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Orientation of Ferrochelatase in Bovine Liver Mitochondria†

Bertille M. Harbin and Harry A. Dailey*

Department of Microbiology, University of Georgia, Athens, Georgia 30602

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ABSTRACT: The orientation of ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1), the terminal enzyme of the heme biosynthetic pathway, was examined in bovine liver mitochondria. The ability of a membrane-impermeable sulfhydryl reagent, 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid, to inactivate ferrochelatase in intact or disrupted mitochondria and mitoplasts was examined. Using succinate dehydrogenase as an internal marker, it was found that ferrochelatase was inactivated only in disrupted mitochondria and mitoplasts, suggesting an internal location for the active site of the enzyme. In addition, antibodies raised against purified ferrochelatase were found to inhibit activity only in disrupted but not in intact mitoplasts. These data demonstrate that in bovine liver mitochondria ferrochelatase is located on the matrix side of the inner mitochondrial membrane. Data obtained with the membrane-impermeable amino reagent isethionyl acetimidate indicate that ferrochelatase physically spans the inner mitochondrial membrane with portions of the protein exposed on both sides of the membrane.

In eukaryotic cells, the enzymes of the heme biosynthetic pathway are distributed between both mitochondrial and cytosolic compartments [see Granick Beale (1978)]. The initial and rate-limiting enzyme in the pathway, δ-aminolevulinate synthase, is located in the mitochondrial matrix. Aminolevulinate is then transported into the cytosol where it is converted first into porphobilinogen, then uroporphyrinogen III, and finally coproporphyrinogen III. The terminal three enzymes responsible for formation of protoheme IX are located in the mitochondria, either associated with or bound to the inner mitochondrial membrane. While the position of coproporphyrinogen oxidase is known to be the cytosolic side of the inner mitochondrial membrane (Elder & Evans, 1978),

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* Address correspondence to this author. He is the recipient of Research Career Development Award AM01038 from NIADDK.

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the orientation of protoporphyrinogen oxidase on the membrane is unknown. At present, there is only a single report on ferrochelatase, the terminal enzyme, suggesting that its active site faces the matrix side of the mitochondrial inner membrane on the basis of latent enzyme activity found with mitoplasts (Jones & Jones, 1969).

The majority of heme produced in mammalian liver cells is required for hemoproteins which are synthesized and/or located extramitochondrially. In rat liver cells, for example, it has been determined that as much as 65% of the heme synthesized is used for the production of microsomal cytochrome P-450 (Kappas et al., 1983). In addition, approximately 15% of the heme produced is utilized for the formation of catalase in the peroxisomes and 8% for cytochrome b_5 in the endoplasmic reticulum. Approximately 6% of the heme synthesized in liver cells is incorporated into the mitochondrial cytochromes, which, with the exception of cytochrome *c* (located in the intermembrane space), are integral proteins of the inner mitochondrial membrane.

Because heme is utilized outside of the mitochondrial matrix, the placement of ferrochelatase inside the mitochondria has significant implications for cellular hemoprotein metabolism. In the present study, we have reexamined the orientation of ferrochelatase by utilizing both chemical modification with impermeant reagents and antibody inhibition.

Materials and Methods

Materials. Fresh beef liver was supplied by Welborn's Abbattoir, Danielsville, GA. The sulfhydryl reagent 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid (DMSD) was purchased from Molecular Probes, Inc. Isethionyl [1-^{14}C]acetimidate was from Amersham, and unlabeled isthionyl acetimidate was from Sigma. Protein A–agarose was also from Sigma. Digitonin and Triton X-100 were purchased from Calbiochem-Behring Corp. Deuteroporphyrin IX dihydrochloride for ferrochelatase assays was from Porphyrin Products, Inc. All other reagents were of the highest purity available.

