Kinetic Studies of Human Liver Ferrochelatase

ROLE OF ENDOGENOUS METALS*

Jean-Michel Camadro, Nader G. Ibrahim$, and Richard D. Levere†

From the New York Medical College, Department of Medicine, Munger Pavilion, Valhalla, New York 10595

The terminal step in heme biosynthesis, the insertion of ferrous iron in the porphyrin ring, is catalyzed by the enzyme ferrochelatase (EC 4.99.1.1). This membrane-bound enzyme is associated with the inner mitochondrial membrane in eu karyotic cells (1, 2). It has been purified to homogeneity from rat liver (3) and bovine liver (4) mitochondria. The membrane-bound and the purified enzymes exhibit a broad affinity for zinc lowers the actual velocity for iron incorporation was 8.7 nmol of protoporphyrin IX (the physiological substrate of ferrochelatase) has shown that ferrous iron, but not ferric iron, is a strong competitive “inhibitor” of the zinc-chelatase activity of yeast ferrochelatase (8).

From the different metalloporphyrins synthesized by ferrochelatase (excluding heme), zinc-protoporphyrin has the greatest pathophysiologic relevance in humans since it has been found in erythrocytes from patients with lead intoxication and iron deficiency anemia (9, 10). On the other hand, unchelated protoporphyrin is accumulated or excreted in patients with erythrohepatic prottoporphyria (9) where a defect in ferrochelatase activity has been demonstrated (11, 12, 14, 15). These observations lead to some important questions about (i) the involvement of ferrochelatase in vivo in the synthesis of zinc-protoporphyrin and (ii) the availability of zinc as a substitute for iron in the chelatase reaction in human liver. Camadro and Labbe (8) emphasized the importance of endogenous metals entrapped within the mitochondrial membranes and the influence of these metals on ferrochelatase activity measurements. Our present kinetic studies of human ferrochelatase have allowed us to point out that human mitochondrial membranes contain large amounts of endogenous metal ions, especially zinc, which act as substrate for ferrochelatase and have to be taken into account in determining ferrochelatase activity.

MATERIALS AND METHODS

Preparation of Liver Mitochondria—Human liver was obtained from the laboratories and Research Department, Westchester County Medical Center, Valhalla, NY, by autopsy a few hours after death. Part of the liver was sliced and homogenized for 3 min in a Waring blender in 5 volumes of 10 mM Tris-HCl buffer, pH 7.6, containing 0.25 M sucrose. The cell-free extract was centrifuged 10 min at 500 × g to remove unbroken cells and large debris. The supernatant (liver mitochondria) was thawed, resuspended in 0.1 M Tris/HCl buffer, pH 7.6, without sucrose, sonicated for 1 min (Arteck Systems Corp. Sonic 300 dismembranator Micropat at 35% of maximum power), and then centrifuged for 90 min at 30,000 × g. The pellet of mitochondrial membranes was resuspended in 0.1 M Tris-HCl buffer, pH 7.6, at a concentration of 20 mg of protein/ml.

Ferrochelatase Assay—Ferrochelatase activity was measured under anaerobic conditions according to Camadro and Labbe (8) with slight modification allowing fluorometric detection of protoporphyrin and zinc-protoporphyrin. The ferrochelatase activity was monitored by direct recording the changes of fluorescence intensity in the incubation medium, i.e. (i) the disappearance of protoporphyrin IX emission signal (λex = 410 nm, λem = 634 nm) or (ii) appearance of zinc-protoporphyrin fluorescence (λex = 420 nm, λem = 590 nm). The Perkin-Elmer MFP 3 spectrophotometer was equipped with a Perkin-Elmer R 100 recorder, a red-sensitive photomultiplier tube.

