Purification and Characterization of Bovine Hepatic Uroporphyrinogen Decarboxylase†

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ABSTRACT: Uroporphyrinogen decarboxylase (EC 4.1.1.37) has been purified to homogeneity from bovine liver by using isoelectric and salt precipitations, followed by chromatography on DEAE-cellulose, phenyl-Sepharose, hydroxylapatite, and Sephacryl S-200. The purified enzyme is a monomer with an $M_r \sim 57000$ and an isoelectric point at pH 4.6. Enzyme activity is optimal in buffers having an ionic strength of ~0.1 M and a pH of 6.8. The purified enzyme has a specific activity (expressed as the disappearance of uroporphyrinogen I) of 936 nmol·h^{-1}·(mg of protein)$^{-1}$. The purified enzyme catalyzes all four decarboxylation reactions in the conversion of uroporphyrinogen I or III to the corresponding coproporphyrinogen. The rate-limiting step in the physiologically significant conversion of uroporphyrinogen III to coproporphyrinogen III is the decarboxylation of heptacarboxylate III. Kinetic data suggest that the enzyme has at least two noninteracting active sites. At least one sulfhydryl group is required for catalytic activity. The enzyme is inhibited by sulfhydryl-specific reagents and by divalent metal ions including Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+}. The pattern of accumulation of intermediate (hepta-, hexa-, and pentacarboxylate porphyrinogens) and final (coproporphyrinogen) decarboxylation products is affected by the ratio of substrate (uroporphyrinogen I or III) concentration to enzyme concentration. Under physiologic conditions where the uroporphyrinogen to enzyme ratio is low, the substrate is nearly quantitatively decarboxylated, and the major product is coproporphyrinogen. If the ratio of uroporphyrinogen to enzyme is high, intermediates accumulate, and heptacarboxylate porphyrinogen becomes the major decarboxylation product. The situation of a high uroporphyrinogen to enzyme ratio, compounded by the inhibitory effect of Fe^{2+}, is analogous to that found in the human disease porphyria cutanea tarda. Patients with that disease have diminished activity of hepatic uroporphyrinogen decarboxylase and increased hepatocellular iron and accumulate and excrete large amounts of uroporphyrin and heptacarboxylate porphyrin.

Uroporphyrinogen (an octacarboxylate porphyrinogen) is converted to coproporphyrinogen (a tetracarboxylate porphyrinogen) by the cytosolic enzyme uroporphyrinogen decarboxylase (EC 4.1.1.37) (Burnham, 1969). The four acetate side chains of uroporphyrinogen are decarboxylated sequentially in a clockwise fashion starting on the "D" ring. Intermediate compounds with seven, six, and five carboxylate substituents are generated (Jackson et al., 1976). Although uroporphyrinogen III is the substrate for decarboxylase under physiologic conditions, both naturally occurring isomers of uroporphyrinogen (I and III) may serve as substrates. Thus, there are a total of eight potential substrates and eight reaction products for the enzyme:

$$8\text{III} \rightarrow 7\text{III} \rightarrow 6\text{III} \rightarrow 5\text{III} \rightarrow 4\text{III}$$
$$8\text{I} \rightarrow 7\text{I} \rightarrow 6\text{I} \rightarrow 5\text{I} \rightarrow 4\text{I}$$

Direct assays of uroporphyrinogen decarboxylase in liver biopsy specimens obtained from patients with porphyria cutanea tarda have revealed subnormal enzymic activity in every case studied (Kushner et al., 1976; Elder et al., 1978; Felsher et al., 1982). In some patients, the activity of uroporphyrinogen decarboxylase in red cell lysates is also subnormal (Kushner et al., 1976; Benedetto et al., 1978; Felsher et al., 1978), but in others, the erythrocyte enzyme activity is normal (Elder et al., 1978; deVerneuil et al., 1978).

Because of the association of subnormal activity of uroporphyrinogen decarboxylase with a human disease, the enzyme has recently come under intense investigation. Partially purified uroporphyrinogen decarboxylase from several sources has been studied (Tomio et al., 1970; Romeo & Levin, 1971; Smith & Francis, 1979). Recently, the enzyme has been purified to homogeneity from normal human erythrocytes, and its physical and kinetic properties have been defined (deVerneuil et al., 1983). The hepatic enzyme, however, has not been purified from any species.

In this report, we describe the purification of uroporphyrinogen decarboxylase from bovine liver and the kinetic properties of the enzyme with each of its eight substrates. The complex decarboxylation reaction is accomplished by a single protein which appears to have multiple catalytic sites. The findings in these studies should be directly applicable to the purification of the enzyme from human liver and to the characterization of the uroporphyrinogen decarboxylase defect responsible for porphyria cutanea tarda.

Materials and Methods

Sephadex G-150 and G-75, Sephacryl S-200, and phenyl-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. DE-52 was purchased from Reeve-Angel, Clifton, NJ. Chemicals and equipment for isoelectric focusing were obtained from LKB-Producter, Pleasant Hill, CA. Hydroxylapatite and materials for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. Trimethyl orthoformate, 14% BF_3 in methanol, ethylene glycol, dextran blue, transferrin, bovine serum albumin, porcine pancreatic ribonuclease, and horse heart cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, MO. Catalase was obtained from Calbiochem-Behring Corp., La Jolla, CA. Ultrapure Tris base and ultrapure ammonium sulfate were purchased from Schwarz/Mann, Spring Valley, NY. Porphobilinogen, octa-, hepta-, hexa-, penta-, and tetracarboxylate porphyrin methyl esters I and III, and porphyrin ester chromatography standards were obtained from Porphyrin Products, Logan, UT. Porphyrin dihydrochlorides were prepared by overnight hydrolysis

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of porphyrin esters in 3.0 N HCl as described by Fuhrhop & Smith (1975).

Assays for Uroporphyrinogen Decarboxylase Activity. Uroporphyrinogen decarboxylase activity was quantified by a modification of the method of Straka et al. (1982a,b) using substrates generated either by enzymic methods (uroporphyrinogen I) or by reduction of the corresponding porphyrin with 3% sodium amalgam. The standard assay was run at 37 °C for 30 min at an ionic strength of 0.10 M, with uroporphyrinogen I (30 μM) as substrate. The method described was modified as follows: all volumes were halved; the decarboxylase reaction was stopped by adding 0.5 mL of 9% $HClO_4$ in absolute methanol (w/v); and porphyrin methyl esters were formed directly by adding 3.5 mL of trimethyl orthoformate plus I mL of BF_3–methanol and heating for 30–60 min at 50 °C in a 16 × 150 mm Pyrex culture tube fitted with a screw cap with a Teflon liner. The esters were extracted and analyzed by high-performance liquid chromatography, (Straka et al., 1982a,b) using an M6000 A pump, M440 detector (405 nm), M730 data module, and a μPorasil column (0.4 mm × 30 cm), all purchased from Waters Associates, Milford, MA. One unit of enzyme activity decarboxylates 1 nmol of uroporphyrinogen I per h at 37 °C.

