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Purification and Characterization of the Membrane-Bound Ferrochelatase from *Spirillum itersonii*

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The membrane-bound enzyme ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1) was purified from isolated membrane fragments of *Spirillum itersonii* approximately 490-fold. Purification was achieved by solubilization with chaotropic salts followed by ammonium sulfate fractionation, diethylaminoethyl-cellulose chromatography, and gel filtration on Sephadex G-200. The purified enzyme has an apparent minimum molecular weight of approximately 50,000, as determined by gel filtration in the presence of 0.1% Brij 35 and 1 mM dithiothreitol but forms high-molecular-weight aggregates in the absence of detergent. Purified ferrochelatase is strongly stimulated in the presence of copper. The apparent K_m for Fe^{2+} is 20 μM in the absence of copper and 9.5 μM in the presence of 20 μM $CuCl_2$. The apparent K_m for protoporphyrin is 50 μM, and it is unaltered by copper. Ferrochelatase has a single pH optimum of 7.50, and it is inhibited 50% by 20 μM heme. Certain divalent cations and sulfhydryl reagents also inhibit the enzyme.

The terminal step in heme biosynthesis, the insertion of iron into the porphyrin ring, is catalyzed by the enzyme ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1). This enzyme has been found in a variety of animal tissues (21) as well as in plants (11), yeasts (8), and bacteria (3, 10). In all cells examined the enzyme is membrane bound. In bacteria it is associated with the cytoplasmic membrane (3, 10), whereas in animal cells it is located on the inner mitochondrial membrane (9), and it is bound to the chloroplast membrane in plants (11). There have been reports of a soluble form of ferrochelatase in some bacteria (7), but this activity does not, apparently, participate in heme biosynthesis in vivo (3).

Ferrochelatase will catalyze the chelation of ferrous, but not ferric, iron, as well as the divalent cations Co^{2+}, Zn^{2+}, and Cu^{2+}, but not Mg^{2+} (3, 10, 11). In addition to protoporphyrin IX, ferrochelatase will also utilize the dicarboxylic mesoporphyrin and deuteroporphyrin, but it will use neither protein-porphyrin complexes nor porphyrinogens as substrates (10, 21, 22).

Ferrochelatase has been studied in a wide variety of organisms and tissues but has not yet been purified to homogeneity. Previously we have made use of chaotropic salts to selectively solubilize ferrochelatase from the cytoplasmic membrane of the gram-negative bacterium *Spirillum itersonii* (3), and the present work describes the purification of ferrochelatase solubilized with chaotropic salts. This purification scheme yields an approximately 1,000-fold increase in specific activity, as compared with crude cell extracts, and an approximately 500-fold increase over the activity in isolated membranes.

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MATERIALS AND METHODS

Chemicals. Protoporphyrin IX was obtained from Porphyrin Products, Logan, Utah; diethylaminoethyl-cellulose was from Bio-Rad Laboratories, Richmond, Calif.; Sephadex G-200 and G-25 were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; Brij 35 was from Atlas Chemical Industries, Inc., Wilmington, Del.; dithiothreitol (DTT) was from Sigma Chemical Co., St. Louis, Mo.; sodium perchlorate was from G. Frederick Smith; and sodium thiocyanate was from J. T. Baker Chemical Co., Phillipsburg, N.J. Handifluor scintillation fluid was from Mallinckrodt Chemical Works, St. Louis, Mo., and ^{59}Fe was from New England Nuclear, Boston, Mass. Sodium dodecyl sulfate (SDS) was from BDH Pharmaceuticals Ltd., London, England; acrylamide was from J. T. Baker; and *N,N'*-methylenebisacrylamide was from Eastman Organic Chemical Div., Eastman Kodak Co., Rochester, N.Y. All other chemicals were of reagent grade.

Growth of *S. itersonii*. Maintenance and growth of *S. itersonii* has been described before (2). Cells were grown in a 40-liter New Brunswick Fermacell fermentor in GGS medium (2) supplemented with 0.2% yeast extract and 2.0 g of KNO_3 per liter. A 36-liter amount of medium was held at 30°C with the agitation at 200 rpm and 0.5 cubic feet (ca. 0.014 m^3)

of air sparged per min. A 1-liter culture (late-log-grown cells) was used as the inoculum, and the culture was harvested after 16 h with a refrigerated Sharples Super Centrifuge. Growth yields were usually between 150 and 250 g (packed wet weight) per 37 liters.

