Organization of the Terminal Two Enzymes of the Heme Biosynthetic Pathway

ORIENTATION OF PROTOPORPHYRINOGEN OXIDASE AND EVIDENCE FOR A MEMBRANE COMPLEX*

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Protoporphyrinogen oxidase (EC 1.3.3.4), the penultimate enzyme of the heme biosynthetic pathway, catalyzes the removal of six hydrogens from protoporphyrinogen IX to form protoporphyrin IX. The enzyme in eukaryotes is associated with the inner mitochondrial membrane. In the present study we have examined requirements for solubilization of this enzyme and find that it behaves as an intrinsic membrane protein that is solubilized only with detergents such as sodium cholate. The in situ orientation of the enzyme with respect to the inner mitochondrial membrane places the active site on the cytosolic face of this membrane rather than the matrix side where the active site of ferrochelatase, the terminal pathway enzyme, is located. Examination of the kinetics of the two terminal enzymes in mitochondrial membranes demonstrates that substrate channeling occurs between these terminal two-pathway enzymes. However, examination of solubilized and membrane-reconstituted enzymes shows no evidence for a stable complex. Based upon these and previous data a model for the terminal three-pathway enzymes is presented.

The enzymes of the heme biosynthetic pathway are distributed between the mitochondria and cytosolic space in eukaryotic cells (1). The terminal three steps of the pathway are catalyzed by enzymes that are either bound to or associated with the inner mitochondrial membrane. Protoporphyrinogen oxidase (EC 1.3.3.4) and ferrochelatase (EC 4.99.1.1) are the two terminal enzymes of the heme biosynthetic pathway. Protoporphyrinogen oxidase, the antepenultimate enzyme, catalyzes the oxidation of protoporphyrinogen IX into protoporphyrin IX (2), while ferrochelatase is responsible for the insertion of ferrous iron into the porphyrin macrocycle to form protoheme (3). With the recent purification of mouse protoporphyrinogen oxidase (4), the three terminal enzymes have now been purified from mammalian sources and partially characterized (4-9). Coproporphyrinogen oxidase (EC 1.3.3.3), the antepenultimate pathway enzyme, has been shown to be loosely associated with the outside of the inner mitochondrial membrane (10, 11), and ferrochelatase has been demonstrated to be an intrinsic membrane protein that spans the inner mitochondrial membrane with its active site present on the matrix side of that membrane (12). Protoporphyrinogen oxidase is known to be bound to the inner mitochondrial membrane (13), but the nature of the membrane association and the orientation of the enzyme with respect to the membrane surface are unclear.

Protoporphyrinogen oxidase and ferrochelatase activities are optimized when the enzymes inserted into a phospholipid environment (14) and coproporphyrinogen oxidase activity are stimulated by phospholipid (6). Their substrates are reactive bulky macromolecules and poorly soluble in aqueous solutions, and one would expect that they would not be present free in the hydrophobic milieu of the phospholipid bilayer. It seems reasonable to hypothesize that if the terminal enzymes of the pathway were arranged in a complex, the product of one enzyme could directly be channeled to the next enzyme, without being diluted in the phospholipid matrix. In this study we examined the characteristics of membrane binding and the orientation of protoporphyrinogen oxidase in mouse mitochondria. The possibility for channeling of substrates/product between the two terminal enzymes of the heme biosynthetic pathway was examined.

EXPERIMENTAL PROCEDURES

Materials—Protoporphyrin IX, mesoporphyrin, hematoporphyrin, and bilirubin ditaurine conjugates were purchased from Porphyrin Products, Logan, UT. $^{57}Fe$ was obtained from Amersham Corp. Soybean phosphatidylcholine, sodium cholate, and trypsin were from Sigma. Reactive Blue CL-6B and Sepharose CL-6B were obtained from Pharmacia LKB Biotechnology Inc. Digitonin was from Behring Diagnostics, and all other reagents and chemicals were of the highest purity available commercially. Mouse liver was purchased from either Bio-Trol, Inc., Indianapolis, IN or Pel-Freez Biologicals, Rogers, AR.

