Purification and Characterization of Murine Protoporphyrinogen Oxidase†

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ABSTRACT: The penultimate enzyme of the heme biosynthetic pathway, protoporphyrinogen oxidase (EC 1.3.3.4), has been purified to apparent homogeneity from mouse liver mitochondria. The purification involves solubilization from mitochondrial membranes with sodium cholate followed by ammonium sulfate fractionation and gel filtration on a Sepharose CL-6B column. The eluate is adjusted to 0.67 M $(NH_4)_2SO_4$ and loaded onto a phenyl-Sepharose column. After salt washes, the enzyme is eluted with 0.5% sodium cholate and 0.5% Brij 35. The final step is high-pressure ion-exchange chromatography on a DEAE-5PW column. The purified protein has a molecular weight of approximately 65000 by gel filtration chromatography on Sepharose CL-6B in the presence of 0.5% sodium cholate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows a single band corresponding to a molecular weight of 65000. The absorption spectrum of the purified enzyme shows no evidence of a chromophoric cofactor. Purified protoporphyrinogen oxidase has a K_m for protoporphyrinogen IX of 5.6 μM with a V_{max} of 2300 nmol mg^{-1} h^{-1}. It utilizes meso- and hematoporphyrinogen at about 10% the level of protoporphyrinogen. The pH optimum is broad with a maximum at 7.1. There is no stimulation or inhibition by any tested divalent cations, and sulfhydryl reagents have no inhibitory effect on the purified enzyme.

The penultimate step in the heme biosynthetic pathway, the removal of six hydrogens from protoporphyrinogen to form protoporphyrin, is catalyzed by the enzyme protoporphyrinogen oxidase (EC 1.3.3.4) (Poulson, 1976; Poulson & Polglase, 1975). This enzyme is bound to the inner mitochondrial membrane in eukaryotic cells and in mammalian cells requires molecular oxygen for activity (Poulson, 1976). Although the reaction may take place nonenzymatically in vitro, convincing data have been presented to show in vivo the step is enzymatically catalyzed (Poulson & Polglase, 1975). Little is known about the enzyme since it has not been purified to homogeneity or kinetically characterized. Previously, it has been solubilized and partially purified from yeast and rat liver mitochondria (Poulson & Polglase, 1975; Poulson, 1976).

The importance of the enzyme to man is made obvious in the genetically inherited disease variegate porphyria. In this disorder, protoporphyrinogen oxidase activity is approximately half of normal (Brenner & Bloomer, 1980a; Deybach et al., 1981). Patients with variegate porphyria may exhibit both neuropsychiatric symptoms and skin lesions in addition to increased levels of excreted fecal porphyrins. In addition, there is a recent report that protoporphyrinogen oxidase activity is decreased in patients with Gilberts syndrome (McColl et al., 1985). While this is probably not an inherited enzyme deficiency, it does point out the possible importance of this one enzyme in two clinically distinct disorders.

In the present study, we report for the first time the purification of protoporphyrinogen oxidase to apparent homogeneity. Some characteristics of the purified enzyme are also presented.

Materials and Methods

Assays. Protoporphyrinogen oxidase was assayed by a modification of the fluorometric assay of Brenner and Bloomer (1980b). The assays were carried out at 37 °C in the dark. The assay mixture contained in 1 mL of 0.1 M tris(hydroxymethyl)aminomethane (Tris)–acetate, pH 8.1, 1.0% (w/v) Brij 35, protoporphyrinogen, and enzyme. At 0, 10, 20, 30, and 60 min, 0.1-mL samples were withdrawn and mixed with 0.9 mL of 10 mM Tris–acetate, pH 8.1, and 0.1% Brij 35 (37 °C), and the fluorescence was determined in a Perkin-Elmer 650-40 spectrofluorometer (wavelength of excitation 405 nm, emission 635 nm). The porphyrinogen substrate was produced just before use by reduction of protoporphyrin with sodium amalgan. One modification that was made to previously described procedures was that 1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) (free acid) was used to adjust the pH rather than solutions of acetic acid or phosphoric acid. This, we found, greatly simplified the pH adjustment since the pK_a (7.2) of MOPS is more favorable than that of acetic or phosphoric acid, thereby decreasing the possibility of decreasing the pH too much. Protoporphyrinogen was stored at 4 °C, under N_2 in the dark, and was used within 1 h.

