Research Article



Insight into the function of active site residues in the catalytic mechanism of human ferrochelatase

[©] Amy E. Medlock^{1,2}, Wided Najahi-Missaoui^{1,*}, Mesafint T. Shiferaw^{1,†}, Angela N. Albetel^{3,‡}, William N. Lanzilotta^{1,3} and Harry A. Dailey, Jr^{1,4}

¹Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, U.S.A.; ²Augusta University/University of Georgia Medical Partnership, Athens, GA 30602, U.S.A.; ³Department of Chemistry, University of Georgia, Athens, GA 30602, U.S.A.; ⁴Department of Microbiology, University of Georgia, Athens, GA 30602, U.S.A.;

Correspondence: Amy E. Medlock (medlock@uga.edu)

Ferrochelatase catalyzes the insertion of ferrous iron into a porphyrin macrocycle to produce the essential cofactor, heme. In humans this enzyme not only catalyzes the terminal step, but also serves a regulatory step in the heme synthesis pathway. Over a dozen crystal structures of human ferrochelatase have been solved and many variants have been characterized kinetically. In addition, hydrogen deuterium exchange, resonance Raman, molecular dynamics, and high level quantum mechanic studies have added to our understanding of the catalytic cycle of the enzyme. However, an understanding of how the metal ion is delivered and the specific role that active site residues play in catalysis remain open questions. Data are consistent with metal binding and insertion occurring from the side opposite from where pyrrole proton abstraction takes place. To better understand iron delivery and binding as well as the role of conserved residues in the active site, we have constructed and characterized a series of enzyme variants. Crystallographic studies as well as rescue and kinetic analysis of variants were performed. Data from these studies are consistent with the M76 residue playing a role in active site metal binding and formation of a weak iron protein ligand being necessary for product release. Additionally, structural data support a role for E343 in proton abstraction and product release in coordination with a peptide loop composed of Q302, S303 and K304 that act a metal sensor.

Introduction

The chelation of metal ions to form coenzymes, including heme, chlorophyll and cobalamin, is essential for most forms of life. These reactions are required for the production of functional coenzymes that are employed in a variety of cellular processes. For heme, an iron containing tetrapyrrole that functions in a number of biological processes including one electron transfer, gas binding, and cell signaling, the enzyme ferrochelatase (E.C. 4.99.1.1) is responsible for the insertion of ferrous iron into the porphyrin macrocycle. For many years this enzyme was viewed as a simple protein platform onto which a porphyrin would bind and distort thereby lowering the energy requirement for metal chelation [1]. The observation that small RNA/DNA molecules [2–4] and some antibodies [5] could catalyze the reaction, albeit at a greatly reduced rate, added support for this hypothesis. However, subsequent crystal structures of ferrochelatase in multiple conformations [6], coupled to biochemical data, demonstrated that the chelation process was not so simple. Interestingly, ferrochelatase is now also recognized as a key regulatory step in heme biosynthesis for some organisms [7,8] and subject to posttranslational modification to regulate its activity *in vivo* [9].

Ferrochelatase is a type II chelatase which does not require ATP for activity [10]. *In vitro* it can catalyze the insertion of a divalent iron, cobalt, zinc, or manganese ion into the porphyrin macrocycle with the release of two protons upon iron chelation [11]. Detailed structural and kinetics studies have been carried out on this enzyme from several sources, including *Bacillus subtilis*, *Saccharomyces cerevisiae* and human, and more recently *Listeria monocytogenes* [12]. The enzyme from for these diverse

*Present address:

Pharmaceutical & Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA 30602, U.S.A. †Present address: Arnold Palmer Hospital, Orlando Health, Orlando, FL 32806, U.S.A.

*Present address: Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, U.S.A.

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organisms has only 10% sequence identity with a majority of conserved residues being located in the active site pocket. Despite the lack of sequence conservation, the three dimensional structures are nearly identical [12–14]. The two most significant differences among ferrochelatases is that some possess a [2Fe-2S] cluster and/or a dozen amino acid loop that extends above the active site [14]. While the role of the cluster is not clearly defined at present, the active site loop or 'lip' segment present on all ferrochelatases other than those of the Gram positive bacteria allows the active site mouth to completely close during the catalytic cycle, thereby restricting the size of the macrocycle that can function as a substrate [15]. The physiological consequence of this is that the enzymes that possess the lip (protoporphyrin ferrochelatases) utilize protoporphyrin IX as substrate, whereas those lacking the lip (coproporphyrin ferrochelatases) utilize coproporphyrin III [15].