Methods—Mouse livers were suspended in a buffer containing 0.25 M sucrose, 10 mM Tris acetate, pH 8.1, 1 mM EDTA, phenylmethylsulfonyl fluoride (10 μg/ml) and homogenized in a glass-Teflon tissue homogenizer. Mitochondria were then isolated by differential centrifugation (15). When necessary, mitoplasts were prepared by the method of Greenewalt (16) and were used immediately. Ferrochelatase was purified from mouse liver mitochondria according to the method described by Dailey and Fleming (7) and Dailey et al. (8). Protoporphyrinogen oxidase was purified by the procedure of Dailey and Karr (4).

Purified ferrochelatase, protoporphyrinogen oxidase, and ferrochelatase with protoporphyrinogen oxidase were reconstituted into soybean phosphatidylcholine vesicles following the method described by Ferreira and Dailey (14).

Ferrochelatase was also assayed using the purine nucleoside phosphorylase procedure as previously described (7, 8). The product concentrations were determined using published extinction coefficients (18). Protoporphyrinogen oxidase was assayed using a modification (4) of the fluorescence assay described by Jacobs and Jacobs (19). Protoporphyrinogen oxidase was prepared just before use by reduction of protoporphyrin IX with sodium amalgam (19). The pH of the protoporphyrinogen stock solution was adjusted to approximately 7 by titration with 0.1 N NaOH.
with a 1.5 M solution of MOPS\(^1\) (free acid). The actual concentration of protoporphyrinogen was determined after photochemical oxidation of the protoporphyrinogen substrate (19).

Total heme production was quantitated using an \(^{57}Fe\) assay (17) with protoporphyrin and/or a combination of protoporphyrinogen with protoporphyrin. The reaction mixtures were identical to those used for the assays described above except that iron was supplied as \(^{57}Fe\) (4 \(\mu\)Ci). At the end of the incubation time 100 \(\mu\)l of 1 M FeSO\(_4\) were added, and the reaction was stopped with 0.5 ml of 2 N HCl. Total heme was extracted into methyl ethyl ketone (0.5 ml). After vortexing and allowing the two phases to separate, an aliquot of 200 \(\mu\)l of the organic phase was withdrawn and counted into a liquid scintillation counter.

In assays where the amount of protoheme formed from protoporphyrinogen and ferrous iron was determined, the assay mixture was identical to that used in protoporphyrinogen oxidase assays except that 100 \(\mu\)M ferrous citrate (4 \(\mu\)Ci of \(^{57}Fe\)) was added. The product was quantitated as radioactive heme formed (8).

**RESULTS**

*Location of Protoporphyrinogen Oxidase—* While it is known that the terminal enzyme of the heme biosynthetic pathway, ferrochelatase, is bound to the inner mitochondrial membrane with its active site facing the matrix side (12), the orientation of protoporphyrinogen oxidase is unknown. Previously its location had been assigned to the inner mitochondrial membrane of rat liver cells based upon subcellular fractionation by centrifugation (20) and its association with isolated mitoplasts (13). Initially we examined the location of this enzyme in mouse mitochondria by formation of mitoplasts via treatment with digitonin. When intact mitoplasts were produced by the method of Greenawalt (16), it was found that both the oxidase and ferrochelatase activities remained with the mitoplast fraction. Since the efficiency of this procedure is dependent upon a variety of experimental variables, a range of concentrations was employed, and ferrochelatase was used as an inner membrane marker. The data shown in Fig. 1 clearly demonstrate that both enzymes exhibit the same sensitivity to solubilization by this detergent.

To discern if protoporphyrinogen oxidase was a peripheral or intrinsic membrane protein, attempts were made to solubilize the enzyme with high salt washes (1 M KCl) and with low salt washes (10 mM Tris acetate) in the presence of up to 10 mM EDTA. Under neither condition was any enzyme solubilized from isolated membranes. Solubilization by sodium cholate followed the same pattern found with ferrochelatase (Fig. 2). Other detergents, such as Triton X-100, which are capable of solubilizing ferrochelatase will also solubilize the oxidase. Triton X-114 has been used in the past to determine the "hydrophobicity" of membrane versus soluble proteins (21), but we were unable to utilize this procedure since this detergent under the conditions employed for this procedure destroyed the activity of the enzymes. However, from the inability to solubilize the enzyme with salt washes or dialysis against solutions containing EDTA and the requirement for detergents to solubilize the enzyme, it is apparent that protoporphyrinogen oxidase can be categorized as an intrinsic membrane protein.

