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Conformational stability of human frataxin and effect of Friedreich's ataxia related mutations on protein folding

Ana R. Correia^{*}, Salvatore Adinolfi[†], Annalisa Pastore[†], Cláudio M. Gomes^{*,§}

^{*} Instituto Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal

[†] National Institute for Medical Research, Medical Research Council, London, United Kingdom

Running title: Stability of wild type and FRDA associated frataxin variants

Abbreviations: FRDA, Friedreich's Ataxia; DSC, Differential scanning calorimetry; FRDA, Friedreich ataxia; GdmCl, guanidinium chloride; GST, glutathione-S-transferase; GuSCN, guanidine thiocyanate; TEV, tobacco etch virus

[§] **To whom correspondence should be addressed:**

C.M. Gomes. Instituto Tecnologia Química e Biológica. Universidade Nova de Lisboa. Av. República 127 2780-756 Oeiras. Portugal

Tel: +351 214469332, Fax: +351 214411277

E-mail: gomes@itqb.unl.pt, URL: <http://www.itqb.unl.pt/pbfs>

Synopsis

The neurodegenerative disorder Friedreich's Ataxia (FRDA) results from a deficiency in frataxin, a putative iron chaperon, and is due to the presence of a high number of GAA repeats in the coding regions of both alleles of the frataxin gene, which impair protein expression. However, some FRDA patients are heterozygous for this triplet expansion, and contain a deleterious point mutation on the other allele. Here we investigate if two particular FRDA-associated frataxin mutants, I154F and W155R, result in unfolded protein as consequence of a severe structural modification. A detailed comparison of the conformational properties of the wild type and mutant proteins combining biophysical and biochemical methodologies was undertaken. We show that the FRDA mutants retain the native fold in physiological conditions but are differentially destabilised as reflected both by their reduced thermodynamic stability and a higher tendency towards proteolytic digestion. The I154F mutant has the strongest effect on fold stability as expected by the fact that the mutated residue contributes to the hydrophobic core formation. Functionally, the iron-binding properties of the mutant frataxins are found to be partly impaired. The apparently paradoxical situation of having clinically aggressive frataxin variants which are folded and are only significantly less stable than the wild type form in a given adverse physiological stress condition is discussed and contextualized in terms of a mechanism determining the pathology of FRDA heterozygous.

Key Words: Protein folding, clinical mutants, iron-binding protein, trinucleotide expansion

Introduction

The possibility of identifying genetic mutations causing human diseases is a revolutionary achievement acquired only within the last 3-4 decades, which has opened new avenues towards our understanding of pathologies. We now know that even a single amino acid mismatch from the wild-type protein sequence may result in major metabolic consequences in an organism thus often leading to severe pathologies. Understanding which effect point mutations have is therefore an important topic essential not only for our basic knowledge of how proteins fold but with direct applications to molecular medicine.

We have recently become interested in single point mutations observed in Friedreich ataxia (FRDA), an autosomal recessive neurodegenerative disease linked to oxidative stress and caused by reduced levels of a small mitochondrial protein, frataxin, encoded by a gene mapped in chromosome 9q13 [1, 2]. Although the exact function of frataxin is still unknown, increasing evidence suggests a role of the protein in iron-sulphur cluster formation [3-9]. The majority of FRDA patients are homozygous for an intronic expansion of a GAA trinucleotide repeat within the frataxin gene [10]. The abnormal triplet expansion does not completely abolish frataxin expression, and a shorter magnitude of the expansion correlates with the late-onset of the disease. However, a few FRDA patients (ca. 4%) are heterozygous, containing an expanded repeat in one allele and a deleterious point mutation in the other [10]. These DNA alterations can either lead to protein truncation or to missense modifications in the mature region of the protein. The latter are particularly interesting as they have different impacts on disease expression: whereas some mutations lead to the normal phenotype of the disease, others result in atypical, milder clinical presentations of the pathology. At the present stage, at least 15 missense frataxin point-mutations have been described in FRDA patients [10]. Reports on these mutations [10] indicate that they do not interfere with splicing and the fact that some compound heterozygous individuals have atypical phenotypic expressions of the disease is suggestive of the cellular presence of frataxin with a partly reduced function [10, 11]. Although mapping these mutations onto the available three-dimensional structures of frataxin [12-15] can provide a framework for predicting its effect on the protein structure, the exact impact of these clinically relevant missense

mutations on the folding pathway of frataxin remains to be experimentally and quantitatively addressed.

Here, we report our studies on the structural stability and folding properties of human frataxin, and on two mutant variants comprising clinically relevant point mutations, I154F and W155R. These modifications involve highly conserved amino acids and are located in structurally distinct regions of the protein [14]. Previous studies have suggested that the two mutations represent two extreme possibilities [10, 13]. The first one, I154F, constitutes the most common clinically occurring mutation and affects a residue which is part of the protein core. This mutant is therefore expected to directly affect the stability of the protein fold and should be structurally important. The second mutation involves instead the conserved W155, which is part of an invariant surface in the exposed region of a β -sheet. The aromatic ring of W155 is fully solvent exposed, suggesting a role of this residue in interaction with a partner. The mutation is expected to be functionally important since conservation of surface residues usually implies that they are directly involved in the protein functions. Both mutations lead to aggressive phenotypic development of the ataxia [10, 13]. In a preliminary report we showed that both these mutants are folded [13]. While this result constitutes little surprise for W155R, the fact that the clinical mutation I154F, which *in vivo* leads to a severe expression of the pathology, results in a protein which is essentially folded under physiological conditions may seem paradoxical in respect to disease expression.