During the purification, a modification of the rapid-fluorometric assay of deVerneuil et al. (1983) was used to identify active fractions. The standard assay system of Straka et al. (1982a,b) was scaled to one-tenth and the reaction stopped with 100 μL of 1.0 N HCl. Under these conditions, only the penta- and tetracarboxylate reaction products can be extracted into diethyl ether. These compounds were then back-extracted into 1.0 N HCl and quantified fluorometrically (λ_{ex} = 402 nm; λ_{em} = 596 nm) with a Perkin-Elmer Model 650-10S recording spectrofluorometer. Coproporphyrin III (0.10 μM) in 1.0 N HCl was used as reference.

Protein concentrations were estimated by the method of Lowry et al. (1951) or by the method of Bradford (1976). Bovine serum albumin was used as a standard in both cases.

Purification of Uroporphyrinogen Decarboxylase. All steps were carried out at 4 °C. Fresh bovine liver, obtained from a local slaughterhouse, was chilled and processed as quickly as possible. If storage was required, fresh liver was washed as described below and frozen at −70 °C. Negligible loss of uroporphyrinogen decarboxylase activity was detected even after 1 year of storage under these conditions.

Crude Homogenate. Liver tissue was cut into thin (~3-mm) slices and was washed at least twice with an isotonic buffer solution to remove blood. The liver slices were blotted dry and weighed. The tissue was homogenized for 1 min at high speed in a Sorvall Omnimix or a Waring Blendor in 10 mM potassium phosphate, pH 6.8 (~3 mL·g^{-1} of tissue). The resulting homogenate was filtered through cheesecloth. The total volume of the filtered homogenate was adjusted with buffer to be equivalent to 4 mL of homogenate per g of tissue (wet weight). This was centrifuged at 10000*g* for 30 min. The supernatant fluid was decanted and the pellet discarded.

Acid Precipitation. Acetic acid (2 N) was added dropwise to the homogenate with vigorous stirring until the solution was pH 5.5. The solution was stirred for an additional 30 min and then centrifuged at 12000*g* for 30 min. The supernatant fluid was decanted and titrated to pH 6.8 with 2 N NH_4OH. The pellets were discarded.

Ammonium Sulfate Fractionation. The resulting solution was brought to about 27% saturation with ammonium sulfate by adding 153 g of solid ammonium sulfate per L of solution. This was stirred for 30–45 min and centrifuged at 10000*g* for 15 min and the precipitate discarded. An additional 153 g of ammonium sulfate per L of the original solution was added slowly to the supernatant liquid. After all the solid had dissolved, the solution was stirred for 30–60 min. The precipitate was collected by centrifugation as described above. This fraction, which precipitated between 27% and 50% saturation, contained most of the decarboxylase activity. The precipitate was redissolved in a minimum volume of 10 mM potassium phosphate buffer, pH 6.8. The resulting solution was dialyzed against three 20-volume changes of the same buffer overnight. The turbid dialysis product was clarified by centrifugation at 39000*g* for 30 min.

DE-52 (DEAE-cellulose) Chromatography. The dialyzed protein solution was applied to a DE-52 column (3.2 cm i.d. × 20 cm) equilibrated with 10 mM potassium phosphate, pH 6.8. The packed column volume was 1.5–2.0 times the sample volume. The sample was washed onto the column with about 1 column volume of buffer. The enzyme was eluted with a linear 0–0.25 M KCl gradient in the same buffer. The total volume of the gradient was equivalent to 5 column volumes. The fractions containing enzyme activity were pooled.

Phenyl-Sepharose Chromatography. The conductivity of the enzyme solution was adjusted with solid KCl to match or slightly exceed that of 0.25 M KCl in 20 mM potassium phosphate buffer, pH 6.8. This solution was applied to a 2.2 cm i.d. × 45 cm phenyl-Sepharose CL-4B column equilibrated with the same KCl–buffer solution. The column was washed with 0.5 column volume of KCl–buffer followed by 0.5 column volume of 20 mM potassium phosphate, pH 6.8. The enzyme was eluted with a 600-mL linear 0–80% ethylene glycol gradient in 20 mM potassium phosphate, pH 6.8.

Hydroxylapatite Chromatography. The pooled active fractions from the phenyl-Sepharose eluate were diluted with an equal volume of distilled water and applied to a 1.6 cm i.d. × 15 cm hydroxylapatite column which had been equilibrated with 10 mM potassium phosphate buffer containing 10% glycerol (w/v). After the column was washed with 1 column volume of the same buffer, the enzyme was eluted with a linear gradient of potassium phosphate (150 mL, 10–300 mM, pH 6.8) containing 10% glycerol.

Sephacryl S-200 Chromatography. The active fractions from the hydroxylapatite column were pooled and concentrated to 6–8 mL by using Ar in an Amicon ultrafiltration apparatus fitted with a PM-10 membrane. The concentrated protein was applied to a 2.2 cm i.d. × 86 cm Sephacryl S-200 column. The column had been equilibrated and the enzyme was eluted with 0.1 M KCl in 50 mM potassium phosphate buffer, pH 6.8. Fractions containing enzyme activity were pooled and concentrated to 6–10 mL in an Amicon ultrafiltration cell as before. The enzyme was stored at −70 °C.

Kinetic Studies. For the kinetic studies, a large volume of a stable enzyme preparation was necessary, but absolute purity was not essential. The preparation used was derived from 12 kg of liver taken through the ammonium sulfate step (see above). The active material was dialyzed and applied in batches of 100–200 mL to DE-52 as described above but was eluted with 0.5 M KCl in 10 mM phosphate buffer, pH 6.8. This concentrated the enzyme activity to a volume of approximately 25 mL which was then applied to a preparative scale Sephadex G-150 column and eluted with 50 mM potassium phosphate, pH 6.8. The active fractions from this column were pooled and frozen at −70 °C for use in the kinetic studies.

Assays for the determination of kinetic parameters employed substrates reduced with sodium amalgam. The amount of

Table I: Purification of Uroporphyrinogen Decarboxylase from Bovine Liver

	volume (mL)	protein concn (mg·mL^{-1})	total protein (mg)	sp act. (units·mg^{-1})	total activity (units)	yield (%)	x-fold purification
crude extract[a]	268	21.7	5821	1.4	7859	100	1
pH 5.5 supernatant	257	17.4	4478	1.7	7702	98	1.3
27–50% ammonium sulfate	300	7.2	2163	2.6	5580	71	1.9
DEAE-cellulose	150	0.6	83	65.6	5430	69	48.6
phenyl-Sepharose	74	0.3	22	131.6	2869	36	97.5
hydroxylapatite	35	0.1	3.4	(79.4)[b]	(270)[b]	(3.4)[b]	(58.8)[b]
Sephacryl S-200	37	0.01	0.5	936.4	418	5	693

[a] Consists of 100 g of liver homogenized in 300 mL of 10 mM potassium phosphate, pH 6.8. [b] Artifactually low values due to the instability of the enzyme at this stage of purification (see text).

enzyme in each series was adjusted so that less than 30% of the substrate was consumed. Data were reduced by the method of Lee & Wilson (1971). Kinetic constants were calculated from double-reciprocal plots by using the method of least squares. Hill plots were drawn and analyzed as described by Atkinson (1966).

