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Backbone resonance assignments and secondary structure of the apo-*Drosophila melanogaster* frataxin homolog (Dfh)

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Abstract

Friedreich's ataxia, the most prevalent hereditary ataxia, is caused by a patient's inability to produce a viable form of the protein frataxin. Frataxin plays an essential role in cellular iron regulation and has been shown to participate in the assembly of iron-sulfur (Fe-S) clusters under a variety of roles, including modulating persulfide production and directing Fe(II) delivery to the assembly scaffold protein. While the activity and structure of multiple eukaryotic frataxin orthologs have been characterized, the fly ortholog has numerous advantages over other orthologs with regards to protein stability, its activity towards Fe-S cluster assembly and its stability for forming stable proteins partner assemblies. Given the obvious advantages for studying the *D. melanogaster* frataxin homolog (Dfh) over its orthologs, we have undertaken a structural characterization of apo-Dfh as the first step towards solving the solution structure of the protein alone and in complex with protein partners within the Fe-S cluster assembly pathway.

Keywords

Friedreich's Ataxia; NMR assignments; Frataxin; Iron Sulfur Cluster; Isu1; IscU; Fe-S Scaffold Protein

Biological context

Friedreich's ataxia (FRDA), a cardio- and neurodegenerative disorder affecting 1 in 30,000 humans, is caused by an inability to produce sufficient levels of a single mitochondrial protein "frataxin" (Campuzano et al. 1996; Pandolfo 1998). Frataxin deficiency in FRDA patients typically results from an atypical GAA trinucleotide intronic expansion in the protein's gene that disrupts transcription, although a subset of patients exhibit frataxin single point mutations (Cossee et al. 1997). Phenotypes of the disorder include deficiency in Fe-S cluster assembly and mitochondrial iron overload, both leading to cell and organism degradation (Foury and Cazzalini 1997; Rotig et al. 1997). Although several roles have been proposed for frataxin's during Fe-S cluster assembly, a specific function(s) has yet to be

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conclusively established; however, interactions between frataxin and several protein partners within the assembly pathway are essential for the successful completion of Fe-S cluster assembly, which occurs within the mitochondria of eukaryotes (Braymer and Lill 2017).

Biochemical and structural studies performed on several frataxin orthologs provide insight into molecular factors that drive frataxin's participation within the Fe-S assembly pathway. Frataxin, originally shown to be an iron binding protein, has ferrous ion affinities within the μM range suggesting the eukaryotic protein is likely iron loaded when present in the high iron concentration environment of the mitochondrial matrix, the location where the mature protein is targeted for placement (Adamec et al. 2000; Yoon and Cowan 2003; Bou-Abdallah et al. 2004; He et al. 2004; Cook et al. 2006). Frataxin's ability to direct Fe delivery to the human cluster assembly scaffold "ISCU" during assembly (Cai et al. 2018), the dependence on Fe for forming a complex between frataxin and the yeast scaffold protein "Isu1" (Cook et al. 2010), and frataxin's ability to regulate the activity of the eukaryotic cysteine desulfurase enzyme that provides activated sulfur for Fe-S assembly (Adinolfi et al. 2009; Tsai and Barondeau 2010), all highlight distinct roles for regions on frataxin's exposed surface likely to direct how the protein interacts with its biomolecular partners. Our laboratory solved the solution structure of the yeast frataxin homologue 1 protein "Yfh1" and completed chemical shift assignments for an intermediate form of the human frataxin ortholog "*HsFtx*"; these data were pivotal for allowing us to clarify frataxin's iron binding site(s) and for mapping the intermolecular surface on frataxin that interacts with the scaffold protein partner (He et al. 2004; Cook et al. 2010; Kondapalli et al. 2010). However, instability in the human frataxin (towards autodegradation), in the fold stability for yeast frataxin and differences seen in structures between the prokaryotic and eukaryotic orthologs have hindered the rate of characterization of this protein or the understanding of the protein in a physiological context. Searching for a more stable frataxin ortholog, our laboratory serendipitously determined that frataxin from *Drosophila melanogaster* (Dfh) has a higher stability than the yeast and human orthologs, making it a perfect protein to pursue additional structural characterization studies for the eukaryotic protein partner system (Kondapalli et al. 2008).

