The Factors Governing the Thermal Stability of Frataxin Orthologues: How To Increase a Protein's Stability[†]

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ABSTRACT: Understanding the factors governing the thermal stability of proteins and correlating them to the sequence and structure is a complex and multiple problem that can nevertheless provide important information on the molecular forces involved in protein folding. Here, we have carried out a comparative genomic study to analyze the effects that different intrinsic and environmental factors have on the thermal stability of frataxins, a family of small mitochondrial iron-binding proteins found in organisms ranging from bacteria to humans. Low expression of frataxin in humans causes Friedreich's ataxia, an autosomal recessive neurodegenerative disease. The human, yeast, and bacterial orthologues were selected as representatives of different evolutionary steps. Although sharing high sequence homology and the same three-dimensional fold, the three proteins have a large variability in their thermal stabilities. Whereas bacterial and human frataxins are thermally stable, well-behaved proteins, under the same conditions yeast frataxin exists in solution as an unstable species with apprechable tracts in a conformational exchange. By designing suitable mutants, we show and justify structurally that the length of the C-terminus is an important intrinsic factor that directly correlates with the thermal stabilities of the three proteins. Thermal stability is also gained by the addition of Fe^{2+}. This effect, however, is not uniform for the three orthologues nor highly specific for iron: a similar albeit weaker stabilization is observed with other mono- and divalent cations. We discuss the implications that our findings have for the role of frataxins as iron-binding proteins.

A large plethora of studies, both theoretical and experimental, has been dedicated for decades to understanding how the primary sequence of a protein determines its tertiary fold (for a recent review, see ref *1*). Recently, the subject has received new attention because the availability of an increasing number of complete genomes has opened new and challenging avenues: comparison of the sequences, structures, and stabilities of a protein from different organisms can provide precious information on how the same fold has adapted through evolution. With this ultimate aim, we have applied a comparative genomic approach to study the stability of frataxin, a small mitochondrial protein involved in Friedreich's ataxia (*2*) (FRDA,[1] OMIM:229300). FRDA is a progressive human neurodegenerative disease caused by expansion of the trinucleotide repeat GAA within the first intron of the gene X25 on human chromosome 9q13 (reviewed in ref *3*). The expansion leads to a severe reduction of the frataxin gene product (*2*, *4*). Frataxins are proteins highly conserved throughout evolution from Gram-negative bacteria to humans (*5*−*8*). Their cellular role is essential, since mice knock-out models show embryo lethality at an early stage of development (*9*). It has been shown experimentally that neuronal death observed in FRDA arises from disregulation of mitochondrial iron homeostasis, with concomitant oxidative damage (*9*−*14*). In vitro studies have shown that frataxins are able to bind iron, although with relatively modest affinities (*15*, *16*). The current working hypothesis, strongly supported by independent lines of evidence, is that frataxin is an iron chaperone involved in the iron−sulfur cluster (Isc) cascade: a specific link with Isc assembly proteins was first suggested by a theoretical phylogenetic study which showed a clear co-occurrence of frataxin with other Isc proteins in all sequenced genomes (*17*) and successively supported experimentally (*18*−*20*).

We have recently carried out a comparative functional study of frataxin orthologues from three organisms of increasing evolutionary complexity: *Escherichia coli* (CyaY), *Saccharomyces cerevisiae* (Yfh1), and *Homo sapiens* (frataxin, hereon abbreviated as hfra) (*21*). We compared the iron incorporation and aggregate formation of the three proteins

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[1] Abbreviations: 2D, two-dimensional; CD, circular dichroism spectroscopy; FRDA, Friedreich's ataxia; GST, glutathione-*S*-transferase; hfra, human frataxin; HSQC, heteronuclear single-quantum coherence; Isc, iron−sulfur cluster; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEV, tobacco etch virus.

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and showed that, although sharing the same fold, they have different tendencies to respond to iron and to aggregate, with Yfh1 being the most susceptible one and the most prone to aggregation. One interesting observation arising from our previous in vitro studies is also that the three orthologues widely differ in thermostability: under the same experimental conditions, hfra and CyaY have stabilities typical of proteins of comparable size from mesophilic organisms, whereas Yfh1 has an unusually low melting temperature (35.8 °C) (*22*). This result poses the question of how a protein that is so unstable can be functional in a host whose optimal growth temperature is around 30 °C. While in vivo environmental factors (such as the viscosity of the cellular compartment or the interaction with another protein or a cofactor) may, of course, compensate for an intrinsic instability, the identification of the factors that convene to stabilize a protein is of great importance to shed light onto the sequence/structure/stability relationship.

