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Characteristics of murine protoporphyrinogen oxidase

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Abstract

Protoporphyrinogen oxidase (EC 1.3.3.4) (PPO) is the penultimate enzyme of the heme biosynthetic pathway. Mouse PPO has been purified in low yield and kinetically characterized by this laboratory previously. A new more rapid purification procedure is described herein, and with this protein we detect a noncovalently bound flavin moiety. This flavin is present at approximately stoichiometric amounts in the purified enzyme and has been identified by its fluorescence spectrum and high performance liquid chromatography as flavin mononucleotide (FMN). Fluorescence quenching studies on the flavin yielded a Stern-Volmer quenching constant of 12.08 M^{-1} for iodide and 1.1 M^{-1} for acrylamide. Quenching of enzyme tryptophan fluorescence resulted in quenching constants of 6 M^{-1} and 10 M^{-1} for iodide and acrylamide, respectively. Plasma scans performed on purified enzyme preparations did not reveal the presence of stoichiometric amounts of protein-bound metal ions, and we were unable to detect any protein-associated pyrroloquinoline quinone (PQQ). Data from circular dichroism studies predict a secondary structure of the native protein consisting of 30.5% alpha helix, 40.5% beta sheet, 13.7% turn, and 15.3% random coil. Denaturation of PPO with urea resulted in a biphasic curve when ellipticity is plotted against urea concentration, typical of amphipathic proteins.

Keywords: circular dichroism; FMN; protoporphyrin; protoporphyrinogen oxidase

Protoporphyrinogen oxidase (EC 1.3.3.4) (PPO) is the penultimate enzyme of the heme biosynthetic pathway. It is an integral membrane protein of the inner mitochondrial membrane that catalyzes the removal of six electrons from protoporphyrinogen IX to form protoporphyrin IX (Poulson & Polglase, 1975; Poulson, 1976; Deybach et al., 1985). Both the mouse and the bovine enzymes have been purified to apparent homogeneity and are single subunit proteins with molecular weights of approximately 65,000 (Dailey & Karr, 1987; Siepker et al., 1987). The mouse enzyme has been kinetically characterized and reconstituted into phospholipid vesicles (Ferreira & Dailey, 1988; Ferreira et al., 1988). In addition, a variety of potential electron acceptors have been examined for their ability to stimulate or inhibit PPO activity. Of all compounds tested with the purified mouse enzyme only oxygen stimulated activity. Oxygen is known to be the terminal electron acceptor for the reaction catalyzed by protoporphyrinogen oxidase in eukaryotic animal cells, and H_2O_2 is believed to be one of the reaction products rather than water (Poulson, 1976; Ferreira & Dailey, 1988; Dailey, 1990). Whereas coupling between the electron transport chain and the protoporphyrinogen oxidizing system has been proposed to function in prokaryotes, no evidence for such has been found in eukaryotes (Jacobs & Jacobs, 1975, 1976; Klemm & Barton, 1985, 1989; Ferreira & Dailey, 1988).

Siepker et al. (1987) reported the identification of flavin adenine dinucleotide (FAD) in their preparations of the purified bovine enzyme although they did not report a stoichiometry for their enzyme preparation. The FAD was reported to have the unusual property of remaining in the reduced state in the absence of substrate and it was tightly bound to the enzyme and required harsh conditions to extract. In the current study we have developed a more rapid purification scheme for the enzyme from mouse liver, which yields higher amounts of the protein than the previously published procedure (Dailey & Karr, 1987). With enzyme from these preparations we have been able to identify a flavin moiety in the purified enzyme. However, unequivocal data in support of any additional electron acceptors such as metals or pyrroloquinoline quinone (PQQ) were not obtained. Data from circular di-

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chroism and denaturation studies show that the enzyme has a biphasic denaturation curve suggestive of an amphipathic protein.

Results

Purification of protoporphyrinogen oxidase

The purification scheme for mouse PPO is detailed below and in Table 1. This scheme is shorter and quicker than earlier protocols (Dailey & Karr, 1987) and works for mouse liver but not for human or pig. It was found that column geometry was important in eluting PPO free from contaminating proteins. A longer column resulted in poor recovery spread over many fractions, and a short column did not eliminate all contaminants. The fractions containing PPO activity coincide with the fractions containing absorbance at 450 nm (due to the presence of flavin). The condition of the frozen mouse livers was another critical factor in the recovery of the active enzyme. Livers with any freezer burn or those more than 1 week old (after isolation from the mice) resulted in low yields of mitochondria and no recovery of PPO. Livers from newly sacrificed mice gave excellent results.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single band with a molecular weight of 65,000 (Fig. 1). The amino acid composition of mouse PPO is shown in Table 2. There are no unusual or distinctive characteristics obvious from the composition data. The ultraviolet/visible spectrum of purified PPO is shown in Figure 2. It has a broad absorbance band in the 400–500-nm region attributable to the presence of a chromophore. The spectrum is suggestive of a flavin moiety but lacks the expected troughs at 300–320 and 400–420 nm. Because this might be attributable to the presence of some residual reduced flavin, attempts were made to completely oxidize the enzyme preparation by bubbling oxygen gas through the enzyme solution. This had no effect on the spectrum. Despite a relatively exhaustive search for any additional chromophoric cofactors or colored contaminants that may be present in the purified enzyme as it elutes from the column, no additional moieties could be identified. However, because the fluorescence and high performance liquid chromatography (HPLC) data (below) were characteristic of FMN, we feel confident in

