Purification and properties of the uroporphyrinogen decarboxylase from *Rhodobacter sphaeroides*

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Uroporphyrinogen decarboxylase (EC 4.1.1.37) was purified 600-fold from *Rhodobacter sphaeroides* grown anaerobically in the light. The enzyme, under both denaturing and non-denaturing conditions, is a monomer of M_r 41 000. The K_m values are 1.8 μ M and 6.0 μ M for the conversion of uroporphyrinogen I and III to coproporphyrinogen I and III respectively. The enzyme is susceptible to inhibition by both uroporphyrinogen and uroporphyrin. The pH optimum is 6.8 and the isoelectric point

is 4.4. The importance of cysteine and arginine residues is implicated from studies with inhibitors. The sequence of the first 29 amino acids of the N-terminus shows a high degree of similarity to the primary structures of other uroporphyrinogen decarboxylases. Studies on the order of decarboxylation of the four acetic acid side chains of uroporphyrinogen III suggest that at high substrate levels a random route is preferred.

INTRODUCTION

Uroporphyrinogen decarboxylase (EC 4.1.1.37) catalyses the decarboxylation of uroporphyrinogen III to give coproporphyrinogen III. The reaction proceeds through intermediate porphyrinogens with seven, six and five carboxyl groups (Scheme 1). Uroporphyrinogen decarboxylase has been purified to homogeneity from human erythrocytes (de Verneuil et al., 1983a; Elder et al., 1983), avian erythrocytes (Kawanishi et al., 1983), bovine liver (Straka and Kushner, 1983) and yeast (Felix and Brouillet, 1990), and has been partially purified from tobacco leaves (Chen and Miller, 1974) and the bacterium *Rhodobacter palustris* (Koopmann et al., 1986).

The identification of oxidized forms of the intermediate porphyrinogens from patients with uroporphyrinogen decarboxylase deficiency, or from rats which have been treated with polychlorinated hydrocarbons, was achieved by comparison with chemically synthesized authentic porphyrins (Jackson et al., 1976). These studies led to the conclusion that the decarboxylation from uroporphyrinogen III to coproporphyrinogen III proceeds by a preferred route, commencing with the decarboxylation of the acetic acid group on ring D followed by those on rings A, B and finally C (Jackson et al., 1976). Recent studies with normal human erythrocytes suggest that the decarboxylation reactions catalysed by this enzyme may proceed by a random route (Luo and Lim, 1990) in which the decarboxylation of all four type-III isomers of the heptacarboxylic acid porphyrinogens (Figure 1) are produced. From these experiments it may be deduced that uroporphyrinogen decarboxylase has a relatively broad substrate specificity and can recognize uroporphyrinogen III and all 13 intermediate type-III porphyrinogens en route to coproporphyrinogen III. The enzyme is also able to catalyse the decarboxylation of uroporphyrinogen I and the unnatural porphyrinogens uroporphyrinogen II and IV (Smith and Francis, 1979).

The uroporphyrinogen decarboxylase from human sources is inhibited by a wide variety of sulphhydryl reagents (de Verneuil et al., 1983a; Elder et al., 1983) and by heavy metals (Kushner et al., 1972; Woods et al., 1981). This has been interpreted as

evidence for the involvement of an essential cysteine group at the catalytic site. The enzyme is also inhibited by oxidized forms of the substrates (Smith and Francis, 1981) and is sensitive to the effects of polychlorinated hydrocarbons *in vivo* (de Verneuil et al., 1983b; Kawanishi et al., 1983). The nature of the groups responsible for catalysis, or those involved in the binding of substrate, is not known. Detailed stereochemical analysis of the reaction has been carried out (Barnard and Akhtar, 1975) and the decarboxylation has been shown to proceed with retention of configuration, with the lost carboxyl group replaced by a hydrogen atom from the solvent in the same spatial orientation. The rat (Romeo et al., 1984), human (Romeo et al., 1986) and *Bacillus subtilis* (Hansson and Hederstedt, 1992) uroporphyrinogen decarboxylase cDNAs and the equivalent gene (*hem12*) from yeast (Garey et al., 1992) have been identified, cloned and sequenced. The derived protein sequences have several regions of

Scheme 1 The conversion of uroporphyrinogen III into coproporphyrinogen III catalysed by uroporphyrinogen decarboxylase

The side-chain substituents are denoted as follows: (A) acetate, (M) methyl, and (P) propionate.

similarity which may assist in the identification of catalytic residues.

Apart from isolated reports on studies of the enzyme from bacteria (Hoare and Heath, 1959; Koopmann et al., 1985), no publications exist describing the purification of any decarboxylase from a bacterial source. This paper reports the purification to homogeneity of uroporphyrinogen decarboxylase from the photosynthetic bacterium *Rhodospirillum rubrum* (previously *Rhodospirillum rubrum*) and describes the properties of the purified enzyme, its N-terminal sequence, the involvement of an active-site cysteine residue and studies on the pathway of decarboxylation.

