



NIH Public Access

Author Manuscript

Biochemistry. Author manuscript; available in PMC 2014 September 03.

Published in final edited form as:

Biochemistry. 2013 September 3; 52(35): . doi:10.1021/bi400443n.

His86 from the N-terminus of frataxin coordinates iron and is required for Fe-S cluster synthesis

Leslie E. Gentry[†], Matthew A. Thacker[†], Reece Doughty[‡], Russell Timkovich[†], and Laura S. Busenlehner^{†,*}

[†]Department of Chemistry, The University of Alabama, Tuscaloosa, Alabama 35487-0336, United States

Abstract

Human frataxin has a vital role in the biosynthesis of iron–sulfur (Fe-S) clusters in mitochondria and its deficiency causes the neurodegenerative disease Friedreich’s ataxia. Proposed functions for frataxin in the Fe-S pathway include iron donation to the Fe-S cluster machinery and regulation of cysteine desulfurase activity to control the rate of Fe-S production, although further molecular detail is required to distinguish these two possibilities. It is well established that frataxin can coordinate iron using glutamate and aspartate side chains on the protein surface; however, in this work we identify a new iron coordinating residue in the N-terminal region of human frataxin using complementary spectroscopic and structural approaches. Further, we demonstrate that His86 in this N-terminal region is required for high affinity iron coordination and iron assembly of Fe-S clusters by ISCU as part of the Fe-S cluster biosynthetic complex. If a binding site that includes His86 is important for Fe-S cluster synthesis as part of its chaperone function, this raises the possibility that either iron binding at the acidic surface of frataxin may be spurious or that it is required for protein–protein interactions with the Fe-S biosynthetic quaternary complex. Our data suggest that iron coordination to frataxin may be significant to the Fe-S cluster biosynthesis pathway in mitochondria.

Keywords

frataxin; ISCU; iron-sulfur cluster synthesis; iron binding

Many neurodegenerative diseases are linked to imbalances in metal homeostasis. The autosomal recessive neuromuscular disorder Friedreich’s ataxia (FA) is one such example. FA is primarily caused by a (GAA)_n triplet repeat expansion in the first intron of the frataxin gene.¹ This results in decreased transcription and/or translation of the gene and, therefore, significantly reduced levels of frataxin in mitochondria of the heart, spinal cord and other tissues. FA is characterized by a marked increase in mitochondrial iron, oxidative stress, decreased activities of enzymes with iron-sulfur clusters (Fe-S) and electron transport chain

*Corresponding Author. Phone: (205) 348-0269. Fax, (205) 348-9104. LSBusenlehner@bama.ua.edu.

[†]Present Addresses

Resident Physician in Internal Medicine, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA 90095.

ASSOCIATED CONTENT

Supporting Information Available. Figure S1 (NMR chemical shift and intensity Co²⁺ binding isotherms with and without Mg²⁺). Figure S2 (Changes in intensity and chemical shift upon addition of Mg²⁺). Figure S3 (HDX-MS rate profiles for all frataxin peptides). Figure S4 (Analytical size exclusion chromatography of frataxin with Co²⁺ and Fe³⁺). Figure S5 (Fluorescence titrations of wild type and H86A frataxin with ISCU). Figure S6 (Frataxin sequence alignment and overlaid human and yeast frataxin structures). Table S1 (HDX-MS rate constants and amplitudes for peptides from Figure S3). Table S2. (Analytical size exclusion calculated molecular weights from Figure S4). This material is available free of charge via the Internet at <http://pubs.acs.org>.

dysfunction. Cells from FA patients indicate cytosolic iron deficiency even though iron levels are highly elevated in mitochondria.²

The function of frataxin has been debated since it was first linked to FA in 1996. By far, the most convincing role is one in Fe-S cluster biosynthesis. Heart biopsies of FA patients reveal decreased activities of Fe-S enzymes in the electron transport chain and in the tricarboxylic acid cycle. The same enzyme deficiencies were observed in *Saccharomyces cerevisiae* strains deficient in Yfh1, the frataxin homologue in yeast, and in other model systems.³ It is apparent that, in some way, frataxin deficiency disrupts production of Fe-S clusters. Fe-S clusters are assembled in the mitochondria of eukaryotic cells through via the ternary Fe-S cluster biosynthetic complex, which consists of the cysteine desulfurase NFS1, the Fe-S scaffold protein ISCU, and the accessory protein ISD11.⁴ Frataxin physically and functionally interacts with ISCU, NFS1 and ISD11; therefore, it is suggested to be a member of the Fe-S complex.⁵ Some research suggests that frataxin is an iron-chaperone that delivers Fe²⁺ to the Fe-S cluster complex.⁶ *In vitro*, frataxin stimulates the rate of formation of [2Fe-2S] clusters on the surface of ISCU with externally supplied sulfide⁷ and photo-activated chemical crosslinking⁸ confirms the interaction between frataxin and ISCU. Other studies indicate that frataxin may regulate Fe-S cluster synthesis through its stimulation of NFS1 cysteine desulfurase activity as part of the ISCU/NFS1/ISD11 complex.^{9,10} For either case, iron is delivered directly or indirectly through NFS1 activation to the ternary ISCU/NFS1/ISD11 Fe-S machinery for cluster synthesis.

The structures of monomeric apo-frataxin have been solved for the human, yeast and bacterial homologues, revealing a unique and conserved fold.¹¹ One striking feature of all frataxin homologues is a clustering of negatively charged aspartate and glutamate residues on one surface of the protein called the “acidic ridge”. NMR studies indicated metal binding at or near these residues in the human^{12,13}, yeast^{14,15}, and bacterial frataxin homologues¹³. The three-dimensional structures of various frataxin proteins have provided much needed structural information, but there is still ambiguity in the coordination environment since there is no structure with iron bound. There is also uncertainty with respect to the Fe²⁺ stoichiometry. Part of the ambiguity lies in the ability of frataxin to coordinate most divalent metal ions using the acidic residues on the surface.¹⁶ Additionally, reducing agents and some buffers also chelate metal ions and many frataxin studies employed these competitors in excess over the frataxin concentration to keep iron in a reduced state. Thus the stoichiometries and measured dissociation constants could be affected by these chemical components. From various experiments, it appears that human frataxin contains 1–2 Fe²⁺ binding sites with nanomolar to micromolar dissociation constants¹⁷. The locations of the actual binding sites are assumed to be along the acidic ridge and into the β1-strand, based on NMR iron titrations of frataxin.^{13,14,18} However, mutation of specific aspartate and glutamate residues within the acidic ridge affect frataxin binding with ISCU, the scaffold for Fe-S cluster synthesis, but does not change the total number of Fe²⁺ binding sites.⁹ This may indicate that metal binding at the acidic ridge is superfluous and that there may be additional, specific Fe²⁺ binding sites that are not readily identifiable by current techniques.

Despite many years of research there are still three main questions about frataxin iron binding: (1) what is the stoichiometry of Fe²⁺ binding to human frataxin?; (2) what residues are involved in coordination?; and (3) which of these Fe²⁺ sites are functionally relevant for Fe-S cluster formation on ISCU? To address these questions, we characterized the metal coordination environment and stoichiometries using Fe²⁺, Fe³⁺ and Co²⁺, which are common surrogates to probe Fe²⁺ sites in proteins because they have characteristic electronic absorptions based on their environment and are not sensitive to aerobic conditions.¹⁹ These properties make Co²⁺ and Fe³⁺ amenable to UV-visible and NMR spectroscopies. Since conformational or dynamics changes in frataxin should accompany

metal binding, backbone amide hydrogen/deuterium exchange mass spectrometry (HDX-MS) was used to characterize structural perturbations in solution. Herein, we report that frataxin binds ~3 metal ions and that one of these binding sites uses a previously unknown residue (His86) from the disordered N-terminal tail, which is functionally required for Fe²⁺ delivery to ISCU for Fe-S synthesis *in vitro*.

EXPERIMENTAL PROCEDURES

Materials

ACS-grade HEPES, dioxane-free IPTG, HPLC grade water and HPLC grade acetonitrile were obtained from EMD Millipore (Billerica, MA). Deuterium oxide (99.99% *at.D*), ACS-grade Bis-Tris and ferrozine were from Acros Organics (Morris Plains, NJ). Ultrapure magnesium chloride and ferric chloride were by Fisher Chemical (Fairlawn, NJ). Deuterated *d*₁₈-HEPES and ¹⁵N-ammonium sulfate were obtained from Cambridge Isotopes (Andover, MA). Other reagents included porcine pepsin (3,200–4,500 units/mg) from Sigma-Aldrich (St. Louis, MO), HPLC grade 2-propanol from Honeywell (Franklin, PA), cobaltous chloride from Macron Chemicals (Center Valley, PA), ferrous ammonium sulfate from MP Biomedical (Santa Ana, CA), and EDTA from BDH Merck (Radnor, PA).

Purification of Human Frataxin

Human frataxin corresponding to residues 81–210, or the mature form, was expressed in *E. coli* BL21(DE3)*pLysS* from *pET81-210Fxn*, as described.⁸ Purification was performed in the presence of 2 mM EDTA in Chelex-treated, metal-free water. All glassware was rinsed with 10% nitric acid and metal-free water to remove trace metals. Frataxin concentrations were determined spectroscopically using $\epsilon_{280\text{nm}} = 26.93 \text{ mM}^{-1}\text{cm}^{-1}$. The amount of zinc and iron in purified frataxin was determined via an AAnalyst 400 atomic absorption spectrometer (Perkin-Elmer, Waltham, MA) and was negligible.

UV-Visible Titrations

Titrations were performed on an Agilent 8453 spectrophotometer (Santa Clara, CA). For cobalt titrations, 300–400 μM frataxin in Chelex-treated 50 mM HEPES (pH 7.4), 400 mM NaCl, and 5% glycerol was titrated with a cobalt chloride stock solution in the same buffer. Co²⁺ was added in 5–10 μL increments, incubated for 5 min at 23°C, and UV-visible spectra were collected from 200–800 nm. Each spectrum was corrected for dilution. Colorimetric Fe²⁺ titrations required ferrozine, a Fe²⁺ specific chelator. All solutions were degassed and prepared in a Vacuum Atmosphere anaerobic glovebox. A 0.53 M stock of Fe²⁺ was made in 25 mM HEPES (pH 7.4), 150 mM NaCl. A 6.5 mM ferrozine stock was prepared in the same buffer with 2.5 M ammonium acetate. Samples of 108 μM with and without 13 μM apo-frataxin were made with increasing Fe²⁺ concentrations and spectra collected from 240–800 nm. The Fe²⁺–ferrozine₃ complex has a molar extinction at 562 nm of 27.9 mM⁻¹cm⁻¹ and a formation constant (K_f) of 3.65×10¹⁵ M⁻³.²⁰

Fluorescence Titrations

Intrinsic tryptophan fluorescence titrations were performed at 23°C using a Spex Fluoromax-3 fluorimeter (Edison, NJ) with ~5 μM frataxin in either 50 mM HEPES (pH 7.4), 400 mM NaCl, 5% glycerol (Co²⁺ titrations) or 50 mM Bis-Tris (pH 7.4), 400 mM NaCl, and 5% glycerol (Fe³⁺ titrations) using an excitation wavelength of 295 nm. The metal titrant concentrations were determined by atomic absorption. After each addition of metal, the sample was equilibrated for 3 min with stirring at 23°C. Fluorescence emission spectra were collected from 270–450 nm and corrected for dilution. Since the addition of Fe³⁺ leads to increased absorbance in this region of the spectrum, the fluorescence data were

corrected for inner filter effect with an equivalent UV-vis titration of frataxin under the same conditions. This procedure was repeated with Co²⁺ for experimental continuity. The corrected fluorescence intensity was determined using eq 1.²¹

$$F_{\text{corrected}} = F_i \times 10^{\left(\frac{\text{Abs}_{290\text{nm}} + \text{Abs}_{343\text{nm}}}{2}\right)} \quad (1)$$

The initial fluorescence intensity (F_0) at the emission maximum wavelength was subtracted from each titration point ($F_i - F_0$), which was plotted against total metal concentration. The binding curves are stoichiometric (*i.e.*, no “free” metal exists during the titration); therefore, the equilibrium binding constants obtained from fitting are not representative of the true K_d values and are not reported.²¹

Nuclear Magnetic Resonance Spectroscopy

One L of M9 minimal media supplemented with 1 g/L 86% ¹⁵N-ammonium sulfate was inoculated, as described, and grown at 37°C to OD₆₀₀ = 0.8, then induced with 1 mM IPTG for 4 hours. Cells were harvested at OD₆₀₀ 1.8–2.0 and purified as described except the final buffer was Chelex-treated 25 mM *d*₁₈-HEPES (pH 7.0) with 100 mM NaCl. MALDI-TOF mass spectrometry confirmed ~85% ¹⁵N-enrichment in the purified NMR sample. Co²⁺, Mg²⁺ and Fe²⁺ titrant stocks were made in the *d*₁₈-HEPES buffer at pH 7.0 and their concentrations determined by atomic absorption.