1 P. Labbe, personal communication.
An aerobic system, needed to prevent ferrous iron oxidation, was achieved by enzymatic consumption of the oxygen dissolved in the incubation medium by the glucose/glucose oxidase/catalase system (13). The incubation medium was 0.1 M Tris-HCl buffer, pH 7.6, 50 mM glucose, 10 units of glucose oxidase, 500 units of catalase. The amount of protoporphyrin IX, metal substrate, and enzyme preparation used is described under "Results." The incubations were carried out at 37°C for 5 min in the presence of the chelators before addition of protoporphyrin IX (1 mM glucose, 10 units of glucose oxidase, 500 units of catalase). The incubation medium was 0.1 M Tris-HCl buffer, pH 7.6, containing 1% (w/v) Tween 80. Glucose oxidase grade II (fungal origin, reference 127019) and catalase (bovine liver, reference 106810) were from Boehringer Mannheim. All other chemicals were of analytical grade.

RESULTS

Ferrochelatase activity from human mitochondrial membranes was assayed at physiological temperature (37°C) and optimum pH (7.6). In view of the foregoing evidence (8), the possible interference with ferrochelatase measurement due to possible contaminating endogenous metals was assayed by incubating mitochondrial membranes with protoporphyrin IX and without exogenous metals. The recording of the emission spectrum of the incubation medium as a function of time (Fig. 1) showed a disappearance of protoporphyrin IX concomitant with, but not equal to, appearance of zinc-protoporphyrin, suggesting the chelation of at least two metals, zinc and ferrous iron. This result led us to study the kinetics of ferrochelatase with these endogenous metals as substrates.

The protein dependence of the chelatase activities is shown in Fig. 2. No linear relationship between velocity and amount of enzyme could be found in the range of protein concentration used (12.5-230 µg of protein/assay). In fact, every increase of the amount of enzyme, i.e., of the volume of membrane fraction, led to a simultaneous increase of the metal ion concentrations (related to the total volume of the incubation medium), as these metal ions are entrapped in the membranes.

It was interesting to study the influence of protoporphyrin IX concentration on the velocity of (i) zinc-protoporphyrin synthesis and (ii) the overall chelate reactions, reflected by the measure of protoporphyrin disappearance. Moreover, to determine the influence of the endogenous metals on the determination of the apparent $K_m$ for protoporphyrin, we measured these apparent $K_m$ values at different concentrations of enzyme containing different concentrations of endogenous metals; there was constant ratio between these metal concentrations. The protoporphyrin concentration was varied from 0.1 to 4 µM. An apparent $K_m$ value of 0.5 µM was found for protoporphyrin when zinc-protoporphyrin synthesis was measured (Fig. 3). We found a dependence of the apparent $K_m$ on protein amount when protoporphyrin disappearance was measured ($K_{m,app}$ = 0.37, 0.5, and 2 µM with 0.23, 0.115, and 0.057 mg of protein, respectively; Fig. 4). The same phenomenon was observed when we replotted the difference of velocity between zinc-protoporphyrin synthesis and protoporphyrin utilization (Fig. 5). Moreover, the Lineweaver-Burk plot (1/v versus 1/s) of the overall activity was performed. Figure 2. Protein dependence of ferrochelatase activities without exogenous metals and 1 µM protoporphyrin IX. $A$, disappearance of protoporphyrin ($λ_{EEXC} = 410$ nm, $λ_{EM} = 634$ nm); $B$, appearance of zinc-protoporphyrin ($λ_{EEXC} = 420$ nm, $λ_{EM} = 580$ nm); $C$, plot of the difference of $A - B$ attributed to heme synthesis.

![Figure 1](http://www.jbc.org/) Repetitive scanning of the incubation medium containing 0.23 mg of mitochondrial protein and protoporphyrin IX (1 µM) without exogenous metal ion. The excitation wavelength ($λ_{EEXC}$) was 420 nm allowing maximum fluorescence emission of zinc-protoporphyrin (ZnPP) ($λ_{EM} = 590$ nm) but not of protoporphyrin IX ($λ_{EEXC} = 410$ nm, $λ_{EM} = 634$ nm). Spectra were recorded at 30 s, 5 min, 10 min, and 15 min after addition of protoporphyrin IX to the incubation medium.

