A structural approach to understanding the iron-binding properties of phylogenetically different frataxins

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Friedreich's ataxia (FRDA), an autosomal recessive cardio- and neurodegenerative disease, is caused by low expression of frataxin, a small mitochondrial protein, encoded in the nucleus. At the biochemical level, the lack of frataxin leads to dysregulation of mitochondrial iron homeostasis and oxidative damage, which eventually causes neuronal death. It is, however, still unclear whether frataxin is directly involved in iron binding, since the yeast orthologue, but not the human protein, has been shown to form large aggregates in the presence of large iron excess. We have compared the properties of three proteins from the frataxin family—the bacterial CyaY from Escherichia coli, the yeast Yfh1 and human frataxin—as representative of organisms of increasing complexity. We show that the three proteins have the same fold but different thermal stabilities and iron-binding properties. While human frataxin has no tendency to bind iron, CyaY forms iron-promoted aggregates with a behaviour similar to that of yeast frataxin. However, aggregation can be competed by chelator agents or by ionic strength. At physiological salt conditions, almost no aggregation is observed. The design of mutants produced to identify the protein surface involved in iron-promoted aggregation allows us to demonstrate that the process is mediated by a negatively charged surface ridge. Mutation of three of these residues is sufficient to convert CyaY in a protein with properties similar to those of human frataxin. On the other hand, mutation of the exposed surface of the β sheet, which contains most of the conserved residues, does not affect aggregation, suggesting that iron binding is a non-conserved part of a more complex cellular function of frataxins.

INTRODUCTION

Friedreich’s ataxia (FRDA, OMIM 229300), the most common form of hereditary ataxia, is a neurodegenerative disorder associated with progressive physical disability, often complicated by hypertrophic cardiomyopathy, which is the usual cause of premature death (reviewed in 1,2). FRDA is inherited recessively. Thus, although the disease incidence is about 1 case out of 50,000 individuals, heterozygotic carriers are as frequent as 1 in 250 individuals (3,4). At the cellular level, the syndrome is caused by the death of peripheral neurons with long axons. Its genetic locus was identified only relatively recently as a single gene mapped on chromosome 9 (5–7). The transcript of the FRDA gene encodes a small (210-amino-acid) protein, frataxin, which is expressed at levels only lower than normal levels in FRDA patients (8). Frataxin is expressed ubiquitously, reaching the highest concentration in heart, spinal cord and dorsal root ganglia (9). The expression levels correlate well with the pattern of neuronal degeneration, cardiomyopathy and increased risk of diabetes observed in the disease.

The protein is encoded in the nucleus, imported into the mitochondria and then processed to its mature form (9–12). All frataxin orthologues have a highly acidic block of 120 amino acids that folds into a compact globular domain (13–15) (Fig. 1). In eukaryotes, the C-terminal domain is preceded by an extension of variable length, part of which is cleaved off during maturation. While the N termini have no homology, the C-terminal domain is highly conserved, with ~22% sequence identity within the family and homology of ~60%. The three-dimensional structure of this domain is, like the sequence, extremely well conserved from bacteria to humans, suggesting that the protein must have similar functions in different organisms. No sequence homology is detected with proteins outside the frataxin family. However, since frataxin orthologues

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are found throughout evolution from purple bacteria to humans, an essential role of this protein for life is expected (16). Knockout studies in mice have shown embryonic lethality at an early stage of development (17).

Consistent evidence shows that FRDA arises from disregulation of mitochondrial iron homeostasis, with concomitant oxidative damage leading to neuronal death (18–21). However, the precise biochemical functions of frataxin are not yet understood. Accumulating evidence suggests that frataxin is involved in iron metabolism (13,22–25). Iron deposits have been reported in cardiac myocytes and in heart, liver and spleen of FRDA patients (21,26,27). Mitochondrial iron is significantly higher in fibroblasts from FRDA patients than in control fibroblasts (18,19). Deletion of the frataxin gene results in decrease of respiration, depletion of mitochondrial DNA, oxidative stress and increase in mitochondrial iron concentration (20,22,23,25,28). No iron accumulation is observed however, in bacterial knockout and in yeast frataxin-deficient cells grown on low-iron medium (24,29). Combined treatment with various anti-oxidants seems to lead to a decrease in the rate of clinical decline in FRDA patients and to recession of myocardial hypertrophy (30). The co-occurrence in genomes of frataxin and proteins of the Isc operon involved in iron–sulfur cluster assembly also strongly suggests an involvement in iron metabolism (31). This suggestion is supported by observation of reduced levels of aconitase and other mitochondrial iron–sulfur enzymes in FRDA patients as in yeast and mouse frataxin-deficient models (24,27,28,32). Does frataxin bind to iron directly? The evidence for direct involvement of frataxin in iron binding is contradictory. It has been reported that the yeast frataxin orthologue shows ferritin-like behaviour in vitro: in the presence of a large iron excess, the protein incorporates iron and forms high-molecular-weight aggregates (33). No direct iron binding is observed, however, for human frataxin under similar conditions (13). When expressed in Escherichia coli, a small fraction of recombinant human frataxin forms high-molecular-weight species that incorporate iron (34).

