

Characterization of the Iron-binding Site in Mammalian Ferrochelatase by Kinetic and Mössbauer Methods*

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All organisms utilize ferrochelatase (protoheme ferriolase, EC 4.99.1.1) to catalyze the terminal step of the heme biosynthetic pathway, which involves the insertion of ferrous ion into protoporphyrin IX. Kinetic methods and Mössbauer spectroscopy have been used in an effort to characterize the ferrous ion-binding active site of recombinant murine ferrochelatase. The kinetic studies indicate that dithiothreitol, a reducing agent commonly used in ferrochelatase activity assays, interferes with the enzymatic production of heme. Ferrochelatase specific activity values determined under strictly anaerobic conditions are much greater than those obtained for the same enzyme under aerobic conditions and in the presence of dithiothreitol. Mössbauer spectroscopy conclusively demonstrates that, under the commonly used assay conditions, dithiothreitol chelates ferrous ion and hence competes with the enzyme for binding the ferrous substrate. Mössbauer spectroscopy of ferrous ion incubated with ferrochelatase in the absence of dithiothreitol shows a somewhat broad quadrupole doublet. Spectral analysis indicates that when 0.1 mM Fe(II) is added to 1.75 mM ferrochelatase, the overwhelming majority of the added ferrous ion is bound to the protein. The spectroscopic parameters for this bound species are $\delta = 1.36 \pm 0.03$ mm/s and $\Delta E_Q = 3.04 \pm 0.06$ mm/s, distinct from the larger ΔE_Q of a control sample of Fe(II) in buffer only. The parameters for the bound species are consistent with an active site composed of nitrogenous/oxygenous ligands and inconsistent with the presence of sulfur ligands. This finding is in accord with the absence of conserved cysteines among the known ferrochelatase sequences. The implications these results have with regard to the mechanism of ferrochelatase activity are discussed.

Ferrochelatase (protoheme ferriolase, EC 4.99.1.1) is the terminal enzyme of the heme biosynthetic pathway (1–3). Its function is to catalyze the chelation of ferrous ion into protoporphyrin IX to form protoheme (1, 2). Although these are the only physiological substrates, the enzyme is capable of utilizing several other divalent transition metals (e.g. Co^{2+} and Zn^{2+}) (4–6) and a wide variety of IX isomer porphyrins (2) *in vitro*. Certain other divalent metals, i.e. Mn^{2+} , Cd^{2+} , and Hg^{2+} , are inhibitors (7). Furthermore, ferric ion is not used as a substrate (8). Deficiencies in ferrochelatase activity cause an accumulation of precursor porphyrins within cells, particularly in those tissues (i.e. liver and bone marrow) where there is a high rate of heme synthesis, and this accumulation results in the disease protoporphyria (9). Because of this and because of heme's importance as a cofactor in a variety of enzymes and proteins (e.g. hemoglobin, cytochromes, NO synthase, peroxidases, catalases), understanding the mechanism and regulation of ferrochelatase activity is of prime importance.

Ferrochelatase is a membrane-associated protein (with the cytoplasmic membrane in prokaryotes and with the inner mitochondrial membrane in eukaryotes) (2), except for the *Bacillus subtilis* enzyme, which is water-soluble (10). As with most mitochondrial proteins, eukaryotic ferrochelatase is synthesized in the cytosol as a larger precursor form and subsequently processed to the mature protein during translocation into the mitochondria (2, 11). Ferrochelatase genes and cDNAs have been isolated and sequenced from *Escherichia coli* (12), *Bradyrhizobium japonicum* (13), *B. subtilis* (14), *Saccharomyces cerevisiae* (15), *Arabidopsis thaliana* (16), barley (17), cucumber (17), mouse (18, 19), and human (20). Human ferrochelatase is encoded by a single gene and has been mapped to chromosome 18q21.3 (21, 22).

Because ferrochelatase is a membrane-associated protein, and hence relatively insoluble, it has been difficult in the past to purify substantial amounts of enzyme from conventional sources (e.g. mammalian liver). This has hindered detailed mechanistic and spectroscopic studies. Recently, however, this problem has been overcome by molecular recombinant DNA techniques. Both mouse (23) and human (24) ferrochelatase have been overexpressed in *E. coli*. For the mouse enzyme, the overexpressed protein remains associated with the soluble bacterial fraction, facilitating and increasing the yields of the purification procedure (23). Adequate amounts of the enzyme are now available, and spectroscopic studies are possible. Since these developments of heterologous overexpression systems, a

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[2Fe-2S] cluster was unexpectedly found in ferrochelatase isolated from mouse livers (25), recombinant (overexpressed) mouse (25), and recombinant human sources (26). The function of the cluster remains to be established, but it has been proposed to be necessary for activity (26). The cluster may or may not be near the ferrochelatase active site (which binds the substrate ferrous ion). The discovery of the cluster has opened new avenues of ferrochelatase research and is changing the way in which ferrochelatase in particular, and iron-sulfur clusters in general, are viewed.