Isolation of Mitochondria and Mitoplasts. Mitochondria were isolated from bovine liver following the previously described procedure of Guerra (1974). Fresh bovine liver was finely minced and homogenized in approximately 250 mL of cold isolation medium [2.0 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, 70 mM sucrose, 200 mM D-mannitol, and 0.5 mg/mL bovine serum albumin (BSA), pH 7.4] per 125 g of liver by using a Brinkmann Polytron at medium speed for 10 s. Mitochondria were obtained by differential centrifugation and subsequently washed twice with isolation medium. After each wash, any loosely packed material on top of the pellet was removed and discarded along with the supernatant. The mitochondria were then suspended in enough isolation medium to give a concentration of 60 mg/mL protein as determined by the Lowry protein method (Lowry et al., 1951).

Mitoplasts were prepared by treating 10 mL of the washed mitochondria with 0.12 mg of digitonin per mg of mitochondrial protein according to the procedure described by Greenawalt (1974). The mitoplasts were suspended in isolation medium to a concentration of 30 mg of protein/mL.

Mitochondria and mitoplasts were disrupted by sonication using a Heat Systems sonicator at 60 W for 2 min. Also, for some preparations, 0.5% Triton X-100 was added to mitochondria or mitoplasts.

Inhibition of Purified Ferrochelatase by 4,4′-Dimaleimidylstilbene-2,2′-disulfonic Acid. Ferrochelatase was purified from bovine liver mitochondria as previously described (Dailey & Fleming, 1983). The sulfhydryl reagent 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid (DMSD) was freshly prepared at a concentration of 50 mM in dimethyl sulfoxide (Me_2SO). To a 1-mL sample of enzyme in 10 mM tris(hydroxymethyl)aminomethane–acetate (Tris–acetate), pH 8.1, was added 20 μL of DMSD in Me_2SO at 24 °C. At various times, samples (100 μL) were removed, and the reaction was stopped by the addition of 100 μL of 50 mL dithiothreitol (DTT). The samples were then assayed for ferrochelatase activity (Dailey, 1982).

Inhibition of Ferrochelatase in Mitochondria and Mitoplasts by DMSD. Intact, sonicated, and Triton-treated mitochondria (60 mg of protein/mL) in 10 mM Tris–acetate pH 8.1, were reacted with 50 mM DMSD (10 μL of reagent per 200 μL of mitochondria) at 24 °C. After a 10-min incubation, the reaction was quenched in the intact and sonicated mitochondria by the addition of 20 μL of 50 mM dithiothreitol. To remove excess dithiothreitol, which interferes with the succinate dehydrogenase assay, the mitochondria were diluted in 10 mL of isolation medium and centrifuged at 100000*g* for 30 min, and the pellet was suspended in 200 μL of isolation medium. The mitochondria were then assayed for ferrochelatase activity. Succinate dehydrogenase, an enzyme located on the inside surface of the inner mitochondrial membrane, was also assayed as an internal control (Singer, 1974). Since the dilution treatment of the Triton-treated mitochondria would result in loss of the solubilized enzyme, dithiothreitol was not added to these samples. Instead, they were assayed immediately after the 10-min incubation period.

Intact, sonicated, and Triton-treated mitoplasts (30 mg of protein/mL) were reacted with DMSD and assayed for ferrochelatase activity following the same procedure as for the mitochondria.

Inhibition of Purified Ferrochelatase by Antibodies. Antibodies against ferrochelatase were raised in male New Zealand white rabbits as described by Schatz & Chan (1979). Samples (100 μL) of purified enzyme were incubated with increasing amounts of both antisera and preimmune serum (5–50 μL) for 15 min at 24 °C. The samples were then assayed for ferrochelatase activity.

Inhibition of Ferrochelatase in Mitoplasts by Antibodies. Samples (100 μL) of intact or sonicated mitoplasts were incubated with an equal volume of antisera for 30 min at 24 °C. Since the normal ferrochelatase assays contain about 0.2% Triton X-100 to keep the porphyrin substrate soluble, any unreacted antibody must be removed prior to the assay since the Triton in the assay mixture would be sufficient to solubilize the intact mitoplasts. To remove uncomplexes antibodies, the mitoplasts were diluted in 10 mL of isolation medium, centrifuged at 100000*g* for 30 min, and resuspended in 100 μL of isolation medium. The mitoplasts were then assayed for ferrochelatase activity.