Ferrochelatase assay. Protoporphyrin stocks (approximately 1 mM) were freshly prepared for each experiment as described previously (3) and kept at 4°C in the dark. The complete assay mixture contained 70 μmol of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6), 0.10 μmol of protoporphyrin, 0.1 μmol of iron citrate (in ^{59}Fe assays, approximately 0.1 μCi), 5.0 μmol of DTT, and enzyme preparation in a final volume of 1.5 ml. Details of the assay have been described (4).

Protein determination. The protein concentration was determined by the method of Lowry et al. (13), using bovine serum albumin as a standard. Columns were monitored for protein by reading the absorbance at 280 nm for each fraction.

Spectrophotometry. Spectrophotometric measurements were made with a Cary 14R recording spectrophotometer, using the 0.0–0.1, 0.1–0.2 A slide wire.

SDS-polyacrylamide gel electrophoresis. Buffer and gel solutions used in SDS-gel electrophoresis were those described by Laemmli (12). Ten-percent gels were used with phenol red as the tracking dye. Slab gels, 1 mm thick, were run on an apparatus similar to that described by Reid and Bieleski (17). Samples were stacked at 10 mA and separated at 15 mA. Gels were stained by the method of Fairbanks et al. (6) and were then soaked in 10% (vol/vol) methanol before being vacuum dried on Whatman no. 1 filter paper.

Purification of ferrochelatase. Step 1: Solubilization of ferrochelatase by chaotropic salts. The procedure used for cell fractionation and ferrochelatase solubilization has been fully described (3). Crude membranes obtained by centrifugation (60 min, 150,000 × *g*) of a French press extract were successively extracted with 1.0 M sodium chloride, 0.2 M sodium perchlorate, and, finally, 0.5 M sodium thiocyanate. Each salt solution was in 25 mM Tris-hydrochloride (pH 7.8), 1 mM DTT. The supernatant solution of the sodium thiocyanate extract contained the solubilized ferrochelatase.

Step 2: Ammonium sulfate fractionation. The solution containing the solubilized enzyme (approximately 1.0 mg/ml) was brought to 20% saturation with ammonium sulfate by the addition of a saturated solution of ammonium sulfate adjusted to pH 8.0. After stirring for 15 min at 20°C the solution was centrifuged (5°C) at 10,000 × *g* for 15 min. The supernatant was removed and brought to 50% saturation with ammonium sulfate. After stirring for 15 min at 20°C this was centrifuged at 10,000 × *g* for 15 min. The supernatant was discarded, and the pellet was suspended in 20 ml of 25 mM Tris-hydrochloride (pH 7.8). Insoluble material present was removed by centrifugation at 10,000 × *g* for 15 min. The gray-colored pellet was washed with 20 ml of the above buffer and centrifuged again. The supernatant fractions were combined, brought to 50% saturation with ammonium sulfate, and centrifuged. The pellet thus obtained was suspended in 5 ml of buffer and desalted by passage through a Sephadex G-25 column (2.5 by 20 cm) equilibrated with 20 mM Tris-hydrochloride (pH 7.8) and 1 mM DTT. This desalting step to remove residual ammonium sulfate is essential since ferrochelatase activity was inhibited by ammonium sulfate.

Step 3: Chromatography on diethylaminoethyl-cellulose. The enzyme preparation from the previous step (about 10 to 12 mg/ml) was applied to a column (2.5 by 25 cm) of diethylaminoethyl-cellulose at 8°C that had been previously equilibrated with 25 mM Tris-hydrochloride (pH 8.2 at 8°C), 1 mM DTT, and 0.1% Brij 35. The column was first washed with 2 volumes of this same buffer, removing most of the cytochromes present in the sample. Three bed volumes of the above buffer containing 0.15 M sodium perchlorate were then passed through the column. The enzyme was eluted with buffer containing 0.21 M sodium perchlorate. Fractions containing activity were pooled and concentrated by pressure using an Amicon XM-50 filter at 4 to 8°C.