MATERIALS AND METHODS

Materials

All porphyrins and porphyrin markers were obtained from Porphyrin Products, Logan, UT, U.S.A. The heptacarboxylic acid porphyrin standards were a kind gift from Dr. C. K. Lim, MRC Toxicology Unit, Carshalton, Surrey, U.K. Acetonitrile (h.p.l.c. grade S) was obtained from Rathburn Chemicals Ltd. Ammonium acetate (AnalaR) and other organic solvents were from Merck Ltd., Poole, Dorset, U.K. The f.p.l.c. system was

purchased from LKB/Pharmacia, and the h.p.l.c. system was from Waters/Millipore, U.K. The Sephacryl S-200 was obtained from Bio-Rad, and all t.l.c. plates were supplied by Anachem. The *N*-ethyl[2,3-¹⁴C₂]maleimide was purchased from Amersham Life Sciences. Non-denaturing-gel molecular-mass standards and general laboratory chemicals were purchased from Sigma Chemical Co. Ltd.

T.l.c. of porphyrin methyl esters

Porphyrins were analysed as their methyl esters by ascending t.l.c. Esterification was achieved by incubation of the porphyrin samples (100 μ l) with 1 ml of 10% (v/v) H₂SO₄ in methanol at 37 °C for 40 min. After the addition of 1 ml of distilled water to quench the reaction, the resulting esters were extracted into 400 μ l of chloroform, and the aqueous layer was discarded. The esters were spotted on to a t.l.c. plate and developed in benzene/ethyl acetate/ethanol (80:18:2, by vol.), using an ascending method as described previously (Seehra et al., 1983). Samples were chromatographed together with standard porphyrin methyl esters of uroporphyrin, coproporphyrin and protoporphyrin. The positions of the porphyrin esters were visualized by their fluorescence under short-wavelength u.v. light. The presence of uroporphyrinogen decarboxylase was shown by the appearance of a band of coproporphyrin tetramethyl ester.

H.p.l.c. analysis of free porphyrin acids

A Waters h.p.l.c. system was used that included a model 440 absorbance detector fitted with a 405 nm filter, two model 6000A pumps controlled by a model 660 solvent programmer and a Waters U6K injector.

Separation of free porphyrin acids (20 μ l injection volume) was carried out with a 5 μ m particle size Shandon Hypersil BDS C₁₈ column using a slightly modified method to that described by Lim and Peters (1984). The mobile phases for the separation of the porphyrin mixtures were: solvent A, 10% (v/v) acetonitrile in 1 M ammonium acetate, pH 5.16; and solvent B, 10% (v/v) acetonitrile in methanol. Separation was achieved by the application of a linear gradient of 0–80% solvent B in solvent A over 17 min, at a fixed flow rate of 1 ml/min. All solutions were made up freshly and filter-degassed under vacuum before use.

The heptacarboxylic acid porphyrins, the type-I and the four type-III isomers, were separated by h.p.l.c. with a mobile phase consisting of 1 M ammonium acetate buffer, pH 5.16, containing 35% (v/v) of acetonitrile/methanol (10:90, v/v). Separation of the porphyrins was carried out with a 5 μ m particle size Shandon Hypersil BDS C₁₈ column, at a flow rate of 1 ml/min. Porphyrins were detected by means of an LKB 2158 UVcord detector at a peak absorbance of 405 nm, and a Perkin-Elmer LS-3 fluorescence spectrophotometer (λ_{ex} , 405 nm; λ_{em} , 618 nm).

The h.p.l.c. system was calibrated with known quantities of uroporphyrin III and coproporphyrin III. The quantities of coproporphyrin III and uroporphyrin III were determined using $\epsilon_{405} = 489 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Falk and Benson, 1953) and $\epsilon_{405} = 548 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Rimington and Sveinsson, 1950) respectively.

Reduction of uroporphyrinogen III with sodium amalgam

Uroporphyrinogen III (approx. 300 μ g), previously purified by h.p.l.c., was dissolved in 500 μ l of 1 M potassium phosphate buffer, pH 7.0, and chemically reduced with one or two small pieces of freshly prepared 5% (w/w) sodium amalgam. The mixture was vortexed for 3–5 min or until the solution became non-fluorescent

Figure 1 The four type-III isomers of the heptacarboxylic acid porphyrinogen and the type-I heptacarboxylic acid porphyrinogen

The side-chain substituents are denoted as follows: (A) acetate, (M) methyl, and (P) propionate. The designation of the intermediates indicates the ring of uroporphyrinogen III that has been decarboxylated e.g. 7IIIA represents the heptacarboxylic acid porphyrinogen in which only the A ring has been decarboxylated, etc.

under u.v. light, indicating that the porphyrin had been reduced to the porphyrinogen. The amalgam was then carefully pelleted by centrifugation in a microfuge for 5 s and the supernatant was transferred to a clean tube at 4 °C and used within 5 min to minimize chemical re-oxidation. The whole preparation was carried out under N₂ in the dark.