Little is known about the ferrous binding site itself. Chemical modification of protein sulfhydryl groups led to the proposal that at least two cysteine residues were responsible for binding the ferrous ion (27). However, comparison of the genes of the sequenced ferrochelatases reveals that not a single cysteine is conserved among all the species (12–20), although there are four cysteines conserved in the mammalian enzymes that have been implicated as ligands of the [2Fe-2S] cluster. In a recent report utilizing site-directed human ferrochelatase mutants, it was observed that the kinetic parameter K_m^{Fe} increased markedly when His-263, which is conserved in all of the sequenced ferrochelatases, was mutated to alanine while the same mutation in three other well conserved histidine residues had little effect (28). It was therefore proposed that His-263 is a ligand of the substrate iron.

Identification of the residue(s) responsible for binding the substrate iron is a critical first step in elucidating the enzymatic mechanism of ferrochelatase. In this paper we report kinetic and Mössbauer data on recombinant mouse ferrochelatase, which are consistent with the proposal that histidine residues are ligands of the ferrous ion and inconsistent with the involvement of sulfur ligands. A modified purification procedure is used and an improved assay for ferrochelatase activity, which eliminates the use of the competitive iron chelator dithiothreitol (DTT),¹ is described. Ferrochelatase activity was monitored by UV-visible and by Mössbauer spectroscopy, and the two techniques yielded consistent results. Mössbauer spectroscopy reveals that the ferrous heme reaction product forms the $S = 0$ bis(pyridine) complex upon addition of base and pyridine followed by sodium dithionite reduction and that the formation of the adduct is complete. The implications of these findings are discussed in relation to the nature of the ferrous substrate binding site.

MATERIALS AND METHODS

Deuteroporphyrin IX dihydrochloride and *N*-methylprotoporphyrin IX were purchased from Porphyrin Products (Logan, UT). Bicinchoninic acid protein assay reagents were obtained from Pierce. ⁵⁷Fe metal foil (> 95% pure) was from Advanced Materials and Technology (New York, NY). Natural-abundance FeSO₄ (containing 92% ⁵⁶Fe) was from Mallinckrodt, and natural-abundance ferrous ammonium sulfate and the sodium citrate were from Fisher. L-Ascorbic acid, ferrozine, and the ferric standard solution were from Sigma. All other chemicals were of the highest purity available.

Enzyme Preparation—Recombinant murine liver ferrochelatase was isolated from hyperproducing *E. coli* cells containing the plasmid pGF42 (23). Cells were grown in a medium containing natural-abundance iron (2.2% ⁵⁷Fe). The protein was purified and concentrated as described previously (23, 25) with the modification that the buffers used throughout the process did not contain DTT. No special anaerobic techniques were used at this stage; the protein was purified and concentrated in air. The purified, concentrated protein (typically 1–2 mM) was frozen and stored in liquid nitrogen until use. Protein concentrations were determined by the bicinchoninic acid assay using bovine serum albumin as the standard (29).

Ferrochelatase Activity Assay—The assays are essentially similar to those reported previously (23), with differences noted below. Ferrochelatase was diluted into a buffer appropriate for the specific experiment

(buffer and pH described for each experiment). In all cases, solutions containing ferrous ion were freshly prepared in previously deaerated distilled water (deaerated by bubbling high purity argon gas directly through).

Reagents containing thiol groups, such as DTT, are well known chelators of both ferric and ferrous ion. Furthermore, thiol reagents are known to destabilize hemes in aerobic environments (30). We therefore sought to determine if DTT, which is commonly used in the ferrochelatase activity assay, could be confounding the activity measurements. In order to assess the effects of aerobic environment and presence of reductant in the activity assays, four experiments were carried out. Four test tubes (soaked overnight in concentrated HCl to remove all traces of iron) were each filled with 0.5 ml of a 100 mM HEPES buffer solution, pH 7.5, containing 20% glycerol, 1.5 M NaCl, and 1% sodium cholate; with 0.1 ml of 2.2 μM protein solution; and with 0.1 ml of 1.88 mM deuteroporphyrin IX solution. Tubes 2 and 3 also contained 0.1 ml of 50 mM DTT solution, while tubes 1 and 4 instead contained an additional 0.1 ml of the buffer solution. All tubes were covered with a rubber stopper and placed at room temperature (22 °C). Tubes 3 and 4 were deaerated for 1 h using cycles of vacuum/ultrapure argon flow. The reactions were then initiated by adding 0.05 ml of 4 mM ferrous sulfate solution. The rubber stoppers were removed from tubes 1 and 2 during the reaction, while tubes 3 and 4 remained under a flow of ultrapure, humidified argon gas. After a 30-min incubation, all tubes were opened and 0.75 ml of 1 M NaOH was added, completely stopping the reaction. The heme content was then measured using the pyridine hemochromogen method (8) using a value of $\Delta\epsilon = 15.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for the reduced – oxidized difference spectra (31).

