Published in final edited form as:

Biochemistry. 2006 June 27; 45(25): 7767–7777.

Monomeric Yeast Frataxin is an Iron Binding Protein†

Jeremy D. Cook[‡], Krisztina Z. Bencze[‡], Ana D. Jankovic[§], Anna K. Crater[‡], Courtney N. Busch[‡], Patrick B. Bradley[‡], Ann J. Stemmler[‡], Mark R. Spaller[§], and Timothy L. Stemmler^{‡,*}

Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, Detroit, MI, 48201, Department of Chemistry, Wayne State University, Detroit, MI, 48202

Abstract

Friedreich's ataxia, an autosomal cardio- and neurodegenerative disorder that affects 1 in 50,000 humans, is caused by decreased levels of the protein frataxin. Although nuclear encoded, frataxin is targeted to the mitochondrial matrix and necessary for proper regulation of cellular iron homeostasis. Frataxin is required for the cellular production of both heme and iron-sulfur clusters. Monomeric frataxin binds with high affinity to ferrochelatase, the enzyme involved in iron insertion into porphyrin during heme production. Monomeric frataxin also binds to Isu, the scaffold protein required for assembly of Fe-S cluster intermediates. These processes (heme and Fe-S cluster assembly) share requirements for iron, suggesting monomeric frataxin might function as the common iron donor.

In order to provide a molecular basis to better understand frataxin's function, we have characterized the binding properties and metal site structure of ferrous iron bound to monomeric yeast frataxin. Yeast frataxin is stable as an iron loaded monomer and the protein can bind 2 ferrous iron atoms with micromolar binding affinity. Frataxin amino acids affected by the presence of iron are localized within conserved acidic patches located on the surfaces of both helix-1 and strand-1. Under anaerobic conditions, bound metal is stable in the high-spin ferrous state. The metal-ligand coordination geometry of both metal binding sites is consistent with a 6 coordinate iron-(oxygen and nitrogen) based ligand geometry, surely constructed in part from carboxylate and possibly imidazole side chains coming from residues within these conserved acidic patches on the protein. Based on our results, we have developed a model for how we believe yeast frataxin interacts with iron.

Keywords

Iron Chaperone; Frataxin; NMR; Iron; Structure; Heme Synthesis; Yfh1 and Iron-Sulfur Cluster Assembly

Friedreich's ataxia (FRDA) is an autosomal recessive cardio- and neurodegenerative disorder that affects approximately 1 in 50,000 people (1-3). The disorder, in the majority of FRDA

[†]This work was supported by the American Heart Association, Midwest Affiliate for T.L.S. (#0130527Z) and by the National Institutes of Health for T.L.S. (DK068139).

* To whom correspondence should be sent: Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201. Telephone: 313-577-5712, Fax: 313-577-2765, E-mail: tstemmler@med.wayne.edu.

[‡]Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201, U.S.A.

[§]Department of Chemistry, Wayne State University, 5101 Cass Ave., Detroit, MI 48202, U.S.A.

Briefs: We present a characterization of monomeric yeast frataxin's iron binding ability. Various spectroscopic techniques were applied to help characterize the iron binding affinity of yeast frataxin, the oligomeric state of the protein, specific amino acids affected by the presence of iron and finally the metal-ligand coordination geometry.

patients, is a direct result of a trinucleotide repeat expansion in the first intron of the gene for the protein frataxin; this expansion disrupts gene transcription leading to a frataxin deficiency in humans (4). The inability to produce frataxin is associated with mitochondrial accumulation of iron that is biologically unavailable. In humans, the breakdown in normal iron regulation pathways resulting from frataxin deficiency is exemplified physiologically by the degeneration of sensory neurons in the dorsal root ganglia, hypertrophic cardiomyopathy and diabetes mellitus (5). In yeast, reintroduction of frataxin into knockout cells results in restored bioavailability of the accumulated metal (6). These data indicate frataxin is required for retaining bioavailable iron within the mitochondria.

While frataxin's exact function regarding mitochondrial iron homeostasis is unclear, numerous reports have suggested the protein participates in various facets of iron metabolism. Frataxin was initially suggested to act as a facilitator or regulator of mitochondrial iron export; thus, loss of function is associated with mitochondrial iron accumulation (6). Frataxin has been implicated in energy conversion and ATP synthesis (7) and in protecting aconitase $[4\text{Fe-4S}]^{2+}$ clusters against disassembly and inactivation (8,9). Frataxin has been shown to form spherical *in vitro* homooligomers under low salt and high iron concentrations, suggesting a possible iron storage capacity for the protein (10-13). Finally, frataxin has been shown to be involved in the *in vivo* production of both heme (14-16) and Fe-S clusters (17-21). Monomeric frataxin binds with nanomolar binding affinity to the ferrochelatase and ISU assembly apparatus enzymes responsible for respective heme and Fe-S clusters production (14,17,22, 23). Activity assays reported using *in vitro* protein show monomeric human frataxin stimulates production of heme and Fe-S clusters (22,23). Similar *in vitro* heme assembly activity was reported for yeast frataxin oligomers (24), however a recent report indicates yeast frataxin mutants with diminished assembly capability are also active for *in vitro* heme assembly (25). *In vivo* studies have also shown that yeast cells containing frataxin mutants that lack the self-assembly ability have normal phenotypes with regards to Fe-cofactor production (26). These data suggest the monomeric form of frataxin controls iron delivery during both heme and Fe-S cluster assembly.

The amino acid sequence and structure of frataxin orthologs are both well conserved and provide insight into where the protein may bind iron. Frataxin orthologs share a general sequence homology (ca. 60%) specifically with regards to conserved acidic residues (27). Structures of the yeast, human and bacterial orthologs show frataxin is a member of the α - β sandwich motif family, constructed predominately by two parallel α -helices on the helical plane and 5 anti-parallel β -strands on the β -sheet protein surface of the protein (28-32). The highly conserved aspartic and glutamic acid residues line the protein's exposed planar edge at the N-terminal α -helix (helix-1) and β -strand (strand-1) interface. NMR analyses indicate chemical environments of these conserved acidic residues are perturbed when iron is present (28,32). A subset of these conserved acidic residues are responsible for the iron-induced assembly and ferroxidase activities reported for *in vitro* frataxin (25-27). A common theme in iron binding metalloproteins is the use of carboxylate oxygen and imidazole nitrogen atoms, from Asp, Glu and His protein side chains, acting as direct ligands to bind metal (33-35). Given the high conservation of Asp and Glu residues in this protein region, the fact these residues are directly affected by the presence of iron and since specific residues in this region have been shown to be required for iron regulation it seems likely these acidic residues are directly utilized by frataxin during iron binding.

The goal of this report is to provide additional structural and biochemical detail to better understand how frataxin binds iron. Although our studies were performed solely on yeast frataxin (Yfh1), given the high sequence homology and structural similarities between protein family members, these results provide additional direct insight into general properties that dictate how frataxin orthologs bind iron. We made specific emphasis to characterize the iron

binding ability of the monomeric form of yeast frataxin due to the mounting evidence that it is the monomeric protein that interacts with protein partners during iron cofactor assembly. Reports showing oligomeric frataxin assists in controlling oxidative stress are also certainly convincing, indicating that frataxin aggregates may regulate reactive oxygen species availability and/or iron induced cellular damage under oxidative stressed conditions. While the iron loaded yeast frataxin trimer has been speculated as being the nucleating species in higher-order oligomer formation by the protein (12), we would argue the first step in oligomer assembly in fact involves the interaction between monomeric frataxin and iron. A direct structural understanding of how monomeric frataxin interacts with iron would therefore also provide insight into the protein's participation in early mechanistic steps during control of cellular iron bioavailability. Solution conditions shown to promote stable iron-loaded yeast frataxin monomers have been published and these conditions were utilized within this study (27). Thermodynamic analyses were used to measure the iron-binding affinity and metal to protein stoichiometry of yeast frataxin. Nuclear magnetic resonance (NMR) spectroscopic analysis was used to further identify amino acids affected by the presence of iron. Finally, X-ray absorption spectroscopic (XAS) studies were used to determine the oxidation and spin-state of iron bound to frataxin, as well as help provide structural detail regarding how monomeric frataxin binds iron.