Both wild-type and variants of the human ferrochelatase have been crystallized in the presence or absence of substrates. Likewise, differentially metallated products have also been captured in crystal structures, which has allowed for detailed structure/function studies. The enzyme is quite dynamic and undergoes large structural changes during catalysis [6,16]. Three distinct conformations have been captured and assigned to distinct steps in the catalytic cycle. These conformations include; an open form in which no substrate or product is bound; a closed form associated with the binding of the porphyrin substrate and; a release conformation in which the metallated porphyrin product is bound [17]. These altered protein conformations also result in distinct changes in the surface electrostatic potentials surrounding the active site. These stage-specific alterations allow for optimization of binding of protein partners [6], including mitoferrin [18] and protoporphyrinogen oxidase [7] for substrate delivery. The largest change observed between these structures is in the release conformation where a conserved π helix is partially unwound and the active site mouth opened [6]. This large structural alteration is consistent with transient kinetic data that identified the slowest step in the reaction as product release after metallation [19]. Additional support for this comes from studies with inhibitory metals showing the stage at which inhibition occurs is product release, not the chelation step, as previously assumed [20]. Specifically, inhibitory metals appear to prevent unwinding of the π helix, a conformational change that is presumably required for product release.

Currently our understanding of the catalytic mechanism, while advanced beyond many enzymes, is far from complete. Kinetic, structural and modeling studies have provided insight in the specific role of several active site residues in the reaction mechanism. However, major questions remain; how does iron access the active site and where in the active site does iron bind prior to chelation? To begin to answer these questions we have analyzed the role of selected active site residues. The first of these is M76 which is found in the active site opposite the catalytic histidine. This residue has been shown to be important for catalysis [19] and proposed to be involved in iron binding. Four variants of this residue have been created and characterized in terms of activity and biophysical properties. In addition, we have interrogated the role of E343 to a greater degree with several structural studies. E343 is involved in a hydrogen bond network with the catalytic histidine H263 and has been shown to be necessary for efficient catalysis with all characterized variants showing significantly decreased activity [21,22]. Finally, the metal sensitive loop composed of residues Q302, S303 and K304 [20] was interrogated further to establish its role in product release. Together these data shed light on the role of several residues, M76, Q302, S303, K304 and E343 in the catalytic mechanism of ferrochelatase.

Materials and methods

Mutagenesis, protein expression and purification, heme content analysis, and assessment of enzyme activity

All M76, Q302, S303 and K304 variants were created using the QuikChange Site-Directed Mutagenesis protocol (Agilent, Santa Clara, CA) in the wild-type background. Construction and initial characterization of both the E343D and E343Q variants have been previously described [21]. The double variant F110A/E343D was created by using a BamHI/HindIII restriction digest followed by a ligation of the E343D variant [21] and F110A variant [6]. All variants were verified by sequencing. Expression and purification of all variants were carried out as previously described [21,42,43]. Heme content of purified proteins was measured via the hemochromogen assay as previously described [44]. Enzyme activity of each variant was assessed by rescue of a ferrochelatase deficient strain of *Escherichia coli* ($\Delta PpfC$ previously known as $\Delta hemH$) [27,28] and by enzyme assays using the continuous direct spectroscopic method using mesoporphyrin and iron [38]. Values reported for kinetic parameters present the average from two separate experiments with a <10% standard deviation.



Crystallization, data collection, model building, refinement and coordinates

Purified protein for crystallization was prepared as previously described [6,16], except the M76H variant concentration required for crystal growth was approximately double that required for crystallization of other human ferrochelatase enzymes reported to date. Both the E343D and E343Q crystals where grown in the presence of cobalt chloride, an equimolar amount and two-fold excess, respectively. Crystals were grown via the hanging drop method at 18°C and typically formed in two to five days. Crystals for all human ferrochelatase variants were obtained using a precipitating solution containing 0.1 M Bis-Tris pH 6.5, 0.05 M ammonium sulfate and a range of pentaerythritol ethoxylate (15/4 EO/OH) varying from 20% to 35%. If necessary the concentration of pentaerythritol ethoxylate (15/4 EO/OH) was increased to 30% before freezing.

All data sets were collected at The Advanced Photon Source and SER-CAT on beamline 22-ID. Phases were obtained by using a monomer of wild-type ferrocheletase (PDB ID 2QD4) as a molecular replacement search model. Molecular replacement was performed using the program CNS [45], version 1.2 as previously described [17]. Iterative rounds of model building and refinement were performed with the programs COOT [46] and CNS, respectively. Data collection, refinement statistic and PDB IDs for all structures are listed in Table 1. Coordinates for all structures have been deposited in the RCSB Protein Data Bank. All structural representations were created using PyMol [47] and Coot [46].