To understand further this apparent paradox, we have extended and complemented previous preliminary data by carrying out an extensive study of the thermodynamic stability of frataxin and its variants using a number of complementary biochemical and biophysical techniques. By exploring different chemical denaturants and experimental conditions, we find that both mutations affect the thermodynamic stability of the proteins but to a very different extent so that the destabilization does not affect the fold of the proteins at physiological temperatures or pH. We show that the structural dynamics of mutant frataxin variants is altered in respect to the wild type protein, resulting in a partly destabilised conformation, more prone to proteolytic processing. Furthermore, both proteins retain partly their iron binding properties. This suggests, in particular, that W155 and the surface around it are implicated in a function distinct from iron binding. On the basis of our results, we suggest a mechanism to explain the strong

pathology associated with I145F. Our study may therefore contribute to a molecular understanding of the frataxin functions and ultimately of its role in FRDA pathology.

Experimental

Chemicals

All reagents were of the highest purity grade commercially available. The chemical denaturant guanidine thiocyanate (GuSCN) was purchased from Promega. Stock solutions were prepared in different buffers and the concentration determined by its density. Guanidinium chloride (GdmCl) was obtained from Sigma and the accurate concentration of the stock solutions in different buffers was confirmed by refractive index measurements.

Gene Expression and Protein purification

The constructs (from the truncated frataxin (91-210), hereon simply Hfra(91-210), and mutant forms) were expressed in *E. coli* (competent cells BLC21 DE3 from Novagen) as fusion proteins with a His and a GST tag with cleavage site for tobacco etch virus (TEV) or PreScission protease as previously described [13, 16]. Briefly the wild type and the two frataxin variants were purified to homogeneity, as follows the cells were grown in LB medium with 30µg/ml of kanamycin. The soluble cell extract was applied on a glutathione Sepharose column (10ml) previously equilibrated with PBS buffer, and eluted with 30ml of PBS buffer, after which TEV was added (2ml of 25µM TEV per litter of cell culture). The column was closed and left overnight at 4°C. The purified protein was eluted by washing the column with PBS buffer. The tags as well as TEV remained bound and were subsequently eluted with reduced glutathione. Protein concentration was determined using the extinction coefficient $\epsilon^{280\text{nm}} = 21030 \text{ M}^{-1}.\text{cm}^{-1}$. Both mutants were found to be stable in solution although susceptible to precipitate upon slow freezing; however, thawed proteins that had been fast frozen retained their spectroscopic properties and melting temperatures.

Spectroscopic methods

UV/Vis Spectra were recorded at room temperature in a Shimadzu UVPC-1601 spectrometer equipped with cell stirring. Fluorescence spectroscopy was performed on a Cary Varian Eclipse instrument ($\lambda_{\text{ex}} = 280\text{nm}$, $\lambda_{\text{em}} = 340\text{nm}$, slit_{ex}: 5 nm, slit_{em}: 10 nm unless otherwise noted) equipped with cell stirring and Peltier temperature control. Far-UV CD spectra were recorded typically at 0.2 nm resolution on a Jasco J-715 spectropolarimeter fitted with a cell holder thermostated with a peltier.

Chemical denaturation

The denaturation curves were measured using the dilutions method, and two solutions with the same protein concentration were prepared, one with no denaturation agent and the other with a high concentration of denaturation agent. These were combined in different proportions yielding different denaturant concentrations. After mixing, the solutions were left equilibrating for two hours. Transitions curves were determined plotting the average emission wavelength versus denaturant concentrations, correcting for the pre and post transitions [17-19]. A non-linear least-square analysis was used to fit the data to a two state model described by equation (1) [19]:

$$Y = \frac{\left((y_f + m_f \cdot [D]) + (y_u + m_u \cdot [D]) \cdot \exp \left[m \cdot \frac{([D] - [D]_{1/2})}{RT} \right] \right)}{\left(1 + \exp \left[m \cdot \frac{([D] - [D]_{1/2})}{RT} \right] \right)} \quad (1)$$

Where y represents the average emission wavelength observed for a given concentration $[D]$, y_f and y_u are the intercepts and m_f and m_u are the slopes of the pre- and post-transition baselines, $[D]_{1/2}$ is the denaturation agent concentration at the curve midpoint and m is from equation 2.

$$\Delta G = \Delta G_{H_2O} - m[\text{Denaturant}] \quad (2)$$

Thermal denaturation

Thermal unfolding was followed by monitoring the intrinsic tryptophan fluorescence ($\lambda_{\text{exc}}=280$ nm, $\lambda_{\text{em}}=340$ nm, slit_{ex}=5 nm and slit_{em}=10 nm) and the ellipticity ($\Delta\epsilon_{\text{mrw}}$ at 222nm) variations. It has been previously demonstrated [20] that identical melting temperatures are obtained for frataxin, by both CD and fluorescence spectroscopies, thus ruling out artefacts resulting from changes of fluorescence quantum yield with temperature. Further, DSC experiments confirmed melting temperatures (data not shown). In all experiments a heating rate of 1°C/min was used, and the temperature was changed from 20 to 90°C. Data were analysed according to a two-state model described by equation 3,

$$Y = \frac{\left((y_f + m_f \cdot [T]) + (y_u + m_u \cdot [T]) \cdot \exp\left[\left(\frac{\Delta H_m}{RT}\right) \cdot \left(\frac{(T - T_m)}{T_m}\right)\right] \right)}{\left(1 + \exp\left[\left(\frac{\Delta H_m}{RT}\right) \cdot \left(\frac{(T - T_m)}{T_m}\right)\right] \right)} \quad (3)$$

where Y is the spectroscopic signal observed, m_f and m_u the slopes of the pre- and post-transition baselines; y_f and y_u correspond to the value of y for folded and unfolded forms, T_m is the midpoint of the thermal unfolding curve, and ΔH_m is the enthalpy change for unfolding at T_m [19]. The fits to the unfolding transitions were made using Origin (MicroCal). For pH variations, the buffers used were 30mM acetate (pH 4.9), 40mM phosphate (pH 6.0, pH 7.9 and pH 11.6), 10mM HEPES (pH 7.0) and 40mM glycine (pH 8.9 and 9.7). The reversibility of the reaction was investigated by downward temperature ramps, as well as repetition of the upward ramps after cooling down of the sample to 25°C.