Purification of uroporphyrinogen decarboxylase from *R. sphaeroides*

Growth of *R. sphaeroides* and preparation of cell-free extracts

Wild-type *R. sphaeroides* (N.C.I.B. 8253) was grown under anaerobic conditions in the light at 30 °C in 10 × 5 litre of MS growth medium (Lascelles, 1959). The bacterial cells were harvested by centrifugation at 10000 *g* using a refrigerated Sharples continuous centrifuge. The cells were resuspended in 100 ml of ice-cold 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM 2-mercaptoethanol, 1 mM benzamidine and 1 mM phenylmethanesulphonyl fluoride (PMSF) and disrupted by sonication using a Soniprep 150 ultrasonicator fitted with a 19-mm-diam. probe. The cells were broken in two batches over 10 min in 30 s bursts at an amplitude of 8 µm (peak to peak), allowing an equal time for cooling using crushed ice. The crude cell extract was then ultracentrifuged at 140000 *g* for 3 h at 0 °C.

(NH₄)₂SO₄ fractionation

Solid (NH₄)₂SO₄ was added to the supernatant (approx. 100 ml) to a level of 35 % satn. The mixture was stirred for 10 min at 4 °C and centrifuged for 8 min at 5000 *g*. The pellet was discarded and further solid (NH₄)₂SO₄ was added to 70 % satn. The precipitate was removed by centrifugation to 7000 *g* for 12 min, and the resulting pellet, containing uroporphyrinogen decarboxylase activity, was resuspended in 20 ml of 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM 2-mercaptoethanol, 1 mM benzamidine and 1 mM PMSF.

Gel-filtration chromatography

The resuspended pellet was applied to a column (100 cm × 4.5 cm) of Sephacryl S-200 that had been equilibrated previously with 25 mM Tris/HCl buffer, pH 7.5, containing 2 mM 2-mercaptoethanol, 1 mM benzamidine and 1 mM PMSF. Fractions containing uroporphyrinogen decarboxylase activity were pooled and concentrated to 50 ml by means of an Amicon Ultrafiltration Cell fitted with a PM-10 membrane.

F.p.l.c.

The uroporphyrinogen decarboxylase was purified to homogeneity by chromatography using an LKB/Pharmacia f.p.l.c. system at two different pH values. The fractions from the previous stage were first loaded on to a Mono-Q (HR 10/10) column equilibrated with 25 mM Tris/HCl buffer, pH 8.0. The protein was eluted with this buffer containing a linear salt gradient of 0–300 mM NaCl (total volume of 160 ml), at a flow rate of 3 ml/min. Fractions containing uroporphyrinogen decarboxylase activity were pooled and desalted by means of a Pharmacia PD-10 desalting column and reappplied to a Mono-Q (HR 5/5) column equilibrated with 30 mM sodium acetate buffer, pH 5.2. The proteins were eluted in this buffer with a linear gradient of 0–200 mM NaCl (total volume of 15 ml) at a flow rate of 1 ml/min. The pure uroporphyrinogen decarboxylase was desalted into 25 mM potassium phosphate buffer, pH 7.0, con-

taining 2 mM 2-mercaptoethanol and stored at –20 °C, at which temperature it was stable for several months.

Assay of protein

An estimate of protein concentration was obtained at 280 nm at which wavelength an absorbance of 1 corresponded to protein of 1 mg/ml. The protein concentration was also routinely determined by the method of Bradford (1976), using BSA as a standard.

Assay of uroporphyrinogen decarboxylase

(a) The coupled assay of uroporphyrinogen decarboxylase with porphobilinogen deaminase

The presence of uroporphyrinogen decarboxylase was determined by a modified method of Hoare and Heath (1959) involving a coupled enzyme system using porphobilinogen deaminase to generate uroporphyrinogen I *in situ*. The decarboxylase enzyme extract described above was incubated with an equal volume of 2 mM porphobilinogen in the presence of 0.1 unit of purified *Escherichia coli* porphobilinogen deaminase (Jordan et al., 1988) at 37 °C in the dark for 1 h. The reaction was terminated by the addition of 30 µl of dimethyl sulphoxide (DMSO)/5 M HCl (1:1, v/v) and the products oxidized in the light for 1.5 h, using a 60 W bulb placed 20 cm away. The porphyrins were esterified and analysed by t.l.c. as their porphyrin methyl esters, as described above. The quantity of porphyrins produced could be determined using h.p.l.c., before esterification.

Porphobilinogen deaminase activity was determined by incubation at 37 °C in the presence of porphobilinogen. The reaction was terminated by the addition of 30 µl of 5 M HCl and 30 µl of benzoquinone (1 mg/ml) in methanol. Samples containing porphobilinogen deaminase produced uroporphyrin I ($\epsilon_{405} = 548 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Rimington and Sveinsson, 1950). One unit of deaminase is equivalent to 4 µmol of porphobilinogen utilized/min under standard assay conditions at 37 °C.