As we have established that DTT is not necessary for enzyme activity (see “Results”), it was not used in any subsequent activity assays. DTT is, however, a reductant that serves the role of keeping the iron in the ferrous form in aerobic assays. Therefore, without DTT, care must be taken to ensure that the assay is carried out under strictly anaerobic conditions from start to finish in order to avoid oxidation of the ferrous substrate. Therefore, the activity assay procedure was slightly modified. In a typical assay, 0.6 ml of 0.1 M Tris-HCl, pH 8.5 (or of varying pH for the pH dependence study), 0.1 ml of enzyme solution, and 0.1 ml of a 2 mM deuteroporphyrin IX solution were mixed in a stoppered test tube and deaerated for 30 min under an ultrapure argon flow. Then, 0.05 ml of a 4 mM ferrous ammonium citrate solution was added. The reaction was allowed to proceed for 20 min at 23 °C and it was stopped by adding 0.5 ml of 1 M NaOH. Heme content was assayed with the pyridine hemochromogen method.

To determine the optimum pH for ferrochelatase activity, activity assays were carried out using a 0.1 mM Tris-HCl buffer at pH values over the range of 6.8 to 9.7. The pH was measured at 20 °C in the final reaction mixture. For the assays of ferrochelatase activity *versus* time of reaction, each assay contained a protein concentration of 0.105 μM.

To examine the inhibition of ferrochelatase under these experimental conditions by the well studied inhibitor *N*-methylprotoporphyrin, stock solutions of *N*-methylprotoporphyrin were freshly prepared in Me₂SO and then diluted into 0.1 M HCl. Each assay contained 235 μM ferrous ammonium sulfate, 235 μM deuteroporphyrin IX, and 0.4 μM ferrochelatase.

Iron Determination—⁵⁷Fe was dissolved from metallic foil into diluted H₂SO₄ as described previously (32). After dissolution, the iron content was determined using a ferrozine colorimetric method (33). A standard curve was made using an atomic absorption ferric standard solution. Total iron content (Fe²⁺ + Fe³⁺) was determined by adding ascorbic acid to the assay mixture, while the ferrous content was determined in an assay that did not include ascorbic acid. In all cases the ferric content was small (<5%).

Mössbauer Spectroscopy—Mössbauer spectroscopy was employed both to describe and quantitate the reaction product in an activity assay and to define the nature of the iron-binding site. To investigate the iron-binding site, a ferrous control was prepared as follows: 0.35 ml of a buffer solution (100 mM MOPS, pH 7.5, containing 10% glycerol, 1.5 M NaCl, and 1% sodium cholate) was placed in a Mössbauer sample cuvette at 23 °C under argon flow and deaerated for 2 h. The lower pH is required here in order to prevent precipitation of Fe(OH)₂. The sample was taken inside an anaerobic glove box (Vacuum Atmosphere Co.), and a stock solution of ⁵⁷FeSO₄ was added to a final concentration of 0.2 mM. The sample was then allowed to incubate for 20 min before freezing in liquid nitrogen. A protein sample was prepared in parallel. Ferrochelatase was purified and concentrated to 1.75 mM in a buffer solution identical to that of the ferrous control described above. The protein sample was placed in a Mössbauer cuvette and deaerated in a stoppered tube under ultrapure argon flow for 2 h while on ice. The sample was taken inside the glove box, and ⁵⁷FeSO₄ was added to a

¹ The abbreviations used are: DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

TABLE I
Comparison of specific activity of recombinant murine ferrochelatase at pH 7.5 in the presence and absence of air and DTT

Experiment	DTT	Air	Specific activity
			nmol deuteroheme produced/(min × mg protein)
1	–	+	7
2	+	+	110
3	+	–	169
4	–	–	323

final concentration of 0.1 mM (the reason for using such a small amount of iron is discussed under "Results"). The solution was stirred and left to incubate for 20 min at 23 °C. The sample was then frozen in liquid nitrogen without exposure to air.

To study the interactions of ferrous ion, ferrochelatase, and DTT, a Mössbauer sample was prepared containing 1 mM $^{57}\text{FeSO}_4$ and 400 mM DTT in a pH 7.5 buffer as described above (sample volume is 0.35 ml). A sample was also prepared containing 310 nmol of ferrochelatase and 280 nmol of $^{57}\text{FeSO}_4$; initially, this sample also contained 390 nmol of DTT. After recording the Mössbauer spectrum, a concentrated DTT stock solution was added to increase the DTT:protein ratio to 23:1 and finally to 90:1. The Mössbauer spectrum was recorded at each ratio.