MATERIALS AND METHODS

Protein Expression and Isolation

The mature full-length yeast frataxin coding sequence, representing residues 52–174 of the complete open reading frame with a Met substituting the Val at position 52, was subcloned into a pET11a bacterial expression vector (Novagen). The growth and isolation strategy for preparing homogeneous yeast frataxin was previously described in detail (28). Unlabeled and ^{15}N -labeled protein were expressed in *Escherichia coli* BL21-(DE3) cells grown at 37°C in Luria-Bertani media (unlabeled protein) or M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ (^{15}N labeled protein). Protein expression was induced with 1 mM IPTG and cells were grown post-induction for 4 hours prior to isolation. Cells were harvested by centrifugation, lysed with a French press, sonicated and finally spun at high speed (18,000 rpm) for 1 hour. Two ammonium sulfate precipitation steps were used to obtain a partially purified frataxin pellet. Solubilized protein was run on anion exchange and phenyl-sepharose columns, each with salt gradients, to obtain pure protein. Average yields amount to 70 and 30 mg/L of pure unlabeled and labeled protein, respectively. Newly isolated protein was stored under an oxygen-depleted argon atmosphere at 4°C in 20 mM HEPES (pH 7.0), 10 mM MgSO_4 . Apo-Yfh1 is susceptible to auto aggregation in a time dependent manner (data not shown) and under these solution conditions monomeric yeast frataxin was stable for only up to two weeks. As a result, all our experiments were performed on protein obtained within a two-week window after isolation.

Size Exclusion Chromatography/ICP-MS Analysis

Size exclusion chromatography and inductively coupled plasma-mass spectroscopy (ICP-MS) studies were used to confirm the solution conditions under which iron loaded yeast frataxin was stable as a monomer. Protein samples were prepared anaerobically in 20 mM HEPES buffer (pH=7.0) and 10 mM MgSO_4 . Iron loaded frataxin samples at 1:1 and 2:1 Fe(II):protein stoichiometries were prepared anaerobically by combining concentrated solutions of buffered protein and buffered ferrous ammonium sulfate (Sigma). All solutions were initially made anaerobic by repeated cycles of vacuum and argon(g) purging on a Schlenk line; samples were capped using septa on all glassware. Samples were mixed within a nitrogen(g) filled glove box (Plas Labs, Lansing, Michigan, USA) to form the iron-protein complexes. Samples were loaded, using an anaerobic syringe, onto a 1.2 meter long Sephacryl S-200 or a 2 meter long Sephacryl S-100 high-resolution size exclusion column, both attached to a BioRAD BioLOGIC

LP system. An argon purged buffer solution of 20 mM HEPES (pH=7.0) and 10 mM MgSO_4 was used, first to equilibrate and wash the column, and then as our running buffer. The column was run at room temperature. A protein control sample (BioRad) of buffered vitamin B-12 (1.3 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), gamma globulin (158 kDa) and thyroglobulin (670 kDa) was used to calibrate molecular size.

ICP-MS analysis was performed on a PE Sciex Elan 9000 spectrometer equipped with a cross-flow nebulizer and Scott type spray chamber. The RF power was held at 1000 W and argon gas flow was optimized at 0.9 L/min. The optimum lens voltage was centered on rhodium sensitivity as counts per second. Samples were prepared by treatment with nitric acid and heated at 70 °C for 1 hour to digest the protein. Final samples were diluted with iron free HPLC grade water. A calibration curve, obtained from diluted stock solutions of 1000 ppm ferrous standards in 2% HNO_3 (high purity acid, OmniTrace EM Science.), was used to correlate protein bound iron to known metal concentrations. All results are determined from three replicate experiments performed on at least two different but independent protein sample preparations.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) measurements were performed to measure the metal binding affinity and maximum metal to protein stoichiometry of monomeric yeast frataxin. Experiments were performed at 30 °C on a VP-ITC titration microcalorimeter (Micro Cal Inc.). Protein and aqueous ferrous ammonium sulfate solutions were prepared anaerobically in 20 mM HEPES (pH = 7.0) and 10 mM MgSO_4 . Experiments were designed to keep a ca. 20 fold excess of iron in the syringe and data were collected at three independent protein concentrations (100 μM , 70 μM and 50 μM) corresponding to aqueous iron solutions of 2.5 mM, 1.4 mM and 0.8 mM, respectively. Protein samples were loaded anaerobically into the sample chamber and after an initial 2 μL iron solution injection, 29 additional injections of 10 μL each were titrated into the protein sample. Spacing between injections was 10 minutes and the stirring speed of the syringe was held constant at 500 rpm. All experiments were repeated in triplicate on independent protein and iron samples to ensure data reproducibility. The Origin 5.0 software package supplied by Micro Cal Inc. was used for data analysis. Origin 5.0 uses non-linear least squares curve fitting algorithm to determine the stoichiometric ratio, dissociation constant and change in enthalpy of the reaction.

NMR Iron Titration

NMR amide chemical shift perturbation experiments were used to identify amino acids affected by the presence of iron under solution conditions that stabilize the monomeric iron loaded frataxin. Spectra were collected on a Varian INOVA 600 MHz spectrometer equipped with a triple resonance $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ Varian cold probe with z-axis pulsed field gradients. Aqueous anaerobic concentrated ferrous ammonium sulfate (Sigma) was titrated into an anaerobic solution of ^{15}N labeled yeast frataxin (protein concentration of ca. 1 mM) to achieve Fe(II) to protein stoichiometric ratios of 1.00 and 2.00. Samples were prepared in 20 mM HEPES buffer (pH 7.0) and 10 mM MgSO_4 buffer at room temperature under an Ar(g) atmosphere. Samples were anaerobically transferred into septa sealed 5 mm NMR tubes using air-tight transfer syringes. Protein samples were placed in the NMR spectrometer and allowed to equilibrate at 30°C for 30 minutes. Full $^1\text{H}/^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) spectra were collected at each titration point with a ^1H sweep width of 9000.9 Hz, with 2048 points and 32 transients and at a ^{15}N sweep width of 2500 Hz, 512 increments (36). Full independent spectra were collected on at least 2 samples to ensure spectral reproducibility. Chemical shifts were referenced using an external DSSH control sample (37). Data were transformed using NMRPipe (38) and amide chemical shifts were identified/measured using the program SPARKY (39). Protein amide resonances that were significantly line broadened by the presence of ferrous iron were identified first. Amide proton and nitrogen chemical shifts were

measured for the remaining residues. Amide chemical shifts changes were calculated and normalized to obtain a chemical shift change (δ) value for each spectral pair using the equation $\delta = 25((\delta_{\text{HN}})^2 + (\delta_{\text{N}}/5)^2)^{0.5}$ (40).

XAS Sample Preparation and Analysis

X-ray absorption spectroscopy was used to study the electronic properties and ligand coordination geometry of iron bound to frataxin. All samples were prepared anaerobically within a glove box using protein and iron solutions that were initially prepared on a Schlenk line and then stored under an Ar(g) atmosphere. XAS samples were prepared at 1 mM protein concentrations in 20 mM HEPES buffer (pH 7.0) and 10 mM MgSO₄ and mixed with buffered anaerobic ferrous aqueous iron in the same manner outlined above. Samples were concentrated to 2 mM iron concentrations using centricon 10 kDa cutoff membranes and diluted to 30% glycerol as a glassing agent. Samples were loaded into Lucite cells wrapped with Kapton tape, flash frozen in liquid nitrogen, removed from the glove box and stored in liquid nitrogen until used. Solid model samples of Fe(NH₄)₂(SO₄)₂•(H₂O)₆, FeS, Fe₂(SO₄)₃, Fe₂O₃, FeCl₃ (all from Sigma) and Fe(imidazole)₆ (prepared in our laboratory (41)) were used as controls for calibrating our protein XAS data analysis. Model samples were diluted with boron nitride in a glove box, loaded directly in Kapton tape wrapped aluminum transmission cells and flash frozen in liquid nitrogen upon removal from the glove box. At least two independent samples were made for each protein and model sample to ensure data reproducibility.