(b) Direct assay of uroporphyrinogen decarboxylase

Uroporphyrinogen decarboxylase was also assayed directly by following the formation of coproporphyrinogen III from the substrate uroporphyrinogen III by means of h.p.l.c. The reaction mixtures contained: 50 µl of the protein sample and 50 µl of 500 mM potassium phosphate buffer (pH 7.0) containing 100 mM dithiothreitol. The reaction was started by the addition of 10 µl of uroporphyrinogen (final concentration of 2–4 µM) (see above). Samples were incubated under N₂ at 37 °C for 3 min in the dark with a suitable non-enzymic control containing buffer alone. The reaction was terminated by the addition of 30 µl of DMSO/5 M HCl (1:1, v/v) to 50 µl of the incubation mixture. The resulting porphyrinogens were oxidized as described above. Samples were then stored in the dark on ice. Precipitated protein was removed by centrifugation for 5 min in a microfuge and a 20 µl sample was analysed by an h.p.l.c. system, previously calibrated with known quantities of the porphyrin. A unit of activity is defined as 1 nmol of coproporphyrinogen III produced/h under standard assay conditions at 37 °C.

M_r determination

(a) Denaturing SDS/PAGE

The M_r value of uroporphyrinogen decarboxylase was determined by SDS/PAGE under denaturing conditions, as described by Weber and Osborn (1969).

(b) Gel-filtration chromatography and non-denaturing PAGE

The M_r value of native uroporphyrinogen decarboxylase was determined by gel-filtration chromatography on a column of Sephacryl S-200 (580 mm $\times$ 6.5 mm) equilibrated with 25 mM Tris/HCl buffer, pH 7.0, eluted at a flow rate of 0.13 ml/min. The column was standardized by means of protein M_r standards. The M_r value was also determined by means of non-denaturing PAGE using a kit (Sigma) based on the modified method of Bryan (1977).

Determination of the isoelectric point of uroporphyrinogen decarboxylase

The isoelectric point of uroporphyrinogen decarboxylase was determined by means of an LKB/Pharmacia flat-bed isoelectric focusing apparatus, using LKB ampholine PAGE plates according to the manufacturer's instructions (pH range 3.5–9.5) and standards of known pI. The gel was focused at 10 mA constant current for 2 h and protein was visualized by staining with Coomassie Brilliant Blue. A standard curve of R_f versus isoelectric point was plotted and the isoelectric point of uroporphyrinogen decarboxylase calculated. The protein standards used were: trypsinogen (pI 9.3), lactic dehydrogenase (pI 8.4), carbonic anhydrase (pI 6.6), β -lactoglobulin A (pI 5.1) and glucose oxidase (pI 4.2).

Sequencing of the N-terminus of uroporphyrinogen decarboxylase

Native uroporphyrinogen decarboxylase (40 μ g) in 25 mM potassium phosphate, pH 7.0, was applied to polybrene filter discs and sequenced using an Applied Biosystems 477A protein sequencer with a PTH 120A analyser.

Titration of free sulphydryl groups

The number of free sulphydryl groups in native uroporphyrinogen decarboxylase was deduced by reaction with 5,5'-dithiobis(2-nitrobenzoate) (DTNB). Uroporphyrinogen decarboxylase (53 μ g) was mixed with 25 μ l of DTNB (4 mg/ml) in a total volume of 700 μ l of 50 mM potassium phosphate buffer, pH 7.0, and the absorbance changes at 412 nm were recorded using a Pye Unicam SP8-100 UV/VIS spectrophotometer, until no further changes were observed.

The number of free sulphydryl groups in the denatured protein were estimated by heating the uroporphyrinogen decarboxylase (53 μ g) at 65 °C for 15 min, before reaction with DTNB. The extinction coefficient ($\epsilon_{412} = 10000 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Seehra et al., 1981) was used to calculate the number of sulphydryl groups/mol of protein.

Modification of uroporphyrinogen decarboxylase by a variety of reagents

Uroporphyrinogen decarboxylase was pre-incubated at 25 °C for the required time in the presence of the reagent at a final concentration of 1–2 mM and the effect on activity was determined as indicated in the legends to Table 3 and Figure 5.

Determination of the level of ^{14}C incorporated into uroporphyrinogen decarboxylase

Uroporphyrinogen decarboxylase was incubated in the presence of *N*-ethyl[2,3- ^{14}C]maleimide (4 mCi/mmol) at 25 °C for 30 min in the presence and absence of porphyrins (5–20 μM). Unreacted

N-ethyl[2,3- ^{14}C]maleimide was removed from uroporphyrinogen decarboxylase by gel-filtration chromatography using a PD-10 column (Pharmacia). The quantity of label incorporated was determined, following the addition of 2 ml of Ready Protein liquid scintillation cocktail (Beckman) to a sample of 'labelled' uroporphyrinogen decarboxylase (500 μ l), using a United Technologies Packard Tri-carb 460 scintillation counter, counting for 1 min.

RESULTS AND DISCUSSION

Purification of uroporphyrinogen decarboxylase from *R. sphaeroides*

To date, there is no published method for the purification to homogeneity of a bacterial uroporphyrinogen decarboxylase. Previously published methods describe only the partial purification of the enzyme from *R. sphaeroides* (Hoare and Heath, 1959) and *R. palustris* (Koopmann et al., 1986). The purification procedure described in this paper gives homogeneous uroporphyrinogen decarboxylase from *R. sphaeroides*, with a 600-fold purification overall (Table 1).