To quantitate and characterize the heme product, a ferrochelatase activity assay was carried out with enough material for quantitation by both the Mössbauer method and by the pyridine hemochromogen UV-visible method. The reaction mixture was prepared as follows. All reagents were deaerated as described above. In a stoppered, acid-cleaned test tube, 0.894 ml of 100 mM HEPES pH 7.5 buffer containing 20% glycerol, 1.5 M NaCl, and 1% sodium cholate was mixed with 6 μl of 74 μM ferrochelatase and 74 μl of 13.6 mM deuteroporphyrin solution. After sufficient deaeration, 26 μl of 39 mM $^{57}\text{FeSO}_4$ was added with a gastight syringe. The reaction was allowed to proceed under argon pressure at 24 °C for 2 h, at which time 0.4 ml was transferred to a Mössbauer sample cuvette and 0.1 ml of pyridine and 0.025 ml of 4 M NaOH added. This sample was stirred and frozen in liquid nitrogen. The remainder was added to 0.75 ml of 1 M NaOH and taken for the pyridine hemochromogen assay.

Mössbauer spectra were recorded on a constant-acceleration spectrometer. The instrument is equipped with a Janis 8DT variable-temperature cryostat and all measurements reported here were collected at 4.2 K. The zero velocity of the Mössbauer spectra is referenced to the centroid of the room temperature spectrum of a metallic iron foil.

RESULTS

During ferrochelatase activity determinations, we noticed that a solution of iron citrate (which is largely ferric in aerobic situations) and DTT turn a brilliant red color when mixed; this red complex slowly turns to a green color, presumably as the excess DTT reduces the ferric ion. The presence of these colored species made us suspect that DTT may be capable of binding substrate iron ions and hence interfering with the enzymatic activity. We therefore investigated this possibility with kinetic measurements and Mössbauer spectroscopy. Table I summarizes the effects of aerobicity and presence of DTT on ferrochelatase activity. Without DTT and in the presence of air, the enzyme activity is minimal. This is understood in terms of the oxidation state of the iron. DTT is a reductant capable of keeping iron in the ferrous state. Without it, in the presence of oxygen, the ferrous ion is expected to oxidize to the ferric form, which is not a substrate for ferrochelatase. When DTT is added to an aerobic assay, activity is observed. However, activity is increased in the anaerobic assays. Importantly, maximum activity is seen in the absence of both oxygen and DTT. The anaerobic assay that includes DTT shows only about half the activity of the assay without DTT. This difference is likely due to the ability of DTT thiol groups to competitively chelate ferrous ion and keep it from the protein. The kinetic experiments summarized here cannot directly confirm this hypothesis, but they do clearly show that DTT is not needed and that it in fact interferes with ferrochelatase activity. The lower activity seen in the aerobic assay with DTT *versus* the anaerobic

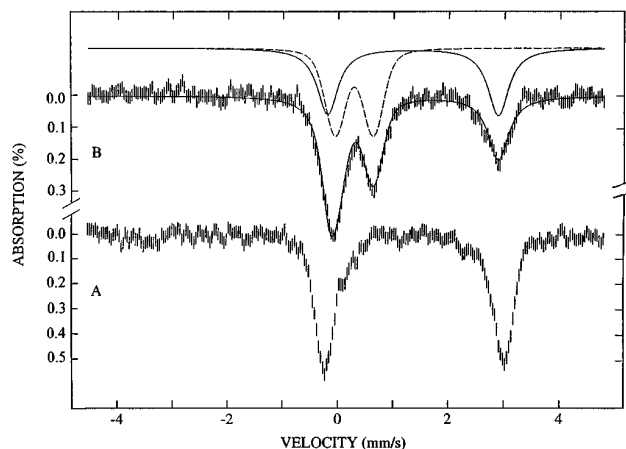


FIG. 1. Mössbauer spectra of ferrous ion binding to ferrochelatase. A, Mössbauer spectrum of 0.2 mM $^{57}\text{FeSO}_4$ in buffer. The sample was prepared as described under "Materials and Methods." The observed quadrupole doublet has parameters indicative of high spin ferrous ions in an ionic coordination environment. B, Mössbauer spectrum of 0.1 mM $^{57}\text{FeSO}_4$ added to 1.75 mM ferrochelatase in a buffer identical to that in spectrum A. Sample preparation is as described under "Materials and Methods."

assay with DTT possibly reflects the documented susceptibility of the heme product to attack by thiols in the presence of oxygen (30). For these reasons we performed all subsequent measurements anaerobically and without DTT.

Mössbauer spectroscopy was employed to investigate the chemical environment of the substrate ferrous ion and to study the binding of ferrous ion to the enzyme. Spectra are shown in Fig. 1 and were collected at 4.2 K in the absence of an external magnetic field. Fig. 1A is the spectrum of the ferrous control in a pH 7.5 buffer. The line shapes are non-Lorentzian and cannot be fit with a single quadrupole doublet. The ferrous ion here is expected to be found in a variety of configurations and its nuclear energy levels would probably be best described with a distribution of energies. Because of this a curve fit to these data is not meaningful and therefore we report only the average values for the isomer shift δ and quadrupolar splitting ΔE_Q (1.39 and 3.25 mm/s, respectively). These values are consistent with high spin ferrous ions in a nitrogenous/oxygenous ligand environment (34) and the lack of ferric species confirms the anaerobicity of our sample preparation.