EPR and UV-Visible spectroscopy

Proteins with imidazole were prepared for electron paramagnetic resonance as they were for crystallization [42,43]. For the removal of imidazole, purified variants were dialyzed against solubilization buffer (50 mM Tris-MOPS buffer pH 8.0, 0.1 mM KCl, 1% sodium cholate) using Slid-A-lyzers dialysis cassettes (Thermo Scientific, Rockford, IL). X-band EPR spectra were obtained using a Bruker ESP-300E spectrophotometer with an Oxford Instruments ESR-9 flow cryostat with the following parameters: temperature 10 K, power 5 mW, modulation amplitude 0.64 mT and frequency 9.6 GHz. UV-Vis spectra were collect using a Cary 1G spectrophotometer (Varian) at room temperature.

Results and discussion

Role of M76 in pre-chelation metal interactions

Considerable attention has been focused on the side of the active site pocket with the highest level of conservation, this includes residues H263 and E343. In contrast, little is known about the role of residues found on the opposite side of the active site pocket. For human ferrochelatase, M76 is found in the active site pocket

nere				
Variant	E343D	F110A/E343D	E343Q	M76H
Resolution range (Å)	50.0–2.6(2.69) ¹	46.2–2.4(2.49)	50.0-2.1(2.18)	50.0–2.5 (2.59)
$R_{\rm sym}$ (%) ²	8.9(42.7)	7.6(39.7)	8.8(35.4)	10.1(54.1)
Ι/σ	13(3)	16(3)	40(8)	8.8(2)
Redundancy	6(3)	7(3)	13(10)	5.5(4)
Unique reflections	27 513	45 871	51 650	31 823
R _{cryst} (%)	21.1	25.7	20.3	20.4
R _{free} (%)	27.1	29.9	25.5	25.9
RMSD bonds (Å)	0.007	0.009	0.011	0.017
RMSD angles (°)	1.3	1.4	1.5	1.5
Average B factor	16.6	32.6	35.1	34.8
PDB ID	4KLA	4KLC	4KLR	4KMM

Table 1 Data collection and refinement statistics for variants of human ferrochelatase presented here

¹Numbers in parenthesis represent the lower resolution limit for the outer resolution shell;

 ${}^{2}R_{sym} = \sum_{hkl} \sum_{l} (|l_{hkl,l}-l|) / \sum_{hkl,l}$, where l_{hkl} is the intensity of an individual measurement of the reflection with indices hkl and

 $< I_{hkl} >$ is the mean intensity of that reflection.



opposite H263, the catalytic histidine. Computational studies [23], amide hydrogen/deuterium exchange mass spectrometry [24], and studies with catalytic antibodies [5] as well as nucleic acids [2-4] suggest that metal insertion occurs on the opposite side of the porphyrin plane relative to proton abstraction. Thus, metal binding and insertion by ferrochelatase would be opposite the previously reported catalytic histidine and on the M76 side of the active site pocket [19,21]. Residue M76 has been observed in multiple conformations within the active site pocket when several human ferrochelatase structures are compared [17,19]. Specifically, this is the case for variants in the active site that disrupt the hydrogen bonding network required for proper function, such as the H263C and H341C variants [19]. While M76 is not strictly conserved in all ferrochelatases, it is generally either a methionine as found in the human and S. cerevisiae forms or a tyrosine as in the B. subtilis and L. monocytogenes forms. Both amino acids are capable of binding metals, specifically the iron atom of heme [25]. It should be noted that B. subtilis and L. monocytogenes ferrochelatases, while also using iron as a substrate, use a different porphyrin substrate and form a different metallated porphryin when compared with human or S. cerevisiase enzymes. Specifically B. subtilis and L. monocytogenes ferrochelatases utilize coproporphyrin III to produce coproheme [12,26]. Thus minor differences in the active site of the enzyme may occur to accommodate a different porphyrin, though if the overall mechanism of metal insertion is the same, conservation of the structure and critical active site residues would be expected. To investigate the role of this residue in the catalytic mechanism, a number of M76 variants were constructed and characterized both in vitro and in vivo.

In previous work, the M76A variant was characterized and shown to have no activity *in vitro* [19]. Four additional substitutions at this residue were created in order understand its function. Mutations were made to convert M76 to the analogous residue in *B. subtilis* and *L. monocytogenes* (M76Y), to another possible weak iron ligand (M76F) and to two strong iron and heme ligands (M76C and M76H). All variants were able to rescue $\Delta PpfC$ (previously known as $\Delta hemH$ [27,28]) (Table 2), a ferrochelatase deficient strain of *Escherichia coli*, demonstrating that *in vivo* all variants have activity. All variants were expressed, purified and found to be as stable as the wild-type enzyme. Activity of all variants was assessed via *in vitro* assays, but kinetic parameters were only measurable for the M76Y variant. The M76Y variant exhibited decreased k_{cat} (0.78 min⁻¹), K_M for mesoporphyrin IX (6 μ M) and K_m for iron (6 μ M) as compared with the wild-type enzyme [6]. To determine if this substitution altered the substrate metal specificity as has been reported for the comparable *B. subtilis* variant [29] which used the non-physiological porphyrin substrate, we carried out the assay with copper as the metal substrate. Previous studies have shown that the human enzyme can use cobalt as a substrate but not copper [30]. The human M76Y variant was not able to utilize copper as a substrate in the *in vitro* assay, thus alteration of this residue did not alter the metal specificity of the enzyme.