In an attempt to understand what determines the different thermodynamic stabilities of frataxins among the species, we have thus performed a comparative characterization of the thermostabilities of CyaY, Yfh1, and hfra using a combination of complementary biophysical techniques. We show that, although the proteins share the same fold, they have remarkably different thermodynamic behavior. We have identified one of the intrinsic factors that govern their stabilities in the length of the C-terminus and quantitatively described the effect. We have also compared the stability of the three proteins when bound to iron, the cofactor that is thought to be bound to frataxin in vivo, and to other ions. We show that the ionic composition has a strong effect on the protein stabilities but in a highly differentiated way which seems to reflect specific differences of the three proteins also at the functional level.

MATERIALS AND METHODS

Sample Preparation. As in previous studies, we have used for hfra the evolutionary conserved domain (residues 91−210, hereafter indicated as hfra(91−210)) rather than the full-length mature protein (*23*). This choice was mainly dictated by the practical consideration that in vitro the mature protein tends to degrade spontaneously to a smaller fragment 15 residues shorter (*6*, *21*). Because of lack of conservation of the N-terminal tail of the protein, the C-terminal domain must anyway be the portion of the protein that is functionally relevant. The other two proteins were used in their full-length form.

Sample preparation was carried out as previously described (*6*, *25*). In short, the constructs were expressed in *E. coli* as fusion proteins with His-tagged glutathione-*S*-transferase (GST) either with tobacco etch virus (TEV) or PreScission protease cleavage sites. The soluble overexpressed proteins were first passed through a nickel column and eluted with imidazole (pH 8) and then further purified by gel filtration chromatography on a Superdex G75 16/60 column (Pharmacia). The purity of the recombinant proteins was checked by SDS−PAGE after each step of the purification and by mass spectroscopy on the final products. In all cases a single species was identified. ^{15}N-labeled samples were produced by growing the bacteria in minimal medium using ammonium sulfate as the sole source of nitrogen.

Far-UV CD Measurements. Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter fitted with a cell holder, thermostated by circulating water from a Neslab RTE-111 water bath. Measurements were carried out in a variety of buffers and ionic strengths using protein concentrations of 10 (for Yfh1 and CyaY) and 16 μM (for hfra) and fused silica cuvettes of 1 mm path length (Hellma). Thermal unfolding was repeated at least twice and up to five times on independent protein preparations to ensure reproducibility of the results. In all cases, we observed no more than 1.5 °C variations. CD spectra were typically recorded with 0.2 nm resolution and were baseline corrected by subtraction of the appropriate buffer spectrum. Thermal unfolding curves were obtained by monitoring the ellipticity at 222 nm using 2 mm path length cells and a heating rate of 1 °C/min for all samples measured in the temperature range 10−90 °C. The temperature of the sample was measured with a thermocouple immersed in the protein solution. The values of melting temperature were obtained directly from the unfolding curves, postulating a two-state mechanism of unfolding and using nonlinear regression analysis, as described in detail elsewhere (*26*). Anaerobic conditions were achieved by performing the measurements under a nitrogen flow. Since the degree of reversibility depends on several different factors, such as the time spent at high temperature before cooling, the way the system is cooled, and the protein concentration, the values given in this paper should be taken only as an indication that an irreversible aggregation phenomenon may be going on. Typically, the degree of reversibility was estimated by cooling the system immediately after reaching the highest temperature and recording a spectrum at 25 °C.

NMR Measurements. NMR measurements were performed on ^{15}N-enriched protein samples of 0.2−0.5 mM concentrations. NMR experiments were recorded at 25 °C on a Varian Unityplus 500 NMR spectometer equipped with pulsed *z*-shielded gradient coils. All NMR spectra were processed using NMRPipe (*27*) and analyzed using XEASY (*28*).

RESULTS

Comparison of the Fold of the Three Orthologues. To check more closely the state of fold of the three proteins, we used nuclear magnetic resonance (NMR) spectroscopy, a powerful technique that can assess protein fold even in the absence of a complete structural description. 2D ^{15}N−^{1}H heteronuclear HSQC spectra were recorded and analyzed (Figure 1). They provide direct local information on the chemical environment of each of the amide protons along the polypeptide chain. The spectra of the three proteins have very different appearances. The spectra of CyaY and hfra(91−210) are well dispersed with clearly defined resonances, thus being indicative of a well-folded globular protein. Peak picking of the spectra gives approximately the expected number of resonances (i.e., 104 resonances for CyaY and 115 for hfra(91−210)). Full assignment of these spectra could be achieved and is described elsewhere (*25*, *29*). In contrast, the quality of the Yfh1 spectrum is poor: sharp, well-dispersed resonances are in co-presence with a dense overlapping cluster of ill-dispersed broad peaks at values expected for a random coil conformation (*30*). Because of the peak broadening, it is very difficult to count the number of resonances. The spectrum could be equally explained by

