Frataxin-mediated Iron Delivery to Ferrochelatase in the Final Step of Heme Biosynthesis*

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Human ferrochelatase, a mitochondrial membrane-associated protein, catalyzes the terminal step of heme biosynthesis by insertion of ferrous iron into protoporphyrin IX. The recently solved x-ray structure of human ferrochelatase identifies a potential binding site for an iron donor protein on the matrix side of the homodimer. Herein we demonstrate Hs holofrataxin to be a high affinity iron binding partner for Hs ferrochelatase that is capable of both delivering iron to ferrochelatase and mediating the terminal step in mitochondrial heme biosynthesis. A general regulatory mechanism for mitochondrial iron metabolism is described that defines frataxin involvement in both heme and iron-sulfur cluster biosyntheses. In essence, the distinct binding affinities of holofrataxin to the target proteins, ferrochelatase (heme synthesis) and ISU (iron-sulfur cluster synthesis), allows discrimination between the two major iron-dependent pathways and facilitates targeted heme biosynthesis following down-regulation of frataxin.

Frataxin is a nuclear-encoded protein that is targeted to the mitochondrial matrix. Reduced frataxin expression, a causative agent of the neurological disorder Friedreich ataxia, results in mitochondrial iron accumulation. Recent evidence has pointed to a functional role for frataxin in mitochondrial iron metabolism, including iron-sulfur cluster (1–5) and heme (6–8) biosynthesis. We have reported earlier that frataxin serves as an iron donor to ISU, the iron-sulfur-cluster scaffold protein (1). Isothermal titration calorimetry and fluorescence quenching experiments demonstrated human frataxin to bind 6 or 7 iron ions with $K_D$ $\approx$ 10–50 $\mu$M for the isolated protein (1). Holofrataxin was further shown to bind to ISU with a $K_D$ $\approx$ 0.15 $\mu$M, and the functional viability of frataxin as an iron donor for assembly of the [2Fe-2S] cluster of ISU in the presence of a sulfur donor was demonstrated through kinetic and spectroscopic studies (1). Iron release by frataxin appeared to be the rate-limiting step. Overall these results correlate well with other published observations concerning a possible role for frataxin in iron-sulfur cluster biosynthesis (2–5).

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To further characterize potential roles for frataxin as a mitochondrial iron donor, we investigated the involvement of Hs frataxin in cellular heme biosynthesis as an iron donor to Hs ferrochelatase. Although the identity of the iron donor protein in heme biosynthesis has not been established, involvement by frataxin has been suggested on the basis of yeast studies that demonstrated mitochondrial iron to be unavailable for heme biosynthesis in cells lacking frataxin (6–8). Dancis and co-workers (6) have recently reported genetics experiments that implicate the involvement of yeast frataxin in heme biosynthesis and have estimated a binding affinity ($K_D$) for frataxin to ferrochelatase of $\approx$ 40 nM by surface plasmon resonance, although no evidence for frataxin-mediated iron delivery in heme biosynthesis was presented. Herein we characterize the interaction of human frataxin with ferrochelatase, and demonstrate holofrataxin to serve as a potential iron donor to ferrochelatase for insertion into the protoporphyrin ring during heme synthesis.

EXPERIMENTAL PROCEDURES

Protein Production and Purification—Human ferrochelatase was expressed and purified as described previously (9). Human frataxin was also obtained as described previously (1), with the following modifications. The expressed protein was purified by nickel-nitrioltriacetic acid chromatography as described (1), and the mature truncated form of frataxin, encompassing residues 81–210, was obtained by an autolysis reaction following incubation at 4 °C for 2 weeks (as observed previously (10–12)) and subsequent fast protein liquid chromatography. The purity of the truncated protein was confirmed by SDS-PAGE, which demonstrated a single protein band with a molecular mass of $\approx$ 14 kDa, consistent with electrospray ionization mass spectrometric analysis that yielded a mass of 14,661 Da. Samples were either used immediately following purification or stored in their final elution buffers at $\approx$ 80 °C. Holofrataxin was prepared under strictly anaerobic conditions as described previously (9) with ferrous iron in 50 mM HEPES, 100 mM KCl, pH 8.0 buffer.