Fluorescence quenching

Quenching experiments were performed using a neutral quencher – acrylamide (Bio-Rad) and an ionic quencher KI (Fluka). The samples were excited at 295nm in order to ensure that the light was absorbed almost entirely by tryptophanyl groups and the fluorescence intensity decrease at 340nm was followed. Results were analysed according to the Stern-Volmer (equation 4).

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (4)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, K_{sv} is the collisional quenching constant and $[Q]$ is the quencher concentration [21].

Trypsin limited proteolysis

Frataxins were incubated with trypsin (bovine pancreas trypsin, sequencing grade, Sigma) at 37°C in 0.1 M Tris-HCl pH 8.5, in a 100 fold excess over the protease. Aliquots (~0.5 nmol protein) were sampled at different incubation periods and the reaction stopped by the addition of 0.2% (v/v) of trifluoroacetic acid (TFA). The products of the proteolysis reaction were analysed by reverse-phase HPLC (Waters Alliance System 2695), using a C18 Column 150 x 3.9 mm (DeltaPak, Waters) run at a 0.5 ml.min⁻¹ flow rate, monitoring the absorbance at 214nm. The peptidic profiles were obtained running a three-step a gradient from (A) 0.1% (v/v) TFA to (B) 80% (v/v) Acetonitrile + 0.1% (v/v) TFA: 0-37% (in 65 min), 37-75% (in 30 min) and 75-100% (in 20 min). The column was regenerated with 0.1% (v/v) TFA.

Results

Thermodynamics of frataxin chemical and thermal unfolding

To evaluate the effect of the introduced clinical mutations on the human frataxin fold, the chemical and thermal unfolding reactions of human frataxin were investigated in detail. Frataxin unfolding can be monitored either by fluorescence or by far UV circular dichroism spectroscopies (Fig. 1); in the present work, intrinsic tryptophan fluorescence changes were routinely used to monitor unfolding transitions (see Experimental). In most denaturation conditions tested, frataxin unfolding was found to be highly reversible. Hfra(91-210) exhibits a considerable stability over the physiological pH range: its thermal unfolding transition is reversible and essentially invariant between pH 6 and 9 (Table 1). However, below pH 5, which corresponds to the isoelectric point of Hfra(91-210) ($pI=5$), as well as at higher pH values, an increasingly extent of irreversibility was observed as a result of protein aggregation. At lower pH values this is expected considering the decreased solubility of the unfolded form of a protein near its isoelectric point [19, 22].

Chemical denaturation experiments were performed at pH 7.9 using urea, guanidinium hydrochloride (GdmCl) and guanidinium thiocyanate (GuSCN) as denaturants. The obtained curves were analysed assuming a two-state mechanism, and the unfolded fraction was plotted as a function of denaturant concentration; from the data in the transition region, and using the linear extrapolation model, the thermodynamic parameters $\Delta G(H_2O)$ and m were determined (Fig. 2, Table 2). Frataxin conformational stability ranges from 23.8 to 35.6 kJ.mol⁻¹ (5.7 to 8.5 kcal.mol⁻¹).

Conformational dynamics of mutant frataxins

The impact of the clinical mutations on the frataxin fold and dynamics was evaluated from fluorescence quenching studies. Fluorescence quenching experiments were performed at 25°C, using acrylamide and iodine (Fig. 3, Table 3). Acrylamide is a neutral quencher molecule, more prone to access to the protein interior, and quench buried residues, whereas iodide is negatively charged. The Stern-Volmer constant for the I154F Hfra(91-210) is comparable to that of the wild type for both quenchers, which shows that this structural core mutation does not result in protein misfolding. In respect to the W155R Hfra(91-210) variant, a lower Stern-Volmer constant is determined, in

agreement with the fact that since the two remaining tryptophans are not superficial, the protein matrix will strongly slow down the penetration of the quencher molecules. For all frataxins studied, the accessibility of the tryptophans is increased in the presence of GdmCl, corresponding to a disrupted tertiary structure. Thus, quenching experiments show that under physiological conditions, the mutant frataxins have a globally stable, wild type like fold and breathing dynamics.

Limited proteolysis experiments evidence destabilization of mutant frataxin forms

The conformational differences between wild type and the mutant frataxin forms were accessed by monitoring the progression of trypsin mediated proteolysis. Trypsin was selected for this comparative mapping as it cleaves with high specificity at the COOH-terminal side of lysine and arginine, unless the following position is occupied by a proline [23]. Inspection of the protein sequence identifies 11 possible digestion sites for the wild type and I154F proteins and an additional one for the W155R mutant, as a result of the inserted arginine. A comparison of these limited proteolysis experiments allows evaluating the degree of destabilization of the mutant forms in respect to the native protein, since we expect that a destabilised fold will have more accessible cleavage sites and undergo a faster proteolysis. The obtained peptide maps clearly show that both mutant frataxins are destabilised in respect to the wild type protein, as evidenced by the appearance of a substantially higher number of peptides during the same incubation period (Fig. 4). This observation indicates that the clinical mutant frataxins under study comprise partly destabilised regions which make them more prone to degradation.

Iron binding properties of frataxin clinical variants are impaired by protein destabilisation

Iron binding was also investigated as this aspect may provide useful insights into the biological impact of the mutated variants, since frataxin has been proposed to act as a cellular iron chaperon. Frataxin is known to be able to bind 6-7 irons per molecule, albeit with low affinity [7, 24]. The FRDA associated mutant frataxins were investigated in respect to their ability to bind iron, as well as the wild type protein, which was used as a positive control. Iron binding monitored by fluorescence

spectroscopy according to a previously reported procedure [7] showed that progressive iron binding destabilises the mutants, resulting in protein precipitation. Although this occurs both with Fe(II) and Fe(III), and under pH controlled conditions, the ferric ion seems to have a more destabilizing effect and precipitation is observed above an iron:frataxin ratio of 2. Concerning ferrous ion binding, precipitation was only observed at iron:frataxin ratios ranging from 5 to 7. Thus, although none of these FRDA associated mutations is directly involved in proposed iron binding region [24, 25], its binding may have a negative functional impact as it likely induces structural modifications which destabilise the frataxin fold leading to the formation of precipitates. In any case, iron-mediated colloidal precipitation of the mutant frataxins cannot be ruled out at this stage.

