Purification and Properties of Uroporphyrinogen I Synthase from Human Erythrocytes

IDENTIFICATION OF STABLE ENZYME-SUBSTRATE INTERMEDIATES*

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Uroporphyrinogen I synthase (EC 4.3.1.8) was purified from human erythrocytes more than 42,000-fold with a 25% yield. The purification procedure included a preparative DEAE-cellulose step followed by sequential chromatography on octyl-Sepharose, phenyl-Sepharose, Sephadex G-100, and DEAE-cellulose. The final anion exchange step resolved uroporphyrinogen I synthase activity into five forms which were designated A, B, C, D, and E. These forms also were separated by polyacrylamide disc gel electrophoresis and isoelectric focusing. The least charged forms, A and B, represented about 75% of total activity and appeared homogeneous by sodium dodecyl sulfate and analytical polyacrylamide gel electrophoresis at pH 6.8 and 8.2. The A and B forms had similar physical and kinetic properties including specific activities of about 2,500 units/mg of protein, pH optima of 6.2, K_m values of ~6 μM, inhibition by sulfhydryl reagents, and identical amino acid compositions. The molecular weights of the A and B activities were 36,000 and 38,000 by gel filtration; under denaturing conditions, both A and B forms had a molecular weight of 37,000, indicating that these forms were monomers. [3H]Porphobilinogen was synthesized and used to demonstrate that the multiple forms represented intermediates in the stepwise conversion of the monopyrrole to the tetrapyrrole. When homogenous A or B enzyme was individually incubated with [3H]porphobilinogen and then subjected to electrophoresis, all five forms were observed; the more anodal bands of activity contained proportionately more radiolabel. These data are consistent with uroporphyrinogen I synthase A being the native enzyme and the B, C, D, and E charge isomers corresponding to the enzyme-substrate (mono-, di-, tri-, and tetrapyrrole) intermediates.

Uroporphyrinogen I synthase (EC 4.3.1.8) is an enzyme in the heme biosynthetic pathway which catalyzes the head-to-tail condensation of 4 molecules of the monopyrrole, porphobilinogen, to form the tetrapyrrole, uroporphyrinogen I (1). Since the reaction is accompanied by the release of 1 mol of ammonia for each mole of porphobilinogen used, the enzyme also is known as porphobilinogen deaminase. The interaction of uroporphyrinogen I synthase with uroporphyrinogen III cosynthase is required for the formation of the asymmetric stereoisomer, uroporphyrinogen III (2–4).

Uroporphyrinogen I synthase has been purified to homogeneity from spinach (specific activity ~1000 units/mg) (5) and Rhodopsseudomonas spheroides (specific activity ~30,000 units/mg) (6,7). To date, the enzyme has been shown to be purified up to 160- to 160-fold with specific activities of 5.4 (8) and 16 units/mg (9), respectively. The molecular weights of the native plant, bacterial, and human enzymes have been estimated to be 40,000 (5), 36,000 (6, 7), and 25,000 (10), respectively. SDS1 electrophoresis of the denatured spinach and bacterial enzymes indicated that they were monomers. The subunit compositions of the bovine and human enzymes have not been reported. Recent interest in the human enzyme has been prompted by the finding of reduced uroporphyrinogen I synthase activity in tissues from individuals with acute intermittent porphyria (11, 12). This dominantly inherited disorder is characterized by acute neurologic symptoms and excretion of the porphyrin precursors, δ-aminolevulinic acid and porphobilinogen (13). Biochemical and immunologic investigations of the enzymatic lesion and the nature of the genetic defect in this disease have been limited by the lack of highly purified human uroporphyrinogen I synthase. Furthermore, studies of the mechanism of mammalian tetrapyrrole biosynthesis would be facilitated by the availability of highly purified enzyme.

In this communication, we describe the purification and properties of homogeneous human uroporphyrinogen I synthase from human erythrocytes. In addition, we report, for the first time, the occurrence of multiple forms of the enzyme and demonstrate that these charge isomers are enzyme-substrate intermediates in the conversion of porphobilinogen to uroporphyrinogen.

