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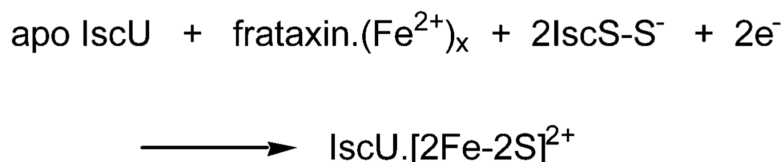

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Iron−Sulfur Cluster Biosynthesis. Characterization of Frataxin as an Iron Donor for Assembly of [2Fe-2S] Clusters in ISU-Type Proteins

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Abstract: ISU (eukaryotes) and IscU (prokaryotes) are a homologous family of proteins that appear to provide a platform for assembly of [2Fe-2S] centers prior to delivery to an apo target protein. The intermediate [2Fe-2S] ISU-bound cluster is formed by delivery of iron and sulfur to the apo ISU, with the latter delivered through an IscS-mediated reaction. The identity of the iron donor has thus far not been established. In this paper we demonstrate human frataxin to bind from six to seven iron ions. Iron binding to frataxin has been quantitated by iron-dependent fluorescence measurements [$K_D(Fe^{3+}) \approx 11.7\ \mu M$; ($K_D(Fe^{2+}) \approx 55.0\ \mu M$] and isothermal titration calorimetry (ITC) [$K_D(Fe^{3+}) \approx 10.2\ \mu M$]. Enthalpies and entropies for ferric ion binding were determined from calorimetric measurements. Both fluorescence (K_D 0.45 μM) and ITC measurements (K_D 0.15 μM) demonstrate holo frataxin to form a complex with ISU with sub-micromolar binding affinities. Significantly, apo frataxin does not bind to ISU, suggesting an important role for iron in cross-linking the two proteins and/or stabilizing the structure of frataxin that is recognized by ISU. Holo frataxin is also shown to mediate the transfer of iron from holo frataxin to nucleation sites for [2Fe-2S] cluster formation on ISU. We have demonstrated elsewhere [*J. Am. Chem. Soc.* **2002**, *124*, 8774−8775] that this iron-bound form of ISU is viable for assembly of holo ISU, either by subsequent addition of sulfide or by NifS-mediated sulfur delivery. Provision of holo frataxin and inorganic sulfide is sufficient for cluster assembly in up to 70% yield. With NifS as a sulfur donor, yields in excess of 70% of holo ISU were obtained. Both UV−vis and CD spectroscopic characteristics were found to be consistent with those of previously characterized ISU proteins. The time course for cluster assembly was monitored from the 456 nm absorbance of holo ISU formed during the [2Fe-2S] cluster assembly reaction. A kinetic rate constant $k_{obs} \approx 0.075\ min^{-1}$ was determined with 100 μM ISU, 2.4 mM Na_2S, and 40 μM holo frataxin in 50 mM Tris-HCl (pH 7.5) with 4.3 mM DTT. Similar rates were obtained for NifS-mediated sulfur delivery, consistent with iron release from frataxin as a rate-limiting step in the cluster assembly reaction.

Frataxin is a nuclear encoded protein that is localized in the mitochondrion and has been implicated in several aspects of mitochondrial iron homeostasis,[1−7] including Fe−S cluster assembly.[6,8] Impairment of frataxin function is the causative agent of the autosomal recessive neurodegenerative disorder termed Friedreich's ataxia[1,9] and is associated with an increase in mitochondrial iron concentration, damage to mitochondrial DNA, respiratory disorders, and impairment of Fe−S cluster formation. Our studies have been performed on a 129 amino acid protein that lacks the N-terminal mitochondrial targeting sequence.[2,10] Preliminary studies of iron binding to human and yeast frataxin have been reported.[2,11]

With the known involvement in mitochondrial iron homeostasis we hypothesized that iron-loaded frataxin might serve as an iron donor to ISU in the early stages of Fe−S cluster assembly. ISU (eukaryotes) and IscU (prokaryotes) are a homologous family of proteins that appear to provide a platform for assembly of [2Fe-2S] centers prior to delivery to an apo target protein.[12−16] ISU-mediated assembly of such clusters is

(1) Bradley, J. L.; Blake, J. C.; Chamberlain, S.; Thomas, P. K.; Cooper, J. M.; Schapira, A. H. V. *Hum. Mol. Genet.* **2000**, *9*, 275−282.
(2) Cavadani, P.; O'Neill, H. A.; Benada, O.; Isaya, G. *Hum. Mol. Gen.* **2002**, *11*, 217−227.
(3) Foury, F. *FEBS Lett.* **1999**, *456*, 281−284.
(4) Cossee, M.; Puccio, H.; Gansmuller, A.; Koutnikova, H.; Dierich, A.; LeMeur, M.; Fischbeck, K.; Dolle, P.; Koenig, M. *Hum. Mol. Genet.* **2000**, *9*, 1219−1226.
(5) Koutnikova, H.; Campuzano, V.; Foury, F.; Dolle, P.; Cazzalini, O.; Koenig, M. *Nat. Genet.* **1997**, *16*, 345−351.
(6) Puccio, H.; Koenig, M. *Hum. Mol. Genet.* **2000**, *9*, 887−892.
(7) Radisky, D. C.; Babcock, M. C.; Kaplan, J. *J. Biol. Chem.* **1999**, *274*, 4497−4499.
(8) Lutz, T.; Westermann, B.; Neupert, W.; Herrmann, J. M. *J. Mol. Biol.* **2001**, *307*, 815−825.
(9) Musco, G.; Stier, G.; Kolmerer, B.; Adinolfi, S.; Martin, S.; Frenkiel, T.; Gibson, T.; Pastore, A. *Structure (London)* **2000**, *8*, 695−707.
(10) Dhe-Paganon, S.; Shigeta, R.; Chi, Y.-I.; Ristow, M.; Shoelson, S. E. *J. Biol. Chem.* **2000**, *275*, 30753−30756.
(11) Adamec, J.; Rusnak, F.; Owen, W. G.; Naylor, S.; Benson, S.; Gacy, A. M.; Isaya, G. *Am. J. Hum. Genet.* **2000**, *67*, 549−562.
(12) Agar, J. N.; Zheng, L.; Cash, V. L.; Dean, D. R.; Johnson, M. K. *J. Am. Chem. Soc.* **2000**, *122*, 2136−2137.
(13) Wu, S.; Wu, G.; Surerus, K. K.; Cowan, J. A. *Biochemistry* **2002**, *41*, 8876−8885.
(14) Mansy, S. S.; Wu, G.; Surerus, K. K.; Cowan, J. A. *J. Biol. Chem.* **2002**, *277*, 21397−21404.
(15) Agar, J. N.; Krebs, C.; Frazzon, J.; Huynh, B. H.; Dean, D. R.; Johnson, M. K. *Biochemistry* **2000**, *39*, 7856−7862.