The ability of the M76A, C, F and H variants to rescue $\Delta PpfC$, but lack of measurable *in vitro* activity suggests these variants either have activity below the limit of detection in the *in vitro* assay or carry out a single turnover in $\Delta PpfC$ with ferrochelatase degradation for heme utilization for bacterial growth. Several interesting properties of the purified M76C, H and F variants were noted and support these suggestions. First, the M76F variant co-purified with a significant amount of porphyrin bound. Upon storage of the protein at 4°C over 24–48 h the bound porphyrin was converted to heme, as detected by UV-Visible spectroscopy and hemochromagen assays. Thus, the M76F activity is likely below the limit of detection for the assay. Second, the H and C

	Rescue ∆PpfC	% Heme bound ¹	λ soret oxidized (nm)	λ soret reduced (nm)
Wild-type	Yes	0–15	429	429
F110A	Yes	10–17	429	429
M76A	Yes	0–3		
M76C	Yes	47–57	416	429
M76F	Yes	0		
M76H	Yes	39–41	417	429
M76Y	Yes	0–3		

Table 2 In vivo and In vitro characterization of M76 variants



variants co-purified with a significant amount of heme bound. Hemochromagen assays were used to quantitate the bound heme. For the M76H variant \sim 40% of the protein had heme bound with the M76C having \sim 50% (Table 2). The UV-Visible spectra of the M76C and M76H variants in the as purified form (oxidized) showed a blue shifted Soret band for the bound heme as compared with heme bound to the wild-type or F110A variant [6]. Upon reduction with sodium dithionite, the Soret band for the M76H and M76C variants was identical with the wild-type and F110A variants. To better understand the heme binding environment in these variants, EPR studies were carried out. The as purified samples contain excess imidazole in the elution buffer so select samples were subject to dialysis before analysis. Both the M76H and M76C samples with imidazole show a mixture of high and low spin 6-coordinate ferric heme, although these species differ in each variant (Figure 1A), indicating that imidazole can access the heme iron, consistent with previous structural work [6]. For the samples lacking imidazole, both samples show a classic high spin species, consistent with pentacoordinate ferric heme. Notably, there are subtle differences in the g values for each of the variants (Figure 1B). While these results do not provide a clear assignment of the heme ligands they confirm differences in the electronic environment that may be attributable to the contribution of the cysteine or histidine to heme coordination, directly or indirectly. So while both the M76C and H variants turnover, they may not readily release the heme and thus may be limited in vivo and in vitro to a single turnover.

To define the altered heme environment of the M76 variants, crystals of the M76H variant were obtained. The crystals of M76H diffracted to 2.5 Å. The overall conformation of this variant is in the open conformation with a root mean square deviation (RMSD) of 0.3 Å for main chain atoms compared with the wild-type enzyme (PDB ID 2QD4). Additional density was observed in both active site pockets that is best modeled with heme at 50% occupancy (Figure 2A). This density is most likely a combination of heme and sodium cholate as both have been previously seen in the active site pocket [6,14,16]. Interestingly, the overall structure is that of the open conformation and not of the closed or release conformation typically associated with an enzyme containing a metallated porphyrin [17]. Initial purification and characterization suggests that the M76H variant has a higher affinity for heme relative to the wild-type enzyme, therefore, it was anticipated that a heme-bound form of the enzyme would be captured in the crystal lattice, potentially ligated by the histidine at position 76. Instead the structure is one of the enzyme with a wound π helix, and the catalytic histidine (H263) in its



Figure 1. M76H and M76C EPR spectra.

EPR spectra of M76H and M76C variants (A) with imidazole and (B) without imidazole.







(A) The A (top) and B (bottom) subunits of M76H with select active site residues and the composite omit map for heme and active site residues displayed. (B) Overlay of M76H variant (tan and colored by atom) with F110A variant (slate) showing location and orientation of active site residues and heme bound in the active site pocket.

resting state hydrogen bonding network, positioned so that it could be a ligand for the heme iron. The introduced histidine (M76H) is best modeled in two conformations each with 50% occupancy (Figure 2A). The orientations observed for the histidine at position 76, both of which are positioned in space distant from the heme iron (closest approach 5.2 Å and 4.9 Å in each subunit), suggest that it is not a direct ligand for the heme.