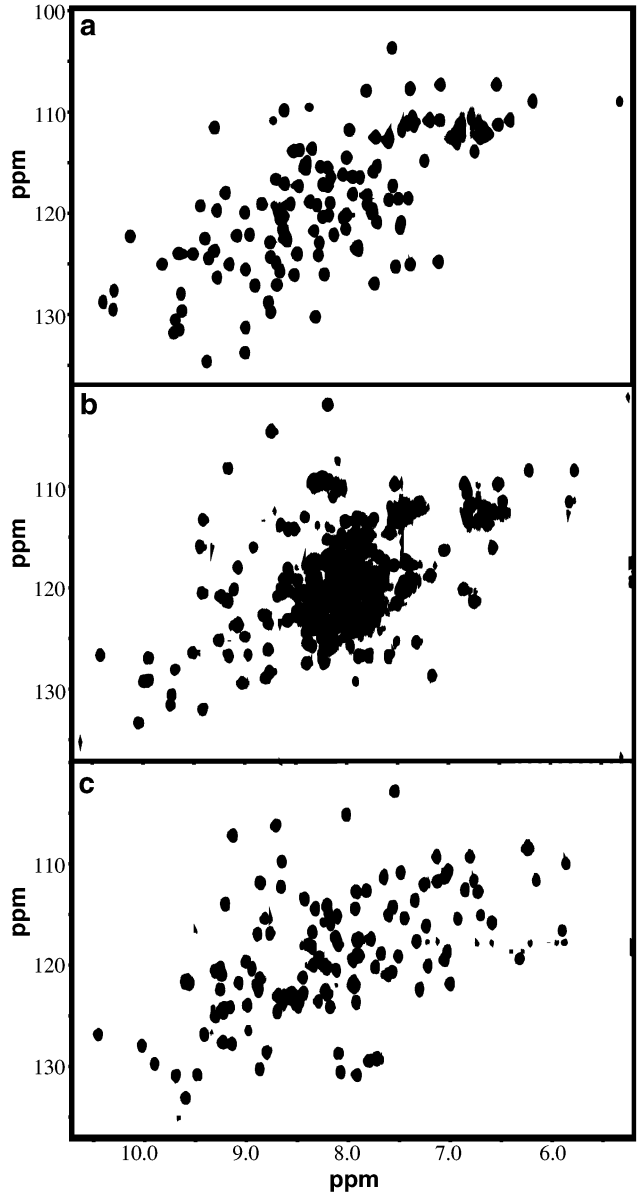

FIGURE 1: Comparison of the NMR spectra of hfra(91−210) (a), Yfh1 (b), and CyaY (c). The spectra were recorded at 25 °C and 500 MHz in 20 mM Tris HCl at pH 8 without additional salt.

the presence of a single, partially unfolded species or by the coexistence of two populations, one folded and the other unfolded. When the temperature was varied over the range 5−30 °C, no appreciable change of the spectral quality was observed, suggesting that this is not related to the experimental conditions being close to the protein melting temperature and that, if two distinct species were present, they are not in mutual exchange within the range of temperatures explored. An alternative and more likely explanation is that the well-dispersed resonances arise from the evolutionary conserved domain, whereas the mitochondrial import tag is flexible and in a conformational exchange. Further increase of the temperature leads to collapse of the spectrum and protein unfolding around 35 °C (data not shown), in agreement with what was previously reported (*21*).

These data confirm a markedly different behavior of the three orthologues within the same structural architecture, with Yfh1 being the most unstable one.

The C-Terminus Length of Frataxin Orthologues Influences Their Thermodynamical Stability. To explore the factors that could influence such large differences in behavior, we first analyzed and compared the sequences and the structures of the three proteins. CyaY, Yfh1, and hfra vary considerably in their lengths (Figure 2). Yfh1 and hfra contain part of the import signals, whereas the bacterial protein only spans the evolutionary conserved domain (*5*). Mature human frataxin spans from 63 to 210 and has the longest N- and C-termini (*23*). In analogy with the observation for protein domains that thermodynamic stability of a domain is often influenced by the choice of the N- and C-domain boundary (*31*−*33*), we hypothesised that the different stabilities could correlate with the length of the proteins. However, the N-terminal tail present in eukaryotes does not seem to contribute to the fold stability, since the melting temperatures of hfra(91−210) and of an N-terminally extended version, hfra(81−210) (the longest form we could produce in bacteria without further degradation), are comparable (*6*). This suggests that the N-terminus does not have a major functional role besides mitochondrial import, as also confirmed by the empirical observation that, among the three proteins, the most unstable is the N-terminal full-length Yfh1.