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thought to occur exclusively in the mitochondrion,[17−20] although the possibility of cytosolic assembly pathways has also been noted.[21] The intermediate [2Fe-2S] ISU-bound cluster is formed by delivery of iron and sulfur to the apo ISU, with the latter delivered through an IscS- or NifS-mediated reaction.[16,22,23] The identity of the iron donor has thus far not been established. In this paper we characterize the iron binding chemistry of frataxin and demonstrate human frataxin[2,10] to complex with human ISU and mediate the transfer of iron from holo frataxin to nucleation sites for [2Fe-2S] cluster formation on ISU. We further demonstrate that provision of holo frataxin and inorganic sulfide (or NifS/Cys) is sufficient for cluster assembly. We have previously demonstrated for a bacterial homologue that this iron-bound form is viable for subsequent IscS-mediated assembly of holo ISU.[22] Consequently, this report fully defines the minimal protein set required to form ISU-bound [2Fe-2S] clusters.

Experimental Procedures

General Chemicals. NTA resin was purchased from QIAGEN (Valencia, CA). DE-52 ion-exchange resin was from Whatman (Aston, PA), and homogeneous-20 precast polyacrylamide gels, G-25, and Superose-12 resins were from Pharmacia (NJ). Bipyridine and tiron were obtained from Acros (NJ).

Protein Expression and Purification. A plasmid containing the cloned frataxin gene, pET-28b(+)(FTX), was transformed into *E. coli* BL21(DE3) by electroporation. For protein hyperproduction an overnight culture (40 mL) was used as an inoculum for 4 L of LB containing 35 mg/mL kanamycin. Cells were grown at 37 °C to an OD of 0.6−0.8, and expression was induced by the addition of IPTG to a final concentration of 1.0 mM. The culture was incubated for an additional 4 h, and then the cells were harvested by centrifugation. The cell pellet was washed with 50 mM Tris-HCl and 50 mM NaCl, pH 7.5, and stored at −80 °C until used.

Human frataxin was purified aerobically at 4 °C. Cell pellets were lysed by sonication and centrifuged at 15 000 rpm, 4 °C, for 15 min. Following centrifugation the supernatant was loaded on a Ni-NTA column that had been equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl). The column was then washed with 5 column volumes of binding buffer and 5 volumes of binding buffer + 25 mM imidazole, and the protein was subsequently eluted with binding buffer + 795 mM imidazole. His-tagged protein was exchanged with 50 mM sodium phosphate, pH 8.0, via repeated ultrafiltration (Amicon). The sample was loaded onto an anion exchange column (DE-52). The column was washed with 3 column volumes of 50 mM sodium phosphate, pH 8.0. The flow-through and wash fractions were combined and concentrated via ultrafiltration. The fractions with a λ_{max} at 278 nm were pooled and confirmed to be pure human frataxin by SDS-PAGE. All apo frataxin samples were stored at either 4 or −80 °C. Prior to holo formation the apo protein was exchanged into an appropriate Tris or HEPES buffer by dialysis and ultrafiltration (Amicon). Holo frataxin was obtained following incubation with excess ferric ion (10 mM $FeCl_3$) in buffer solution at room temperature for 5 h with subsequent passage through a Sephadex G-25 column to remove unbound ferric ions. Holo frataxin with bound ferric ion was kinetically stable over the time frame of these experiments.

Iron Assay. Human holo frataxin (1 mL) was mixed with 0.3 mL of 12 M HCl, in Ependorff tubes. The tubes were stoppered and heated to 100 °C for 15 min. Distilled water (0.4 mL) was added to each tube. The precipitated material was removed by centrifuging 4 min in an Ependorff 5415C centrifuge. Aliquots from the supernatants were transferred to disposable plastic tubes and diluted to 1.5 mL with 0.5 M Tris-HCl buffer, pH 8.5. Sodium ascorbate (0.1 mL, 5% in water) and 0.4 mL of bathophenanthrolinedisulfonate (0.1% in water) were subsequently added. Finally, after 1 h, the absorbance was measured at 535 nm against a control containing the buffer and the reagents. The molar extinction coefficient of iron−bathophenanthrolinedisulfonate complex was taken as 22 140 $M^{-1}cm^{-1}$. Calibrations with standard $FeSO_4$ solutions confirmed this value. Optical spectra were obtained with an HP diode array spectrophotometer.