In assays where metals or alternate electron acceptors were used, control assays were run with all components except the enzyme fraction. The rate of any nonenzymatic oxidation was subtracted from the enzymatic rate.

Purification of Protoporphyrinogen Oxidase. The initial steps in the purification of the oxidase are identical with those previously described for purification of mammalian ferrochelatase (Dailey & Fleming, 1983; Dailey et al., 1986), so they are only outlined here. The procedure described below is effective in purifying protoporphyrinogen oxidase from both mouse and beef liver although all of the data presented are for the murine enzyme.

Mitochondria are isolated from 100–200 g of fresh beef liver or frozen mouse livers by differential centrifugation (Guerra, 1974). Unless otherwise noted, the buffer used is 20 mM Tris–acetate, pH 8.1, 1 mM dithiothreitol, 20% glycerol, and 10 μg/mL phenylmethanesulfonyl fluoride (PMSF). All buffers contained 10 μg/mL PMSF which was added from a stock solution (10 mg/mL) in propanol immediately before the buffer was used. Following sonication and isolation of membrane fragments, the enzyme is solubilized in buffer

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containing 1.0% sodium cholate and 0.1 M KCl. The solubilized enzyme is fractionated with ammonium sulfate between 35% and 55% saturation.

A Sepharose CL-6B gel filtration column (1.5 × 100 cm) was equilibrated with 20 mM Tris–MOPS, pH 6.9, 0.5% sodium cholate, 0.5 mM dithiothreitol, and 10 μg/mL PMSF at 4 °C. The 55% ammonium sulfate precipitate was dissolved in the above buffer, and a 5-mL sample was loaded onto the column. Fractions which contain enzyme activity elute at M_r 65 000.

The fractions containing enzyme activity from the Sepharose CL-6B column (about 20 mL) are pooled and their volume measured. One-half volume of a 2 M ammonium sulfate solution is added to yield a final concentration of 0.67 M. After setting in ice for 10 min, this is centrifuged at 10000*g* for 10 min, and the supernatant is loaded onto a phenyl-Sepharose CL-4B column (10-mL bed volume in a 10 cm^3 syringe). The phenyl-Sepharose column is equilibrated with 20 mM Tris–MOPS, pH 6.9, 1 M KCl, 0.5% sodium cholate, and 10 μg/mL PMSF. After the sample is loaded, the column is washed with 200 mL of equilibration buffer with 0.2 M ammonium sulfate, followed by 100 mL of equilibration buffer. The enzyme is eluted with 20 mM Tris–MOPS, pH 6.9, 0.5% sodium cholate, 0.5% Brij 35, and 10 μg/mL PMSF.

Fractions from the phenyl-Sepharose CL-4B elution that contain activity were loaded onto a Sepherogel diethylaminoethyl (DEAE) high-performance liquid chromatography (HPLC) column (1EX-DEAE-5PW; Beckman Instrument Co.) equilibrated at room temperature with 20 mM Tris–MOPS, pH 6.9, and 0.1% Brij 35 (buffer A). A 4-mL sample was loaded, and the column was washed at 1 mL/min with buffer A for 15 min. During this time, a small peak containing little or no protoporphyrinogen oxidase activity appears. After 15 min, a gradient (0–25% buffer B over 25 min) was started with buffer containing 20 mM Tris–MOPS, pH 6.9, 0.1% Brij 35, and 1.0 M KCl (buffer B). A single, sharp peak eluted which contains the enzyme activity. The column was recycled by washing with 100% buffer B for 20 min during which time a small protein peak elutes that does not contain any enzyme activity.