In order to test the working hypothesis of a direct involvement of frataxin in iron binding and to investigate further its ion-binding properties, we have carried out a comparative study of the behaviour of three frataxin orthologues from E. coli (CyaY), yeast (Yfh1) and human (hfra). Using these proteins, selected as representative of organisms of increasing phylogenetic complexity, we have characterized their fold and thermodynamic stabilities, and compared their ion-binding specificity and their tendency to aggregate. The structural information available was used as a guide to understand and rationalize the results. Structural considerations led to the design of a number of

Figure 1. Multiple alignment of currently known frataxin orthologues, displayed with ClustalX colours to emphasize conserved sequence features (45). The sequences are identified by their SWISS–PROT entry names. The numbering refers to hfra. The red boxes and cyan arrows indicate the positions of helices and β strands respectively. The CyaY mutations discussed in this work are indicated with the corresponding letter.
RESULTS

The frataxin orthologues have similar folds but different stabilities

The recombinant proteins were expressed in E. coli and purified. The bacterial and yeast orthologues span the full-length proteins. Since the full-length mature human frataxin as produced in E. coli persistently degrades after purification to a truncated form spanning residues 63–210 (13,34), we decided to work with a shorter construct [residues 91–210, hereinafter referred to as hfra(91–210)]. This construct spans the conserved C-terminal globular domain (Fig. 1) and corresponds to the fra_short construct described in (13). The lack of conservation of the N-terminus strongly suggests that our data should not be biased by this choice. Previous exploratory data produced comparable behaviour of the short and the extended (though cleaved) human constructs (data not shown).

The proteins were first characterized for their 3D fold and their thermal stability. Their circular dichroism (CD) spectra are comparable and characteristic of globular proteins with an αβ fold (Fig. 2A). However, the thermal denaturation curves of CyaY and hfra(91–210) have a melting point of 50.1 ± 0.7°C and 69.4 ± 0.04°C respectively, whereas Yfh1 is much less stable, with a melting point of 35.8 ± 1.2°C (Table 1). These results give experimental support to the conservation throughout evolution of frataxin tertiary structure although the proteins from different organisms are characterized by different thermodynamic stabilities.

Unlike hfra, CyaY has a similar behaviour towards iron-promoted aggregation as the yeast orthologue

It has already been noted that aggregation and iron binding are observed for Yfh1, but not for hfra (13,34). When testing the behaviour of CyaY, we used the other two orthologues for comparison. Iron (ii) ammonium sulfate [Fe(NH₄)₂(SO₄)₂] was added at neutral pH under aerobic conditions to solutions of CyaY, Yfh1 and hfra(91–210) [protein concentrations of 8–20 μM and protein-to-iron ratio 1:20—it has been shown previously that iron binding is influenced by the protein-to-cation ratio rather than by the absolute protein or iron concentration (33)]. The same quantity of Fe(NH₄)₂(SO₄)₂ was also added to the buffer in the absence of protein. Iron precipitation was observed in the form of a yellow precipitate in the control without protein and in the hfra(91–210) solution. No iron precipitation was observed in the solutions containing CyaY and Yfh1, reproducing the work of Isaya and co-workers (33) and suggesting that the bacterial protein behaves like the yeast orthologue. Accordingly, iron could be detected by atomic absorption in CyaY and Yfh1 solutions, whereas only background levels of iron were observed in hfra(91–210).