In comparing the position of the heme (partial occupancy) in the M76H variant with the heme (full occupancy) in the F110A variant [6], the heme is found in a similar orientation and location although significant differences exist (Figure 2B). First, in the M76H variant propionate 6 of the bound heme is best modeled in an orientation similar to that of the enzyme with substrate bound and not that of the product bound form, i.e. extended and not bent towards the center of the macrocycle. The only other structures with metallated porphyrins bound to ferrochelatase with the propionate 6 in this orientation are of the manganese inhibited form in which the Mn-porphyrin is modeled at full occupancy [20] and the B monomer of the wild-type structure crystallized in the presence of ammonium acetate in which the heme is modeled at 50% occupancy [6]. In both of these structures the conserved π helix is wound even in the presence of a metallated porphyrin. In all other ferrochelatase structures containing a metallated porphyrin, the π helix is partially unwound and propionate 6 is in an altered orientation [6,20]. Second, for the M76H variant, the heme is best modeled in a more distorted conformation than other bound metallated porphyrins studied to date. In addition, additional density in the difference map is seen at the back of the active site pocket and on both sides of the macrocycle (Figure 2A). The density at the back of the active site pocket into which an alternate conformation of histidine 76 is modeled could indicate the iron atom in transit to the active site or bound just prior to chelation. This density is in line with a proposed pathway for iron delivery from the surface of the protein [17]. While it is tempting



to view this data as support of metal active site entry via the channel and insertion on the M76 side of the active site, it is important to remember that occupancy of the heme is at best 50% and that this density could arise from another molecule being bound also at partial occupancy.

Role for E343 in proton abstraction and product release

Opposite M76 and adjacent to the other face of the planar macrocycle is a hydrogen bond network consisting of several conserved residues, including H263 and E343 [19]. The E343 residue is one of the most extensively studied in all ferrochelatases. All variants of this residue that have been characterized, independent of the source of the enzyme, have decreased catalytic activity [16,19,21,31-35]. This residue has been shown to play a central role in the active site hydrogen bonding network that reorients during the catalytic cycle and is proposed to be involved in proton abstraction via its interaction with the imidazole side chain of the catalytic histidine, H263 [19]. It has also been suggested that E343 and H341 may participate in the later steps of catalysis by serving as a conduit for at least one of the pyrrole protons that is initially donated to H263. This is significant since protonation of E343 would disrupt its interaction with H263 and H341 leading to helix unwinding and formation of the release conformation. Residue H341 serves as the cap residue for the π helix and its movement may destabilize the π helix [6,19]. It has also been shown that the movement of E343 influences, or is influenced by the movement of the conserved residue F337 found at the back of the active site pocket which suggests an integral connection between the spatial orientation of the two residues [17]. Both steady-state and transient kinetic studies have investigated the role of E343 in human ferrochelatase and have demonstrated that enzyme variants (K, D, Q, V, A and H) at this conserved residue are either inactive or have an altered k_{cat} with no change in the $K_{\rm M}$ for either substrate suggesting that this residue may be involved in proton abstraction and/or product release, but not in metallation per se [19,21,33,35]. All of the evidence indicates that the H263-E343 pair is essential to the activity of all ferrochelatase enzymes and a similar arrangement, an acid base pair, has been observed in other enzymes where deprotonation is an essential first step in enzyme catalysis [36,37].

The human E343D variant has been previously characterized by steady state [21] and transient kinetic studies [19]. This variant is able to rescue $\Delta PpfC$ and has normal $K_{m}s$ for both substrates, but a decreased k_{cat} [21]. To better understand the role that E343 may play, we have determined the crystal structure of the human ferrochelatase E343D variant. In the 2.6 Å resolution structure of the E343D variant, the overall conformations of the A and B monomers resemble the open conformation of wild-type ferrochelatase. However, while the active site pocket of the A monomer is occupied by cholate molecules as previously observed in multiple other human structures, the B monomer is best modeled with protoporphyrin IX at 50% occupancy (Figure 3A). While the orientation of the porphyrin macrocycle in the B monomer is similar to that observed for porphyrin in the closed conformation [6,16], it is found slightly titled and not as deep in the active site. These differences are likely a result of the overall open conformation of the enzyme, thus may represent porphyrin interaction prior to the closing of the active site mouth.

The overall orientation of active site residues in both monomers of the E343D variant is similar to that of the wild-type enzyme with some exceptions. First, the imidazole of H263 is slightly more distant from the porphyrin binding site. This is likely due to hydrogen bonding with the mutated aspartate of residue 343 (Figure 3B). The shorter side chain of aspartate at position 343 shortens the distance between H263 and residue 343 by approximately half an Å and lengthens the distance to H341 compared with the wild-type model. Second, the conserved F337 is in a different orientation in the A subunit and N75 is also found in an altered orientation in both subunits as compared with the wild-type enzyme in the open conformation (Figure 3B). As minor as these changes and the one carbon shortening of the E343 side chain are, the fact that the catalytic efficiency of the E343D variant is significantly diminished from the wild-type enzyme [19,21] demonstrates the importance of the active site hydrogen bonding network and orientation of active site residues on catalytic efficiency. Previous transient kinetic studies on the human E343D variant showed a 3-fold decrease in the rate of chelation and a 7-fold decrease in the k_{cat} [19]. The movement of H263 away from the porphyrin may provide some structural evidence as to the cause in the decreased rate of chelation.