Procedures. For the determination of the molecular weight by gel filtration chromatography, a Sepharose CL-6B column (1.5 × 100 cm) was equilibrated with 20 mM Tris–acetate, pH 8.1, 1.0% sodium cholate, and 10 μg/mL PMSF. The column was run at 4 °C and was calibrated with aldolase (M_r 158 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), and cytochrome *c* (M_r 12 500). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was done either in a Hoeffer Protean slab gel or in a Bio-Rad minigel apparatus. The discontinuous buffer system of Laemmli (1970) was used for both SDS and urea gel electrophoresis. For urea gels, a final concentration of 8 M urea was present in the polyacrylamide gels. Proteins were detected by silver staining (Merril et al., 1981).

Protein concentrations were determined with the Pierce BCA protein reagent. Since free sulfhydryls, such as dithiothreitol, react with the reagent, protein samples were first incubated with an equal volume of 50 mM iodoacetamide.

Porphyrinogen concentrations were determined after photochemical oxidation to the porphyrin (Jacobs & Jacobs, 1982). Extinction coefficients used were those previously listed (Furhop & Smith, 1976). To quantitative the amount of porphyrin formed in the enzymatic reaction, a standard curve of protoporphyrin fluorescence was made for each determination.

Table I: Purification of Mouse Protoporphyrinogen Oxidase[a]

fraction	protein (mg/mL)	recovery (%)	sp act. (RFU mg^{-1} h^{-1})[b]
mitochondria	55	100	1.0
cholate solubilized	10	68	2.4
$(NH_4)_2SO_4$ precipitate (35–55%)	35	70	6.8
Sepharose CL-6B	1.5	37	63
phenyl CL-4B	0.2	19	860
HPLC ion exchange	0.06	12	2436

[a] Details of the purification are given under Materials and Methods. [b] Specific activity is expressed as relative fluorescent units per milligram per hour. Details of the assay are given under Materials and Methods.

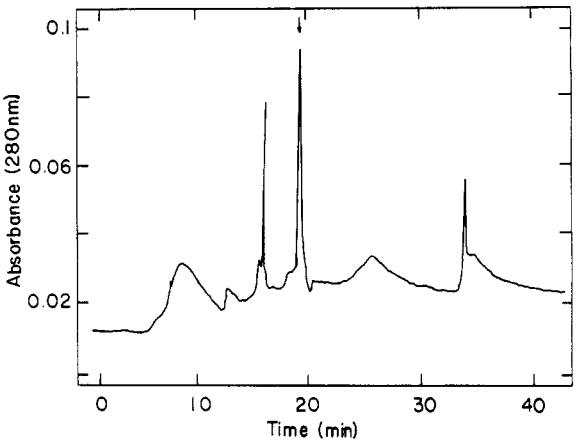

FIGURE 1: HPLC ion-exchange column elution profile. The experimental details are in the text. Protoporphyrinogen oxidase activity (peak shown with the arrow) eluted in the second sharp peak at approximately 15% buffer B. The broad early peak is nonadsorbed material.

Materials. Mouse livers were obtained from either Pel Freez Biologicals, Rogers, AR, or Bio Trol, Inc., Indianapolis, IN. Beef livers were obtained from a local slaughterhouse. Sepharose CL-6B and phenyl-Sepharose CL-4B were from Pharmacia, and the high-pressure liquid chromatography anion-exchange column was a Spherogel IEX-DEAE-5PW from Beckman Instruments, Inc. All porphyrins were from Porphyrin Products, Logan, UT, and all other reagents were of the highest quality available.

RESULTS

Purification of Protoporphyrinogen Oxidase. The purification scheme is detailed above, and the results are shown in Table I. This purification scheme is effective for both bovine and mouse liver protoporphyrinogen oxidase. Overall, an average purification of 2500-fold with about 10% recovery is achieved. Similar yields are found for both animals. Inclusion of a protease inhibitor in all buffers is critical for good recovery. It was found that buffer composition, column geometry, and pH are critical to get the enzyme to interact with both the HPLC DEAE and phenyl columns. Of a wide variety of buffers tested at various pH values, *Tris*–MOPS, pH 6.8 proved to be the most effective. The elution profile of the HPLC DEAE column is shown in Figure 1.