*Orientation of Protoporphyrinogen Oxidase in the Inner Mitochondrial Membrane—* Since it has not yet been possible to produce antibodies against protoporphyrinogen oxidase that inhibit the enzyme's activity and because the only effective chemical inhibitors of the enzyme that we have found are membrane-permeable,\(^2\) the only valid approach to determine the membrane orientation of the active site of the enzyme was to use a membrane-impermeant inhibitor of the enzyme's activity. The inhibitor used was the ditarurle conjugate of bilirubin. This compound and unconjugated bilirubin are competitive inhibitors of the oxidase with \(K_i\) values of about 25 \(\mu\)M.\(^3\) Since this compound is small enough to pass through the outer mitochondrial membrane it was possible to use intact mitochondria for these experiments. To check the

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\(^1\)The abbreviation used is: MOPS, 3-(N-morpholino)propanesulfonic acid.

\(^2\)G. C. Ferreira, T. L. Andrew, S. W. Karr, and H. A. Dailey, unpublished data.

\(^3\)G. C. Ferreira and H. A. Dailey, manuscript in preparation.
integrity of the mitochondria the inability of dimaleimidyl-stilbene disulfonic acid, a membrane-impermeant sulffhydryl reagent, to inactivate ferrochelatase (12, 22) was monitored. The data (Fig. 3) show that the oxidase activity was inhibited in intact mitochondria as well as in sonicated mitochondrial fractions by low concentrations of diatine bilirubin indicating that the enzymes' active sites must face the cytosolic side of the inner mitochondrial membrane.

Incubation of isolated intact mitoplasts with 20 μg/ml trypsin for 30 min at 25 °C resulted in the loss of 88% of the oxidase activity.

Evidence for Substrate Channeling between Protoporphyrinogen Oxidase and Ferrochelatase—Usually for the determination of enzyme activities and kinetic parameters relatively high concentrations of substrate are used. However, we reasoned that because of their poor solubility and high chemical reactivity, intracellular porphyrin(ogen) concentrations should be low, and for these same reasons it would not be unexpected to find some sort of substrate channeling between the terminal membrane-bound enzymes of this pathway. As a first approach to this question we examined the conversion by mitochondrial membranes of protoporphyrinogen to protoporphyrinogen as varied from about 1 to 10 μM.

It was proposed that if channeling occurs between the two terminal enzymes in intact mitochondria, then exogenously supplied protoporphyrin may not compete well with endogenously produced porphyrin. It was possible to test this hypothesis since ferrochelatase activity is in excess over oxidase activity (when assayed in vitro) in mitochondria, and, thus, in the absence of channeling one would expect to see protoporphyrin production dependent upon the concentration of both endogenously produced and exogenously supplied protoporphyrin. Specifically the following experiments were conducted: 1) protoporphyrinogen and ferrous iron (or protoporphyrinogen alone) were added as substrates, and protoporphyrin (or protoporphyrinogen) production was followed in assays containing either cholate-solubilized enzymes or isolated mitochondrial membranes to determine the level of individual enzyme activity; 2) protoporphyrinogen and ferrous iron were added to solubilized enzymes or mitochondrial membrane fractions, and protoporphyrin production was measured; 3) protoporphyrinogen, ferrous iron, and protoporphyrin were added to assays containing either soluble or membrane-bound enzyme, and protoporphyrin was measured. In all of these experiments ranges of porphyrin(ogen)s were examined, and the ratio of porphyrin to porphyrinogen was varied.