Frataxin mutants I154F and W155R have reduced thermodynamic stability

The conformational properties of the two Hfra(91-210) mutants were spectroscopically investigated using fluorescence and far-UV CD. The differences in conformational stability between the wild type Hfra(91-210) and the two mutants were determined from analysis of their chemical and thermal denaturation transitions, at pH 7 (Fig. 5 and 6). In both cases, cooperative unfolding transitions were observed and, under the experimental conditions used, the unfolding reactions were found to be reversible. Concerning chemical unfolding, the order of the midpoint unfolding urea concentrations was determined to be Hfra wild type > W155R > I154F (Table 4). The slightly higher *m* values determined for the two mutants, also shows that a larger fraction of buried residues is exposed upon unfolding. The mutants midpoint denaturation concentrations was about one unit below the observed for the wild type and this corresponded to a destabilization of 5.9-7.1 kJ (1.4 – 1.7kcal) as a result of the mutations. Thermal unfolding experiments also revealed that the mutant I154F is more destabilized than the W155R having a *T_m* ~16°C lower than the wild type protein, whereas the mutant W155R shows a decrease of only about 5°C. Altogether these data reveal that both mutations under study reduce the thermodynamic stability of frataxin and that the mutation I154F is more aggressive.

Discussion

We have reported here a detailed comparison of the thermodynamic properties of wild type frataxin and of two clinically relevant mutations (I154F and W155R) in FRDA. We show that under chemically or thermally destabilizing conditions, the two mutants show distinct behaviours, but that they both are folded at physiological temperature and pH.

The mutation W155R, which can be fit to the category of functional mutations, shows only some destabilization as compared to the wild-type. Destabilization is expected from the destruction of a π interaction between a tryptophan and the contiguous arginine (Arg165) (Fig. 7), an interaction often observed in protein structure [26]. Additionally, introduction of another positively charged residue near Arg165 introduces further destabilization due to electrostatic repulsion. Observation of these properties is therefore consistent with the location of the tryptophan on the external surface of the β -sheet, with its aromatic moiety completely exposed to the solvent, which is highly suggestive of a role in recognition or interaction with a partner.

A significant perturbation was detected instead for the I154F mutant whose fold is substantially destabilised, as determined from the high number of peptides resulting from partial tryptic digestions and from a red shift in its fluorescence emission maximum. While this is reasonable since the mutation affects a buried residues which takes part of the hydrophobic core (Fig. 7) [13], the interesting observation is that the destabilization effect becomes evident only under stressing conditions. At room temperature, this mutant shows features very similar to those of the wild-type, suggesting that despite the mutation the protein can be correctly folded. Accordingly, I154F, as W155R, partly retains its iron binding capability, which nevertheless depends strongly on the specific three-dimensional scaffold. Correct folding would be essential to keep in spatial proximity the acidic residues found to be involved in iron binding, which are distributed along the first helix and in the first beta strand [24, 25].

These observations are interesting because the severe effects of both mutations in FRDA patients would have possibly suggested a much stronger effect on the frataxin fold. What is then the mechanism that determines the pathology in heterozygous cases? The effect seems to be more subtle and could occur at two different levels. On the one hand, because of a higher destabilization, the folding efficiency once the protein is

detached from the ribosome or migrates through the mitochondrial membrane could be reduced so that a lower concentration of 'functional' protein is released to the cell. On the other hand, the increased rate of proteolytic degradation also points out to a more efficient degradation of the mutant proteins. At the present stage, however, we do not have evidence of what the cellular fate of the mutant variant proteins upon their synthesis may be but much more efforts should be put to follow up these ideas. *In vitro* studies on mutant variants can therefore provide new experimental insights towards the understanding of the structural basis of the disease. The frataxin variants, which result to milder forms of the disease, are particularly interesting and are currently under investigation in our laboratories.

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Figure Legends

Figure 1 – Spectroscopic characterisation of wild type Hfra(91-210) in different conformational states

Panel A. Fluorescence spectroscopy. Emission spectra of the native form (25°C), and the chemically (7M GdmCl) and thermally (80°C) denaturated forms. **Panel B.** Far-UV CD spectroscopy. Spectra of the native and thermally denaturated forms.

Figure 2 – Chemical denaturation curves for Hfra(91-210) at pH 7.9

Hfra(91-210) typical denaturation curves with (○) GdmCl, (□) GuSCN and (△) Urea. Lines represent fits to the two state model, see Table 2 for parameters.

Figure 3 - Stern–Volmer plots of acrylamide and iodide quenching of frataxin tryptophan fluorescence. The figures show plots of F_0/F versus concentration of acrylamide (**A**) and iodide (**B**) for the native wild type Hfra(91-210) (○), W155R Hfra(91-210) (△) and I154F Hfra(91-210) (□). See Table 3 for parameters.

Figure 4 – Peptide maps resulting from partial tryptic digestions.

Wild type (wt) and mutant variants (I154F and W155R) after being incubated with trypsin for 100 min at 37°C.

Figure 5 – Comparison of chemical denaturation of FRDA Hfra(91-210) mutants, pH 7.0

The wild type Hfra(91-210) (○) denaturation curve with urea is compared to that of Hfra(91-210) W155R (△) and Hfra(91-210) I154F (□). See Table 4 for parameters characterizing the urea denaturation.

Figure 6 – Comparison of thermal denaturation of FRDA Hfra(91-210) mutants, pH 7.0

The wild type Hfra(91-210) (○) denaturation curve with urea is compared to that of Hfra(91-210) W155R (△) and Hfra(91-210) I154F (□).