Gel-filtration chromatography using a Sephacryl S-200 column was placed uncharacteristically early in the procedure because it gave a reasonable degree of purification and also desalted the sample for immediate application on to the Mono-Q anion-exchange f.p.l.c. column. In the early stages of the purification it was noted that porphobilinogen deaminase and the decarboxylase co-purified, the deaminase being separated only during the first f.p.l.c. stage. Thus the more rapid porphobilinogen deaminase assay was used in preference to the more laborious decarboxylase assay to locate the enzyme during the early stages of the purification.

The final stages of the purification procedure included two anion-exchange-chromatography steps using f.p.l.c. The selection of pH 8.0 for the first separation using f.p.l.c. was made because it gave very good separation of porphobilinogen deaminase from the uroporphyrinogen decarboxylase. The second f.p.l.c. step, at pH 5.2, removed the remaining traces of protein contamination from the uroporphyrinogen decarboxylase. The purified decarboxylase had a specific activity of 754 units/mg of protein with a typical u.v.-absorbance spectrum having a maximum at 280 nm. No evidence for the presence of a chromophore or the involvement of a co-enzyme was found. The enzyme could be conveniently stored frozen in 25 mM potassium phosphate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol. The enzyme lost

Table 1 The purification of uroporphyrinogen decarboxylase from 50 litres of photosynthetically grown *R. sphaeroides*

Stage	Volume (ml)	Protein content (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (-fold)
Crude extract	129	1651	2080	1.26	100	1.0
Ultracentrifugation extract	110	1210	2057	1.70	99	1.4
(NH_4) ₂ SO ₄ (35–70%)	29	783	1990	2.54	96	2.0
Gel filtration F.p.l.c.	65	224	1792	8.00	86	7.0
pH 8.0	24	5.5	1780	324.0	85	260
pH 5.2	4	1.16	872	754.0	42	600

Figure 2 A sample of purified uroporphyrinogen decarboxylase

Uroporphyrinogen decarboxylase was subjected to PAGE in the presence of SDS (Weber and Osborn, 1969). Lane 1 contains homogeneous uroporphyrinogen decarboxylase. Lane 2 contains monomeric protein M_r standards: A, BSA (66 000); B, ovalbumin (45 000); C, glyceraldehyde-3-phosphate dehydrogenase (36 000); D, carbonic anhydrase (29 000); E, trypsinogen (24 000); F, trypsin inhibitor (20 100); and G, α -lactalbumin (14 200).

activity very rapidly in the absence of the 2-mercaptoethanol. The enzyme was unstable at pH values below pH 5.5.

M_r determination of uroporphyrinogen decarboxylase

The uroporphyrinogen decarboxylase from *R. sphaeroides* was judged to be homogeneous by use of denaturing PAGE in the presence of SDS, followed by staining with Coomassie Brilliant Blue (Figure 2). The M_r of uroporphyrinogen decarboxylase was found to be $41\,000 \pm 3\,000$, by comparison with standard protein M_r markers. The determination of the M_r value of the native protein under non-denaturing conditions was important to determine whether the uroporphyrinogen decarboxylase exists as a monomer or an oligomer. Gel-filtration chromatography, through a Sephacryl S-200 column, yielded a value of $39\,000 \pm 4\,000$, while non-denaturing PAGE yielded a value of $38\,000 \pm 3\,000$. These values, when taken with the value obtained from denaturing PAGE, suggest that uroporphyrinogen decarboxylase from *R. sphaeroides* is a monomeric protein. The finding is consistent with observations on uroporphyrinogen decarboxylases purified from other human (de Verneuil et al., 1983a), yeast (Felix and Brouillet, 1990) and bovine (Straka and Kushner, 1983) sources, where the M_r values ranged from 39 000 to 46 000. The chicken enzyme appears to be an exception, being a dimer with a reported M_r of 72 000 (Kawanishi et al., 1983).

Kinetic properties of uroporphyrinogen decarboxylase

The apparent K_m values for the conversion of uroporphyrinogen I and III into coproporphyrinogen I and III were determined, using substrate concentrations of 0.5–5.0 μM , and found to be 1.8 μM and 6.0 μM respectively. These values are of a similar

order to those reported for uroporphyrinogen III for the decarboxylase from human erythrocytes, 0.35 μM (de Verneuil et al., 1983a); rat liver, 2.7 μM (Smith and Francis, 1981); and bovine liver, 0.46 μM (Straka and Kushner, 1983).

In separate experiments, increasing concentrations of uroporphyrinogen III have been shown to decrease uroporphyrinogen decarboxylase activity, an effect attributed to substrate inhibition (see Figure 3a). The decrease in coproporphyrinogen production was accompanied by an accumulation of the heptacarboxylic acid porphyrinogen intermediate(s). Similar observations have been reported by Kawanishi et al. (1983) with preparations of uroporphyrinogen decarboxylase isolated from chicken erythrocytes.