Step 4: Gel filtration through Sephadex G-200. The concentrated enzyme preparation from step 3 (approximately 10 to 12 mg) was applied to a Sephadex G-200 column at 8°C (1.5 by 85 cm) equilibrated with 25 mM Tris-hydrochloride (pH 8.2), 1 mM DTT, and 0.1% Brij 35. Fractions (1.5 ml) were collected. A small peak (approximately 20% of the total recovered activity) eluted in a volume corresponding to a molecular weight of approximately 200,000. The major peak of activity eluted at a volume corresponding to a molecular weight of 48,000 to 50,000. The six peak fractions were pooled and concentrated using an Amicon XM-50, which retained all the activity.

RESULTS

Purification and stability of ferrochelatase. The purification (outlined in Table 1) resulted in a 490-fold increase in specific activity over the activity of the membrane fractions and approximately 1,000-fold purification in terms of the crude cell extract. The course of the purification was followed by SDS-polyacrylamide gel electrophoresis (Fig. 1).

The solubilization scheme using chaotropic reagents was reproducible and gave recoveries of 60% with a 10-fold increase in specific activity. Fractionation with ammonium sulfate gave recoveries of 70 to 90% with purification of from 2.5- to 3.6-fold. (Fractionation within narrower limits led to poorer recoveries without substantial increases in purification.) This step includes the fractionation as well as the subsequent removal of ammonium sulfate by passage through a Sephadex G-25 column. The material obtained after this step was stable for several months if kept in 25 mM Tris-hydrochloride (pH 7.8) and 1 mM DTT at −70°C.

The diethylaminoethyl-cellulose chromatog-

TABLE 1. *Purification of ferrochelatase*[a]

Step	Fraction	Total units[b]	Volume (ml)	Sp act (U/mg of protein)		Purification (fold)[c]
				$-CuCl_2$	$+CuCl_2$	
1	Chaotropically solubilized	287	500	0.62	0.60	10
2	Ammonium sulfate (20–50%)	220	10	1.85	1.90	32
3	Diethylaminoethyl-cellulose chromatography	147	6	10.40	13.52	225
4	Gel filtration	35	2	2.0	29.40	490

[a] Details of the purification scheme are in the text.
[b] Enzyme units are expressed as nanomoles of protoheme formed per minute.
[c] Purification is based upon the specific activity (with $CuCl_2$ in the reaction mixture) of isolated membrane fragments.

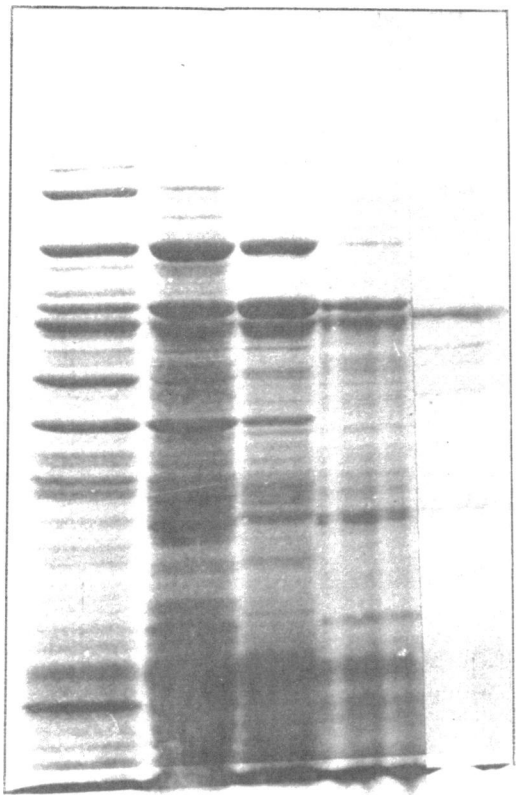

FIG. 1. *SDS-polyacrylamide gel electrophoresis of purified ferrochelatase. Samples of each step from the purification scheme (outlined in Table 1) were subjected to electrophoresis on 10% polyacrylamide gels as described in the text. From left to right: crude membrane fraction, 15 μg; step 1 material, 20 μg; step 2 material, 8 μg; step 3 material, 5 μg; step 4 material, 2 μg.*

raphy step gave 50 to 70% recovery, with purification ranging from 5- to 11-fold. Elution of ferrochelatase required the presence of detergent, and either Triton X-100 or Brij 35 was effective. Brij 35 was used since it does not absorb as strongly in the ultraviolet region as does Triton X-100 and is, therefore, more suitable for monitoring column fractions by absorbance at 280 nm. It was found that a step gradient of 0.15 to 0.21 M sodium perchlorate in the presence of 1 mM DTT, 0.1% Brij 35, and 25 mM Tris-hydrochloride (pH 8.2 at 8°C) was most effective in eluting ferrochelatase with the highest purification and yield. NaCl, at concentrations up to 1 M, was ineffective in eluting the enzyme.