¹H-¹⁵N HSQC spectra were collected for 0.56 mM apo-¹⁵N-frataxin with 5% (v/v) D₂O. Resonances were assigned according to reference 22. Titrations from 0.2–6.0 molar equivalents of Co²⁺ and Mg²⁺ were performed aerobically. Individual NMR samples containing 1.0–4.0 molar equivalents of Fe²⁺ were prepared anaerobically. NMR data were collected at 298 K on a Bruker Avance 600 MHz spectrometer (Fremont, CA) with a triple resonance ¹H/¹³C/¹⁵N probe equipped with *z*-axis pulsed field gradients. The fingerprint main chain amide region was recorded by the two dimensional ¹H-¹⁵N-HSQC experiment²³ using the standard Bruker pulse program. Data collection time per spectrum ranged from 1.5 hr to 20 hr to improve signal-tonoise ratios. Data were processed using NMRPipe²⁴ and SPARKY (T. D. Goddard and D. G. Kneller, University of California, San Francisco). The data matrix consisted of 2048 points in the proton dimension centered on the water resonance and 128 points in the nitrogen dimension centered at 117 ppm. Data in both dimensions were apodized with a shifted squared sine bell function and zero-filled prior to Fourier transformation. After transformation, data in both directions were baseline corrected with the default polynomial function of NMRPipe. Normalized amide chemical shifts (δ_{NH}) were calculated using the change in chemical shift from apo-frataxin values of the ¹H (δ_{H}) and ¹⁵N (δ_{N}) resonances upon each addition of metal as shown in eq 2, modified from ref²⁵.

$$\delta_{\text{NH}} = \sqrt{\left(\delta_{\text{H}}\right)^2 + \left(\frac{\delta_{\text{N}}}{5}\right)^2} \quad (2)$$

Amide Hydrogen/Deuterium Exchange Mass Spectrometry

Twenty micrograms of frataxin was added to 100 μL 0.1 M potassium phosphate (pH 2.3) containing 20 μg pepsin. After a 5 min digestion on ice, the sample was separated on a 1 mm × 50 mm C18 column (Phenomenex, Torrence, CA) equilibrated at 0.1 mL/min by a ThermoFinnigan Surveyor HPLC connected to a TSQ Quantum mass spectrometer (San Jose, CA). Peptides were eluted with a 2%–50% acetonitrile gradient in water with 0.4% formic acid over 20 min and subjected to data-dependent tandem MS/MS collision-induced dissociation in positive-ion mode. Peptide assignments were made through Protein

Prospector (<http://prospector.ucsf.edu/>) and Peaks.²⁶ The complete map is shown in Figure 1.

The aerobic HDX reactions were performed in triplicate with frataxin containing 0, 1, 2 and 3 molar equivalents of either Co²⁺ or Fe³⁺. Deuterium exchange reactions in 0.2 mL thin-walled tubes containing 5 µL of 580 µM frataxin (30 µM) were initiated with 45 µL deuterium oxide (99.99% *at.* D₂O). Samples were incubated at 23°C between 15s–6h. Exchange was quenched with 50 µL of 0.1 M potassium phosphate (pH 2.3; 0°C) and placed on ice. After 10 sec, 2 µL of 20 µg/µL pepsin was added and incubated 5 min at 0°C. For HDX with reduced Fe²⁺, we performed all steps in an anaerobic glovebox (<0.1 ppm O₂) using highly degassed stock solutions. Iron was prepared fresh (from solid) inside the glovebox and the concentration of reduced Fe²⁺ was measured anaerobically with a bathophenanthrolinedisulfonate (BPS) colorimetric assay. The total concentration of iron was determined by atomic absorption. Our Fe²⁺ stocks contained <5% of contaminating Fe³⁺. Deuterium exchange reactions in 0.2 mL thinwalled tubes containing 5 µL of 580 µM frataxin (30 µM) with 1, 2, 3 and 4 equivalents of Fe²⁺ were initiated with 45 µL deuterium oxide (99.99% *at.* D₂O). Samples were immediately sealed with parafilm and removed from the glovebox. Addition of quench solution and pepsin digestion was as above. The exposure to air upon addition of acidic quench is appropriate since the exchange reaction will have essentially stopped. As a control test for iron oxidation, mock samples of Fe²⁺-frataxin in H₂O were incubated for 15 min, returned to the glovebox for BPS analysis which revealed, on average, that 16% Fe³⁺ was present. Additional, longer incubations were not performed.

Pepsin-generated peptides were separated by HPLC, as described, except that the solvent bottles, injector and column were submerged in an ice bath and the elution was 2%–35% acetonitrile over 10 min. Mass spectra were recorded using positive ion electrospray ionization with a capillary temperature of 190°C and a spray voltage of 3.5 kV. A control to account for natural isotope abundance (m_0) was prepared as other samples except that H₂O was used instead of D₂O.²⁷ Another control to correct for deuterium loss during chromatography (m_{100}) required frataxin to be completely exchanged for deuterium (m_{100}). This was done by incubating 5 µL of 580 µM frataxin with 45 µL of D₂O for at least 5 hr at 60°C.²⁷ The sample was then quenched and digested, as described. Deuterium loss during HPLC varied from 18–36%. HDX–MS data were averaged and processed using by HDExaminer (Sierra Analytics, Modesto, CA). The percent deuterium incorporation (%D) was calculated using eq 3

$$\%D = \left(\frac{m_t - m_0}{m_{100} - m_0} \right) \times 100 \quad (3)$$

where m_t is the centroid value of the peptide at time t , m_0 is the non-deuterated peptide and m_{100} is the fully deuterated peptide.²⁷ The percentage of deuterium incorporated was plotted and fit to single-, double-, or triple-exponential equations to give the percentage of amide protons exchanging for deuterons (%D_n) at a given rate constant (k_n).²⁸ Amides that exchange faster than $k_n = 4 \text{ min}^{-1}$ are considered the fast phase. Those than have rate constants slower than $k_n = 1 \times 10^{-5} \text{ min}^{-1}$ are considered “non-exchangeable” under our experimental conditions.

Mutagenesis of H86A in Frataxin

The codon for His86 in *pET81-210Fxn* was mutated to Ala via site-directed mutagenesis using QuikChange Lightning (Agilent, Santa Clara, CA). H86A frataxin was expressed in BL21(DE3)*pLysS* and purified as for the wild-type protein.⁸ Co²⁺ and Fe³⁺ titrations monitored by UV-visible spectroscopy of H86A frataxin were performed as for wild-type.

The Fe^{2+} titration of H86A frataxin in the presence of ferrozine chelator was also as described for the wild-type frataxin protein.

Purification of D37A ISCU

The *pET-mISCU* plasmid²⁹ was graciously obtained from Dr. Tracey Rouault (National Institutes of Health, Bethesda, MD). The codon for Asp37 was mutated to that for Ala and a stop codon was introduced after the codon for Lys133 using QuikChange mutagenesis (Agilent, Santa Clara, CA). D37A ISCU was expressed in BL21(DE3)*pLysS* and purified as described for native ISCU.⁸ D37A ISCU samples were anaerobically dialyzed into Chelex-treated 50 mM HEPES (pH 7.4), 150 mM NaCl with 10% glycerol. The concentration of ISCU was determined spectrophotically using $\epsilon_{280\text{nm}} = 9.97 \text{ mM}^{-1}\text{cm}^{-1}$.

Fe-S Cluster Assembly Assays

All reactions were performed in an anaerobic cuvette fitted with a gas-tight syringe before removal from the glove box. The reaction buffer was 25 mM HEPES (pH 7.4) with 150 mM NaCl. Frataxin or H86A frataxin (100 μM) was incubated with 200 μM ferrous ammonium sulfate (2 Fe^{2+} :1 frataxin) for 30 minutes at 25°C. The reaction containing 100 μM D37A ISCU and 2.4 mM sodium sulfide was initiated by frataxin with 2 equivalents of Fe^{2+} and was monitored at 426 nm using an Agilent 8453 spectrophotometer (Santa Clara, CA) in kinetics mode.⁷ Controls included D37A ISCU with 200 μM ferrous ammonium sulfate (no frataxin), as well as with 200 μM Fe^{2+} bound to bovine serum albumin (BSA). The data were fit to a first order rate equation (eq 4) to determine the initial rate of Fe-S cluster assembly,

$$\text{rate} = A_1 \cdot e^{-k_1 t} \quad (4)$$

where A_1 is the amplitude of the first order rate, k_1 , as a function of time, t .

RESULTS

Metal coordination environment and stoichiometry

Mature human frataxin (residues 81–210) lacks cysteine residues, therefore the Fe^{2+} -frataxin complex does not have electronic transitions within the visible range.³⁰ Experiments with ferrous iron require rigorous anaerobic or reducing conditions. Because many reducing agents can coordinate metals and exhibit metalbased absorptions, they should be excluded in binding experiments.³¹ However, Co^{2+} is a common surrogate to aerobically probe Fe^{2+} binding sites in proteins and has electronic absorptions in the UV-visible region.¹⁹ The Co^{2+} titration of frataxin reveals broad, overlapping transitions centered at 532 nm and 485 nm after 1 equivalent of Co^{2+} , which is consistent with oxygen and/or nitrogen-coordination.³² As additional Co^{2+} binds to frataxin, there is a blue shift to 511 nm and 466 nm, which could indicate more oxygen-based coordination.³³ The low molar absorptivities for the Co^{2+} *d-d* transitions ($\epsilon < 15 \text{ M}^{-1}\text{cm}^{-1}$) indicate octahedral geometry.³² The Co^{2+} binding isotherm for frataxin is more linear in shape than hyperbolic, which is characteristic for stoichiometric binding, so ~3 Co^{2+} binding sites were estimated (Figure 2). To further confirm the stoichiometry, metal titrations of frataxin, which contains 3 tryptophan residues, were followed by intrinsic tryptophan fluorescence. The emission (λ_{max} at 343 nm) is quenched by Co^{2+} and saturates at ~3 equivalents (Figure 3A), consistent with UV-visible titrations. Frataxin was also titrated with Fe^{3+} and displayed saturation after ~3 molar equivalents of Fe^{3+} (Figure 3B).

To estimate apparent affinities for ferrous iron, samples containing the Fe^{2+} -specific colorimetric chelator ferrozine, with and without frataxin, were incubated with increasing

concentrations of Fe^{2+} (Figure 4). The Fe^{2+} -ferrozine₃ (1:3) complex absorbs at 562 nm ($\epsilon_{562} = 27.9 \text{ mM}^{-1}\text{cm}^{-1}$) with an apparent K_f of 10^{15} M^{-3} .²⁰ The sample containing frataxin clearly binds one equivalent of Fe^{2+} before the purple color of Fe^{2+} -ferrozine₃ forms (Figure 4). This indicates that frataxin possesses one Fe^{2+} site of higher affinity than ferrozine, but the remaining two sites are of lower affinity than ferrozine.