EXPERIMENTAL PROCEDURES

Materials

Oxidized human erythrocytes were obtained from the Greater New York Blood Center, Sepharose 4B, octyl-Sepharose CL-4B, phenyl-Sepharose CL-4B, and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. DEAE-cellulose was obtained from Sigma Chemical, St. Louis, MO. Cytochrome c, aldolase, "M"-dihydroalcoholase, "M"-dihydroalcoholase, δ-aminolevulinic acid, hydrochloride, coproporphyrin, and low fluorescence imidazole were prepared in this laboratory. 1SDS1 electrophoresis of the denatured spinach and bacterial enzymes indicated that they were monomers. The subunit compositions of the bovine and human enzymes have not been reported. Recent interest in the human enzyme has been prompted by the finding of reduced uroporphyrinogen I synthase activity in tissues from individuals with acute intermittent porphyria (11, 12). This dominantly inherited disorder is characterized by acute neurologic symptoms and excretion of the porphyrin precursors, δ-aminolevulinic acid and porphobilinogen (13). Biochemical and immunologic investigations of the enzymatic lesion and the nature of the genetic defect in this disease have been limited by the lack of highly purified human uroporphyrinogen I synthase. Furthermore, studies of the mechanism of mammalian tetrapyrrole biosynthesis would be facilitated by the availability of highly purified enzyme.

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chased from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin was obtained from Schwarz/Mann Research Laboratories, Orangeburg, NY. Catalase was purchased from Worthington Biochemical Corp., Freehold, NJ. Chymotrypsinogen was obtained from Calbiochem, La Jolla, CA. Dowex 1-X8 ion exchange resin and materials used for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. NCS tissue solubilizer was obtained from Amersham Corp., Arlington Heights, IL. Amphot- 
ylates were purchased from LKB Instruments, Inc., HICKsville, NY.

Polyporphyrin standards were obtained from Polyporphyrin Products, Logan, UT. Cyanogen bromide, 3-aminopropylthiolicysines, 1,6-di-

amino-1,6-dihydroxy-6-nitroadipic acid, 1-ethyl-3-(3-dimeth-ylaminopropyl)-carboxydime hydrochloride were obtained from Pierce Chemical Co., Rockford, IL. Amicon ultrafiltration and hollow fiber cartridges were purchased from Amicon Corp., Lexington, MA. All other chemicals were of highest grade obtainable.

Methods

Synthesis of Porphobilinogen—Porphobilinogen was synthesized by modification of the procedure of Gurne and Shemin (14) using immobilized human δ-aminolevulinate dehydratase purified from human red cells. The enzyme was covalently bound to Sepharose 4B using the procedure of March et al. (16) or, alternatively, the enzyme was hydrobiologically immobilized on phenyl-Sepharose (16). The immobilized enzyme was maintained at 37°C in a column (6 x 10 cm) and a solution of 2 mM δ-aminolevulinate chloride in 20 mM potassium phosphate buffer, pH 6.7, containing 0.3 mM dithiothreitol was pumped through the column at a rate of 0.7 ml/min. In order to separate unreacted δ-aminolevulinate from porphobilinogen, a second column (2.6 x 40 cm) containing Dowex 1-X8 ion exchange resin (acetate form) was placed in series with the enzyme reactor. When the effluent from the Dowex 1-X8 column containing δ-aminolevulinate was washed with distilled water (400 ml) followed by 4 M acetic acid to elute the bound porphobilinogen. Lyophilization resulted in a white powder of porphobilinogen acetate. A portion of salt was dissolved in distilled water and converted to porphobilinogen hydrate by adjusting the pH to 6 with M NaOH; the hydrate was lyophilized to remove ammonium acetate. Using a 1-butanol/water/acetic acid (45:45:1) solvent system, freshly synthesized porphobilinogen co-migrated with an authentic sample provided by Dr. David Shemin (14). The porphobilinogen was also converted to uroporphyrin by incubation at 100°C.

Stock Solutions—A 1 ml sample containing 1 μl of δ-aminolevulinate in 0.1 M potassium phosphate, pH 6.7, and 25 units of human δ-aminolevulinate dehydratase was incubated at 37°C for 1 h and then placed immediately on ice. Porphobilinogen was then separated from δ-aminolevulinate and isolated as the acetate salt by ion exchange chromatography on Dowex 1-X8 and lyophilized as described above.