Isoelectric Focusing. Partially purified uroporphyrinogen decarboxylase was subjected to preparative isoelectric focusing in flat beds of granulated gel. The gel bed containing sample was prepared by using Sephadex G-75 as described by Winter (1977), except that 10% (w/v) glycerol was included in the gel slurry. Focusing was carried out at 4 °C for 12–16 h at 6 W (constant power). The resulting pH gradient was determined by eluting 0.5 × 0.5 cm^2 sections of the gel in 1.0 mL of distilled water and determining the pH of the resulting eluate.

Gel sections were eluted over at least 4 h with 1.5–2 volumes of 0.1 M potassium phosphate buffer, pH 6.8, containing 10 mM dithiothreitol and 10% (w/v) glycerol. The pH was adjusted to 6.8–7.0 when necessary with 1 M K_2HPO_4. Ampholines were removed by desalting on Sephadex G-25 into the desired final buffer.

Analytical Polyacrylamide Gel Electrophoresis. Analytical disc gel electrophoresis was performed in 7.5% acrylamide [30:1 acrylamide:bis(acrylamide)] by using the Davis buffer system as modified by Fairbanks et al. (1971). For systems at other pH values, the formulations described by Gabriel (1971) were used. The gels were stained in Coomassie brilliant blue G-250 (0.07% in 4% $HClO_4$) for 1–2 h and then destained in several changes of 7% (w/v) acetic acid (Reisner et al., 1975).

Two-dimensional electrophoresis was performed essentially as described by O'Farrel (1973), except that the first dimension was run as described above rather than in isoelectric focusing gels.

Correlation of Enzymic Activity with Protein Bands on Agarose Slab Gels. A partially purified sample of decarboxylase was electrophoresed in 2% agarose slab gels (3 mm thick) in sodium barbital buffer, pH 8.6 (μ = 0.1). The gels were sectioned perpendicular to the direction of electrophoresis, and each section was eluted with 0.5 mL of 50 mM potassium phosphate buffer. Each eluate was assayed for decarboxylase activity and electrophoresed in polyacrylamide gels as described above.

Molecular Weight Determination. The molecular weight of uroporphyrinogen decarboxylase was determined by gel filtration on a Sephadex G-150 column (1.5 cm i.d. × 74 cm) developed in 50 mM potassium phosphate buffer, pH 6.8. Standards used were catalase (M_r 232 000), transferrin (M_r 74 000), bovine serum albumin (M_r 65 000), hepatic porphobilinogen deaminase (M_r ~32 000), bovine pancreatic ribonuclease (M_r 13 700), and horse heart cytochrome *c* (M_r 12 400). The sample volume was 4.0 mL. The column was developed ascending at 8.5 cm·h^{-1}. The determination was repeated with a Sephacryl S-200 column (2.2 cm i.d. × 80 cm), developed in 50 mM potassium phosphate buffer (pH 6.8) containing 0.1 M KCl, ascending at 3.2 cm·h^{-1}.

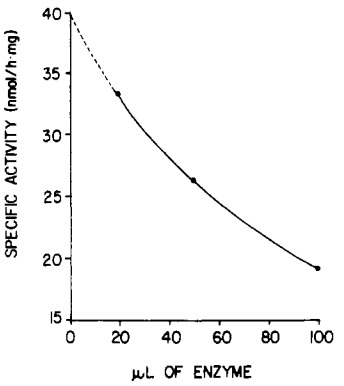

FIGURE 1: Decrease in specific activity of bovine hepatic uroporphyrinogen decarboxylase with the addition of excess enzyme to the standard assay mixture. The enzyme source was eluted from DEAE-cellulose, protein concentration 3.16 mg·mL^{-1}.

Results

Purification of Uroporphyrinogen Decarboxylase. The results of a representative purification of uroporphyrinogen decarboxylase from bovine liver are shown in Table I. At all steps of purification, optimal activity was obtained at pH 6.8–7.0 in buffers with an ionic strength of approximately 0.1 M. Addition of pyridoxal phosphate, biotin, avidin, thiamin pyrophosphate, or heated rat or bovine liver cytosol (60 °C, 5 min) had no effect on enzymic activity.

To accurately determine specific activity at each step of purification, it was important to add less than 4 units of enzymic activity to each assay. Calculated specific activity fell when excess enzyme was added to the assay mixture (Figure 1). This effect, presumably due to protein–protein interactions, was most marked at the earlier steps in the purification scheme.

The enzyme is cytosolic and was separated from organelles and membrane components by centrifugation. Recovery of enzymic activity from the pH 5.5 supernatant could be increased to greater than 95% by washing the precipitate with dilute sodium acetate buffer, pH 5.5. Recovery from the ammonium sulfate precipitation step averaged approximately 75%.

Chromatography on DEAE-cellulose provided a 25-fold purification and was highly reproducible with enzymic activity eluting at about 0.18 M KCl. Chromatography on phenyl-Sepharose provided an additional 2-fold purification. Enzyme activity eluted at approximately 30–40% ethylene glycol.

Between runs, phenyl-Sepharose was regenerated by thorough washing with distilled water only. The yield of enzyme decreased dramatically when the phenyl-Sepharose was fresh or had been washed with alcoholic solutions.

Chromatography on hydroxylapatite was unpredictable but required if pure enzyme was to be obtained. The enzyme, which eluted at the beginning of the phosphate gradient, was unstable and had to be concentrated and immediately run on Sephacryl S-200. Concentration was done by using an Amicon PM-10 membrane in a pressure dialysis cell under Ar. No enzyme appeared in the dialysate, but there was always a loss of enzyme activity during this process. Washing the membrane with solutions varying in ionic strength, glycerol content, or 2-mercaptoethanol content failed to elute activity from the membrane. Sephacryl S-200 chromatography yielded from two to five protein peaks. Enzyme activity appeared in a single symmetrical peak. Recovery from the hydroxylapatite and Sephacryl S-200 steps together rarely exceeded 50%.

Enzyme recovered from either the DEAE-cellulose or the ammonium sulfate steps was stable for prolonged (greater than 2 years) periods when stored at −70 °C in 10–50 mM potassium phosphate buffer, pH 6.8. Following extended storage, any apparent decrease in activity could be restored by incubation with 10 mM dithiothreitol just prior to assay. At higher states of purity, stability was enhanced by 10% glycerol (w/v) or 0.1 M KCl. Exposure of the enzyme, even for brief periods, to alkaline buffers (pH ≥8.5) resulted in marked and irreversible loss of activity. At any stage of purification, freezing the enzyme in the presence of 5–10 mM 2-mercaptoethanol or dithiothreitol resulted in protein precipitation and nearly complete loss of decarboxylase activity. In addition, enzyme recovered from hydroxylapatite, regardless of the presence or absence of glycerol or 2-mercaptoethanol, was very unstable under any storage conditions. This instability accounts for the low activity and recovery values for this step recorded in Table I since the high-pressure liquid chromatography (HPLC) assay was usually done 24–48 h after active fractions had been identified fluorometrically.