HSQC NMR Co^{2+} and Fe^{2+} Titrations

To gain more insight into the localization of the surrogate metal compared to the native metal, ¹H-¹⁵N HSQC spectra were collected from uniformly ¹⁵N-labeled apo-frataxin titrated with Co^{2+} (aerobic) and with Fe^{2+} (anaerobic). Residues 81–90 were not observed due to intrinsic disorder.²² Co^{2+} and Fe^{2+} are paramagnetic so through-bond coupling of the metal free electrons and protein nuclei lead to large chemical shifts for amide nitrogens and protons near a bound metal, as well as line broadening of nuclei in close proximity.³⁴ It is assumed that MgCl_2 , which is often used to reduce adventitious metal binding, could be included in Co^{2+} and Fe^{2+} titrations because diamagnetic metals like Mg^{2+} do not strongly affect chemical shifts.

Aerobic Co^{2+} binding to frataxin with and without 10 mM Mg^{2+} was investigated to assess its ability to alleviate non-specific metal binding. It is observed that inclusion of 10 mM Mg^{2+} alters Co^{2+} binding isotherms for several residues within $\alpha 1/\beta 1$ (Figure S1, Supporting Information). Thus, all subsequent NMR titrations were performed *without* Mg^{2+} . With increasing concentrations of Co^{2+} in the absence of Mg^{2+} , frataxin NMR resonances show significant line broadening. By ~3 equivalents of Co^{2+} , resonances from the acidic $\alpha 1$ helix (D104, S105, E108, D112, L113, A114, D115) and others near it (Y118, V125, F127, V131) are no longer observable (Figure 5A). These residues are most likely within 8–15 Å of the bound Co^{2+} ions.³⁴ A plot of the change in normalized amide chemical shift (δ_{NH}) shows clustered shifting between residues 103–117 and 122–133, which are localized to $\alpha 1$ -loop- $\beta 1$. There are also smaller shifts for residues at the beginning of $\alpha 1$ (D91, A99).

We then compared the Co^{2+} HSQC spectra to those of Fe^{2+} collected anaerobically. High-spin Fe^{2+} broadens and shifts resonances within 5–7 Å of bound iron.³⁴ As shown in Figure 5B, the largest changes in normalized amide chemical shift after 2 equivalents of Fe^{2+} localize to $\alpha 1$ (D104, S105, D112, L113, A114, D115) and into $\beta 1$ (T119, D122, D124, V125). With 3 equivalents of Fe^{2+} , D91, D104, and D122 undergo further chemical shifting. Many residues in $\alpha 1$ broaden but only D112, L113, and D115 broaden beyond detection at 3 molar equivalents of Fe^{2+} . It is important to note that no significant changes in resonance intensities or chemical shift are observed beyond 3 equivalents of Co^{2+} or Fe^{2+} .

HDX-MS

While NMR experiments have yielded valuable information about the localization of metal binding sites, it is still unclear whether frataxin undergoes changes in conformation or dynamics upon metal binding since there is no three dimensional structure of any frataxin with metal bound. These changes could be important for interactions with potential protein partners such as ISCU and NFS1. We performed HDX-MS on apo-frataxin and frataxin with 1, 2, and 3 molar equivalents of Co^{2+} , Fe^{2+} , or Fe^{3+} . HDX-MS monitors the rate of deuterium incorporation into amides within the protein backbone. The amount of deuterium exchanged into the backbone within the first 15 sec of incubation mainly reports on accessibility of the amide hydrogen to D_2O and is a good indicator of secondary structure content (*i.e.* amide hydrogen bonding). Amides that exchange on a slower timescale are best attributed to dynamic motions and local unfolding/folding processes.²⁸ Deuterium

incorporation as a function of time is detected through increased masses (*m/z*) of pepsin-digested peptides from exchange of ^1H with ^2H .

First, we investigated whether Co^{2+} , Fe^{2+} (~5% Fe^{3+}), and Fe^{3+} altered the solvent accessibility of amide hydrogens and whether the effects were metal specific. In general, decreased deuterium incorporation with one equivalent of each metal was slight compared to that of apo-frataxin. The differences in HDX become more pronounced with the second and third equivalents of metal, which indicates distribution between all three binding sites. No additional changes in HDX are observed beyond 3 equivalents of metal. The percent change amide deuteration with 2 and 3 equivalents of metal are shown in Figure 6. Peptides from the N-terminus (residues 81–91), in $\beta 1$ (residues 122–127), and within the $\beta 4$ – $\beta 5$ strands (residues 156–172) showed reduced deuterium incorporation for all metal ions. Fe^{3+} also displayed a change within residues 128–132 that was not observed with Fe^{2+} or Co^{2+} . The third equivalent of metal further protected amides within $\alpha 1$ and $\beta 1$ (81–91, 91–98, 110–121 and 122–127). These peptides are mapped on the frataxin structure with the exception of residues 81–90, which were not observed in the NMR structure (Figure 6 B–G).¹² It is also interesting to note that metal binding deuterium exchange in the $\beta 4$ – $\beta 5$ strands, which are thought to interact with the Fe-S cluster machinery.¹² This indicates that metal coordination affects frataxin conformation beyond the immediate binding vicinity.

In addition to changes in amide D_2O accessibility, HDX rates can be correlated to altered local dynamics or structure upon metal binding that could be functionally relevant. We compared the HDX progress curves for only Co^{2+} and Fe^{3+} -frataxin samples. The signal to noise of the mass spectra for Fe^{2+} -frataxin decreased as a function of time presumably from iron oxidation, and was not pursued further. HDX deuterium incorporation rate profiles for all pepsin generated peptides with both Co^{2+} and Fe^{3+} were comparable (Figure S3 in Supporting Information), but only Fe^{3+} progress curves are shown (Figure 7). Addition of 2 and 3 molar equivalents of Fe^{3+} to frataxin leads to a conformational change in the $\beta 4$ – $\beta 5$ sheets (peptide 156–172) that affects protein dynamics and solvent accessibility. Other notable decreases in rate were observed for the N-terminus (peptide 81–91) and $\alpha 1$ – $\beta 1$ (peptides 91–98, 99–103, 110–123). Given that the N-terminus is disordered, we sought to better understand why residues 81–91 displayed such a large change in solvent accessibility and conformational dynamics upon metal binding. It is possible that residues in the N-terminus could be involved in metal-promoted aggregation, as observed in Yfh1 studies³⁵, or direct metal coordination.

Mutagenesis of His86 in the N-terminal region

Analytical size exclusion chromatography with frataxin pre-incubated with up to 10 molar equivalents of Co^{2+} or Fe^{3+} does not indicate stable oligomerization (Figure S4, Supporting Information), consistent with other studies.³⁶ To test for direct metal coordination, site-directed mutagenesis of potential metal binding ligands was pursued. Within residues 81–91, His86 is the only residue to commonly coordinate metals so His86 was mutated to alanine and characterized. Co^{2+} absorption spectroscopy is sensitive enough to detect changes in coordination environment.³² The *d*-*d* transitions for H86A frataxin with Co^{2+} shift to higher energies and with decreased molar absorptivity, compared to wild-type frataxin (Figure 8A). Analysis of the Co^{2+} binding isotherm for H86A frataxin reveals saturation after ~2 equivalents (Figure 8B); therefore, substitution of His86 leads to loss of one metal binding site. H86A frataxin was also titrated with Fe^{2+} in the presence of ferrozine chelator (Figure 8C,D). In contrast to wild-type, ferrozine out-competes H86A frataxin for Fe^{2+} . This demonstrates that His86 is a ligand in the higher affinity Fe^{2+} site observed for the wild-type protein.

Fe-S Cluster Assembly

Although mutagenesis of His86 resulted in a loss of a single metal binding site, it was not clear whether this had functional relevance. ISCU is a Fe-S scaffold protein that assembles [2Fe-2S] clusters on its surface using free Fe^{2+} and sulfide. The presence of iron-bound frataxin enhances the rate of cluster assembly *in vitro*.⁷ This assay was used to determine if mutation of His86 affected the rate of Fe-S cluster synthesis (Figure 9). In the presence of free Fe^{2+} , ISCU assembled a cluster with a basal initial rate of 0.018 s^{-1} ; however, the rate was ~4-fold greater in the presence of wild-type frataxin pre-incubated with 2 equivalents of Fe^{2+} (0.074 s^{-1}). When H86A frataxin with 2 equivalents of Fe^{2+} was used, the rate of Fe-S formation is similar to the basal level (0.018 s^{-1}). Incubation of H86A frataxin with >2 equivalents of Fe^{2+} did not increase the rate of Fe-S synthesis (data not shown). These data suggest that the H86A mutation negates the stimulatory effect by frataxin on Fe-S cluster biogenesis. To elucidate if the loss of Fe-S rate enhancement from the H86A mutation could be from decreased affinity for ISCU, intrinsic tryptophan fluorescence quenching titrations of ISCU into frataxin were performed with and without Fe^{3+} , as described.³⁷ The fluorescence of wild type and H86A frataxin was quenched by ISCU only in the presence of Fe^{3+} (Figure S5, Supporting Information). H86A only had a 2-fold lower K_D for ISCU than for wild type frataxin. Together, these studies show that His86 is important for iron binding and in the formation of Fe-S clusters by ISCU *in vitro*, but does not seem to play a direct role in ISCU binding.

DISCUSSION

Human frataxin is a unique Fe^{2+} -binding protein in that the mature form does not contain cysteine residues, which are preferred based on hard-soft acid base theory.³⁸ Instead, a cluster of aspartate and glutamate residues along one face of $\alpha 1$ helix creates multiple binding sites for Fe^{2+} (Figure 1).¹¹ This region has hampered direct determination of frataxin metal stoichiometry from a variety of approaches since its interactions with metals could also be non-specific. For a better understanding of frataxin molecular interactions, metal coordination as related to structure and function was investigated. From this study, we propose that His86 is a previously unidentified iron ligand and that it plays a functional role in Fe-S synthesis by the scaffold protein ISCU.

Localization of metal binding sites in frataxin using paramagnetic NMR and HDX-MS

Our studies cumulatively suggest that mature frataxin can bind up to 3 metal ions. One Fe^{2+} binding site has higher affinity than the other 2 sites based on chelator competition assays. In these studies, a significant population of “free” metal exists so differences in affinity can be discerned. In other experiments, the frataxin concentration is high enough that stoichiometric binding was observed. It also led to metal distribution among all 3 sites. At this time, it is not possible to determine if the two weaker metal binding sites observed in this study are nonspecific because actual equilibrium binding constants have not been determined. Further functional roles for these residues must be ascertained. The HSQC NMR metal titrations in the presence of Mg^{2+} attempted to control non-specific metal coordination, but Mg^{2+} competed with Co^{2+} for binding at or near acidic residues within $\alpha 1$ -loop- $\beta 1$ (Figure S1, Supporting Information). This suggests that electrostatic interactions with the acidic $\alpha 1$ helix are possible.¹⁸ Some of these acidic residues are important for interaction with ISCU and other components of the Fe-S machinery, and not for iron binding.⁹

When the Co^{2+} and Fe^{2+} NMR results with 81–210 frataxin are compared with those for truncated human frataxin (residues 91–210), *S. cerevisiae* Yfh1, and *E. coli* CyaY, similarities are observed that allow for the assignment of several potential ligands (Figure

10). D112 and D115 from the α 1 helix are likely metal coordinating residues since their NMR resonances were broadened beyond detection with Co^{2+} and Fe^{2+} in our experiments and other homologues (Figure 10).^{13,14,18} Acidic residues at positions 112 and 115 are conserved in most eukaryotic frataxins.¹¹ HDX–MS studies showed that the peptide containing D112 and D115 (110–123) incorporated less deuterium into the backbone in the presence of metal (Figure 7). In combination with NMR results, this is consistent with reduced solvent accessibility and dynamics from metal coordination.³⁹

HDX–MS also revealed that amides within residues 122–127 in β 1 were protected from D_2O in the presence of metal without a measurable change in the rate of uptake. In other words, metal binding does not significantly perturb structure in β 1. Several residues in this region had altered NMR chemical shifts and broadening in the presence of Co^{2+} and Fe^{2+} , including D122 and D124, but the magnitude was smaller than D112 and D115. D122 and D124 are highly conserved and appear to coordinate Co^{2+} and Fe^{2+} in human, *S. cerevisiae*, and *E. coli* frataxins (Figure 10).^{11,13,14} Interaction studies of mutant frataxin proteins with the ISCU/NFS1/ISD11 ternary complex showed that D124 of frataxin was essential for interaction.⁹ It is not apparent if iron coordination to D124 mediates the interaction or if the residue is available to coordinate iron when frataxin is bound to the Fe-S machinery. It has been noted that “free” frataxin binds less iron than and frataxin in complex with ISCU/NFS1/ISD11, supporting the latter prediction, but more specific binding studies are required.¹⁰

Large decreases in solvent accessibility and slower rates of deuterium incorporation were also observed for peptides 81–91 (N-terminus) and 91–98 (beginning of α 1). In fact, 81–91 had the largest overall decrease in D_2O accessibility of any peptide, especially at 2 equivalents of metal. Resonances of residues 81–90 from the disordered N-terminus were not observed in NMR structures of human frataxin so metal coordination to residues within 81–90 could not be obtained.¹² Thus, further studies were required to ascertain if residue(s) in the N-terminal region can coordinate metals.