Uroporphyrinogen I Synthase Assay—The enzyme was assayed by quantitation of uroporphyrin fluorescence using a Turner model 111 fluorometer equipped with a 405 nm primary filter and a 515 nm secondary filter. For routine assays, the standard reaction mixture contained 50 μl of appropriately diluted sample, 750 μl of 0.1 M Tris/ HCl buffer containing 0.1 mM dithiothreitol, and 100 μl of 0.1 M δ-aminolevulinate. The solution was mixed and incubated in the dark at 37°C for 20 min. The reaction was then stopped by the addition of 100 μl of 5 N HCl and exposed to light for 20 min to oxidize the uroporphyrinogen to uroporphyrin. When large amounts of hemoglobin were present, the procedure was modified by the addition of 100 μl of 50% trichloracetic acid to terminate the reaction. Precipitated protein was then pelleted by centrifugation at 2000 × g for 10 min. If samples to be assayed had protein concentrations of less than 0.5 mg/ml, bovine serum albumin (2 mg/ml) was included in the assay buffer. The assay was linear from 0.005 to 0.0125 μmol of uroporphyrin produced/ml. One unit of uroporphyrinogen I synthase activity represents the amount of enzyme activity present at 1 μg/ml that results in the synthesis of 1 nmol of uroporphyrin per minute at 25°C. Analysis of porphyrins and porphyrin isomer composition was performed by standard methods (17, 18).

Protein Assay—Protein concentrations were determined using the fluorescamine method described previously (13).

Analytical Polyacrylamide Gel Electrophoresis—Samples were electrophoresed on polyacrylamide disc gels using a constant current of 1 mA/gel in a pH 6.8, low fluorescence imidazole/sodium 4-(2-
hydr oxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer system (15) or in the pH 8.2, Tris buffer system described below. A 2 cm 25% polyacrylamide stacking gel and a 10 cm 7% running gel were prepared from a 2.6 × 40 cm column containing 0.3 mM dithiothreitol was pumped through the column at 0.2 mM porphobilinogen in 0.1 M Tris/HCl buffer, pH 8.1, at 37°C for 12 min followed by incubation of uroporphyrinogen to uroporphyrin with ultraviolet light. The red fluorescence of the uroporphyrin permitted direct visualization of bands of enzyme activity.

Isoelectric Focusing—Flat bed isoelectric focusing was performed on an LKB Multiphor apparatus using pH 4.5 to 8 polyacrylamide gels prepared according to the instruction manual (21). Polyethylene glycol (20%) also was incorporated into the gel. A constant voltage (800 V) was applied for 16 h and the gel was maintained at 4°C. The pH was determined with a surface electrode. Uroporphyrinogen I synthase activity was identified by a filter paper overlay containing 0.1 mM porphobilinogen in 0.1 M Tris/HCl buffer, pH 8.1, and visualizing uroporphyrinogen as described above.

Uroporphyrinogen I Synthase Activity Measurements—Each peak of enzymatic activity after the final DEAE-cellulose purification step was rechromatographed on DEAE-cellulose. Uroporphyrinogen I synthase A, B, C, D, and E were chromatographed separately on Sephadex G-100 to estimate native molecular weights. Molecular weight standards used were: bovine serum albumin (M, = 64,000), ovalbumin (M, = 44,000), chymotrypsinogen (M, = 25,700) and cytochrome c (M, = 12,400). Enzyme and 5 μg of each standard were applied to a column of Sephadex G-100 (superfine) previously equilibrated with 0.01 M potassium phosphate buffer, pH 8.1, containing 0.05 M KCl. Fractions (2 ml) were collected and assayed for protein and uroporphyrinogen I synthase activity. The molecular weights were estimated from the elution volumes.

The molecular weight of desaturated uroporphyrinogen I synthase was performed according to the method of Weber and Osborn (22). In addition to the calibration of proteins mentioned above, aldolase (subunit M, = 40,000) was also employed.

Amino Acid Analysis—Amino acid composition was determined following hydrolysis of 0.5 μg of purified enzyme in 0.5 ml of 3 M toluenesulfonic acid containing 10 μl of 12-ethanediol and 10 μl of phenol in evacuated sealed tubes at 110°C for 24 h. The amino acid analysis was performed on a Beckman 119 CL amino acid analyzer.