Enzyme recovered from DEAE-cellulose or Sephacryl S-200 could be preincubated up to 30 min at 45 °C with only slight loss of activity. However, heating decarboxylase at 60 °C for 5 min resulted in a 60% loss of activity; after 10 min at 60 °C, no detectable decarboxylase activity remained.

Isoelectric Point. The isoelectric point of uroporphyrinogen decarboxylase was determined by isoelectric focusing of samples of partially purified enzyme in a flat-bed apparatus. Decarboxylase activity focused in a single band at pH 4.60 ± 0.10.

Molecular Weight. The molecular weight of uroporphyrinogen decarboxylase was estimated by molecular seive chromatography on Sephadex G-150 (in 50 mM potassium phosphate, pH 6.8) and on Sephacryl S-200 (in 50 mM potassium phosphate, pH 6.8, containing 0.1 M KCl). In both cases, enzyme activity eluted as a single symmetrical band with an apparent M_r of (56–58) × 10^3.

Polyacrylamide Gel Electrophoresis of Uroporphyrinogen Decarboxylase. Discontinuous polyacrylamide gel electrophoresis of the active fraction obtained from Sephacryl S-200 chromatography revealed a single component with a relative mobility of about 0.5. Varying either the gel density (5–12% acrylamide) or the pH of the resolving gel (7.5–9.5) gave no evidence for multimeric or charge isomer forms of the enzyme (Hedrick & Smith, 1968). Activity could be recovered from a single band of protein on 2% agarose gels (see Materials and Methods). Activity recovered was less than 5% of the activity applied (perhaps related to the alkaline pH at which the gel was run). Electrophoresis of this active fraction on polyacrylamide gels also revealed a single band with a relative migratory aptitude of about 0.5. Two-dimensional polyacrylamide gel electrophoresis (see Materials and Methods) showed the component associated with enzyme activity had a molecular weight of ~56 000, confirming the data from molecular sieve chromatography (see above).

Table II: Effect of Inhibitors on Uroporphyrinogen Decarboxylase Activity[a]

inhibitor	concn (mM)	% control activity
none		100
2,2′-dipyridyl disulfide	*b*	0
5,5′-dithiobis(2-nitrobenzoic acid)	*b*	<5
iodoacetamide	5	<5
NH_2OH	100	80
Mg^{2+}	10	101
Al^{3+}	10	27
Ca^{2+}	10	97
Mn^{2+}	10	70
Fe^{2+}	0.5	88
	10	55
Co^{2+}	10	32
Cu^{2+}	10	<5
Zn^{2+}	10	15
Pb^{2+}	10	14

[a] Activity was measured in the standard Tris-phosphate-buffered mixture with enzymically generated uroporphyrinogen I (30 μM) as substrate. Inhibitors were added to the enzyme solution at 4 °C prior to assay. Metals were chloride salts, except lead acetate and ferrous ammonium sulfate. [b] Enzyme was mixed with these agents (3.0 mM) and then dialyzed against 50 mM potassium phosphate buffer, pH 6.8, to remove unbound reagent.

Effects of Inhibitors on Enzyme Activity. Reaction of the enzyme with sulfhydryl reagents resulted in the loss of enzymic activity (Table II). Inhibition by 2,2′-dipyridyl disulfide or 5,5′-dithiobis(2-nitrobenzoic acid) could be reversed by incubation with 10 mM dithiothreitol at 4 °C prior to assay. Several metal ions (Al^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+}) were also found to inhibit enzymic activity in the standard assay mixture (Table II).

Factors Affecting Distribution of the Reaction Products. The primary substrates for uroporphyrinogen decarboxylase in vivo are the octacarboxylate uroporphyrinogens III and I. The ultimate reaction products are the tetracarboxylate coproporphyrinogens III and I. Porphyrinogens with seven, six, and five carboxylate groups are both intermediate reaction products and substrates for the enzyme. The composition of the reaction products was affected by the ratio between the concentrations of primary substrates (octacarboxylate porphyrinogens) and enzyme in the assay mixture as well as the reaction time.

A series of experiments was done in which the concentration of either uroporphyrinogen I or III was varied from 0.5 to 20 μM in the presence of a low but fixed concentration of enzyme (1.1 unit·mL^{-1}). Under these conditions, less than 30% of the initial substrate was consumed. Only the heptacarboxylate product accumulated to any extent (0.7–0.8 μM maximal concentration) with either uroporphyrinogen I or III as substrate. The other products appeared in only trace amounts (less than 0.1 μM).

At high enzyme concentrations (11 units·mL^{-1}), greater than 50% of substrate initially present was consumed, and a very different distribution of reaction products was observed (Figure 2). At low substrate concentrations (<5 μM), the predominant product was coproporphyrinogen I or III. At uro-

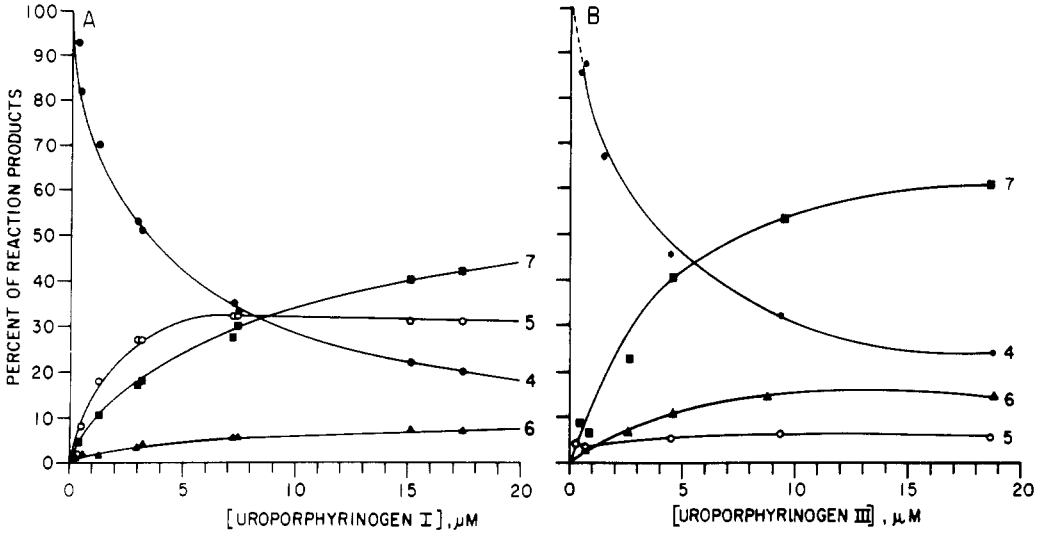

FIGURE 2: Effect of substrate concentration on the distribution of reaction products. Each assay contained 11 units·mL^{-1} bovine hepatic uroporphyrinogen decarboxylase. (A) Uroporphyrinogen I substrate. (B) Uroporphyrinogen III substrate. (■) Heptacarboxylate porphyrinogen; (▲) hexacarboxylate porphyrinogen; (○) pentacarboxylate porphyrinogen; (●) tetracarboxylate porphyrinogen (coproporphyrinogen).

porphyrinogen I concentrations greater than 10 μM (Figure 2A), or at uroporphyrinogen III concentrations greater than 5 μM (Figure 2B), the heptacarboxylate became the predominant product.