ITC Measurements of Binding—ITC1 measurements were carried out at 25 °C using a MicroCal Omega ultrasonic titration calorimeter. The sample solutions were made from the stock buffer solution (50 mM HEPES, 100 mM KCl, 1% sodium cholate, pH 8), and the titrant solution was made up in 50 mM Tris (pH 7.5), 100 mM NaCl. The purity of the truncated protein was confirmed by SDS-PAGE, which demonstrated a single protein band with a molecular mass of $\approx$ 14 kDa, consistent with electrospray ionization mass spectrometric analysis that yielded a mass of 14,661 Da. Samples were either used immediately following purification or stored in their final elution buffers at $\approx$ 80 °C. Holofrataxin was prepared under strictly anaerobic conditions as described previously (9) with ferrous iron in 50 mM HEPES, 100 mM KCl, pH 8.0 buffer.

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Stoichiometric Titration by Fluorescence Quenching—The tryptophan fluorescence of human ferrochelatase solution was measured in...
Role for Frataxin in Heme Biosynthesis

Frataxin and binding to ferrochelatase and ISU.

A. Kinetic Measurements of Ferrochelatase Activity—The disodium salt of protoporphyrin IX was obtained from Aldrich. A protoporphyrin stock solution was prepared in 2 M NH₄OH (0.1 ml) followed by the addition of 1.9 ml of a buffered solution containing 50 mM Tris–HCl, 100 mM KCl, 1% sodium cholate, pH 8.0. Stock solutions were diluted with the same Tris–HCl buffer to make a desirable concentration of protoporphyrin before each experiment. Ferrochelatase activity was measured anaerobically at 20 °C by monitoring the rate of disappearance of the protoporphyrin IX band at 506 nm. A 2-ml solution containing 11.8 μM protoporphyrin, 200 nM ferrochelatase, 2.5 mM dithiothreitol, and various concentrations of apofrataxin in 50 mM Tris–HCl, 100 mM KCl, 1% sodium cholate, pH 8.0, was degassed and argon-purged for at least 20 min. To trigger the activity of ferrochelatase, buffered solutions of ferrous ion and citrate were added by injection to the reaction mixture in the cuvette to achieve a final ferrous concentration of 5.8 μM and a citrate concentration of 2 mM.

RESULTS

To demonstrate the potential involvement of frataxin in heme biosynthesis, we have quantitatively investigated complex formation between human holofrataxin and human ferrochelatase. The cloned frataxin gene incorporated a His₆ tag and residues following the second mitochondrial processing peptidase cleavage site (11) (residues 47–210 were included). Cloned His₆-tagged frataxin was expressed and purified as described (1); however, over a period of several days this autocleaves to a truncated protein (residues 81–210) that lacks the N-terminal His₆ tag. The formation of a truncated protein has been documented previously (10–12) and is the form of the protein that has been structurally characterized in the apo form (12). Earlier studies have demonstrated this truncated human holofrataxin to be monomeric (1).

Isothermal titration calorimetry provided a measured KD ~17 nM, with binding both enthalpically (∆H = −3.7 kcal/mol) and entropically (∆S = 23 cal/K·mol) favorable (Fig. 1A), and parameters are defined per mol of either frataxin or ferrochelatase dimer. No detectable binding response was measured in the absence of iron ion, consistent with observations made in binding studies of apofrataxin and apoISU (1). For the latter study it was deduced that iron ion might mediate a bridging contact between the two proteins rather than a structural

Fig. 1. Frataxin and binding to ferrochelatase and ISU. A, experiments were carried out at 25 °C on a MicroCal Omega ultrason-
transition, because iron binding does not influence frataxin structure. Certainly the proposed frataxin (13) and ferrochelatase (14, 15) binding sites show a number of acidic residues that might coordinate to bridging iron ions. High affinity binding of holofrataxin and ferrochelatase was also confirmed by fluorescence quenching experiments (Fig. 1C). Although binding was too tight for accurate quantitation, both data sets are consistent with the nM affinity of one frataxin to a ferrochelatase dimer.