Fig. 1B is the spectrum of 0.1 mM ferrous ion incubated with ferrochelatase. Sample preparation was similar to that of the ferrous control described above, except that the buffer solution also contained 1.75 mM ferrochelatase. The decision to use such a small amount of iron was based on the following considerations. If we assume a simple equilibrium model with three components (ferrochelatase, ferrous iron, and the enzyme-substrate complex) and define the dissociation constant for Fe(II) as

$$K_D^{\text{Fe}} = \frac{[E][\text{Fe(II)}]}{[E-\text{Fe(II)}]} \quad (\text{Eq. 1})$$

where $[E]$ and $[\text{Fe(II)}]$ are concentrations of the free ferrochelatase and unbound ferrous ion, respectively, and $[E-\text{Fe(II)}]$ is the concentration of the complex, the ratio R of bound iron to total iron can be written as

$$R = \frac{1}{2} \left\{ 1 + \frac{K_D^{\text{Fe}}}{[\text{Fe(II)}]_0} + \frac{[E]_0}{[\text{Fe(II)}]_0} - \sqrt{\left(1 + \frac{K_D^{\text{Fe}}}{[\text{Fe(II)}]_0} + \frac{[E]_0}{[\text{Fe(II)}]_0} \right)^2 - \frac{4[E]_0}{[\text{Fe(II)}]_0}} \right\} \quad (\text{Eq. 2})$$

Here $[\text{Fe(II)}]_0$ is the total concentration of iron and $[E]_0$ represents the total concentration of enzyme capable of binding iron.

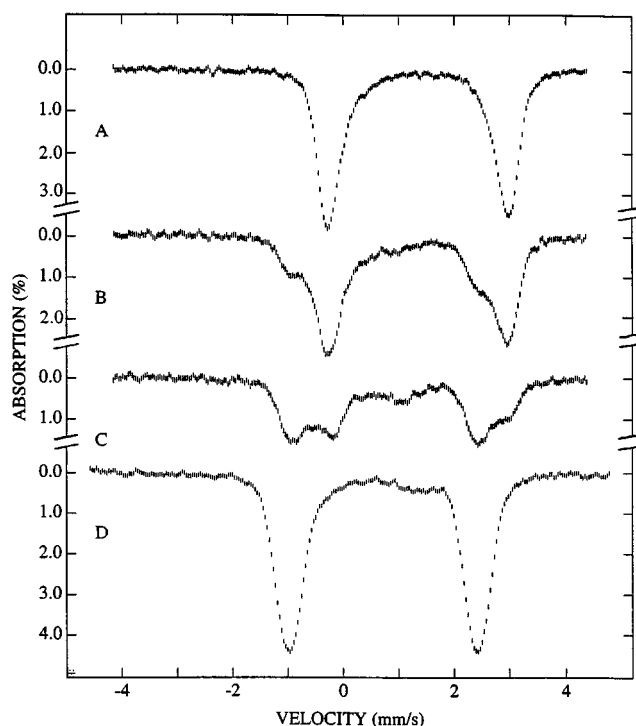


FIG. 2. Mössbauer spectra of ferrous ion binding to DTT. Conditions are as described in the text. In spectrum A the sample has a 1:1 ratio of DTT:protein, in B the ratio is 23:1, and in C the ratio is increased to 90:1. Spectrum D is that of a sample containing only 0.4 mM DTT and 1 mM $^{57}\text{FeSO}_4$. The relative amount of the DTT-bound ferrous species clearly increases as the DTT:protein ratio is increased.

It may be clearly seen from Equation 2 that, for a given $[E]_0$ and K_D^{Fe} , R will be greatest for a small $[\text{Fe(II)}]_0$. Approximating K_D^{Fe} with the previously determined K_m^{Fe} (112 μM) (23), which may be taken as a measure of the affinity of the active site for ferrous ion, R approaches 1.0 for $[E]_0 \approx 2 \text{ mM}$ and $[\text{Fe(II)}]_0 \approx 10^{-1} \text{ mM}$. Consequently, under such conditions, the Mössbauer spectrum will represent the protein-bound Fe(II) species.

Two distinct species, both quadrupole doublets, are seen in the spectrum of Fig. 1B. The first, a species with $\delta = 0.28 \text{ mm/s}$ and $\Delta E_Q = 0.69 \text{ mm/s}$, represents the $[\text{2Fe-2S}]^{2+}$ cluster (25). The protein was isolated from bacteria grown on natural abundance Fe, and this spectral species arises from the 2.2% natural abundance of ^{57}Fe in the cluster. The spectrum of this component is plotted as a *dashed line* above the experimental spectrum. The second species, with a well resolved high energy line at 2.88 mm/s, represents high spin Fe(II) bound to the active site of ferrochelatase. A simulation of this species is plotted as a *solid line* above the experimental spectrum. The resulting parameters, $\delta = 1.36 \pm 0.03 \text{ mm/s}$ and $\Delta E_Q = 3.04 \pm 0.06 \text{ mm/s}$, are consistent with nitrogen/oxygen ligands for the high spin ferrous ion. Note especially the positions of the right lines of the ferrous doublets: in the control, this peak is at 3.01 mm/s; in the ferrochelatase-bound ferrous ion, the peak has shifted to 2.88 mm/s. The bound ferrous ion is thus distinguishable from the free ferrous ion. Analysis of this peak in Fig. 1B indicates that in this sample the added iron is completely bound to the ferrochelatase active site; at the very most, only 10% of the added ferrous ions can be considered free. The two species that comprise this spectrum are plotted together as the *solid line* over the experimental data.