The length of the C-terminus also varies in different species. Human and murine frataxins are the longest known sequences; bacterial orthologues have intermediate lengths; *S. cerevisiae* Yfh1 is the shortest, including only four residues after the last element of secondary structure. This implies the absence of two very conserved hydrophobic residues (Leu198 and Leu200 in hfra). In the structures of hfra(91−210) and CyaY, the C-terminus inserts into the groove formed between helices 1 and 2 (Figure 3). The two hydrophobic residues anchor the C-terminus to the groove by pointing directly into the hydrophobic core, like a zipper. In longer sequences such as human, murine, and fly frataxins, two more hydrophobic residues (Leu203 and Tyr205 in hfra) could provide an additional contribution. Although this

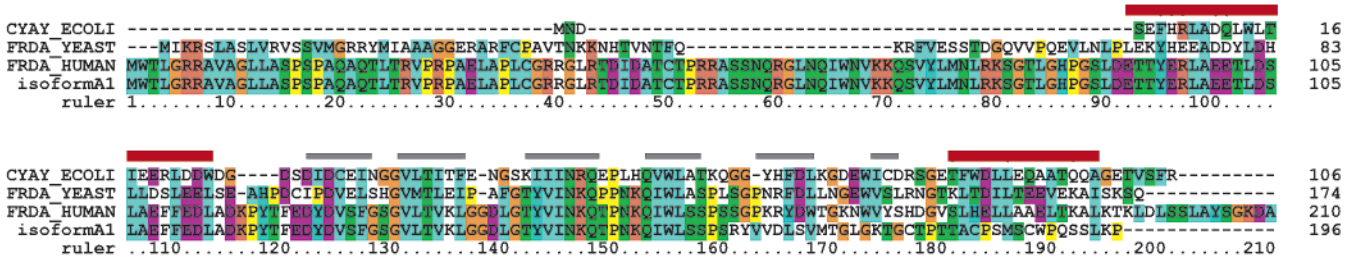

FIGURE 2: Multiple alignment of the three frataxin orthologues, color-coded according to the ClustalX conventions to emphasize conserved sequence features (*41*). The sequences are identified by their SWISSPROT entry names and correspond from top to bottom to CyaY, Yfh1, and hfra. The sequence of human isoform A1 (*39*) is shown in the last line of the alignment. Red and gray rectangles indicate the positions of α-helices and *β*-sheets in the structure of hfra(91−210) (*6*, *8*).

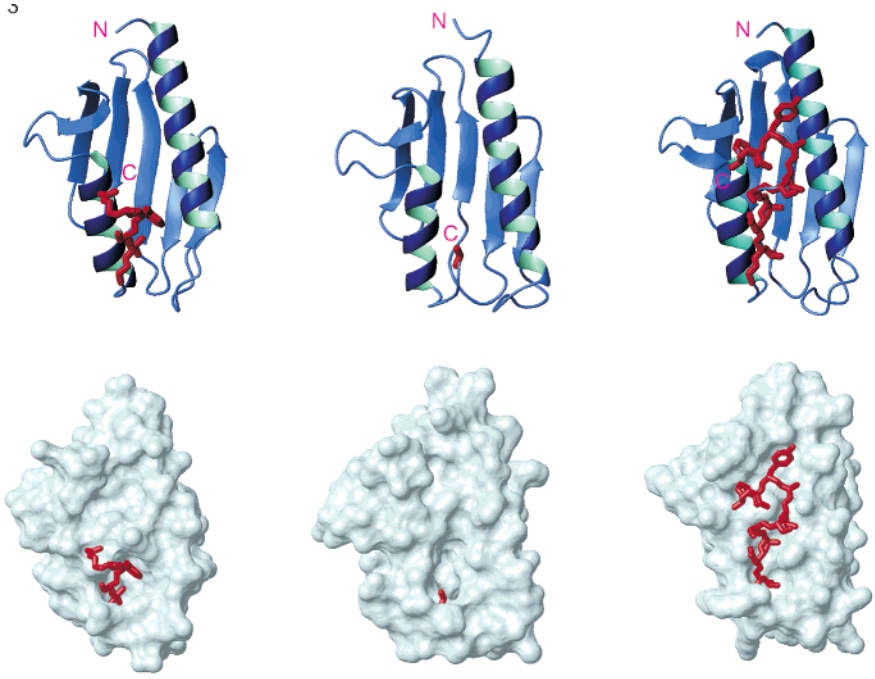

FIGURE 3: Ribbon (top) and contact surface representations (bottom) of the structures of CyaY (left), Yfh1 (center), and hfra(91−210) (right). The structures of CyaY and hfra(81−210) have been experimentally determined (*6-8*), whereas the structure of Yfh1 was modeled by homology from the coordinates of hfra(81−210) (*21*). The C terminal tails, represented as red neons, are $V^{103}SFR^{106}$, Q (backbone atoms), and $L^{198}DLSLAYSGK^{208}$ for CyaY, Yfh1, and hfra, respectively. Models were generated with Molmol (*42*).

feature is not shared in some other yeasts (e.g., in *S. pombe*), a similarly short C-terminus is observed in other species (Figure 2 and Gibson, personal communication). We therefore hypothesized that the thermal stabilities could directly correlate with the length of the C-terminus.