His86 is a potential metal ligand and is required for Fe-S synthesis by ISCU

Investigation into why the N-terminus showed a conformational response with metal suggested that it was from direct metal coordination. Specifically, mutation of His86 to alanine led to loss of one of the three identified metal binding sites and a shift in the Co^{2+} $d-d$ transitions, suggesting a change in coordination environment such as loss of a nitrogen-containing ligand.^{32,33} Ferrozine competition assays suggested that the higher affinity Fe^{2+} binding site was lost in the H86A mutant. It has been assumed that because the N-terminal “extension” before α 1 is not found in bacteria and has weak conservation in eukaryotes, that residues within it are not functionally relevant.¹¹ Our studies revealed a functional role for His86 in Fe-S synthesis *in vitro* since H86A frataxin did not stimulate Fe-S cluster biogenesis by ISCU. This could be due, in part, to impaired binding to ISCU. Sequence alignments reveal a lack of conservation of His86 across all frataxin homologues. Some higher eukaryotes and yeast have a metal coordinating residue such as histidine, glutamate, or aspartate aligned with human frataxin residue His86 (Supporting Information Figure S6A), which is intriguing, but unclear if it is related to intracellular function at this time.¹¹ Clearly, research into the *in vivo* relevance of His86 is required.

It is well established that the N-terminus of eukaryotic frataxins is processed for mitochondrial localization, leaving the N-terminal extension before α 1.⁴⁰ The intermediate processed form of frataxin (residues 46–210) undergoes iron-mediated self-cleavage to produce mature frataxin. It is intriguing that the intermediate form had one high affinity Fe^{2+} binding site ($K_d = 300 \text{ nM}$).⁴¹ Iron-dependent cleavage of the N-terminal domain to form 78–210 frataxin led to additional, lower affinity iron binding sites. Yoon *et al.*

proposed that the unprocessed N-terminus in 46–210 frataxin could interact with the α 1 carboxylate residues to block iron binding along the acidic ridge.⁴¹ This observation is somewhat consistent our results showing ~3 Fe²⁺ binding sites in 81–210 frataxin, one with higher affinity. There is no structure of the human intermediate with the extended N-terminus; however, a structure of the intermediate form of yeast frataxin, Yfh1 (residues 53–174, yeast numbering), revealed that the disordered N-terminus (residues 53–69, yeast numbering) extended over α 1 (Supporting Information, Figure S6B).¹⁴ Based on this structure and a protein alignment (Supporting Information, Figure 6A), His86 in human 81–210 frataxin would be too far away from residues D112, D115, D122, and D124 to form a binding site, but it is within interaction distance to residues at the beginning of α 1 such as D91. Our Co²⁺ and Fe²⁺ NMR titrations indicated broadening and shifting of resonances at the beginning of α 1 (D91, T93) of about the same magnitude as those for D122 and D124. Our results warrant further study into the role of His86 and potentially other residues near the N-terminus that could form an iron binding site.

Conclusions

Using a variety of spectroscopic and structural approaches, we describe the identification of His86 as a new iron coordinating residue in the unstructured N-terminus of human frataxin. Further studies revealed that His86 is required for efficient Fe-S cluster synthesis by ISCU; however, additional mutagenesis and *in vivo* characterization is required to further clarify the biological role of this residue in the synthesis of Fe-S clusters. Our results question the functional relevance of iron binding at residues within the acidic α 1 helix and support the possibility that residues in the N-terminus could be relevant to Fe-S cluster synthesis as part of the quaternary complex with ISCU/NSF1/ISD11.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the Department of Chemistry Mass Spectrometry Resource Facility manager, Dr. Qiaoli Liang, and NMR Facility manager, Dr. Ken Belmore for assistance with instrumentation. We also thank Prof. Richard Armstrong for allowing initial HDX-MS studies with frataxin to be conducted at Vanderbilt University.

Funding Sources

This work was supported by National Science Foundation grant MCB 0845273 (L.S.B), National Science Foundation CRIF grant CHE 0639003, and National Institutes of Health grant R01 GM030910 (to Richard N. Armstrong).

ABBREVIATIONS

$\epsilon_{280\text{nm}}$	molar extinction coefficient at 280 nm
Bis-Tris	2,2-bis(hydroxymethyl)-2',2"-nitrilotriethanol
CyaY	bacterial frataxin homologue
$d_{18}\text{-HEPES}$	deuterated 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid
FA	Friedreich's Ataxia
Fe-S	cluster iron–sulfur cluster

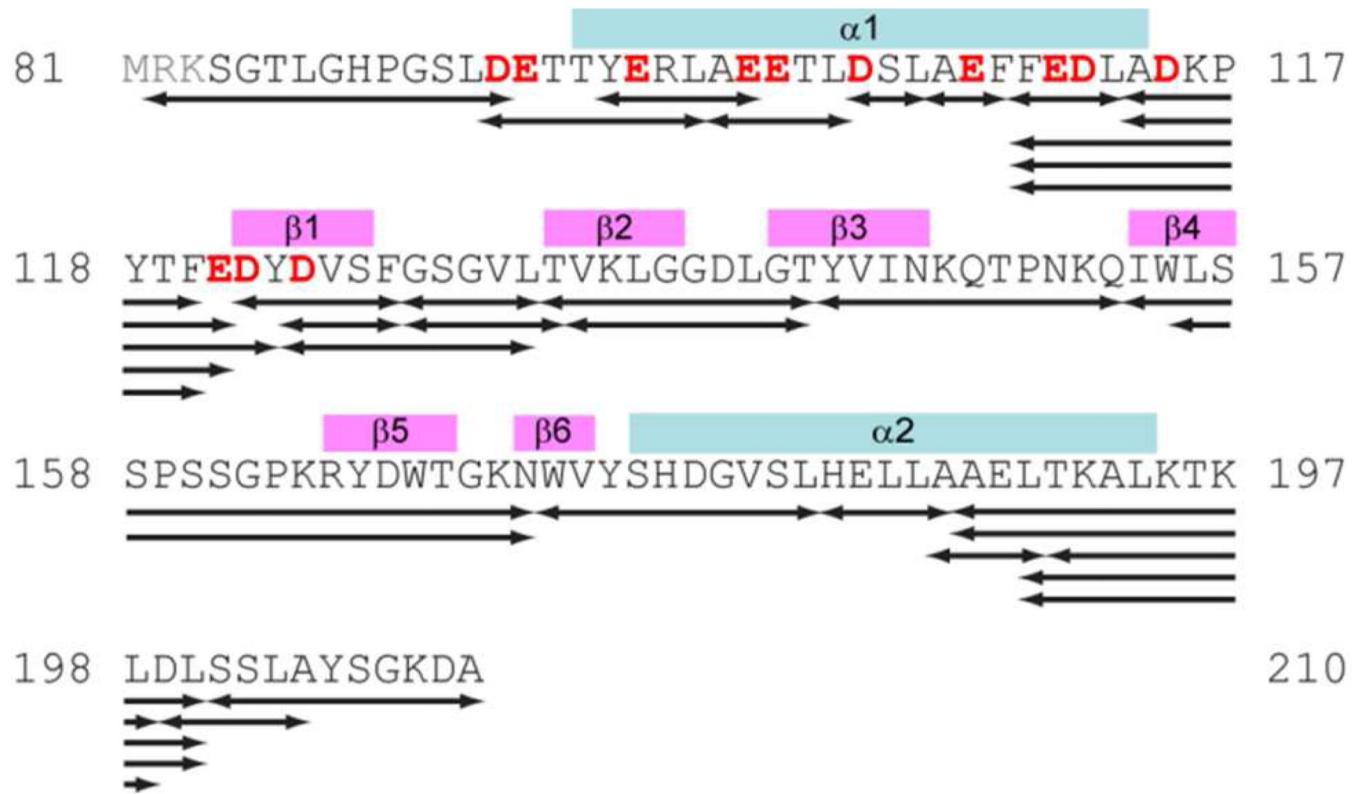
HDX-MS	hydrogen/deuterium exchange mass spectrometry
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	high performance liquid chromatography
HSQC	heteronuclear single-quantum coherence
IPTG	isopropyl β -D-1-thiogalactopyranoside
ISCU	human iron-sulfur cluster scaffold protein
ISD11	human iron-sulfur cluster accessory protein
K_D	equilibrium dissociation constant
K_f	formation constant
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NFS1	human cysteine desulfurase
NMR	nuclear magnetic resonance
OD_{600nm}	optical density at 600 nm
Yfh1	yeast frataxin homologue

REFERENCES

1. 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, Fillia A, De Frutos R, Palau F, Patel PI, Di Donato S, Mandel JL, Cocozza S, Koenig M, Pandolfo M. Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science*. 1996; 271:1423–1427. [PubMed: 8596916]
2. Schmucker S, Puccio H. Understanding the molecular mechanisms of Friedreich's ataxia to develop therapeutic approaches. *Hum. Mol. Genet.* 2010; 19:R103–R110. [PubMed: 20413654]
3. Gonzalez-Cabo P, Llorens JV, Palau F, Molto MD. Friedreich ataxia: an update on animal models, frataxin function and therapies. *Adv. Exp. Med. Biol.* 2009; 652:247–261. [PubMed: 20225031]
4. Rouault TA, Tong WH. Iron-sulfur cluster biogenesis and human disease. *Trends Genet.* 2008; 24:398–407. [PubMed: 18606475]
5. Ye H, Rouault TA. Human iron-sulfur cluster assembly, cellular iron homeostasis, and disease. *Biochemistry*. 2010; 49:4945–4956. [PubMed: 20481466]
6. 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; 11:2025–2036. [PubMed: 12165564]
7. Kondapalli KC, Kok NM, Dancis A, Stemmler TL. Drosophila frataxin: an iron chaperone during cellular Fe-S cluster bioassembly. *Biochemistry*. 2008; 47:6917–6927. [PubMed: 18540637]
8. Watson HM, Gentry LE, Asuru AP, Wang Y, Marcus S, Busenlehner LS. Heterotrifunctional chemical cross-linking mass spectrometry confirms physical interaction between human frataxin and ISU. *Biochemistry*. 2012; 51:6889–6891. [PubMed: 22897349]
9. Schmucker S, Martelli A, Colin F, Page A, Wattenhofer-Donze M, Reutenauer L, Puccio H. Mammalian frataxin: an essential function for cellular viability through an interaction with a preformed ISCU/NFS1/ISD11 iron-sulfur assembly complex. *PLoS One*. 2011; 6:e16199. [PubMed: 21298097]
10. Colin F, Martelli A, Clemancey M, Latour JM, Gambarelli S, Zeppieri L, Birck C, Page A, Puccio H, Ollagnier de Choudens S. Mammalian Frataxin Controls Sulfur Production and Iron Entry