Double Labeling of Ferrochelatase with Isethionyl [1-^{14}C]-Acetimidate and DMSD. To remove peripheral membrane proteins that are ionically bound to the outside of the mitoplast membrane, mitoplasts (2 mL) were washed once with 20 mL of 0.5 M NaCl in isolation medium, centrifuged at 10000*g* for 15 min, and resuspended to the original volume in isolation medium. These mitoplasts were divided into two equal fractions, and one fraction was sonicated as described above. To remove soluble matrix proteins, the sonicated mitoplasts were centrifuged at 100000*g* and resuspended in cold isolation medium to the original volume. A fresh solution of 250 mM isethionyl [1-^{14}C]acetimidate (specific activity 58.1 mCi/mmol) neutralized with NaOH was prepared, and immediately

Table I: Inhibition of Ferrochelatase and Succinate Dehydrogenase in Intact, Sonicated, or Triton-Treated Mitochondria by DMSD

	mitochondria								
	intact			sonicated			+Triton		
	activity		inhibition	activity		inhibition	activity		inhibition
enzyme	-DMSD	+DMSD	(%)	-DMSD	+DMSD	(%)	-DMSD	+DMSD	(%)
ferrochelatase[a]	9.5	9.5	0	11.2	4.5	60.4	10.7	5.3	50.7
succinate dehydrogenase[b]	8.53	8.53	0	7.14	0.595	91.7	11.9	0.595	95

[a] Ferrochelatase activity is expressed as nanomoles of deuteroheme per milligram per hour. [b] Succinate dehydrogenase activity is expressed as nanomoles of 2,6-dichlorophenolindophenol (reduced) per milligram per minute.

a 5-μL aliquot of this solution was added to 75 μL of intact or sonicated mitoplasts or to 200 μL of purified ferrochelatase. The solutions were mixed thoroughly and incubated at 24 °C for 30 min before the reaction was stopped by the addition of 10 μL of 1 M ammonium bicarbonate.

Ferrochelatase of intact and sonicated mitoplasts was solubilized by the addition of 1% (v/v) Triton X-100. All three fractions were incubated with 5 μL of 50 mM DMSD at 24 °C for 15 min. This reaction was terminated by the addition of 10 μL of 50 mM dithiothreitol. The remaining membrane fractions were pelletized in a Beckman airfuge at 150000*g* for 10 min and discarded. The supernatants were retained.

Immunoprecipitation of Ferrochelatase. The labeled ferrochelatase in each of the above fractions was immunoprecipitated according to a modification of the procedure described by McAda & Douglas (1983). To each fraction was added 5 μL of ferrochelatase-specific antisera. This mixture was incubated for 30 min at 24 °C with occasional stirring followed by the addition of 25 μL of Staph protein A–agarose. After 30 min of additional incubation, the protein A–antibody–enzyme complex was pelletized by using the airfuge at 10000*g* for 15 s. The supernatant was carefully removed, and the pellet was washed 5 times with 150 μL of buffer containing 1% Triton, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris-HCl, pH 7.5. After the final wash, the supernatant was removed, and 75 μL of buffer containing 2% sodium dodecyl sulfate (SDS), 2 mM EDTA, 0.2% 2-mercaptoethanol, and 100 mM Tris-HCl, pH 7.5, was added to each pellet. The tubes were placed in a Pierce Reacti-Therm heating module at 95 °C for 2 min to release the protein A–agarose. The agarose was then pelletized at 10000*g* for 15 s, and the supernatants were carefully removed and saved for quantification of the labels.

Quantification of ^{14}C and Fluorescent Labels. To determine the amount of enzyme-bound [^{14}C]imidate, a 5-μL sample of each labeled ferrochelatase fraction was counted in Fisher Scinti-Verse cocktail by using a Searle Analytic 92 scintillation counter. To ensure the purity of the immunoprecipitated enzyme, a 40-μL aliquot of each fraction was run on a 10% SDS–polyacrylamide gel. The protein bands were cut off the gel, minced finely with a razor blade, and counted in the ^{14}C channel. Another 5-μL sample of each fraction was diluted to 0.5 mL in 1% Triton buffer and the fluorescence of the bound DMSD measured in a Perkin-Elmer 650-40 fluorescence spectrophotometer.