![Figure 2](http://www.jbc.org/) Protein dependence of ferrochelatase activities without exogenous metals and 1 µM protoporphyrin IX. $A$, disappearance of protoporphyrin ($λ_{EEXC} = 410$ nm, $λ_{EM} = 634$ nm); $B$, appearance of zinc-protoporphyrin ($λ_{EEXC} = 420$ nm, $λ_{EM} = 580$ nm); $C$, plot of the difference of $A - B$ attributed to heme synthesis.

![Figure 3](http://www.jbc.org/) Double reciprocal plot of the effect of protoporphyrin IX concentration on zinc-chelatase activity measured at different enzyme concentrations. $A_1$, 57.5 µg of protein/assay; $B_1$, 115 µg of protein/assay; $C_1$, 230 µg of protein/assay ($λ_{EEXC} = 420$ nm, $λ_{EM} = 580$ nm).
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Fig. 4. Double reciprocal plot of the effect of protoporphyrin IX concentration on the overall reactions measured at different enzyme concentrations (λ_exc = 410 nm, λ_em = 634 nm). Proteins are (micrograms/assay) A, 57.5; B, 115; and C, 239.

Chelatase reaction deviated significantly from linearity at high concentrations of protoporphyrin IX. The experimental value of the maximum velocity in the present report was found to be 8.7 nmol/mg of protein/h (Fig. 5), which was lower than the estimated value reported by Elder (16) taken from the data of Bonkowsky et al. (17), but is in close agreement with the value reported for bovine liver mitochondrial membranes (4).

As it is clearly established that ferrochelatase reaction occurs according to a random bi-bi mechanism, it is not absolutely necessary to work with saturating concentrations of the second substrate, the metal ion, to determine the apparent K_m for protoporphyrin IX. However, we studied the effects of exogenous metal ions on these chelatase activities. As shown in Fig. 6, variations of added metal concentrations from 0.25 to 10 μM did not lead to a large increase of the activities, indicating that both endogenous zinc and iron are almost saturating. The maximum velocities reached were 7.5 nmol of protoporphyrin used per h/mg of protein and 2.9 nmol of zinc-protoporphyrin synthetized per h/mg of protein.

Using the method of Kato and Inoue (20), we estimated the concentrations of endogenous iron and zinc to be approxi-
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In the presence of 1 μM protoporphyrin IX, A, αα'-dipyridyl; B, EDTA.

FIG. 9. Effect of metal chelators concentration on zinc-chelatase activity of 0.115 μg of mitochondrial protein/assay in presence of 1 μM protoporphyrin IX. A, αα'-dipyridyl; B, EDTA.

The presence of 1 approximately 0.75 μM each. We plotted our experimental data (shown in Figs. 7 and 8) to try to determine the order of magnitude of the kinetic constants of ferrochelatase for the metal substrates.

Fig. 7 showed the Lineweaver-Burk plot of heme synthesis (Δprotoporphyrin = Δzinc-protoporphyrin) as a function of iron concentration, with two concentrations of enzyme and two concentrations of the competitive inhibitor zinc. Using the values of the intercepts with 1/s axis = 1/(Km(1 + (I/Ki))), and 1/(Km(1 + (2/I/Ki))), we determine $K_{m}^{\text{Fe}} = 0.35$ μM and $K_{m}^{\text{FeZn}} = 0.58$ μM.

On the other hand, using a combination of the Dixon plot of zinc-chelatase inhibition by iron (Fig. 8) (intercept with I axis = Ki(1 + 8/Km)), curve not shown) and Lineweaver-Burk representation of zinc-chelatase activity as a function of zinc concentration (intercept with 1/s axis = 1/(Km(1 + (I/Ki))), we determine $K_{m}^{\text{Zinc}} = 0.082$ μM and $K_{m}^{\text{FeZn}} = 0.123$ μM.