Gel filtration with Sephadex G-200 in the presence of 0.1% Brij 35 and 1 mM DTT gave only about a 20 to 30% recovery with about a two- to threefold increase in specific activity. Total activity recovered in all fractions was less than 40%. The enzyme was labile after the G-200 step, particularly in the absence of DTT, and this lability may be attributable to the low protein concentration (less than 1 mg/ml) of this sample. The addition of 50 μM $CuCl_2$ to this enzyme preparation did not prevent further loss of activity.

Estimation of molecular weight. The molecular weight of native ferrochelatase was estimated by gel filtration on Sephadex G-200 calibrated with the following commercially available soluble proteins: apoferritin, catalase, aldolase, bovine serum albumin, ovalbumin, myoglobin, and cytochrome *c*. In the presence of 0.1% of Brij 35 the molecular weight was estimated to be 48,000 to 50,000. A small amount of activity (20%) eluted at a position corresponding to a molecular weight of approximately 200,000. In the absence of detergent the enzyme eluted in the void volume, suggesting the presence of high-molecular-weight aggregates.

SDS-polyacrylamide gel electrophoresis showed a single major band, although several minor bands were visible (Fig. 1). Comparisons of the R_f value for this band with those of proteins of known molecular weight gave an estimated molecular weight of 53,000.

The visible and ultraviolet spectrum of step 4 enzyme (0.5 mg/ml) showed no evidence for the presence of any heme, pyridoxal, or flavin com-

pounds (using the 0.1 full scale on a Cary 14R recording spectrophotometer).

Kinetic properties. The apparent K_m values and pH optima for purified ferrochelatase were the same as those reported for the membrane-bound enzyme (3). Variation in ionic strength (produced by addition of NaCl) between 0.01 and 0.25 had no measurable effect on enzyme activity.

Sulfhydryl reagents. Purified ferrochelatase was allowed to react with the sulfhydryl blocking reagents *N*-ethylmaleimide and iodoacetamide (Table 2). Unreacted reagent was inactivated by addition of DTT before adding the enzyme preparation to the assay mixture. Iodoacetamide at concentrations up to 10 mM inhibited the purified enzyme (step 3) 50%, whereas 10 mM *N*-ethylmaleimide inhibited activity 94%. The presence of either protoporphyrin (100 μM) or iron citrate (100 μM) did not prevent inactivation by iodoacetamide or *N*-ethylmaleimide. In the interpretation of the data concerning iron citrate's inability to prevent inactivation, it must be remembered that ferrochelatase requires ferrous iron for activity and that in the *N*-ethylmaleimide and iodoacetamide reactions (which are carried out aerobically in the absence of added reducing compounds) iron is present mainly in the ferric state.

TABLE 2. *Effect of sulfhydryl reagents on ferrochelatase*[a]

Inhibitor[b]	Addition[c]	Activity (%)[d]
–	–	100
NEM	–	6
NEM	Fe^{3+} citrate	6
NEM	Protoporphyrin	6
Iodoacetamide	–	53
Iodoacetamide	Fe^{3+} citrate	56
Iodoacetamide	Protoporphyrin	59

[a] Enzyme obtained after step 3 was used with the ^{59}Fe assay. –, None.

[b] The enzyme preparation was incubated with *N*-ethylmaleimide (NEM) or iodoacetamide at 10 mM concentration in 25 mM Tris, pH 7.6, in a total volume of 0.5 ml for 15 min at 23°C. At the end of this incubation time, unreacted NEM or iodoacetamide was neutralized by the addition of excess DTT. This mixture containing the enzyme was then assayed as described in the text. The control was treated exactly the same as the test samples, except no sulfhydryl reagent was added.