Immobilization of Porphobilinogen—Carboxyethyl-Sepharose 4B and aminohexyl-Sepharose 4B were prepared by coupling 6-aminocaproic acid or 1,6-diminohexane to Sepharose 4B using the method of March et al. (16). Aminopropyl porous glass beads were separated from Sephadex G-50 by filtration and assayed as described by Weetall and Filbert (23). To attach the carboxyl group(s) of porphobilinogen to the support, the amino group of the pyrrole was first blocked using trifluoroacetic anhydride and then the blocked pyrrole was attached to aminohexyl-Sepharose 4B using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (24). The trifluoroacetic acid group was removed with 0.1 M NaOH. To attach the amino group of porphobilinogen to carboxyethyl-Sepharose 4B, the support was activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, the gel was washed with 0.01 M sodium phosphate containing 0.5 M NaCl. The coupling agent, and then 0.02 M δ-aminolevulinate was added to the activated gel. The coupling of porphobilinogen through the pyrrole nucleus to a zirconium-clad arylamin porous glass support was accomplished via the diazo linkage procedure described by Weetall and Filbert (23). All supports contained significant quantities of porphobilinogen as measured by radioimmunoassays of 10 ml of the eluate from labeled porphobilinogen into the support and 2) a positive Ehrlich’s test (25) which produced magenta beads.

Production and Identification of Enzyme-Substrate Intermediate—A sample (80 μg) of homogeneous human uroporphyrinogen I synthase (subunit M, = 55,700) was incubated with 0.005 M p-labeled porphobilinogen for 10 min at 37°C and then placed in an ice bath. The mixtures were immediately applied in triplicate to polyacrylamide disc gels at 4°C and electrophoresed as described above. Control gels with only enzyme or substrate were electrophoresed. The gels were stained for

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activity and protein, sliced into 2-mm sections, and stained for uroporphyrinogen I synthase activity. Bands containing enzymatic activity and protein, sliced into 2-mm sections, and stained for liquid scintillation counter.

**Purification of Uroporphyrinogen I Synthase**

All purification procedures were carried out at 4°C. Although outdated human erythrocytes contained less uroporphyrinogen I synthase activity than fresh erythrocytes, pilot studies indicated that enzyme isolated from 21-day-old erythrocytes had the same Km, pH optimum, and molecular weight values as enzyme isolated from fresh cells. Therefore, outdated human blood was used for large scale purification procedures. After centrifugation at 2,000 x g, the buffy coat and plasma were removed and the erythrocytes were washed twice with cold isotonic NaCl. Osmotic lysis of 10 liters of packed erythrocytes was performed in liter aliquots by the addition of 3 volumes of cold deionized water containing 0.3 mM dithioerythritol. Stroma and incompletely lysed cells were removed by centrifugation at 12,500 x g for 90 min. The resultant supernatant was dialyzed against 7 mM potassium phosphate buffer, pH 6.80, containing 0.1 mM dithioerythritol and 0.017% sodium azide.

**Preparative DEAE Cellulose Chromatography**—A column (10 x 60 cm) containing DEAE-cellulose was thoroughly washed with the above dialysis buffer. It was important to use DEAE-cellulose which had been precycled in both acid and base. The pH was maintained below 6.87 to elute the hemoglobin in the buffer wash in order to prevent backup pressure and above 6.75 in order to bind the enzyme. The dialyzed erythrocyte lysate was pumped through the column at a flow rate of 10 ml/min and the column was washed with 3 liters of buffer. The enzyme was eluted batchwise in the same buffer containing 0.12 M NaCl and was concentrated to a volume of approximately 700 ml using an Amicon CH-4 hollow fiber concentration unit.

**Octyl-Sepharose Chromatography**—The concentrated uroporphyrinogen I synthase was passed through a column (5 x 20 cm) of octyl-Sepharose CL-4B in the same buffer used for elution of the preparative ion exchange column. The enzyme was only slightly retarded on the hydrophobic support and was easily eluted with 20 mM potassium phosphate, pH 8.0, containing 0.25 M KCl and 0.1 mM dithioerythritol.

**Phenyl-Sepharose Chromatography**—The eluate from the octyl-Sepharose column was applied directly to a column (10 x 10 cm) of phenyl-Sepharose CL-4B at a flow rate of 100 ml/h and the column was washed with 500 ml of the above buffer. The enzyme was eluted using a linear gradient of 0 to 80% ethylene glycol gradient which was constructed in a 5-chamber (2,000 ml) apparatus with equal volumes of 0, 40, 60, 80, and 80% ethylene glycol. The last chamber was held in reserve until approximately 1,500 ml of the gradient had been pumped onto the column. To achieve good yields of human uroporphyrinogen I synthase, it was necessary to adjust the pH of the alkaline ethylene glycol from pH 9.2 to 8.0 with 50 mM phosphoric acid. Fractions containing uroporphyrinogen I synthase activity were pooled and concentrated in an Amicon ultrafiltration cell using a PM-10 filter.