Quantitation of Iron Binding to Frataxin by Fluorimetry. The tryptophan fluorescence of human frataxin solution was measured in 1 mL quartz cuvettes at room temperature with a Perkin-Elmer LS50B luminescence spectrometer. The excitation and monitoring wavelengths were 291 and 341 nm, respectively. Corrections for the inner filter effect were considered but were negligible as a result of the weak absorbance at the emission wavelength. Intensity data from a control titration, consisting of a similar titrant solution but containing only the buffer solution in the sample cell, were subtracted from the experimental data obtained from the ISU titration to account for the minimal background fluorescence from the titrant solution. For ferric ion binding, spectra were obtained on a 24 μM solution of apo frataxin and ferric ion was titrated from a stock solution of $FeCl_3$ over the concentration range shown. For ferrous ion binding, spectra were obtained on a 15 μM solution of apo frataxin and ferrous ion was titrated from a stock solution of $FeSO_4 \cdot 7H_2O$ over the concentration range shown. Protein and iron samples were prepared in argon-purged 100 mM HEPES, pH 7.5, and 50 mM NaCl. For ferrous ion titrations the buffer solutions also contained 5 mM DTT. The quenching of tryptophan fluorescence induced by the binding ferric and ferrous ions (Figures 1 and 2) was used to calculate the fraction of binding sites occupied, f_a:

$$f_a = (y - y_f)/(y_b - y_f) \quad (1)$$

where y is the fluorescence intensity at a given concentration of ferric and ferrous ions and y_b and y_f are the intensities when the binding sites are fully occupied and unoccupied, respectively. The stoichiometry, p, and apparent dissociation constant, K_d, were then obtained from the intercept and slope of a linear regression of the data transformed as described by Winzor and Sawyer[24] in terms of eq 2:

$$C_s/f_a = pC_a + K_d/(1 - f_a) \quad (2)$$

where C_s is the total concentration of metal ions and C_a is the total concentration of human frataxin. Consistent values for dissociation constants were determined from an alternative fitting procedure from a plot of binding function r versus C_s according to eq 3,[24] where the binding function r is defined by eq 4 and the fractional saturation f_a is defined by eq 5.

$$r = pC_s/(K_1 + C_s) + pC_s/(K_2 + C_s) \quad (3)$$

$$r = f_a p \quad (4)$$

$$f_a = (y - y_f)/(y_b - y_f) \quad (5)$$

Quantitation of Iron Binding to Frataxin by Isothermal Titration Calorimetry. ITC measurements of ferric ion binding to apo frataxin

(16) Urbina, H. D.; Silberg, J. J.; Hoff, K. E.; Vickery, L. E. *J. Biol. Chem.* **2001**, *276*, 44521−44526.
(17) Muhlenhoff, U.; Lill, R. *Biochim. Biophys. Acta* **2000**, *1459*, 370−382.
(18) Lill, R.; Diekert, K.; Kaut, A.; Lange, H.; Pelzer, W.; Prohl, C.; Kispal, G. *Biol. Chem.* **1999**, *380*, 1157−1166.
(19) Lill, R.; Kispal, G. *Trends Biochem. Sci.* **2000**, *25*, 352−356.
(20) Lange, H.; Kaut, A.; Kispal, G.; Lill, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1050−1055.
(21) Tong, W. H.; Rouault, T. *EMBO J.* **2000**, *19*, 5692−5700.
(22) Nuth, M.; Yoon, T.; Cowan, J. A. *J. Am. Chem. Soc.* **2002**, *124*, 8774−8775.
(23) Zheng, L.; Cash, V. L.; Flint, D. H.; Dean, D. R. *J. Biol. Chem.* **1998**, *273*, 13264−13272.

were carried out at 27 °C on a MicroCal OMEGA ultrasensitive titration calorimeter (Figure 3). The titrant and sample solutions were made up in the same stock buffer solution (100 mM HEPES buffer, pH 7.5), and both experimental solutions were thoroughly degassed before each titration. The solution in the cell was stirred at 200 rpm by syringe to ensure rapid mixing. Typically, 5−12 μL of titrant was delivered to a solution of 20 μM frataxin over a period of 15 s with an adequate interval (10−15 min) between injections to allow complete equilibration. Titrations continued until 10−20 equiv had been added to ensure no further complex formation following addition of excess titrant. A background titration, consisting of the identical titrant solution, but containing only the buffer solution in the sample cell, was subtracted from each experimental titration to account for heat of dilution.

The data were collected automatically and subsequently analyzed with a one-site binding model by the Windows-based Origin software package supplied by MicroCal. The Origin software uses a nonlinear least-squares algorithm (minimization of χ^2) and the concentrations of the titrant and the sample to fit the heat flow per injection to an equilibrium binding equation, providing best fit values of the stoichiometry (n), change in enthalpy (ΔH), and binding constant (K).

Quantitation of Frataxin Binding to ISU by Fluorimetry. Tryptophan fluorescence from human frataxin was measured in 50 mM HEPES, pH 7.5, buffer solution in a quartz cuvette at room temperature by use of a Perkin-Elmer LS50B luminescence spectrometer. Aliquots of holo frataxin with bound ferric ion were added to 3 μM human ISU in 50 mM Tris-HCl, pH 7.5, HEPES buffer at room temperature. The excitation and monitoring wavelengths were 291 and 341 nm, respectively. Fluorescence data was analyzed following Winzor.[24] Corrections for the inner filter effect were considered but were negligible as a result of the weak absorbance at the emission wavelength. Intensity data from a control titration, consisting of a similar titrant solution but containing only the buffer solution in the sample cell, were subtracted from the experimental data obtained from the ISU titration to account for the minimal background fluorescence from the titrant solution. The dissociation constants determined from the quenching of tryptophan fluorescence induced by holo frataxin binding (Figure 5) were determined from fitting the binding function r versus C_s according to eqs 3−5.[24] Parameters r and f_a have been earlier defined, signals y_b and y_f are from the emission intensities of the acceptor sites when fully occupied and unoccupied, respectively, y is the emission at any defined holo frataxin concentration (C_s), and p is the deduced number of binding sites (using the ITC defined stoichiometry with $p \approx 1$).