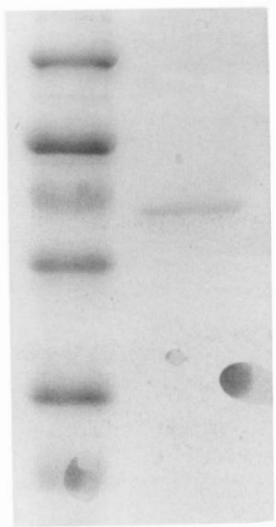

Fig. 1. Polyacrylamide gel electrophoresis of purified mouse protoporphyrinogen oxidase. Molecular weight standards are shown to the left and 1.3 μg of purified enzyme is to the right. The standards are myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor. Acrylamide content of the gel was 10%, and the proteins were visualized by staining with Fast Stain purchased from Zoion Research, Allston, Massachusetts.

Table 1. *Purification of mouse protoporphyrinogen oxidase*[a]

Fraction	Volume (mL)	Protein (mg/mL)	Enzyme activity[b]
Sonicated mitochondria	124	45	1.0
Solubilized enzyme	60	14	2.4
$(NH_4)_2SO_4$ (35–55%) fraction	35	7.2	6.5
Phenyl-Sepharose	20	0.2	1,980

[a] Details of the purification are in the text.
[b] Enzyme activity is expressed as relative fluorescence units mg^{-1} min^{-1}.

Table 2. *Amino acid composition of murine protoporphyrinogen oxidase*

Amino acid	No. residues/mol PPO[a]
Aspartic acid[b]	94
Glutamic acid[b]	82
Serine	43
Glycine	93
Histidine	23
Arginine	27
Threonine	29
Alanine	52
Proline	26
Tyrosine	11
Valine	29
Isoleucine	20
Leucine	42
Phenylalanine	15
Lysine	30
Tryptophan[c]	10

[a] The number of residues per mole is based upon a molecular weight of 65,000 and is rounded off to the nearest whole integer.
[b] Aspartic and glutamic acids represent the combination of both free acids and the corresponding aminated acids, since this composition was obtained from an acid hydrolysis of the protein.
[c] Tryptophan concentration was determined spectrally.

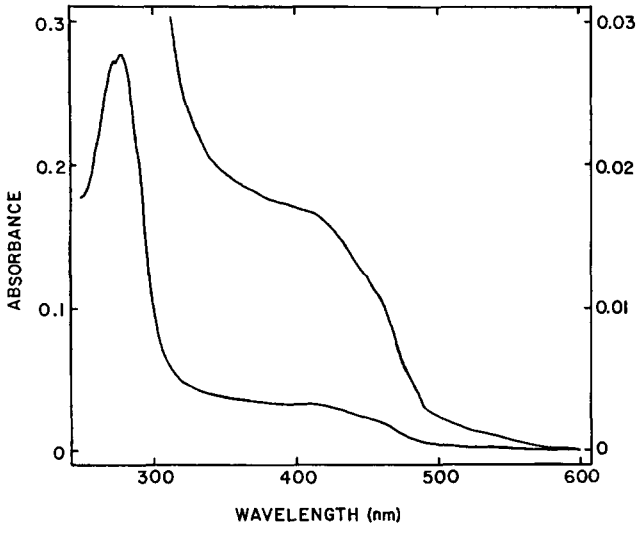

Fig. 2. Absorption spectrum of purified mouse PPO. The cuvette that contained the blank held 20 mM Tris-MOPS, pH 6.9, 0.5% sodium cholate, 0.5% Brij 35. The sample held the above buffer and 0.19 mg of purified mouse PPO. The absorbance scale on the left-hand side of the figure corresponds to the lower spectrum and that on the right-hand side corresponds to the upper spectrum.

