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Purification and Characterization of Human Uroporphyrinogen III Synthase Expressed in *Escherichia coli*

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The side-chain asymmetry of physiological porphyrins is produced by the cooperative action of hydroxymethylbilane synthase and uroporphyrinogen (uro'gen) III synthase. Although the role of uro'gen III synthase is essential for the chemistry of porphyrin biosynthesis, many aspects, structural as well as mechanical, of uro'gen III synthase have yet to be studied. We report here an expression system in *Escherichia coli* and a purification procedure for human uro'gen III synthase. The enzyme in the lysate was unstable, but we found that glycerol prevents the activity loss in the lysate. The purified enzyme showed remarkable thermostability, particularly when kept in phosphate buffer containing DTT or EDTA, indicating that the enzyme activity may depend on its oxidation state. Examination of the relationship between the number of Cys residues that are accessible to 5,5′-dithiobis(2-nitrobenzoic acid) and the remaining activity during heat inactivation showed that a particular Cys residue is involved in activity loss. From the crystal structure of human uro'gen III synthase [Mathews *et al.* (2001) *EMBO J.* 20, 5832–5839], this Cys residue was considered to be Cys73, which is buried deep inside the enzyme, suggesting that Cys73 of human uro'gen III synthase plays an important role in enzyme activity.

Key words: congenital erythropoietic porphyria (CEP), porphyrin synthesis, hydroxymethylbilane synthase, uroporphyrinogen III synthase.

Abbreviations: CEP, congenital erythropoietic porphyria; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HMB, hydroxymethylbilane; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; PBG, porphobilinogen; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; uro'gen, uroporphyrinogen.

Porphyrin, one of the most common materials in the living world, is utilized to form heme, chlorophyll, or corrin. Heme biosynthesis proceeds by a series of sequential reactions catalysed by eight enzymes (*1*). Hydroxymethylbilane (HMB) synthase [EC 2.5.1.61], the third enzyme, condenses four porphobilinogens (PBG) successively in a head-to-tail fashion forming a linear tetrapyrrole, HMB. Next, uroporphyrinogen (uro'gen) III synthase [EC 4.2.1.75], the fourth enzyme, converts the linear tetrapyrrole accompanying D-ring inversion into uro'gen III, a molecule whose acetate and propionate side chains are asymmetric with respect to the center of the molecule as shown in Fig. 1. Because the asymmetry is not restored in the subsequent biosynthetic pathway, all the natural and physiological porphyrins and their derivatives bear asymmetric side chains. In the absence of uro'gen III synthase, HMB is spontaneously converted to uro'gen I, which has symmetric acetate and propionate side chains. The uro'gen I thus formed also can be a substrate for uro'gen decarboxylase [EC 4.1.1.37], the fifth enzyme, to produce coproporphyrinogen I, which is never used by the organism. Accumulated coproporphyrinogen I, because of its photosensitivity, is the cause of the various skin lesions found in congenital erythropoietic porphyria (CEP) patients, whose uro'gen III synthase activity in erythroid is markedly deficient, though not absent (*2*, *3*).

For each of the first three of the eight enzymes that participate in porphyrin biosynthesis there are two isozymes, an erythroid form and a ubiquitous form expressed in the liver and other organs. In contrast, no isozymes have been found for the latter five enzymes thus far. In the case of human HMB synthase (*4*), the erythroid and ubiquitous forms are produced by alternative splicing of the primary transcript of a single gene that has 15 exons. The mRNA of the ubiquitous enzyme consists of exons 1 and 3–15, and that of the erythroid form, of exons 2–15. The initiation codons do not reside in exon 2 but in exons 1 and 3; translation of the ubiquitous and erythroid enzymes is initiated at the initiation codons in exon 1 and in exon 3, respectively. As a result, the erythroid-specific enzyme lacks the 17 amino acid residues present at the N-terminus of the ubiquitous enzyme, but the remaining amino acid sequences are the same in the two forms. The regulation of transcription and splicing of the primary transcript of uro'gen III synthase is similar to that of HMB synthase. The human uro'gen III synthase gene is a single gene with 10 exons, and it carries promoters specific to the ubiquitous and erythroid forms upstream exon 1 and exon 2, respectively (*5*). The ubiquitous form utilizes exons 1, 2B and 3–10, and the erythroid form utilizes exons 2A, 2B and 3–10. Unlike HMB synthase, the initiation codon in uro'gen III

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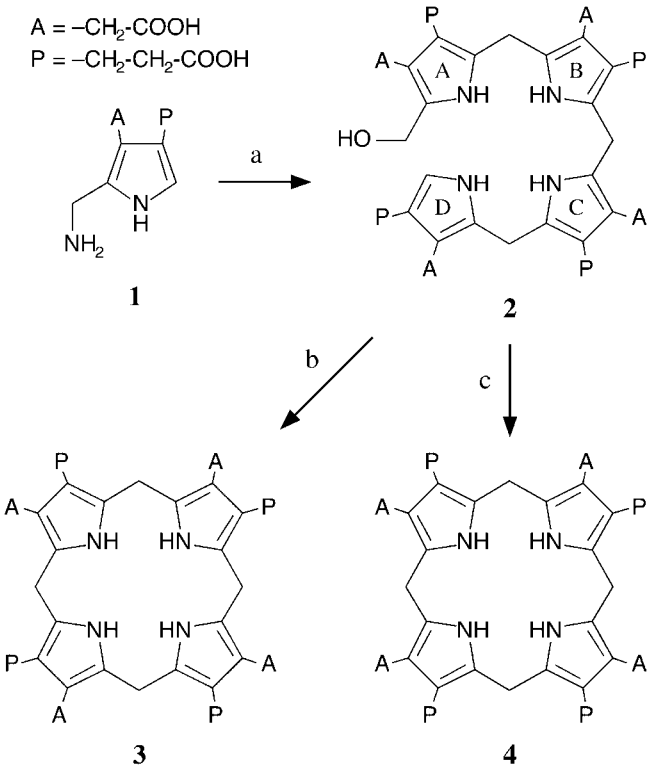

Fig. 1. **The third and fourth steps in the porphyrin biosynthetic pathway. 1**, porphobilinogen (PBG); **2**, hydroxymethylbilane (HMB); **3**, uroporphyrinogen I (uro'gen I); **4**, uroporphyrinogen III (uro'gen III). a, hydroxymethylbilane synthase; b, non-enzymatic (spontaneous); c, uroporphyrinogen III synthase.

synthase is only in exon 2B, which is common to both forms. Transcription of uro'gen III synthase, therefore, is regulated by different promoters in a tissue-specific manner, but the resulting enzymes have identical amino acid sequences.