Quantitation of Frataxin Binding to ISU Isothermal Titration Calorimetry. ITC measurements were carried out at 27 °C on a MicroCal OMEGA ultrasensitive titration calorimeter (Figure 4). The titrant and sample solutions were made from the same stock buffer solution (50 mM HEPES buffer, pH 7.5), and both experimental solutions were thoroughly degassed before each titration. The solution in the cell (a 0.03 mM solution of apo frataxin or holo frataxin with bound ferric ion) was stirred at 200 rpm by syringe to ensure rapid mixing. Typically, 7 μL of titrant (a 0.5 mM solution of human ISU) was delivered over 10 s with an adequate interval (10 min) between injections to allow complete equilibration. Titrations continued until 5 equiv had been added to ensure no further complex formation following addition of excess titrant. A background titration, consisting of the identical titrant solution but only the buffer solution in the sample cell, was subtracted from each experimental titration to account for heat of dilution. The data were collected automatically and subsequently analyzed, as described earlier, by use of a one-site binding model by the Windows-based Origin software package supplied by MicroCal.

Iron−Sulfur Cluster Reconstitution. Iron−sulfur reconstitution was performed with D37A human ISU, since this derivative stabilizes the bound cluster.[25] A solution of 50 μM apo-D37A HISU in 50 mM Tris-HCl, pH 7.5, was argon purged. DTT was added from a concentrated stock to a final concentration of 50 mM. Subsequently an equivalent volume of an argon-purged solution of 50 μM holo frataxin and 100 μM Na_2S was added. After 45 min the product holo ISU was isolated from the reaction solution by passage through a G-25 desalting column and subsequent ion exchange chromatography on a DE-52 column, as previously described.[13,25] For reconstitution reactions using iron ion in place of holo frataxin, a buffered solution that contained the same total iron concentration was used. For reconstitution reactions using NifS-mediated sulfur delivery a catalytic amount of NifS (1 μM) and 100 μM Cys were used. In each case the yield of holo ISU was quantitated by use of optical spectroscopy and published extinction coefficients.[25]

The cluster in the isolated reconstituted ISU was also characterized by circular dichroism spectroscopy using an Aviv model 202 circular dichroism spectrometer with 10 mm path-length cuvette. Spectra were obtained at 25 °C and a scan rate of 35 nm/min. Control buffer spectra were subtracted, and the data shown in Figure 6 are the average of four different spectra.

Kinetics of Iron Sulfur Cluster Reconstitution. All components for D37A ISU reconstitution were prepared under anaerobic conditions. To a solution of 100 μM ISU, 4.3 mM DTT, and 2.4 mM Na_2S in 50 mM Tris-HCl buffer (pH 7.5) was added holo frataxin to a final concentration of 40 μM. Holo frataxin was the only iron source for cluster reconstitution, and cluster formation was monitored by UV−vis absorption spectroscopy. The time course for reaction progress was monitored at 456 nm, a characteristic absorption band for the $[2Fe\text{-}2S]^{2+}$ iron sulfur cluster. A control experiment was carried out in the absence of ISU. Similarly, NifS[22] was also used as the sulfur donor with 1 μM enzyme and 2.4 mM cysteine in place of the 2.4 mM Na_2S.

The kinetics of iron release was also determined spectrophotometrically using bipyridine as a ferrous ion binding ligand ($\lambda \approx 520$ nm) and tiron for ferric ion binding ($\lambda \approx 480$ nm). In each case 4.6 μL of a 3 mM stock solution of the ligand was added to a 400 μL solution of 35 μM holo frataxin, all in 50 mM HEPES buffer at pH 7.5. For ferrous ion the solution also contained 10 mM DTT to maintain the reduced state.

Table 1. Iron Binding Stoichiometry to Human Frataxin

method	Fe:protein
iron assay	6.2 ± 0.1
fluorescence	6.4 ± 0.2
ITC	6.5 ±0.2

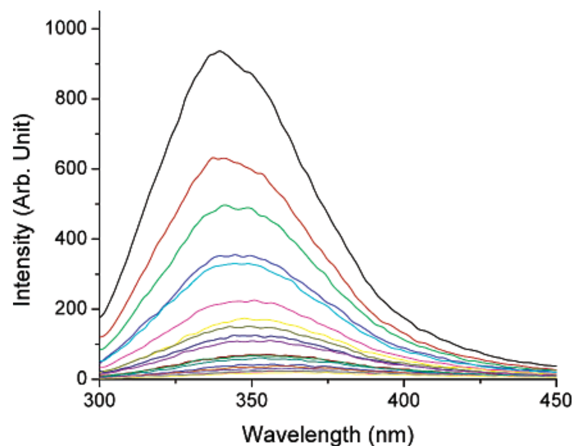
Results and Discussion

Stoichiometry and Thermodynamics of Iron Binding to Frataxin. Consistent with prior reports,[2,10,26] we observed the mature expressed and purified form of frataxin to undergo autoproteolysis to a shorter protein extending from residue 81 to 210. The apo form of this 14.3 kDa protein has been the subject of prior NMR[9] and X-ray crystallographic[10] structure determinations. Holo frataxin was readily formed by addition of at least a 10-fold excess of ferric ion to the apo protein and excess iron removed by gel filtration chromatography. No significant release of iron was observed during several cycles of dilution and ultrafiltration or following further gel filtration chromatography. The iron binding stoichiometry for holo frataxin was subsequently determined by a variety of methods (Table 1). By use of a colorimetric assay the iron content of a sample of holo frataxin was determined by complex formation

(24) Winzor, D. J.; Sawyer, W. H. *Quantitative Characterization of Ligand Binding*; Wiley-Liss: New York, 1995.

(25) Foster, M. W.; Mansy, S. S.; Hwang, J.; Penner-Hahn, J. E.; Surerus, K. K.; Cowan, J. A. *J. Am. Chem. Soc.* **2000**, *122*, 6805−6806.

(26) Cho, S.-J.; Lee, M. G.; Yang, J. K.; Lee, J. Y.; Song, H. K.; Suh, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8932−8937.



