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Analysis of human ferrochelatase iron binding via amide hydrogen/deuterium exchange mass spectrometry

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ABSTRACT

Human ferrochelatase (E.C. 4.99.1.1) is a membrane-associated enzyme that catalyzes the last step in the heme biosynthetic pathway, the insertion of ferrous iron into protoporphyrin IX. Crystallographic structures have revealed that protoporphyrin binds in a cleft between two domains; however, the entry pathway and location of the iron binding site(s) is still contested. In an effort to address this issue, the structural elements involved in binding substrate iron were studied by amide hydrogen/deuterium exchange mass spectrometry. The deuterium incorporation rates into the backbone of apo- and iron-ferrochelatase in the absence of porphyrin substrate were measured. For the first time, it is demonstrated how the binding of ferrous iron specifically modulates ferrochelatase structure in solution. The distinct regions affected by the presence of iron provide insight into the mechanism by which iron is transported to the active site.

1. Introduction

Ferrochelatase (E.C. 4.99.1.1) catalyzes the terminal step of heme biosynthesis by catalyzing the insertion of ferrous iron in protoporphyrin IX to form protoheme IX (Fig. 1) [1]. The newly formed heme is incorporated into various proteins and enzymes that perform vital functions in the human body such as cellular respiration and the transport of electrons and oxygen. The disruption of the heme biosynthetic pathway leads to several disorders and diseases. Mutations resulting in the diminished activity of ferrochelatase cause the inherited disorder erythropoietic protoporphyria (EPP) [2,3]. Individuals with EPP experience painful dermatologic photosensitivity due to tissue damage that occurs when accumulated protoporphyrin IX in the skin is exposed to visible light [4].

Human ferrochelatase exists as an inner mitochondrial membrane-associated homodimeric enzyme *in vitro* and each monomer contains a [2Fe–2S] cluster [5]. X-ray crystallographic structures of ferrochelatase from different organisms have been solved and all structures displayed two similar Rossmann-type domains with a porphyrin binding cleft between them [6–11]. In all published crystallographic structures of ferrochelatases, only the 1.7 Å resolution structure from *Bacillus subtilis* contained the Fe(II) substrate [11]. Crystallographic evidence with surrogate metals such as Hg(II) and Co(II) suggest there is an outer metal binding site (His231, Asp383) near the matrix-exposed surface of ferrochelatase as part of a channel leading from the surface of the protein to the catalytic site where protoporphyrin is bound [7,9]. In this pocket, His263 is suggested to abstract a proton from the porphyrin macrocycle during catalysis [12]. However, mutational studies of the human and yeast enzymes and the recent structure of *B. subtilis* ferrochelatase support that this invariant His residue is involved in Fe(II) binding and insertion into protoporphyrin IX [9,11,13]. Thus, position of the catalytic metal binding site in human ferrochelatase and the pathway involved in metal insertion is still a matter of debate.

Structures of various ferrochelatases with and without substrate metals do not show remarkable changes in conformation upon metal coordination in the absence of porphyrin [7,9]. This could be the result of soaking apo-ferrochelatase crystals in a metal solution and not having initially crystallized the Fe(II)-bound form of the enzyme. In addition, these static structures give little insight into the structural dynamic component of catalysis for most enzymes [14]. Recent structures indicate that porphyrin binding to ferrochelatase in the absence of iron causes the active site "mouth" to close, resulting in the reorientation of the binding pocket [10,12,15]. Many lines of evidence support that protein dynamics are critical to controlling steps in catalysis such as substrate binding or product release [15–18]. Thus amide hydrogen/deuterium exchange mass spectrometry (H/D-exchange MS)[1]

Abbreviations: HD-exchange MS, hydrogen/deuterium exchange mass spectrometry; β-ME, β-mercaptoethanol; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate; MPIX, mesoporphyrin IX; ddH_2O, doubly distilled H_2O; D_2O, deuterium oxide.

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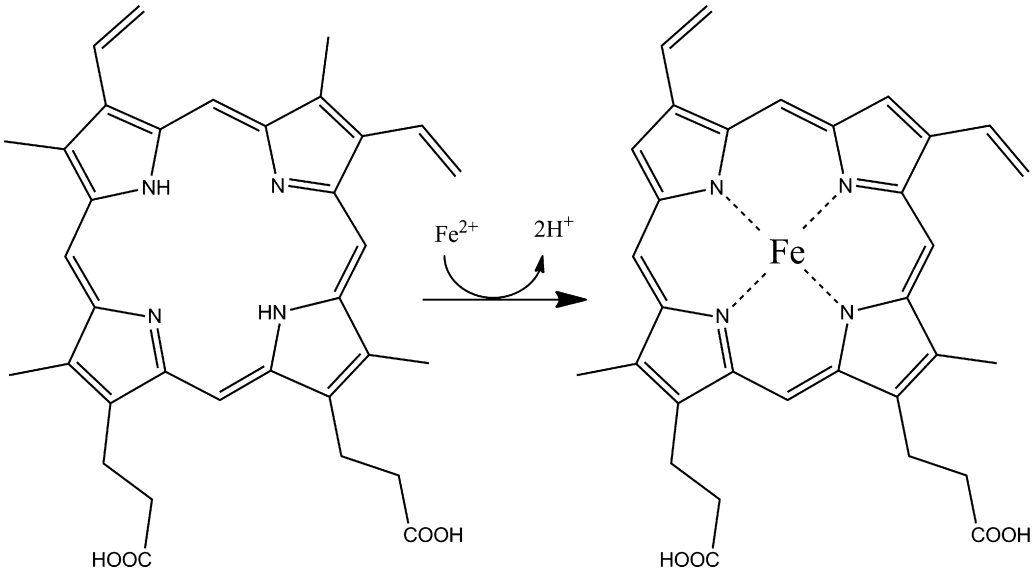