When very high concentrations of uroporphyrinogen I (30–50 μM) were used, the amount of pentacarboxylate intermediate formed exceeded that of coproporphyrinogen. This resulted in a product distribution where hepta- > penta- > tetra- > hexacarboxylate porphyrinogen. When uroporphyrinogen III (30–50 μM) was used as substrate, the product distribution was hepta- ≫ coproporphyrinogen > hexa- > pentacarboxylate porphyrinogen.

The decarboxylations of uroporphyrinogens I and III (30 μM) were also examined as a function of time. Experiments were done at enzyme concentrations of 1.1 or 11 units·mL^{-1}. The results of a representative experiment with the higher enzyme concentration with uroporphyrinogen I as a substrate are shown in Figure 3A. Under these conditions, intermediate reaction products accumulated until they reached apparent steady-state levels. Very little coproporphyrinogen I appeared until its immediate precursor (pentacarboxylate porphyrinogen I) attained a concentration of about 1.5–2.0 μM. After this point, the appearance of coproporphyrinogen I was linear with time. The distribution of the intermediate reaction products varied markedly over the 60-min time course of these experiments.

Reducing the concentration of enzyme had the effect of expanding the initial phase of the time course experiments. The composition of the reaction products as a function of time under these conditions is shown in Figure 3B. With either the I or III isomer as substrate, the major reaction product throughout the time course was the heptacarboxylate porphyrinogen.

Effects of Ionic Strength on Enzyme Activity. When the decarboxylase reaction was carried out in buffers of very low ionic strength (μ < 10 mM), enzymic activity was less than one-fourth that measured under standard conditions (μ = 100 mM). Enzymic activity was studied as a function of the concentration of monovalent cations and mono- and divalent anions. In these experiments, 0.01 M tris(hydroxymethyl)-aminomethane–*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Tris–Hepes) buffer, pH 6.8, was substituted for the 0.1 M Tris–phosphate buffer system of the standard assay mixture.

Total decarboxylase activity varied with monovalent cation concentration (Na^+, K^+, or NH_4^+), regardless of the anion present. Total activity was maximal at potassium ion concentrations between ~60 and 100 mM with either the chloride or the sulfate salts (panel A or panel C, respectively, of Figure 4). Identical results were obtained with NaCl, NH_4Cl, and sodium, potassium, or ammonium acetate. Product distribution, however, appeared sensitive to anion concentration. Below 30 mM chloride (Figure 4A) or sulfate (Figure 4B) ion concentrations, coproporphyrinogen was the predominant product. At higher anion concentrations, heptacarboxylate porphyrinogen was the predominant product. These results suggest that the net rate of decarboxylation of uroporphyrinogen is sensitive to monovalent cation concentration while the relative decarboxylation rates for intermediate porphyrinogens are sensitive to anion concentration. Optimal enzymic activity occurred at an ionic strength near 0.10 M with all salts tested. This observation led to the selection of the buffer system used in the standard assay.

Effect of Metal Ions. The effect of divalent metal ions on decarboxylase activity was examined, again substituting 0.01 M Tris–Hepes, pH 6.8, containing 0.1 M KCl for the phosphate buffer system in the standard assay mixture. The Tris–Hepes–KCl buffer has the requisite ionic strength (see above) and pH for optimal enzyme activity but, because of the very small amount of Tris present, is less likely to form metal complexes than is the phosphate buffer. Metal ions were added as their chloride salts with the exception of Pb^{2+}, which was added as the acetate salt. Metal ion solutions were made in Ar-flushed distilled water immediately before addition to the assay mixture.

Metal ions inhibited decarboxylase activity when assayed in the Tris–Hepes buffer system. Two patterns of inhibition were observed. Most metal salts (Pb^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}) inhibited the enzyme such that almost no uroporphyrinogen was decarboxylated (Figure 5A). In contrast, Mn^{2+} (and to a lesser extent Mg^{2+}) resulted in the accumulation of the heptacarboxylate product. The net decrease of enzymic activity was due to a decrease in the amount of penta- and tetracarboxylate products (Figure 5B), implying inhibition of the conversion of hepta- to pentacarboxylate porphyrinogen. Ferrous iron appeared to inhibit in both ways (Figure 5C). At low concentrations (<1.5 mM), the effects of Fe^{2+} resembled those of Mn^{2+}. At higher concentrations of Fe^{2+}, the

Table III: Kinetic Parameters for Partially Purified Porphyrinogen Decarboxylase

porphyrinogen substrate	0.5 μM < [S̄] < 5 μM			5 μM < [S̄] < 20 μM			0.5 μM < [S̄] < 20 μM		
	K_m (μM)	V_{max}[a]	r^2[b]	K_m (μM)	V_{max}	r^2	K_m (μM)	V_{max}	r^2
octacarboxylate III[c]							0.46	25	0.9686
heptacarboxylate III[c]							0.24	15	0.8363
hexacarboxylate III	0.64	44	0.7803	5.2	85	0.9590	1.2	58	0.7989
pentacarboxylate III	0.43	53	0.8248	3.9	91	0.9653	0.81	66	0.6730
octacarboxylate I	0.79	32	0.9392	3.5	47	0.8275	1.0	36	0.9271
heptacarboxylate I	0.44	42	0.9139	1.7	52	0.6387	0.59	46	0.8851
hexacarboxylate I	0.42	47	0.8971	3.4	78	0.9101	0.80	59	0.8399
pentacarboxylate I[c]							0.85	10	0.9956

[a] Nanomoles per hour per milligram of protein. [b] r^2 = coefficient of determination for the line from which the kinetic constants were derived. [c] These substrates gave linear double-reciprocal plots throughout the concentration range tested.