XAS data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL), beamlines 7–3 and 10–2, and at the National Synchrotron Light Source (NSLS), beamline X9-b. Both SSRL beamlines were equipped with Si(220) double crystal monochromators detuned 50% for harmonic rejection. NSLS beamline X9-b was equipped with a Si(111) single crystal monochromator equipped with a harmonic rejection mirror. Samples were maintained at 10 K using Oxford Instruments continuous-flow liquid helium cryostats at SSRL and at ca. 24 K using a He Displex Cryostat at NSLS. Protein fluorescence excitation spectra were collected using 30-element Ge solid-state array detectors at SSRL and a 13-element Ge solid-state detector at NSLS. Transmission data were recorded on model compounds using ionization detectors filled with nitrogen gas, placed before and after the cryostat. XAS spectra were measured using 5 eV steps in the pre-edge regions (6900–7094), 0.25 eV steps in the edge regions (7095–7135 eV) and 0.05 Å⁻¹ increments in the extended X-ray absorption fine structure (EXAFS) region (to $k = 13.5 \text{ Å}^{-1}$), integrating from 1s to 20s in a k^3 weighted manner for a total scan length of approximately 40 min. X-ray energies were calibrated by collecting an iron foil absorption spectrum simultaneously with collection of the protein and model data. The first inflection point for the Fe foil edge was assigned at 7111.3 eV. Each fluorescence channel of each scan was examined for spectral anomalies prior to averaging and spectra were closely monitored for photoreduction. SSRL protein data represent the average of 5 to 6 scans, while NSLS protein data represent the average of 8 to 9 scans.

XAS data were processed using the Macintosh OS X version of the EXAFSPAK program suite integrated with the Feff v7.2 software for theoretical model generation (42,43). XAS data reduction utilized a Gaussian function in the pre-edge region and a three-region cubic spline in the EXAFS region. EXAFS data were converted to k -space using an E_0 value of 7130 eV. The k -cubed weighted EXAFS was truncated at 1.0 and 12.85 Å⁻¹ for filtering purposes and Fourier transformed. Data were Fourier-filtered to isolate the EXAFS for each peak in the Fourier transform and then fit. The final fitting results listed in Table 1 are from analysis of the raw unfiltered data.

Analysis of the XANES 1s→3d transitions was completed using the EDG_FIT subroutine within EXAFSPAK (43). Only spectra collected using the higher resolution [Si220] monochromator crystals were subjected to edge analysis. Pre- and post-transitions splines were

fit to the spectra within the energy range of 7090 to 7100 eV and 7120 to 7125 eV, respectively. The extrapolated line was then subtracted from raw data to obtain baseline corrected spectra. The pre-edge features were modeled using pseudo-Voigt line shapes (simple sums of Lorentzian and Gaussian functions), and the energy position, the full width at half-maximum (FWHM) and the peak heights for each transition were varied. A fixed 50:50 ratio of Lorentzian to Gaussian functions successfully reproduced the spectral features of the pre-edge transitions. Global peak transition areas were determined for all spectra over the energy range of 7109 to 7117 eV using the program Kaleidagraph. Pre-edge intensity values were multiplied by 100 to match published procedure and area values shown for our models are nearly identical to published values (44).

EXAFS data fitting analysis performed on both Fourier filtered and raw/unfiltered data gave equivalent structural results. Model and protein EXAFS data were fit using both single and multiple scattering amplitude and phase functions calculated using Feff v7.2. Single scattering Feff v7.2 models were calculated for carbon, oxygen, sulfur and iron coordination to simulate possible iron-ligand environments in our systems. Multiple scattering Feff v7.2 models were generated for iron-imidazole interactions utilizing crystallographic coordinates from characterized model compounds. Fits to the crystallographically characterized model compounds were used to calibrate the scale factor and ΔE_0 values which we used in our protein fits. A scale factor of 0.95 and a threshold shift (ΔE_0) -11.5 eV were used during our protein data analysis. The S_0 and E_0 were not allowed to vary as they were calibrated from the Fe(II) model compounds matching the oxidation state of the ferrous iron we found in our protein samples. When simulating empirical data, metal-ligand coordination numbers were fixed at half-integer values while only the absorber-scatterer bond length (R) and Debye-Waller factor (σ^2) were allowed to freely vary. The criteria for judging the best fit simulation, and for adding ligand environments to the fit, included: (A) the lowest mean square deviation between data and fit (F'), a value corrected for number of degrees of freedom in the fit, (B) individual shell bond distances must be outside the spectral resolution ($> 0.13 \text{ \AA}$), and finally (C) all Debye-Waller factors in the ligand system must have values less than $0.006 \text{ \AA}^2$ (35,45).

RESULTS

Iron Loaded Yeast Frataxin is a Stable Monomer

In order to characterize the *in vitro* iron binding properties of monomeric yeast frataxin, we employed solution conditions that stabilize monomeric iron-bound yeast frataxin (27). The presence of magnesium or calcium salts stabilize iron- loaded monomeric yeast frataxin and the absence of oxygen helps to stabilize iron bound to frataxin in the ferrous oxidation state. We prepared frataxin samples anaerobically with 0, 1 and 2 iron bound per monomer in 20 mM HEPES buffer (pH=7.0) in the presence of 10 mM MgSO_4 . Samples were subjected to size exclusion chromatography to ensure protein with the bound iron was stable as a monomer.

Under these solution conditions, apo-yeast frataxin elutes from the sizing column as a single band, consistent with apo-protein existing as a stable monomer (Figure 1A-III). Full-length mature apo-yeast frataxin (123 amino acids) consistently runs at ca. 17 kDa on sizing columns. Protein samples were characterized by mass spectrometry to ensure no degradation was taking place following extensive exposure to iron. Surprisingly, *in vitro* isolated apo-protein was susceptible to homooligomerization in progressively increasing amounts, in a buffer and iron independent fashion, after about 2 weeks post isolation (data not shown). As a result, all experiments were performed immediately (or up to 2 weeks) after the protein was isolated. Protein was stored anaerobically at 4°C when not in use and discarded after 2 weeks. The addition of ferrous iron at either a 1:1 or 2:1 metal to protein stoichiometric ratio had no effect on the oligomeric state of the protein under these solution conditions (Figure 1A-II and 1A-I, respectively). In each case, the protein elutes from the column at the same position as

monomeric apo-frataxin band in the chromatograph. ICP-MS analysis coupled to a Bradford assay was used to confirm that metal is still bound to the protein following separation (Figure 1B).

Yeast Frataxin Binds Two Iron Atoms at Micro-Molar Affinity

ITC was used to provide insight into monomeric yeast frataxin's iron binding affinity and the maximal number of ferrous iron atoms that the protein can bind. ITC measurements were performed anaerobically under solution conditions that stabilized the monomeric iron loaded protein. Changes in heat resulting from iron binding are displayed in $\mu\text{cal/sec}$ on the raw ITC data (Figure 2A). These data show a single strong exothermic reaction that follows exponential behavior in the early injection points. Heats at the end of the titration equal those obtained from Fe(II) alone, indicating metal binding is complete following the early titration points. Initially, data sets were treated using a model with one set of binding sites (Origin 5.0). The calculated stoichiometry from the fitted curve provided for an average of 1.4–1.6 iron atoms per monomeric frataxin. However, upon analyzing the diagram, it was apparent that complete saturation is achieved for a 2:1 Fe(II) to protein ratio. Considering also the nature of binding sites in yeast frataxin, an advanced curve fitting of two sets of binding sites was employed. The theoretical curve for this model resulted in a better fit to the original data represented in the metal binding isotherm (Figure 2B), and used to determine the protein's metal binding affinity. After curve fitting analysis, binding affinities for both sites was determined to be in the micromolar range ($K_{d1} = 3.0 \pm 0.4 \mu\text{M}$ and $K_{d2} = 2.0 \pm 0.2 \mu\text{M}$). These results indicate the two metal binding sites are independent, although they have nearly identical binding affinities.