To further investigate the behaviour of the three proteins with respect to iron incorporation, we analysed the samples in the absence and in the presence of iron by gel filtration using a HiLoad Superdex 75 column (fractionation range 3–70 kDa). In the absence of iron, the gel filtration profile of each of the three proteins presents a single peak at a position consistent with the monomeric species. After incubation of the proteins with Fe(NH₄)₂(SO₄)₂ (1:20 protein-to-iron ratio) for 1 hour at 30°C, hfra(91–210) remains an unperturbed monomer with absorbance comparable to that recorded in the absence of iron (peak 1, Fig. 2B). The other two proteins form high molecular weight aggregates. The elution profile of Yfh1 presents a single peak at high molecular weight that exceeds the size range of the column (peak 3). In agreement with the work of Isaya and co-workers (35), who observed an aggregate form of YFH1 of 840 kDa. The CyaY profile contains three peaks: the monomer (peak 1), an intermediate species (peak 2) and a high-molecular-weight species (peak 3). These results show that, as for Yfh1, aggregation of CyaY is promoted by iron binding. In comparison with CyaY, Yfh1 seems to have a higher tendency to form high-molecular-weight aggregates, as suggested by the absence of the monomer and of the intermediate in Yfh1 at the same protein-to-iron ratio and the larger size of the aggregates. However, the sharper appearance of the aggregate peak suggests a more homogenous population of this species in CyaY. To exclude the possibility that aggregation could be affected/mediated by the presence of exposed cysteines, Yfh1 was carboxy-methylated by iodoacetamide (36) and subsequently incubated with Fe(NH₄)₂(SO₄)₂ (1:20 protein-to-iron ratio). This treatment did not influence the gel filtration results.

Only under strongly destabilizing conditions (i.e. incubating the protein at 57°C in the presence of Fe(NH₄)₂(SO₄)₂, 1:20 ratio), does hfra(91–210) form a minor quantity of aggregate, which readily precipitates. No precipitation or aggregation was observed when the protein was incubated at the same temperature in the absence of iron.

Since the iron-binding properties of CyaY and Yfh1 are similar regardless of the presence of an N-terminal extension in the yeast protein and of exposed sulphhydryl groups, we can rule out these factors as determining the different behaviour of human frataxin. Unless otherwise specified, further studies were carried out on the least-characterized CyaY, since this protein is better behaved and much more thermostable than Yfh1.

Table 1. Summary of properties of the three frataxins relevant for the work presented here

<table>
<thead>
<tr>
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<th>ρf</th>
<th>Tm (°C)</th>
<th>No.</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>His</td>
<td>Cys</td>
<td>Asp</td>
</tr>
<tr>
<td>CyaY</td>
<td>4.1</td>
<td>50.1 ± 0.7</td>
<td>3</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>CyaY, 181922</td>
<td>4.5</td>
<td>55.3 ± 1.2</td>
<td>3</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>CyaY, 776</td>
<td>4.2</td>
<td>46.8 ± 0.6</td>
<td>2</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>CyaY, 61</td>
<td>4.2</td>
<td>40.1 ± 0.7</td>
<td>3</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>CyaY, 1833</td>
<td>4.1</td>
<td>47.2 ± 0.8</td>
<td>3</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Yfh1</td>
<td>4.2</td>
<td>35.8 ± 1.2</td>
<td>4</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>hfra(91–210)</td>
<td>4.4</td>
<td>69.4 ± 0.05</td>
<td>3</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>hfra(mature)</td>
<td>5.7</td>
<td>n.d.</td>
<td>3</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
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The columns indicate the isoelectric point, the experimental melting point as determined by CD, and the numbers of histidines, cysteines and negatively charged groups, which are the amino acids potentially able to chelate iron.

aThe mature form spans residues 58–210 of the transcript.

bNot determined, since this species could not be obtained in a stable form.
CyaY can absorb a large excess of iron with relatively slow kinetics

Aggregation of CyaY was studied by exploring the chromatographic profile for increasing protein-to-iron ratios at fixed protein concentration (Fig. 3A). From 1:1 to 1:60 ratios, the aggregate forms and progressively moves towards the excluded volume. At a 1:1 ratio, only the CyaY monomer (peak 1, Fig. 3A) and a small amount of intermediate (peak 2) are detected. At a 1:20 ratio, the monomer and the intermediate are in co-presence with a heterogenous population of a higher-molecular-weight species (peak 3). At 1:40, the peak of the aggregate sharpens and the aggregate becomes the only species detectable in the void volume of the column. At a 1:60 ratio, the protein starts precipitating. Although the relative peak intensities seem to suggest a large predominance of the aggregate already at low protein-to-iron ratios, the much higher molar extinction coefficient at 280 nm of Fe$^{3+}$ leads to an overestimate of the aggregate. An estimate obtained by colorimetric methods shows that at a 1:20 protein-to-iron ratio, only $\sim 60\%$ of the total protein is in non-monomeric form (distributed between high-molecular-weight aggregate and intermediate form) (Fig. 3B). Iron incorporation by CyaY was further confirmed by atomic absorption performed on the gel filtration elution fractions (Fig. 3C). Iron was detected in the aggregate and, to a minor extent in the intermediate species,
but not in the CyaY monomer (detected iron content within the background noise).