**Gel Filtration**—The concentrated enzyme was applied to a Sephadex G-100 column (5 x 100 cm) which was equilibrated previously with 10 mM potassium phosphate, pH 8.0, containing 0.05 M KCl and 0.1 mM dithioerythritol. Slow flow rates (0.4 ml/min) resulted in the separation of the enzyme from several visible protein bands. Fractions containing the highest specific activity were pooled, concentrated as described above, and dialyzed against 10 mM potassium phosphate, pH 8.0, containing 0.1 mM dithioerythritol.

**DEAE-Cellulose Chromatography**—The dialyzed uroporphyrinogen I synthase was applied to a column (1.6 x 40 cm) of DEAE-cellulose which had previously been equilibrated with dialysis buffer. The enzyme was eluted using a linear gradient of 0 to 0.12 M sodium chloride pumped at a rate of 1 ml/min. Each of the five peaks of enzymatic activity were concentrated and stored at -20°C.

**RESULTS**

**Purification of Uroporphyrinogen I Synthase**—Table I summarizes the results of a typical purification of uroporphyrinogen I synthase from 10 liters of outdated human erythrocytes. The enzyme was purified approximately 42,000-fold with a 25% overall yield. The initial preparative DEAE-cellulose chromatographic step resulted in separation of the enzyme from hemoglobin with a recovery of over 90%. A hollow fiber apparatus was used to concentrate the preparative DEAE-cellulose eluate, since ammonium sulfate concentration required 80% saturation and resulted in less than 50% yield.

Although uroporphyrinogen I synthase did not bind to octyl-Sepharose, this step was used to remove strongly hydrophobic proteins prior to chromatography on phenyl-Sepharose. A slight, but reproducible, stimulation of enzymatic activity was observed after the octyl-Sepharose step. The enzyme was tightly bound to the less hydrophobic phenyl-Sepharose (Fig. 1) and was eluted with either distilled water or 80% ethylene glycol, the latter providing better recovery and a 25-fold purification. Subsequent gel filtration removed the ethylene glycol and resulted in an 8-fold purification (Fig. 2). The final step, DEAE-cellulose chromatography, resolved the total uroporphyrinogen I synthase activity into five distinct peaks, designated A, B, C, D, and E (Fig. 3). When the two major peaks, A and B, were combined, the yield for this step was approximately 50%.

Attempts to construct an immobilized porphobilinogen support for the affinity purification of uroporphyrinogen I synthase were unsuccessful. The pyrrole was attached through its carboxyl groups to aminoethyl-Sepharose 4B or to aminopropyl porous glass beads and also was covalently linked by its amino group to carboxyhexyl-Sepharose 4B. The partially purified enzyme did not bind to either support even after ionic strength, buffer, pH, and temperature conditions were varied. Another affinity support, constructed by the diazo linkage of the pyrrole nucleus of porphobilinogen to aminopropyl porous glass beads, bound 100% of purified enzyme. Unfortunately, the enzyme could not be eluted even with 10 mM substrate and required either 8 M urea or 80% ethylene glycol for elution. These findings suggested that the porphobilinogen-arylamine support acted as an efficient hydrophobic support but not as an affinity column.

**Purity**—The uroporphyrinogen I synthase A and B activities appeared to be homogeneous by polyacrylamide disc gel electrophoresis at pH 6.8 and 8.2. Although the A activity migrated slightly slower than the B enzyme, each migrated as a single band which stained for both protein and activity. After SDS-polyacrylamide disc gel electrophoresis, the A and B activities exhibited identical mobilities and a single protein band as illustrated in Fig. 4

**Molecular Weight and Subunit Composition**—Each of the purified uroporphyrinogen I synthase activities resolved by the final anion exchange step was subjected to analytical gel filtration to determine the respective approximate molecular weight of the purified enzyme.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Purification of uroporphyrinogen I synthase from human erythrocytes</th>
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<tbody>
<tr>
<td>Step</td>
<td>Volume</td>
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<tr>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Outdated erythrocytes</td>
<td>10,000</td>
</tr>
<tr>
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<td>Phenyl-Sepharose</td>
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<tr>
<td>Sephadex G-100</td>
<td>13.3</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>A form</td>
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</table>

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Fig. 1. Phenyl-Sepharose chromatography of human uroporphyrinogen I synthase. The buffer wash from the preceding octyl-Sepharose chromatographic step was applied to a column (5 x 10 cm) of phenyl-Sepharose. The column was washed with 0.02 M potassium phosphate, pH 8.0, and eluted with ethylene glycol using a gradient constructed as described under “Experimental Procedures.”