Fig. 2 shows the effect of DTT in the reaction mixture. Fig. 2A is the spectrum of a sample containing 0.76 mM ferrochelatase, 0.84 mM $^{57}\text{FeSO}_4$, and 1 mM DTT (DTT:protein ratio of 1.3:1). Gross inspection reveals a single major quadrupole dou-

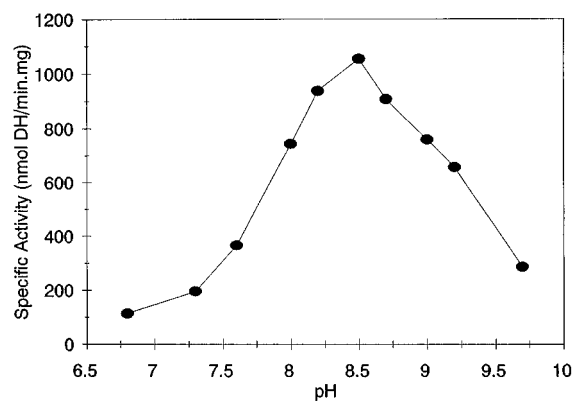


FIG. 3. Ferrochelatase specific activity as a function of pH. Deuteroheme concentrations were measured by the pyridine hemochromogen method. Assay conditions are as described under "Materials and Methods." All assays were conducted anaerobically and without DTT. The results shown are the averages of triplicate data.

plet and, except for the decrease in the relative intensity of the $[\text{2Fe-2S}]^{2+}$ cluster spectrum, the spectrum is similar to that shown in Fig. 1B. In Fig. 2B, the DTT:protein ratio has been increased to 23:1. In addition to a quadrupole doublet similar to that of Fig. 2A, an extra component having parameters $\delta = 0.73 \pm 0.02 \text{ mm/s}$ and $\Delta E_Q = 3.39 \pm 0.03 \text{ mm/s}$ is observed. The relative absorption of this latter component increases further when the DTT:protein ratio is increased to 90:1 (Fig. 2C), and in Fig. 2D, which is the spectrum of a sample containing 400 mM DTT and 1 mM $^{57}\text{FeSO}_4$ in the buffer solution, it is seen to be the only component. The parameters given above are consistent with tetrahedral sulfur coordinated high spin ferrous ions (34). This component therefore represents ferrous ion chelated by DTT, and these spectra clearly demonstrate the competition between enzyme and DTT for ferrous ion binding. It is important to realize that in the procedures commonly used to assay ferrochelatase activity, DTT:protein ratios of 10^4 :1 are not uncommon (35).

Kinetic studies of purified ferrochelatases have generally been reported in the presence of DTT. In light of our finding that DTT competes with the protein active site for binding ferrous ion, we sought to characterize recombinant murine ferrochelatase kinetically using the new experimental conditions. Under anaerobic conditions and in the absence of DTT, the pH dependence of the murine ferrochelatase specific activity (Fig. 3) showed a single optimum value at pH 8.5. This is a slightly more basic pH value compared to that previously obtained (pH 7.5) from rat liver, under aerobic conditions and in the presence of DTT (35). The difference might be due to heme degradation induced by the thiol or simply to differences between the species.

At pH 8.5, the amount of ferrochelatase-catalyzed heme product increased linearly with time (Fig. 4A) and with enzyme concentration up to 0.5 μM (Fig. 4B). Without DTT, the enzyme exhibits the same type of inhibition by *N*-methylprotoporphyrin IX (Fig. 5) as seen in assays containing DTT (36). In addition, the measured specific activity values are about 2-10-fold those of the purified enzyme measured under aerobic conditions in the presence of DTT (36).

To check whether the heme product can be quantified by Mössbauer spectroscopy as well as UV-visible spectroscopy, we performed an activity assay in which the heme was quantitated by the two techniques. At the end of the assay, the reaction mixture was split into two aliquots; one was measured by the previously described pyridine hemochromogen method, and the other by Mössbauer spectroscopy. The pyridine hemochromogen method indicated that $63 \pm 12\%$ of the added $^{57}\text{Fe}^{2+}$ ions