- during de Novo Fe(4)S(4) Cluster Assembly. *J. Am. Chem. Soc.* 2013; 135:733–740. [PubMed: 23265191]
11. Pandolfo M, Pastore A. The pathogenesis of Friedreich ataxia and the structure and function of frataxin. *J. Neurol.* 2009; 256(Suppl 1):9–17. [PubMed: 19283345]
 12. Musco G, Stier G, Kolmerer B, Adinolfi S, Martin S, Frenkel 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]
 13. 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.* 2004; 12:2037–2048. [PubMed: 15530368]
 14. 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; 43:16254–16262. [PubMed: 15610019]
 15. Cook JD, Bencze KZ, Jankovic AD, Crater AK, Busch CN, Bradley PB, Stemmler AJ, Spaller MR, Stemmler TL. Monomeric yeast frataxin is an iron-binding protein. *Biochemistry.* 2006; 45:7767–7777. [PubMed: 16784228]
 16. Lane DJ, Richardson DR. Frataxin, a molecule of mystery: trading stability for function in its iron-binding site. *Biochem. J.* 2010; 426:e1–e3. [PubMed: 20141512]
 17. Huang J, Dizin E, Cowan J. Mapping iron binding sites on human frataxin: implications for cluster assembly on the ISU Fe–S cluster scaffold protein. *J. Biol. Inorg. Chem.* 2008; 13:825–836. [PubMed: 18425540]
 18. Pastore C, Franzese M, Sica F, Temussi P, Pastore A. Understanding the binding properties of an unusual metal-binding protein--a study of bacterial frataxin. *FEBS. J.* 2007; 274:4199–4210. [PubMed: 17651435]
 19. Maret W, Vallee BL. Cobalt as probe and label of proteins. *Methods Enzymol.* 1993; 226:52–71. [PubMed: 8277880]
 20. Thomsen JC, Mottola HA. Kinetics of the Complexation of Iron(II) with Ferrozine. *Anal. Chem.* 1984; 56:755–757.
 21. van de Weert M. Fluorescence quenching to study protein-ligand binding: common errors. *J. Fluoresc.* 2010; 20:625–629. [PubMed: 19997966]
 22. Musco G, de Tommasi T, Stier G, Kolmerer B, Bottomley M, Adinolfi S, Muskett FW, Gibson TJ, Frenkel TA, Pastore A. Assignment of the 1H, 15N, and 13C resonances of the C-terminal domain of frataxin, the protein responsible for Friedreich ataxia. *J. Biomol. NMR.* 1999; 15:87–88. [PubMed: 10549137]
 23. Kay L, Keifer P, Saarinen T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J. Am. Chem. Soc.* 1992; 114:10663–10665.
 24. 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; 6:227–293. [PubMed: 22910847]
 25. Grzesiek S, Stahl SJ, Wingfield PT, Bax A. The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. *Biochemistry.* 1996; 35:10256–10261. [PubMed: 8756680]
 26. Ma B, Zhang K, Hendrie C, Liang C, Li M, Doherty-Kirby A, Lajoie G. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2003; 17:2337–2342. [PubMed: 14558135]
 27. Engen JR, Smith DL. Investigating protein structure and dynamics by hydrogen exchange MS. *Anal. Chem.* 2001; 73:256A–265A.
 28. Busenlehner LS, Armstrong RN. Insights into enzyme structure and dynamics elucidated by amide H/D exchange mass spectrometry. *Arch. Biochem. Biophys.* 2005; 433:34–46. [PubMed: 15581564]
 29. Tong WH, Rouault TA. Functions of mitochondrial ISCU and cytosolic ISCU in mammalian iron-sulfur cluster biogenesis and iron homeostasis. *Cell Metab.* 2006; 3:199–210. [PubMed: 16517407]

30. Solomon EI, Brunold TC, Davis MI, Kemsley JN, Lee SK, Lehnert N, Neese F, Skulan AJ, Yang YS, Zhou J. Geometric and electronic structure/function correlations in non-heme iron enzymes. *Chem. Rev.* 2000; 100:235–350. [PubMed: 11749238]
31. Krezel A, Lesniak W, Jezowska-Bojczuk M, Mlynarz P, Brasuñ J, Kozlowski H, Bal W. Coordination of heavy metals by dithiothreitol, a commonly used thiol group protectant. *J. Inorg. Biochem.* 2001; 84:77–88. [PubMed: 11330484]
32. Bertini I, Luchinat C. High spin cobalt(II) as a probe for the investigation of metalloproteins. *Adv. Inorg. Biochem.* 1984; 6:71–111. [PubMed: 6442958]
33. Dian C, Vitale S, Leonard GA, Bahlawane C, Fauquant C, Leduc D, Muller C, de Reuse H, Michaud-Soret I, Terradot L. The structure of the *Helicobacter pylori* ferric uptake regulator Fur reveals three functional metal binding sites. *Mol. Microbiol.* 2011; 79:1260–1275. [PubMed: 21208302]
34. Otting G. Protein NMR using paramagnetic ions. *Annu Rev Biophys.* 2010; 39:387–405. [PubMed: 20462377]
35. Li H, Gakh O, Smith DYt, Isaya G. Oligomeric yeast frataxin drives assembly of core machinery for mitochondrial iron-sulfur cluster synthesis. *J. Biol. Chem.* 2009; 284:21971–21980. [PubMed: 19491103]
36. Gakh O, Bedekovics T, Duncan SF, Smith DYt, Berkholz DS, Isaya G. Normal and Friedreich ataxia cells express different isoforms of frataxin with complementary roles in iron-sulfur cluster assembly. *J. Biol. Chem.* 2010; 285:38486–38501. [PubMed: 20889968]
37. 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; 125:6078–6084. [PubMed: 12785837]
38. Pearson RG. Hard And Soft Acids And Bases HSAB 1. Fundamental Principles. *J. Chem. Educ.* 1968; 45:581-&.
39. Asuru AP, Busenlehner LS. Analysis of human ferrochelatase iron binding via amide hydrogen/deuterium exchange mass spectrometry. *Intl. J. Mass Spectrom.* 2011; 302:76–84.
40. Cavardini P, Adamiec J, Taroni F, Gakh O, Isaya G. Two-step processing of human frataxin by mitochondrial processing peptidase. Precursor and intermediate forms are cleaved at different rates. *J. Biol. Chem.* 2000; 275:41469–41475. [PubMed: 11020385]
41. Yoon T, Dizin E, Cowan JA. N-terminal iron-mediated self-cleavage of human frataxin: regulation of iron binding and complex formation with target proteins. *J. Biol. Inorg. Chem.* 2007; 12:535–542. [PubMed: 17285345]
42. Dhe-Paganon S, Shigeta R, Chi YI, Ristow M, Shoelson SE. Crystal structure of human frataxin. *J. Biol. Chem.* 2000; 275:30753–30756. [PubMed: 10900192]
43. Larkin MA, Blackshields G, Brown NP, Chenna R, McGgettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. *Bioinformatics.* 2007; 23:2947–2948. [PubMed: 17846036]

**Figure 1.**

Identified peptides from pepsin digestion of frataxin. Frataxin (residues 81–210) was digested with pepsin and subjected to tandem mass spectrometry. Peptide amino acid sequences are indicated with arrows. The secondary structure as determined from PDB: 1LY7 is indicated, where α -helices are in light blue and β -strands are in pink. Residues in red are acidic residues from α_1 and β_1 .¹²