The effect of porphyrins on activity

The incubation of uroporphyrinogen decarboxylase with uroporphyrinogen III in the presence of a variety of porphyrins showed that inhibition of coproporphyrinogen III production occurs with all the porphyrins tested (Table 2). The degree of inhibition of activity by uroporphyrin I and III was shown to be concentration dependent (Figure 3b). The most effective inhibitors of activity were coproporphyrin I and III. Inhibition to a lesser degree was also exhibited by protoporphyrin IX, haemin and synthetic porphyrins, such as mesoporphyrin IX and haematoporphyrin IX. Inhibition of activity by these porphyrins appeared to be dependent upon the nature of β -substituents at positions 3 and 8, on the A and B rings respectively. The more hydrophilic the side chain the greater the degree of inhibition. The order of inhibition observed was haematoporphyrin > mesoporphyrin > protoporphyrin, where the hydrophilicity of R is $-\text{CHOHCH}_3 > -\text{CH}=\text{CH}_2 > -\text{CH}_2\text{CH}_3$. This is consistent with the requirement that the active-site region binding these side chains must be hydrophilic or charged to accommodate the propionic acid side chains of the substrates.

Porphobilinogen, the precursor of uroporphyrinogen III, was also an effective inhibitor of uroporphyrinogen decarboxylase activity. Presumably the acetic acid and propionic acid side chains of porphobilinogen can interact with the active site in a similar way to the uroporphyrinogen.

Table 2 The effect of porphyrins on purified uroporphyrinogen decarboxylase activity

Uroporphyrinogen decarboxylase (0.2 nmol) was incubated with uroporphyrinogen III in the presence of a variety of porphyrins (concentrations as shown) and the effects upon activity were determined, as described in the Materials and methods section. All results are from a minimum of three determinations.

Porphyrin	Concentration (μM)	Activity (%)
Control	0	100
Porphobilinogen	8	65
Uroporphyrin I	8	54
Uroporphyrin III	8	49
Coproporphyrin I	8	29
Coproporphyrin III	8	25
Protoporphyrin IX	8	87
Mesoporphyrin IX	8	68
Haematoporphyrin IX	8	38
Haemin	8	81

Figure 3 Properties of purified uroporphyrinogen decarboxylase

(a) The effect of substrate concentration on the activity of uroporphyrinogen decarboxylase. Pure uroporphyrinogen decarboxylase was assayed for activity, as described in the Materials and methods section, with a range of uroporphyrinogen III concentrations. The quantity of enzyme used for each incubation was 11.7 μ g. (b) The effect of uroporphyrin I and III on the activity of purified uroporphyrinogen decarboxylase. Aliquots of uroporphyrinogen decarboxylase were incubated with uroporphyrinogen III, as described in the Materials and methods section, in the presence of a range of concentrations of uroporphyrin I and III. (c) The pH optimum for purified uroporphyrinogen decarboxylase. The pH optimum of pure uroporphyrinogen decarboxylase was determined by the assay of activity, as described in the Materials and methods section, using uroporphyrinogen III as substrate, at a range of different pH values, in phosphate buffer. (d) The effect of prolonged incubation at 55 °C on the activity of purified uroporphyrinogen decarboxylase. Pure uroporphyrinogen decarboxylase was pre-incubated at 55 °C for various time intervals. Aliquots were removed and assayed for activity, as described in the Materials and methods section, using uroporphyrinogen III as substrate. All results are from a minimum of three determinations.

Determination of the pH optimum and isoelectric point of uroporphyrinogen decarboxylase

The effect of pH on the activity of uroporphyrinogen decarboxylase gave a pH optimum between pH values of 6.7 and 6.9, and yielded a 'bell-shaped' curve (Figure 3c) showing a more rapid loss of activity at acidic pH values. Similar values for the pH optimum (pH 6.8) have been reported for the decarboxylases from human (de Verneuil et al., 1983a), chicken (Kawanishi et al., 1983) and yeast (Felix and Brouillet, 1990).

The isoelectric point of uroporphyrinogen decarboxylase, determined by isoelectric focusing, was found to be pH 4.4. A single protein band was obtained on the gel, confirming that the uroporphyrinogen decarboxylase was homogeneous. The pI

determined for uroporphyrinogen decarboxylase from other sources are 4.6 for the bovine enzyme (Straka and Kushner, 1983), and 6.0–6.2 for both the yeast (Felix and Brouillet, 1990) and the avian enzymes (Kawanishi et al., 1983).