If there is no substrate channeling, at a set concentration of protoporphyrinogen, exogenous protoporphyrin should result in increased protoporphyrin production, and increasing the amount of protoporphyrin should yield increasing amounts of protoporphyrin. Thus, a plot of protoporphyrin produced versus the ratio of protoporphyrin to protoporphyrinogen should, in this case, show a line with a positive slope. If, however, there is substrate channeling, then one would expect to obtain a line with a slope of zero. In the present experiment the concentration of both exogenously supplied porphyrin and porphyrinogen was varied to eliminate possible system artifacts that might occur if only a single concentration were examined. Thus, the enzyme activity is expressed as a ratio of protoporphyrin produced in the presence of endogenously produced and exogenously supplied protoporphyrin divided by the protoporphyrin produced from protoporphyrinogen alone. Under these conditions channeling would be expected to yield a line with a slope of zero that has an activity ratio of about one. The data for this experiment (Fig. 4) clearly show that at low (1-10 μM) porphyrin concentrations there is no increase in proto-

![Fig. 3. Inhibition of protoporphyrinogen oxidase by diatine bilirubin.](image)

![Fig. 4. Production of protoporphyrin from protoporphyrinogen.](image)
heme production upon the addition of exogenous protoporphyrin. At high (100 μM) porphyrin concentrations, however, one does find a significant increase in product formed. When solubilized enzymes are used with these same substrate concentrations there is no evidence for substrate channeling, thus suggesting that solubilization may either break apart any stable bifunctional enzyme complex or dilute out the separate enzymes to such an extent that the probability of efficient protein-protein interactions occurring is greatly diminished.

Interactions between Ferrochelatase and Protoporphyrinogen Oxidase—Since the kinetic data shown above are strongly suggestive of some type of protein-protein interactions involving ferrochelatase and protoporphyrinogen oxidase, other approaches to this question were tried. The first approach was to examine the kinetics of protoheme formation in an in vitro reconstituted phospholipid vesicle system that contained both purified enzymes (14). When this reconstituted vesicle system was examined in the same fashion as described above for isolated mitochondrial membranes, the data (Fig. 4, open circles) showed no evidence for substrate channeling. When soluble purified enzymes were assayed under the same conditions, they gave data identical (within experimental limits) to those obtained with the reconstituted system.

The second approach was to try selective solubilization and reconstitution. It is known that both ferrochelatase and protoporphyrinogen oxidase are solubilized by 1% sodium cholate during their respective purifications (4, 7). If a stable complex exists, then solubilization at low concentrations of detergent may release a complex such as is found for components of the electron transport chain. The data show that both enzymes are solubilized at 0.4% sodium cholate in the presence of 0.1 M KCl (Fig. 2). These solubilized preparations do not efficiently convert protoporphyrinogen and iron into protoheme. If this solubilized preparation is dialyzed overnight against 10 mM Tris MOPS, 0.5 mM dithiothreitol, 10 μg/ml phenylmethylsulfonyl fluoride, and centrifuged to pellet the reformed membrane vesicles (sodium cholate solubilizes both protein and phospholipid), it is found that over 90% of the ferrochelatase is in the vesicle fraction, but less than half of the protoporphyrinogen oxidase is pelleted. Addition of either 10 mM EDTA or 1.0 M KC1 to the dialysis buffer did not have a significant effect on the efficiency of reconstitution of the oxidase. The reformed vesicles which did contain both of the enzyme activities did not, however, efficiently catalyze the formation of protoheme from porphyrinogen and ferrous iron. Addition of soybean phospholipids to the solubilized preparations (as described in Ref. 14) results in the formation of a larger vesicle pellet upon centrifugation but does not significantly alter these results. When (NH4)2SO4 or Mg2+ was included there was no change. Inclusion of Ca2+ in the dialysis buffer resulted in an increase recovery of enzyme activity in the membrane pellet, but this was apparently due to precipitation or aggregation stimulated by Ca2+ since subsequent dialysis against buffer with 2 mM EDTA resolubilized some activity.

We are unable to study the possibility of complex formation by the use of chemical cross-linking (23) since the enzymes are present at levels that are too low to individually detect on polyacrylamide gel electrophoresis and the use of Western blot analysis yielded equivocal results due to the low immunogenicity of both proteins.