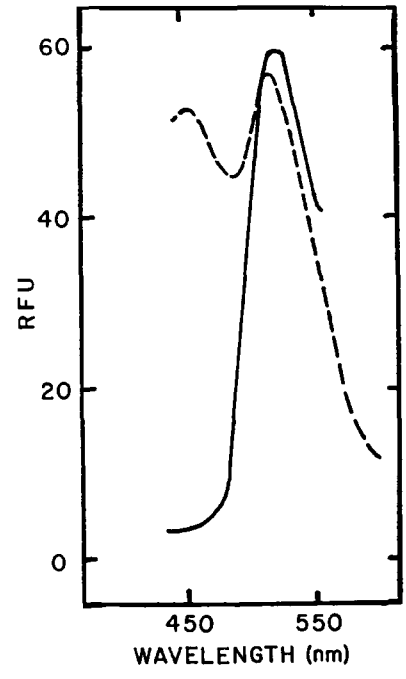

Fig. 3. Fluorescence emission spectrum of PPO. ------, Native PPO emission spectrum (λ_{ex} 377 nm), emission maxima at 450 and 516 nm; ———, fluorescence emission spectrum of digested PPO (λ_{ex} 377 nm), emission maximum at 526. FMN standard showed the same emission spectrum as digested PPO.

this assignment. Using a millimolar extinction coefficient of 83 at 278 nm for PPO and a value of 10.6 for FMN (Kozoil, 1971), the average molar ratio of FMN to PPO for three preparations was calculated to be 1.1.

Identification of flavin in PPO

The fluorescence emission spectrum of native PPO had a maximum at 516 nm when excited at 377 nm. When the protein is extensively digested with trypsin to release the chromophore the emission maximum is shifted to 526 nm, the same as an FMN standard emission maximum (Fig. 3). This shift upon digestion of the protein is typical of protein-bound fluorophores when they become more exposed to the aqueous solvent (Lakowicz, 1983). There is no change in the fluorescence emission maximum of the native or the denatured protein upon saturation of the solutions with oxygen, suggesting that the chromophore already exists in the oxidized state in the purified protein. The excitation spectra of native PPO, denatured PPO, O_2-saturated PPO, and FMN standard solution are all the same. HPLC was used to identify the chromophore in PPO as FMN. The chromatograms resulting from these studies are depicted in Figure 4. FAD standard solution was eluted with a retention time of 5 min, FMN at 7.8 min (Fig. 4A). When the chromophore released from PPO was injected, a peak occurred at a retention time of 7.8 min (Fig. 4B). Figure 4C shows the single peak resulting from the coinjection of the chromophore released from PPO and FMN standard. That the two samples when coinjected eluted as a single peak strongly implicates FMN as the chromophoric factor in PPO.

PQQ content of PPO

PPO was treated as described by van der Meer et al. (1987) to determine if the cofactor PQQ was present in the enzyme. We were unable to detect any protein-associated PQQ under conditions that clearly show 1 mol PQQ/1 mol PPO (data not shown). We were also unable to detect any quinoid cofactors associated with PPO by the nitrocellulose-bound assay of Paz et al. (1991) as shown in Figure 5. In Figure 5A, only bovine serum albumin (BSA) covalently modified with hematoxylin, which is known to carry out redox-cycling (Gallop et al., 1990), stained with nitroblue tetrazolium (NBT)/glycinate. In Figure 5B PPO, BSA, and BSA–hematoxylin all stained with amido black, which stains any protein.

Metal content of PPO

Plasma scans were performed on four separate preparations of purified PPO to determine if there were any metals present at stoichiometric amounts. A series of metals commonly found in metal-containing proteins (Mo, Mg, Mn, Ni, Co, Fe, Zn, Ca, Cu) were tested for, and none were found to any significant extent in purified enzyme preparations (data not shown).

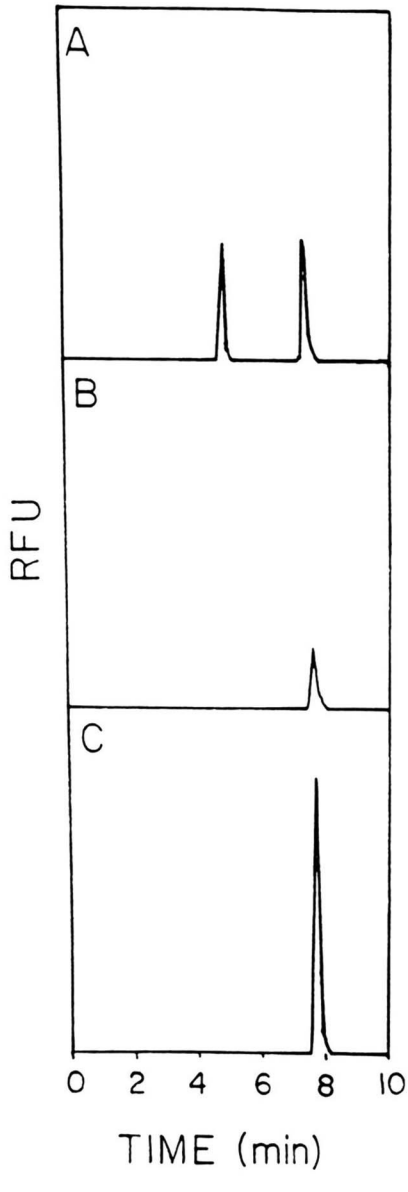

Fig. 4. HPLC chromatogram of FMN, FAD, and digested PPO. **A**: FAD and FMN standards: 100 μL of FAD and FMN (10^{-7} M) dissolved in 20 mM Tris-MOPS, pH 6.9, 0.5% sodium cholate, and 0.5% Brij 35 injected. **B**: 200 μg of PPO after digestion with trypsin to release the flavin. **C**: 200 μg of digested PPO and 100 μL 10^{-7} M FMN coinjected. All samples were filtered through a 2-μm filter prior to injection.