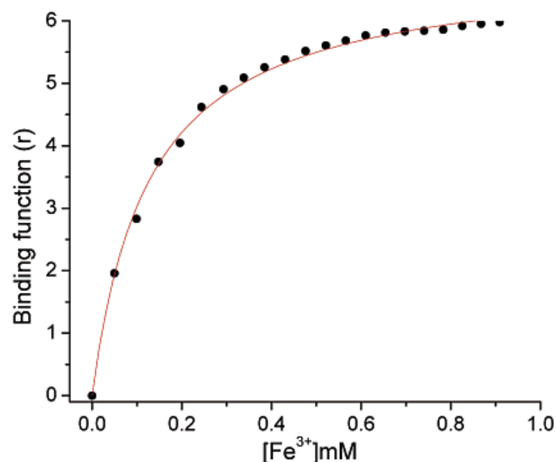
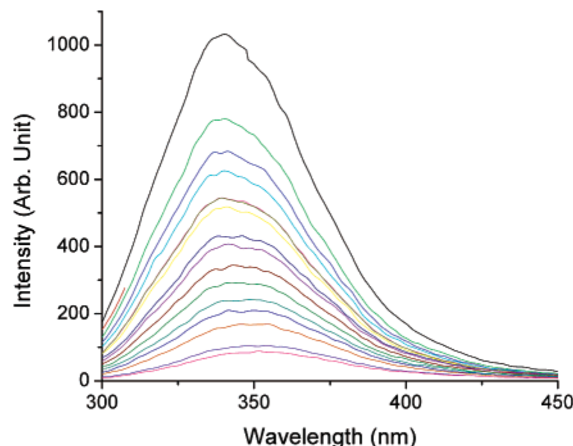


Figure 1. (top) Fluorescence spectra showing the quenching of natural Trp fluorescence for apo frataxin following titration with ferric ion. Spectra were obtained on a 24 μM solution of apo frataxin, and ferric ion was titrated from a stock solution of $FeCl_3$ over the concentration range shown. Protein and iron samples were prepared in argon-purged 100 mM HEPES, pH 7.5, and 50 mM NaCl. Excitation and monitoring wavelengths were 291 and 341 nm, respectively. (bottom) Binding curve generated from analysis of the fluorescence quenching measurements following the method of Winzor.[24] Fitted parameters (stoichiometry and apparent binding affinity) are summarized in Tables 1 and 2.



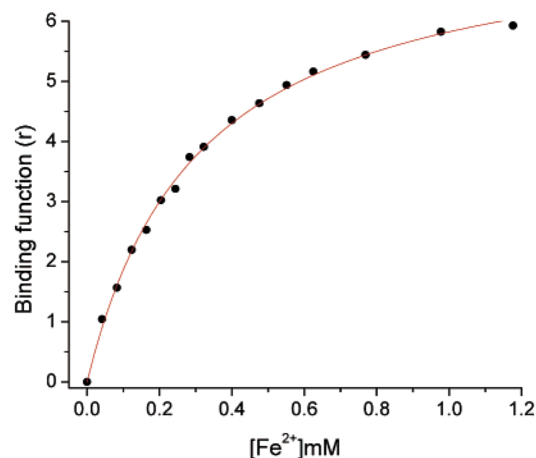


Figure 2. (top) Fluorescence spectra showing the quenching of natural Trp fluorescence for apo frataxin following titration with ferrous ion. Spectra were obtained on a 15 μM solution of apo frataxin, and ferrous ion was titrated from a stock solution of $FeSO_4 \cdot 7H_2O$ over the concentration range shown. Protein and iron samples were prepared in argon-purged 100 mM HEPES, pH 7.5, 50 mM NaCl, and 5 mM DTT. Excitation and monitoring wavelengths were 291 and 341 nm, respectively. (bottom) Binding curve generated from analysis of the fluorescence quenching measurements following the method of Winzor.[24] Fitted parameters (stoichiometry and apparent binding affinity) are summarized in Tables 1 and 2.

with bathophenanthrolinedisulfonate (from its 535 nm absorbance), while protein was quantitated by use of a standard Bradford assay. Data were obtained and compared with control calibrant standards. A stoichiometry of ~6.2 iron ions to frataxin was determined.

Further evidence for the formation of an iron−frataxin complex was provided by fluorescence quenching experiments. Tryptophan fluorescence of human frataxin in 100 mM HEPES buffer (pH 7.5) was quenched by addition of either ferric or ferrous ion (Figures 1 and 2). Human frataxin contains three tryptophans, and two of them are conserved across a variety of species. Following excitation at 291 nm, the variation of emission intensity was analyzed following the procedure of Winzor and Sawyer (Figures 1 and 2).[24] In each case the data yielded mean values for the stoichiometry of ~6.4, and apparent dissociation constants for ferrous and ferric ions were measured as 55.0 and 11.7 μM, respectively.

Iron binding to human frataxin was also studied by isothermal titration calorimetry (ITC) at 27.0 °C in 100 mM HEPES (pH 7.5) buffer solution with 1.0−2.5 mM ferric ion in the same buffer (Figure 3). Ferric ion solutions were prepared by adding 0.5 M $FeCl_3$ in 0.05 M HCl into 100 mM pH 7.5 HEPES buffer to obtain a desirable range of ferric ion concentration. To avoid solubility problems for ferric ion, a concentrated stock was prepared in acidic solution. No change of pH was observed after mixing into 100 mM HEPES buffer. Moreover, a background titration, consisting of the identical titrant solution but only the buffer solution in the sample cell, was subtracted from each experimental titration to account for heat of dilution. All samples were thoroughly degassed before each titration. The ITC determined stoichiometry and dissociation constants for ferric ion binding experiments are ~6.5 and 10.2 μM, respectively (Table 2). Consistent results have therefore been determined for a variety of experimental methods. The data obtained show a stoichiometry of ~6−7 iron ions per mole of frataxin, with a dissociation constant in the range 10−55 μM.