Results

Inactivation of Purified Ferrochelatase by DMSD. It has previously been shown that a variety of sulfhydryl-specific reagents are capable of inactivating ferrochelatase (Dailey, 1984). In this study, inhibition of ferrochelatase by a membrane-impermeable sulfhydryl reagent, DMSD, was used to localize the enzyme in bovine liver mitochondria. The ability of this compound to rapidly inactivate purified ferrochelatase

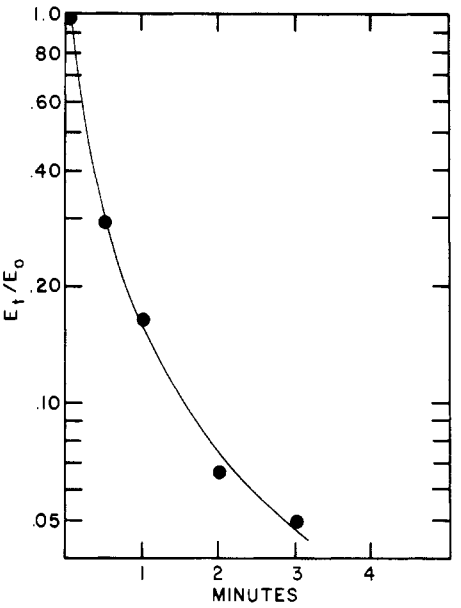

FIGURE 1: Inactivation of bovine ferrochelatase by 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid (DMSD). Purified ferrochelatase (1 μM) was reacted with DMSD as described under Materials and Methods. E_0 is enzyme activity at 0 min, and E_t is enzyme activity at time t.

is shown in Figure 1. The rate of inactivation by this compound is similar to that of other maleimidyl reagents studied previously (Dailey, 1984; Hanson & Dailey, 1984).

Localization of Membrane-Bound Ferrochelatase Using DMSD. The experimental approach utilized to localize the position of the active site of ferrochelatase with respect to the inner mitochondrial membrane was based upon the fact that DMSD, which was shown to inactivate ferrochelatase, cannot cross intact biological membranes (Cabantchik & Rothstein, 1974). Reaction of intact mitochondria or mitoplasts would only inactivate ferrochelatase if its active site is on the exterior cytosolic surface of the inner mitochondrial membrane. Sonicated mitochondria and sonicated mitoplasts will be oriented mainly inside out so that the matrix side of the membrane will be reacted. Triton X-100 treatment solubilizes ferrochelatase so that it will react with the reagent regardless of its original orientation. As an internal control, succinate dehydrogenase activity was monitored in parallel with ferrochelatase. Succinate dehydrogenase is located on the matrix side of the inner mitochondrial membrane and is also inactivated by sulfhydryl reagents (Singer, 1974). Inactivation of ferrochelatase by DMSD in intact mitochondria as compared to that in sonicated or Triton-treated mitochondria was examined. As shown in Table I, it was found that the membrane-impermable reagent is incapable of inhibiting ferrochelatase when reacted with intact mitochondria; however, when the mitochondria are disrupted by sonication or detergent prior to the addition of DMSD, a significant increase in inhibition is seen. These results suggest that the active site of ferrochelatase is situated

Table II: Inhibition of Ferrochelatase in Mitoplasts by DMSD or Antibodies

	mitoplasts								
	intact			sonicated			+Triton		
	activity[a]		inhibition	activity		inhibition	activity		inhibition
treatment	-treatment	+treatment	(%)	-treatment	+treatment	(%)	-treatment	+treatment	(%)
DMSD	4.8	3.4	30	4.5	1.4	69	5.6	1.2	79
antibodies	5.3	3.8	28	4.8	2.9	60	5.6	1.4	75

[a] Ferrochelatase activity is expressed as nanomoles of deuteroheme per milligram per hour.