Physicochemical Properties. The estimated molecular weight of mouse protoporphyrinogen oxidase is 65 000 as determined by gel filtration on a Sepharose CL-6B column in buffer containing 1% sodium cholate. The presence of 0.5 mM dithiothreitol (DTT) had no effect on the molecular weight. We were unable under any conditions to detect any enzyme activity in fractions corresponding to a molecular

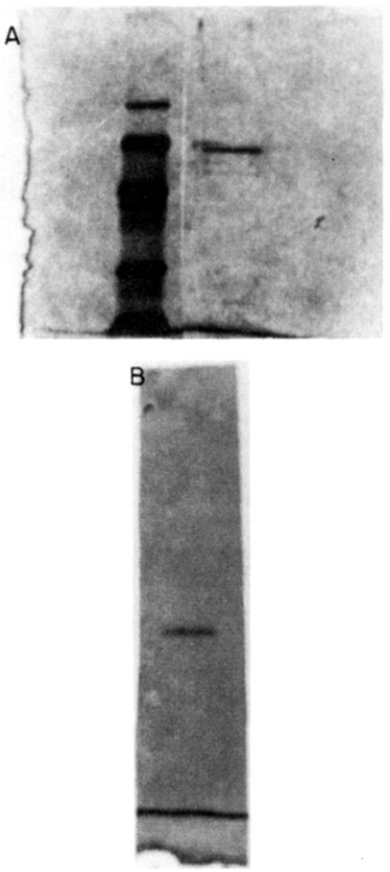

FIGURE 2: Polyacrylamide gel electrophoresis of purified mouse protoporphyrinogen oxidase. (A) SDS–polyacrylamide gel of purified enzyme. Molecular weight standards are shown to the left, and approximately 1 μg of purified enzyme is to the right. (Standards are phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin, inhibitor, and α-lactalbumin.) (B) 8 M urea–polyacrylamide gel of purified enzyme. Approximately 2 μg of purified enzyme was run on an 8 M urea gel as described in the text.

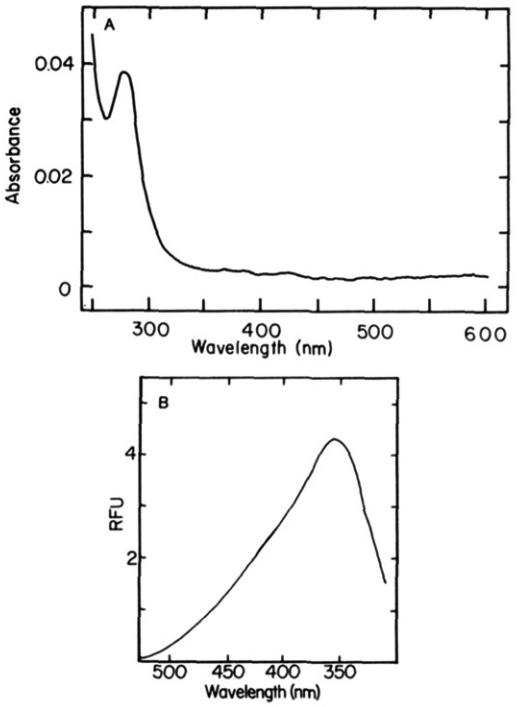

FIGURE 3: (A) Ultraviolet–visible light absorption spectrum of purified mouse protoporphyrinogen oxidase. The protein concentration was 20 μg/mL, and the buffer was 20 mM Tris–MOPS, pH 6.9, 1 M KCl, and 1% sodium cholate. The spectrum was recorded against a buffer blank using a Cary 219 split-beam spectrophotometer. (B) Fluorescence emission spectrum of purified mouse protoporphyrinogen oxidase. The excitation wavelength was 288 nm, the protein concentration was 20 μg, and the temperature was 25 °C. The excitation and emission slits were 2 nm. The spectrum was recorded on a Perkin-Elmer 650-40 spectrofluorometer. RFU is relative fluorescence units.