Dfh shares a high degree of sequence homology and the same projected fold with other frataxin orthologs (Bencze et al. 2006; Stemmler et al. 2010). A direct role in controlling cellular oxidative stress has been shown for Dfh, with activity towards aconitase repair and stability also noted in this report (Kondapalli et al. 2008). Our laboratory has shown biochemically that Dfh is highly stable against degradation, under anaerobic conditions it exists as a stable iron-loaded protein monomer, Fe(II)-loaded Dfh binds to Isu1 with sub μM binding affinity, and ferrous ions bind to Dfh in a pseudo-symmetric 6-coordinate structure constructed exclusively by oxygen and nitrogen based ligands. In addition, we showed Dfh prevents reactive oxygen species-induced oxidative damage to DNA in the presence of both Fe (II) and H_2O_2 . Finally, we showed Dfh mediates delivery of Fe(II) to Isu1 promoting Fe-S cluster assembly *in vitro* when combined with the yeast assembly machinery (Dzul et al. 2017). The biochemical importance of Dfh during Fe-S assembly has been well explored, however a lack of structural details for the protein prevents an understanding of how the molecule's structure dictates its function as a protein partner and directly during Fe-S assembly.

Here we present the assignment of the backbone ^1H , ^{15}N and ^{13}C NMR resonance assignments for apo-Dfh protein residues 1 – 133 (gene construct residues 59–190, including an N-terminal Met), along with chemical shift based secondary structural and random coil index order parameters determined for the protein. These data serve as the foundation for solving the protein's 3-dimensional structure and will also serve as a reference when determining what amino acids serve at the iron and protein binding interfaces on the molecule through future chemical shift perturbation studies.

Methods and experiments

Labeling, expression, and purification of *Drosophila* Frataxin

Uniformly ^{15}N -labeled and $^{13}\text{C}/^{15}\text{N}$ doubly labelled Dfh, spanning residues 1 – 133, were produced in bacteria grown in M9 minimal media with $^{15}\text{NH}_4\text{Cl}$ (1g/L) and $^{13}\text{C}_6$ D-glucose (4g/L) as the sole nitrogen and carbon sources, respectively. The construct was expressed in the *E. coli* BL21 (DE3) strain transformed with a modified pET 101/D-TOPO vector DNA. For protein expression, *E. coli* competent cells were grown at 37° C, Dfh expression was induced at an OD₆₀₀ of 0.4 with 1 mM IPTG for 5 hours, cells were lysed using a French press and sonicated to liberate protein and disrupt DNA. Protein at ca 99% purity (as judged by gel analysis) expressed at the mass of 50 mg/L in cell media. Two ammonium sulfate precipitation steps (40% using the supernatant, 65% using the precipitate) were performed and samples centrifuged to obtain a partially purified Dfh pellet. This pellet was dialyzed twice against a 1:200 volume dilution of Buffer A (25 mM Tris-Cl, 10 mM EDTA, 5 mM β -mercaptoethanol, pH = 8) over 6 hours. Following dialysis, protein was subjected to anion exchange chromatography using a Q-Sepharose column (Pharmacia) equilibrated in Buffer A, and eluted using a sodium chloride salt gradient (also in Buffer A, but with salt added) up to a final salt concentration of 1 M. Dfh eluted at a sodium chloride concentration of 500 mM. Following 2 additional dialysis steps with Buffer A, the protein was subjected to a Phenyl-Sepharose column (Pharmacia) run using Buffer A and a reverse 1M ammonium sulfate gradient. Dfh eluted at 800 mM ammonium sulfate. Following an additional dialysis step, the final purification stage utilized a Sephacryl 75 size-exclusion column equilibrated with 20 mM HEPES, 150 mM NaCl at pH=7.5. Freshly isolated protein was degassed on a Schlenk line and stored under positive argon pressure at 4°C until required.

Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra used for resonance assignments were acquired at 293 K on the Varian INOVA 720 MHz spectrometer located at the National High Field Magnet Laboratory (Tallahassee, FL), with subsequent identical data collected using an in-house Varian INOVA 600 MHz spectrometer. The 720 MHz spectrometer was equipped with a triple resonance gradient room temperature probe while the 600 MHz spectrometer utilized a Varian triple resonance gradient cold probe. Frataxin samples utilized for backbone assignments were prepared at 1.2 mM concentration in NMR buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 90% H₂O and 10% D₂O). Backbone assignments were accomplished using the following experiments: ^{15}N -HSQC, HNCA, HN(CO)CA, HNCO, HN(CA)CO, HNCACB and CBCA(CO)NH (Cavanagh 1996). Collection parameters for these experiments are outlined in Table 1. When combined, these data allowed for the sequential assignments of Dfh backbone resonances.

All spectra were transformed and analysed according to established lab protocols (He et al. 2004) using the processing programs NMRPipe (Delaglio et al. 1995) and NMRView (Johnson 2004). Chemical Shift Indices (CSI) analysis was performed within NMRView using backbone residue assignments utilizing C α , C β , C O and N H chemical shifts.