To address the means by which product release is affected in the E343D variant we created a double variant E343D/F110A. The F110A human ferrochelatase variant was previously shown to be active, but purifies with heme bound. The latter observation indicates that the thermodynamically-favored conformation for the F110A variant is the product-bound state [6]. Given that the E343D variant has a low k_{cat} [19,21], the double variant F110A/E343D was produced and analyzed with the hope that it would provide a snapshot of the enzyme with metallated porphyrin bound or an enzyme trapped with iron bound pre-chelation. Kinetic parameters were





Figure 3. E343D variant structure.

(A) B monomer of E343D variant with composite omit map showing partial occupancy of porphyrin in active site and select active site residues. (B) Altered location and orientation of E343D active site residues (orange) compared with enzyme with resting state hydrogen bond network (grey).

determined for the double variant and were similar to those obtained for the E343D variant; $K_{\rm M}$ for iron of 17 μ M, $K_{\rm M}$ for mesoporphyrin of 11 μ M and $k_{\rm cat}$ of 0.51 min⁻¹.

The F110A/E343D variant was then crystallized and its structure determined to 2.4 Å. In the structure, density was observed in both active sites that was best modeled with heme at 50% occupancy (Figure 4). The overall conformation of the F110A/E343D is more similar to the open conformation, although there are some differences in the lip region. Like the M76H active site heme, the metallated porphyrin found in the E343D/ F110A has its 6-propinate extended and interacting with Y123 and a wound π helix thus making the structure similar to the previously described Mn-deuteroporphyrin [20] and wild-type with heme found at partial occupancy [6]. To better understand slow product release attributable to E343D [19], the orientation of active site residues was investigated in the F110A/E343D variant. Few differences were observed, but of note were the altered orientation of several residues including H263, M76, S303, H341, E347 and the E343D variant. In the F110A/E343D structure, the catalytic histidine, H263, is found in the orientation it is observed prior to substrate binding or metal insertion, the resting state hydrogen bond network [19]. This orientation would be stabilized by metal histidine interactions and, thus, may represent the structure of the enzyme that has reset for another round of catalysis, but without product loss. The interaction of H263 with the metallated porphyrin likely influences the location of the variant aspartate residue which is found to be moved by almost 1 Å (0.7 Å) from its location in the single variant. This would in turn cause a change in residue E347, also a residue in the π helix, which is moved by 1.6 Å.

E347 is normally found in the open and closed conformation interacting with both side chain residues as well as backbone atoms of residues Q302 and S303, both part of a metal sensitive loop [20]. The hydrogen bonding between E347, Q302 and S303 likely provide some stability to the π helix in the wild-type enzyme. In the F110A/E343D double variant the interaction between E347 and Q302 is lost and instead E347 is found within 2.9 Å of E343D. A close distance between E343D and E347 suggests that one of these residues is protonated, most likely E343D. The altered orientation of E343D also positions it to interact with the side chain of





Figure 4. F110A/E343D active site pockets.

Stick representation and composite omit map showing F110A/E343D variant structure with heme at partial occupancy in the active site of the A subunit (A) and the B subunit (B).

residue S303. The interaction of E343D with both E347 and S303 may provide extra stability to the amino end of the π helix. These changes would decrease unwinding of the π helix, preventing product release and thereby explain the decrease in k_{cat} reported for the E343D variant [19].

To further define the role of E343 another variant, E343Q, was crystallized and its structure determined to a resolution of 2.1 Å. The human E343Q variant does not rescue $\Delta PpfC$ and has unmeasurable activity *in vitro* with iron and protoporphyrin as substrates. Interestingly this variant co-purifies with a significant amount of porphyrin bound [21]. The analogous variant of the *B. subtilis* and mouse enzymes has also been produced and characterized. The *B. subtilis* variant E264Q (*B. subtilis* numbering) resulted in only 21% of wild-type activity with zinc and protoporphyrin IX as substrate [35]. Similarly the mouse variant E287Q (mouse numbering) has been shown to bind porphyrin more tightly and to have a decreased rate of product release [32,33]. The E343Q variant in our study was crystallized in the presence of excess cobalt which resulted in a metallated porphyrin bound at full occupancy in both active sites and the enzyme in the release conformation. Neither fluorescence nor anomalous data were collected on these crystals and therefore the identity of the chelated metal ion is unknown, but most likely cobalt or iron. Overall the structure of the E343Q variant is similar to the heme bound F110A variant [6] with a RMSD for the C α atoms of 0.222 Å. While few differences were noted, a





Figure 5. Comparison of E343Q active sites.