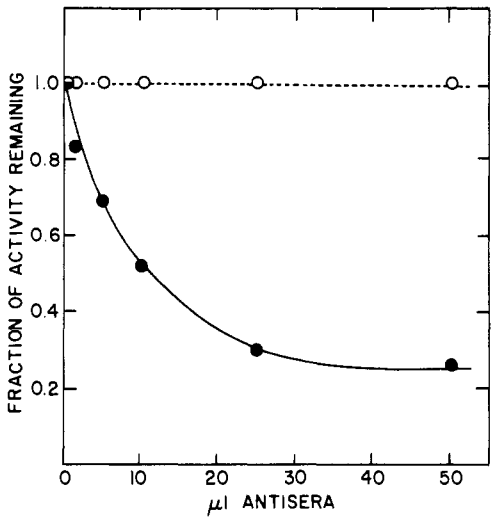

FIGURE 2: Inhibition of bovine ferrochelatase by specific antibodies. Purified ferrochelatase (1 μM) was reacted with antisera as described under Materials and Methods. Immune sera (●); nonimmune sera (○).

in the inner surface of the inner mitochondrial membrane. This is supported by the similarity between the data for ferrochelatase and those for succinate dehydrogenase, an enzyme which is known to be located on the inside of the inner mitochondrial membrane.

The effect of DMSD on intact, sonicated, and Triton-treated mitoplasts was also examined. The results, as shown in Table II, were similar to those for the mitochondria except that some inhibition of the enzyme is seen even before the mitoplasts are sonicated or Triton is added. This inhibition can be attributed to a fraction of the membranes which were broken during the mitoplast preparation procedure. After repeated attempts, we were unable to obtain greater than 70% intact bovine liver mitoplasts using the digitonin method of Greenawalt (1974). Nonetheless, there is a significant increase in the amount of inactivation of ferrochelatase in mitoplasts after sonication or the addition of Triton.

Inhibition of Purified Ferrochelatase by Antibodies. Ferrochelatase-specific antibodies were used to localize the active site of the enzyme in bovine liver mitochondria. The ability of these antibodies to inhibit the activity of purified ferrochelatase is shown in Figure 2. As these data indicate, the maximal inhibition of ferrochelatase by the antibody preparation is approximately 75%. One hundred percent inhibition was never achieved.

Localization of Ferrochelatase in Mitoplasts Using Antibody Inhibition. Since antibodies are too large to penetrate the outer mitochondrial membrane, it was necessary to employ mitoplasts rather than intact mitochondria for the antibody inhibition studies. Inhibition of ferrochelatase by antibodies in intact mitoplasts as compared to that in sonicated mitoplasts was examined. As shown in Table II, inhibition of the enzyme is significantly greater in mitoplasts which have been inverted by sonication. These results are consistent with the chemical modification data above and once again indicate that the active site of ferrochelatase is located on the matrix of the inner mitochondrial membrane. The small amount of inhibition seen in the intact mitoplasts is again attributed to a fraction of the membranes which were broken during the mitoplast preparation procedure.

Table III: Labeling of Ferrochelatase with Isethionyl [1-^{14}C]Acetimidate and DMSD

fraction	^{14}C cpm	RFU[a]	cpm/RFU
intact mitoplasts	21 000	27	777
sonicated mitoplasts	14 000	20	700
purified enzyme	8 400	8	1050

[a] RFU = relative fluorescence units.

Determination of the Transverse Distribution of Ferrochelatase. Isethionyl acetimidate, a membrane-impermeable lysyl group reagent (Whiteley & Berg, 1974), was used to investigate the transverse distribution of ferrochelatase in the inner mitochondrial membrane. To determine whether or not ferrochelatase completely spans the inner membrane, samples of intact or sonicated mitoplasts and purified enzyme were labeled with isethionyl [^{14}C]acetimidate. DMSD (a fluorescence sulfhydryl reagent) was used as an internal reference. The samples were reacted first with the imidate, and after termination of this reaction and solubilization of the membrane fractions with Triton X-100, DMSD was used to label the enzyme in each sample. After immunoprecipitation of ferrochelatase, the ratio of ^{14}C to fluorescence in each fraction represents the relative amount of isethionyl acetimidate bound to the enzyme.