In view of the foregoing evidence, that iron and zinc content play a significant role in chelatase activity, it had to be demonstrated whether the level of zinc-protoporphyrin was a consequence of a reduction in ferrous iron in mitochondrial membrane. To assess this problem, we utilized dipyridyl maneuvers, which lower mitochondrial iron (21) without removing zinc, and EDTA maneuvers, which chelate several metals including zinc and iron. As shown on Fig. 9, 10 μM EDTA completely inhibits zinc-chelatase activity, while addition of αα'-dipyridyl led to an increase of zinc-chelatase activity (maximum increase at 7.5 μM αα'-dipyridyl with 150% of control activity). Higher concentrations of αα'-dipyridyl inhibited zinc-chelatase activity. The enhancement of zinc-protoporphyrin synthesis by αα'-dipyridyl correlated well with a decrease of protoporphyrin utilization (data not shown), indicating a preferential chelation of the other endogenous metal, ferrous iron.

DISCUSSION

Properties of human liver ferrochelatase were assessed in its physiological environment, i.e. the mitochondrial membranes. We have applied the fluorometric method for enzyme assay, which not only allowed for a continuous recording of protoporphyrin fluorescence disappearance due to its enzymatic chelation catalyzed by ferrochelatase, but also allowed detection of side products of the overall reaction, such as zinc-protoporphyrin, without extraction of porphyrin or metalloporphyrin from the incubation medium. In the experimental design, specific consideration was given to the endogenous metals of the mitochondrial membrane. As reported previously for yeast mitochondrial membranes (8), human mitochondrial membranes contain large amounts of endogenous metals that are substrates for ferrochelatase. These metal ions are trapped in the membranes, so every increase of the volume of membrane preparation in the assay leads to an increase of both the enzyme and its substrates. It is clear that this situation provides a nonlinear relationship between the velocity of product formation with the amount of enzyme. A schematic approach of that problem can be shown with a Michaelis enzyme with one substrate; if $v_{0} = E_{0}k_{cat}S_{0}/(K_{m} + S_{0})$ is the velocity of the enzyme for a volume $V_{0}$ of membrane containing $E_{0}$ and $S_{0}$, then with $V_{0} = nV_{0}$, we have $v_{0} = n^{2}E_{0}k_{cat}S_{0}/(K_{m} + nS_{0})$ and $v_{0} \neq n^{2}v_{0}$. It is expected that saturating concentration of substrate $S \gg K_{m}$ will allow $nS_{0} \gg K_{m}$ and then $v_{0} = n^{2}E_{0}k_{cat}S_{0}/(K_{m} + nS_{0}) = n^{2}E_{0}k_{cat}S_{0}/(nS_{0})$ so $v_{0} \approx n^{2}v_{0}$, giving a linear relationship between velocity and protein concentration. Unfortunately, in our case, every volume of membrane contains the enzyme protein and two endogenous metal substrates that have been shown to be present at almost saturating concentrations; moreover, excess of both metal ions leads to an inhibition of ferrochelatase activities (Fig. 6). By estimating the endogenous metal concentrations by the method of Kato and Inoue (20), we have been able to plot our experimental data to determine the kinetic constants of ferrochelatase for its metal substrate. Since the range of the substrate concentration used for the calculation of the kinetic constant is very narrow (Figs. 7 and 8), the graphical analysis of the statistical data for these estimations is rather poor, but a good correlation of these kinetic values was obtained for the different experimental results. The general mechanism of human ferrochelatase regulation by metals is similar to that described for the yeast enzyme (8), i.e. ferrous iron is a more effective inhibitor of zinc incorporation than zinc is on iron incorporation ($K_{m}^{\text{FeZn}} = 0.12$ μM versus $K_{m}^{\text{FeZn}} = 0.58$ μM) in spite of the fact that the $K_{m}^{\text{Zinc}}$ is smaller than the $K_{m}^{\text{Fe}}$ (0.08 versus 0.35 μM).