[c] In the specified experiments either Fe^{3+} citrate (0.1 mM final concentration) or protoporphyrin (0.1 mM final concentration) was present during the 15-min incubation period. Fe^{3+} citrate and protoporphyrin were added 1 min before NEM or iodoacetamide.

[d] All data are normalized so that 100% is equivalent to 10 U/mg.

Effect of heme. Heme, the product of the reaction catalyzed by ferrochelatase, inhibited the enzyme 50% at a concentration of 20 μM (Fig. 2). Complete inhibition never occurred, even a concentration as high as 100 μM.

Metal ions. Step 3 purified enzyme was used to test the effect of divalent cations on ferrochelatase activity (Table 3). Since ferrochelatase will also catalyze the insertion of some of the cations (Co^{2+}, Zn^{2+}, and Cu^{2+}) into protoporphyrin, the ^{59}Fe assay was used. Ba^{2+}, Ca^{2+}, and Mg^{2+} had no significant effect, whereas Zn^{2+}, Ni^{2+}, and Mn^{2+} had minimal effects on enzyme activity at the concentration tested. Ferrochelatase was significantly inhibited by Co^{2+} and Pb^{2+} at 50 μM. In contrast to other cations, copper was the only metal found to increase activity and, therefore, its stimulatory effect was examined in more detail. The monovalent cations Na^+ and K^+ had no effect on ferrochelatase activity.

The most marked stimulatory effect of copper was observed with enzyme purified to step 4. It was found that after gel filtration on G-200 very little enzyme activity was recovered if the assay was performed without added copper. Figure 3 shows the stimulation of ferrochelatase by copper. Half-maximal stimulation of the most purified preparation of ferrochelatase occurred at 2.5 μM copper. Copper was found to alter the apparent K_m for Fe^{2+} without changing the V_{max} (Fig. 4). Copper, however, had no effect on the apparent K_m for protoporphyrin.

DISCUSSION

Since its discovery ferrochelatase has been studied in a wide variety of organisms and tissues. In no instance, however, has the enzyme been purified to homogeneity. An approximately 200-fold purification from duck erythrocytes was achieved mainly by the removal of hemoglobin (21), and a 20- to 40-fold purification was reported for rat liver mitochondria (14). Previously, this laboratory has purified ferrochelatase from *S. itersonii* about 10-fold by solubilization with chaotropic salts (3), and in this study we report the 1,000-fold purification of this enzyme.

One major difficulty in purifying the enzyme has been the rapid loss of activity during purification procedures. In the present study ferrochelatase was stable up to step 3. During gel filtration, however, a substantial loss of activity occurred, and the enzyme preparation at step 4 was labile, with total recovery of enzyme after this step of only about 30%. The addition of copper restored some, but not all, of the lost

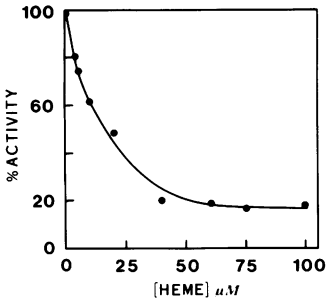

FIG. 2. *Inhibition of purified ferrochelatase by heme. An activity of 100% is equivalent to 11.0 nmol of protoheme formed/min per mg of protein. Fifty percent inhibition occurs at 20 μM heme. Solutions of heme were prepared and quantified by the pyridine hemochromogen method (3). Step 3 enzyme was used in these experiments.*

TABLE 3. *Effect of metal ions on ferrochelatase*[a]

Metal added (50 μM)[b]	Activity (%)[c]
None	100
$BaCl_2$	100
$CaCl_2$	100
$MgCl_2$	95
$ZnCl_2$	83
$NiCl_2$	82
$MnCl_2$	72
$CoCl_2$	42
$Pb(NO_3)_2$	33
$CuCl_2$	132

[a] Enzyme obtained after step 3 was used in all of these experiments, and the ^{59}Fe ferrochelatase assay was used. These values are averages of three experiments, and none of the values varied more than ±10%.

[b] The metal solutions were added to the complete assay mixture just before evacuation of the tubes. No precipitation occurred in any of the assays at final metal concentrations of 50 μM. Iron citrate was present in the assay mixture at 100 μM.