Figure 7 – Ribbon representation of the Hfra structure.

Two views differing by a 180 rotation around the y axis are displayed. The side chains of I145 and W155 are shown (top panel). The hydrophobic side chains of the residues which surround I145 and of R165 which packs against W155 are also marked (bottom panel).

Tables

Table 1 – Parameters for Hfra thermal denaturation as a function of pH.

pH	T_m (°C)	ΔH_m (kJ.mol ⁻¹)	ΔT_m (°C)	% refolding
4.9	59.0 ± 0.1	275.1 ± 1.7	-7.3 ± 0.1	20
6.0	65.4 ± 0.2	371.8 ± 2.1	-0.9 ± 0.3	80
7.0	62.5 ± 0.2	250.4 ± 1.3	-3.8 ± 0.3	95
7.9	66.3 ± 0.1	373.5 ± 1.7	-	100
8.9	62.1 ± 0.2	362.2 ± 3.3	-4.2 ± 0.3	100
9.7	59.0 ± 0.1	282.2 ± 1.3	-7.3 ± 0.1	20

Table 2 - Parameters for the chemical denaturation of wild type hfra, pH 7.9

Denaturant	$[D]_{1/2}$ (M)	m kJ.mol ⁻¹ .M. ⁻¹)	ΔG_{H_2O} (kJ.mol ⁻¹)
GuSCN	1.1	22.4 ± 1.4	24.6 ± 1.7
GdmCl	2.1	16.8 ± 1.4	35.3 ± 2.1
Urea	4.3	5.7 ± 0.2	24.5 ± 1.3

Table 3 – Effect of FRDA mutations on quencher accessibility, Stern-Volmer constants using different quenchers.

Protein	K_{sv} (M ⁻¹)	
	Acrylamide (close symbols)	KI (open symbols)
Wild Type	11.2 ± 0.2	5.0 ± 0.2
W155R	4.5 ± 0.1	3.2 ± 0.1
I154F	10.5 ± 0.2	5.9 ± 0.2

Table 4 – Parameters for urea denaturation of wild type and mutant forms (I154F and W155R). Comparing the differences in stability.

Protein	ΔG_{H_2O} (kJ.mol ⁻¹)	m (J.mol ⁻¹ .M. ⁻¹)	$[Urea]_{1/2}$ (M)	$\Delta[Urea]_{1/2}$ ^a	$\Delta(\Delta G)$ ^b (J.mol ⁻¹)	T_m (°C)	ΔT_m (°C)
Wild type	24.3 ± 1.3	5665 ± 264	4.3			66.3 ± 0.1	-
W155R	21.4 ± 0.8	6209 ± 327	3.4	0.9	-7172	61.4 ± 0.4	- 4.9
I154F	24.7 ± 0.8	7687 ± 281	3.2	-1.1	-5866	50.7 ± 0.1	-15.6

^a Difference between the $[urea]_{1/2}$ for the wild type and the mutant forms.

^b $\Delta(\Delta G)$ = From $\Delta[urea]_{1/2}$ x average of the three m values [18].