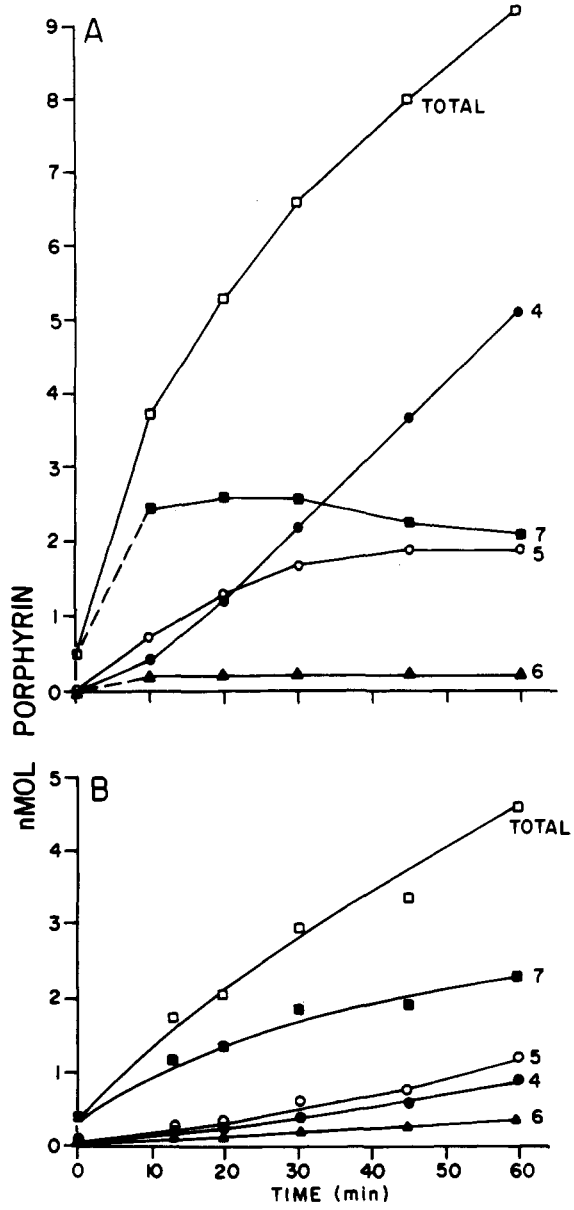

FIGURE 3: Activity and distribution of reaction products as a function of time at two concentrations of bovine hepatic uroporphyrinogen decarboxylase. The standard assay mixture (see Materials and Methods) was used to measure activity. (A) Enzyme concentration 11 units·mL^{-1}. (B) Enzyme concentration 1.1 units·mL^{-1}. (□) Total decarboxylation products; (■) heptacarboxylate porphyrinogen; (▲) hexacarboxylate porphyrinogen; (○) pentacarboxylate porphyrinogen; (●) tetracarboxylate porphyrinogen (coproporphyrinogen).

decarboxylation of uroporphyrinogen to heptacarboxylate porphyrinogen was also inhibited.

Kinetics of the Decarboxylase Reaction. In addition to uroporphyrinogens I and III, the enzyme also decarboxylates hepta-, hexa-, and pentacarboxylate porphyrinogens of each isomer type. Kinetics of the enzyme reaction were studied with each of these substrates. Initial substrate concentrations were varied between 0.7 and 20 μM.

Double-reciprocal plots produced straight lines for octa- and heptacarboxylate III and pentacarboxylate I substrates. Octa- and heptacarboxylate I and pentacarboxylate III substrates yielded plots which curved downward with increasing substrate concentration. Both hexacarboxylate substrates (I and III) yielded plots which were biphasic with strong downward curvature at high (>5 μM) substrate concentrations.

Simple plots of v vs. [S̄] were constructed from the data obtained for substrates showing curvature in the double-reciprocal plots. In each case, the data best fit two hyperbolic curves: one constructed from data with [S̄] < 5 μM and the other with [S̄] > 5 μM. Double-reciprocal plots from data within either concentration range were linear.

The results of the kinetic experiments are summarized in Table III. At low substrate concentrations, the apparent K_m's for all substrates were in the range of 0.24–0.85 μM. Under the same conditions, the values for V_{max} were in the range of 15–55 nmol·h^{-1}·mg^{-1}. A comparison of the kinetic constants for uroporphyrinogens I and III revealed that the catalytic activity of decarboxylase toward these substrates is similar (V_{max} = 32 and 25 nmol·h^{-1}·mg^{-1}, respectively). The K_m and V_{max} values obtained for heptacarboxylate III were relatively low, suggesting that the rate-limiting step in the conversion of uroporphyrinogen III to coproporphyrinogen III is the conversion of hepta- to hexacarboxylate porphyrinogen III.

The K_m's for substrates showing the greatest downward curvature in the double-reciprocal plots (hexacarboxylates I and III and pentacarboxylate III) increased 8–9-fold with increasing substrate concentrations. An approximate 2-fold increase in the V_{max} was also observed as substrate concentration was increased. Hill plots were constructed for these substrates in the low (<5 μM), high (>5 μM), and full (0.5–20 μM) concentration ranges by using the V_{max} calculated from the corresponding double-reciprocal plots. Linear Hill plots were obtained from data in both the low and high ranges of substrate concentration. Sigmoidal plots were obtained for the full concentration ranges. The slope of the inflection point was used to calculate n_{app}. For each of the substrates, regardless of concentration range, the calculated n_{app} was in the range of 0.72–1.20.

Discussion

Uroporphyrinogen decarboxylase has been purified over 700-fold from bovine liver. The purified protein, with an apparent mass of 5.7 × 10^4 daltons, catalyzes all four decarboxylation reactions in the conversion of uroporphyrinogen to coproporphyrinogen. The relationship between product

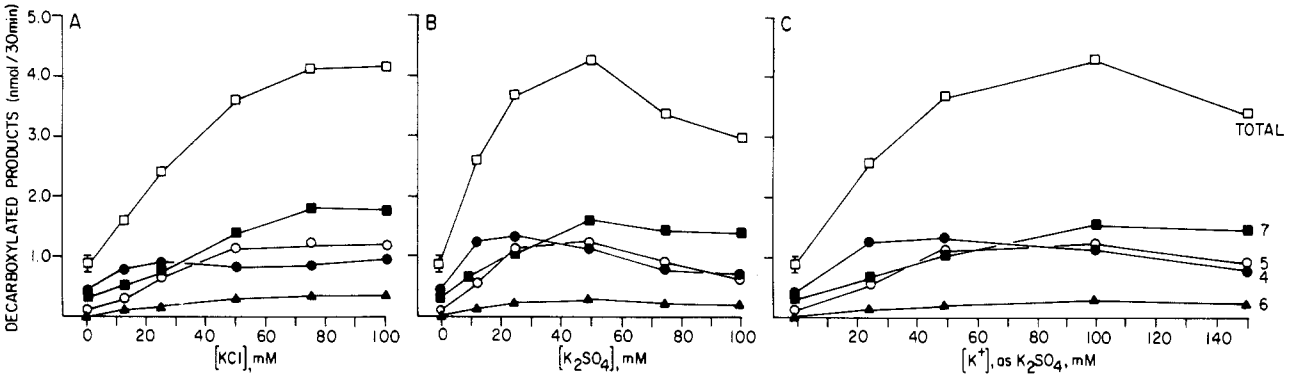

FIGURE 4: Effect of salts on the activity of uroporphyrinogen decarboxylase. Tris–Hepes buffer (μ = 0.01 M, pH 6.8) was substituted for the Tris–phosphate buffer of the standard assay mixture (see Materials and Methods). Uroporphyrinogen I (30 μM) was substrate. (A) Activity as a function of [KCl] (=[K^+] + [Cl^-]). Identical results were obtained with the chloride and acetate salts of K, Na, or NH_4. (B) Activity as a function of [SO_4^{2-}]. Identical results were obtained with K_2SO_4, Na_2SO_4, or $(NH_4)_2SO_4$. (C) Activity as a function of [K^+] added as K_2SO_4. (□) Total decarboxylation products; (■) heptacarboxylate porphyrinogen; (▲) hexacarboxylate porphyrinogen; (○) pentacarboxylate porphyrinogen; (●) tetracarboxylate porphyrinogen (coproporphyrinogen).