Stoichiometry and Thermodynamics of ISU Binding to Frataxin. Complex formation between holo frataxin and apo human ISU was established by two independent methods. A

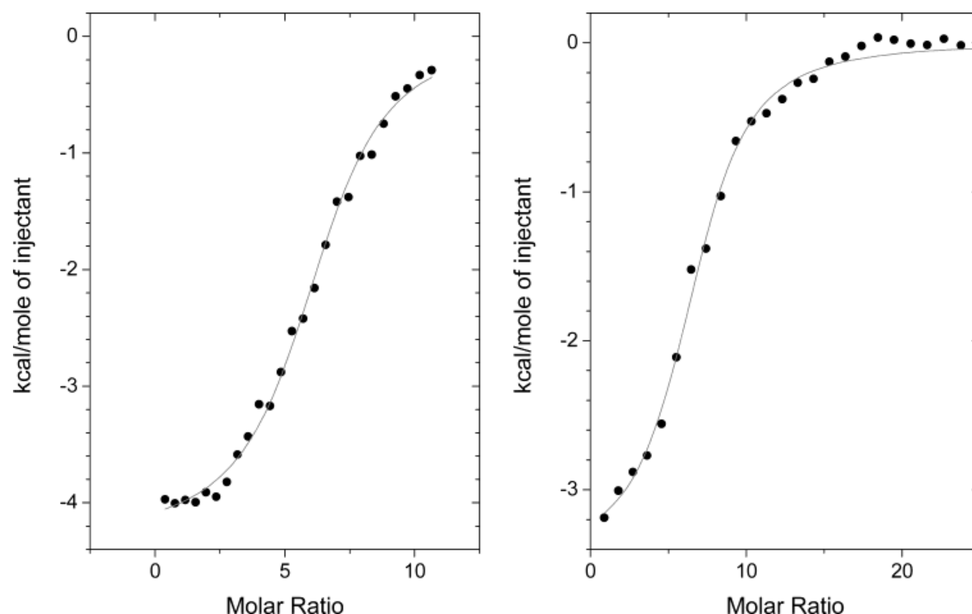


Figure 3. ITC measurements of ferric ion binding to apo frataxin carried out at 27 °C on a MicroCal OMEGA ultrasensitive titration calorimeter. All samples were in degassed 100 mM HEPES (pH 7.5) buffer solution and measurements made over two concentration ranges. (left) A 1 mM stock of Fe^{3+} was titrated into a solution of 20 μM frataxin. (right) A 2.5 mM stock of Fe^{3+} was titrated into a solution of 20 μM frataxin. Data were collected automatically and subsequently analyzed with a Windows-based Origin software package supplied by MicroCal.

Table 2. Calorimetric and Fluorescence Quantitation of Iron Binding to Human Frataxin

ITC[a]		fluorescence	
ion	K_D (μM)	ion	K_D (μM)
Fe^{3+}	10.2	Fe^{3+}	11.7
Fe^{2+}		Fe^{2+}	55.0

[a] For ferric ion binding the stoichiometry was determined as ~6.5 with measured ΔH of -17.9 kJ mol^{-1} and ΔS of 34.6 J K^{-1} mol^{-1} at 300 K.

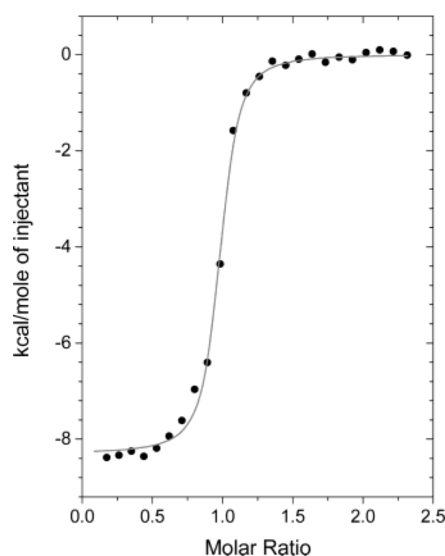


Figure 4. ITC measurements of holo frataxin binding to D37A HISU were carried out at 27 °C on a MicroCal OMEGA ultrasensitive titration calorimeter. All samples were in degassed 50 mM HEPES (pH 7.5) buffer. A 7 μL aliquot of a 0.5 mM solution of titrant was delivered over 10 s to 0.03 mM holo frataxin with an adequate interval (10 min) between injections to allow complete equilibration. Data were collected automatically with a software package supplied by MicroCal.

$K_D \approx 0.15$ μM was determined by isothermal titration calorimetry (Figure 4) with binding both enthalpically and entropically favorable (Table 3). Significantly, no response was

Table 3. Frataxin Binding to ISU Monitored by Isothermal Titration Calorimetry (ITC)

ISU	frataxin	n	ΔH (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})	K_D (μM)
D37A apo ISU	holo frataxin	0.94	-8.3	3.6	0.15
D37A apo ISU	apo frataxin	nd	nd[a]	nd	nd
apo ISU	apo frataxin	nd	nd	nd	nd

[a] nd = no detectable binding.

obtained for apo frataxin binding to apo ISU, and so either complex formation does not occur, or the enthalpy change for apo to apo complex formation was beyond detection levels. To distinguish these two possibilities and confirm complex formation by holo frataxin, binding affinities were further studied through fluorescence quenching experiments.

Human ISU contains one Trp with $\lambda_{em}^{max} \approx 341$ nm (Figure 5). While frataxin also contains three Trp residues,[10] the emission from these is strongly quenched by the presence of bound iron (Figures 1 and 2). Following complex formation with holo frataxin the emission from ISU was quenched in a concentration-dependent manner until available binding sites were populated (Figure 5). Measured binding affinities (Table 4) were found to be similar to those determined by calorimetry (Table 3). Substitution of Asp37 of ISU with Ala, which has previously been shown to stabilize the ISU-bound Fe−S cluster,[25] was found to have no significant effect on complex formation with frataxin (Tables 3 and 4). N-terminal His- and non-His-tagged versions of frataxin were both found to behave similarly in binding experiments, and so the His tag does not interfere with complex formation. Again, no evidence was found for complex formation between apo frataxin and apo ISU. Both fluorescence binding experiments and ITC therefore support this conclusion, which strongly implicates frataxin-bound iron in promoting association with ISU, possibly by stabilizing a contact surface with negatively charged residues on the latter.