DISCUSSION

The terminal three enzymes of the heme biosynthetic pathway are associated with the inner mitochondrial membrane (5, 11–13). Previously it has been demonstrated that coproporphyrinogen oxidase is associated with the cytosolic side of the membrane (10, 11) while ferrochelatase has its active site present on the matrix side (12). From the initial work of Poulson's laboratory (20) it had been assumed protoporphyrinogen oxidase was an intrinsic membrane protein; however, the actual data presented for the rat mitochondrial enzyme only demonstrated that detergent and sonication released the enzyme from intact mitochondria (2). Deybach et al. (13) clearly demonstrated that in the rat the enzyme is associated with the inner mitochondrial membrane when they found protoporphyrinogen oxidase activity was associated with isolated rat mitoplast membranes. While they reported that sonication did not release the enzyme, they did not characterize the interaction of the protein and membrane. Ample evidence exists to show that coproporphyrinogen oxidase is loosely associated with the mitochondrial membrane (10, 11) and that ferrochelatase is an intrinsic membrane protein that spans the inner mitochondrial membrane (12). Above we have provided evidence that this enzyme is firmly associated with the inner mitochondrial membrane and that it requires detergents to solubilize the enzyme from isolated membranes. Salt washes or extensive dialysis do not release the enzyme. Indeed, the enzyme behaves in a fashion similar to ferrochelatase when it comes to detergent solubilization.

The only evidence previously available concerning the orientation of protoporphyrinogen oxidase was from proteolysis studies which indicated that some of the activity of protoporphyrinogen oxidase in intact and sonicated mitoplasts could be destroyed by digestion with trypsin (13). Active site antibodies are not available, and the only known chemical modification reagents that strongly affect the enzyme's activity are membrane-permeable, so a water-soluble competitive inhibitor was used with intact mitochondria. The data presented herein clearly show that the activity of protoporphyrinogen oxidase is effectively inhibited by a membrane-impermeant compound which is consistent with an orientation of the enzyme with its active site facing the cytosolic compartment.

In the present paper we have looked for evidence for or against the presence of some sort of protein-protein interaction between the terminal two enzymes. When examining the terminal two reactions two possible models may be envisioned (Scheme I). In the first of these there is no interaction and protoporphyrin, the product/substrate, is "free" in solution. In the second there is some sort of protein-protein interaction between the two enzymes. Another possibility that might also

![Scheme I. Possible models for interactions of the terminal two enzymes.](image-url)
be considered is the presence of an unidentified intermediate carrier between the enzymes. While there is no experimental evidence for such a component, there is also no data available to rule out such a possibility.

The kinetic data obtained from solubilized mitochondrial membrane-bound and vesicle-reconstituted enzymes suggest that in situ on the mitochondrial membrane substrate channeling between the two terminal enzymes does occur. The fact that this channeling is readily destroyed by solubilization procedures and is not regained upon reformation of membrane vesicles with the solubilized enzymes suggests that a complex of ferrochelatase and protoporphyrinogen oxidase may exist in the membrane milieu but that this complex is not a stable one.

Certainly the idea of an enzyme complex in biosynthetic pathways is not unique given the large number of examples now available (see Refs. 24 and 25). In the present case the possibility of a complex is an attractive one because such a complex with substrate channeling would seem optimal for the cell. Channeling would overcome the need for accumulation of free porphyrin in the cell. Due to the high chemical reactivity of both the porphyrin macrocycle and vinyl group, the accumulation of micromolar concentrations of free protoporphyrinogen oxidase would not be ideal for the cell.

The presence of a complex may also help to explain the variety of observations reported in variegate porphyria. Some controversy exists over whether only protoporphyrinogen oxidase alone (26, 27) or it and ferrochelatase both (28-30) are affected in this disorder. Since the disease is dominantly inherited it would be unusual to have two separate enzymes affected. If, however, a single mutation on the oxidase not only decreased its activity but also affected the interaction with ferrochelatase, then one may expect to see some effect on heme synthase activity in membrane fractions. With the controversy exists over whether only protoporphyrinogen oxidase alone (26, 27) or it and ferrochelatase both (28-30) are involved with this aspect. Current approaches to this problem have been slowed by the low levels of the heme synthetic pathway enzymes and by the propensity of protoporphyrin to spontaneously intercalate into phospholipid bilayers.

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**SCHEME II. A proposed model for the terminal three membrane-associated enzymes of the heme biosynthetic pathway.**

CPO, coproporphyrinogen oxidase; PPO, protoporphyrinogen oxidase; FC, ferrochelatase; Copro'gen, coproporphyrinogen; OM, outer mitochondrial membrane; IM, inner mitochondrial membrane.