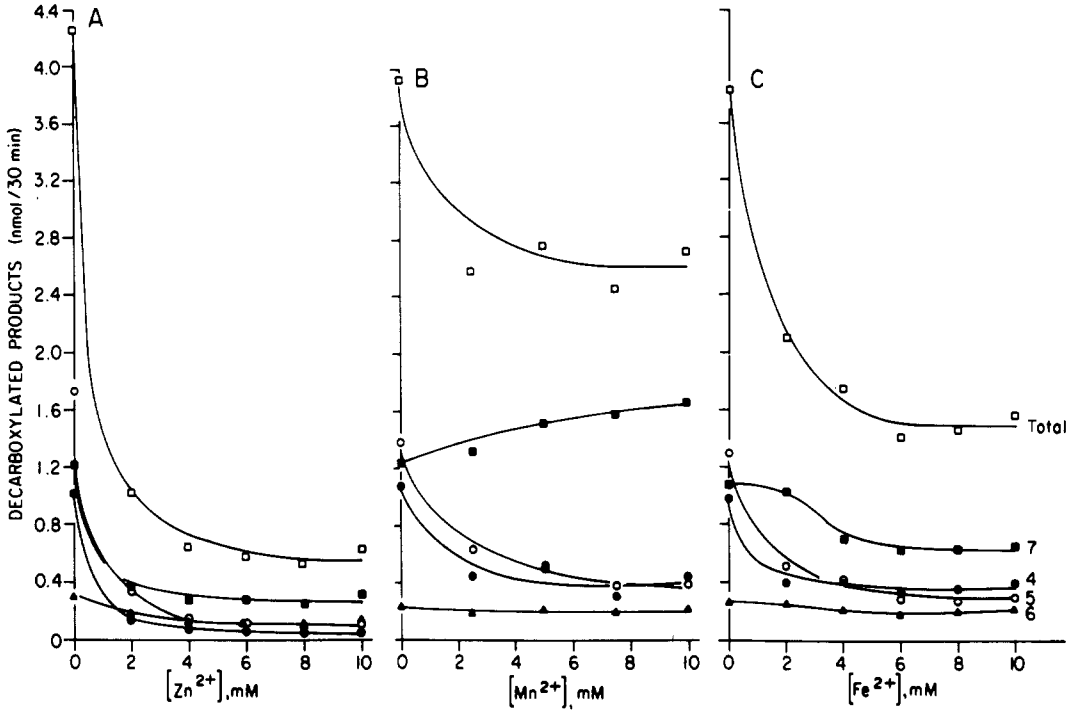

FIGURE 5: Effects of metal ions on enzyme activity and product composition. Assay conditions were as in Figure 4. (A) Activity as a function of [$ZnCl_2$]. Similar results were obtained with lead acetate, $CuCl_2$, and $CoCl_2$. (B) Activity as a function of [$MnCl_2$]. (C) Activity as a function of [$FeCl_2$]. (□) Total decarboxylation products; (■) heptacarboxylate porphyrinogen; (▲) hexacarboxylate porphyrinogen; (○) pentacarboxylate porphyrinogen; (●) tetracarboxylate porphyrinogen (coproporphyrinogen).

distribution and enzyme concentration remained constant throughout the purification. Electrophoresis on denaturing gels gave no evidence for the existence of subunits. The enzyme has optimal activity at pH 6.8–7.0 and at an ionic strength near 0.1 M. No evidence was found for a cofactor requirement.

Partially purified enzyme was quite stable. Enzyme recovered from the ammonium sulfate or DEAE-cellulose steps was stable for several years at −70 °C. At higher degrees of purity, the enzyme was less stable, but stability could be improved by the addition of glycerol. Freezing in the presence of thiols resulted in protein precipitation and irreversible loss of enzymic activity. At high states of purity, the enzyme appeared to adhere to membranes and glass. This property markedly affected yields in the final steps of purification. Enzyme activity was markedly diminished after even a brief exposure to high pH (>8.5). In contrast, the enzyme appeared quite stable at acidic pH (down to pH 4.6).

Inhibition of uroporphyrinogen decarboxylase by sulfhydryl reagents suggests that at least one sulfhydryl group is required for catalytic activity. Slight losses of activity observed after prolonged storage could be restored by preincubation of the enzyme with dithiothreitol. The sulfhydryl group(s) appear to be in the interior of the protein since the enzyme failed to bind to thiopropyl-Sepharose (data not shown).

Uroporphyrinogen decarboxylase is an acidic protein with an isoelectric point at pH 4.6. At neutral pH, the protein bears a net negative charge. At least a portion of its negative charge likely resides on the surface since the enzyme has a high affinity for positively charged DEAE-cellulose. The enzyme also binds to phenyl-Sepharose, suggesting that a hydrophobic domain is accessible.

The substrates for uroporphyrinogen decarboxylase are large, multivalent anions with from five to eight negatively charged carboxylate groups surrounding a relatively hydrophobic tetrapyrrole core. When uroporphyrinogen de-

carboxylase is assayed in buffers of very low ionic strength (<10 mM), very little activity is detected. Net enzymic activity increases with increasing ionic strength, primarily as a function of monovalent cation concentration. This cation effect may result from neutralization of negative charges on the enzyme surface. Anion concentration, on the other hand, primarily affected product distribution. This suggests that the active catalytic site(s) may contain positively charged residues required for proper orientation of the appropriate substrate.

More than one K_m could be calculated for some substrates of uroporphyrinogen decarboxylase, but for all substrates, calculations yielded a Hill coefficient of approximately 1. These kinetic data suggest that the enzyme has at least two noninteracting active sites. Three substrates (octa- and heptacarboxylates III and pentacarboxylate I) "fit" only one site as reflected by linear double-reciprocal plots. Biphasic double-reciprocal plots generated for the other five substrates suggest that these substrates could occupy at least two active sites, characterized as low- and high-affinity sites. These sites are likely substrate selective since metal salts inhibited enzyme activity in two different ways. Some metals (Pb^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+}) inhibited the conversion of uroporphyrinogen to heptacarboxylate porphyrinogen. Others (Mn^{2+}, or Mg^{2+}, and Fe^{2+} at low concentration) primarily affected the decarboxylation of heptacarboxylate porphyrinogen.

Under physiological conditions, uroporphyrinogen decarboxylase activity exceeds the activities of the rate-determining enzymes of the heme biosynthetic pathway, 5-aminolevulinic acid synthase and porphobilinogen deaminase (Bishop & Desnick, 1982). In a normal cell, the substrate concentration remains far below saturating levels. Under conditions in which the ratio of enzyme to substrate is high, the kinetic data presented here predict that the predominant reaction product would be coproporphyrinogen, which is in fact the case in vivo. Relatively small amounts of intermediate porphyrinogen substrates accumulate or are excreted in normal subjects.