Mapping the Iron-Binding Surface of Yeast Frataxin

NMR spectroscopy was used to identify frataxin amino acids perturbed by the presence of ferrous iron under solution conditions that stabilize the monomeric protein. Our goal was to locate the region of the monomeric protein affected by the presence of iron with the intent to determine which amino acids may be directly involved in iron binding. We recently reported a similar titration on yeast frataxin in sodium phosphate buffer, corresponding to conditions we utilized when solving the protein's solution structure (28). However, we repeated these studies in HEPES/MgSO₄ buffer to confirm the protein with metal bound was a stable monomer and also to provide a better representation of frataxin's iron binding ability under solution conditions similar to those seen *in vivo* that surely contain salt. Buffered anaerobic ferrous ammonium sulfate was added to anaerobic ¹⁵N-labeled yeast frataxin at 1:1 and 2:1 metal to protein stoichiometries. ¹⁵N-filtered HSQC spectra were used to identify amide chemical shift perturbations of residues affected by the presence of iron (36). As observed in our phosphate buffered titration, residues localized within the helix-1 and strand-1 regions of frataxin were significantly perturbed by the presence of iron (Figure 3) (28). However, unlike our initial phosphate buffer report, a second set of residues underwent substantial amide line broadening in the iron-loaded spectra. Additional residues in the β -sheet and H2 regions were also affected in the presence of iron under these new buffer conditions. Residues with significant amide chemical shift perturbations (i.e., greater than 2 times resolution of the data) include: E24, E25, D27, L33, S36, L37, E38, E39, L40, S41, E42, A43 in helix-1, G56 in the strand-1/-2 connecting loop, K77 in the strand-3/-4 connecting loop, W80 in strand-4, and E116, A119 and K121 in helix-2. The second residue set, with amide resonance that were line-broadened beyond recognition, include: D28, D31, H32, L33, D35 in helix-1, E52 in strand-1, E61 in strand-2, T67 in strand-3 and Q123 at the C-terminus. Residue numbers represent the sequence position corresponding to the mature processed protein.

Iron Coordination Geometry of Iron-Loaded Monomeric Frataxin

XAS studies were used to obtain an electronic and structural characterization of iron bound to monomeric yeast frataxin. Full XAS data sets were collected on multiple independent yeast frataxin samples with one iron (1Fe-Yfh1) and two iron (2Fe-Yfh1) bound. Samples were again prepared under solution conditions that stabilized the iron bound protein monomer. XANES spectra for the different protein samples are compared to Fe(II) and Fe(III) controls in Figure 4. General edge features from the protein samples closely resemble those obtained from the ferrous ammonium sulfate control spectrum (Figure 4A). The first inflection edge energies measured for 1Fe-Yfh1 and 2Fe-Yfh1 were at 7122.40 ± 0.02 and 7122.75 ± 0.03 eV's, respectively. The averaged edge inflection energy for the two signals in our ferrous ammonium sulfate control occurs at 7122.90 ± 0.03 eV, while the first inflection energy for our ferric ammonium sulfate control occurs at 7126.30 ± 0.02 eV. Our protein edge inflection energies more closely resemble values seen for our ferrous control, indicating bulk iron bound to monomeric yeast frataxin is predominately in the ferrous oxidation state. The pre-edge features, centered at ca. 7112 eV in the Fe(II) containing XANES spectra, arise from the $1s \rightarrow 3d$ electronic transitions in the XAS absorption edge (44,46-48). The $1s \rightarrow 3d$ signals in the protein XANES spectra were compared to model spectra to identify the spin state and ligand symmetry of Fe(II) bound to Yfh1. Three independent $1s \rightarrow 3d$ transitions were measured for both 1Fe-Yfh1 and 2Fe-Yfh1 at 7111.27 ± 0.02 , 7112.28 ± 0.03 and 7113.38 ± 0.02 eVs. The global area for these transitions were measured in unitless values for both 1Fe-Yfh1 and 2Fe-Yfh1 at 5.46 ± 0.07 and 5.56 ± 0.01 , respectively. A comparison of these spectral features to published iron model data indicate ferrous iron bound to yeast frataxin is most likely high-spin, based on the intensity, number and energy of the observed transitions (44,46). Global $1s \rightarrow 3d$ transition areas for the protein samples, when compared to published ferrous model data, indicate the iron-ligand geometry in frataxin is highly symmetric, based on the low values obtained compared to the value of 5.18 ± 0.015 from the highly symmetric $[\text{Fe}(\text{H}_2\text{O})_6](\text{NH}_4)_2(\text{SO}_4)_2$ octahedral sample (44,46). Our XANES analysis therefore confirms that iron bound to monomeric yeast frataxin is high-spin and that ferrous iron is coordinated in a highly symmetric metal-ligand geometry, regardless the metal to protein stoichiometry when up to two iron atoms bound.

The protein EXAFS data was used to provide the metal-ligand geometric parameters for one and two ferrous iron bound to monomeric yeast frataxin. Raw EXAFS data for both 1Fe-Yfh1 and 2Fe-Yfh1 show a single frequency pattern with maximum amplitude at $5.5 \text{ \AA}^{-1}$, suggesting symmetric Fe-nearest neighbor coordination geometry constructed by oxygen and/or nitrogen based ligands (Figure 5A and 5C, black lines, respectively). The first large oscillation in both raw EXAFS data sets show a "camel back" feature that is often attributed to imidazole scattering from histidine coordination to the metal (49). Fourier transforms of the EXAFS data show a predominate ligand environment is localized in a symmetric pattern at a phase shifted bond length ($R + \Delta$) of ca. $1.8 \text{ \AA}$ in both samples (Figure 5B and 5D). Minimal long-range scattering (above $2.5 \text{ \AA}$) is observed in both spectra. Spectral simulations show ligands directly coordinated to the iron are completely oxygen/nitrogen based (Table 1). In both 1Fe-Yfh1 and 2Fe-Yfh1 data sets, the nearest neighbor environment is best fit with a single nearest neighbor Fe-oxygen/nitrogen ligand environment that is partially disordered and with coordination numbers close to six (Figure 5, simulations in grey). These data indicate iron bound to yeast frataxin is in a fairly symmetric Fe-(oxygen and/or nitrogen) ligand environment that is most likely six coordinate. There is no evidence for Fe-S ligation, consistent with that fact that only amino acids with side chain carboxylates and histidines were identified as residues perturbed in our NMR iron into protein titration. Furthermore, long-range scattering can only be fit with carbon scattering, so there is no evidence for any $\text{Fe} \cdots \text{Fe}$ scattering in either reduced iron sample. Finally, a comparison of the 1:1 and 2:1 Fe:protein data indicates there is no major

structural rearrangement at the metal center upon addition of the second protein bound iron atom.

DISCUSSION

The goal of this report was to help characterize the structural and electronic properties of iron bound to monomeric yeast frataxin. Frataxin has been suggested to play a variety of roles in helping to maintain cellular iron homeostasis, most of which involve direct binding to iron. Frataxin facilitates *in vivo* heme and Fe-S cluster production and the monomeric protein binds to enzyme partners that complete the assembly of these iron-containing cofactors, suggesting monomeric protein may play a direct role in both processes by acting as the iron chaperone (14-24). Fluctuations in mitochondrial iron or Fe-cofactor levels, and the differences in binding affinities between partners, have been proposed to serve as the trigger for which cofactor assembly system is delivered iron (23). Frataxin has also been shown to oligomerize *in vitro* in high iron conditions and the formation of these oligomers is highly dependent on the solution ionic strength (10,27). Given that magnesium and calcium salts stabilize iron loaded frataxin monomers, it has been also been suggested that changes in mitochondrial ion concentrations may trigger frataxin to shift from an iron delivery to iron storage capacity (27). During the process of frataxin's iron induced oligomerization, while the trimeric protein appears to be the nucleating species for higher order assembly (10-12), it is surely the initial interaction of soluble monomeric protein with iron that instigates trimer formation. In addition, iron bound to frataxin multimers is predominately bound in a multinuclear form, while that bound to monomeric frataxin under low oxygen conditions appears to be as isolated atoms (13,50). Since a single iron atom is transferred during heme and Fe-S cluster assembly, it seems further unlikely that frataxin oligomers would participate as the source of metal during delivery. Given this rationale, having a structural and biochemical understanding of iron bound to monomeric frataxin would help propel the general understanding of how frataxin functions towards controlling iron bioavailability in the cell.