[c] All data are normalized so that the control, which had 10 U/mg, has 100% activity.

activity. Mazanowska et al. (14) found that the addition of a crude lipid extract to preparations of their purified rat liver ferrochelatase stimulated activity; even so, their total recoveries were less than 3% with a 40-fold purification. Activity of our purified preparation of ferrochelatase from *S. itersonii* was not stimulated by additions of crude lipid extracts; however, enzyme-associated lipid may be present, or the small amount of detergent present in the assay mixture may satisfy any possible lipid requirement.

The stimulatory effect of phospholipids has been reported for ferrochelatase of avian erythrocytes (18) and *Rhodopseudomonas sphaeroides* (15). This observed stimulation has led some investigators to propose that phospholipids may function by either attracting Fe^{2+} ions or providing a favorable environment for protoporphyrin, which is poorly soluble in aqueous solutions (15, 18). Though these functions may be possible, it seems reasonable that the lipid stimulation of ferrochelatase activity may reflect a more general lipid requirement characteristic of many membrane-bound enzymes. Obviously, this question cannot be entirely resolved until ferrochelatase can be purified free of all other proteins and lipids.

Wagner and Tephly (20) have reported that detergent-solubilized ferrochelatase from rat

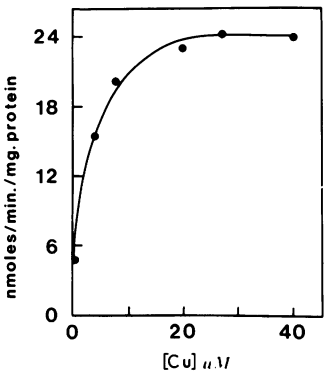

FIG. 3. *Stimulation of purified (step 4) ferrochelatase activity by copper. Copper was supplied as $CuCl_2$. This particular preparation of enzyme had 5 U of activity without added copper. One preparation was obtained that contained no activity without added copper. Units of activity are nanomoles of protoheme formed per minute per milligram of protein.*

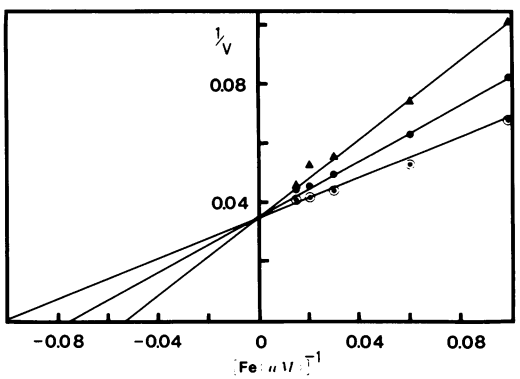

FIG. 4. *Lineweaver-Burk plot showing the effect of copper on the K_m for Fe^{2+}. Enzyme preparation and experimental details are the same as those in Fig. 3. Symbols: no copper added (▲), 5 μM copper (●), 10 μM copper (⊙).*

liver mitochondria lost activity upon dialysis but that activity was restored by addition of copper ion to the assay mixture. We have found that the activity of purified ferrochelatase of *S. itersonii* is strongly stimulated by copper. The stimulation of activity in both rat and bacterial systems appears to be specific for copper, since no other cation tested caused increased activity. It is difficult to assess the role copper may play since the enzyme preparation is not homogeneous, but in vivo observations lend credibility to a role for copper in ferrochelatase activity. These observations are that severe copper deficiency in animals causes anemia (5) and that low copper concentrations in *Paracoccus denitrificans* cultures result in elevated δ-aminolevulinic acid synthase (19), which might be attributable to decreased intracellular heme levels (due to diminished ferrochelatase activity) leading to derepression of δ-aminolevulinic acid synthase. The function of copper, as with the lipid requirement, will not be resolved until ferrochelatase can be fully purified.

It is well documented that ferrochelatase in vitro requires ferrous iron for activity. Recent studies with rat liver mitochondria (1) and bacterial extracts (4, 16) have shown that a ferric iron-reducing system exists that can provide ferrous iron for heme biosynthesis. Although it is tempting to speculate that ferrochelatase in vivo may itself be able to reduce iron for heme biosynthesis, presently available evidence suggests that ferric reductase is an entity separate from ferrochelatase.

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