The kinetics of CyaY aggregation was studied by injecting fixed amounts of protein incubated with Fe(NH$_4$)$_2$(SO$_4$)$_2$ (protein concentration 8 $\mu$M, 1:20 protein-to-iron ratio) at increasing incubation time (1, 30 and 60 minutes and 24 hours). The process of aggregation can be considered complete ~30 minutes after mixing the two components (data not shown).

Iron-promoted aggregation does not involve conformational changes

To check whether the protein binds Fe(II) or Fe(III) selectively or both, the experiments were repeated in the presence of increasing quantities of FeCl$_3$ at pH 7.4 and of Fe(NH$_4$)$_2$(SO$_4$)$_2$ at pH 6. When Fe(III) was used, upon increasing the protein-to-iron ratio from 1:1 to 1:60, the formation of the

Figure 3. Effect of Fe(II) titration on the gel filtration elution profile of CyaY. (A) Titration of CyaY with increasing quantities of Fe(NH$_4$)$_2$(SO$_4$)$_2$. The peaks of the monomer, intermediate species and aggregate are labelled progressively. The total protein concentration in all experiments was 8 $\mu$M using protein-to-iron ratios as indicated. (B) Protein concentration in the eluted fractions as estimated by colorimetric measurements for CyaY. A known amount (as determined by UV spectroscopy) of purified protein was used for calibration. The fractions refer to those shown for the elution profile in (A) except that the initial total CyaY concentration was increased to 80 $\mu$M to allow more accurate measurement. (C) Iron concentration in the eluted fractions, as determined by atomic absorption, given as percentage of the total iron detected. A 1:20 protein-to-iron concentration ratio was used.
A high-molecular-weight aggregate was detected but not that of the intermediate species, suggesting a different kinetic mechanism for iron aggregation (Fig. 4A). At each protein-to-iron ratio, a smaller percentage of aggregate was observed with respect to the same experiment performed with Fe(II), due to the competition of the precipitation of the iron (III) hydroxide at this pH. At pH 6, where the oxidation of Fe(II) to Fe(III) is slowed down, CyaY remained monomeric, but precipitation started at a low protein-to-iron ratio (1:10) (data not shown). A pH dependence in the range pH 6–7 could, however, also suggest an involvement of histidines, whose pKₐ value is within this range.

Figure 4. (A) Titration of CyaY with FeCl₃. The monomeric and aggregate species are labelled 1 and 2 respectively. (B) Effects of different cations on CyaY. The curves correspond to additions of aluminium, copper, cobalt and zinc sulfates [Al₂(SO₄)₃, CuSO₄, CoSO₄ and ZnSO₄].
Far-ultraviolet (UV) circular dichroism (CD) spectroscopy was used to check if aggregation is associated to a conformational change. Fixed amounts of CyaY were titrated with increasing quantities of Fe(NH$_4$)$_2$(SO$_4$)$_2$ (up to a ratio of 1 : 20). Within the whole range explored, the spectral appearance remains unchanged and the individual curves are superimposable within experimental error (data not shown), showing that aggregation has no effect on the secondary structure of the protein. This in turn strongly suggests that the three-dimensional structure is retained.

**Aggregation of CyaY is mostly iron-specific**

The effect on aggregation of a number of divalent and trivalent cations was studied and compared. All salts were used as sulfates. Aggregation seemed to be mostly iron-specific, since only Al$^{3+}$ and Co$^{2+}$ showed a minor quantity (20–30%) of aggregate (Fig. 4B). The other divalent cations, even those belonging to the same row of the periodic table, did not show tendency to aggregation. However, the protein solubility varied from cation to cation. The protein remained soluble and monomeric up to a protein-to-cation concentration ratio of 1 : 800 in the presence of cobalt, whereas it precipitated with zinc, copper and aluminium already at ratios of 1 : 20, 1 : 40 and 1 : 60 respectively. Calcium and Magnesium ions did not affect solubility.

As a control, increasing quantities of iron were added to chymotrypsin A (37) and to a domain from the protein FMR1 (spanning region 1–160) (38), two well-characterized proteins that have no known iron-binding properties. They precipitated at protein-to-iron ratios of 1 : 20 and 1 : 100 respectively, suggesting that precipitation is a phenomenon due to colloidal precipitation and is distinct and independent from iron binding and aggregation.

When chloride salts were used instead of sulfates, minor effects either in the solubility or in the relative ratio of the aggregate populations were observed, suggesting that the anion has some, but not a dominant, influence on the aggregation. These results show a high specificity towards iron.
Iron binding can be competed by increasing the ionic strength or adding iron chelators

Competition experiments were followed using two different techniques. First, aggregation was studied by gel filtration using citrate and ethylenediamine tetraacetic acid (EDTA) as mild and strong ion chelators, small divalent cations (calcium and magnesium), and performing the experiment at higher and more physiological ionic strengths (KCl). When CyaY (8 μM) was first incubated with Fe(NH₄)₂(SO₄)₂ (1 : 20), and then an excess of citrate or EDTA (1 : 100) was added, aggregation could not be reversed. However, when iron and citrate were added at the same time, the protein remained as a monomer (data not shown). These results show that aggregation can be competed by the presence of chelators even as mild as citrate.