The characteristic action of uro'gen III synthase is D-ring inversion. The mechanism of this inversion has been investigated extensively by Battersby and co-workers (*6*) using chemically synthesized analogues of the substrate and intermediate. They demonstrated that a spiro-pyrrolenine is an intermediate (*7*, *8*) and proposed a reaction scheme (*9–11*). Compared to the progress of such chemical studies, much remains to be learned about the enzymology of uro'gen III synthase. This enzyme has been purified from several sources; *Euglena gracilis* (*12*), human erythrocytes (*13*), rat liver (*14*), and *Escherichia coli* (*15*). A characteristic common to these enzymes is instability against heat treatment. The *Bacillus subtilis* enzyme expressed in *E. coli* is relatively thermostable compared to the enzymes from other sources (*16*). The full-length cDNA of human uro'gen III synthase also has been cloned and expressed in *E. coli*, but the enzyme has not been purified (*17*, *18*). More recently, a human enzyme was expressed with a His-tag, and its crystal structure was determined (*19*). Detailed expression and purification procedures for the native enzyme, however, have yet to be reported.

To determine the biological significance of porphyrin asymmetry in living systems, it is necessary to delineate the detailed mechanism of D-ring inversion during catalysis by uro'gen III synthase. Unfortunately, an attempt at co-crystallization of uro'gen III synthase with an analogue of the substrate, intermediate, or product failed (*19*). A steady supply of the enzyme must be obtained in order to clarify the enzyme's nature. We here report the details of an expression system for human uro'gen III synthase in *E. coli* and of a rapid purification procedure to obtain a native enzyme.

MATERIALS AND METHODS

The polymerase used in the PCR was AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Porphobilinogen, uroporphyrin I, and uroporphyrin III were purchased from Frontier Scientific (Logan, UT, USA). BSA was the product of Pierce (Rockford, IL, USA). All other chemicals used were of reagent grade and obtained commercially.

Construction of Expression Vectors—The DNAs encoding uro'gen III synthase and HMB synthase were amplified by PCR with the human liver cDNA library (Clontech, Palo Alto, CA, USA) as the template. The PCR primers, designed according to the published sequences (for uro'gen III synthase (*17*) and for the ubiquitous form of HMB synthase (*20*)), included an *Nde* I restriction site at the initiation codon and an *Sal* I restriction site downstream the termination codon to facilitate the subsequent construction of expression vectors. The PCR products were digested with *Nde* I and *Sal* I, ligated to the cloning site of an expression vector, pBAce (*21*), then transformed and cloned into *E. coli* JM109. The mutation of uro'gen III synthase was performed with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The resulting sequences were confirmed by an automated DNA sequencer at Kyurin Omtest (Kitakyushu, Japan) or FASMAC (Atsugi, Japan).

Expression and Purification of Uro'gen III Synthase—The *E. coli* strain transformed with the expression vector was pre-cultured in Luria-Bertani broth at 37°C for 10 h, after which 1 ml of the pre-culture was inoculated into 15 liters of low phosphate induction medium (*21*) in a jar. The cells were grown at 30°C for 40 h with continuous aeration. After harvest by centrifugation, the cells were washed with isotonic potassium phosphate buffer (pH 7.4) and lysed for 1 h on ice with lysozyme (2 mg/g wet cell) in 200 ml of 50 mM Tris-HCl (pH 8.0) containing 2 mM EDTA, 2 mM dithiothreitol (DTT), 25% glycerol, and 0.15 mM phenylmethylsulfonyl fluoride (PMSF). The lysed cells were disrupted by sonication, and the soluble fraction was obtained by centrifugation. Purification was carried out at 4°C, and all buffers used contained 25% glycerol and 1 mM DTT. The soluble part of the lysate was fractionated with ammonium sulfate at 277 to 430 g/liter. The term "saturation" is incorrect when describing the ammonium sulfate concentration of solutions that contain glycerol at high concentration, because the solubility of ammonium sulfate in water is altered by glycerol. The collected precipitate was dissolved in 60 ml of 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, and then dialyzed against the same buffer (the ammonium sulfate fraction). All chromatographic procedures were

done with medium-pressure glass columns attached to a Gilson 305 HPLC Pump System equipped with titanium pump heads. The ammonium sulfate fraction was applied at a flow rate of 5 ml/min to a 50-ml POROS HQ anion exchange column (PerSeptive Biosystems, Framingham, MA, USA) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. After the column was washed with the same buffer, elution was achieved with a 200-ml linear gradient from 0 to 0.25 M NaCl in the same buffer. Fractions containing uro'gen III synthase, which eluted around 0.1 M NaCl, were combined, and 144 g/liter ammonium sulfate was added. This combined fraction was applied at a flow rate of 5 ml/min to a 50-ml Phenyl Sepharose 6 Fast Flow column (Amersham Biosciences, Piscataway, NJ, USA), which had been equilibrated with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 144 g/liter ammonium sulfate. After the column was washed with the same buffer, the bound protein was eluted with a 250-ml decreasing linear gradient from 144 to 0 g/liter ammonium sulfate in 20 mM Tris-HCl (pH 7.4). Uroporphyrinogen III synthase-rich fractions eluted around 40 g/liter ammonium sulfate were combined and dialyzed against 5 mM potassium phosphate buffer (pH 7.4). Then the dialyzed solution was applied at a flow rate of 2.5 ml/min to a 20-ml Ceramic Hydroxyapatite Type I column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 5 mM potassium phosphate buffer (pH 7.4). After the column was washed with the same buffer, uro'gen III synthase was eluted with a 150-ml linear gradient from 5 to 50 mM potassium phosphate buffer (pH 7.4). Fractions containing uro'gen III synthase, which eluted around 20 mM potassium phosphate, were concentrated by ultrafiltration with Centriprep YM-10 and stored at –80°C.