W, uroporphyrinogen I synthase activity; C, protein concentration.

Fig. 2. Gel filtration of human uroporphyrinogen I synthase. Concentrated post-phenyl-Sepharose enzyme containing ethylene glycol was applied to a column (5 x 100 cm) of Sephadex G-100. The enzyme was eluted as a single peak which only produced uroporphyrinogen of the type I isomer. W, uroporphyrinogen I synthase activity; C, protein.

weigths. As illustrated in Fig. 5a, the purified A, B, C, D, and E activities had native molecular weights between 36,000 and 42,900; the more negatively charged forms had increasingly higher molecular weights. When electrophoresed under denaturing conditions, the A and B activities had subunit molecular weights of approximately 37,000 (Fig. 5b) indicating that human uroporphyrinogen I synthase is a monomeric protein.

Characterization of the Multiple Enzyme Forms—The final DEAE-cellulose elution profiles of uroporphyrinogen I synthase activity from fresh or outdated erythrocytes were nearly identical. The A, B, C, D, and E activities were stable and maintained their integrity when stored at -20°C for up to 18 months in 10 mM potassium phosphate buffer, pH 8.0, containing 0.2 mM dithioerythritol. When a mixture of uroporphyrinogen I synthase A and/or B and radiolabeled porphobilinogen was incubated at 37°C and then immediately electrophoresed at 4°C, all five forms of the enzyme were observed (Fig. 6). Increasing amounts of radiolabel were associated with the more anodal enzyme forms and a band of uroporphyrin containing about 5 x 10^7 dpm was detected near the dye front. Similarly, when the purified B enzyme was incubated with substrate and then electrophoresed, all five forms were generated. The proportional increments of radioactivity observed were consistent with the B, C, D, and E forms being stable enzyme-substrate intermediates in the stepwise condensation of 4 molecules of radiolabeled porphobilinogen to uroporphyrinogen.

Fig. 3. Chromatographic profile of human erythrocyte uroporphyrinogen I synthase on DEAE-cellulose. The post-Sephadex G-100 enzyme was applied and eluted from a column (1.6 x 40 cm) of DEAE-cellulose as described under “Experimental Procedures.” The five peaks of uroporphyrinogen I synthase activity were designated A, B, C, D, and E from least to most charged. W, uroporphyrinogen I synthase activity; C, protein.

Fig. 4. Analytical polyacrylamide disc gel electrophoresis of human uroporphyrinogen I synthase. Eight micrograms of enzyme was applied. A single band of enzymatic activity coincided with protein in gels run at pH 6.8 (left) and pH 8.2 (center). After SDS-electrophoresis, a single band (M, = 37,000) stained for protein. Electrophoretic conditions are described under “Experimental Procedures.”
subjected to analytical polyacrylamide disc gel electrophoresis at 4°C was incubated at 37°C with 0.1 μmol of purified preparation of homogenous uroporphyrinogen I synthase.

Partially purified uroporphyrinogen I synthase activity from human erythrocytes was described under "Experimental Procedures." The reaction was linear with time and protein. The K₅₀ was 200 units/mg. The calculated turnover number, assuming one active site/mol of enzyme was 15 mol of enzyme-substrate intermediates and per-mitted their electrophoretic separation at 4°C.

Isoelectric Focusing—Flat-bed isoelectric focusing of the partially purified uroporphyrinogen I synthase activity from the Sephadex G-100 step revealed the presence of five bands of enzymatic activity with pI values of 6.2, 6.3, 6.5, 6.6, and 6.8. These results were consistent with the presence of five charge isomers as demonstrated by anion exchange chromatography.

Effect of pH—Fig. 7 shows the effect of pH on the activity of uroporphyrinogen I synthase. The enzyme had a pH optimum of about 8.2; activity was not detected below pH 6.0. The activity profiles were similar in phosphate, Tris, and barbital buffers; somewhat less activity was noted with barbital buffer.