Overlay of E343Q A and B monomers showing differences in the metal sensitive loop. Loop residues Q302, S303, and K304 as well as H263, E343Q and H341 are shown for the **A** (green) and **B** (turquoise) subunits.

comparison of the A and B monomer of E343Q showed the largest difference in residues 302–304 (Figure 5). This region is a metal sensitive loop which connects β strand 7 to α helix 12 [14] and is composed of residues Q302–Q314. Several heavy metal inhibited structures showed altered conformations in this region suggesting that it may be necessary for the release of the metallated porphyrin [20]. The altered orientation of the metal sensitive loop in the E343Q variant structure may explain the decreased product release in this variant.

Role of metal sensitive loop in product release

Data from the E343 variants presented herein as well as previous studies with inhibitory metals has suggested that the loop composed of residues Q302–Q314 is important for product release and may play a role in triggering the unwinding of the π helix [20]. Analysis of all structures in the open or closed conformation in which the π helix is wound show that E347 is within hydrogen bonding distance with both the side chain and the backbone amine group of residue S303 and the side chain of Q302. To better understand the function of this region we constructed and characterized several variants of Q302, S303 and K304. It was of interest that most variants had *in vivo* activity, as assessed by the rescue of $\Delta PpfC$, with the exception of S303P (Table 3). Since proline is an imino acid, the S303P variant lacks both side chain and backbone hydrogen binding capacity and is thus unable to interact with E347 and stabilize the π helix. In addition, proline will also limit the flexibility of this loop region. All Q302, S303 and K304 variants were purified and had an intact [2Fe-2S] as assessed by UV-Vis spectroscopy. *In vitro* activity was assessed using the direct assay [38] with iron and mesoporphyrin IX

metal sensitive loop variants					
	Rescue <i>∆PpfC</i>	Activity (% WT \pm S.D.) ¹			
Wild-type	Yes	100			
Q302A	Yes	40 ± 2.5			
Q302D	Yes	BDL^2			
S303A	Yes	47 ± 2.0			
S303T	Yes	BDL			
S303P	No	BDL			
K304L	Yes	7.0 ± 0.63			

Table 3 Assessment of in vivo and in vitro activity of

¹Values reported represent the average activity for six separate experiments. S.D. is standard deviation;



as substrates. The Q302D, S303 T, S303P variants had no measurable activity. The S303A and Q302A variants retained 47% and 40% *in vitro* activity, respectively, while the K304L variant only had 7% activity as compared with the wild-type enzyme (Table 3). With the exception of K304L, all variants were less stable upon short term storage, suggesting that interactions between this metal sensitive loop region and the π helix are important for overall protein stability. The complete lack of activity *in vitro* for the Q302D, S303T and S303P variants demonstrated the importance of the steric constraints and overall charge of this region for maintaining the interactions with the π helix. The introduction of a negative charge (Q302D), a bulkier side chain (S303T), or a residue not capable of hydrogen bonding (Q302A, S303A, S303P) likely results in an enzyme that is predominately in the release conformation and thus as observed has either significantly decreased or no activity. The large decrease in activity of the K304L variant is of interest. K304 has been found in several conformations specifically in the Hg-protoporphyrin inhibited structure [20]. The K304L variant removes the ability of the side chain of this residue to interact with other charged or polar side chains such as Q314 or D316. Given the distance of K304 from the active site, but proximity to the π helix, it seems likely that this residue plays a role in product release. Further structural and kinetic analysis of these and other loop variants is necessary to confirm their role in catalysis.

Conclusions

Crystal structures for three distinct conformational states of the human ferrochelatase enzyme have provided snap shots that support a putative enzyme mechanism for porphyrin macrocycle metallation. Additional support of this model comes from molecular dynamics (MD) [39,40], high level quantum mechanical/molecular mechanics (QM/MM) and quantum mechanical thermodynamic cycle perturbation (QTCP) calculations [41]. These structure-based theoretical simulations elucidate molecular motions and structural interactions that occur for human ferrochelatase in going from the open, to closed, and finally release conformations. Furthermore, they provide strong support for the role of H263 in porphyrin proton extraction and M76 in ferrous iron delivery/insertion, and support previous studies [2–5,23,24].