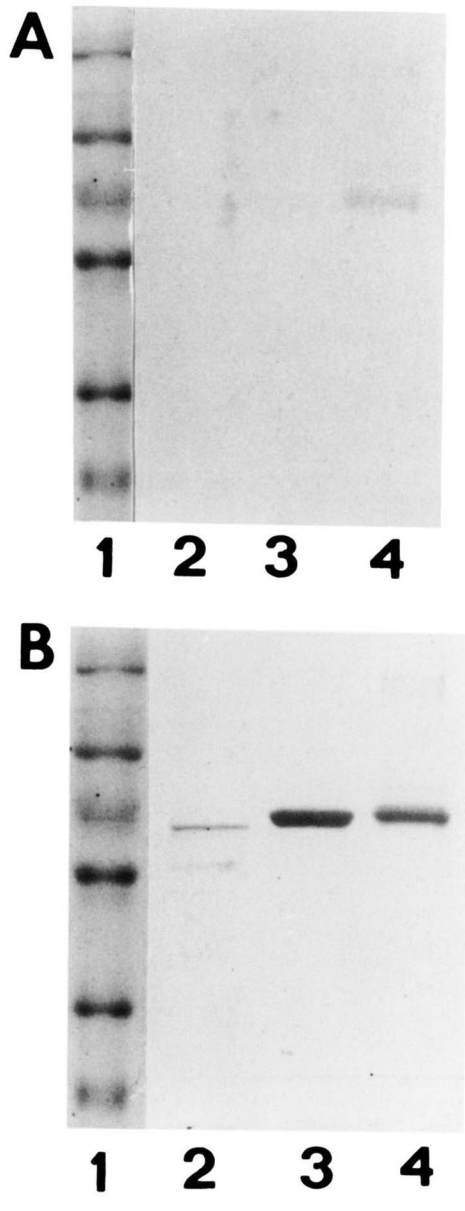

Fig. 5. NBT/glycinate staining of quinoprotein(s) on nitrocellulose. **A**: Nitrocellulose blot stained with NBT/glycinate. Lane 1, prestained molecular weight standards—myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor; lane 2, purified PPO; lane 3, BSA; lane 4, BSA-hematoxylin. **B**: Nitrocellulose stained with amido black. Lane 1, molecular weight standards; lane 2, purified PPO; lane 3, BSA; lane 4, BSA-hematoxylin.

Quenching of tryptophan and FMN fluorescence in PPO

KI and acrylamide were used as fluorescence quenching compounds in an attempt to probe the environment surrounding the cofactor and enzyme tryptophanyl residue(s) in the native protein. Both compounds are highly effectively contact quenchers with quenching efficiencies of 1.0 (Eftink & Ghiron, 1981). Their differing charge characteristics, and hence their differing abilities to penetrate the internal structure of the protein, allow one to make qualitative estimates about the environment of a protein-bound fluorophore.

The quenching of the FMN fluorescence in PPO by both KI and acrylamide resulted in the linear Stern-Volmer plots shown in Figure 6. A linear Stern-Volmer plot is indicative of a single class of fluorophores all equally accessible to the quencher (Lakowicz, 1983). The Stern-Volmer quenching constant ($K_{S\text{-}V}$) for acrylamide quenching of FMN was 1.1 M^{-1} and for KI the value was 12.08 M^{-1}. The inverse of these values, 0.91 M and