Frataxin's metal binding affinity is similar to those seen for the copper chaperones suggesting a common theme in cellular metal delivery if frataxin is in fact acting as an iron chaperone. The micromolar dissociation constants measured for yeast frataxin, with respect to ferrous iron (K_d 's of 2.0 and 3.0 μ M), resembles those obtained for the human and bacterial orthologs (K_d 's of 55.0 and 3.8 μ M, respectively) (22,51). A comparison of the frataxin iron dissociation constants to those measured for the copper chaperones Cox17 and Atox1 (K_d 's of ca. 0.17 and 10 μ M, respectively) place these proteins within roughly the same affinity range (52,53). Ferrous iron binding affinities measured for ferrochelatase are also within the micromolar range indicating frataxin's metal binding affinity is closely matched by its protein partners that receives metal (54). It is therefore reasonable to suggest frataxin can act as an iron chaperone during Fe-cofactor production. Given the high binding affinity between frataxin and its protein binding partners ferrochelatase and ISU (K_d 's in the nM range), it is also reasonable to suggest that although frataxin binds ferrous iron weakly, regulation of metal transfer events could be driven by the high affinity for forming a multiprotein complex (14,22,23). A general question is, given that cellular iron regulation is so tightly controlled and that mitochondrial free iron levels are surely below the micromolar range, why would cells utilize such a weak metal chaperone to help control the production of these essential iron-cofactors, especially given the high reactivity of the metal?

Metal binding site(s) on monomeric frataxin must accommodate the binding of multiple iron atoms. Recent spectroscopic studies indicate frataxin residues within the conserved acidic region at the helix-1 and strand-1 regions are affected by iron binding (28,32,51). In these studies, NMR spectroscopy proved to be a sensitive tool for probing which frataxin amino acids are affected by the presence of bound ferrous iron. Iron titrations into yeast, human and

bacterial frataxin consistently show residues within this conserved acidic region have their amide chemical shifts perturbed in the presence of iron. In organic buffers, all three frataxin orthologs also show a set of residues that have their amide resonances line-broadened beyond identification when protein is in the presence of the paramagnetic metal. Our original NMR iron titration into yeast frataxin showed no amide line broadening, suggesting phosphate buffer somehow can alter iron binding to the protein or that iron binding is transient under those conditions (28). While amide chemical shift perturbations may indicate a variety of occurrences (metal binding, localized change in the protein's fold, changes in the chemical nature of the residue environment, etc.) it has been shown that protons located within a 10 Å radius of the bound paramagnetic metal can have their NMR line widths significantly broadened (55). Therefore, frataxin residues with acidic side chains that undergo significant amide line broadening in the presence of ferrous iron are most likely the amino acids in closer proximity to the metal, as compared to the residues that have only amide resonance chemical shift changes. Therefore, the broadened residues are more likely the amino acids that act as ligands to the metal.

A comparison of the amide NMR resonances significantly line broadened in the presence of iron show a subset of these residues are well conserved. Amide resonances for yeast frataxin D31, D35, corresponding to human residues D104 and E108 and bacterial residues L15 and E19, are significantly broadened in the human and yeast orthologs, and, in the case of residue E19, in the bacterial ortholog (this report and (32)). Acidic residues have side chain carboxylate groups that are often found to serve as iron ligands within metalloproteins (33,34,56,57). In all three cases, additional residues in close proximity to the acidic residues listed above are also broadened, suggesting if metal binds directly to their carboxylate side chain, the additional residues in close proximity should also be affected. In yeast frataxin, additional amide line-broadened residues in proximity to D31 and D35 include D28 and H32. Interestingly, yeast frataxin residues D28 and D31 have been shown to be required for the formation of the ferroxidase center observed in yeast frataxin oligomers by the Isaya laboratory, while D35 was implicated by the Craig group as assisting homooligomer formation and by the Isaya group for assisting in iron binding (25,26). In the bacterial and human orthologs, residues C-terminal to D35 in yeast frataxin are also broadened (32); these residues in yeast frataxin are simply perturbed in their amide chemical shift values. These differences between reports may suggest that human and bacterial orthologs have a more expansive iron-binding surface on their helix-1 region, or that the divalent magnesium ions we used to stabilize the yeast protein as a monomer could be blocking weak transient metal binding sites identified on the other orthologs. Yeast frataxin contains a second region of multiple residues that are line-broadened at their amide position in the presence of iron; this site includes residues E52 and E61 on the protein's β -sheet surface. In this case, the broadened signals are not observed in the bacterial and human orthologs and only E52 is conserved. Interestingly, D50 in yeast frataxin is completely conserved, and this amide signal is broadened in both the human and bacterial orthologs suggesting again that the β -sheet region of all frataxins act as an additional metal binding sight on the protein on the opposite side of the helix-1 region of the molecule (32).

XAS studies of ferrous iron bound yeast frataxin are consistent with a metal-ligand environment constructed by residues that were broadened in our NMR studies. Our XANES analysis indicate, based on first inflection point analysis of the iron edges, that iron is stable in the ferrous state when iron-loaded frataxin is kept anaerobic. Given that oxygen levels in the mitochondria are highly controlled, we suggest the environment within the mitochondria may, under normal conditions, resemble the *in vitro* conditions we report in our anaerobic studies. The 1s→3d features indicate that ferrous iron bound to frataxin is high-spin and held within a coordination geometry that is nearly octahedral in its metal-ligand geometric symmetry. There are minimal differences in the edges when the protein has one or two metals bound, indicating the two metal binding sites on frataxin are either very similar (as expected since both appear

to be constructed by acidic residues) or that at stoichiometric metal to protein concentration, the two sites are being evenly populated (consistent with the very similar binding constants). EXAFS analysis confirms ferrous iron bound to frataxin is coordinated by six oxygen/nitrogen based ligands. The metal-ligand nearest neighbor coordination environments in frataxin are nearly identical independent of if there are one or two metals bound. The predominate ligand scattering environment is constructed of approximately six metal ligands at 2.12Å. Analysis of the metal-ligand coordination environments for six coordinate ferrous iron models in the Cambridge Structural Database (CSD) suggest average Fe(II)-O₆ distances from oxygens that are either protonated (resembling water) or in a carboxylate like environment (representing Asp and Glu side chain ligands) are on average 2.11Å and 2.15Å, respectively (58). Distances obtained in the 2.12Å average iron-nearest neighbor environment of iron bound to frataxin closely match these six-coordinate model values, indicating the majority of the protein bound iron ligands most likely originate from acidic residue carboxylate side chains, water or more likely a combination of both. In addition, the long-range (>2.5Å) scattering observed in the Fourier transforms of the protein iron EXAFS would certainly be consistent with carboxylate carbon atom scattering. Interestingly, the appearance of the imidazole camel-back feature in the first bead pattern of the protein EXAFS data suggests there may be direct ligation of a histidine to the bound iron. The presence of long-range scattering features in our protein data could also be suggestive of Fe-imidazole scattering. In our NMR analysis, we identified H32 as a possible ligand that is directly coordinated to iron. Scattering from H32 could contribute to the apparent imidazole EXAFS scattering we see in our data, although an equally likely scenario exists that the apparent imidazole scattering actually may come from carboxylate carbon scattering. Additional experiments are underway to test if H32 directly binds to iron. There is no evidence in our reduced samples of the Fe•••Fe scattering observed in ferric loaded frataxin oligomers, and the general Fe-O bond distance in our monomeric samples are longer than those reported in the oligomer study as expected given the difference in metal oxidation state (13). These data suggest that under reducing conditions, iron bound to monomeric frataxin does not exist in a ferrihydrite form, although the absence of a Fe•••Fe signal does not rule out the possibility that the two bound metals in the 2Fe-Yfh1 sample are not in close proximity to each other (35,59).