To test this hypothesis, three mutants were designed. First, we produced two CyaY and hfra deletion mutants (CyaY-(1−103) and hfra(91−198)), in which the last 3 and 12 amino acids were deleted, respectively, to lead to proteins of the same C-terminal length as Yfh1. The two constructs showed a behavior different from that of the parental proteins, which were soluble already at the level of *E. coli* expression. CyaY-(1−103) expressed only partially in a soluble form, with a high percentage of the protein forming inclusion bodies. Hfra(91−198) was all produced into inclusion bodies, and all attempts to vary the experimental conditions to obtain a soluble protein failed. The thermal stability of the bacterial mutant drops by ca. 14 °C, becoming comparable to that of the Yfh1 (Table 1).

As a positive control, we tried to stabilize Yfh1 by extending it C-terminally. On the basis of a detailed analysis of the interactions formed by the C-termini in the structures of hfra and CyaY (*6*−*8*), we introduced in Yfh1 a hydrophobic-X-hydrophobic motif (where X stands for any amino acid) by mutating the last Gln into a Leu and adding three amino acids taken from the hfra sequence (Asp-Leu-Ser). Not only could the mutant protein (Yfh1(LDLS)) be expressed in a soluble form, but its thermostability increased by 6 °C (under the same experimental conditions).

These results show that the C-terminal length of Yfh1 is one of the important intrinsic factors that determines the fold instability of this protein in vitro.

Screening the Environmental Factors Influencing the Thermostabilities. The protein length is, of course, an intrinsic factor. We then questioned whether and to what extent stability could be gained by environmental factors and performed a systematic search by varying pH, ionic strength, and salt type (Table 1). First, the pH was varied in the range 5−8 to explore the effect on stability of small variations around the physiological range, above the protein's pI (for all three in the range 4.1−5.0). The proteins tend to aggregate and precipitate around pH 5. Under all other conditions (except those explicitly stated below), the thermal unfolding was highly cooperative and almost completely reversible (more than 95%), an indication that there is no (or negligible) aggregation brought about by thermal denaturation of the proteins under these conditions. Of the three proteins, Yfh1 is the only one which has a small but appreciable pH-dependent stability within the pH range explored, being less unstable at more acidic pH.

Increasing the ionic strength using the same buffer (phosphate) leads to a strong stabilization effect that increases regularly with the buffer concentration up to a plateau (data not shown) for all three orthologues. The same trend is also reproduced for the mutants.

The effect of specific cations/anions was then tested in detail for the least stable Yfh1. An effect is expected since it is well known that different cations/anions influence differently protein stability (Hofmeister series) (*34*, *35*). We observed that the influence of the anion type follows the Hofmeister series, so that at the same concentration, chloride anions have a less stabilizing effect than sulfate and phosphate buffers (Table 1, "other ions" section). Conversely, the cations follow a trend opposite to that expected from the Hofmeister series. According to this empirical rule, divalent cations are expected to have a destabilizing effect as compared to equal concentrations of monovalent cations: for instance, measurements of Yfh1 in Na_2SO_4 and $CaCl_2$ at the same concentration lead to an appreciable stabilization in the presence of calcium cations ($\Delta T_m = 6$ °C). While the

Table 1: Screening of the Factors Influencing Thermal Stability of Frataxin Orthologues[a]