A theoretical treatment of this complex enzymology has been described (18) without reference to the substrate inhibition (see "Appendix"). A complete description of the kinetic parameters of ferrochelatase should take into account (i) the $K_{m}$ values for protoporphyrin, iron, and zinc, (ii) the $K_{i}$ values of metal substrate competition, and (iii) inhibition by excess of substrates. Other investigators have also reported an inhibition of ferrochelatase by excess of hemin (19). The endogenous metals present in the samples of human tissues have never been taken into account when measuring ferrochelatase. Although the presence of a substantial amount of zinc-protoporphyrin has been reported in pathological situations of red blood cells (9), the mechanism of its synthesis has been ignored. Camadro and Labbe (8) postulated that the ability of human ferrochelatase to synthesize zinc-protoporphyrin could be dependent on depletion of a mitochondrial ferrous iron pool that, in normal conditions, ensures an inhibition of zinc incorporation.

To have more insight into this crucial problem of ferrochelatase activity regulation, we used two approaches. First, we incubated the mitochondria with αα'-dipyridyl, which is described as a specific ferrous iron chelator (21, 22). Second, we measured the enzyme activity in presence of a general chela-
tor; for this purpose, we utilized EDTA that has a broad specificity for divalent or trivalent metal ions. Our results show that lowering mitochondrial iron content by addition of $\alpha\alpha'$-dipyridyl to the incubation medium leads to increase of zinc-chelatase activity (Fig. 9), while EDTA inhibits this activity. It has been demonstrated that $\alpha\alpha'$-dipyridyl is highly lipid soluble (22), while its ferrous iron complex is very water-soluble (22). That means that the chelator penetrates the mitochondrial membrane and chelates ferrous iron, and subsequently metal-chelator complex is released into the aqueous phase of the incubation medium. Although other interpretations are possible, these data confirm our two main assumptions that the endogenous substrates are entrapped in the mitochondrial membranes and that zinc incorporation into protoporphyrin IX is actually inhibited by ferrous iron at the level of the membranes.

Our results must be related to those found in patients with iron deficiency anemia or lead poisoning, where high levels of zinc-protoporphyrin but not protoporphyrin have been found in the red blood cells. The role of human ferrochelatase in zinc-protoporphyrin synthesis demonstrated in this paper suggests that, in both types of patients mentioned, a deficiency in available iron is present. We suggest that, in lead poisoning, there is no direct inhibition of ferrochelatase in vivo but rather inhibition of some step(s) of iron metabolism (transport, accumulation, and/or reduction), which produces the observed increase in zinc-protoporphyrin. Our data are supported by the findings in the inherited disease protoporphyria, where a true defect in ferrochelatase activity has been described (11, 12) with resultant accumulation of protoporphyrin but not zinc-protoporphyrin.

**APPENDIX**

The complete reaction system can be described as follows:

\[
\begin{align*}
EBA & \xrightarrow{k_1} EA \\
EB & \xrightarrow{k_2} AB \\
E & \xrightarrow{k_3} A + B \\
EAB^* & \xrightarrow{k_4} B^*
\end{align*}
\]

where $E =$ ferrochelatase, $A =$ protoporphyrin IX, $AB =$ heme, $B =$ iron (II), $AB^* =$ zinc-protoporphyrin, and $B^* =$ zinc (II). $B$ and $B^*$ can be taken as alternative substrates for ferrochelatase reaction. The velocity equation for the one substrate (or one product) approach is given in Ref. 18 as:

\[
\frac{v}{v} = \frac{1 - \frac{k_1 k_3}{k_2 k_4} + \frac{h_t}{B} (1 + \frac{B^*}{k_2}) + \frac{k_e}{AB (k_1 + B^*)}}{1 + \frac{k_1 k_3}{k_2 k_4} + \frac{h_t}{B} (1 + \frac{B^*}{k_2}) + \frac{k_e}{AB (k_1 + B^*)}}.
\]

This equation does not take into account the inhibition constants by excess of substrates.

**REFERENCES**

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J M Camadro, N G Ibraham and R D Levere


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