Similarly, when iron was added to protein solutions that contained increasing quantities of CaCl₂ or MgSO₄, aggregate formation was competed out (Fig. 5A and B). Already at low Ca²⁺ concentrations (0.1 mM), part of the monomeric form is subtracted from the aggregate: as estimated by colorimetric measurements, the monomer concentration, which accounts for ~40% of the total protein concentration in the absence of competitor, increases to 70% (Fig. 5A). At intermediate salt concentrations (1 mM), the monomer further increases to 80%, and at high salt concentrations (10 mM), the aggregate disappears completely (100% monomer). Mg²⁺ has a similar but stronger competing effect as compared with Ca²⁺, consistently with its higher charge density (Fig. 5B). Since competition occurs already at 0.1 mM Ca²⁺/Mg²⁺ concentrations, which are lower than that of iron, this must imply that the two cations have a higher affinity for the protein than iron itself. When Fe(NH₄)₂(SO₄)₂ was added to protein solutions with increasing concentrations of KCl, the aggregate disappears almost completely at 150 mM, that is at concentrations close to physiological conditions (Fig. 5C).

Second, the kinetics of these experiments were followed by fluorescence (Fig. 5D). Upon addition of iron, there was an instantaneous decrease in the fluorescence intensity at 340 nm. This corresponded to 5–10% of the total intensity, and was irreversible. This rapid quenching was followed by a further slow decrease in the fluorescence intensity as aggregation proceeded. The half-life (100–500 seconds) and amplitude (10–70% of total intensity) of this process depended on the iron and protein concentrations. The addition of an excess of competitor (K⁺, Ca²⁺, Mg²⁺ or Zn²⁺) or a chelator (EGTA) restored the signal to the level reached at the end of the rapid quenching step.

The 3D structure provides a rationale for understanding the different behaviour of frataxin orthologues

Analysis was attempted to identify regions of the protein surface able to bind to cations and/or regions that would explain the different behaviour of hfra(91–210) and the other two orthologues. The structures of the evolutionary conserved domain from hfra and CyaY have been solved (13–15), and show that this region adopts a compact fold, which superposes with an rmsd of 0.17 nm. This degree of structural similarity confirms that all members of the family have the same fold. As a corollary, the structures of other members of the family can be modelled by homology using the known structures as templates. The structure of Yfh1 was modelled from hfra(91–210) and compared with that of the other two orthologues (Fig. 6A). The frataxin fold consists of an αβ fold.
in which two helices pack against an antiparallel 5–7 strand β sheet (in CyaY, only the first five strands are present). The C terminus inserts between the helices, but its length varies in the different species and is shortest in Saccharomyces cerevisiae. A longer C terminus might stabilize the fold, in agreement with the correlation between the melting points and the C-terminus length.

As for regions that could be important for iron binding, the domain does not have cavities or other features (e.g. the presence of conserved exposed histidines or cysteines) that could suggest a direct involvement in iron binding or a storage function. However, the protein surface is highly charged, with a predominance of negatively charged residues (Table 1). A highly conserved ridge of negatively charged residues clusters along the first helix and the first strand of the β sheet and comprises residues 18, 19, 22 and 33 of CyaY (Fig. 6A). A second negative patch is also present on the same face, but involves semiconserved residues (e.g. Asp89). A non-conserved cysteine in Yfh1 is expected to be highly exposed to the solvent, whereas both cysteines of CyaY are buried inside the hydrophobic core. None of the exposed histidines is completely conserved.

We then focused on the features that could explain the difference in behaviour of hfra from that of CyaY and Yfh1. We searched for residues potentially able to bind to iron that are conserved in CyaY and Yfh1 but not in hfra(91–210). We identified two potential regions. The first one is the patch formed by the four negatively charged sites at positions 18, 22, 31 and 33 of CyaY. Two of these positions, 18 and 33, are replaced in hfra by an alanine and a serine respectively. The second region involves His7 and Asp76, which are replaced in hfra by a glutamate and a lysine respectively.

### Design of mutants helps identify the surfaces responsible for iron binding and aggregation

Using the structural information, we settled to identify the surface and the residues involved in iron-promoted aggregation. Four CyaY mutants were designed (Fig. 6B), chosen according to the following rationale:

(i) A CyaY_181922 mutant was designed to affect three of the most conserved negatively charged residues: Glu18, Glu19 and Asp22. These residues are spatially adjacent in the structure, and are part of the conserved negatively charged patch mentioned above. They were mutated into lysines in order to affect drastically the electrostatic surface potential of the protein.