Expression and Purification of HMB Synthase—E. coli cells were transformed with the expression vector, cultured, and lysed as described for uro'gen III synthase in the previous section, except that the cells were grown in 10 liters of induction medium at 37°C for 24 h, and then lysed with lysozyme in 50 mM Tris-HCl (pH 8.0) containing 2 mM EDTA and 0.15 mM PMSF. The enzyme was purified from the soluble fraction of the lysate principally according to the procedures reported previously (*22*) with modifications as described below. To increase the HMB synthase content and to reduce the sample volume, fractionation by ammonium sulfate at 33–80% saturation was performed. The precipitate was dissolved in 15 ml of 50 mM Tris-HCl (pH 7.4) and then dialyzed against the same buffer. The uro'gen III synthase of bacterial origin was heat-denatured at 70°C for 15 min, and insoluble materials were removed by centrifugation. The supernatant was passed through a Sephadex G-100 column (5 × 70 cm) in 50 mM Tris-HCl (pH 7.4). Fractions showing high HMB synthase activity were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 8.0). Then the dialyzed solution was applied to a DE-52 column (2.5 × 8 cm, Whatman, Kent, UK) equilibrated with the dialysis buffer. After the column was washed with the same buffer, the enzyme was eluted with a 400-ml linear gradient from 0 to 0.12 M NaCl in the same buffer. Fractions containing HMB synthase were concentrated and stored at –80°C.

Protein and Enzyme Assays—The molecular masses of the purified enzymes were determined by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectroscopy with an Applied Biosystems Voyager RP Mass Spectrometer at the National Institute of Advanced Industrial Science and Technology Kyushu (Tosu, Japan). Protein concentration was determined by the BCA Protein Assay (Pierce) using BSA as the standard.

The activity of HMB synthase was measured by optical quantification of the amount of uroporphyrin produced (*23*). The assay mixture (0.9 ml) contained 0.1 M Tris-HCl (pH 7.4), 0.1 mM DTT, 2 mg/ml BSA, 0.1 mM PBG, and HMB synthase (equivalent to 0.1–0.5 μg of the purified enzyme per assay). The mixture was incubated in the dark at 37°C for 30 min with shaking. The reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid (TCA), and the mixture was illuminated for 20 min to completely oxidize the uro'gens into uroporphyrins. After removal of the precipitated protein, the amount of uroporphyrin in the supernatant was determined with a JASCO V-560 Spectrophotometer using $\varepsilon = 528\ mM^{-1}\ cm^{-1}$ at 406 nm (*24*). One unit of activity was defined as the amount of enzyme that produces 1 nmol of uroporphyrin per h under the reaction conditions.

The activity of uro'gen III synthase was measured by the coupled enzyme assay as first described by Jordan (*25*) and then modified by Desnick and co-workers (*26*) and by Hart and Battersby (*12*), whereby the substrate of uro'gen III synthase, HMB, is generated by HMB synthase from PBG. The standard assay mixture (0.75 ml) consisted of 0.2 M sodium phosphate buffer (pH 8.0), 0.6 mM EDTA, 100 units of HMB synthase, 0.55 mM PBG, and uro'gen III synthase (equivalent to *ca*. 5 ng of purified enzyme per assay). The uro'gen III synthase solution was diluted with the appropriate buffers as indicated (*vide infra*), which contained 1 mg/ml BSA to avoid denaturation of the enzyme by dilution. The reaction mixture without PBG was pre-incubated at 37°C for 5 min, and the reaction was initiated by the addition of PBG that had been warmed to 37°C. After 3 min, 0.4 ml of 0.5% I_2 in 1% KI was added to terminate the reaction and at the same time to oxidize the uro'gens formed to uroporphyrins; by the addition of I_2/KI, linear oligopyrrole intermediates bound to HMB synthase were degraded and the remaining HMB was converted to non-porphyrin products. Excess I_2 was quenched with 0.1 ml of 1% sodium disulfite, and proteins were precipitated with 1.75 ml of 7.1% TCA. The supernatant was analysed by HPLC or optical measurement. Analysis by HPLC was done to separate uroporphyrins I and III on a reversed-phase column, L-column ODS (4.6 × 150 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan), attached to a Waters 600E HPLC System. Elution was carried out with a 1-ml/min isocratic flow of 1 M ammonium acetate (pH 5.15) containing 0.27 mM EDTA and 12.5% acetonitrile. The eluate was monitored with a Waters 996 photodiode array detector, and the Soret band maximum at 400 nm was extracted. For routine assay, the amounts of uroporphyrins in the supernatant were determined from the absorbance at 406 nm. Uroporphyrins I and III are indistinguishable from their absorption spectra, but the rate of spontaneous cyclization of HMB to uro'gen I is much

slower than that of the enzyme-catalysed conversion of HMB to uro'gen III (*27*). Therefore the amount of uro'gen III produced by uro'gen III synthase could be estimated fairly well by subtracting the absorbance of a reaction without uro'gen III synthase (corresponding to the amount of uro'gen I) from that of a reaction with uro'gen III synthase (corresponding to the sum of uro'gens I and III). One unit of activity was defined as the amount of enzyme that catalyses the formation of 1 μmol uroporphyrin III per h under the reaction conditions used.

The K_m for HMB of uro'gen III synthase was determined by measuring the concentration of HMB and the initial rate of the uro'gen III synthase reaction with the modified assay system with varying amounts of PBG and HMB synthase. To obtain individual points on the kinetic curve of the uro'gen III synthase reaction, four reaction tubes were prepared as follows: A) the reaction of the standard assay mixture without uro'gen III synthase was terminated by I_2/KI 3 min after the addition of PBG; B) the reaction of the same mixture as in A was terminated by TCA after 3 min and then I_2/KI was added; C) the reaction of the same mixture as in A was terminated by I_2/KI after 3.5 min; and D) the reaction of the same mixture as in A was allowed to proceed for 3 min, then 2.8 ng (0.1 pmol) of uro'gen III synthase was added and the reaction was terminated at 3.5 min by I_2/KI. The amounts of uro'gen I produced spontaneously during 3 and 3.5 min were determined by reactions A and C, respectively. HMB in reaction B was converted to uro'gen I by TCA then to uroporphyrin I by I_2/KI, because nonenzymatic conversion of HMB to uro'gen I is very rapid under acidic conditions (*28*). Thus, the sum of the uro'gen I formed spontaneously and the HMB produced by HMB synthase for 3 min was determined as uroporphyrin I in reaction B. The initial HMB concentration, when the uro'gen III synthase reaction was initiated at 3 min in reaction D, was calculated from the difference between reactions A and B (B minus A). The sum of the uro'gen III produced by uro'gen III synthase between 3 and 3.5 min and the uro'gen I formed during 3.5 min was determined from reaction D. The amount of uro'gen III formed between 3 and 3.5 min in reaction D was obtained from the difference between reactions C and D (D minus C). A similar strategy was adopted for the crude enzyme (*26*). A series of reactions A and B were carried out to determine the time course of the formation of HMB by HMB synthase, *i.e.*, reactions A and B were terminated at the desired times by the respective sequential additions of I_2/KI then TCA and of TCA then I_2/KI.