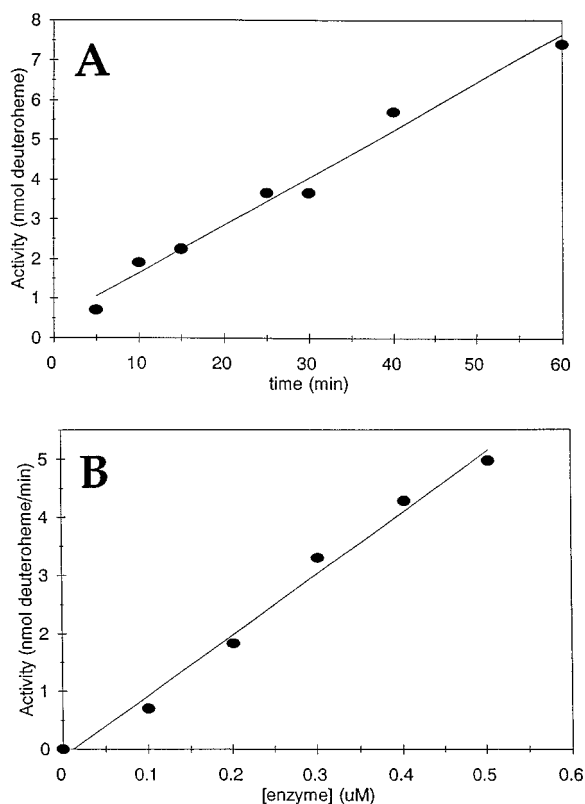


FIG. 4. **Ferrochelatase activity assayed at pH 8.5.** The plot in *A* shows linear formation of deuteroheme with time. Ferrochelatase concentration was $0.105 \mu\text{M}$ in each assay. Plot in *B* shows that total activity is linear with enzyme concentration up to $0.5 \mu\text{M}$. Assay conditions are as described under "Materials and Methods."

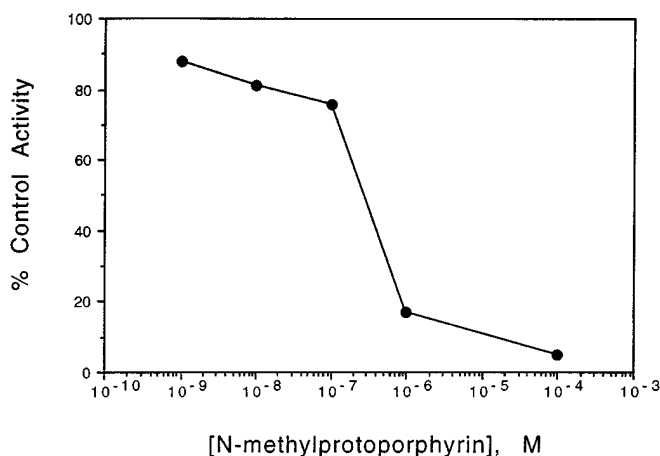


FIG. 5. **Inhibition of ferrochelatase activity by *N*-methylprotoporphyrin IX.** Percent of ferrochelatase activity versus *N*-methylprotoporphyrin IX concentration. Each assay contained $235 \mu\text{M}$ deuteroporphyrin, $234 \mu\text{M}$ ferrous ion, and $14.4 \mu\text{g}$ ($0.4 \mu\text{M}$) of purified enzyme. The assay was done anaerobically, and activity was measured by the pyridine hemochromogen method as described under "Materials and Methods."

had been incorporated into heme, giving an activity of 280 nmol of deuteroheme/(min \times mg of ferrochelatase).

Because the ferrous heme product is expected to be found in a variety of forms (monomeric, multimeric, with or without aquo axial ligands, etc.; Refs. 37–39), pyridine was added to the Mössbauer sample under alkaline conditions in order to form the well defined bis(pyridine) heme complex. The Mössbauer spectrum of the sample after reduction with sodium dithionite was recorded at 4.2 K in the presence of a 60-millitesla mag-

netic field oriented parallel to the γ -beam (data not shown). The major species was a doublet with parameters $\delta = 0.45 \pm 0.01$ mm/s and $\Delta E_Q = 1.12 \pm 0.02$ mm/s, indicative of the low spin ($S = 0$) bis(pyridine) heme adducts (39). This species represented $59 \pm 10\%$ of the total spectral area, in agreement with the UV-visible pyridine hemochromogen determination.

DISCUSSION

The data summarized in Table I clearly demonstrate that reducing agents (*e.g.* DTT) are not needed for ferrochelatase activity. Indeed, DTT is shown to compete with ferrochelatase for binding free ferrous ion. It has been postulated previously that the role of DTT in aerobic assays is to keep the substrate iron in the ferrous form (40), and our data are consistent with this hypothesis. Other groups have reported finding ferrochelatase activity using purified enzyme preparations in assays that supposedly did not include DTT (27, 40); however, careful inspection of their purification procedures reveals that DTT was present (at higher concentrations than the enzyme) as it was used in the purifications. When the iron-chelating ability of DTT is considered together with the fact that hemes are known to degrade in the presence of thiol-containing species and oxygen, it becomes evident that the activity assays commonly used are inaccurate and reflect a complex set of chemical reactions, rather than a single enzymatic activity. The procedure reported here has corrected these problems, and we recommend that it be used whenever measuring ferrochelatase activity.