effect	orthologue	pH	buffer	T_m (°C)
intrinsic stability	CyaY	7.0	phosphate 20 mM	53.8 ± 0.1
		7.0	Hepes 10 mM	50.4 ± 0.1
		7.4	Hepes 10 mM	51.0 ± 0.1
	Yfh1	7.0	phosphate 20 mM	35.9 ± 0.2
		7.4	Hepes 10 mM	35.5 ± 0.1
	hfra(91−210)	7.0	phosphate 20 mM	60.0 ± 0.1
		7.0	Hepes 10 mM	58.0 ± 0.1[b]
	hfra(91−198)			nd
	CyaY(1−103)	7.4	Hepes 10 mM	36.8 ± 0.5
	Yfh1(LDLS)	7.0	phosphate 20 mM	42.7 ± 0.1
phosphate and pH	CyaY	6.0	phosphate 20 mM	53.5 ± 0.1
		8.0	phosphate 20 mM	53.8 ± 0.1
		6.0	phosphate 150 mM	64.1 ± 0.2
	Yfh1	6.0	phosphate 20 mM	39.9 ± 0.1
		7.0	phosphate 20 mM	35.9 ± 0.2
		8.0	phosphate 20 mM	33.0 ± 0.2
		6.0	phosphate 150 mM	49.0 ± 0.1
		7.0	phosphate 150 mM	48.6 ± 0.1
		8.0	phosphate 150 mM	45.9 ± 0.1
	hfra(91−210)	7.0	phosphate 20 mM	60.0 ± 0.1
		6.0	phosphate 150 mM	64.9 ± 0.1
		7.0	phosphate 150 mM	65.7 ± 0.1
	CyaY(1−103)	8.0	phosphate 200 mM	45.8 ± 0.8
	Yfh1(LDLS)	7.0	phosphate 20 mM	42.7 ± 0.1
		7.0	phosphate 150 mM	53.6 ± 0.2
iron effect	CyaY	7.0	Hepes 10 mM	50.4 ± 0.1
		7.0	Hepes 10 mM, 1:6 protein:Fe^{2+}	61.2 ± 0.1
		7.5	Hepes 10 mM, 1:6 protein:Fe^{2+}	60.5 ± 0.1[c]
		7.5	Hepes 10 mM, 1:20 protein:Fe^{2+}	64.1 ± 0.1
	Yfh1	7.2	Hepes 10 mM	34.3 ± 0.2[d]
		7.2	Hepes 10 mM, 1:6 protein:Fe^{2+}	37.9 ± 0.3
		7.2	Hepes 10 mM, 1:8 protein:Fe^{2+}	38.7 ± 0.2
		7.2	Hepes 10 mM, 1:20 protein:Fe^{2+}	42.3 ± 0.3
		7.2	Hepes 10 mM, 1:40 protein:Fe^{2+}	44.8 ± 0.4[e]
		7.2	Hepes 10 mM, 1:20 protein:Fe^{2+}, 150 mM NaF	46.9 ± 0.2
	hfra(91−210)	7.0	Hepes 10 mM	58.0 ± 0.1[b]
		7.0	Hepes 10 mM, 1:8 protein:Fe^{2+}	68.6 ± 0.1
		7.0	Hepes 10 mM, 1:20 protein:Fe^{2+}	69.3 ± 0.5
other ions effect	CyaY	7.5	Hepes 10 mM, 1:5 protein:$CaCl_2$	54.8 ± 0.1
		7.5	Hepes 10 mM, 1:20 protein:$CaCl_2$	58.3 ± 0.1
		7.0	Hepes 10 mM, 1 mM $CaCl_2$	62.7 ± 0.3
		7.0	Hepes 10 mM, 150 mM $CaCl_2$	precipitation
	Yfh1	7.2	Hepes 10 mM, EDTA	32.1 ± 0.2
		7.0	Hepes 10 mM, NaCl 150 mM	44.6 ± 0.2
		7.0	Hepes 10 mM, NaF 150 mM	44.8 ± 0.2
		7.0	Hepes 10 mM, Na_2SO_4 150 mM	46.9 ± 0.2
		7.0	phosphate 150 mM	48.6 ± 0.1
		7.0	Hepes 10 mM, $CaCl_2$ 1 mM	51.1 ± 0.1
		7.0	Hepes 10 mM, $CaCl_2$ 150 mM	52.9 ± 2.0
	Hfra(91−210)	7.0	Hepes 10 mM, $CaCl_2$ 1 mM	65.9 ± 0.1
		7.0	Hepes 10 mM, NaCl 150 mM	65.0 ± 0.1

[a] The melting temperatures (T_m in °C) have been clustered according to the effect probed. "nd" stands for "not determined": the construct was expressed but in inclusion bodies. [b] Due to a typographical error, this value was incorrectly reproduced as 69.4 °C in ref *21*. [c] The experiment was performed under anaerobic conditions. [d] To allow direct comparison, the value refers to the same batch of Yfh1 protein used for all the thermal denaturations in the presence of iron. [e] Some precipitation occurred.

melting temperature of this orthologue undergoes a dramatic increase by ca. 12 °C when 150 mM NaCl is added to the buffer, the most prominent effect resulted from addition of $CaCl_2$ ($\Delta T_m = 19$ °C already in the presence of 1 mM, which corresponds to a ca. 1:100 protein:Ca^{2+} ratio). Further increase of the calcium concentration leads to a negligible additional stabilization.

A similar but not equally pronounced stabilization effect (of 12 °C and 8 °C) is observed for CyaY and hfra(91−210), respectively, at 1 mM $CaCl_2$.

The CyaY(1−103) and Yfh1(LDLS) mutants follow the same trend as the corresponding wild-types and are stabilized by increasing ionic strength, with a stronger preference for the phosphate buffer.