weight lower than 65 000. SDS–polyacrylamide gel electrophoresis revealed a single band with a molecular weight of 65 000, suggesting that the oxidase is a monomer (Figure 2). Gel electrophoresis in the presence of 8 M urea, which allows proteins to separate on the basis of size and charge, yielded a single protein band. It was found that the protein is very sensitive to proteolytic nicking, and chromatography in the absence of protease inhibitors always results in lowered enzyme recovery and the appearance of lower molecular weight protein bands in SDS gel electrophoresis. The protein elutes at low protein concentrations, and attempts to concentrate the enzyme have proven to be unsuccessful. Dialysis against aqueous solutions without detergents resulted in loss of protein. As a result, it was not possible to obtain sufficient protein free from detergents and salts to do an accurate metal determination. Repeated attempts using a gas phase amino acid sequencer to obtain amino-terminal sequence information yielded no results. Under identical conditions, proteins with an unblocked amino terminus did sequence. These data suggest that protoporphyrinogen oxidase, like ferrochelatase,[1] may have a blocked amino terminus.

The ultraviolet/visible spectrum of purified protoporphyrinogen oxidase is shown in Figure 3A. There is no spectral evidence for any chromophoric cofactor. Even at the low protein concentrations (about 0.5 μM) with which we are forced to work with the native enzyme, it would be expected that a compound such as FAD or FMN (ϵ_{mM} = 13 at 445 nm) would be detectable. The fluorescence emission spectrum is shown in Figure 3B. The excitation maximum and the excitation wavelength used for this spectrum are 288 nm. The spectrum is characteristic of a typical protein with tryptophan and tyrosine residues. Attempts to detect flavin fluorescence in either oxidizing or reducing conditions, in the presence or absence of 1% SDS, proved fruitless. Excitation at 450 nm did not yield an emission spectrum characteristic of flavin ($\lambda_{emission}$ ~500–520 nm).

Enzymatic Properties of Purified Protoporphyrinogen Oxidase. The ability of a variety of porphyrinogen IX isomers to serve as substrates for the purified enzyme was examined. These included the porphyrinogens of the commercially available proto-, meso-, deutero-, and hematoporphyrin, deuteroporphyrin bisacetal, and deuteroporphyrin bisglycol. Of these, only proto-, meso-, and hematoporphyrinogen were substrate, with protoporphyrinogen having 10 times higher activity than the other two. The apparent K_m of the purified enzyme for protoporphyrinogen was 5.6 μM with a V_{max} of 2300 nmol mg^{-1} h^{-1}. The apparent K_m's for meso- and hematoporphyrinogen are both approximately 90 μM.

The ability of *N*-methylprotoporphyrin to inhibit enzyme activity was examined. The inhibition observed was minimal with only 11% inhibition at 0.1 μM. Ferrochelatase, the terminal enzyme, is inhibited by this compound with a K_i = 7 nM (Dailey & Fleming, 1983).

The effects of a variety of divalent cations were examined. The metals tested were $CaCl_2$, $CoCl_2$, $Fe[(NH_4)_2SO_4]_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, $Pb(NO_3)_2$ and $ZnSO_4$. At the concentration tested (100 μM), there was no significant stimulation or inhibition by any of these. This is in contrast to

[1] H. A. Dailey and T. Andrew, unpublished observations.

Table II: Effect of Potential Electron Acceptors on Protoporphyrinogen Oxidase Activity[a]

electron acceptor	concn (μM)	relative activity[b]
oxygen (air-saturated buffer)	241	100
FMN	10	65 ● 18
FAD	10	143 ± 9
NADP	10	97 ± 1
NAD	10	96 ± 3
heme	0.1	64

[a] All assays, except with buffers saturated with O_2 or N_2, were done in tubes open to the air at 37 °C. [b] Activities are expressed relative to the activity found in air-saturated buffers without added cofactors. The values listed are the means of three determinations with standard deviations listed except with heme which was done only once.

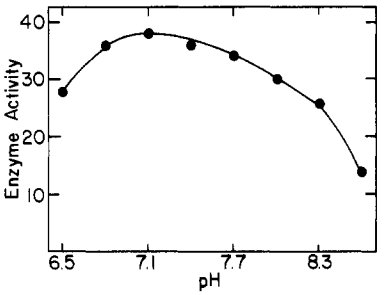

FIGURE 4: pH optimum for purified protoporphyrinogen oxidase. The assay was run in 0.1 M Tris–MOPS adjusted to the indicated pH at 37 °C. Enzyme activity is expressed as relative fluorescence units per milligram per hour.

ferrochelatase which is strongly inhibited by several of these metals at a 10-fold lower concentration (Dailey & Fleming, 1983). Inclusion of 0.1 mM EDTA in the assay buffers also had no effect on the observed activity of the purified enzyme.

