Identification and Characterization of an Inhibitory Metal Ion-binding Site in Ferrochelatase

Gregory A. Hunter and Gloria C. Ferreira

Ferrochelatase catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme. The severe metal ion substrate inhibition observed during in vitro studies of the purified enzyme is almost completely eliminated by mutation of an active site histidine residue (His-287, murine ferrochelatase numbering) to leucine and reduced over 2 orders of magnitude by mutation of a nearby conserved phenylalanine residue (Phe-283) to leucine. Elimination of substrate inhibition had no effect on the apparent activity for Ni$^{2+}$, but the apparent $K_m$ for Ni$^{2+}$ was increased 100-fold, indicating that the integrity of the inhibitory binding site is important for the enzyme to turn over substrates rapidly at low micromolar metal ion concentrations. The inhibitory site was observed to have a $pK_a$ value of 8.0, and this value was reduced to 7.5 by the F283L mutation and to 7.4 in a naturally occurring positional variant observed in most bacterial ferrochelatases, murine ferrochelatase H287C. A H287N variant was also found to be substrate-inhibited, but unlike the H287C variant, pH dependence of substrate inhibition was largely eliminated. The data indicate that the inhibitory metal ion-binding site is composed of multiple residues but primarily defined by His-287 and Phe-283 and is crucial for optimal activity at low metal ion concentrations. It is proposed that this binding site may be important for ferrous iron acquisition and desolvation in vivo.

Despite these expectations, there is currently no definitive kinetic or structural evidence for direct channeling of iron to ferrochelatase. It was recently shown that human ferrochelatase can form an immuno-precipitable complex with mitoferrin (7), an inner mitochondrial membrane iron transporter (9, 10), and yet the mechanism whereby a trans-membrane oriented into the inner mitochondrial membrane (8). This suggests that the iron atom must be transported directly through the membrane or that the active site intermittently reorients away from the membrane and toward a matrix-soluble iron chaperone. In either case, this hypothetical channeling mechanism is expected to involve an iron-specific transporter protein, which would explain the substrate specificity observed in vivo.
usual location of this binding site within a conformationally dynamic active site $\pi$-helix may offer important clues as to how iron acquisition for heme biosynthesis is realized in vivo.

**EXPERIMENTAL PROCEDURES**

Reagents—MOPS, Tween 80, sodium chloride, cobalt chloride hexahydrate, zinc chloride, nickel chloride hexahydrate, and cupric chloride dihydrate were from Sigma. Ferrous porphyrin IX was from Frontier Scientific. Blue Sepharose ride tetrahydrate was obtained from Fisher Chemical. Protoporphyrin IX was from GE Healthcare Life Sciences. PIPBS\textsuperscript{3} buffer was from GFS Chemicals.

Construction, Overexpression, Purification, Storage, Handling, and Analysis of Murine Ferrochelatase—The H287L and H287N variants were produced using "megaprimer" mutagenesis (17), whereas the H287L and F283L variants were produced and isolated from a random mutagenesis library designed and screened for directly evolved variants of ferrochelatase with elevated activity at high metal ion concentrations (data not shown). The coding regions of the variants were sequenced, and the mutation-encoding fragments were subcloned (18) into the vector backbone. Wild-type recombinant murine ferrochelatase and the variants were overexpressed, purified, stored, and handled as previously described (18). Protein concentrations were determined spectrophotometrically using the calculated extinction coefficient of 48,500 M$^{-1}$cm$^{-1}$ at 280 nm based on the primary amino acid sequence encoded by the cDNA for the recombinant wild-type enzyme (19). Reported enzyme concentrations are thus in terms of monomers or active sites.

Buffers—Buffers were devoid of strong metal-complexing agents such amines and sulfhydryls to avoid complex equilibria effects on activity measurements (16, 20–23). Buffer A was defined as 0.1 M MOPS, 0.4 M sodium chloride, and 0.2% (v/v) Tween 80, pH 7.00, whereas buffer B, which was utilized for pH variation studies, was 20 mM MOPS, 20 mM PIPBS, 0.4 M sodium chloride, and 0.2% (v/v) Tween 80, at pH 5.75–9.25.