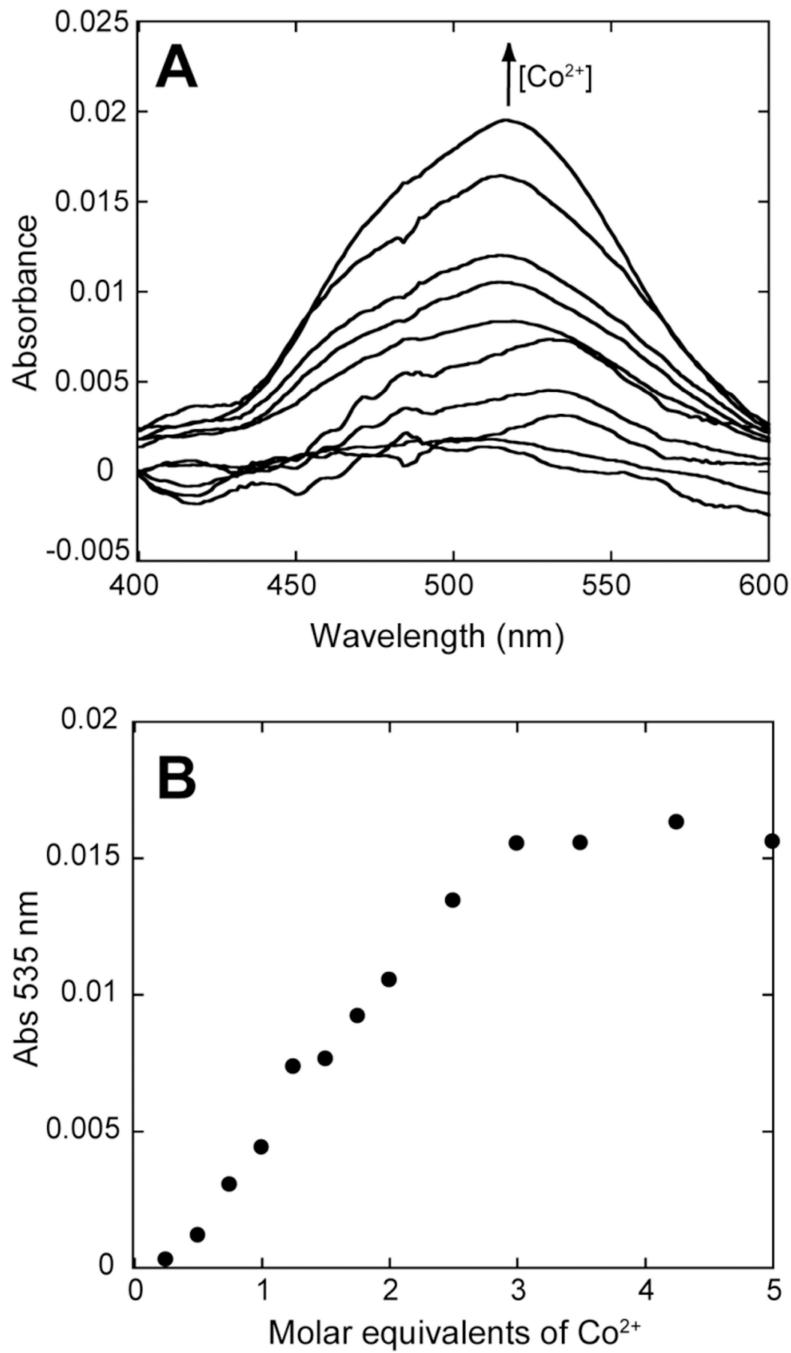
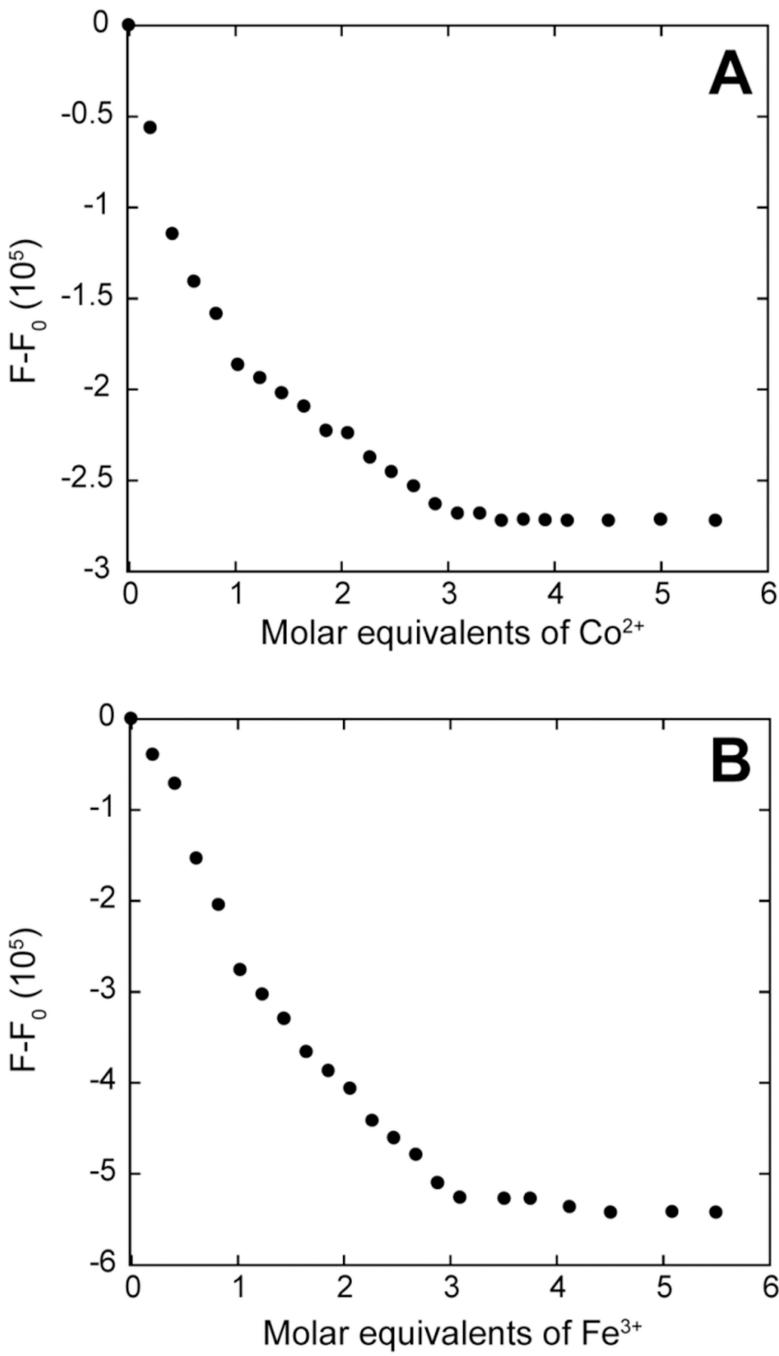
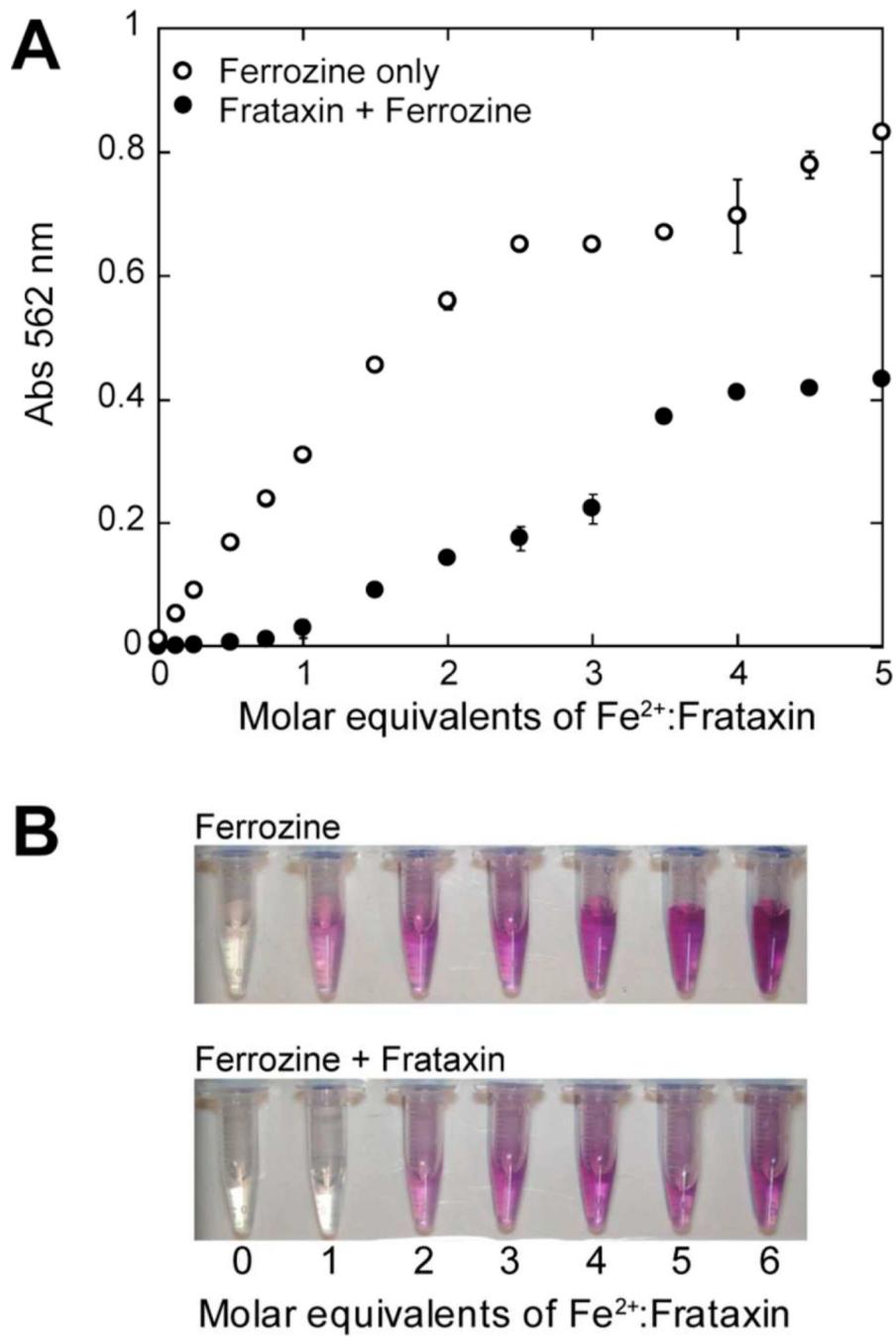


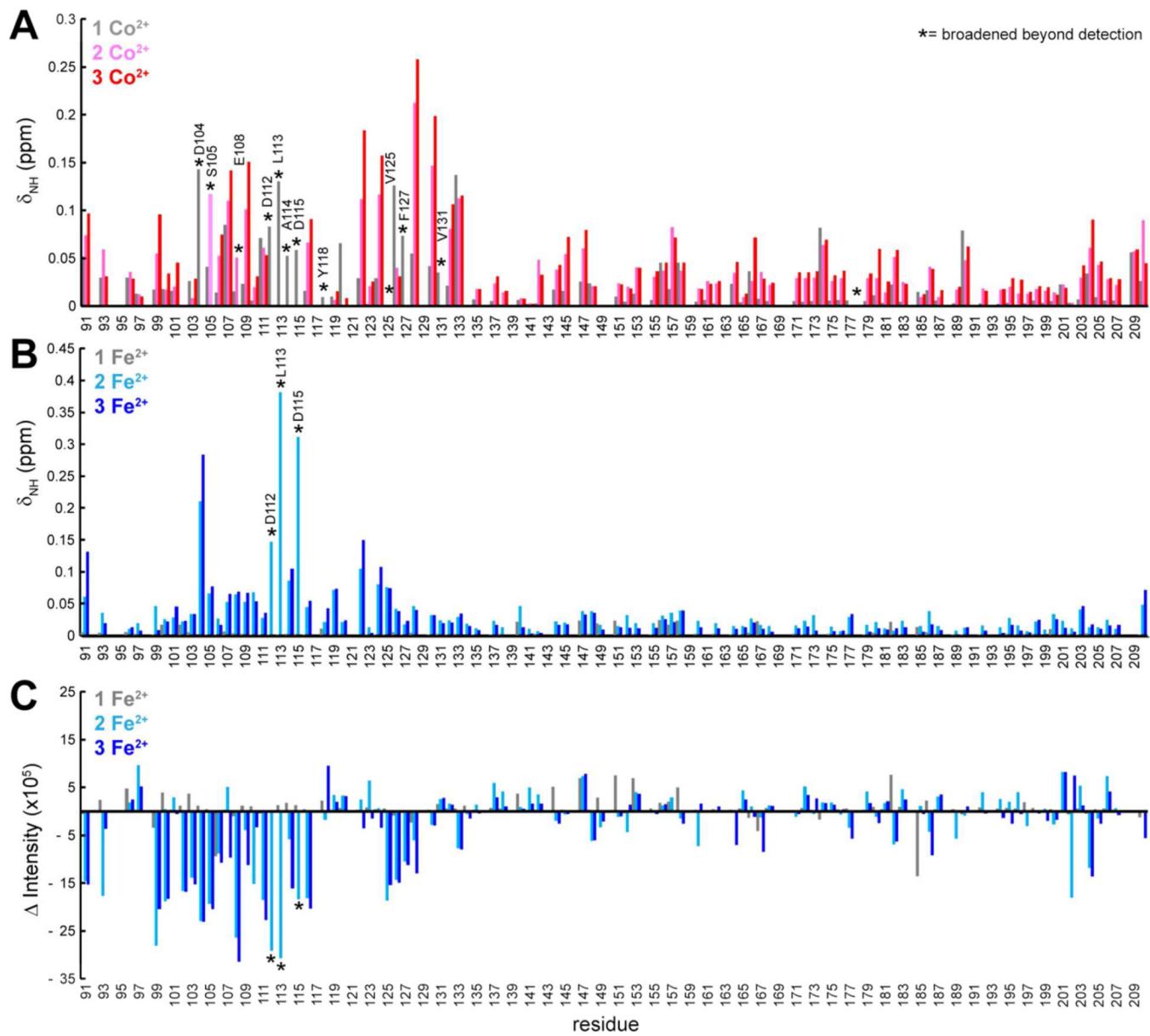
Figure 2.
UV-visible cobalt titration of frataxin. (A) Shown are representative spectra from a Co^{2+} titration of 300 μ M frataxin. Broad $d-d$ transitions from 450–550 nm are observed with a λ_{max} of 535 nm. (B) The isotherm indicates saturation at ~3 equivalents of Co^{2+} per frataxin protein.

**Figure 3.**

Fluorescence iron and cobalt titrations. Fluorescence emission spectra were recorded for metal titrations of 5 μM frataxin with an excitation at 295 nm. The corrected fluorescence intensity was plotted against the molar equivalents of Co^{2+} (A) and Fe^{3+} (B). In both cases, stoichiometric binding was observed with saturation at ~3 equiv of metal.

**Figure 4.**

Ferrozine iron competition titration. (A) The binding isotherms at 562 nm for the ferrozine titrations without (○) and with 13 μM frataxin (●) are shown. (B) Shown are representative samples from a Fe^{2+} titration of 108 μM ferrozine (top) and 13 μM frataxin with 108 μM ferrozine (bottom). The ferrozine $_{3}/\text{Fe}^{2+}$ complex is purple in color. Up to 1 mol equiv of Fe^{2+} , the metal preferentially binds to frataxin as shown by the lack of purple color and absorbance at 562 nm.

**Figure 5.**

NMR titrations of frataxin with cobalt and iron. (A) The change in normalized amide chemical shift (δ_{NH}) of 560 μM ^{15}N -frataxin with 1, 2 and 3 molar equivalents of Co^{2+} are in gray, pink, and red, respectively. (B) The change in normalized amide chemical shift (δ_{NH}) of 560 μM ^{15}N -frataxin with 1, 2 and 3 molar equivalents of Fe^{2+} are in gray, cyan, and dark blue, respectively. Samples were made anaerobically. (C) The change in intensity of ^1H - ^{15}N -frataxin resonances upon addition of 1, 2 and 3 molar equivalents of Fe^{2+} are in gray, cyan, and dark blue, respectively. Asterisks denote the positions of the amino acids whose resonances broadened beyond detection during the titrations.