Since it had previously been suggested that protoporphyrinogen oxidase might have catalytically important sulfhydryl groups, the effects of several sulfhydryl reactive reagents were tested. We found no inhibition of enzyme activity after reaction for 30 min in 2.5 mM *N*-ethylmaleimide, iodoacetamide, or 4,4′-dimaleimidostilbene-2,2′-disulfonate. This along with the lack of inhibition by 100 μM $HgCl_2$ argues strongly against sulfhydryl group involvement. Furthermore, the inclusion of dithiothreitol, glutathione, or 2-mercaptoethanol in the reaction mixture had no stimulatory effect on the enzyme's activity.

Since protoporphyrinogen oxidase catalyzes the removal of six electrons, various electron acceptors were included in the assay to see if they stimulated enzyme activity (Table II). Of the compounds tested, only FAD was stimulatory, and the stimulation seen was slight. These same compounds when tested at 10 times higher concentration also had no effect except that at that concentration FAD was no longer stimulatory. Saturation of the buffer with oxygen resulted in higher activity as would be expected since oxygen is the terminal acceptor.

The pH optimum of the purified enzyme is 7.1 (Figure 4). No effect was seen when different Goods buffers were used.

Discussion

Above we have presented the purification of mouse protoporphyrinogen oxidase. This enzyme, the penultimate in the pathway, is the last enzyme in the heme biosynthetic pathway to be purified. Previously, a partial purification of the enzyme from rat liver mitochondria was reported (Poulson, 1976). The rat enzyme was reported to have a molecular weight of 35 000 while we find a molecular weight of about 65 000 for mouse protoporphyrinogen oxidase. We have no explanation for this large discrepancy in reported size although we did find that the absence of the protease inhibitor during purification resulted in decreased recovery of enzyme and the appearance of a 35 000 molecular weight band on SDS gels. By gel filtration, it was never possible to recover any enzyme activity in fractions corresponding to a weight of 35 000.

Several other properties of purified mouse protoporphyrinogen oxidase differed from those reported for the rat enzyme. One of these was the sensitivity of the enzyme to sulfhydryl reagents. We were unable to obtain any significant inhibition by *N*-ethylmaleimide or iodoacetamide. No stimulation of activity was found by 2-mercaptoethanol or dithiothreitol. Both of these observations are contrary to those previously reported for the partially purified rat enzyme (Poulson, 1976).

One similar finding was for the substrate specificity of the enzymes. Protoporphyrinogen IX was an effective substrate with an apparent K_m = 5.6 μM. Mesoporphyrinogen and hematoporphyrinogen were poor substrates with an apparent K_m of 90 μM, and at the usual concentration of porphyrinogen in the assay system, they had about 10% of the activity found with protoporphyrinogen. No other 2,4-substituted porphyrinogen tested served as a substrate. *N*-Methylprotoporphyrin, which is a tight-binding competitive inhibitor of ferrochelatase (Dailey & Fleming, 1983), was not an inhibitor or protoporphyrinogen oxidase. This was of interest since the in vivo administration of certain compounds to laboratory animals causes an accumulation of *N*-methylprotoporphyrin and the resultant development of a chemically induced porphyria (Onisawa & Labbe, 1963; DeMatteis et al., 1982). Interestingly, the symptoms of the porphyria resemble variegate porphyria more than protoporphyria since the marked photosensitivity of this second disorder is not present. This suggested that in vivo protoporphyrinogen oxidase, rather than ferrochelatase, may be affected by the *N*-methylprotoporphyrin, but an alternate explanation is that the organ affected (hepatic in variegate porphyria, erythropoietic in protoporphyria) is more crucial than the actual enzyme for expression of these disease conditions. The finding of no inhibition of protoporphyrinogen oxidase by *N*-methylprotoporphyrin would support the latter alternative.