Fig. 1. Ferrochelatase reaction. Ferrochelatase (E.C. 4.99.1.1) catalyzes the formation of heme (protoheme IX) by insertion of ferrous iron into protoporphyrin IX.

presents an excellent method to probe the structural dynamics of ferrochelatase in relation to its catalytic mechanism [14]. Here, the technique was used to investigate the regions of the enzyme that experience changes in structure and/or dynamics in the presence of ferrous iron substrate. These studies provide structural insight into how ferrochelatase binds iron and transports it to the active site in the absence of protoporphyrin IX. In addition, this work has indicated new regions of ferrochelatase as of yet unexplored in terms of metal binding. The significance of these results is discussed.

2. Materials and methods

2.1. Protein expression and purification

Escherichia coli HB101 cells were transformed with pHisTF20E provided by Dr. Harry Dailey (University of Georgia) and plated on LB agar with 100 μg/mL ampicillin [19]. A single colony was used to inoculate 2 L of Circlegrow medium (Bio101, Vista, CA) that was supplemented with 50 μg/mL carbenicillin. The cells were incubated at 37 °C and 225 rpm until the cells reached stationary phase. The harvested cells were stored at a temperature of −20 °C.

The cells were resuspended in 10 mL of solubilization buffer (Buffer S) composed of 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM β-mercaptoethanol (β-ME), 10 mM imidazole, and 3% CHAPS. Phenylmethanesulphonyl fluoride (1 mg/mL) and lysozyme (1 mg/mL) were then added and the homogenate was lysed with a Branson Sonifier 250 (Danbury, CT) on ice with 30 s pulses at 60% saturation power. Following centrifugation at 14,000 × *g* for 15 min at 4 °C, the lysis supernatant was diluted with solubilization buffer (no CHAPS) to lower the CHAPS concentration to 1% for metal affinity chromatography.

Ferrochelatase was purified using 1 mL packed Ni-NTA Superflow resin (5 Prime, Gaithersburg, MD) equilibrated with Buffer S (10 mM imidazole). The resin and lysis supernatant were combined and allowed to incubate overnight at a temperature of 4 °C. Following incubation, the resin was loaded into a column and the eluant collected. The resin was washed with ∼1 mL of Buffer S, then ∼5 mL of Buffer S with 50 mM imidazole. Ferrochelatase was eluted with ∼2 mL of Buffer S plus 200 mM imidazole. Following purification, an additional 20 mM β-ME was added to the elution. Purified ferrochelatase was dialyzed against 2 L of chelexed 25 mM HEPES (pH 7.4), 100 mM NaCl, 20 mM β-ME, and 0.1% CHAPS at 4 °C. The concentration of ferrochelatase was estimated at 278 nm (ε_{278} = 46,910 M^{-1} cm^{-1}) and the percentage of bound [2Fe–2S] cluster was estimated by the absorbance at 330 nm (ε_{330} = 12,000 M^{-1} cm^{-1}) [19]. The iron content of purified apoferrochelatase was measured by atomic absorption spectroscopy and found to be 1.8 ± 0.1 mol iron per mol ferrochelatase, as expected if each monomer contains the [2Fe–2S] cluster.

2.2. Ferrochelatase activity measurements

The activity of ferrochelatase in the zwitterionic detergent CHAPS was accessed since previous purification schemes used the anionic detergent cholate which is not amenable to electrospray ionization mass spectrometry [19–21]. Activity measurements monitored the conversion of mesoporphyrin IX dihydrochloride (MPIX; Frontier Scientific, Logan, UT) to protoporphyrin IX through a decrease of the Soret band at 496 nm [22]. A 0.78 mM MPIX stock solution was made by dissolving 2.5 mg MPIX with 30 μL 2N ammonium hydroxide, 500 μL 10% Triton X-100 in 4.5 mL doubly distilled H_2O (ddH_2O). A 1 mM ferrous ammonium sulfate solution with 5 mM ascorbate in ddH_2O was prepared immediately prior to activity measurements. The assay mixture had a total volume of 1 mL and contained 100 mM Tris–HCl (pH 8.0), 0.5% Tween-20, 10 μM MPIX, 10 mM β-ME and 0.5 μM ferrochelatase at room temperature. Ferrous ammonium sulfate (0.5–30 μM) was added to start the reaction. The initial velocities were used to generate the Michaelis–Menten plot which yielded K_m^{Fe} = 5.8 ± 1.9 μM and a k_{cat} = 1.11 ± 0.04 min^{-1}, which are similar to reported values with sodium cholate as the detergent [22].

2.3. Pepsin peptide mapping

The protein sample for digestion contained ∼1 μM ferrochelatase in 50 mM potassium phosphate buffer (pH 2.3). Approximately 40 μg of porcine pepsin (Sigma Aldrich, St. Louis, MO) was added and the sample was incubated on ice for either 5 or 8 min. The 8 min digest did not yield a significantly different digestion result. The pepsin digest (20 μL) was loaded onto a Phenomenex microbore 1 mm × 50 mm C18 reverse-phase column (Torrence, CA) that had been equilibrated with HPLC-grade aqueous solvent (98% H_2O, 2% acetonitrile, 0.4% formic acid). The column was washed with aqueous solvent for 2 min at 60 μL/min