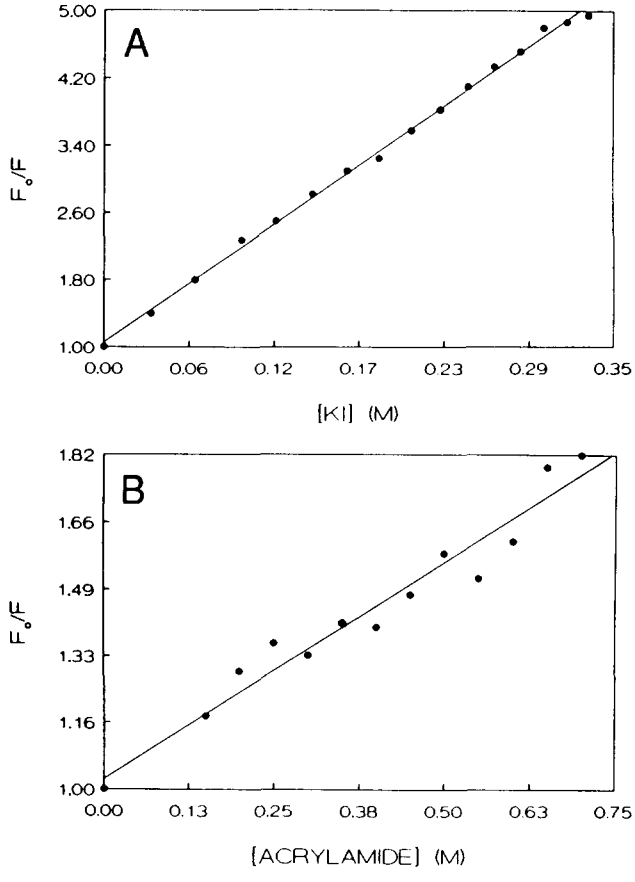

Fig. 6. Quenching of protein FMN fluorescence. **A**: Quenching by KI. **B**: Quenching by acrylamide. Concentrated stock solutions of the quenchers were added to protein in the cuvette, mixed, and the fluorescence measured. There was no decrease in fluorescence due to dilution of the protein as tested by addition of 20 mM Tris-MOPS.

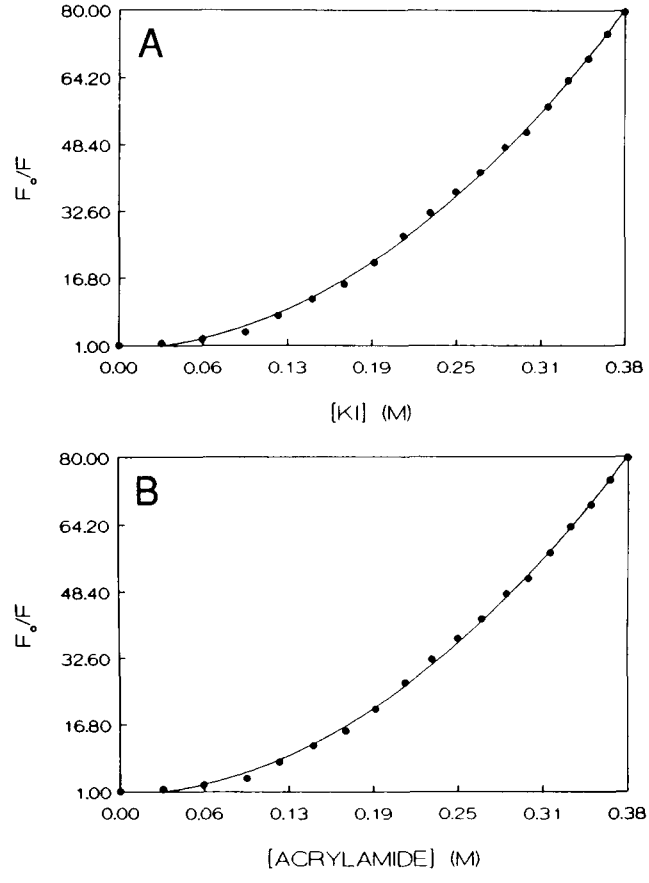

Fig. 7. Quenching of protein tryptophan fluorescence. **A**: Quenching of KI. **B**: Quenching by acrylamide.

0.08 M, respectively, are those concentrations of the quenchers at which 50% of the total fluorescence was quenched. The finding that KI was better able to quench FMN fluorescence than acrylamide suggests that the protein-bound flavin is exposed on the surface of the protein and that it may exist in a positively charged environment, thereby attracting the negatively charged iodide ion to it.

Quenching of protein tryptophan fluorescence by both acrylamide and iodide yielded upward sloping Stern-Volmer plots (Fig. 7). Such plots are indicative of quenching occurring by both static and dynamic mechanisms both of which require molecular contact between the fluorophore and the quencher. The $K_{S\text{-}V}$ for the quenching of tryptophan fluorescence by acrylamide was 10 M^{-1} and that for KI was 6 M^{-1}. Their inverses were 0.10 and 0.17 M, respectively. Because acrylamide was able to cause 50% quenching at about one-half the concentration required for KI to have the same effect, it is possible that tryptophan in PPO may be buried in the structure of the protein where it is more accessible to the uncharged acrylamide molecule than to the iodide ion.

Circular dichroism characteristics of PPO

Circular dichroism spectra of purified, native PPO yielded the following estimations of secondary structure: 30.5% alpha helix, 40.5% beta sheet, 13.7% turn structure, and 15.3% random structure (Fig. 8).