The fact that reducing agents are not required for ferrochelatase activity will have implications for any proposed mechanistic model. DTT is commonly used to keep protein cysteinyl residues in the reduced (sulfhydryl) form (41), and the mechanism proposed previously (27) required the cysteinyl residues responsible for binding substrate ferrous ion to be in the reduced form. DTT (or other reductant) has been presumed to play just such a role in the commonly used procedures to purify ferrochelatase and measure its activity. Without a comparable reducing agent, a pair of vicinal, accessible cysteinyl residues is usually expected to be in the oxidized (disulfide) form upon aerobic purification and hence inactive in this proposed mechanism. Instead, we observe greater activity than that reported for assays using DTT. The findings reported here suggest that this mechanism (27) be re-examined.

The biochemical and kinetic behavior of the ferrochelatases purified from various sources are found to be quite similar (2). This observation, and evolutionary considerations, make it rather unlikely that the active site residues responsible for binding substrate should differ from species to species. Cysteine residues are not conserved among the sequenced species, but there are 3 aspartyl, 3 tyrosyl, 2 glutamyl, 5 seryl, and 1 histidyl residue which are conserved. All of these amino acids contain side groups known to chelate ferrous ion in proteins. Kinetic studies utilizing site-directed human ferrochelatase mutants led to the proposal that His-263, the conserved histidine, is a ligand of the ferrous substrate (28). The possibility exists that other residues also serve as ligands.

The Mössbauer data also are inconsistent with sulfur coordination of the substrate ferrous ion. In general, the isomer shift decreases with decreasing coordination number and spin state and with increasing covalency and oxidation state. The high isomer shift of the ferrochelatase-bound iron (1.36 mm/s) is indicative of an ionic coordination environment, consisting of nitrogen- and/or oxygen-containing ligands, for the high spin ferrous ions. As nitrogen/oxygen ligands are replaced by cysteine sulfurs, the isomer shift decreases substantially. The effect of replacing a single nitrogen/oxygen ligand with a cysteine sulfur is observable in the Mössbauer spectrum: binding

of one cysteine S to the active-site ferrous ion in isopenicillin-N-synthase reduces the isomer shift from 1.3 to 1.1 mm/s (42). For reduced rubredoxin, an iron-sulfur protein containing a tetrahedral sulfur coordinated Fe(II)S₄ center, the isomer shift is 0.7 mm/s (43); such a species, whose spectrum resembles the DTT-bound ferrous ion, is absent from the spectrum of Fig. 1B. The model proposed for the ferrochelatase mechanism in which cysteine residues were responsible for binding the ferrous ion (27) was based on experiments which demonstrated that sulfhydryl reagents were capable of blocking enzymatic activity. Those findings suggest rather convincingly that cysteinyl residues are of great importance to enzymatic activity but do not necessarily mean that these residues are directly involved in binding substrate ferrous ion. The data reported here, coupled with the available ferrochelatase sequences, imply that the importance of cysteinyl residues is not due to their ability to ligate substrate ferrous ion.

A question that might be raised in our interpretation of the Mössbauer spectra is the fact that many proteins are known to bind ferrous ion in a nonspecific, adventitious manner. It may be argued that the protein-bound ferrous component seen in Fig. 1B is indeed such an adventitiously bound species. Although it may be possible that the species we have assigned as "bound ferrous" is such an adventitious species or is a mixture of ferrous ions bound in the active site and in nonspecific sites, it is important to note that samples prepared in this manner are active. The fact that this preparation is functional indicates that the active site of the enzyme in these samples is capable of binding iron. With the protein and iron concentrations of the sample in Fig. 1B, and assuming the K_m^{Fe} value of 112 μM (which was determined under aerobic conditions and in the presence of DTT) as an upper value for K_D^{Fe} , Equation 2 predicts that virtually all of the added ferrous ion should be bound to the active site. Therefore, the species in Fig. 1B represents substrate iron bound in the ferrochelatase active site and not adventitiously bound iron.

In conclusion, our findings are consistent with the proposal that histidine residues are ligands of the ferrous substrate, although we do not rule out other ionic ligands (e.g. aspartate, glutamate, tyrosinate). We do rule out the direct involvement of any cysteine residues in binding ferrous ion; however, cysteine residues in mammalian ferrochelatases are ligands of the [2Fe-2S] cluster and may play other important roles in enzyme function or regulation. Further studies are needed to conclusively identify specific residues ligating the substrate iron. Investigations in progress using site-directed mutants should prove most helpful. Mössbauer spectroscopy should provide detailed information on the binding environment of the ferrous substrate and has been shown to be accurate in quantitating heme product and will continue to be employed in our investigations. We also suggest that iron chelators such as DTT are unnecessary for ferrochelatase activity and complicate the measurement and should be eliminated from further usage.

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Characterization of the Iron-binding Site in Mammalian Ferrochelatase by Kinetic and Mössbauer Methods

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