Preparation of Protoporphyrin IX and Metal Ion Solutions—The details of these procedures have been described elsewhere (16).

Determination of Steady-state Kinetic Parameters—Enzyme activities were determined by monitoring the change in porphyrin absorbance, as described previously (16). In experiments where the protoporphyrin IX concentration was held constant at 20 $\mu$M, nickel-protoporphyrin formation was monitored at 562 nm, and zinc-protoporphyrin formation was monitored at 584 nm, where the extinction coefficients were determined to be 20,720 and 8060 M$^{-1}$cm$^{-1}$, respectively. Determination of the extinction coefficients was as described previously (6, 16).

Data points were fitted using SigmaPlot (Systat Software) and either the Michaelis-Menten equation (Equation 1) or a substrate-inhibited reaction equation (Equation 2).

![Graph](https://via.placeholder.com/150)

**FIGURE 1.** Activity profiles of murine ferrochelatase variants with Ni$^{2+}$ as substrate. The data points correspond to the following: o, murine ferrochelatase; A, F283L murine ferrochelatase variant; and ■, H287L murine ferrochelatase variant. Each enzyme was at 0.2 $\mu$M, whereas protoporphyrin IX was held constant at 3.0 $\mu$M. The lines represent the best fit to Equation 2 (wild-type and F283L ferrochelatase) or Equation 1 (H287L). Specific activity is in units of micromoles metalloporphyrin produced per minute per micromolecule enzyme.

**TABLE 1**

<table>
<thead>
<tr>
<th>Wild-type ferrochelatase</th>
<th>F283L</th>
<th>H287L</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{app}}$</td>
<td>53 ± 11</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>$K_{\text{app}}$</td>
<td>1.0 ± 0.4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>$K_{\text{app}} / V_{\text{app}}$</td>
<td>7 ± 3</td>
<td>2400 ± 800</td>
</tr>
<tr>
<td>$K_{\text{app}} / V_{\text{app}}$ min$^{-1}$</td>
<td>53 ± 21</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

In these equations, $[\text{Me}^{2+}]$ corresponds to the metal ion concentration, and $K_{\text{app}}$ represents the apparent inhibitory constant under the conditions described. Uncompetitive inhibition is assumed in Equation 2, with the numerically inconsequential caveat that in the case of ferrochelatase, it appears most likely that the inhibitory metal ion binds to the product-bound enzyme, as described by Reaction 1, rather than the Michaelis complex (6).

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\textsuperscript{3}The abbreviation used is: PIPBS, piperazine-$N,N$'-bis(4-butanesulfonic acid).
**RESULTS**

**Activity Profiles for Ferrochelatase Variants**—Purified ferrochelatase is subject to substrate inhibition by \( \text{Zn}^{2+}, \text{Ni}^{2+}, \text{Co}^{2+}, \) and \( \text{Cu}^{2+} \) ions when assayed in the absence of metal ion-complexing buffer components (6, 16). \( \text{Zn}^{2+}, \text{Co}^{2+}, \) and \( \text{Cu}^{2+} \) ions are highly inhibitory, and precise determination of kinetic and inhibitory constants is difficult, but Ni\(^{2+}\) ion is

\[
K_{\text{app}} = \frac{1 + (K_o[H^+])^2}{K_1[H^+]} \quad \text{(Eq. 4)}
\]

where \( K_o \) represents the dissociation constant for the inhibitory metal ion-binding site, \( K_i \) is the pH-independent value of the apparent \( K_i \), at high pH, and \([H^+]\) is the hydronium ion concentration. This equation assumes that the inhibitory metal ion binds exclusively to a deprotonated form of the enzyme.

**pH Dependence of Substrate Inhibition**—Apparent inhibitory constants were determined at several pH values using Equation 2 and then fit to Equation 4 using SigmaPlot (25),

\[
E + P \overset{K_{\text{cat}}}{\longrightarrow} EP + M \overset{K_{\text{cat}}}{\longrightarrow} EPM \overset{K_{\text{cat}}}{\longrightarrow} EPr \overset{K_{\text{cat}}}{\longrightarrow} E + Pr
\]

**REACTION 1**

Here, \( E \) represents the enzyme, \( P \) is protoporphyrin IX, \( M \) is metal, and \( Pr \) is metalloporphyrin product. The catalytic rate is defined by the rate of product release, as demonstrated in stopped-flow experiments (24).