The Thermal Stabilities of the Three Orthologues Are Strongly Influenced by Fe^{2+}. Frataxins have been reported to bind iron directly (*15*, *16*, *36*). Therefore, we explored the effect of this cation on the stabilities of the complexes (Figure 4 and Table 1). While no tendency to aggregation has been reported for hfra, CyaY and Yfh1 have been reported to form aggregates in the presence of relatively high iron:protein ratios (*21*, *36*, *37*), thus making the interpretation of the results more complicated. An appreciable stabilization effect was observed both for CyaY ($\Delta T_m = 11$ °C) and for

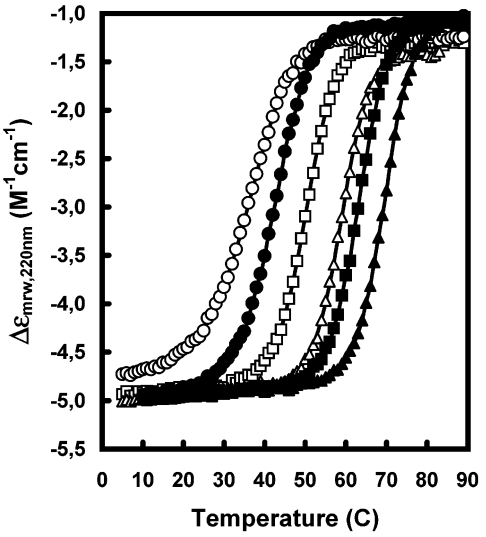

FIGURE 4: Comparison of the thermal denaturation curves followed by CD of the three orthologues in Hepes at pH 7 in the absence (open symbols) and in the presence (close symbols) of a 1:20 ratios of Fe^{2+}. Circles, squares, and triangles are used for Yfh1, CyaY, and hfra(91−210), respectively.

hfra(91−210) ($\Delta T_m = 10$°C) already in the presence of 1:6 protein:Fe^{2+} ratios, which represents the binding stoichiometry reported for hfra (1:6 hfra:iron) (*15*, *16*). Under these conditions, only a minor percentage of aggregation is present at room temperature (*16*, *21*), although aggregate formation could be promoted by temperature, as suggested by the lower degree of reversibility of the unfolding process (86% for CyaY and 88% for hfra(91−210)). Interestingly, at the same protein:Fe^{2+} ratios, Yfh1 gains less than 4 °C, having a degree of reversibility comparable to those of the other two proteins (86%). At protein:Fe^{2+} concentration ratios of 1:20, the thermostabilities of CyaY and Yfh1 increase further but not linearly, whereas the stability of hfra(91−210) remains practically unchanged. Under these conditions, the percentage of aggregate present at room temperature is significantly relevant (*16*, *21*, *36*−*38*), suggesting that the further stabilizing effect may be also influenced by aggregation. Accordingly, the degree of reversibility of the unfolding process decreases (72% for CyaY but only 47% for Yfh1). At 1:40 protein:Fe^{2+} concentration ratios, Yfh1 precipitates at high temperatures. No differences in the melting temperatures were observed when the experiments were performed under anaerobic conditions, to prevent oxidation of Fe^{2+} into Fe^{3+}.

To check to what extent the effect of iron binding is selective, we compared the protein stability of CyaY in the presence of increasing concentrations of Fe^{2+} and Ca^{2+} (Table 1). At the same protein:cation ratios, iron has a consistently stronger effect than calcium. However, even small protein:calcium ratios (1:6) lead to ca. 4 °C stabilization, suggesting that also the effect of calcium is due to specific, albeit weaker interactions.

All together, these results indicate that formation of iron/protein complexes leads to a strong stabilizing effect.

DISCUSSION

We have explored systematically the factors that influence the thermal stability of the frataxin fold, using three orthologues selected as representatives of different evolutionary steps. Although sharing the same fold, the three proteins have in vitro a remarkable variation of melting temperatures under the same conditions. Yfh1 is by far the least stable and seems to contain in solution an appreciable region in a conformation exchange. Analysis of several unfolding CD curves of Yfh1 gives in fact a ΔH of 34.8±1.2 kcal/mol. Using this value in the Gibbs−Helmholtz equation with an estimated ΔC_p of 1.7 ± 0.3 kcal (K mol)$^{-1}$ for a protein of 139 residues gives ΔG(20 °C) = 1.1 ± 0.12 kcal/mol, suggesting that Yfh1 is only 87 ± 4% folded at 20 °C. These results may have important implications for the use of *S. cerevisiae* as a model system to understand the functions of frataxin. Despite the several excellent reasons that make *S. cerevisiae* an easy and ductile model system in vivo, particular caution should be used when trying to rationalize the effects of mutations in Yfh1, especially in in vitro studies. Because of its strong intrinsic instability, even small alterations of its amino acid composition could have a dramatic effect on its fold. This could then make it particularly difficult to discriminate whether a given residue is essential for the three-dimensional scaffold or directly involved in the protein functions, e.g., by being part of the interaction surface with other molecules.