Stability of Uro'gen III Synthase—Harvested bacteria cells expressing uro'gen III synthase were lysed in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 0–50% glycerol in the presence or absence of 2 mM DTT. The soluble fraction of the lysate was kept at 4°C, and an aliquot was stored at –80°C on each day indicated for later assay. The stored aliquots were diluted several hundred times to adjust the enzyme concentration in the assay mixture with 20 mM potassium phosphate buffer (pH 7.4) containing 25% glycerol, 1 mM DTT, and 1 mg/ml BSA, and were then subjected to the standard assay.

The stability of purified uro'gen III synthase was examined as follows. The stock solution of purified enzyme was diluted several thousand times with 50 mM Tris-HCl or 20 mM potassium phosphate buffer (pH 7.4) containing 1 mg/ml BSA in the presence of 1 mM EDTA, 1 mM DTT, or 25% glycerol, and kept at 4, 37, 45, or 60°C for up to 14 d, 72 h, 24 h, or 30 min, respectively. An aliquot was stored at –80°C at each time point indicated for later assay. The stored aliquots were diluted several hundred times to adjust the enzyme concentration in the assay mixture with 20 mM potassium phosphate buffer (pH 7.4) containing 25% glycerol, 1 mM DTT, and 1 mg/ml BSA, and were then subjected to the standard assay.

Analysis of Cys Residues in Uro'gen III Synthase—The stock solution of the purified enzyme was dialyzed extensively against 20 mM potassium phosphate buffer (pH 7.4) to remove any DTT present in the stock solution, which might disturb quantification of the thiol groups of uro'gen III synthase, and the enzyme concentration was adjusted to 1.4 mg/ml with the same buffer. The number of Cys residues and the residual enzyme activity were measured during heat treatment at 45°C. The Cys residues in the enzyme were quantified by the use of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (*29*). The reaction was carried out at 25°C in 0.1 M sodium phosphate buffer (pH 7.27) containing 2.5 μM uro'gen III synthase and 1 mM DTNB in the presence or absence of 0.2% SDS. The absorbance change at 412 nm was recorded, and the total number of thiols was calculated based on an extinction coefficient of 14,150 M^{-1} cm^{-1} (*29*). The molar concentration of uro'gen III synthase was calculated from its molecular mass of 28,631 Da. A part of each heat-treated sample was diluted 1,200-fold with 20 mM potassium phosphate buffer (pH 7.4) containing 25% glycerol, 1 mM DTT, and 1 mg/ml BSA, and then used for the assay of the remaining enzyme activity.

RESULTS

Characterization of the Expressed HMB Synthase—Expressed HMB synthase, which was purified by ammonium sulfate fractionation, gel filtration, and anion exchange column chromatography, contained a slight impurity on SDS-PAGE (Fig. 2), but no further purification was attempted. The specific activity of the purified HMB synthase was 2,100 units/mg protein, similar to the 2,300 units/mg that has been reported for the HMB synthase purified from human erythrocytes (*22*). Ninety milligram of purified enzyme could be obtained routinely from a 10-liter bacterial culture with a yield of 37%.

Characterization of the Expressed Uro'gen III Synthase—The DNA sequence encoding uro'gen III synthase in the expression vector was confirmed to be identical to that reported (*17*). *E. coli* strains JM109, HB101, DH5 (TOYOBO, Osaka, Japan), XL1-Blue, Sure, and XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) were transformed with the expression vector carrying the uro'gen III synthase gene. Most of the protein expressed was found in the precipitates of the lysates of the *E. coli* strains tested, indicating the formation of inclusion bodies. Recovery in the soluble fraction was not improved by changing the culture temperature and period. Denaturation with urea followed by renaturation by stepwise dilution by dialysis was performed to recover the enzyme from the inclusion bodies. The yield of active enzyme, however, was unsatisfactory, and the attempt to recover

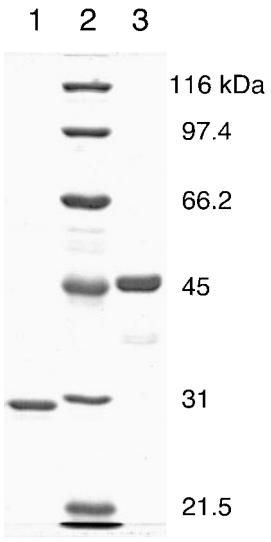

Fig. 2. **SDS-PAGE of purified enzymes.** The purified uro'gen III synthase and HMB synthase were applied on lanes 1 and 3, respectively. Lane 2 shows marker proteins; their molecular masses are indicated.

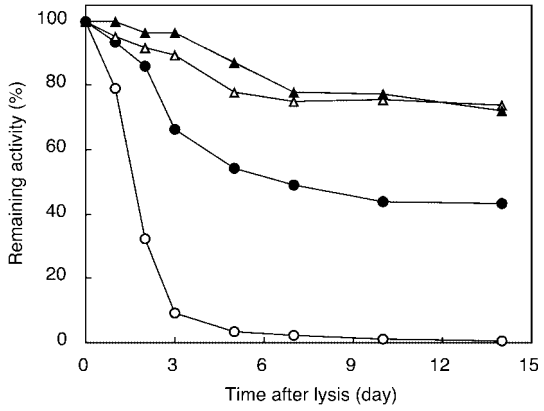

Fig. 3. **Activity of uro'gen III synthase in the soluble fraction stored at 4°C.** JM109 cells expressing the enzyme were lysed in 50 mM Tris-HCl (pH 8.0) containing 1mM EDTA and 2 mM DTT along with 0 (open circles), 5 (closed circles), 10 (open triangles), or 20% (closed triangles) glycerol. Remaining activities on the days indicated are plotted as fractions of the initial activity on day 0.

the enzyme from the inclusion bodies was abandoned. Of the host cells examined, JM109 cultured at 30°C expressed the highest amount of enzyme in the soluble fraction, therefore the enzyme was purified from the soluble fraction of the JM109 lysate.