An overview of the ferrochelatase catalytic cycle can be broken into three stages; (i) porphyrin binding, (ii) metallation, and (iii) metallated porphyrin release. In the first step, porphyrin entry into the open conformation is accompanied by rotation of H263 by ~90° to form hydrogen bonds with a pyrrole hydrogen which alters the resting state hydrogen bond network with E343 and H341. Water exits the pocket as porphyrin enters via a solvent-filled channel gated internally by the side chain of F337 and opening on the back surface of human ferrochelatase at H240. Once the porphyrin is bound in the active site the side chain of F337 rotates to close the H240 channel and open the Q139 channel [17]. The upper lip of the active site mouth closes down as R115 rotates to form a hydrogen bond with a porphyrin propionate. The porphyrin macrocycle is held in position by side chains of R115, S130 and Y123. On the opposite side of the active site pocket from H263, a hydrogen bond between Y191 and R164 breaks allowing R164 to rotate more than 90 degrees away from active site pocket to form a hydrogen bond with D95. The side chain of M76 also rotates into the pocket being positioned over the center of the porphyrin. Movement of residues on both sides of the active site [22].

The molecular route of entry for iron has not been experimentally defined, but available biochemical data are consistent with iron entry via the H240 aqueous tunnel [17]. Whatever the route, the side of the pocket from which desolvated iron is inserted into the porphyrin macrocycle is the side containing M76 and not H263. Theoretical calculations that considered both M76 and H263 as potential iron donating residues, are clearly consistent with iron coming from M76 and pyrrole proton abstractions occurring via H263. Two protons must be removed from the porphyrin to allow iron insertion. As outlined above when the porphyrin binds in the active site pocket H263 rotates into position to accept the first proton from the porphyrin. As this happens the iron forms its first bond with a pyrrole nitrogen. The proton on H263 is then transferred to E343 which had moved into position to allow such a transfer to occur. This allows the second pyrrole proton to be abstracted by H263, thereby allowing the second iron-porphyrin bond to form. As is seen in ferrochelatase structures with metallated porphyrins bound, the side chain of H263 moves away from its position directly under the macrocycle center to a position that is outside of the meso carbon between rings B and C. The movement of the protonated H263 may be due to charge repulsion between the cationic metal and the protonated imidazole of H263. The porphyrin propionate 6 releases from hydrogen bonding with the side chains of \$130 and Y123 and bends back over the center of the macrocycle to a position where it can form a salt bridge with the protonated H263. It has been proposed that the reorientation of H263 is the initial trigger for unwinding of



the π helix and reopening of the mouth for product release. Following metallation M76 moves away from its position over the porphyrin ring in the active site pocket and the side chain of R164 rotates 90° out of the pocket forming a hydrogen bond with the carboxylate of D95 and S201. As this occurs, the metallated macrocycle moves out of the active site pocket.

Overall our studies reported herein support this model for iron binding in the active site, proton abstraction, disruption of the active site hydrogen bonding network, chelation, and rearrangement of active site residues for product release. Variants of M76 are consistent with this residue being a weak iron/heme ligand for transient binding of iron and heme in the active site. Alteration to change M76 to a strong iron/heme ligand results in the creation of an enzyme with disruption in product release. Even though all the M76 variants rescued $\Delta PpfC$, only the M76Y had measurable activity in vitro. Since this is the amino acid found in the B. subtilis and L. moncytogenes coproheme producing ferrochelatases this was not surprising and suggests that weak iron ligands are necessary for transient binding of iron as a substrate and release of the final product. Analysis of E343 on the opposite side of the active site pocket support a role for this residue in proton abstraction and product release. The structures of the E343D, F110A/E343D and E343Q are consistent with decreased turnover rates due to alternations of the active site hydrogen bonding network, specifically with H263 which is necessary for catalysis. Finally, the interactions between the metal sensitive loop and the H263 and E343 side chains in the active site pocket provides some information on how product release occurs upon metal insertion and alteration in the interactions of the metal sensitive loop region (Q302–Q314) are translated to the conversed π helix which unwinds for product release. Further structural studies of additional variants as well as alternate substrates will further shed light on the remaining unanswered questions on the mechanism of ferrochelatase.

Data Availability

The coordinates and structure factors for all four structures of the human ferrochelatase variants have been deposited to the PDB. The PDB codes for each structure are as follows: E343D - 4KLA, F110A/E343D - 4KLC, E343Q - KLR, and M76H - 4KMM.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Amy E. Medlock: Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Investigation, Visualization, Writing — original draft, Writing — review and editing. Wided Najahi-Missaoui: Investigation, Writing — review and editing. Mesafint T. Shiferaw: Formal analysis, Investigation. Angela N. Albetel: Investigation, Writing — review and editing. William N. Lanzilotta: Data curation, Formal analysis, Funding acquisition, Investigation, Visualization, Writing — review and editing. Harry A. Dailey: Conceptualization, Funding acquisition, Investigation, Writing — original draft, Writing — review and editing.

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Abbreviations

MD, molecular dynamics; QTCP, quantum mechanical thermodynamic cycle perturbation; RMSD, root mean square deviation.

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