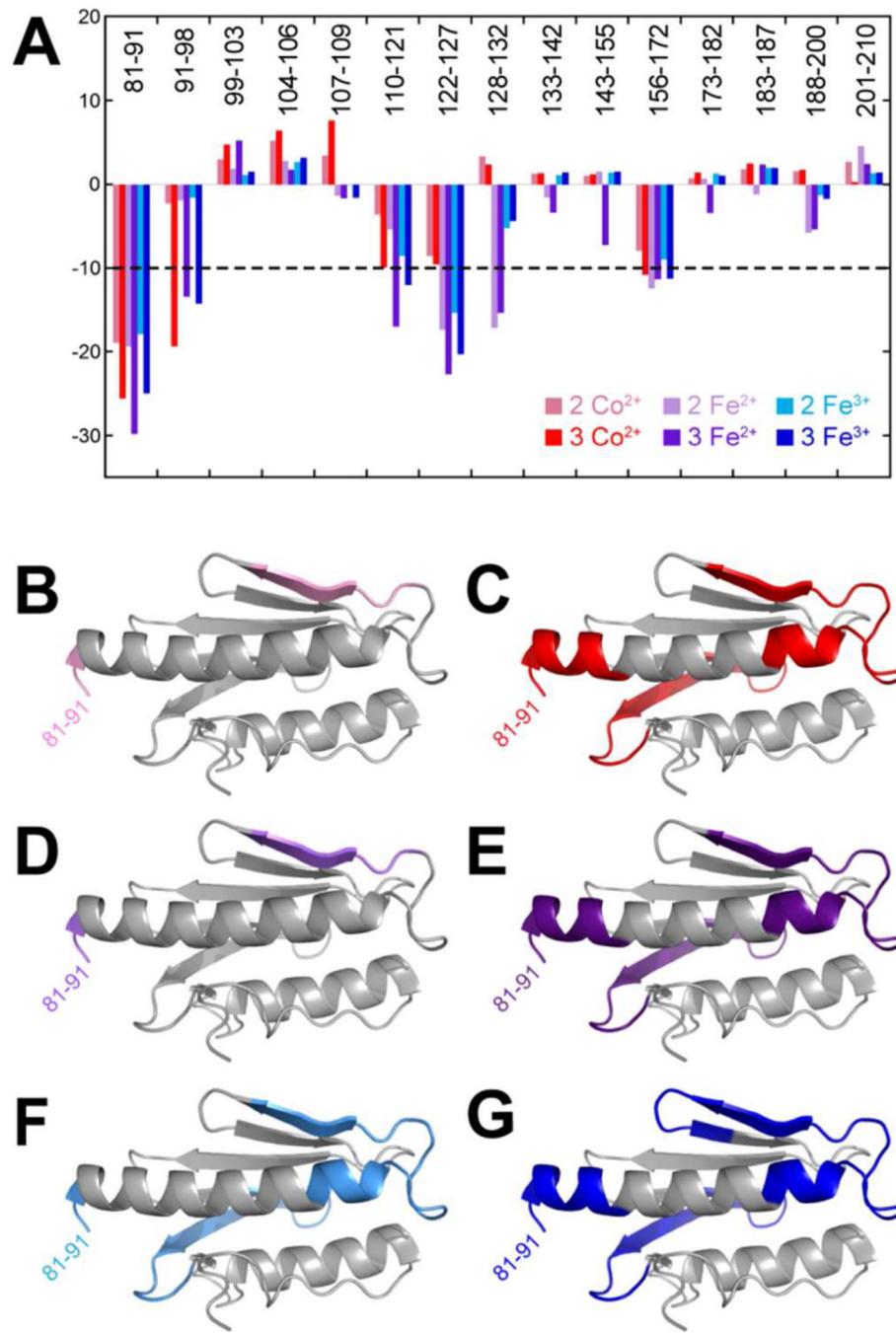
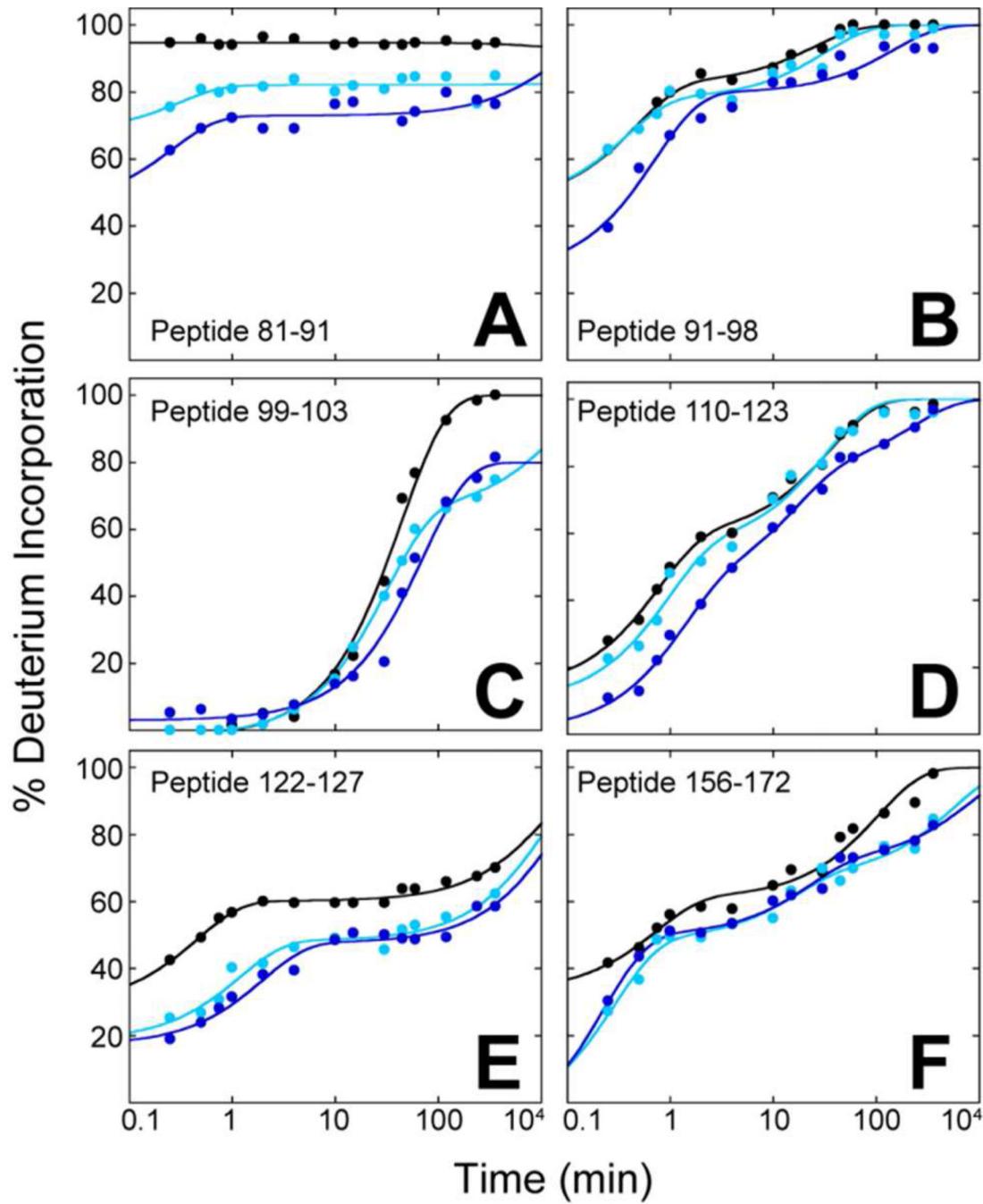
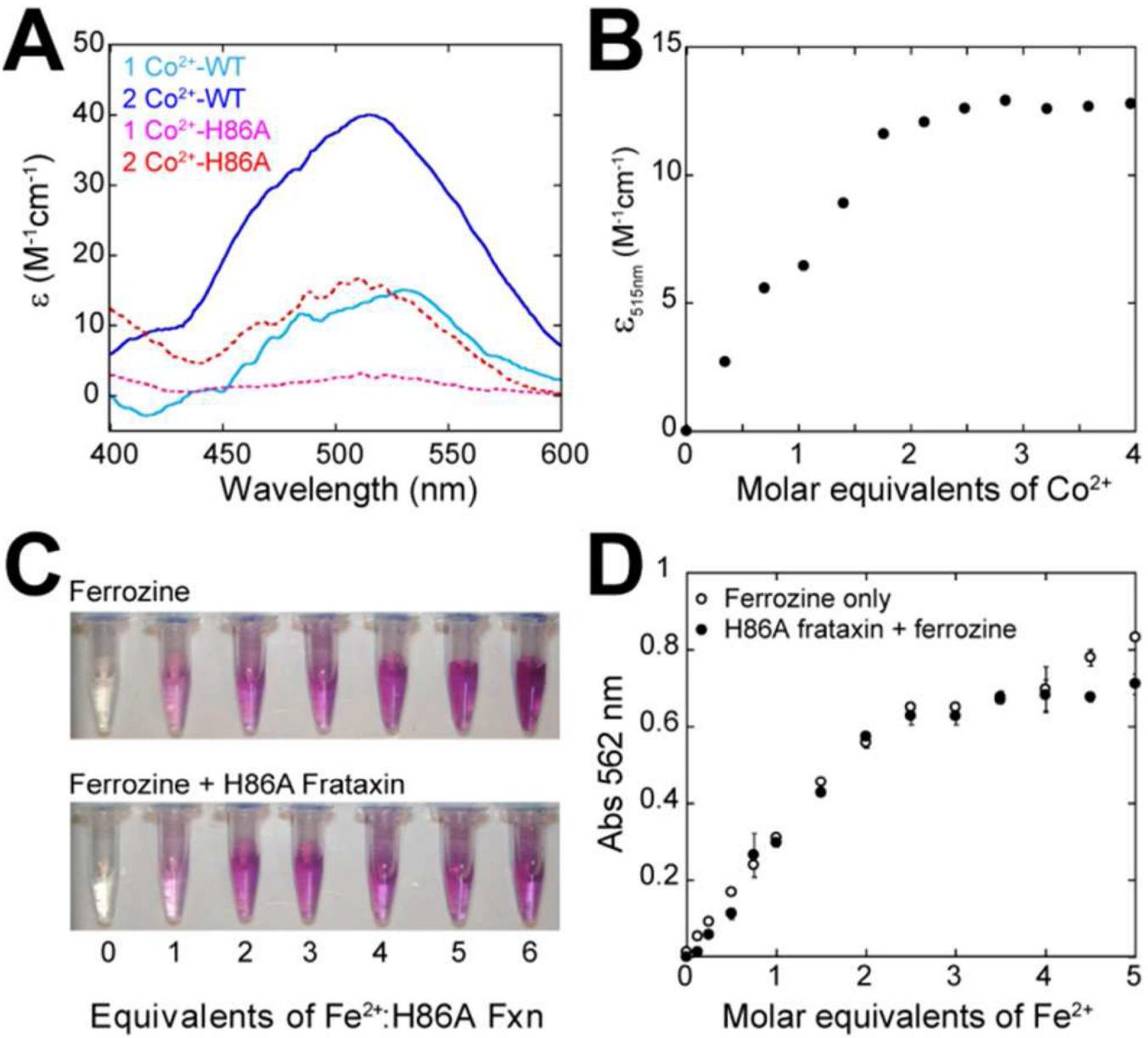


Figure 6.
HDX-MS titrations with cobalt and iron. The D₂O accessibility of backbone amides was determined by the percentage of amides exchanging with rates greater than 4 min⁻¹ for each pepsin-generated peptide. The percentage exchanged for apo-frataxin was subtracted from the percentage obtain with 2 and 3 equivalents of metal: 2Co²⁺ (pink); 3Co²⁺ (red); 2Fe²⁺ (light purple); 3Fe²⁺ (purple); 2Fe³⁺ (cyan); 3Fe³⁺ (blue). (B–G) Peptides that have HDX changes greater than 10% at 3 equivalents of metal are mapped to the structure of 91–210 frataxin (PDB:1EKG)⁴² using the color scheme in panel A. Note the HDX data includes peptide 81–91, which is not observed in the structure.

**Figure 7.**

HDX-MS rate profiles for selected frataxin peptides. Shown are the kinetic traces for the % deuterium incorporated into peptides derived from apo-(black), 2Fe³⁺-(cyan), and 3Fe³⁺-(dark blue) frataxin as a function of incubation time in D₂O. (A) Peptide 81–91. (B) Peptide 91–98. (C) Peptide 99–103. (D) Peptide 110–123. (E) Peptide 122–127. (F) Peptide 156–172. The data are averaged from 3 experiments and were fit to single or double exponential equations to obtain the rate constants for exchange (k_n) and the corresponding percentage of deuterons in each phase (%D_n). The fitted parameters are given in Table 1. The corresponding HDX-MS plots for 2Co²⁺- and 3Co²⁺-frataxin samples are in Supporting Information.