When the purified protein was denatured with urea and the percent ellipticity at 230 nm remaining after denaturation was plotted against the concentration of urea, the biphasic curve shown in Figure 9 was obtained. At a urea concentration of 3 M the calculated secondary structure was 23.9% alpha helix, 42.1% beta sheet, 14.5% turn, and 19.5% random coil. At 6 M urea these values were 18.6%, 59.4%, 20.1%, and 1.9%, respectively.

Discussion

It is known that the terminal acceptor for the six-electron transfer catalyzed by PPO is molecular oxygen (Ferreira & Dailey, 1988; Dailey, 1990). In the six-electron transfer reaction catalyzed by xanthine oxidase the protein accepts two electrons each at a Mo and a flavin site and one electron at each of two Fe-S sites (Hille & Massey, 1981; Malmstrom, 1982). The identification of a single FMN

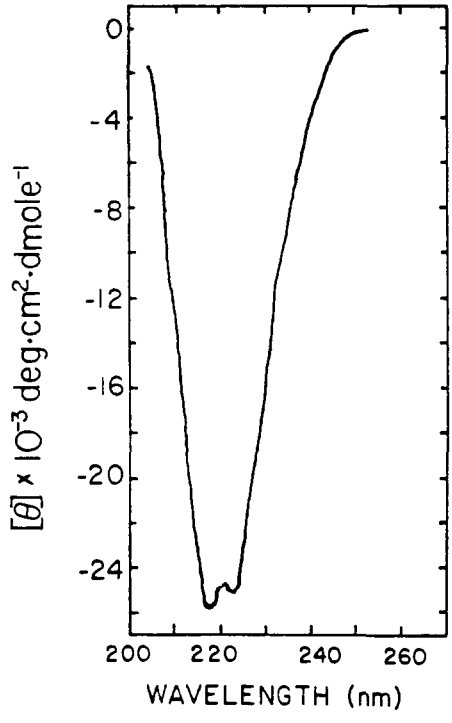

Fig. 8. Circular dichroism spectrum of PPO. Measurements taken at room temperature using a quartz cuvette with a 1-cm pathlength. The protein concentration was 0.3 mg/mL.

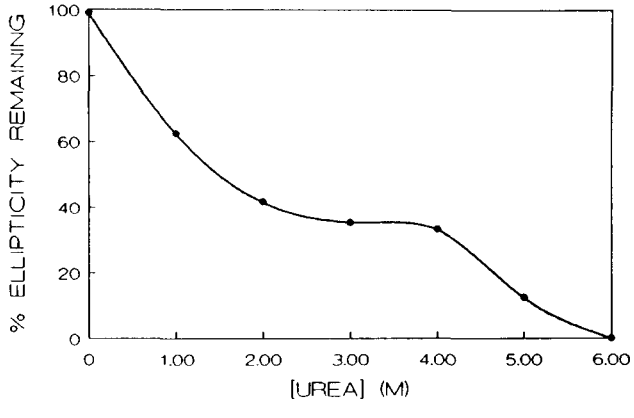

Fig. 9. Urea denaturation curve. Aliquots of a stock solution of urea were added to the protein to give the desired concentrations and allowed to come to equilibrium at room temperature for about 12 h. There was no alteration of secondary structure due to dilution of the protein as measured by the addition of 20 mM Tris-MOPS. Percent ellipticity was measured at 230 nm.

moiety in mouse PPO described above explains how the enzyme can accept two of the electrons it transfers, but the lack of any detectable metals or PQQ in active, purified preparations still leaves the fate of the four other electrons in question. The possibility also exists that the enzyme catalyzes three, two-electron transfers. Identification of such a mechanism is currently complicated by the low protein concentrations, which prevents accurate single turnover experiments that may answer this question. Previous kinetic studies of PPO shed no additional light on this process (Ferreira & Dailey, 1987, 1988). In the reaction catalyzed by coproporphyrinogen oxidase, the antepenultimate enzyme of the heme biosynthetic pathway, tyrosyl residues have been reported to play a part in the shuttling of electrons during the oxidation (Yoshinaga & Sano, 1980). Although the amino acid analysis of PPO indicates that there are 11 tyrosyl residues that may be available to play a role in the reaction, we currently have no data to support such a hypothesis.

In the bovine PPO preparation a flavin was detected and identified as FAD (Siepker et al., 1987), whereas in our mouse enzyme the flavin is FMN. We have no explanation for this difference other than it may be simply a species difference. Because PPO has not been purified to homogeneity from any other higher eukaryote, and because we have been unable to reconstitute activity with exogenously supplied FMN or FAD, we are unable to say if one flavin is preferred. One discrepancy between our data and those reported by Siepker et al. (1987) is that in our preparations the protein-associated FMN exists in an oxidized state but the FAD in the purified bovine protein was reported to be not fully oxidized even after extensive treatment with known oxidizing agents. One would expect that the bound flavin would be reduced only in the presence of substrate and not in purified fractions in the absence of substrate and the presence of oxygen. Unfortunately, because of the spectral overlap of the product, protoporphyrin IX, and FMN it was not possible to follow the reduction and oxidation of FMN as PPO turns over during catalysis.