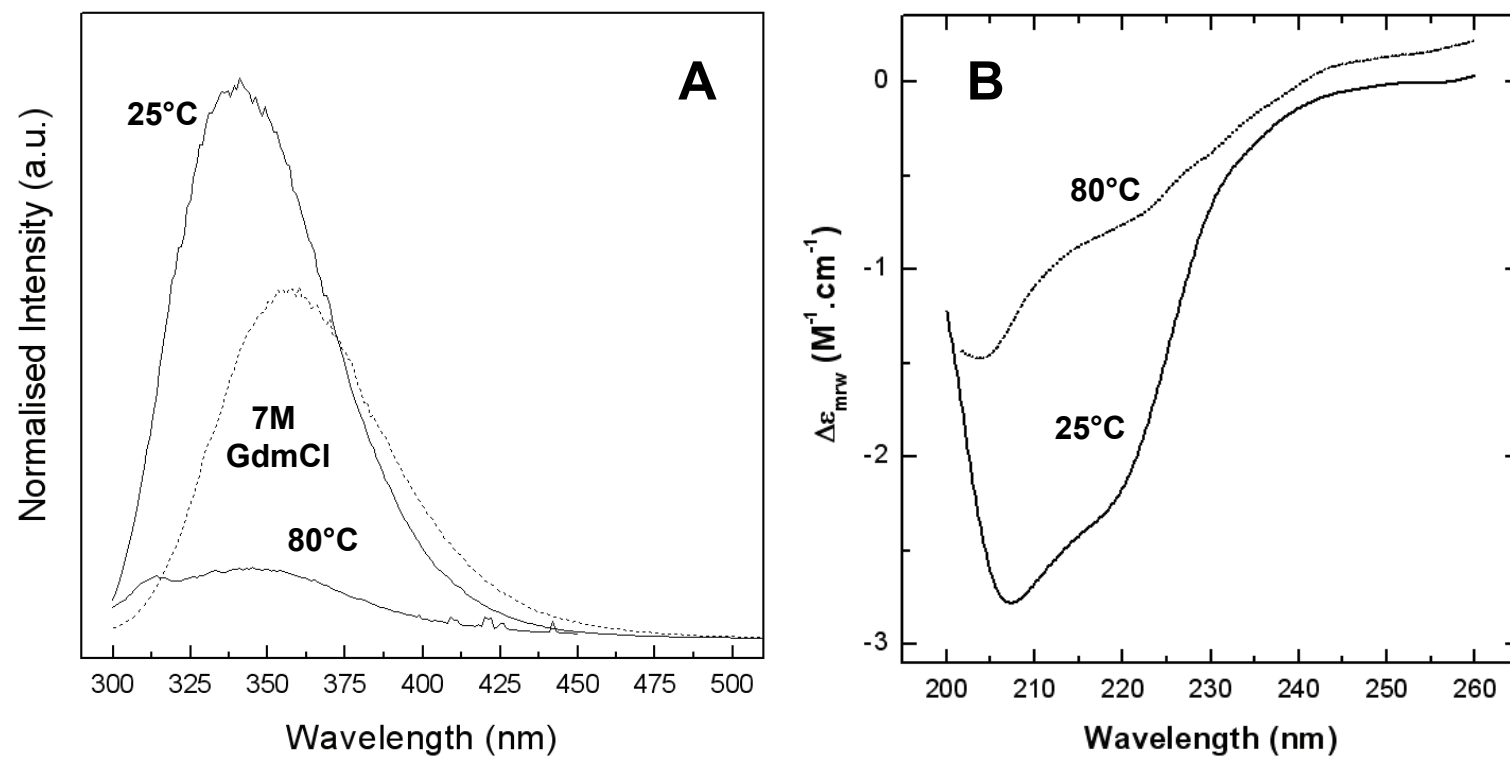


Figure 1
Correia et al (2006)

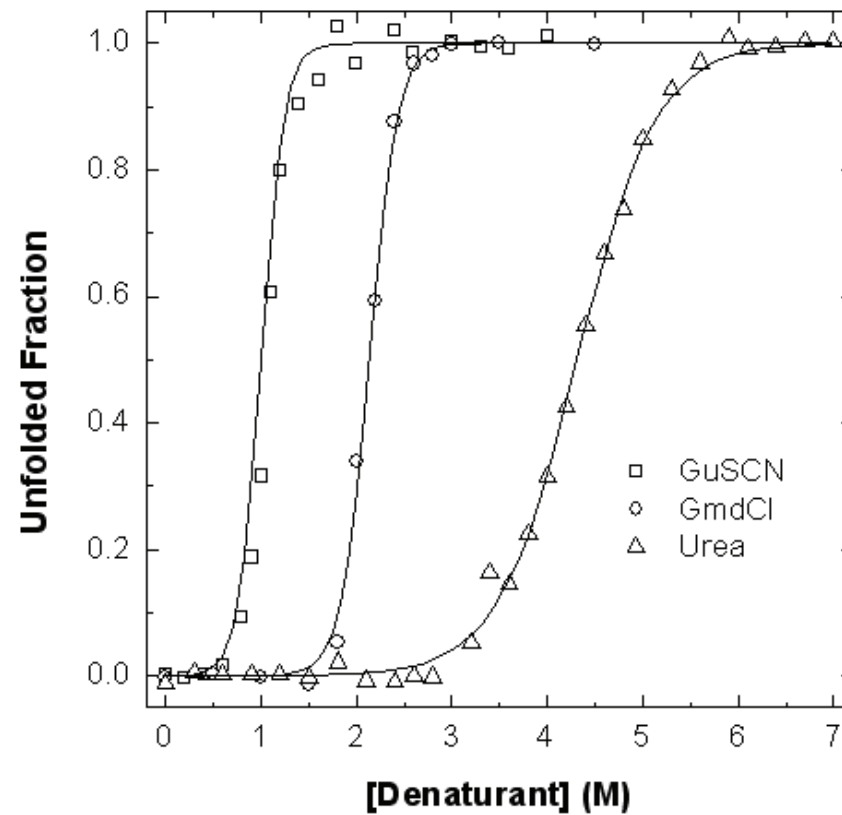


Figure 2
Correia et al (2006)