(ii) Two mutants were designed to affect residues common in CyaY and Yfh1 but not in hfra, aiming at reproducing the behaviour of the human protein in CyaY. Glu18 and Glu33 were mutated to alanine and serine respectively (mutant CyaY_1833). CyaY_1833 is similar to CyaY_181922, but the mutations are more conservative and the two glutamates are replaced by the corresponding residues in hfra. His7 and Asp76, potentially able to be involved in iron binding and present in a different region of the protein face, were mutated into lysines (mutant CyaY_776).

(iii) Trp61 was mutated into an arginine (mutant CyaY_61). This is the equivalent in CyaY of a clinically important mutation (39), and should affect a highly conserved exposed residue that is at the centre of the β-sheet surface (13).

The fold of the mutants was first probed by far-UV CD to ensure that the proteins retained their structures (Fig. 7A and Table 1). The spectra showed that the mutants are folded and have a secondary structure content similar to that of the wild type.
The thermodynamic stabilities vary, with a stabilization effect for CyaY_181922. This effect is probably due to the introduction of positively charged groups (lysines) in a negatively charged environment, thus possibly allowing formation of salt bridges. The stabilities of both CyaY_1833 and CyaY_776 are slightly lower than, but comparable to, that of the wild type. Destabilization of the fold by a Trp→Arg mutation, as in CyaY_61, has also been observed for the corresponding hfra(91→210) Trp155→Arg mutant (13). Mutation of the conserved tryptophan into an arginine can cause disruption of a π interaction between a positive charge and an aromatic ring due to the proximity of a spatially close Arg (Arg165 in hfra) and electrostatic repulsion between the two positively charged residues.

The aggregation properties of the mutants were then checked by gel filtration (Fig. 7B). CyaY_181922 is mostly monomeric up to a 1:20 protein-to-iron concentration, with the appearance of a small amount of the aggregate at a 1:10 ratio. The protein starts precipitating at a 1:40 ratio. CyaY_1833 shows similar behaviour: aggregation is not completely abolished, but is strongly diminished. CyaY_776 has a stronger tendency to aggregate, because at a 1:1 ratio, formation of the aggregate is already observed together with an intermediate species. The full aggregate and the intermediate are both present up to a 1:40 ratio, at which the protein precipitates. The behaviour of CyaY_61 is practically indistinguishable from that of the wild type.

The percentage of protein found in the aggregate state for the different mutants was estimated by colorimetry performed on the column fractions collected at a 1:20 protein-to-iron ratio. It is about 60% for CyaY_61 (comparable to that of the wild-type protein), 11% for CyaY_1833, 9% for CyaY_776 and 0% for CyaY_181922, thus strongly suggesting which surfaces are directly responsible for aggregation. Accordingly, the different mutants incorporate drastically different amounts of iron. The total iron concentration as measured by atomic absorption in all the eluted fractions is 44%, 19% and 18% that of the wild type for for CyaY_776, CyaY_1833 and CyaY_181922 respectively. The iron content measured in the eluted fractions containing the non-monomeric species is 27%, 2% and 1% that measured for the wild type for CyaY_776, CyaY_181922 and CyaY_1833 respectively.

DISCUSSION

The main aim of this work was to compare the aggregation and iron-binding properties of three frataxin orthologues that are phylogenetically distant. The results were rationalized using the structural information and extended to the whole protein family in order to understand the functions of the protein. In particular, we have characterized in detail the aggregation and iron-binding properties of CyaY from E. coli. While the human and yeast frataxins have so far attracted much attention, relatively little is known about CyaY. Yet, because of the high conservation of frataxins throughout evolution (16), the information acquired for an organism with a genome much less complex than the eukaryotic one can be expected to be much simpler to interpret.

We have screened the conditions under which aggregation occurs, showing that CyaY, like Yfh1, has a clear tendency to bind cations and to aggregate. Under the same conditions, human frataxin is either in a monomeric state or precipitates under a large excess of iron, as previously reported (13,34). The similar behaviour of CyaY and Yfh1 allows us to localize the observed properties into the evolutionarily conserved C-terminal globular domain, independently of the nature of the non-conserved N-terminal extensions that are involved in the specific process of mitochondrial import and maturation in eukaryotes.