The uro'gen III synthase activity present in the soluble fraction of the lysate decreased rapidly at 4°C, when cells were lysed in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and then the fraction was kept in the same buffer. SDS-PAGE showed no proteolysis in the sample whose activity had been diminished. In fact, the addition of a combination of several protease inhibitors to the lysis buffer had no effect on the activity loss. In order to maintain enzyme activity during purification, the effects of such stabilizing reagents as DTT and glycerol were examined. The activity was not preserved when cells were lysed in the buffer containing 1 to 2 mM EDTA or 1 to 5 mM DTT. However, the addition of glycerol to the lysis buffer was found to be remarkably effective in preventing the loss of activity in a concentration-dependent manner (Fig. 3). In the presence of 25% glycerol, irrespective of the presence or absence of DTT, more than 95% and about 75% of the initial activity remained 2 and 14 d after lysis, respectively. Similar preventive effects against activity loss were observed with 20–50% glycerol (data not shown). Thus, glycerol was able to prevent the activity loss almost completely for the first 2 d after lysis, but failed to prevent the gradual loss after day 2. Therefore, the entire enzyme purification process was carried out within 2 days after lysis in the presence of 25% glycerol.

Typical results of uro'gen III synthase purification are summarized in Table 1. Routinely, *ca*. 15 mg of purified enzyme could be obtained from a 15-liter bacterial culture with a yield of 22%. The final preparation was homogenous as judged by SDS-PAGE (Fig. 2). The observed molecular mass was 28,807 Da on MALDI-TOF mass spectroscopy, whereas the value deduced from the amino acid composition is 28,631 Da.

Authentic uroporphyrins I and III were eluted from a reversed-phase column, L-column ODS, with retention times of 6.1 and 7.4 min, respectively; by using the L-column, separation of the two isomers was accomplished in a shorter period compared with previous studies (*26*, *27*). HPLC analysis of the products produced in the standard reactions with the purified enzymes showed only uroporphyrin I in the absence of uro'gen III synthase, whereas uroporphyrin III in addition to uroporphyrin I was formed in the presence of uro'gen III synthase. It should be noted that the amount of uroporphyrin I formed was the same whether or not uro'gen III synthase was included in the reaction. This means that the spontaneous conversion of HMB to uro'gen I takes place at constant rates depending on the HMB concentration, and indicates, therefore, that the substrate for uro'gen III synthase, HMB, was sufficiently supplied by

Table 1. **Purification of uro'gen III synthase from a 15-liter of bacterial culture.**

	Protein (mg)	Activity[a] (unit)	Specific activity[a] (unit/mg)	Yield (%)	Purification (fold)
Lysate, soluble	1,183	224,000	189	100	1
Ammonium sulfate	542	116,000	214	51.7	1.1
POROS HQ	37.7	49,300	1,310	22.0	6.9
Phenyl Sepharose	16.6	49,000	2,950	21.9	15.6
Hydroxyapatite	14.7	48,100	3,280	21.5	17.4

[a]Activity units as μmol uroporphyrin III/h.

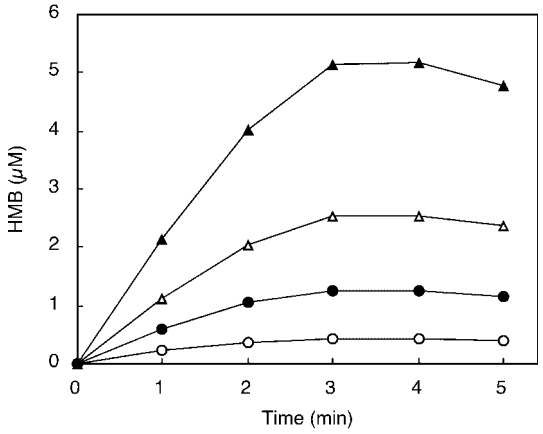

Fig. 4. **Time course of HMB formation by HMB synthase.** The concentration of HMB formed at the times indicated was determined in the modified assay system in the absence of uro'gen III synthase. The amounts (units) of HMB synthase used and the initial concentrations (μM) of PBG were: 22.5 and 2.5 (open circles), 33.5 and 7.5 (closed circles), 50 and 15 (open triangles), and 83 and 30 (closed triangles), respectively.

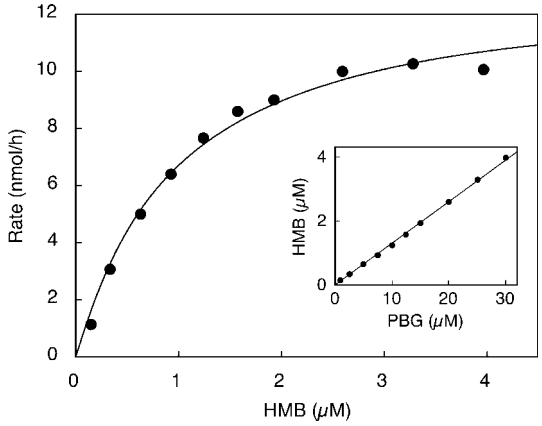

Fig. 5. **Kinetics of the uro'gen III synthase reaction.** The initial rates of the uro'gen III synthase reaction are plotted against the concentration of HMB. The reactions were performed in the modified assay system. The inset shows the linear relationship between the concentration of HMB at 3 min after the addition of PBG and the concentration of the added PBG.

HMB synthase in the standard assay. Thus, the coupled enzyme assay proved to reflect the uro'gen III synthase activity quantitatively. The specific activity of the purified uro'gen III synthase was 10- and 2-fold higher than those of the enzymes purified from human erythrocytes (*13*) and from *E. coli* (*15*), respectively.