Based on the paramagnetically broadened residues in the NMR titrations, and on the geometric parameters obtained in our XAS studies, our results are consistent with yeast frataxin having two unique iron binding regions. The first site involves side chains from residues D28, D31, H32 and D35 on helix-1 of the protein (Figure 6A). At this site, only the acidic residues are conserved while the histidine is a serine in the human protein. This first site is close to the additional conserved acidic residues, C-terminal but still on helix-1 in position, which are affected by the presence of iron. The second site may involve residues E52 and E61 on the β-sheet surface of the molecule, possibly also including D50 a residue that is consistently broadened in the human and bacterial NMR iron titrations and shifted in our Yfh1 iron/phosphate buffer titration (Figure 6B). The fact that these two unique sites are located on distant and opposite regions of the frataxin structure may indicate each could be selectively responsible for iron delivery to a different protein binding partner. *In vivo* and *in vitro* studies are currently underway to dissect the role of each site in the different proposed iron delivery function of frataxin.

Additional conserved acidic helix-1 residues C-terminal to amino acids that construct our first proposed metal binding site are also affected by the presence of iron in the NMR titrations and line broadened in the bacterial and human studies (32). Interestingly, human frataxin has been shown to bind 6 iron atoms while the bacterial homolog binds 2 iron atoms in the micromolar range, with multiple additional residues at an even weaker (unmeasurable by ITC) binding affinity (22,23,32). We believe these additional helix-1 residues C-terminal to those identified in the first binding site (including yeast frataxin residues E38, E39 and E42 shifted in our iron

titration) may construct a possible third (but transient) binding site for additional metals during aerobic/iron overloaded conditions. These acidic conserved helix-1 residues are substantially broadened in the human and bacterial iron NMR titrations and E38, E39 and E42 have been shown to be important for iron binding and the ferroxidase chemistry noted in yeast frataxin oligomers (25). In our yeast iron binding analysis, this site may also be a transient site for weaker binding salt divalent ions (i.e., Mg^{2+} , which we have in abundance in our samples), and when these salts are in high abundance, they out-compete iron for binding.

In summary, all three structurally characterized frataxin orthologs bind multiple bind iron atoms with a micromolar binding affinity when the protein exists in the monomeric state, consistent with a functional role of monomeric protein being involved in iron binding and delivery. N-terminally conserved acidic residues spanning the helix-1 and strand-1 regions of the protein are significantly perturbed in their chemical environments upon binding of paramagnetic iron, implicating residues in these regions are performing selective iron binding. Ferrous iron bound to monomeric frataxin is high-spin and coordinated in a highly symmetric metal-ligand geometry constructed of only oxygen and nitrogen ligands. These ligands are presumably donated from carboxylate and imidazole containing amino acid side chains and from coordinated water molecules. The weak nature of the protein's metal binding affinity and the ligand environment utilized by the protein to bind metal would be consistent with frataxin acting as an iron chaperone; the protein must be able to form a favorable interface with its protein partners and also easily release the metal. Frataxin's iron binding helix-1 and strand-1 regions have already been implicated in assisting the intermolecular interface when the protein binds to ferrochelatase (28,60) and to ISU (unpublished results). Additional structural characterization will be critical to help elucidate how frataxin binds iron and docks with its protein partners to promote metal delivery.

ACKNOWLEDGEMENT

Portions of this research were carried out at both the Stanford Synchrotron Radiation Laboratory (SSRL) and at the National Synchrotron Light Source (NSLS). SSRL is a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and by the NIH, National Center for Research Resources, Biomedical Technology Program. NSLS, located at Brookhaven National Laboratory, is supported by the U.S. Department of Energy, Division of Materials Sciences and Division of Chemical Sciences, under Contract No. DE-AC02-98CH10886.

Abbreviations

Yfh1, Yeast frataxin homolog 1 protein; NMR, Nuclear Magnetic Resonance; XAS, X-ray absorption spectroscopy; XANES, X-ray absorption near edge structure; EXAFS, Extended X-ray absorption fine structure; ITC, Isothermal titration calorimetry; ICP-MS, Inductively coupled plasmid mass spectroscopy.

REFERENCES

1. Skre H. Friedreich's ataxia in western Norway. *Clin. Genet* 1975;7:287–98. [PubMed: 1126051]
2. Winter RM, Harding AE, Baraitser M, Bravery MB. Intrafamilial correlation in Friedreich's ataxia. *Clin. Genet* 1981;20:419–27. [PubMed: 7337957]
3. Pandolfo M, Sirugo G, Antonelli A, Weitnauer L, Ferretti L, Leone M, Dones I, Cerino A, Fujita R, Hanauer A. Friedreich ataxia in Italian families: genetic homogeneity and linkage disequilibrium with the marker loci D9S5 and D9S15. *Am J Hum Genet* 1990;47:228–35. [PubMed: 2378348]
4. Campuzano V, Montermini L, Molto MD, Pianese L, Cossee M, Cavalcanti F, Monros E, Rodius F, Duclos F, Monticelli A, Zara F, Canizares J, Koutnikova H, Bidichandani SI, Gellera C, Brice A, Trouillas P, De Michele G, Filla A, De Frutos R, Palau F, Patel PI, Di Donato S, Mandel JL, Coccozza S, Koenig M, Pandolfo M. Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science* 1996;271:1423–7. [PubMed: 8596916]

5. Koutnikova H, Campuzano V, Foury F, Dolle P, Cazzalini O, Koenig M. Studies of human, mouse and yeast homologues indicate a mitochondrial function for frataxin. *Nat Genet* 1997;16:345–51. [PubMed: 9241270]
6. Radisky DC, Babcock MC, Kaplan J. The Yeast Frataxin Homologue Mediates Mitochondrial Iron Efflux: Evidence for a mitochondrial iron cycle. *J. Biol. Chem* 1999;274:4497–4499. [PubMed: 9988680]
7. Ristow M, Pfister MF, Yee AJ, Schubert M, Michael L, Zhang CY, Ueki K, Michael MD 2nd, Lowell BB, Kahn CR. Frataxin activates mitochondrial energy conversion and oxidative phosphorylation. *Proc Natl Acad Sci U S A* 2000;97:12239–43. [PubMed: 11035806]
8. Bulteau AL, O'Neill HA, Kennedy MC, Ikeda-Saito M, Isaya G, Szewda LI. Frataxin acts as an iron chaperone protein to modulate mitochondrial aconitase activity. *Science* 2004;305:242–5. [PubMed: 15247478]
9. Bulteau AL, Lundberg KC, Ikeda-Saito M, Isaya G, Szewda LI. Reversible redox-dependent modulation of mitochondrial aconitase and proteolytic activity during in vivo cardiac ischemia/reperfusion. *Proc Natl Acad Sci U S A* 2005;102:5987–91. [PubMed: 15840721]
10. Adamec J, Rusnak F, Owen WG, Naylor S, Benson LM, Gacy AM, Isaya G. Iron-dependent self-assembly of recombinant yeast frataxin: implications for Friedreich ataxia. *Am J Hum Genet* 2000;67:549–62. [PubMed: 10930361]
11. Cavadini P, O'Neill HA, Benada O, Isaya G. Assembly and iron-binding properties of human frataxin, the protein deficient in Friedreich ataxia. *Hum Mol Genet* 2002;11:217–27. [PubMed: 11823441]
12. Gakh O, Adamec J, Gacy AM, Westen RD, Owen WG, Isaya G. Physical evidence that yeast frataxin is an iron storage protein. *Biochemistry* 2002;41:6798–804. [PubMed: 12022884]
13. Nichol H, Gakh O, O'Neill HA, Pickering IJ, Isaya G, George GN. Structure of frataxin iron cores: an X-ray absorption spectroscopic study. *Biochemistry* 2003;42:5971–6. [PubMed: 12755598]
14. Lesuisse E, Santos R, Matzanke BF, Knight SA, Camadro JM, Dancis A. Iron use for haeme synthesis is under control of the yeast frataxin homologue (Yfh1). *Hum Mol Genet* 2003;12:879–89. [PubMed: 12668611]
15. Schoenfeld RA, Napoli E, Wong A, Zhan S, Reutenauer L, Morin D, Buckpitt AR, Taroni F, Lonnerdal B, Ristow M, Puccio H, Cortopassi GA. Frataxin deficiency alters heme pathway transcripts and decreases mitochondrial heme metabolites in mammalian cells. *Hum Mol Genet* 2005;14:3787–99. [PubMed: 16239244]
16. Zhang Y, Lyver ER, Knight SA, Lesuisse E, Dancis A. Frataxin and mitochondrial carrier proteins, Mrs3p and Mrs4p, cooperate in providing iron for heme synthesis. *J Biol Chem* 2005;280:19794–807. [PubMed: 15767258]
17. Gerber J, Muhlenhoff U, Lill R. An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. *EMBO Rep* 2003;4:906–11. [PubMed: 12947415]
18. Stehling O, Elsasser HP, Bruckel B, Muhlenhoff U, Lill R. Iron-sulfur protein maturation in human cells: evidence for a function of frataxin. *Hum Mol Genet* 2004;13:3007–15. [PubMed: 15509595]
19. Muhlenhoff U, Gerber J, Richhardt N, Lill R. Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *Embo J* 2003;22:4815–25. [PubMed: 12970193]
20. Muhlenhoff U, Richhardt N, Ristow M, Kispal G, Lill R. The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. *Hum Mol Genet* 2002;11:2025–36. [PubMed: 12165564]
21. Ramazzotti A, Vanmansart V, Foury F. Mitochondrial functional interactions between frataxin and Isu1p, the iron-sulfur cluster scaffold protein, in *Saccharomyces cerevisiae*. *FEBS Lett* 2004;557:215–20. [PubMed: 14741370]
22. Yoon T, Cowan JA. Iron-sulfur cluster biosynthesis. Characterization of frataxin as an iron donor for assembly of [2Fe-2S] clusters in ISU-type proteins. *J. Am. Chem. Soc* 2003;125:6078–84. [PubMed: 12785837]
23. Yoon T, Cowan JA. Frataxin-mediated iron delivery to Ferrochelatase in the final step of Heme biosynthesis. *J Biol Chem* 2004;279:25943–6. [PubMed: 15123683]
24. Park S, Gakh O, O'Neill HA, Mangravita A, Nichol H, Ferreira GC, Isaya G. Yeast frataxin sequentially chaperones and stores iron by coupling protein assembly with iron oxidation. *J. Biol. Chem* 2003;278:31340–51. [PubMed: 12732649]