In the presence of increasing cation concentrations, two distinct phenomena are observed. One is an aggregation of otherwise-monomeric proteins. The second is precipitation at higher ion concentrations. The first phenomenon is mostly iron-specific; the second occurs with other cations from the transition metal series, and is also observed with completely unrelated proteins. We must therefore conclude that precipitation is a non-specific phenomenon caused by colloidal precipitation promoted by cations. Iron-promoted aggregation can, however, be competed out by increasing the ionic strength of the solution or by the presence of iron chelators, showing that aggregation is extremely labile. Only a minor percentage of aggregate is present at physiologic ionic strength.

It is tempting to correlate the tendency towards aggregation of the three proteins with their thermal stability, since yeast frataxin, which has an unusually low melting point, is also the most sensitive to the ionic environment. However, CD studies tell us that aggregation occurs without alteration of secondary structure, excluding a mechanism in which the tendency of the protein to unfold correlates with its aggregation properties. This conclusion is also confirmed by the similarity in behaviour of CyaY and Yfh1, despite the ~15°C difference in thermal stability.

The results obtained for the mutants allow us to pin down the protein surface involved in aggregation and to understand the nature of the process. Mutation of residues potentially able to bind iron into positively charged ones strongly alters the aggregation properties of CyaY. Aggregation must therefore be mostly of an electrostatic nature, with iron ions acting as bridging elements between the different monomers. Competition of an excess of mono- or divalent cations shows that these compete for the same sites but are unable to promote aggregation. More than one protein surface is involved, as expected from the large size of the aggregates. Among other sites, the conserved negatively charged patch involving Glu18, Glu19, Asp22 and Glu33 is very important. Mutation of the non-conserved Glu18 and Glu33 positions of CyaY into the corresponding hfra amino acids seems to be sufficient to produce an almost complete loss of the aggregation properties, thus explaining the different behaviour of the human protein and why this protein is intrinsically iron-insensitive. However, mutation of Trp61 leads to a protein with properties very similar to those of the wild type. Aggregation cannot therefore be achieved by formation of an amorphous aggregate in which each monomer is surrounded by others. These properties are strongly reminiscent of the ordered spherical assembly of ferritins (reviewed in 40). In particular, frataxin could aggregate with a mechanism similar to that of the bacterial ferritin from Listeria innocua in which the ferroxidase site is formed by sidechains belonging to different subunits (41). An important difference is, however, that apo-ferritins are either stable
24mers or 12mers, whereas frataxin forms highly soluble monomers except in the presence of a large excess of iron.

At this point, two scenarios are in principle possible: (i) frataxins are iron scavengers and transporters finely tuned to respond with high selectivity to very specific ionic environments, or (ii) the tendency of yeast and bacterial frataxins to aggregate and to have ferritin-like iron storage properties, as suggested by Adamec et al. (33), is a side-function lost during evolution but is not the main function of the family, even though it might be related to this and might influence it. In favour of the first possibility is the strong tendency to respond to cation excess of proteins from species as far separated in evolution as E. coli and S. cerevisiae. A role in ion binding would also explain the unusual instability of apo-Yfh1 and the acidic pI common to all frataxin orthologues (Table 1). However, a number of considerations would favour the second hypothesis. Iron binding is observed only at very low ionic strength and is competed out at salt concentrations close to physiologic conditions. A mere involvement of frataxin as a storage protein would not explain the pattern of conservation of hfra in yeast (43) and the sequence and structure of hfra: a role in iron binding is suggested by Isaya and co-workers (42), but this mechanism must be common to all frataxin orthologues and not only true for hfra: a common function is required to explain the rescuing experiments of hfra in yeast (43) and the sequence and structure conservation. The aggregation that we observe could therefore be only the ‘echo’ of the formation of a heterogeneous complex. If the protein partner was one or more of the proteins from the lsc operon, this hypothesis would also explain the pattern of co-occurrence with proteins involved in iron–sulfur cluster formation (31). Frataxin could participate in this complex machinery as part of a chaperone complex or as a molecular adaptor. We are currently testing this hypothesis.

**MATERIALS AND METHODS**

**Protein production**

Human frataxin was cloned as previously described; Yfh1 and CyaY were subcloned by PCR from plasmids kindly provided by F. Foury and S.J. Cho. The constructs were cloned into pET-derived plasmid vectors as fusion proteins with Histagged glutathione-S-transferase (GST). The mutants were derived, also as GST-fusion proteins, from CyaY using megaprimer. The constructs were expressed in E. coli strain BL21(DE3). For protein expression, the cells were inoculated in LB medium with ampicillin (100 mg/l) or kanamycin (30 mg/l), induced for 3–4 h by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) after the cultures reached an optical density (OD) of 0.6–0.8 at 600 nm. The cell pellets were harvested and frozen. The frozen cells were thawed in a lysis buffer and subsequently sonicated and centrifuged. The proteins were purified by affinity chromatography (using either Ni-NTA gel or a glutathione-S-sepharose) and, when necessary, further purified using gel filtration chromatography on a G-75 column (Pharmacia). The purity of the recombinant proteins was checked by SDS–PAGE after each step of the purification and by mass spectroscopy of the final products.