Determination of the K_m for HMB of Uro'gen III Synthase—To determine the K_m, the concentration of HMB should be known accurately, and the initial rate of the uro'gen III synthase reaction should be measured precisely. Currently, HMB, however, is unavailable commercially, and it is technically difficult for most biochemistry laboratories to prepare HMB. Even if HMB were available, the exact concentrations of HMB in assay mixtures would not be knowable due to the non-enzymatic conversion of HMB to uro'gen I. Prior to measuring the K_m, therefore, we investigated the relationship between the amount of HMB synthase and the PBG concentration during the HMB synthase reaction in the modified assay system. As shown in Fig. 4, we found that the concentration of HMB remained constant between 3 and 4 min irrespective of the initial concentration of PBG, when the amount of HMB synthase employed fit the following equation.

HMB synthase (unit) = 2.2 (unit) × PBG concentration in μM + 17 (unit)

Although the equation was derived empirically, as far as satisfying the equation, the HMB concentration at 3 min was proportional to the PBG concentration up to 30 μM as shown in the inset of Fig. 5. The uro'gen III synthase reaction was initiated 3 min after the addition of PBG in the modified reaction D; therefore, the HMB concentration at 3 min could be regarded as the concentration of substrate at time 0 in the uro'gen III synthase reaction. The initial rate of the reaction could be determined from the combination of the modified reactions C and D. The results of these kinetic experiments are shown in Fig. 5. An apparent K_m for HMB of uro'gen III synthase of 1.01 ± 0.11 μM and a V_{max} of 13.5 ± 0.6 nmol/h were obtained by non-linear curve fitting. The turnover number was estimated to be 1,800 min^{-1}.

Stability of the Purified Uro'gen III Synthase—The purified uro'gen III synthase was much more stable at 4°C (Fig. 6) than the enzyme in the lysate (Fig. 3, open circles). Only 20% of the activity was lost by day 14 when the enzyme was stored in potassium phosphate buffer (pH 7.4) without any additions. The enzyme was somewhat less stable in Tris-HCl than in potassium phosphate (data not shown). The preservative effect of glycerol on the purified enzyme was not so conspicuous as that for the enzyme in the lysate (Fig. 3). Instead of glycerol, EDTA and DTT were found to have distinct preservative effects on the purified enzyme, although they did not work for the enzyme in the lysate. In potassium phosphate buffer (pH 7.4) containing EDTA or DTT, the initial activity of the purified enzyme was retained for 14 d with almost no activity loss (Fig. 6). Combined additions of glycerol, EDTA, or DTT showed no additive effect in any combination. The enzyme activity did not change after several cycles of freeze-and-thawing in potassium phosphate buffer (pH 7.4) containing DTT and glycerol.

The stability of the purified uro'gen III synthase against heat treatment was examined at 37, 45, and 60°C in potassium phosphate buffer (pH 7.4) with or without such preservatives as DTT, EDTA, or glycerol as shown in Fig. 7. Of the three preservatives examined, DTT exhibited a marked preservative effect at those temperatures. In the presence of DTT, the enzyme retained 20% of the initial activity after treatment at 60°C for 30 min. The effect of EDTA was the second best at 37 and 45°C, but it showed no effect at 60°C. A slight additive effect was observed at 60°C for the combination of glycerol and DTT (data not shown).

Relationship between Uro'gen III Synthase Activity and Cys Residues—Because of the remarkable preservative effects of DTT and EDTA against the heat denaturation of uro'gen III synthase, there was a possibility that the

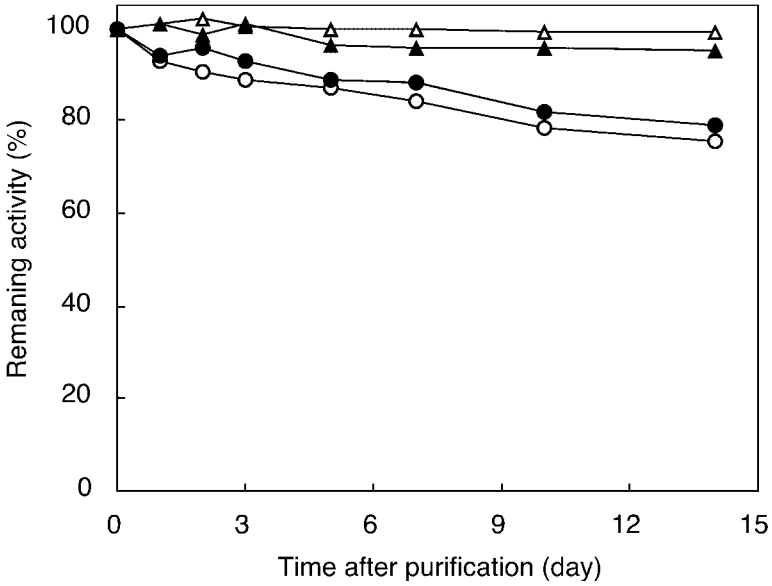

Fig. 6. **Stability of the purified uro'gen III synthase under various conditions.** The purified uro'gen III synthase was diluted to *ca*. 1 μg/ml and stored at 4°C in 20 mM potassium phosphate buffer (pH 7.4). The enzyme was diluted with the buffer alone (open circles), with the buffer containing 25% glycerol (closed circles), with the buffer containing 1 mM DTT (open triangles), or with the buffer containing 1 mM EDTA (closed triangles). All buffers contained 1 mg/ml BSA. The remaining activities on the days indicated are plotted as fractions of the initial activity on day 0.

enzyme activity might depend on the state of the thiol groups of Cys residues in the enzyme. Hence, the number of total thiol groups detected in the presence of SDS and the number of exposed thiol groups detected in the absence of SDS were determined. Figure 8 shows the numbers of thiol groups and remaining activities when the enzyme was treated at 45°C and at 4°C as a control. The enzyme has a total of 8 Cys residues as deduced from its nucleotide sequence. In the native enzyme at time zero, however, only 7 thiol groups were detected as a total in the presence of SDS. This discrepancy in the total number would be due to the two adjacent Cys residues at the *C*-terminus; they might be counted as one probably because of steric hindrance in binding to the labeling reagent, DTNB. Likewise, the number of exposed thiol groups observed in the absence of SDS was 6. These findings indicate that one thiol group is not exposed; in other words, it is buried inside the enzyme and is not accessible to DTNB. Whereas the number of total thiol groups decreased gradually on treatment at 45°C, the number of exposed thiol groups remained 6 during the initial 2 h. Thereafter, the numbers of thiol groups detected in the presence and absence of SDS were equal and decreased at the same rate. It should be noted that the remaining activity of the enzyme decreased to only 20% of the initial activity during the first 2 h at 45°C. Thus it was considered that the thiol group of the buried Cys residue might be affected by some type of structural or functional change before the oxidation of the other Cys residues. This buried Cys residue may play some key role in the uro'gen III synthase reaction. Neither the activities nor numbers of the thiol groups were changed at 4°C for 24 h.