**FIGURE 2. Metal ion inhibition is pH-dependent.** Activity profiles at pH 7.0 (●) and 9.0 (■) were determined for murine ferrochelatase variants. Each enzyme was at 0.2 \( \mu \text{M} \), whereas protoporphyrin IX concentration was held constant at 20.0 \( \mu \text{M} \) and \( \text{Ni}^{2+} \) concentration was varied. A, wild-type murine ferrochelatase; B, H287L variant; C, H287N variant; D, F283L variant; and E, H287C variant. The H287C variant was assayed with \( \text{Zn}^{2+} \) as substrate due to the absence of detectable activity with \( \text{Ni}^{2+} \) (not shown). Specific activity is in units of micromoles metalloporphyrin produced per minute per micromole enzyme.
less inhibitory, with an inhibition constant of $70 \pm 10 \, \mu M$ at pH 7.0, and this metal ion is therefore more suitable for robust characterization of the inhibitory metal ion-binding site (16). As seen in Fig. 1, the F283L mutation partially alleviated metal ion substrate inhibition by Ni$^{2+}$, whereas the H287L mutation eliminated inhibition altogether. Alleviation or elimination of substrate inhibition resulted in higher absolute levels of activity being observed for these variants at higher concentrations of Ni$^{2+}$, as seen in Fig. 1A. However, examination of the low micromolar range, as in Fig. 1B, revealed that the mutations resulted in substantially less activity at these more physiologically relevant metal ion concentrations. Fits of data at pH 8.0 indicated that the mutations not only increased $K_i$ (F283L) or eliminated inhibition (i.e. H287L, $K_i \rightarrow \infty$) but also increased the apparent $K_{m}$, whereas maximal velocities were not affected (Table 1). These data suggested that the primary player in the inhibitory binding site was His-287, with the nearby Phe-283 having a marked influence on binding at His-287, and that the capacity to bind metal ion tightly at His-283/Phe-287 might correlate or translate into stronger binding at the catalytic site, which is defined by the His-209/Glu-289 pair (26).

Ph Dependence of Metal Ion Substrate Inhibition—Metal ion substrate inhibition was pH-dependent for the wild-type, F283L, and H287C variants, partially pH-dependent for the H287N variant, and pH-independent for the H287L variant (Figs. 2–4). Inhibition increased with pH for each titratable variant, consistent with tighter binding to a deprotonated inhibitory site, with the important caveat that the H287C variant was unique in that the specific activity increased at more acidic pH values, indicating that this mutation converted the higher activity form of the enzyme from deprotonated to protonated. Direct comparison of activities and inhibition constants for H287C with the other variants was complicated by a lack of detectable activity with Ni$^{2+}$ as substrate, which necessitated the use of Zn$^{2+}$ to assess the kinetic characteristics of H287C. The H287N and H287C mutations both resulted in substantial changes in the activity profiles, but these naturally occurring positional variants, like the wild-type enzyme, were strongly substrate-inhibited. The weaker binding at the secondary site in the H287L and F283L variants can be viewed in Fig. 2, where the metal-ion concentrations necessary to complete the reaction were generated across a range of pH values, and the apparent $pK_a$ for the inhibitory binding site, as depicted in Fig. 3. Activity profiles were generated across a range of pH values, and the apparent $pK_a$ values were determined at each pH to construct a log-log plot of $pK_a$ values versus pH. Substrate (Ni$^{2+}$) inhibition of the wild-type enzyme increased with pH and then plateaued above pH 8.0 at less than $10^{-3} \, M$. The inflection point defining the $pK_a$ for inhibition was estimated to be 8.0. The F283L variant was similar in shape, but the $pK_a$ was lowered by about 0.5 pH units, and inhibition plateaued at only $10^{-4} \, M$, more than an order of magnitude lower than the unaltered enzyme.