The fluorescence quenching studies performed on the FMN in mouse PPO suggested that the flavin is exposed to the solvent and not buried within the structure of the protein. The blue shift of FMN fluorescence subsequent to trypsin digestion of the protein, however, indicates that there is some protein shielding of the FMN by the protein and digestion exposes it completely to the aqueous solvent.

Assuming that the FMN accepts electrons from protoporphyrinogen and donates them directly to O_2, it would not be unexpected to find the FMN exposed on the surface. The lack of complete FMN fluorescence quenching by the protein would add further support for an aqueous exposure of the FMN. The quenching of protein tryptophan fluorescence by acrylamide and KI indicates that, although it is accessible to the quenchers in solution, it is shielded to some extent by the protein structure.

Protein secondary structure estimates obtained by circular dichroism studies suggest an amphipathic structure for PPO. This is not surprising considering that this is common to many membrane-bound proteins, a class of which PPO is a member. The biphasic denaturation curve resulting from treatment of PPO with urea is typical

of amphipathic proteins such as cytochrome b_5 (Tajima et al., 1976). Overall, the secondary structure composition (e.g., 30% alpha helix, 40% beta sheet, etc.) is not unusual, and denaturation appears to result in a major decrease in alpha helix with a concomitant increase in beta structure.

Materials and methods

Purification of mouse protoporphyrinogen oxidase

Mitochondria were isolated from 100 g of frozen mouse livers by differential centrifugation (Guerra, 1974). The buffer used consisted of 0.25 M sucrose, 10 mM Tris(hydroxymethyl)aminomethane-3-(*N*-morpholino)propanesulfonic acid (Tris-MOPS), pH 7.8, and 1 mM ethylenediaminetetraacetic acid (EDTA). All buffers also contained 10 μg/mL phenyl methanesulfonyl fluoride (PMSF), which was added from a stock solution of 10 mg/mL in isopropanol immediately before the buffer was used. The mitochondria were then disrupted by sonication for four 30-s intervals with a Heat Systems sonicator set at 20 W. The mitochondrial membranes were then isolated by centrifugation at 100,000 × *g* for 90 min. The membrane fraction was resuspended in a buffer consisting of 20 mM Tris-MOPS, pH 7.8, 1 mM dithiothreitol, 20% glycerol, 1% sodium cholate, and 0.1 M KCl, and the enzyme was solubilized by sonication for two 30-s intervals and then stirring for 30 min. The remaining membranes were pelleted by centrifugation at 100,000 × *g* for 90 min.

The solubilized enzyme was fractionated with ammonium sulfate between 35% and 55% saturation. The 55% ammonium sulfate precipitate was dissolved in 30–40 mL of a buffer consisting of 20 mM Tris-MOPS, pH 6.9, 1 M KCl, and 0.5% sodium cholate. This was loaded onto a Phenyl-Sepharose CL-4B column (2.5 × 15 cm) with a bed volume of 45 mL that had been equilibrated with the above buffer. The column was then washed with 300 mL of the equilibration buffer before PPO was eluted with 20 mM Tris-MOPS, pH 6.9, 0.5% sodium cholate and 0.5% polyoxyethylene lauryl ether (Brij 35). The protein (usually 0.1–0.4 mg/mL) was used as it eluted from the column. All attempts to concentrate PPO lead to poor recovery of both activity and protein. The purified PPO could be stored at 4 °C for up to 3 days with a loss of about 50% of activity. Freezing destroyed activity.

Enzyme assays and determinations

Protoporphyrinogen oxidase activity was assayed by a modification of the fluorometric assay of Jacobs and Jacobs (1982). The assays were carried out at 37 °C in the dark. The assay mixture contained, in 1 mL, 0.1 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.4, 1 mM EDTA, 5 mM glutathione, 0.1% Tween 20, and enzyme. At 0-, 10-, 20-, 30-, and 40-min intervals 0.1 mL of the samples was withdrawn, mixed with 0.9 mL of the above buffer at 37 °C without the detergent, and the fluorescence determined in a Perkin-Elmer 650-40 spectrofluorometer (excitation wavelength of 405 nm, emission 635 nm), which was thermostated at 37 °C. The porphyrinogen substrate was produced immediately before use by reduction with sodium amalgam. Protein concentrations were determined with the Pierce BCA (bicinchoninic acid) protein reagent using bovine serum albumin as a standard. Protein samples were incubated with at least an equal volume of 50 mM iodoacetamide to prevent solution sulfhydryls from reacting with the BCA reagent.