Holofrataxin was previously shown to bind to the iron-sulfur scaffold protein, ISU, with a $K_D = 0.15 \mu M$. To allow a comparison with holofrataxin binding to ferrochelatase, the former was determined under similar solution conditions to that reported here for frataxin binding to ferrochelatase, including the presence of 1% sodium cholate. Under similar solution conditions to those used for ferrochelatase binding (Fig. 1A), ITC experiments provided a measured $K_D = 0.48 \mu M$ for frataxin binding to human apoISU, with a binding stoichiometry of 0.95, $\Delta H = -2.1$ kcal/mol, and $\Delta S = 22$ cal/K/mol (Fig. 1B).

To understand the role of frataxin in heme biosynthesis, we performed additional experiments to measure ferrochelatase activity as a function of frataxin concentration at fixed ferrous ion concentration. Ferrochelatase activity was measured by following the absorbance change at 506 nm that results from heme production. Activity was found to increase with frataxin concentration and was optimal at a ratio of 1 frataxin molecule/ferrochelatase dimer (Fig. 2). These results support the hypothesis that frataxin recruits iron ion and mediates delivery to ferrochelatase. Free ferrous iron can also be delivered to ferrochelatase by nonspecific collision; however, free iron is not a bioavailable species. The sharply defined optimal frataxin/ferrochelatase ratio reflects the high affinity interaction and stoichiometric complex formation.

The observed decrease in ferrochelatase activity with increasing frataxin concentration may be explained in several ways. Excess frataxin might bind elsewhere on ferrochelatase and inhibit its function. However, our previous ITC and fluorescence experiments show no evidence for binding of additional frataxin molecules in the concentration range used. A more likely explanation stems from the iron binding properties of frataxin that removes “free” iron from solution, lowering the available iron concentration and inhibiting ferrochelatase activity.

**DISCUSSION**

The three-dimensional structure of human ferrochelatase (14, 15) demonstrates the active form of human ferrochelatase to be a homodimer, similar to that for Saccharomyces cerevisiae ferrochelatase (16). These studies show that the binding and release pathways for protoporphyrin IX and heme, respectively, are located on the inner membrane side of a homodimeric form of ferrochelatase. However, the putative iron-binding site is found on the opposite side of the protein, which is exposed to the mitochondrial matrix (15), and forms an appropriate domain for docking of an iron donor protein. The reaction stoichiometry observed by ITC and fluorescence quenching experiments is consistent with this hypothesis. The possibility that iron delivery from holofrataxin to ferrochelatase occurs via a specific intermolecular interaction was discussed, because heme levels from ferrochelatase activity did not significantly change with excess of the iron chelator, citrate. These results are consistent with findings for copper delivery from the carrier protein (Atx1) to a target protein (Ccc2) (17), a process that was not influenced by the presence of the copper chelator, glutathione.

Finally, although deletion of frataxin does not eliminate cluster or heme synthesis (low molecular weight cellular iron species or other donor proteins might serve) (18), the evidence reported here does support a direct role for frataxin in the biosynthesis of hemes. It is significant that holofrataxin functions as an iron donor in both heme and iron-sulfur cluster biosynthesis, since this allows for a simple control mechanism for utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of holofrataxin. Thus, in the case of a heme-deficient condition, holofrataxin should be used mainly for the biosynthesis of hemes. It is significant that holofrataxin prevents utilization of heme synthesis at the same developmental stage with increasing concentration of protoporphyrin (7). The published data can be explained in terms of a general model for iron utilization in mitochondria (Fig. 3). The binding affinity between holofrataxin and ferrochelatase ($K_D = 17 \text{ nm}$) is 28 times greater than the affinity between holofrataxin and ISU ($K_D = 0.48 \mu M$), and so reduced frataxin levels will have a
more significant impact on iron-sulfur cluster biosynthesis. Although the complete details of the molecular mechanism of iron regulation in mitochondria remain unknown, Fig. 3 shows a plausible model from the perspective of iron utilization.

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Fig. 3. Cellular model for the regulation of frataxin chemistry in iron-sulfur cluster and heme biosynthesis. Holofrataxin ([Hftx]) is used as an iron donor for both heme and iron-sulfur cluster biosynthetic pathways. Under normal cell growth conditions the frataxin concentration is sufficient for both heme and iron-sulfur cluster syntheses. The level of frataxin is down-regulated in erythroid differentiation, as is the iron-sulfur cluster biosynthesis pathway. However, heme biosynthesis remains essentially normal as a consequence of the distinct binding affinities of frataxin to ISU and ferrochelatase.