Aided by the structural knowledge of the frataxin fold, we have identified the intrinsic length of the proteins as an important factor influencing their thermostabilities: deletion mutants of hfra(91−210) and CyaY lead to strongly destabilized proteins, whereas C-terminal extension of as few as three amino acids produces an appreciable stabilizing effect in Yfh1. At the molecular level, this behavior can be explained (and predicted) by noticing that, in the known frataxin structures, the C-terminus has an important structural role in inserting between the two helices and packing against the hydrophobic core (*6*−*8*). Truncation of this tail must therefore have an influence on the fold stability. Following this reasoning, we succeeded in designing not only mutations that destabilize CyaY and hfra but also mutations that stabilize the unstable CyaY. The latter result is particularly exciting because, whereas it is relatively simple to introduce destabilizing mutations, it is much more difficult to obtain the opposite effect: millions of years of evolution are normally needed to select the "optimal" sequence. It can be expected that further stabilization could be achieved by a more detailed screening of the amino acid sequence of the extension.

A role of the C-terminus in the fold stability is very interesting also in view of the recently identified A1 isoform of hfra, an alternatively spliced transcript of the frataxin gene (*39*). This variant encodes a hfra protein 196 residues long, expressed at low levels as compared with the predominant A1 isoform. The sequences of the two isoforms are identical up to residue 160 and have no homology from there on (Figure 2). The length of the C-terminus of A1 is comparable to that of Yfh1, thus suggesting, according to our results, that the fold of this variant could be strongly compromised. It is, however, still possible that the drastic sequence divergence from 160 on could lead to a major fold rearrangement that is able to compensate for the shorter C-terminus. Further structural studies will be needed to test this hypothesis.

The role of N- and C-terminal extensions in protein modules has been studied for a long time: it is well known,

when producing isolated domains, that the choice of the domain boundaries can greatly influence the protein stabilities (*31*−*33*). More striking is to notice a similar effect in frataxins, which are small single-domain full-length proteins (leaving aside the nonconserved N-terminal tail needed for mitochondrial import in eukaryotes). While we do not, of course, claim that the different sequence lengths are the only cause of a different stability, an important point worthy of consideration is that the protein length, like other contributing factors not examined here, such as the presence of salt bridges, hydrogen bonds, and/or minor structural rearrangements, is an intrinsic factor (that is solely related to the amino acid sequence of the proteins). In vivo, the intrinsic instability may be compensated by other "environmental" factors, whose identification may be directly related to the protein function.

An obvious stabilizing factor is iron, since all three orthologues studied here have been reported to bind iron, although with different mechanisms. Both hfra and CyaY have been shown to bind to iron with a 1:6 stoichiometry (*15*, *16*). At large iron:protein ratios (i.e., 20:1), Yfh1 and CyaY have been reported to be involved in iron-promoted aggregation (*21*, *36*−*38*), which could, however, be competed out by increasing the ionic strength (*21*). We have explored the effect of this cation on the fold stability and compared it with that of other metals. We observed a strong influence of the ionic strength, emphasising the need for using physiological salt concentrations in in vitro studies. A stabilization is induced by both mono- and divalent cations. This should not be surprising since frataxins are small acidic proteins with a pI around 4.2. However, the inverse correlation with the Hofmeister cation series (*34*, *35*) suggests that the effect is caused not by unspecific salting in but by direct binding of divalent cations to the protein.

Iron ions induce the strongest stabilization, with an effect maximal for a 1:6 protein:iron ratio with almost no further increase at higher iron concentrations, in agreement with the reported binding stoichiometries (*15*, *16*). A similar stabilization effect is well described for other metal-binding proteins, such as calmodulin which, when in its holo form, gains more than 50 °C stability as compared to the apo form (*40*). It is interesting, though, that the observed effect is not uniform for the different orthologues: hfra and CyaY present a stronger stabilizing effect, whereas Yfh1 is only marginally (at least by comparison) affected. This could reflect either different iron binding affinities of the different orthologues or the interference of an additional differential contribution from protein aggregation. It is, in fact, known that the tendency toward iron-promoted aggregation first reported for Yfh1 (*36*−*38*) strongly differs among the three orthologues (*21*). Formation of aggregates is also reflected in our studies by the different degree of reversibility observed.

Comparison of the stabilization effect induced by iron with that of calcium suggests that, although selectivity of iron binding is high, Ca^{2+} can bind to the same binding sites, in agreement with the reported ability of this cation to compete for iron-induced aggregation (*21*).

In conclusion, we have identified some of the intrinsic and environmental factors that stabilize proteins from the frataxin family and correlated them both with their sequence and structures and with their iron-binding properties. How these properties correlate with the role of frataxin as an iron chaperone involved in the Isc assembly remains to be established by further investigations.

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