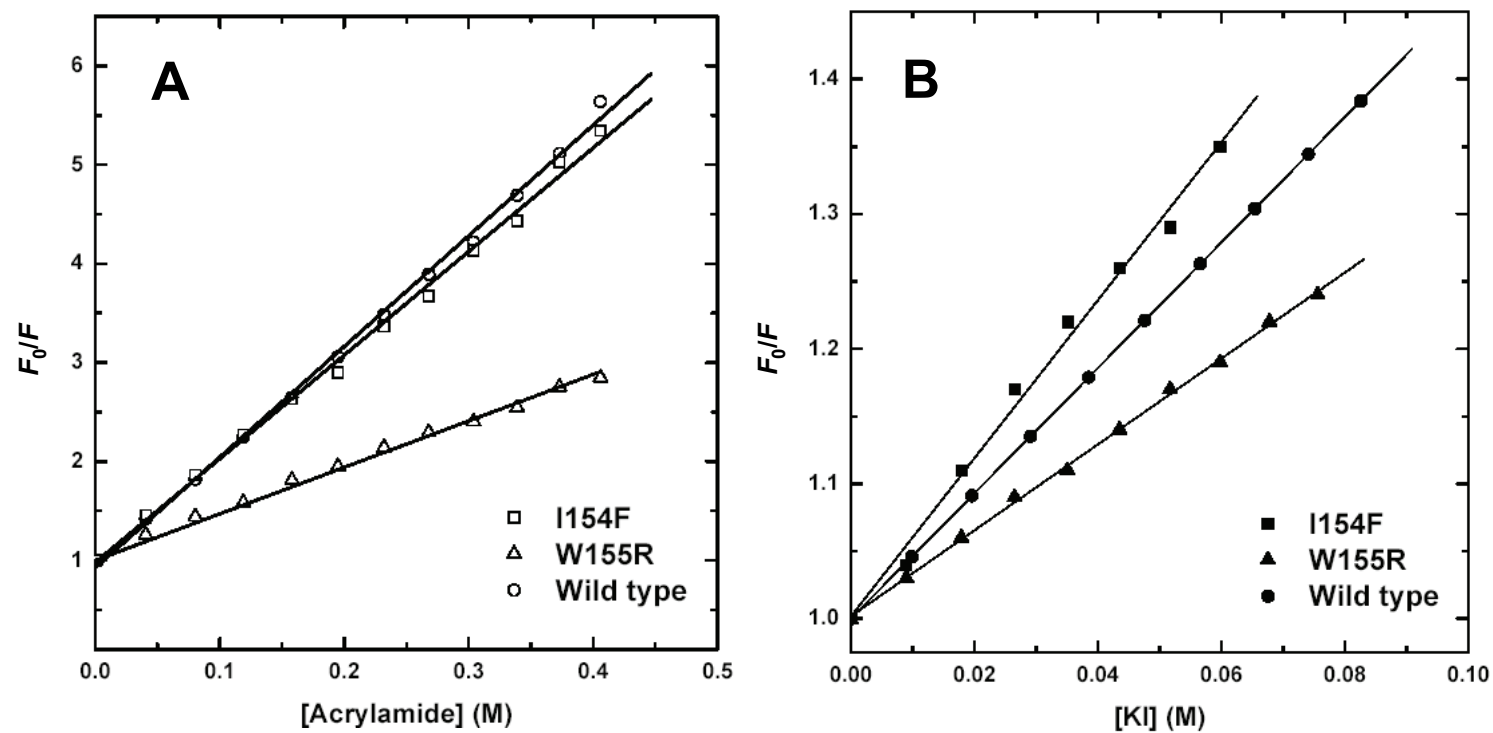


Figure 3
Correia et al (2006)

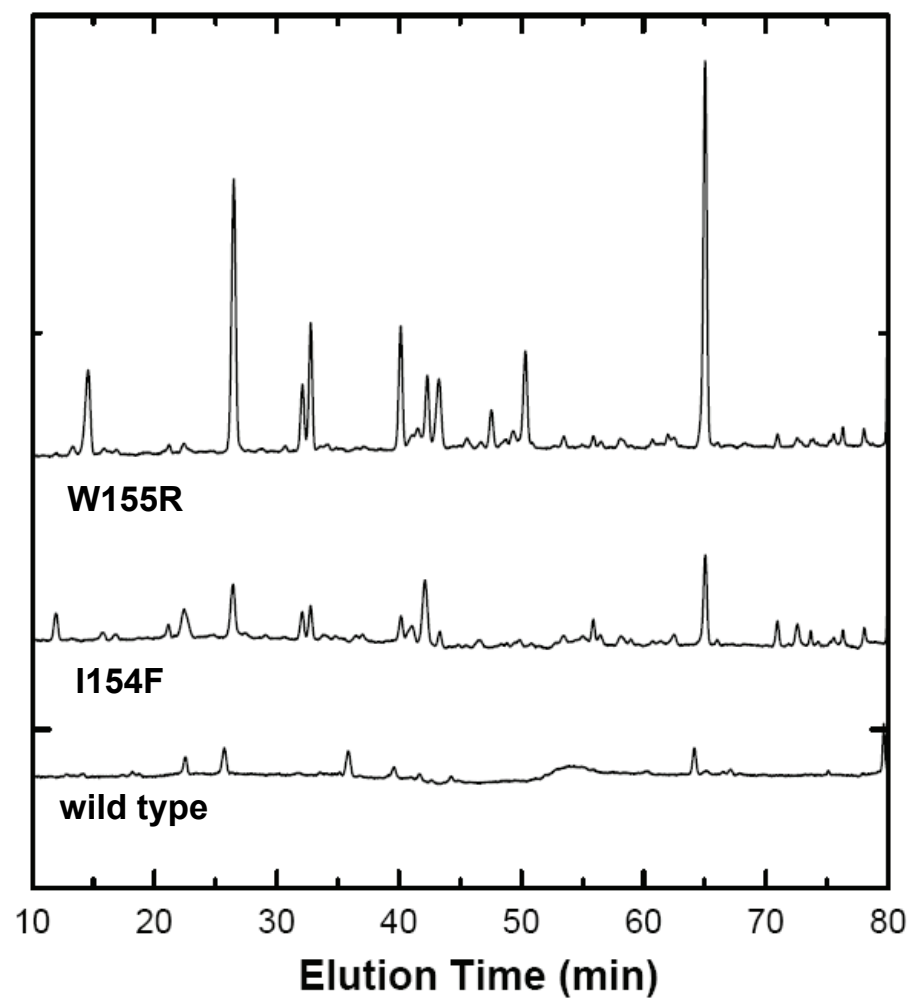


Figure 4
Correia et al (2006)