Enzyme Activities of Uro'gen III Synthase Mutants—Table 2 shows the activity of purified uro'gen III synthase with a point mutation at Cys73. The Cys73Arg mutant that is found most commonly in CEP patients exhibited only 9% of the wild type activity. On the other hand, other mutants, Cys73Ala and Cys73Ser retained 99% and 78% activity, respectively. These mutants showed thermostability at 45°C similar to that of the wild type enzyme as shown in Fig. 7B; that is, their activities decreased gradually in the absence of DTT and EDTA, but the activity

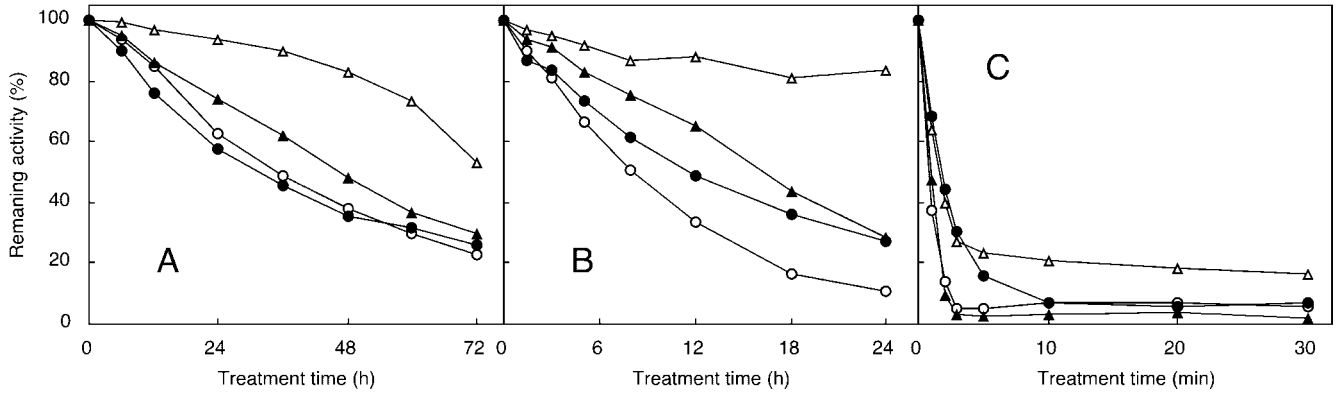

Fig. 7. **Thermostability of the purified uro'gen III synthase under various conditions.** The purified uro'gen III synthase was diluted to *ca*. 1 μg/ml with 20 mM potassium phosphate buffer (pH 7.4) alone (open circles), with the buffer containing 25% glycerol (closed circles), with the buffer containing 1 mM DTT (open triangles), or with the buffer containing 1 mM EDTA (closed triangles). All buffers contained 1 mg/ml BSA. After dilution, the samples were stored at 37 (A), 45 (B), or 60°C (C). The remaining activities at the times indicated are plotted as fractions of the initial activity at time 0.

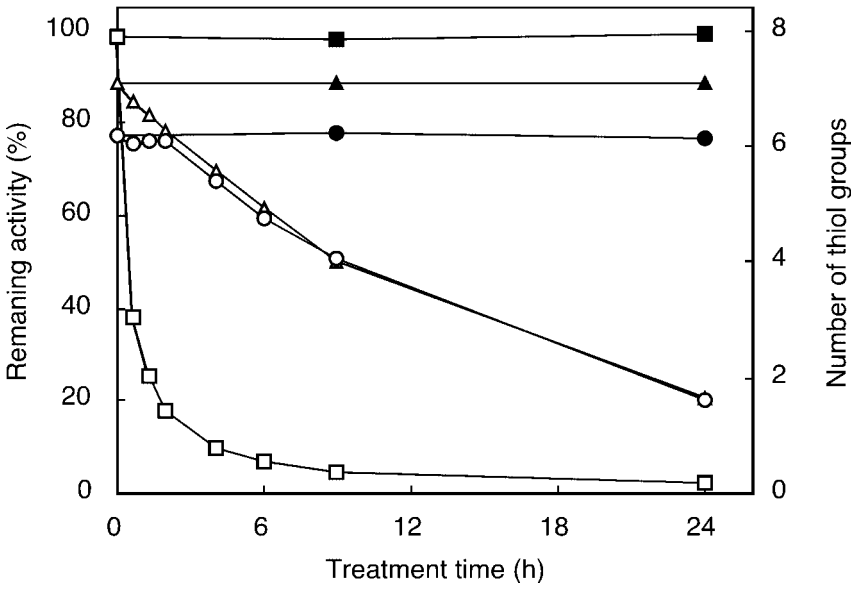

Fig. 8. **Changes in activity and the number of thiol groups of uro'gen III synthase during heat treatment at 45°C.** The purified uro'gen III synthase was dialyzed against 20 mM potassium phosphate buffer (pH 7.4), and its concentration was adjusted to 1.4 mg/ml. The sample was kept at 45°C (open symbols) or 4°C (closed symbols). The remaining activity (squares) at each time is expressed as a fraction of the initial activity at time 0 (left axis). The number of thiol groups was determined in the presence (triangles) or absence (circles) of 0.2% SDS (right axis).

losses were prevented mostly in the presence of DTT and moderately in the presence of EDTA.