Frataxin-Mediated Reconstitution of ISU. Reconstitution of human ISU was readily carried out with holo frataxin as iron

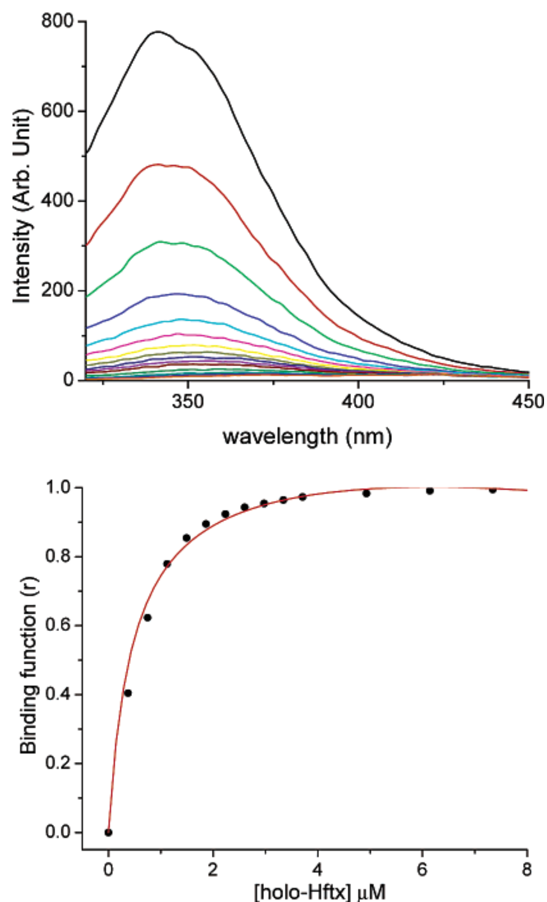


Figure 5. (top) Fluorescence spectra showing the quenching of natural Trp fluorescence for apo D37A human ISU following titration with holo frataxin. A 2.8 mL volume of a 3 μM solution of D37A ISU was titrated with a 0.35 mM solution of holo frataxin. Both protein samples were prepared in argon-purged 50 mM HEPES, pH 7.5. Excitation and monitoring wavelengths were 291 and 341 nm, respectively. (bottom) Binding curve generated from analysis of the fluorescence quenching measurements following the method of Winzor.[24] Fitted parameters are summarized in Table 4.

Table 4. Frataxin Binding to ISU Monitored by Fluorescence

ISU[a]	frataxin	K_D (μM)
apo ISU	holo frataxin	0.67
apo ISU	apo frataxin	nd[b]
D37A apo ISU	holo frataxin	0.45
D37A apo ISU	apo frataxin	nd

[a] Data obtained with $(His)_6$-tag ISU samples gave similar results. [b] nd = no detectable binding.

source and inorganic sulfide, producing ~70% of holo ISU based on published extinction coefficients.[25] We have previously characterized the Fe−S cluster in human ISU as a $[2Fe-2S]^{2+}$ center.[25] The cluster formed by frataxin-mediated reconstitution was verified as such by the similarity in UV−vis and circular dichroism spectra (Figure 6) to previously published examples.[12,14,16,25] With NifS[22] as a sulfur donor in reconstitution experiments, yields in excess of 70% were obtained.

Direct transfer of iron to apo ISU is supported by several key observations. First, neither ferric nor ferrous ion is released from holo frataxin on the time scale of the reconstitution reaction in the absence of an external acceptor ligand, while addition of similar concentrations of free iron and sulfide results in no observable reconstitution under otherwise similar reaction conditions. The presence of reducing agents such as DTT

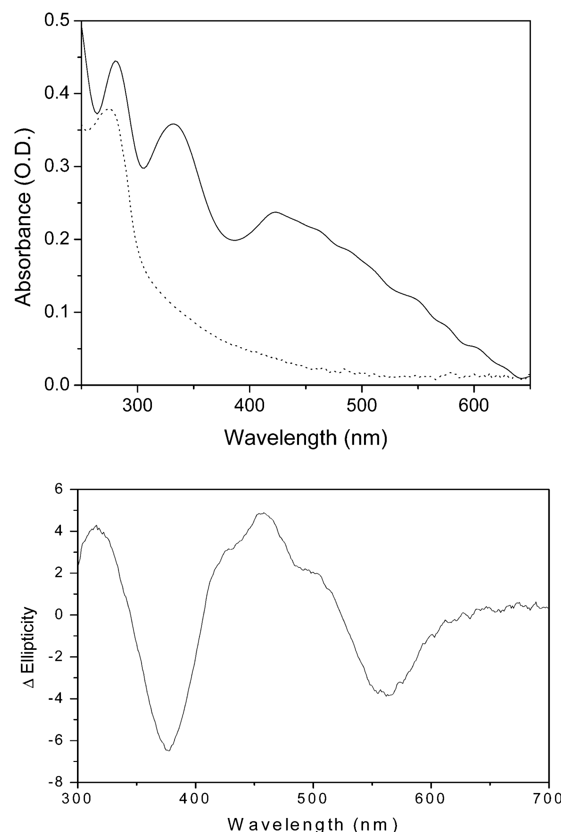


Figure 6. UV−vis (top) and CD (bottom) spectra for apo and isolated holo ISU following frataxin-mediated assembly of the [2Fe-2S] cluster in ISU and subsequent chromatographic purification. Spectra were obtained in 50 mM Tris buffer, pH 7.5, as described in the experimental procedures.

facilitates the reaction. Presumably these serve to prevent disulfide bond formation on ISU, but it is also possible that ferrous ion is the preferred oxidation state for transfer to ISU.