In contrast to the wild-type ferrochelatase and F283L variant, the inhibitory constant of the H287N variant was only mildly pH-dependent, and no inflection point was observed over the range in which the enzyme was active. This result indicated that the H287N mutation largely eliminated the titratable nature of the inhibitory binding site. H287L was not substrate-inhibited by Ni$^{2+}$ at any pH tested but was inhibited by Co$^{2+}$, Zn$^{2+}$, and Cu$^{2+}$ (Table 2 and Fig. 5). The relative errors in the determined inhibitory constants increased substantially at higher $K_i$ values, but clear differences were observed.

The pH dependence of inhibition for the H287C variant with Zn$^{2+}$ as substrate was similar to the wild-type and F283L variant with Ni$^{2+}$ (Fig. 4), with the estimated $pK_a$ being lowered by about 0.6 pH units as a result of the mutation. The H287C mutation resulted in an enzyme that was more active than wild-type ferrochelatase at lower pH values, consistent with cysteine being found at this position in most bacteria, but this was not investigated further.

A Metal Ion-binding Side Chain at the Position of His-287 Is Not Absolutely Necessary for Substrate Inhibition to Be Observed—As noted above, although the H287L variant was not inhibited at any pH by Ni$^{2+}$, it was observed to be weakly inhibited by Co$^{2+}$ and Zn$^{2+}$. This can be observed in Fig. 5,
where at pH 7.0 an inhibitory constant of 250 $\pm$ 30 $\mu$M was observed. This result suggests that the inhibitory metal ion-binding site is composed of one or more other residues, in addition to His-287.

**DISCUSSION**

The *in vivo* substrate of ferrochelatase is Fe$^{2+}$ (1). Ferric iron (Fe$^{3+}$) is not a substrate, and a key outstanding question in the biochemistry of ferrochelatase is the mechanism whereby the oxidatively unstable ferrous iron substrate is acquired *in vivo*. The orientation of the enzyme active site into the periphery of the inner mitochondrial membrane (8) suggests that a membrane transporter such as mitoferrin might deliver ferrous iron to ferrochelatase, whereas other studies have implicated the matrix-soluble iron chaperone frataxin in interacting with ferrochelatase with iron delivery for heme biosynthesis (11–15).

We and others previously reported that ferrochelatase is subject to metal ion substrate inhibition during *in vitro* assays when complexing agents are eliminated from the assay buffer, which facilitated the discovery of the existence of a second catalytically important metal ion-binding site on the enzyme (6, 16). In this report, we identify murine ferrochelatase active site residues Phe-283 and His-287 as key components of the second binding site observed during *in vitro* assays and demonstrate that this inhibitory site may not be inhibitory at all *in vivo* as it actually enhances activity at low $\mu$M metal ion concentrations.

A compelling argument can be made that this second ferrochelatase metal ion-binding site is conserved in nature and possibly even fine-tuned to support the unique ecological niche of the host organism. Phe-283 is one of only six ferrochelatase amino acid residues known to be perfectly conserved (16) and resides at the bottom of the active site cleft at the end of a conformationally dynamic $\pi$-helix (5, 27). The absolute biological requirement of a phenylalanine residue at this position is somewhat perplexing because several other amino acids have similar chemical properties, and the results presented here indicate that Phe-283 is not essential for high levels of activity *in vitro*, yet no replacements have been observed in nature. Four amino acids toward the carboxyl terminus from this residue is a histidine that is also conspicuously located at the back of the active site, about 10 $\AA$ away from the invariant histidine-glutamate pair reported to form the catalytic insertion site (26). Cadmium-soaked crystals of *Bacillus subtilis* ferrochelatase were found to have metal ion bound at both of the equivalent histidines simultaneously, clearly demonstrating the metal binding capacity of the secondary site proposed here (28). Murine ferrochelatase His-287 is conserved in eucaryotes (with the exception of the parasitic *Plasmodium* and *Toxoplasma*, which have a cysteine at this position; Table 3) and many bacteria but is replaced by a cysteine in most prokaryotes and by an asparagine in a small number of infectious bacteria including *Rickettsia*, *Campylobacter*, and *Helicobacter*, as well as symbiotic nitrogen-fixing cyanobacteria. No other amino acids are observed at this position, and this in itself is intriguing from an evolutionary perspective because interconversion of either histidine or asparagine to cysteine requires at least two point mutations, and no single point mutation intermediates are currently observed in nature, suggesting an unlikely double mutational event or a now extinct primordial intermediate. In either case, there would appear to be an unusually strong selective pressure toward these three particular metal ion-binding amino acids within the organisms that harbor them. The unusual natural distribution makes it tempting to speculate that the presence of cysteine or asparagine at this position confers some infectious capacity to many organisms, perhaps via iron-related oxidative toxicity or higher activity in more acidic pH environments, but direct experiments have not yet been con-