Thermal denaturation of uroporphyrinogen decarboxylase activity

Heat treatment leads to a progressive loss in uroporphyrinogen decarboxylase activity. Above 40 °C the accumulation of the heptacarboxylic acid porphyrinogen intermediate was evident. This suggests that heat-induced conformational changes lead to a reduction in the uroporphyrinogen decarboxylase:uroporphyrinogen III ratio, resulting in the production of the hepta-

Human MEANGLGPQGFPPELKNDFLRAAWGEETDYTFVWCMRQAGRYLP.....
 Rat MEANGLGLQNFPELKNDFLRAATGEETDYTFVWCMRQAGRYLP.....
 Yeast MGNFPAPKNDLILRAAKGEKFERPPCWIMRQAGRYLP.....
Synechococcus sp. MVASSSLPRLLRAARGEVLDRFPVMMRQAGRYMK.....
B. subtilis MSKRETFNETLKRARGEKADHTFVWYMRQAGRSQP.....
R. sphaeroides TMLRALKGETLPTPSIWLMRQAGPYLP.....
 * ** * *****

Figure 4 Comparison of the N-terminal amino-acid sequence of *R. sphaeroides* with the sequences derived from the cDNA of *hemE* of human, rat and *B. subtilis*, *hem13* of yeast, and *ORF2* of *Synechococcus*

Identical amino acids in the sequences are indicated by asterisks.

carboxylic acid porphyrinogen rather than coproporphyrinogen III (Kawanishi et al., 1983; Straka and Kushner, 1983).

Incubation of uroporphyrinogen decarboxylase at 55 °C for various periods of time showed a steady loss in coproporphyrinogen production over the first 20 min, with a $t_{1/2}$ at 55 °C of 8 min (Figure 3d). The effects of heat upon activity are similar to those made on the uroporphyrinogen decarboxylases from human erythrocytes (Elder et al., 1983; de Verneuil et al., 1983a). In contrast, the uroporphyrinogen decarboxylase partially purified from *R. palustris* (Koopmann et al., 1986) was reported to be heat stable and this property was exploited during its partial purification.

Determination of the N-terminal sequence of uroporphyrinogen decarboxylase

The sequence of the first 29 amino acids from the N-terminus of uroporphyrinogen decarboxylase from *R. sphaeroides* was determined by Edman degradation. Comparison of the amino-acid sequences of the N-terminus of the uroporphyrinogen decarboxylase from *R. sphaeroides* with those derived from the cDNA of human (Romeo et al., 1986), rat (Romeo et al., 1984), yeast (Garey et al., 1992), the open reading frame, ORF2, from *Synechococcus* (Kiel et al., 1990) and the *hemE* gene from *Bacillus subtilis* (Hansson and Hederstedt, 1992) show remarkable areas of similarity (Figure 4). The sequence of the N-terminus of the *R. sphaeroides* enzyme is 50% identical with the human enzyme and 32% identical when compared with all known sequences. Of particular note is the sequence MRQAG in which a conserved arginine is evident (see below).

Analysis of the human, rat and yeast cDNA-derived sequences show a high degree of similarity throughout, with a notable conservation of positively charged amino acids. In addition to these residues, there is a single conserved cysteine residue. It would be interesting to determine whether the cysteine group modified in the *R. sphaeroides* enzyme is structurally related.

Investigation of sulphhydryl groups in uroporphyrinogen decarboxylase

(i) Titration of sulphhydryl groups with DTNB

Reaction of DTNB (4 mg/ml) with native uroporphyrinogen decarboxylase indicated the presence of five sulphhydryl groups/molecule of enzyme (Figure 5). Two groups titrated in the first 5 min, with the remaining three groups reacting more slowly. The reaction of DTNB completely inactivated the enzyme.

When the DTNB reaction was carried out with heat-denatured enzyme, a more rapid reaction occurred in which three groups

Figure 5 The titration of sulphhydryl groups of purified uroporphyrinogen decarboxylase with DTNB

The free cysteine residues of pure uroporphyrinogen decarboxylase were reacted with DTNB, and the change in absorbance followed to completion. Trace A shows the titration of native uroporphyrinogen decarboxylase, and Trace B shows heat-denatured enzyme.

Table 3 The effect of various reagents on the activity of uroporphyrinogen decarboxylase

Purified uroporphyrinogen decarboxylase (0.2 nmol) was pre-incubated with various reagents (concentrations as shown) for 1 h at 25 °C. The metals were all chloride salts except for Cu^{2+} , where the sulphate salt was utilized. The effect on the activity was determined as described in the Materials and methods section, using uroporphyrinogen III as substrate in the absence of dithiothreitol. All results are from a minimum of three determinations.

Reagent	Concentration (mM)	Activity (%)
None	0	100
Iodoacetic acid	2	80
Iodoacetamide	2	43
N-Ethylmaleimide	2	0
Phenyglyoxal	2	0
Dithiothreitol	2	150
Fe^{3+}	1	83
Mn^{2+}	1	125
Ni^{2+}	1	96
Na^+	1	97
Hg^{2+}	1	0
Cu^{2+}	1	57
Co^{2+}	1	97
Mg^{2+}	1	105
Zn^{2+}	1	98
EDTA	25	96

titrated within the first 5 min. These findings suggest that more sulphhydryl groups are accessible than in the native enzyme, presumably due to a heat-induced conformational change during denaturation.