Assignments and data deposition

Residue assignments for mature Dfh are provided in the high-resolution ^{15}N -HSQC spectrum presented in Figure 1. Full backbone assignments were achieved for 128 out of the 133 residues in the protein; we were unable to completely assign the 5 proline residues or the 3 tryptophan $\epsilon 1$ resonances. The complete amino acid sequence for full-length Dfh can be found at <http://www.uniprot.org/uniprot/Q9W385>. Chemical shift assignments for Dfh backbone atoms were deposited in the Biological Magnetic Resonance Bank (<http://www.bmrb.wisc.edu/>) under the accession code BMRB - 17135.

Reasonable dispersion for amide resonances for Dfh, displayed in the ^{15}N HSQC spectrum (Figure 1), confirm the majority of residues in the protein are likely folded. The narrow amide proton line widths (average half-height widths of 7 Hz) indicate the protein likely exists as a monomer in solution. Prediction of secondary structural elements for Dfh in solution, performed using CSI analysis within NMRView (Figure 2), indicate the protein has α -helical folds predominately at the N- and C-termini, with β -strands located in between, in a manner characteristic of the α – β sandwich motif 3D structure seen for other frataxin orthologs. The suggested secondary structure for Dfh appears to lack the N-terminal 3_{10} helix observed in the yeast frataxin solution structure (He et al. 2004), however the predicted Dfh structure contains a β -strand covering residues (10 – 12). The two α -helices seen in the predicted structure occurs between residues 13–38 (Helix-1) and 108–120 (Helix-2). Six additional β -strands, likely forming the β -sheet plane of the protein, are seen between residues 46–48 (Strand-1), 53–57 (Strand-2), 72–74 (Strand-3), 77–79 (Strand-4), 88–92 (Strand-5) and 96–100 (Strand-6).

Backbone assignments of Dfh provide the controls for future proposed experiments directed at monitoring structural changes coupled with Fe(II) and/or partner protein binding. Using this predicted secondary structure model, we found the majority of the iron binding residues observed for iron-loaded yeast frataxin (He et al. 2004), corresponding to Dfh residues Ala 26, Asp 29, Glu 36, Asn 37, Ala 38, Asp 45, Ala 47, Tyr 48, and Ser 49, lie within or border the helix-1 and strand-1 elements of the protein, and this places potential Dfh binding residues in the same proximity as those seen for Yfh1. In addition, conserved residues Leu 105 to Glu 112 lie close to and within the helix-2 region as shown in other orthologs. These results may provide significant insight in protein elements utilized for Fe delivery by Frataxin when in complex with the scaffold protein.

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Figure 1.

Assigned ^1H , ^{15}N HSQC spectrum of ^{15}N labeled Dfh. Data was collected on the National Magnetic Resonance Facility (NMRFAM) at Madison on a Bruker 900 MHz spectrometer at 293 K. Dashed lines connect peaks from N/Q sidechain NH_2 groups. Peaks in yellow are folded. Three unassigned Trp(ϵ_1) resonances exist in spectrum.

Figure 2.

Consensus secondary structure prediction, assembled from combined analysis of the C_{α} , C_{β} , and N_H chemical shifts using CSI analysis within the program NMRView. Single letter residue designations with residue number are provided (top). Predicted secondary structure displayed for helical residues in red, unfolded residues in grey, and β -strand residues in blue (middle); lines missing a dot were undecided. Consensus secondary element shown in grey (bottom).

Table 1.

Experimental details for 2D and 3D NMR spectra collected at 720 MHz and used for backbone assignments.

Experiment	# scans	Spectral window (ppm) $^1\text{H} \times ^{13}\text{C} \times ^{15}\text{N}$	Complex points $^1\text{H} \times ^{13}\text{C} \times ^{15}\text{N}$	Offset (ppm) $^1\text{H} \times ^{13}\text{C} \times ^{15}\text{N}$
^{15}N -HSQC	32	12.5×41.1	2048×128	4.73, 119.0
HNCA	16	$12.5 \times 27.0 \times 41.1$	$2048 \times 64 \times 32$	4.73, 56.0, 119.0
HN(CO)CA	16	$12.5 \times 27.0 \times 41.1$	$2048 \times 64 \times 32$	4.73, 56.0, 119.0
HNCO	8	$12.5 \times 16.0 \times 41.1$	$2048 \times 64 \times 40$	4.73, 176.0, 119.0
HN(CA)CO	8	$12.5 \times 16.0 \times 41.1$	$2048 \times 64 \times 40$	4.73, 176.0, 119.0
HNCACB	16	$12.5 \times 60.0 \times 41.1$	$2048 \times 128 \times 64$	4.73, 46.0, 119.0
CBCAC(CO)NH	16	$12.5 \times 60.0 \times 41.1$	$2048 \times 128 \times 64$	4.73, 46.0, 119.0