As the results in Table III show, the amount of isethionyl [^{14}C]acetimidate bound to ferrochelatase in intact mitoplasts is similar to that found in sonicated mitoplasts and significantly less that that found in the purified protein sample. Since the entire enzyme was exposed to the reagent in the purified protein sample, it was expected that the maximum amount of labeling would occur in this fraction. The ability of the membrane-impermeable reagent to label the protein in intact mitoplasts as well as mitoplasts which were inverted by sonication indicates that ferrochelatase spans the inner mitochondrial membrane with a portion of the enzyme exposed on both sides of the bilayer.

Discussion

Prior to the present study, Jones & Jones (1969) presented data showing that ferrochelatase activity in rat liver mitochondria was latent and, thus, suggested that ferrochelatase was located on the matrix side of the inner mitochondrial membrane. The localization of ferrochelatase to the inside of the mitochondrion is an interesting feature when one considers that a major portion of the heme produced in the cell is utilized external to the inner mitochondrial membrane. In addition, it requires that both iron and porphyrin substrates be transported across the membrane to get to the active site of the enzyme. Because of the importance of the location of ferrochelatase, and the difficulty in interpreting enzyme la-

tency, we reexamined the orientation of the enzyme using less equivocal methods that were previously unavailable to researchers. Inhibition of enzymes by specific antibodies is a technique commonly used to determine the orientation of enzymes in biological membranes (Schatz & Chan, 1979); only recently has this approach been made possible for ferrochelatase by the availability of the purified protein. We took advantage of this technique, as well as chemical modification by a membrane-impermeable reagent, to reevaluate the topography of ferrochelatase in bovine liver mitochondria.

The data obtained in this study clearly demonstrate that ferrochelatase completely spans the inner mitochondrial membrane and is oriented such that its active site is located on the matrix side of the membrane. The present data place the first enzyme, δ-aminolevulinate (ALA) synthase, and the last enzyme of the pathway inside the mitochondrion with the remainder (except possibly protoporphyrinogen oxidase, whose orientation on the inner mitochondrial membrane is unknown at present) in the cytoplasm or associated with the outside of the inner mitochondrial membrane. It has been suggested that the significance of this orientation of ferrochelatase might possibly be explained with respect to a regulatory role in the cell [see Kappas et al. (1983)]. Placing ferrochelatase in close proximity with ALA synthase, the rate-limiting enzyme of the pathway, may allow for fine control of ALA synthase by heme. Although this mechanism of control seems quite reasonable, recent in vitro studies on the inhibitory effect of heme on ALA synthase make the importance of this regulatory role uncertain (Pirole et al., 1984).

Information on the distribution of ferrochelatase in the inner mitochondrial membrane is important not only in understanding the overall regulation of heme biosynthesis but also in gaining a better understanding of how this enzyme functions with respect to the other two terminal membrane-bound enzymes of the pathway. For example, it would be interesting to discover if these three enzymes carry out their reactions completely independent of each other or whether they are present as a stable complex in the membrane. Because the substates for these enzymes are relatively large, highly reactive, nonpolar compounds, the existence of such a complex would provide an efficient means of processing these compounds by allowing the product of one enzyme to immediately become the substrate for the next without ever having to leave the membrane environment.

Since it has now been established that coproporphyrinogen oxidase is associated with the cytoplasmic side of the inner mitochondrial membrane (Elder & Evans, 1978), and ferrochelatase with the matrix side, the next step to understanding the relationship of these enzymes is to determine the orientation of protoporphyrinogen oxidase in the inner membrane. Current studies in this laboratory are aimed at the purification and localization of bovine liver protoporphyrinogen oxidase as well as further characterization of the three terminal enzymes of the pathway.

Registry No. Ferrochelatase, 9012-93-5.

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