Kinetic Studies—For kinetic studies, homogeneous enzyme was dialyzed against 5 mM sodium phosphate buffer, pH 8. The reaction was linear with time and protein. The K₅₀ was determined to be 6 μM and the V₅₀ was approximately 2,300 units/mg. The calculated turnover number, assuming one active site/mol of enzyme was 15 mol of product formed/min. Enzymatic activity was temperature-dependent; little or no activity was detected at 20°C or less which prevented interconversion of the enzyme-substrate intermediates and permitted their electrophoretic separation at 4°C.

Inhibitors—The effects of several known inhibitors of uroporphyrinogen I synthase (6, 7, 9) are summarized in Table II. Sulphydryl reactive agents and lead were particularly effective inhibitors at micromolar concentrations. Iron and copper cations were effective inhibitors when employed at millimolar concentrations, whereas calcium and magnesium were the least effective inhibitors studied.

Amino Acid Composition—Table III summarizes the results of the amino acid analysis of the homogeneous A or B enzyme. The enzyme had little, if any, methionine. Despite use of protective conditions, no evidence was obtained for the presence of tryptophan and only 1 tyrosine residue/Mr = 37,000 was detected. The ultraviolet absorption spectrum of the enzyme (Fig. 8) was compatible with these results. When the hydrolysate from the homogeneous B enzyme was chromatographed, a red fluorescent peak with an absorption maximum in the Soret region was eluted just before cystathionine.

Stability—Human uroporphyrinogen I synthase A, B, C, D, and E were extremely heat-stable. When the enzyme was incubated and assayed in the presence of adequate protein
stored at high concentrations (>1 mg/ml), no enzymatic activity was lost after incubation at 56°C for 2 h and more than 90% of initial activity remained after incubation at 60°C for 2 h. Homogeneous uroporphyrinogen I synthase was stable for at least 12 months when stored at high concentrations (>1 mg/ml) in 5 mM potassium phosphate, pH 8.0, containing 0.2 mM dithioerythritol at -20°C. Erythrocyte lysates were stored in 1 mM potassium phosphate, pH 7.6, containing 0.05% Triton X-100, 1 mM dithiothreitol, and 1 mM MgCl₂ with little or no loss of activity after 12 months.

**Table II**
Inhibition of uroporphyrinogen I synthase

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<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
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<tr>
<td>HgCl₂</td>
<td>0.0004</td>
<td>80%</td>
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<tr>
<td>p-Chloromercuric benzoate</td>
<td>0.002</td>
<td>74%</td>
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<tr>
<td>Pb(NO₃)₂</td>
<td>0.005</td>
<td>35%</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1</td>
<td>80%</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>1.0</td>
<td>38%</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>1.0</td>
<td>87%</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>1.0</td>
<td>62%</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>1.0</td>
<td>58%</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>50.0</td>
<td>55%</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>50.0</td>
<td>73%</td>
</tr>
</tbody>
</table>

**Table III**
Amino acid composition of uroporphyrinogen I synthase from human erythrocytes

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol/37,000 g protein</th>
<th>Nearest integer of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>36.4</td>
<td>36</td>
</tr>
<tr>
<td>Threonine</td>
<td>18.0</td>
<td>18</td>
</tr>
<tr>
<td>Serine</td>
<td>20.7</td>
<td>21</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>40.1</td>
<td>40</td>
</tr>
<tr>
<td>Proline</td>
<td>12.0</td>
<td>12</td>
</tr>
<tr>
<td>Glycine</td>
<td>29.9</td>
<td>30</td>
</tr>
<tr>
<td>Alanine</td>
<td>22.9</td>
<td>23</td>
</tr>
<tr>
<td>Valine</td>
<td>17.0</td>
<td>17</td>
</tr>
<tr>
<td>Half cystine</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.1</td>
<td>0</td>
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<tr>
<td>Isoleucine</td>
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<td>15</td>
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<tr>
<td>Leucine</td>
<td>39.1</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
<td>7.8</td>
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<td>Lysine</td>
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<tr>
<td>Histidine</td>
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<td>3</td>
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<tr>
<td>Arginine</td>
<td>10.2</td>
<td>10</td>
</tr>
<tr>
<td>Tryptophan</td>
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</tr>
</tbody>
</table>

**Fig. 8.** Ultraviolet absorption spectrum of uroporphyrinogen I synthase A. Prior to optical spectroscopy, homogeneous A enzyme was desalted on a column (0.9 x 30 cm) of Sephadex G-50 (medium) and equilibrated with 0.05 M sodium phosphate buffer, pH 8.0. Determinations of ultraviolet absorbance were performed using a Gilford model 2000 spectrophotometer.