25. Gakh O, Park S, Liu G, Macomber L, Imlay JA, Ferreira GC, Isaya G. Mitochondrial iron detoxification is a primary function of frataxin that limits oxidative damage and preserves cell longevity. *Hum Mol Genet* 2006;15:467–79. [PubMed: 16371422]
26. Aloria K, Schilke B, Andrew A, Craig EA. Iron-induced oligomerization of yeast frataxin homologue Yfh1 is dispensable in vivo. *EMBO Rep* 2004;5:1096–101. [PubMed: 15472712]
27. Adinolfi S, Trifuoggi M, Politou AS, Martin S, Pastore A. A structural approach to understanding the iron-binding properties of phylogenetically different frataxins. *Hum. Mol. Genet* 2002;11:1865–77. [PubMed: 12140189]
28. He Y, Alam SL, Proteasa SV, Zhang Y, Lesuisse E, Dancis A, Stemmler TL. Yeast frataxin solution structure, iron binding, and ferrochelatase interaction. *Biochemistry* 2004;43:16254–62. [PubMed: 15610019]
29. Musco G, Stier G, Kolmerer B, Adinolfi S, Martin S, Frenkiel T, Gibson T, Pastore A. Towards a Structural Understanding of Friedreich's Ataxia: The Solution Structure of Frataxin. *Structure* 2000;8:695–707. [PubMed: 10903947]
30. Dhe-Paganon S, Shigeta R, Chi YI, Ristow M, Shoelson SE. Crystal structure of human frataxin. *J Biol Chem* 2000;275:30753–6. [PubMed: 10900192]
31. Cho SJ, Lee MG, Yang JK, Lee JY, Song HK, Suh SW. Crystal structure of Escherichia coli CyaY protein reveals a previously unidentified fold for the evolutionarily conserved frataxin family. *Proc Natl Acad Sci U S A* 2000;97:8932–7. [PubMed: 10908679]
32. Nair M, Adinolfi S, Pastore C, Kelly G, Temussi P, Pastore A. Solution structure of the bacterial frataxin ortholog, CyaY: mapping the iron binding sites. *Structure (Camb)* 2004;12:2037–48. [PubMed: 15530368]
33. Rosenzweig AC, Frederick CA, Lippard SJ, Nordlund P. Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. *Nature* 1993;366:537–43. [PubMed: 8255292]
34. Nordlund P, Eklund H. Structure and function of the Escherichia coli ribonucleotide reductase protein R2. *J Mol Biol* 1993;232:123–64. [PubMed: 8331655]
35. Riggs-Gelasco PJ, Stemmler TL, Penner-Hahn JE. XAFS of Dinuclear Metal Sites in Proteins and Model Compounds. *Coord. Chem. Review* 1995;114
36. Mori S, Abeygunawardana C, O'Neil Johnson M, van Zijl PCM. Improved Sensitivity of HSQC Spectra of Exchanging Protons at Short Interscan Delays Using a New Fast HSQC (FHSQC) Detection Scheme that Avoids Water Saturation. *J. Magn. Res. B* 1995;108:94–98.
37. Wishart, DS.; Bigam, CG.; Yao, J.; Abildgaard, F.; Dyson, HJ.; Oldfield, L.; Markley, JL.; Sykes, BD. *J. Biomol. NMR.* 6. 1995. 1H, 13C and 15N chemical shift referencing in biomolecular NMR.; p. 135-40.
38. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–93. [PubMed: 8520220]
39. Goddard, TD.; Kneller, DG. University of California; San Francisco: 2001.
40. Grzesiek S, Bax A, Clore GM, Gronenborn AM, Hu JS, Kaufman J, Palmer I, Stahl SJ, Wingfield PT. The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase. *Nat Struct Biol* 1996;3:340–5. [PubMed: 8599760]
41. Garrett TPJ, Guss JM, Freeman HC. Hexakis(imidazole)manganese(II) Dichloride Tetrahydrate, [Mn(C₃H₄N₂)₆]Cl₂•4H₂O. and Hexakis(imidazole)zinc(II) Dichloride Tetrahydrate, [Zn(C₃H₄N₂)₆]Cl₂•4H₂O. *Acta Cryst* 1983;C39:1027–1031.
42. Rehr JJ, Ankudinov AL. Progress and challenges in the theory and interpretation of X-ray spectra. *J Synchrotron Radiat* 2001;8:61–5. [PubMed: 11512868]
43. George, GN.; George, SJ.; Pickering, IJ. 2001. <http://www.ssrsl.slac.stanford.edu/~george/exafspak/exafs.htm>.
44. Westre TE, Kennepohl P, DeWitt JG, Hedman B, Hodgson KO, Solomon EI. A Multiplet Analysis of the Fe K-Edge 1s→3d Pre-Edge Features of Iron Complexes. *J. Am. Chem. Soc* 1997;119:6297–6314.
45. Lee PA, Citrin PH, Eisenberger P, Kincaid BM. Extended x-ray absorption fine structure - its strengths and limitations as a structural tool. *Reviews of Modern Physics* 1981;53:769–806.