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**TABLE 2**

Inhibitory constants for ferrochelatase variants with several divalent metal ions

<table>
<thead>
<tr>
<th>FC variant</th>
<th>Fe$^{2+}$</th>
<th>Zn$^{2+}$</th>
<th>Co$^{2+}$</th>
<th>Ni$^{2+}$</th>
<th>Cu$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>NO</td>
<td>12 $\pm$ 2</td>
<td>22 $\pm$ 3</td>
<td>70 $\pm$ 10</td>
<td>$&lt;$1</td>
</tr>
<tr>
<td>H287L</td>
<td>NO</td>
<td>100 $\pm$ 80</td>
<td>250 $\pm$ 30</td>
<td>NO</td>
<td>$&lt;$1</td>
</tr>
<tr>
<td>H287C</td>
<td>NO</td>
<td>12 $\pm$ 3</td>
<td>NO</td>
<td>ND</td>
<td>$&lt;$1</td>
</tr>
<tr>
<td>H287N</td>
<td>500 $\pm$ 200</td>
<td>300 $\pm$ 100</td>
<td>20 $\pm$ 10</td>
<td>100 $\pm$ 30</td>
<td>4 $\pm$ 1</td>
</tr>
<tr>
<td>F283L</td>
<td>500 $\pm$ 300</td>
<td>26 $\pm$ 7</td>
<td>44 $\pm$ 17</td>
<td>300 $\pm$ 100</td>
<td>Inconclusive</td>
</tr>
</tbody>
</table>

**TABLE 3**

Evolutionary examination of the position occupied by murine ferrochelatase histidine 287

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Notable species</th>
<th>Ecological niche</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucaryotic consensus</td>
<td>FXXHXE</td>
<td><em>Plasmodium</em>, <em>Toxoplasma</em></td>
</tr>
<tr>
<td>Exceptions in eucaryotic</td>
<td>FXXHXXE</td>
<td><em>Vibrio</em>, <em>Yersinia</em>, <em>Salmonella</em>, <em>Haemophilus</em>, <em>Burkholderia</em>, <em>Shigella</em>, <em>Bordetella</em></td>
</tr>
<tr>
<td>Prokaryotic consensus</td>
<td>FXXH/XXE</td>
<td></td>
</tr>
<tr>
<td>Notable divergences from</td>
<td>FXXHXXE</td>
<td><em>Helicobacter</em>, <em>Rickettsia</em>, and <em>Campylobacter</em></td>
</tr>
<tr>
<td>eucaryotic sequences</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All others</td>
<td>FXXHXXE</td>
<td></td>
</tr>
</tbody>
</table>
ducted to test these possibilities. The murine ferrochelatase H287C mutation increases activity at lower pH values, which suggests a clear selective advantage to placing cysteine in this position in anaerobic organisms, whereas binding at the secondary site in the H287N variant is almost pH-independent, which allows it to remain a functional metal ion-binding site at lower pH as well.