Protoporphyrinogen oxidase activity is decreased to approximately half of normal levels in patients suffering from variegate porphyria (Brenner & Bloomer, 1980a). Several investigators have suggested that this single disorder is the basis for the disease (Deybach et al., 1981; Brenner & Bloomer, 1980a) while others have proposed that both protoporphyrinogen oxidase and ferrochelatase may be involved (Viljoen et al., 1983; Becker et al., 1977). While the data presented above do not answer this question directly, they do exclude the proposed possibility that the terminal two enzymes share a common subunit (Viljoen et al., 1983). Ferrochelatase has a molecular weight of approximately 40 000 and is composed of a single polypeptide chain while protoporphyrinogen oxidase is M_r 65 000 and, therefore, does not have a component of the same molecular weight as ferrochelatase.

Other unanswered questions that may be approached now with the purified enzyme are the in vivo electron acceptor, the role of phospholipids in enzyme activity, and possible protein–protein interactions between protoporphyrinogen oxidase and ferrochelatase. The absorption spectrum shows no evidence of a chromophoric cofactor such as a flavin, and since the enzyme carries out a six-electron step, it will be of interest to determine how the protein catalyzes this step. In addition, it may now also be possible to approach questions about the

molecular basis of variegate porphyria and to examine the possible role of this enzyme during erythropoietic differentiation.

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Phosphorylation of the Sarcoplasmic Calcium-Activated Adenosinetriphosphatase As Studied by ^{31}P Nuclear Magnetic Resonance

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ABSTRACT: A reinvestigation of a study of Fossel et al. [Fossel, E. T., Post, R. L., O'Hara, D. S., & Smith, T. W. (1981) *Biochemistry 20*, 7215–7219] in which the ^{31}P nuclear magnetic resonance (NMR) signal of the phosphointermediate of the sarcoplasmic (Ca^{2+},Mg^{2+})-ATPase has been identified shows that the signal they describe most probably originates from free Mg·ATP but not from the phosphoenzyme itself. It was possible to detect the ^{31}P NMR signal of the phosphoenzyme in peptic fragments of sarcoplasmic ATPase phosphorylated either by ATP or by inorganic phosphate. The two products exhibit the same spectral characteristics in ^{31}P NMR, implying that most probably both reaction pathways yield the same chemical product. Chemical shifts at low pH (−6.5 ppm) and high pH (−1.4 ppm) of the phosphoryl group are indicative of a β-phosphoaspartyl moiety, thus confirming independently the results from chemical analysis. The relatively low pK value of 4.3 of the phosphoryl group suggests an interaction with a positively charged group of the enzyme.

The amino acid sequence of Ca^{2+}-activated ATPase from rabbit muscle sarcoplasmic reticulum and the amino acid sequence of sheep kidney (Na^+,K^+)-ATPase have been deduced previously from their complementary DNA sequence (MacLennan et al., 1985; Shull et al., 1985). Both enzymes show significant homologies in their amino acid sequences and are probably the most intensively studied model systems for active ion-transport processes. During enzymatic catalysis, the pump protein is phosphorylated by its substrate ATP. The phosphoryl group is probably covalently bound to an aspartyl residue as Post and Kume (1973) and Bastide et al. (1973) could show by phosphorylation of the enzyme with radioactively labeled ATP and subsequent digestion of the phosphorylated protein. The active-site phosphopeptides could be isolated; they contain the sequence-Cys-Ser-Asp-Lys-Thr- (Bastide et al., 1973; Allen & Green, 1976).

A more direct approach for the study of the activity of enzymes during catalysis is the detection of phosphoenzyme intermediates by ^{31}P nuclear magnetic resonance (NMR).[1] However, the observation of phosphorylated intermediates of (Ca^{2+},Mg^{2+})-ATPase should be rather difficult due to the low concentrations obtainable. Moreover, the sarcoplasmic Ca^{2+} pump is embedded in the phospholipid matrix of the mem-

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; NMR, nuclear magnetic resonance; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

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