The procedure described by van der Meer et al. (1987) was used to assay for PQQ in purified preparations of PPO. Purified PPO was derivatized overnight with dinitrophenylhydrazone (DNPH) and then digested with trypsin to release protein-bound cofactors. The sample was concentrated, filtered, and then injected onto a reverse-phase (Econosphere C18) HPLC column. The eluant consisted of a 30-min linear gradient of 7–63% methanol in 10 mM sodium phosphate, pH 7.0, containing 10 mM ammonium chloride and was monitored by absorbance at 450 nm with a Beckman model 160 absorbance detector. The C-5 hydrazone of PQQ and DNPH was prepared as described (van der Meer et al., 1987) for use as a model compound.

In a parallel experiment PPO was assayed by the redox-cycling staining method as outlined by Paz et al. (1991). In this experiment PPO, BSA, and BSA covalently modified with hematoxylin were run on an SDS-polyacrylamide gel and then electrophoretically transferred onto nitrocellulose. The apparatus used for electrophoresis and blotting was a Bio-Rad Mini-Protean II system and the blotting buffer used was 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. Duplicates of all samples were run on one gel, blotted to nitrocellulose, and after blotting the membrane was cut in half. One half of the membrane was stained with a 0.1% solution of amido black in 45% methanol, 10% acetic acid to detect proteins and the other half was stained in the dark with a 0.24 mM NBT solution in 2 M potassium glycinate, pH 10, to detect quinoproteins.

Spectra

The absorption spectrum of PPO was determined on a Cary 219 spectrophotometer at room temperature using quartz cuvettes with a 1-cm pathlength. The calculated extinction coefficient for PPO was based upon the BCA-derived protein concentration. Accurate dry ash weights could not be obtained due to the presence of detergents, the insolubility of the protein in the absence of added detergents, and the low concentration of protein.

Fluorescence measurements were performed on a

Perkin-Elmer 650-40 spectrofluorometer equipped with a thermostated cell holder. All fluorescence measurements were done in the ratio mode to compensate for variations in source lamp intensity. Fluorescence emission and excitation spectra were measured using a quartz cuvette at 15 °C with slit widths of 6 nm.

Fluorescence quenching measurements were carried out at 15 °C with slit widths of 4 nm. Solutions of KI and acrylamide were prepared in distilled water before use. Data are expressed as Stern–Volmer plots. The Stern–Volmer quenching constant was calculated as $F_0/F = 1 + K_{S\text{-}V}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, and $[Q]$ is the molar concentration of the quencher.

Plasma scan

Plasma scans to determine the metal content of the protein were performed at the Soil Testing and Plant Analysis Laboratory of the University of Georgia Cooperative Extension Service, Athens, Georgia. Four separate enzyme preparations were analyzed, and the quantity of material analyzed was sufficient to identify 0.1 equivalents of any metal.

Isolation and identification of chromophore

The chromophore was released from the protein by extensive trypsin digestion as described previously (Kozoil, 1971). The digested sample was injected onto the reverse-phase HPLC column (Econosphere C18) that had been equilibrated with 10 mM ammonium phosphate, pH 5.5. Elution of the column was with 10 mM ammonium phosphate:acetonitrile 100:12 (v/v), pH 5.5. Peaks were detected with an Applied Biosystems fluorescence detector (model 980) equipped with a 470-nm filter. The excitation wavelength used was 440 nm. The data shown represent the average of three determinations.

Circular dichroism

A Jasco 500-C spectropolarimeter was used for circular dichroism studies. Data were analyzed using a Jasco DP501-N data processor in conjunction with an IBM PC XT equipped with Jasco software. All measurements were repeated on at least two separate preparations of enzyme.

Amino acid composition

The amino acid composition (except for Trp) was determined following acid hydrolysis by the Molecular Genetics Facility at the University of Georgia. Tryptophan was determined fluorometrically on the denatured protein as described by Pajot (1976).

Materials

Amido black, NBT, FMN, FAD, PQQ, and BSA were purchased from Sigma. BSA covalently modified with hematoxylin was generously donated by Dr. Paul M. Gallop, the Children's Hospital, Boston, Massachusetts. Acrylamide was purchased from BDH Chemicals Ltd., Poole, England and potassium iodide from J.T. Baker Chemical Co., Phillipsburg, New Jersey. Mouse livers were obtained from Bioproducts For Science Inc., Indianapolis, Indiana. The BCA reagent and Tween 20 were purchased from Pierce, Rockford, Illinois. Protoporphyrin IX was purchased from Porphyrin Products of Logan, Utah. Phenyl-Sepharose CL-4B was purchased from Pharmacia, and the HPLC column that was used was an Econosphere C18 reverse-phase column obtained from Alltech Associates, Inc., Deerfield, Illinois. All other reagents were of the highest quality available.

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