46. Randall CR, Shu L, Chiou Y-M, Hagen KS, Ito M, Kitajima N, Lachicotte RJ, Zang Y, Que L Jr. X-ray absorption pre-edge studies of high-spin iron(II) compounds. *Inorg. Chem* 1995;34:1036–9.
47. Roe AL, Schneider DJ, Mayer RL, Pyrz JW, Widon J, Que L Jr. X-ray absorption spectroscopy of iron-tyrosinate proteins. *J. Am. Chem. Soc, USA* 1984;106:1676–81.
48. Shulman RG, Yafet Y, Eisenberger P, Blumberg WE. Observation and interpretation of x-ray absorption edges in iron compounds and proteins. *Proc. Natl. Acad. Sci., USA* 1976;73:1384–8. [PubMed: 5720]
49. Feiters MC, Navaratnam S, Al-Hakim M, Allen JC, Spek AL, Veldink GA, Vliegthart JFG. EXAFS of poly[m-hexakis(2-methylimidazolato-N,N')triiron(II)]: Implications for metalloprotein studies. *J. Am. Chem. Soc* 1988;110:7746–50.
50. Park S, Gakh O, Mooney SM, Isaya G. The ferroxidase activity of yeast frataxin. *J. Biol. Chem* 2002;277:38589–95. [PubMed: 12149269]
51. Bou-Abdallah F, Adinolfi S, Pastore A, Laue TM, Dennis Chasteen N. Iron binding and oxidation kinetics in frataxin CyaY of *Escherichia coli*. *J Mol Biol* 2004;341:605–15. [PubMed: 15276847]
52. Abajian C, Yatsunyk LA, Ramirez BE, Rosenzweig AC. Yeast cox17 solution structure and Copper (I) binding. *J Biol Chem* 2004;279:53584–92. [PubMed: 15465825]
53. Wernimont AK, Yatsunyk LA, Rosenzweig AC. Binding of copper(I) by the Wilson disease protein and its copper chaperone. *J Biol Chem* 2004;279:12269–76. [PubMed: 14709553]
54. Shi Z, Ferreira GC. A continuous anaerobic fluorimetric assay for ferrochelatase by monitoring porphyrin disappearance. *Anal Biochem* 2003;318:18–24. [PubMed: 12782026]
55. La Mar, GN.; de Ropp, JS. NMR Methodology for Paramagnetic Proteins. In: Berliner, LJ.; Reuben, J., editors. *Biological Magnetic resonance*. Plenum Press; New York: 1993. p. 1-73.
56. Holmes MA, Stenkamp RE. Structures of met and azidomet hemerythrin at 1.66 Å resolution. *J Mol Biol* 1991;220:723–37. [PubMed: 1870128]
57. Holmes MA, Le Trong I, Turley S, Sieker LC, Stenkamp RE. Structures of deoxy and oxy hemerythrin at 2.0 Å resolution. *J Mol Biol* 1991;218:583–93. [PubMed: 2016748]
58. Centre, CCD. Cambridge: 2002.
59. Stemmler TL, S. J. TM, Goldstein JI, Ash DE, Elgren TE, Kurtz DM Jr. Penner-Hahn JE. EXAFS Comparison of the Dimanganese Core Structures of Manganese Catalase, Arginase and Manganese-Substituted Ribonucleotide Reductase and Hemerythrin. *Biochemistry* 1997;36:9847–9858. [PubMed: 9245417]
60. Bencze KZ, Yoon T, Bradley PB, Cowan JA, Stemmler TL. Human frataxin: Iron structure and ferrochelatase binding surface. *J. Am. Chem. Soc.* Submitted. 2006

Figure 1. Oligomeric state and iron to protein stoichiometry for yeast frataxin samples
 (A) Size exclusion chromatographs for yeast frataxin with 2, 1 or 0 iron atoms bound shown in (I), (II) and (III) respectively, coupled with protein standards (IV). Samples were run anaerobically on a S-200 Sephacryl size exclusion column at 27° C. (B) Representative comparison of the ICP-MS iron quantitation (squares) versus the protein concentration from a Bradford assay (circles) for 1:1 iron loaded yeast frataxin on data collected on a high resolution S-100 Sephacryl column under identical conditions as when we ran the S-200 column.

Figure 2. Raw isothermal titration calorimetry (A) and binding isotherm data (B) for ferrous iron to yeast frataxin

Grey line in panel (A) shows baseline and in panel (B) shows the simulated fit to the binding isotherm data. Data were collected anaerobically at 30° in 20 mM HEPES (pH 7.0) and 10 mM MgSO_4 . These data were collected by injecting small aliquots of a 0.8 mM ferrous iron solution into a 50 μM Yfh1 sample. Spacing between injections was 10 minutes. Syringe stirring speed was kept at 500 rpm.

Figure 3. Frataxin residues perturbed at the amide position by the presence of iron

(A) ^{15}N filtered HSQC spectra for 500 μM apo- (blue) and 2Fe-Yfh1 (red) in 20 mM HEPES buffer (pH 7.0), 10 mM MgSO_4 at 600 MHz. NMR data collected anaerobically at 30°C. Inset: expanded region marked in dashed lines with residues identified, “§” indicates side chain or unassigned peaks. (B) Summary of yeast frataxin normalized amide chemical shift changes in the presence and absence of 2 equivalents of iron. Residues identified in black correspond to paramagnetically broadened amide signals. Residues identified in red correspond to amide signals with chemical shift changes. Residues indicated with a “*” in the x-axis indicate Pro positions. Horizontal black line indicates chemical shift cutoff, which corresponds to 2x data resolution. Top: secondary structural elements for yeast frataxin with helices and strands labeled as H and S, respectively. (C) Location of residues on apo-yeast frataxin structure with amide backbone resonances that are significantly line broadened (yellow) or have substantial chemical shift changes (red) in the presence of Fe(II). Note: the protein's structure represents an updated PDB file for yeast frataxin (PDB accession number 2GA5), currently available for download.

Figure 4. XANES comparison of iron-yeast frataxin with ferrous and ferric models

Full XANES spectra for yeast frataxin with 1 iron (solid) and 2 iron bound (bold, solid) with ferrous ammonium sulfate (dashed line) and ferric ammonium sulfate (dotted line). Inset: expansion of background subtracted $1s \rightarrow 3d$ region of the XANES spectra for all samples.

Figure 5. EXAFS and Fourier transforms of iron loaded yeast frataxin XAS data
EXAFS spectra in black for yeast frataxin with 1 iron bound (A) and 2 iron bound (C), along with the corresponding Fourier transforms (B and D, respectively). Simulations for EXAFS and FT data are shown in grey.

Figure 6. Location of yeast frataxin residues strongly perturbed by the presence of iron

A) Apo-protein structure with helix-1 amino acid side chain atoms from line broadened amide resonances in the yeast frataxin NMR iron titration. Expanded view to the right of arrows. B) Apo-protein structure with β -sheet amino acid side chain atoms from line broadened amide resonances in the yeast frataxin NMR iron titration and D50. Expanded view to the right of the arrow. Note: the protein's structure represents an updated PDB file for yeast frataxin (PDB accession number 2GA5), currently available for download.

Table 1
Summary of EXAFS fitting results for iron loaded yeast frataxin
Data fit over a k range of 1 to 12.85 Å⁻¹. Values are averaged from at least 2 independent samples.

Sample/ Fit#	Atom ^b	Ligand Environment ^d R(Å) ^c	C.N. ^d	σ^2	Atom ^b	Ligand Environment ^d R(Å) ^c	C.N. ^d	σ^2	Atom ^b	Ligand Environment ^d R(Å) ^c	C.N. ^d	σ^2	R^2
1Fe- Yfh1 1 ^g	O/N	2.12	4.75	5.80									0.98
2 ^g	O/N	2.12	4.75	5.79	C	3.06	1	2.46					0.95
3 ^g	O/N	2.12	4.75	5.78	C	3.07	1.75	4.30	C	3.30	1.25	4.47	0.92
2Fe- Yfh1 1 ^g	O/N	2.12	5.25	5.72									0.54
2 ^g	O/N	2.12	5.25	5.71	C	3.36	1	3.93					0.52
3 ^g	O/N	2.12	5.25	5.72	C	3.00	1	3.49	C	3.42	1.25	2.82	0.50

^aIndependent metal-ligand scattering environment
^bScattering atoms: O (Oxygen), N (Nitrogen)
^cMetal-ligand bond length
^dMetal-ligand coordination number
^eDebye-Waller factor in Å² × 10³
^fNumber of degrees of freedom weighted mean square deviation between data and fit
^gFit using only single scattering Feff 7 theoretical models