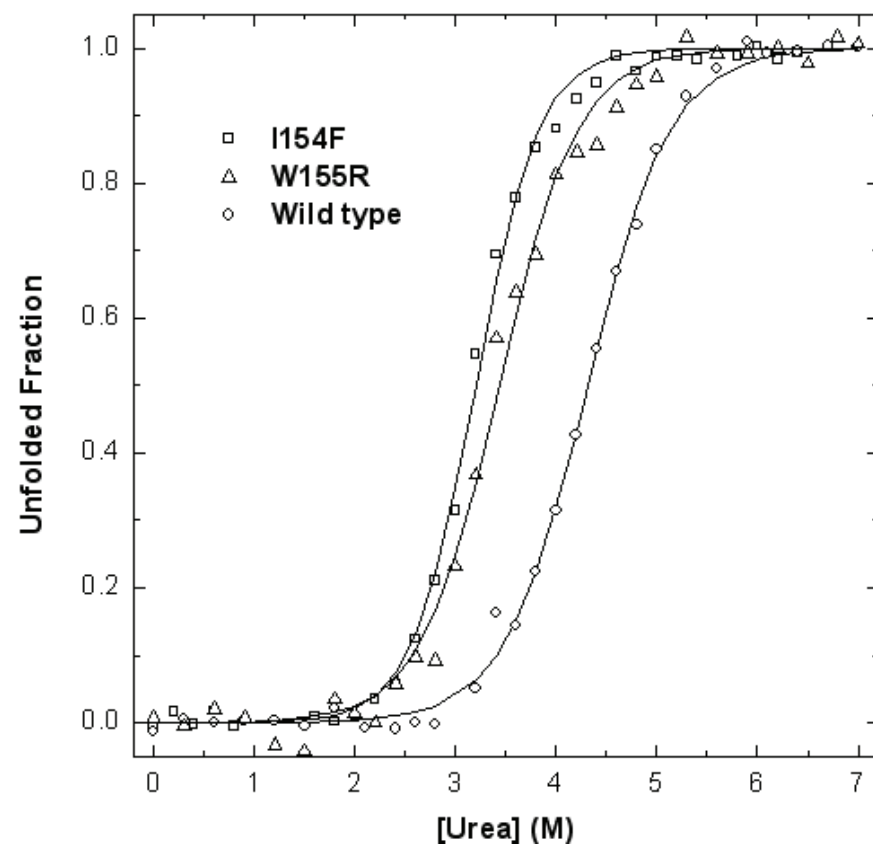


Figure 5
Correia et al (2006)

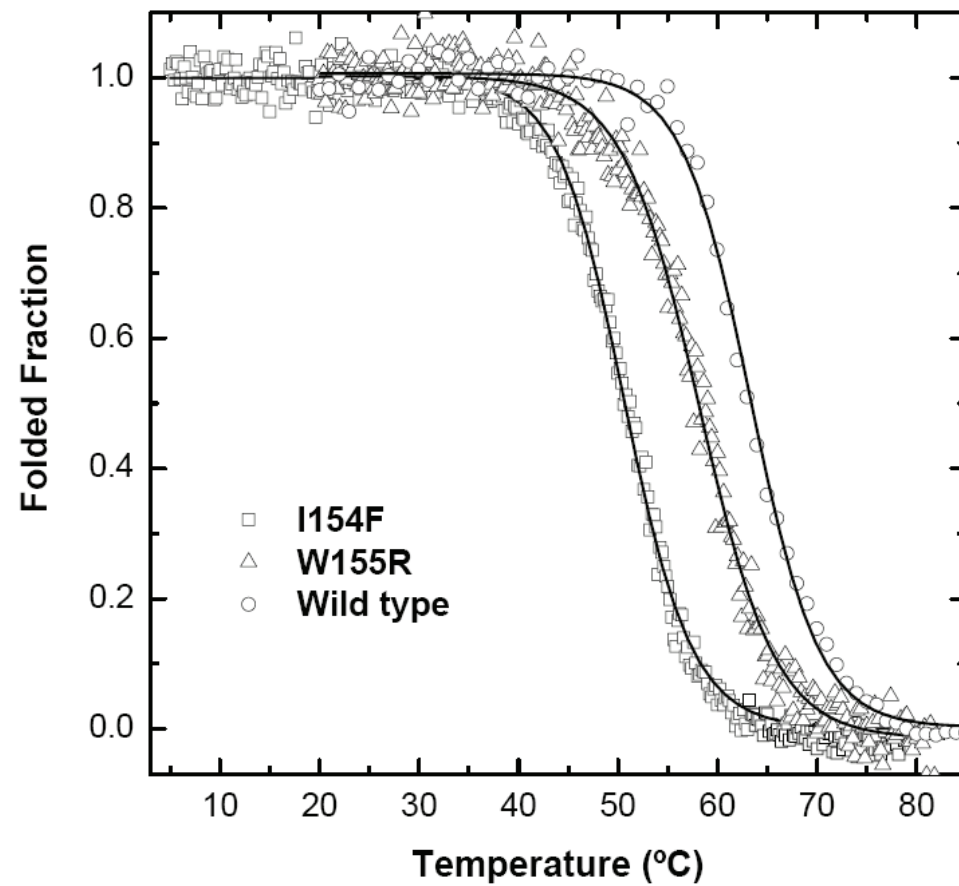


Figure 6
Correia et al (2006)

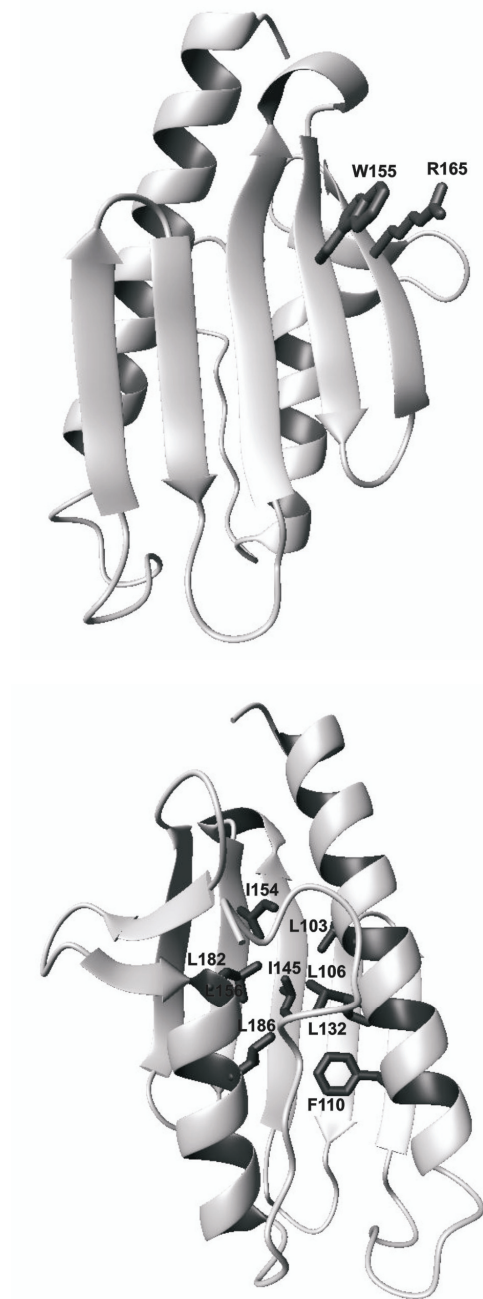


Figure 7
Correia et al (2006)