DISCUSSION

In the present study, we have established an expression and purification system for human uro'gen III synthase. Most of the uro'gen III synthase expressed in *E. coli* was present in the precipitate of the cell lysate, and an attempt to retrieve the enzyme from the pellet failed. This obliged us to purify uro'gen III synthase from the soluble fraction. Consequently, 1 mg of purified uro'gen III synthase could be obtained from a 1-liter bacterial culture. Fortunately, the specific activity of the purified uro'gen III synthase was markedly high and, with this yield, we were able to carry out all of the biochemical studies reported here without experiencing a shortage of enzyme. On the other hand, we were able to purify HMB synthase from the soluble fraction with a yield of 9 mg/liter culture.

A remarkable preservative effect of glycerol on uro'gen III synthase activity in the lysate was noted for the first time in this study; a glycerol concentration above 20% maintained the enzyme activity for at least 2 d. We, therefore, decided to purify the enzyme in the presence of 25% glycerol and to accomplish the three column chromatographic manipulations within 48 h after lysis. Glycerol might be unnecessary for the later purification steps, because the presence of glycerol has no effect on the stability of the purified enzyme. We also found that the enzyme is more stable in phosphate buffer than in Tris-HCl. Phosphate buffer, however, is not preferable for anion exchangers as POROS HQ, which was employed for the first column chromatography. This is the reason we lysed the cells in Tris-HCl instead of phosphate buffer.

Table 2. **Activities of purified uro'gen III synthase mutants.**

Mutant	Specific activity (unit/mg)	Relative activity (%)
Wild type	3,130	100
Cys73Ala	3,100	99
Cys73Arg	280	9
Cys73Ser	2,450	78

With the purified human uro'gen III synthase, we obtained an apparent K_m of 1 μM for HMB and a turnover number of 1,800 min^{-1}. For the human erythrocyte enzyme, the apparent K_m and turnover number have been reported to be 5–20 μM and 350 min^{-1}, respectively (*13*). It should be noted, however, that the kinetic parameters may differ depending on the assay conditions. A human enzyme in the hemolysate gave an apparent K_m of 1.9 μM for HMB when assayed by the direct method in which HMB was added directly to the reaction mixture; but when assayed by the coupled enzyme assay, the value was 0.13 μM (*26*). Most of the previous studies adopted the direct assay method. In spite of the difference in assay methods, the enzyme we obtained seems to exhibit the highest specific activity compared to the enzymes purified from human erythrocytes (*13*), rat liver (*30*), *Euglena gracilis* (*12*), and *E. coli* (*15*). The specific activity of the human uro'gen III synthase expressed in *E. coli*, which was crystallized (*19*), has not been described. The preservative effect of glycerol was not examined in the previous studies, which may partly explain why the activities of the purified uro'gen III synthases reported are relatively low compared with the activity of our purified enzyme.

Another feature of the purified human uro'gen III synthase is its remarkable stability against heat treatment. The enzymes purified from various sources have been reported to be unstable to heat; the half-life of the human erythrocyte enzyme at 37, 45, and 60°C was 30, 4, and 1 min, respectively (*13*), and the *E. coli* enzyme was completely inactivated at 60°C in 1 min (*15*). The *Bacillus subtilis* enzyme expressed in *E. coli* seems to be an exception. It has features similar to our enzyme: remarkable thermostability and a low K_m for HMB (0.33 μM) (*16*). The half-life of our human enzyme in potassium phosphate buffer at 37 and 45°C is estimated to be 36 and 8 h, respectively, and the further addition of DTT markedly lengthens its half-life (Fig. 7). The heat denaturation curves at 45°C in potassium phosphate buffer that are

shown in Fig. 7B (open circles) and in Fig. 8 (open squares) differ greatly. This might be due to the presence of 1 mg/ml BSA in the former experiment, whereas BSA was avoided in order to quantify the Cys residues in the latter one.

The molecular mass of human uro'gen III synthase is calculated to be 28,631 from its amino acid composition. However, MALDI-TOF mass spectrometry of the purified enzyme gave a molecular mass of 28,807. The difference in mass of about 180 between the calculated and observed values could be considered to be within the range of error of MALDI-TOF mass spectrometry, but the observed value might indicate the actual molecular mass of the enzyme. The reason for this discrepancy is not clear at present. A couple of decades ago, it was suggested that folate was a non-covalently bound cofactor of rat liver uro'gen III synthase (*31*), but the presence of folate is denied today (*12*, *14*).

By DTNB labeling, we found that one Cys residue is buried inside human uro'gen III synthase and considered that this residue may play key role in catalysis. Based on the crystal structure of human uro'gen III synthase (*19*), we infer the residue to be Cys73 from a computer analysis of the atomic coordinates deposited in the Protein Data Bank (1JR2). The involvement of this residue in enzyme activity is supported by the facts that the mutation of Cys73 to Arg is most common in CEP and has been found in the allele of 33% of CEP patients (*32*), and that a crude human mutant enzyme, Cys73Arg, shows less than 2% of the activity of wild type (*18*). We also confirmed this with the purified Cys73Arg mutant; it exhibited only 9% of the wild type activity (Table 2). The change that occurs on Cys73 remains to be determined. Disulfide formation, however, can be negated, because a loss of two Cys residues was not observed during this period. It has been suggested that the structure in crystal may not reflect an active form of human uro'gen III synthase in solution, but the flexibility of the enzyme may be important in catalysis (*19*). Since Cys73 is far from the putative active site in the crystal structure, the effect of Cys73 on the enzyme activity would be indirect. In fact, Cys73Ala and Cys73Ser mutants showed more than 80% of the activity of the wild type (Table 2). Thus the Cys73Arg mutation may have to be considered in relation to the correct folding of the enzyme. It should be noted that the results of thermostabilities of these mutants indicate that the preservative effects of DTT and EDTA are not considered in relation to Cys73 only.

In conclusion, we established for the first time an expression and purification system for human uro'gen III synthase in its native form. The enzyme expressed in *E. coli* was purified to homogeneity in the presence of glycerol. The purified enzyme was markedly stable. The side-chain asymmetry of the physiological porphyrins is introduced by the cooperative actions of HMB synthase and uro'gen III synthase. Although the role of uro'gen III synthase is essential for the chemistry of porphyrin biosynthesis, structural and mechanical studies of uro'gen III synthase have been hampered partly due to the instability of the enzyme preparations thus far purified. The thermostable uro'gen III synthase produced in our system will facilitate further studies of this enzyme.

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