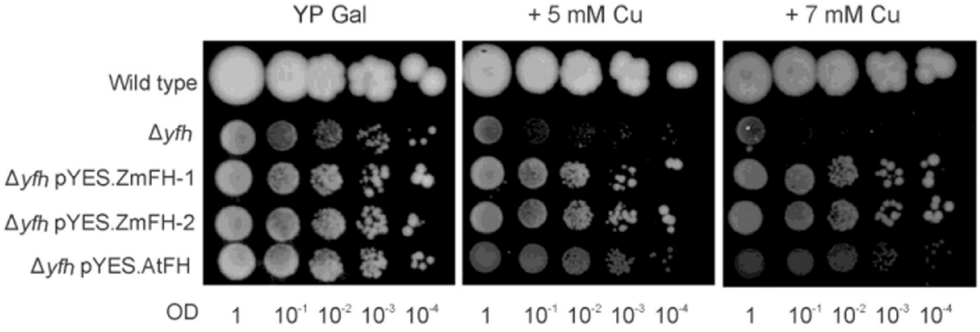

Fig. 5. Complementation of YFH1 deficiency by plant frataxins. The sensitivity of wild type *S. cerevisiae* DY150, Δyfh and Δyfh transformed cells with pYES.ZmFH-1, pYES.ZmFH-2 and pYES. AtFH cells to copper excess conditions was evaluated. 3 μL of each culture 1/10 serial dilutions were spotted on YP 2% (w/v) galactose medium supplemented or not with the indicated amounts of $CuSO_4$. Photographs were taken after 4 days of growth at 30 °C.

resulting mixture was treated with DTT 1 mM (Dithiothreitol, from Sigma-Aldrich) for 1 h and then analyzed again by ESI-MS.

4.4. ESI-TOF MS molecular mass determinations

Samples for ESI-MS were prepared at protein concentration of 20 μM. All samples were analyzed under the following conditions: 20 μL of protein solution injected at 40 μL min^{-1}; a capillary counter-electrode voltage of 5 kV; desolvation temperature between 90 and 110 °C. The carrier buffer was a 5:95 mixture of acetonitrile:ammonium acetate/ammonia (15 mM, pH 7.0). The equipment used was a Micro TOF-Q instrument (Bruker) interfaced with an Agilent Series 1200 HPLC pump, both controlled using the Compass Software. Calibration was attained with ESI-L Low Concentration Tuning Mix (Agilent Technologies). All the spectra were recorded at least 3 times in order to ensure the reproducibility of the experiments. In all cases the spectra were perfectly matching among different experiments.

4.5. UV–Vis spectroscopy

Bathocuproinedisulfonic acid disodium salt (BCS) was used to detect the Cu^{+} generated by the reduction of Cu^{2+} by frataxins. Experiments were done with frataxins (40 μM) in 20 mM of TRIS buffer pH 7.4 with an excess of BCS (200 μM). Samples were purged with Argon and then, copper(II) chloride (80 μM) was added. Cu^{+} was detected after 5 min of reaction by UV–Vis spectroscopy. Absorbances were collected at 480 nm due to the formation of $[Cu^{I}(BCS)_2]^{+}$ complex (ε^{480} = 14,000 M^{-1}). The spectra were collected with Spectronic Unicam UV300.

4.6. Fluorescence spectroscopy

Spectra were taken with a Cary Eclipse fluorescence spectrophotometer. The monochromator slit widths for excitation and emission were both set to 10 nm. For fluorescence titrations, the protein sample was excited at 295 nm to minimize the fluorescence contribution of tyrosine residues and the emission spectra were acquired from 305 nm to 500 nm. Titrations were performed in 20 mM TRIS buffer pH 7.4 at a protein concentration of 3 μM. Fluorescent emission spectra (protein auto-fluorescence) were taken then and as successive additions of 1 μL of Cu^{2+} (1 mM) until a Cu^{2+}/protein ratio of 3 were carried out. Fluorescence emission spectra were collected after every addition. Finally, a two-fold excess of DAHK (with respect to copper) was added to every copper treated sample and the fluorescence emission spectra were collected.

4.7. Labelling of frataxins with 2-iodoacetamide

Reaction with iodoacetamide (IAM) was done in phosphate buffer (20 mM, pH 8) with a frataxin concentration of 45 μM. An excess of 2-iodoacetamide (200 μM) was added to frataxin and the mixture was incubated for 3 h at 37 °C. Finally, the mixture was exhaustively dialyzed against Milli-Q water using a Spectra/Por Float-A-Lyzer with a molecular weight cut-off (MWCO) of 8 kDa to remove excess of iodoacetamide.

4.8. Yeast functional complementation assays

Chimeric constructs containing the *S. cerevisiae* cytochrome oxidase subunit 4 (COX4) transit peptide fused to the mature domain of each of the frataxins under study were obtained as described in [24]. *Saccharomyces cerevisiae* wild type cells (DY150) and frataxin knock out cells (Δyfh::His3) transformed with AtFH, ZmFH-1 and ZmFH-2 chimerical constructs cloned in the pYES3CT vector system [17,24] were used for complementation assays. Yeasts were grown in YP raffinose 2% *W/V* and induced protein expression with galactose 2% W/V. To evaluate the sensitivity of these cells to copper, serial dilutions of these cells were spotted in YP galactose medium supplemented or not with different amount of $CuSO_4$ (5–7 mM) and grown for 4 days at 30 °C.

Author's contribution

M.S., C.B. and L.S. performed the experiments. M.A.P., D.F.C. and S.A. synthesized and characterized the recombinant frataxin proteins. O.P. led the ESI-MS experiments and their interpretation. All authors analyzed the experiments performed in their laboratories. M.C. and J.M.D.-V. designed the experiments and were primarily responsible for writing the manuscript. All authors edited and approved the final version of the manuscript.

Abbreviation list

AtFH frataxin from *Arabidopsis thaliana*
BCS bathocuproinedisulfonic acid disodium salt
DAHK Asp-Ala-His-Lys peptide
DTT dithiothreitol
ESI-MS electrospray ionization mass spectrometry
IAM iodoacetamide
PAGE polyacrylamide gel electrophoresis
TRIS tris(hydroxymethyl)aminomethane
UV–Vis Ultraviolet-visible
ZmFH-1 frataxin 1 from *Zea mays*
ZmFH-2 frataxin 2 from *Zea mays*

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We would like to dedicate this paper in memory of geneticist and co-author Silvia Atrian, who passed away last year. She is sorely missed.

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