**Fig. 9.** Proposed reaction scheme for the biosynthesis of uroporphyrinogen by uroporphyrinogen I synthase. E₁, E₂, E₃, E₄, and E₅ represent enzyme-substrate intermediates. RC-NH₂ is the substrate porphobilinogen. The overall Kₐ of the reaction is 6 μM.

**DISCUSSION**

This is the first report on the purification to homogeneity of mammalian uroporphyrinogen I synthase. The human enzyme was purified from erythrocytes approximately 42,000-fold with a recovery of 26%. The physiologic low activity of the enzyme in human erythrocytes necessitated the use of preparative scale procedures in order to obtain milligram quantities of homogeneous enzyme. The initial DEAE-cellulose chromatographic step provided over 80-fold purification primarily by the removal of hemoglobin, the major protein contaminant. In addition, this preparative procedure could be employed simultaneously to purify, over 600-fold (15), the preceding enzyme, δ-aminolevulinate dehydratase, in the heme biosynthetic pathway.

Subsequent chromatography of uroporphyrinogen I synthase on hydrophobic supports provided a 30-fold purification. Although the enzyme did not bind to octyl-Sepharose even in the presence of 0.25 M potassium chloride, it bound tightly to phenyl-Sepharose (Fig. 1). Exploitation of these hydrophobic properties resulted in an enzyme preparation with a specific activity more than 8 times greater than the highest value previously achieved for the human enzyme (9). Gel filtration not only removed the ethylene glycol, but also separated the uroporphyrinogen I synthase from higher molecular weight proteins including any contaminating uroporphyrinogen III cosynthase. The final anion exchange step resolved the uroporphyrinogen I synthase activity into five forms, designated A through E (Fig. 3).

The following experimental findings demonstrated that the A form was the native enzyme and that the charge isomers B, C, D, and E were enzyme-substrate intermediates in the stepwise condensation of four monopyrrole substrates to the tetrapyrrole, uroporphyrinogen. 1) Incubation of the A enzyme with radiolabeled porphobilinogen resulted in the generation of the B, C, D, and E forms, each with a proportionately increased amount of radioactivity. Similarly, when the B enzyme was incubated with the radiolabeled substrate, all five forms were observed; the A form had minimal radioactivity, presumably due to substrate diffusion in the polyacrylamide gel or H₂O in the sample. 2) Coproporphyrin was detected when the homogeneous B enzyme hydrolysate was subjected to amino acid analysis. In contrast, no porphyrin was detected when the A enzyme hydrolysate was analyzed. This was consistent with the fact that porphobilinogen can be nonenzymatically condensed to uroporphyrin and subse-
The quently decarboxylated to coproporphyrin by heat (17, 18). The A, B, C, D, and E forms had similar properties and native molecular weights (Fig. 4). The monomeric structure of the enzyme forms precluded subunit interactions or aggregate formations. These findings are most consistent with the occurrence of stable enzyme-substrate intermediates which may be described by the equilibrium equation illustrated in Fig. 9. In support of this reaction scheme, recent studies of the mechanism of the enzymatic synthesis of uroporphyrinogen by NMR concluded that the product is formed by the stepwise head-to-tail condensation of monopyrroles to form the tetrapyrrrole (4). Since milligram quantities of homogeneous stable intermediates can be isolated, the nature of enzyme-substrate binding and the reaction stereochmistry can be investigated by similar techniques such as x-ray diffraction and NMR.

Finally, the availability of homogeneous uroporphyrinogen I synthase will permit the production of monoclonal antibodies for the immunochemical investigation of the enzymatic deficiency in families with acute intermittent porphyria. In addition, the stable and highly purified uroporphyrinogen I synthase will permit the production of monospecific antibodies for the immunochemical investigation of the enzymatic biosynthesis in cultured cells.

Acknowledgments—We wish to express our appreciation to Dr. David F. Bishop for experimental advice and review of the manuscript, to Dr. P. G. Katsayannis and Mr. Carlos Barreda for the amino acid analyses, to Mr. Philip Giampietro for the isoelectric focusing studies, to Mr. Raman Reddy for expert technical assistance, and to Ms. Minnie Woodson for clerical assistance.

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