Kinetics of Frataxin-Mediated Reconstitution of ISU and Iron Release from Frataxin. The kinetics of [2Fe-2S] cluster formation was directly monitored from the time dependence of the 456 nm absorbance of the $[2Fe-2S]^{2+}$ cluster in holo ISU (Figure 7). A kinetic rate constant $k_{obs} \approx 0.075$ min^{-1} was determined with 100 μM ISU, 2.4 mM Na_2S, and 40 μM holo frataxin in 50 mM Tris-HCl (pH 7.5) with 4.3 mM DTT. Control experiments were carried out in the absence of ISU to confirm the observed time course for iron sulfur cluster formation in D37A ISU. Moreover, when free iron ion was used as an iron source for cluster assembly on ISU, rather than frataxin-mediated iron delivery, a negligible rate of cluster formation was observed under these solution conditions. Nevertheless, it is important to note that Fe−S clusters (including the ISU-bound cluster) can be assembled or reconstituted in vitro with no accessory proteins under appropriate (typically higher and nonphysiological) concentration conditions. The fact that the cluster can be reconstituted in the absence of protein mediators provides an important indicator of the intracellular roles for many proteins on the Fe−S cluster assembly pathway. Indeed such proteins are probably more correctly viewed as carrier proteins, rather than as catalysts for the reaction, that both prevent the toxicity associated with free iron and sulfide and allow delivery at lower intracellular concentrations of these species.

As noted earlier, holo frataxin is stable to iron release in the absence of external ligands. By following the appearance of

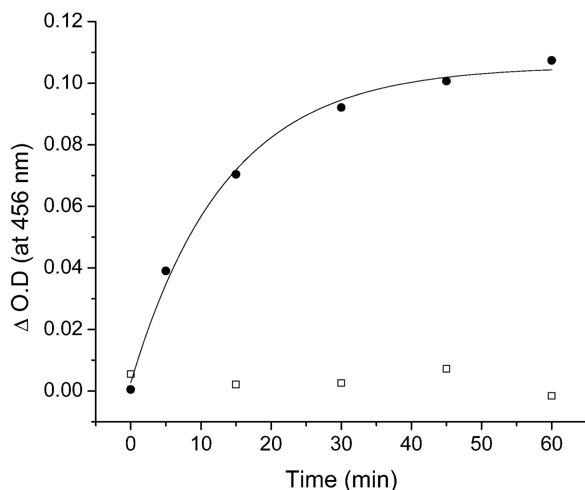
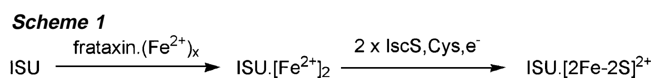


Figure 7. (filled circles) Time dependence for formation of the $[2Fe-2S]^{2+}$ cluster of holo D37A ISU. The solution contained 100 μM ISU, 4.3 mM DTT, and 2.4 mM Na_2S in 50 mM Tris-HCl buffer (pH 7.5), to which was added holo frataxin to a final concentration of 40 μM. Reaction progress was monitored at 456 nm. (empty squares) Control experiment with no ISU added.

characteristic absorbance bands for each complex, apparent rate constants were determined in the presence of acceptor ligands (bipyridine for Fe^{2+} and tiron for Fe^{3+}) under conditions similar to those used for frataxin-mediated reconstitution of human ISU. Both reactions followed pseudo-first-order profiles at 1:1 ligand to holo frataxin (or 1:7 ligand:iron) with $k_{obs} \approx 0.15 \pm 0.01$ min^{-1} for Fe^{2+} and 0.16 ± 0.01 min^{-1} for Fe^{3+}. The experiment with ferrous ion was carried out in the presence of 10 mM DTT to maintain the reduced state; however, DTT does not competitively remove ferrous ion from the protein. Assuming similar rates of release to ISU, such transfer reactions could be rate limiting for ISU cluster assembly (compare the rate constants noted above for cluster formation). The similarity in rate constants for ferrous ion and ferric ion release suggests that either state could be transferred to the apo ISU; however, under physiological conditions it is likely that the ferrous state is most relevant.

Model for [2Fe-2S] Cluster Biosynthesis. Human frataxin is a mitochondrial protein that has been implicated with both iron metabolism and mitochondrial iron homeostasis; however, the function of human frataxin has been unclear. To address this issue, we have characterized the iron binding properties of human frataxin using a variety of experimental methods and further demonstrated complex formation between holo frataxin and apo human ISU by two independent methods. Holo frataxin is herein shown to mediate reconstitution of ISU in a kinetically reasonable time frame. As a whole these data allow us to further develop the working model that we have earlier proposed for assembly of the [2Fe-2S] cluster in ISU-type proteins.[22] In this model (Scheme 1) two iron ions bind to apo ISU and establish a nucleation site for [2Fe-2S] cluster formation. Subsequent sulfide delivery is mediated through the persulfide form of IscS that complexes with ISU. Two $2e^-$-electron reductive cleavages of IscS persulfide bonds yield the 2 equiv of inorganic S^{2-} for cluster formation.

Scheme 1



Here we establish holo frataxin as a functional iron donor for the initial step of the reaction pathway for mitochondrial Fe−S cluster assembly. In the case of the yeast *Saccharomyces cerevisiae* Kaplan and co-workers have documented some evidence for the presence of clusters in cytosolic proteins even when the frataxin gene had been deleted.[7] Such an observation may reflect either an additional, and yet to be identified, mitochondrial protein that can also mediate iron delivery and the formation of Fe−S clusters or the presence of a direct cytosolic cluster assembly pathway.[21] The distinct iron-promoted aggregation properties of the yeast and human frataxins,[2,11] together with their distinct influence on their respective mitochondrial respiratory processes,[27,28] do, however, provide a warning that the yeast model may not carry over to human cell lines. Nevertheless, the prevalence of frataxin and homologues in eukaryotic and prokaryotic cell lines,[9,10,26] taken with the evidence that we describe in this report, does demonstrate an important, direct, and central role for frataxin in the biosynthesis of Fe−S clusters as an iron donor protein to ISU.

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(27) Chen, O. S.; Kaplan, J. *FEBS Lett.* **2001**, *509*, 131−134.
(28) Ristow, M.; Pfister, M. F.; Yee, A. J.; Schubert, M.; Michael, L.; Zhang, C. Y.; Ueki, K.; Michael, M. D.; Lowell, B. B.; Kahn, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12239−12243.