Patients with porphyria cutanea tarda have an inherited defect in hepatic uroporphyrinogen decarboxylase activity (Kushner et al., 1976), and clinical expression of the disease requires the presence of excess iron in hepatocytes (Felsher & Kushner, 1977). Based on the results presented here, this would be expected to result in a decreased enzyme to substrate ratio and the accumulation of uroporphyrinogen and heptacarboxylate porphyrinogen. The low K_m and low catalytic activity of the enzyme for heptacarboxylate porphyrinogen III predict that this would be the only decarboxylation product to accumulate to any significant degree. This is exactly what is observed in patients with porphyria cutanea tarda (Kushner, 1981). Thus, the pattern of porphyrin accumulation and excretion in porphyria cutanea tarda is consistent with the kinetic situation in which the ratio of decarboxylase activity to uroporphyrinogen substrate concentration is low.

Uroporphyrinogen decarboxylase has recently been purified from human erythrocytes (deVerneuil et al., 1983). The Michaelis constants for the human erythrocyte and bovine hepatic enzymes are quite similar, although the specific activity for the bovine hepatic enzyme is about 10-fold lower than that of the human erythrocyte enzyme. Other similarities include the molecular weights, the isoelectric points, and the requirements for sulfhydryl groups. The enzyme from erythrocytes stored at 4 °C appears to undergo proteolysis, resulting in the loss of a portion of the molecule of mass 10000 daltons and a 10-fold decrease in K_m for the pentacarboxylate substrate. This was not observed in our preparation as liver was processed quickly after removal from the animal.

deVerneuil et al. (1983) reported only slight inhibition of the erythrocyte enzyme by Fe^{2+}. Their studies were done in 100 mM phosphate buffer, which would be expected to complex with ferrous ions. In our studies done in 100 mM phosphate buffer, 0.5 mM Fe^{2+} produced only ~12% inhibition of enzyme activity (Table II), a result nearly identical with that of deVerneuil et al. In the presence of 100 mM phosphate, we observed a 45% inhibition of enzyme activity with 10 mM Fe^{2+}. In our studies in the Tris–Hepes buffer system, however, ~50% inhibition occurred with 2 mM Fe^{2+} (Figure 5C). Our findings support the hypothesis that iron plays an important role in the pathogenesis of porphyria cutanea tarda.

Uroporphyrinogen decarboxylase is an unusual enzyme in several ways. First, the enzyme does not appear to require a coenzyme. All other decarboxylation reactions in intermediary metabolism utilize pyridoxal phosphate, thiamin pyrophosphate, or biotin as cofactors, with the exception of coproporphyrinogen III oxidase (Jackson et al., 1976) and histidine decarboxylase (Chang & Snell, 1968). Histidine decarboxylase has an N-terminal pyruvyl residue which functions in a manner analogous to pyridoxal phosphate. Second, the substrates for uroporphyrinogen decarboxylase have no α-heteroatom substituents. Decarboxylating enzymes generally act on substrates which are α-amino or α-keto acids. Uroporphyrinogen decarboxylase is unique in that it is a single polypeptide which catalyzes four distinct reactions in the conversion of a substrate to its final (and only physiologically important) product. We know of no other enzyme which catalyzes such a conversion. Purification of this enzyme should permit structural and mechanistic studies on the catlytic mechanism.

The purification of the enzyme from bovine liver has provided a scheme applicable to the purification of the enzyme from human liver. This should allow a comparison of the properties of the enzyme derived from normal human liver with that obtained from persons with porphyria cutanea tarda, thereby defining the specific nature of the enzymic defect in this disease.

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Registry No. EC 4.1.1.37, 9024-70-8; octacarboxylate III, 1976-85-8; heptacarboxylate III, 86689-80-7; hexacarboxylate III, 31922-78-8; pentacarboxylate III, 31922-77-7; octacarboxylate I, 1867-62-5; heptacarboxylate I, 66365-86-4; hexacarboxylate I, 86689-81-8; pentacarboxylate I, 72698-25-0.

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Isolation and Covalent Structure of the Aspirin-Modified, Active-Site Region of Prostaglandin Synthetase†

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ABSTRACT: Aspirin (acetylsalicylic acid) inhibits prostaglandin synthesis by acetylating a single internal serine residue of the initial enzyme in the biosynthetic pathway, prostaglandin synthetase. In this study, the region of the enzyme that is modified by aspirin has been isolated, and its amino acid sequence has been determined. Sheep vesicular gland [*acetyl*-^{3}H]prostaglandin synthetase was purified following treatment with [*acetyl*-^{3}H]aspirin and digested with pepsin. An acetyl-^{3}H-labeled peptic peptide of approximately 25 residues was isolated by high-pressure liquid chromatography, and its amino acid sequence was determined to be Ile-Glu-Met-Gly-Ala-Pro-Phe-Ser-Leu-Lys-Gly-Leu-Leu-Gly-Asn-Pro-Ile-Glu-Ser-Pro-Glu-Tyr. The acetylated serine residue was located at position 8 in this sequence. The current study marks this polypeptide sequence as a region related to an active site of the enzyme.

Prostaglandin (PG)[1] synthetase (cyclo-oxygenase, prostaglandin endo-peroxide synthetase) catalyzes the first step in prostaglandin synthesis: the oxygenation of arachidonic acid to PGG_2 and the subsequent peroxidase-mediated reduction of PGG_2 to PGH_2 (Sammuelsson, 1972; Nugteren & Hazelhof, 1973; Miyamoto et al., 1976; Van der Ouderaa et al., 1977). The enzyme has been purified and characterized as a labile, membrane-bound, heme protein, M_r 70000, which requires an indole or phenol cofactor for full enzymatic activity in vitro (Rollins & Smith, 1980; Roth et al., 1981; Ohki et al., 1979; Smith & Lands, 1971).

Aspirin (acetylsalicylic acid) inhibits the oxygenase activity of cyclo-oxygenase by acetylating a single internal serine residue located somewhere within the polypeptide chain (Roth et al., 1975, 1980; Roth & Siok, 1978). Van der Ouderaa et al. have isolated a dipeptide, Phe-Ser, that contains the acetylated serine residue (Van der Ouderaa et al., 1980), but no other published sequence information is available for this region of the enzyme. In this study, we have used pepsin digestion to obtain a peptide of approximately 25 residues that contains the acetylated serine at position 8. The sequence data from this study document the fact that the acetylated serine is an internal residue and serves to mark this portion of the polypeptide chain as an active-site region of the enzyme.

Experimental Procedures

Preparation of Acetyl-^{3}H-Labeled Peptic Peptide. Prostaglandin synthetase was purified in the acetylated, ^{3}H-labeled form as described (Roth, 1982) through the first DEAE-

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‡ Established Investigator of the American Heart Association.

[1] Abbreviations: PG, prostaglandin; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin.

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