It had been posited that the secondary binding site might function to confer some specificity for iron over other metal ion substrates (16), but this is not supported by the work presented here, and it now appears more likely that the additional binding site functions to strengthen overall metal binding affinity and thereby enhance activity. This is more consistent with another functional role we had postulated, which was that the second metal ion-binding site is part of a substrate-processing pathway that also enhances overall binding (16). The spatial positioning of His-287 within a \( \pi \)-helix that unwinds during product release may offer important clues as to the true significance of this secondary binding site. As delineated in Fig. 6, the function of the second metal ion-binding site \textit{in vivo} could be to assist in transport of Fe\(^{2+} \) by Glu-289 into the catalytic insertion site, in a way that not only precludes the toxicity that would be associated with release and oxidation but also promotes desolvation as a means to enhance reactivity with protoporphyrin IX (see supplemental video). In this model, which is based on interpolations of known crystal structures, Phe-283 “sweeps” around His-287 and the Fe\(^{2+} \) atom while it is within binding distance of both Glu-289 and His-287, immediately prior to its arrival at the catalytic site. In this way, Phe-283 might promote reactivity via desolvation of the Fe\(^{2+} \) atom or modulate the interaction of the metal ion with His-287 such that it is tightly bound initially but then rapidly released from His-287 into the catalytic site upon reformation of the \( \pi \)-helix. It is even possible that the His-287/Phe-283-binding site acts as part of a molecular switch that delivers iron to the active site. For instance, it is possible that binding of protoporphyrin IX, which is believed to involve direct channeling from protoporphyrinogen oxidase in the inner mitochondrial membrane, triggers unwinding of the \( \pi \)-helix, creating a transitory matrix-oriented binding site for an iron chaperone, which then channels iron to the active site via a pathway that involves Glu-289 and the His-287/Phe-283-binding site. This mechanistic model would require expansion and refinement of Reaction 1 to reflect the conformational status of the enzyme, particularly about the \( \pi \)-helix region, as described by Reaction 2.

\[
\begin{align*}
E + P & \rightleftharpoons E_P + M \\
& \xrightleftharpoons{K_{01}} E_{PM} \\
& \xrightleftharpoons{K_{12}} E_P + M \\
& \xrightleftharpoons{K_{23}} E_{Pr} + M \\
& \xrightleftharpoons{K_{34}} E + P \\
\end{align*}
\]

Here, it is the open conformation of the protoporphyrin IX-bound enzyme (\( E_P \); unwound \( \pi \)-helix) that binds the iron atom at Glu-289, stimulating reversion to the closed Michaelis complex conformation (\( E_{PM} \)). His-287 transiently complexes the metal ion during this structural transition, and Phe-283 enhances the binding affinity and possibly assists in
metal ion desolvation. Following catalysis, the product-bound enzyme (EPr) reverts to the open conformation (Ep), as supported by recent crystallographic data (5), and the metalloporphyrin is slowly released. However, at the higher metal ion concentrations utilized during in vitro experiments with the purified enzyme, a second metal ion binds to the product-bound enzyme prior to product release, resulting in a conformational complex (EPrM) wherein product release is inhibited. This admittedly speculative but testable catalytic model suggests that the inhibited complex should be found to resemble that shown in Fig. 7, where the metal ion is bound by His-287 and the catalytic residues, with Phe-283 stabilizing the complex. Ironically, the substrate inhibition observed during in vitro enzyme assays may not be operable in vivo, where a channeling mechanism would ensure that only one iron atom enters the active site each catalytic cycle, but could serendipitously tell us something important about iron acquisition and processing in vivo.

The H287L mutant is subject to some degree of substrate inhibition, leading us to further suggest that other residues may also be coordinately involved in metal ion binding at the secondary site. The identity of any other binding residues is somewhat speculative, but the nearest conserved residue capable of complexing a metal ion is Glu-293 in the π-helix, the function of which is currently unknown.

In summary, based on the data presented here, we propose that metal ion substrate inhibition is an in vitro phenomenon only observable with unnaturally high substrate concentrations, and in vivo, the second binding site is part of an evolutionarily conserved multiresidue substrate delivery system that transports Fe2+ to the active site while simultaneously preventing oxidation and promoting desolvation. The orientation of the catalytic site residue Glu-289 into the matrix in the unwound π-helix conformation further identifies a specific and novel docking site for an iron chaperone and implies that the sought after iron transport protein should be found in the mitochondrial matrix rather than the membrane.

Acknowledgments—We acknowledge Arianna Mangravita-Novo, Matthew P. Sampson, and W. Christopher Adams for preliminary work with the F283L and H287L murine ferrochelatase variants.

REFERENCES