The proteins were desalted, dialysed against the final buffer (either 10 mM HEPES–KOH pH 7.4 or MES pH 6) and concentrated with an Amicon concentrator (model 8050). Carboxymethylation was achieved according to the protocol described in (36).

**CD studies**

CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a cell holder thermostatted by circulating water from a Neslab RTE-111 water bath. All measurements were performed in 20 mM Tris–HCl buffer at pH 8 using protein concentrations of 7 μM. The spectra were recorded in fused silica cuvettes of 1 mm pathlength. Ten scans were averaged, and the appropriate buffer baseline was subtracted. Iron titration of CyaY was performed using 7 μM protein concentration and adding increasing quantities of FeSO₄ (up to a 1 : 20 protein-to-iron ratio). The spectra are reported in terms of mean residue weight ellipticity θ/deg cm dmol⁻¹. CD changes as a function of temperature were studied in 10 mm cuvettes with direct temperature monitoring with an immersed thermocouple. Heating rates of 1°C/min were used over the range 10–85°C.

**Gel filtration**

A prepacked HiLoad 10/30 Superdex 75 column (Pharmacia) was equilibrated with HEPES buffer (pH 7.4) or MES buffer (pH 6) and 100 mM NaCl. Ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) were used as molecular standards for the mass calibration. Samples (1 ml) were loaded using a static loop (1 ml) and were eluted with the same equilibrating buffer. Samples were prepared by incubating 8 or 20 μM protein concentrations in the presence of a given salt for 1 h at 30°C. After centrifugation at 13 000 g for 5 min, the supernatant was loaded on the column. The experiments were performed by varying the added salt, the temperature, the protein-to-salt ratio, and the pH. Kinetics of aggregation were followed by mass spectroscopy of the final buffer sample of CyaY (1 : 20 protein-to-iron ratio) by varying the incubation time (1, 30 and 60 min and 24 h).

Competition experiments were performed either by using increasing competitor concentrations in the initial buffer or by adding the equivalent quantity after incubation of the protein in the presence of a 20- and a 60-fold excess of Fe(NH₄)₂(SO₄)₂. Calcium chloride or magnesium sulfate were added to obtain final concentrations of 0.1, 1 and 10 mM. Competition experiments using KCl were performed using 50, 100 and 150 mM concentrations. Citrate and EDTA were added using 1 : 100 protein-to-competitor ratios. Each gel filtration experiment was repeated at least three times to test for reproducibility. For the colorimetric assay, 100 μl of each column fraction were added to 900 μl of BIORAD reagent (BIORAD) solution diluted 1 : 5. The absorbance was measured as OD at 595 nm after 15 min incubation at room temperature.
Fluorescence

Fluorescence spectra were recorded with a SPEX Fluoromax spectrometer with excitation at 290 nm (bandwidth 1.7 nm) and emission recorded from 300 to 450 nm (bandwidth 2 nm). Measurements were performed at 25°C. The protein concentration used was 0.65 μM, using a 1:20 protein-to-iron ratio. Competition experiments were performed using Zn²⁺ (5, 10 and 25 μM), Ca²⁺ (0.1, 0.5 and 2.4 mM), Mg²⁺ (0.5 and 2.4 mM), EGTA (0.5 mM) and KCl (10 and 30 mM). The experiments were done both by adding the competitor first and then the iron and vice versa. The salts were all used as chlorides.

Atomic absorption

Solutions were analysed for total metal contents using standard procedures (44). Metals were determined by flame atomic absorption spectrometry. Solutions were directly aspirated into an air-acetylene flame with no prior treatment. Concentrations were obtained both by comparison with a calibration curve and by standard addition. No differences were observed that overcome experimental uncertainty. The background level of iron was measured for a solution prepared with the same conditions used otherwise but in the absence of protein. A Varian SpectrAA 220 atomic absorption spectrometer equipped with an MK7 burner was used.

Modelling

Modelling of the region 64–174 of Yfh1 (spanning the conserved globular domain) was based on a multiple sequence alignment calculated by CLUSTALX (45) and checked with a structural alignment achieved by Dali (46). The three-dimensional model was generated using the BLDPSQ option in WHATIF (47), using the coordinates of hfra(91–210) as a template. The two sequences differ only by two single-residue deletions in Yfh1 that occur in loops. No attempt to model deletions was made.

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REFERENCES