**Figure 8.**

Cobalt and iron binding to H86A frataxin. (A) Shown are representative spectra plotted in molar absorptivity (ϵ) of wild-type and H86A frataxin with Co^{2+} bound. Wild-type frataxin (solid lines) with 1 and 2 equivalents of Co^{2+} are in cyan and blue, respectively. H86A frataxin (dashed lines) with 1 and 2 equivalents of Co^{2+} are in pink and red, respectively. Both a loss of molar absorptivity and a shift in the λ_{max} is observed with H86A frataxin. (B) After correcting the data for non-specific binding, the isotherm indicates H86A frataxin coordinates ~2 equivalents of Co^{2+} per protein. (C) Shown are representative samples from a Fe^{2+} titration of 108 μM ferrozine (top panel) and 13 μM H86A frataxin with 108 μM ferrozine (bottom panel). The ferrozine₃: Fe^{2+} complex is purple in color. (D) The binding isotherms at 562 nm for the ferrozine titrations without (○) and with H86A frataxin (●) described in (C) are shown. Fe^{2+} preferentially binds to ferrozine even in the presence of H86A frataxin.

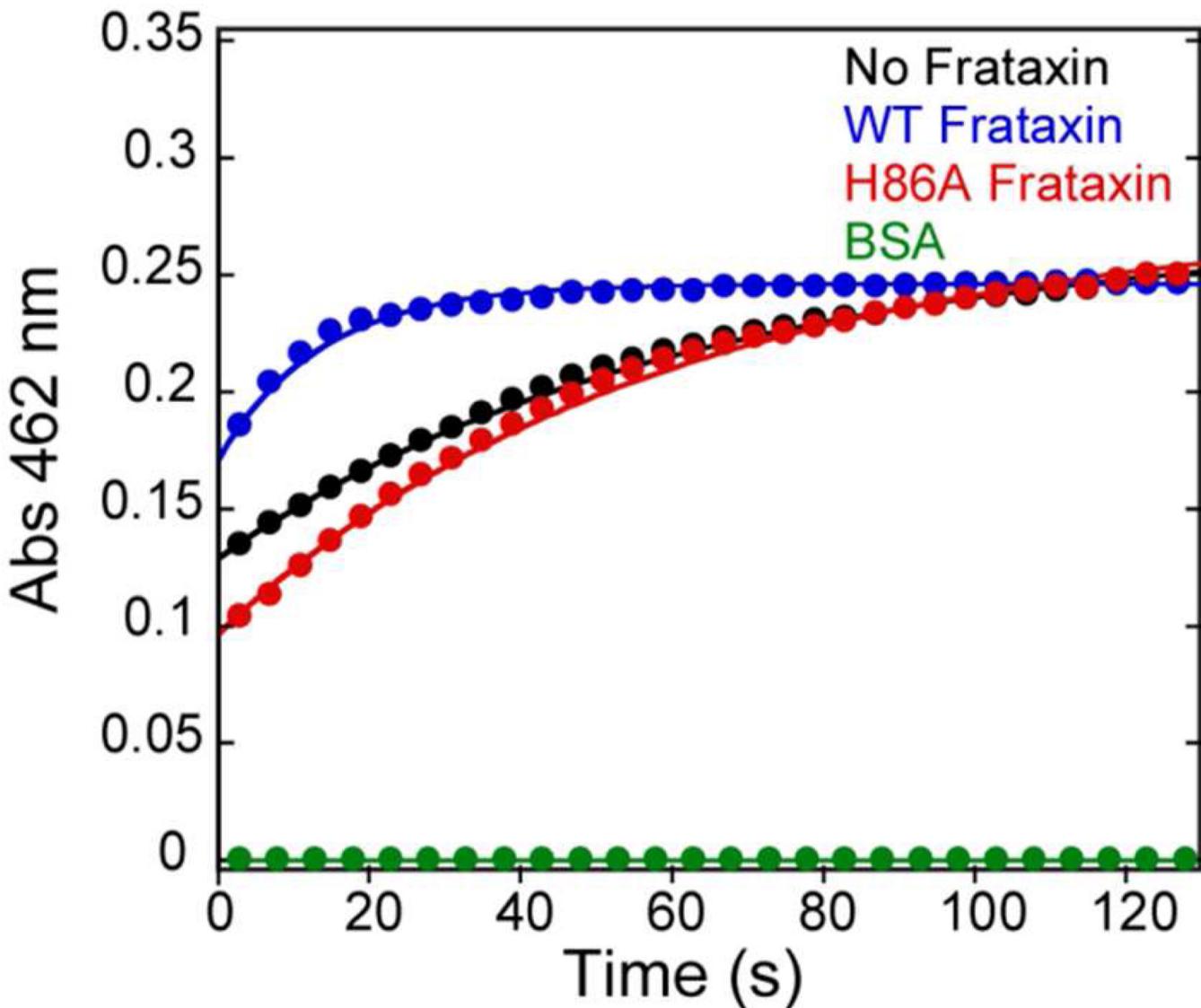


Figure 9.

Functional and physical interaction between frataxin and ISCU. (A) Shown are averaged progress curves for the assembly of [2Fe–2S] clusters by D37A ISCU (100 μ M) using various Fe^{2+} donors in excess sodium sulfide. The anaerobic reaction progress curves were followed at 426 nm and were initiated by 200 μ M Fe^{2+} as “free” metal in the absence of protein (black), 2Fe^{2+} –frataxin (blue), 2Fe^{2+} –H86A frataxin (red), or 2Fe^{2+} –BSA (green). The curves were fit to first order rate equations to obtain the initial rate of Fe–S cluster assembly.

Human	Co²⁺	81	-----SGTLG--HPGSLDETTYERLAEETLDS LAEFFEDLADKPYTFEDYDVSGSGVLTVKLGGLGTY	143
Human	Fe²⁺	81	-----SGTLG--HPGSLDETTYERLAEETLDS LAEFFEDLADKPYTFEDYDVSGSGVLTVKLGGLGTY	143
Human	Fe²⁺	91	-----DETTYERLAEETLDS LAEFFEDLADKPYTFEDYDVSGSGVLTVKLGGLGTY	143
Yeast	Fe²⁺	52	VESSTDGQVVPQEVLNLPLEKYHEEADDYL DHLLDSLEELSEAH PDCIP-D VELSHGVMTLEIP-AFGTY	119
E.coli	Fe²⁺	1	-----MNDSEFHLRADQLWLTI E RLLLDDWD GDS -----D IDCEINGVLTITFE-NGSKI	49
E.coli	Co²⁺	1	-----MNDSEFHLRADQLWLTI E RLLLDDWD GDS -----D IDCEINGVLTITFE-NGSKI	49
: :... *::: : : :::: * . . . * *::*: : ..				
144	VINKQTPNKQIWLSSPSSGPKR Y DWTGKNWVYSHDGVL L HELLAAELTKALKTKLDLSS L AYSGKDA	210		
144	VINKQTPNKQIWLSSPSSGPKR Y DWTGKNWVYSHDGVL L HELLAAELTKALKTKLDLSS L AYSGKDA	210		
144	VINKQTPNKQIWLSSPSSGPKR Y DWTGKNWVYSHDGVL L HELLAAELTKALKTKLDLSS L AYSGKDA	210		
120	VINKQPPNKQIWLASPLSGP N RFDLLNGEWVSLRNGTKLTDILTEVEKAISKSQ-----	174		
50	IINRQEPLHQVWLATKQGG-YHFDLKGDEWICDRSGETFWDLLEQAATQQAGETVSFR-----	106		
50	IINRQEPLHQVWLATKQGG-YHFDLKGDEWICDRSGETFWDLLEQAATQQAGETVSFR-----	106		
:***:*** :*:***::: .* :*: . :*: :.* . : :* :				

Figure 10.

Summary of NMR titrations of frataxin homologues. An alignment of mature *H. sapiens* frataxin, *S. cerevisiae* Yfh1, and *E. coli* CyaY proteins was created with ClustalW⁴³ and the residues shown to broaden beyond detection (magenta) or have large chemical shifts (cyan) in Fe²⁺ or Co²⁺ NMR titrations are indicated. The 1st and 2nd entries (81–210 human frataxin) are from this study, the 3rd entry (91–210 human frataxin) is from Nair *et al.*¹³, the 4th entry (52–174 yeast Yfh1) is from He *et al.*¹⁴, and the 5th and 6th entries (1–106 *E. coli* CyaY) are from Pastore *et al.*¹⁸ The lines under the amino acid sequence indicate peptides that had decreased deuterium incorporation in HDX–MS experiments with Fe³⁺ (blue) and Co²⁺ (red).

Table 1HDX-MS rate constants and amplitudes for peptides from Figure 7.^a

Peptide	%D _{Pre-Ex} ^b	%D ₁	k ₁ (min ⁻¹) “Fast”	%D ₂	k ₂ (min ⁻¹) “Intermediate”	%D ₃	k ₃ (min ⁻¹) “Slow”
81–91							
Apo	~100	—	—	—	—	—	—
2 Fe ³⁺	~81	—	—	—	—	17.8 (±1.0)	1×10 ⁻⁴
3 Fe ³⁺	~73	—	—	—	—	27.0 (±1.4)	1×10 ⁻⁴
91–98							
Apo	~47	35.7 (±4.5)	2.2 (±0.4)	17.5 (±1.3)	0.040 (±0.007)	—	—
2 Fe ³⁺	~47	31.5 (±11.2)	2.7 (±1.2)	21.9 (±2.2)	0.031 (±0.007)	—	—
3 Fe ³⁺	~26	53.3 (±8.8)	1.4 (±0.4)	20.3 (±2.4)	0.007 (±0.002)	—	—
99–103							
Apo	0	—	—	100 (±1.2)	0.022 (±0.001)	—	—
2 Fe ³⁺	0	—	—	67.1 (±2.6)	0.032 (±0.002)	~33	1×10 ⁻⁴
3 Fe ³⁺	0	—	—	76.9 (±1.5)	0.014 (±0.001)	~23	1×10 ⁻⁴
110–123							
Apo	~23	36.8 (±5.0)	1.1 (±0.3)	40.1 (±2.3)	0.018 (±0.002)	—	—
2 Fe ³⁺	~12	34.3 (±5.0)	1.4 (±0.4)	38.1 (±3.6)	0.06 (±0.01)	15.6 (±3.3)	0.002 (±0.001)
3 Fe ³⁺	~2	28.7 (±7.5)	2.0 (±1.0)	32.5 (±3.9)	0.10 (±0.02)	37.4 (±2.4)	0.0030 (±0.0005)
122–127							
Apo	~29	31 (±4)	2.2 (±0.4)	—	—	40 (±0.5)	1×10 ⁻⁴
2 Fe ³⁺	~18	30 (±4)	0.9 (±0.2)	—	—	52 (±1)	1×10 ⁻⁴
3 Fe ³⁺	~18	30 (±3)	0.5 (±0.1)	—	—	52 (±1)	1×10 ⁻⁴
156–172							
Apo	~33	28 (±7)	1.4 (±0.6)	39 (±2)	0.010 (±0.002)	—	—
2 Fe ³⁺	~25	24 (±9)	2.1 (±0.5)	19 (±4)	0.06 (±0.04)	32 (±4)	0.0017 (±0.0005)
3 Fe ³⁺	~29	21 (±2)	2.3 (±0.5)	22 (±3)	0.05 (±0.01)	28 (±3)	0.0012 (±0.0005)

^a Parameters obtained from fitting the H/D-exchange kinetics of frataxin peptides with and without metal (Figure 7 A–F) according to a single, double or triple exponential equations. The rates have been loosely grouped in to fast, intermediate and slow exchange.

^b The amount of exchange before the first time point is estimated from the fit parameters and is assigned a rate of exchange > 4 min⁻¹.²⁸