(ii) The effect of sulphhydryl reagents on uroporphyrinogen decarboxylase activity

The results of pre-incubating uroporphyrinogen decarboxylase with a variety of potential protein-modifying reagents are shown in Table 3. Incubation of uroporphyrinogen decarboxylase with uroporphyrinogen III after treatment with the cysteine-modifying reagents resulted in an inactivation of the enzyme consistent with the order of chemical reactivity of the reagent (i.e. *N*-ethylmaleimide > iodoacetamide > iodoacetic acid). The inactivation

Figure 6 The effect of protein-modification reagents on the activity of purified uroporphyrinogen decarboxylase

Uroporphyrinogen decarboxylase was pre-incubated with a range of reagent concentrations for 1 h at 25 °C. The effect on the activity was determined using uroporphyrinogen III as substrate, in the absence of dithiothreitol. The reagents used were: ■, iodoacetic acid; ▲, *N*-ethylmaleimide; and ●, phenylglyoxal. All results are from a minimum of three determinations.

Figure 7 The effect of dithiothreitol concentration on the activity of purified uroporphyrinogen decarboxylase

Purified uroporphyrinogen decarboxylase was assayed for activity, as described in the Materials and methods section, using uroporphyrinogen III as substrate, in the presence of a range of dithiothreitol concentrations as indicated. All results are from a minimum of three determinations.

resulted in the increased accumulation of the heptacarboxylic acid porphyrinogen intermediate. The degree of inactivation was concentration-dependent (Figure 6). The involvement of a cysteine residue is consistent with the need for a reducing agent, such as dithiothreitol or 2-mercaptoethanol, for maximal enzyme activity. Incubation of uroporphyrinogen decarboxylase in the presence of dithiothreitol (up to 50 mM) resulted in the stimulation of activity (Figure 7).

(iii) The determination of the number of sulphydryl groups which are important for activity, and the protective effect of porphyrins on the modification of uroporphyrinogen decarboxylase by *N*-ethylmaleimide

The above experiments highlighted the importance of sulphydryl residues for activity and prompted further investigation to determine the nature of the inactivation. The number of cysteine

Table 4 The effect of porphyrins upon the modification of uroporphyrinogen decarboxylase by *N*-ethyl[2,3-¹⁴C₂]maleimide

Pure uroporphyrinogen decarboxylase (12 μg) was incubated with *N*-ethyl[2,3-¹⁴C₂]maleimide (0.5 μCi) in the presence and absence of various concentrations of uroporphyrin III and coproporphyrin III (as indicated), and the effect upon the incorporation of label determined. All results are from a minimum of three determinations.

Porphyrin	Concentration (μM)	Incorporation of label (%)
Control	0	100
Uroporphyrin III	4	20
Uroporphyrin III	20	10
Coproporphyrin III	4	10
Coproporphyrin III	20	5

residues modified during inactivation was thus determined after modification of the enzyme with radiolabelled *N*-ethyl[2,3-¹⁴C₂]maleimide. A total of 7200 ± 200 d.p.m. of *N*-ethyl[2,3-¹⁴C₂]maleimide (specific radioactivity 4 mCi/mmol) was incorporated into 37 μg of uroporphyrinogen decarboxylase (7900 ± 200 d.p.m./nmol). Modification resulted in the complete inactivation of the enzyme. The quantity of radioactivity associated with the protein indicated that 0.9 mol of labelled reagent is bound/mol of inactivated protein, suggesting that a single cysteine residue is important for activity.

Modification of uroporphyrinogen decarboxylase with *N*-ethyl[2,3-¹⁴C₂]maleimide in the presence of either uroporphyrin III or coproporphyrin III resulted in a much reduced incorporation of label into the enzyme (Table 4). Uroporphyrin III, the oxidized substrate, and coproporphyrin III, the oxidized product, have been shown to be effective inhibitors of decarboxylation of uroporphyrinogen III (Table 2). The degree of 'protection' was dependent on the concentration of porphyrin present, although significant protection was seen at porphyrin concentrations close to the *K_m* value for uroporphyrinogen III. Coproporphyrin III, the more effective inhibitor of activity, gave the greater protection from modification by *N*-ethylmaleimide. This observation can be satisfactorily explained if the modified cysteine residue is located at the active site of the enzyme and is therefore obscured from the attacking sulphydryl reagent in the presence of porphyrin. Another possibility, although less likely, is that the binding of the porphyrin to uroporphyrinogen decarboxylase results in a conformational change that makes the cysteine residue inaccessible to attack by the *N*-ethylmaleimide.

Modification of uroporphyrinogen decarboxylase activity by other reagents

In addition to the effects of sulphydryl reagents, uroporphyrinogen decarboxylase was completely inactivated by phenylglyoxal, suggesting the participation of arginine residues (Table 3). Modification of uroporphyrinogen decarboxylase from rat liver with arginine-selective reagents has also been shown to result in the complete inactivation of the enzyme (Billi de Catabbi et al., 1991). It is generally accepted that arginine is one of the prime residues in proteins involved in the interaction with carboxyl groups. For instance, X-ray studies on porphobilinogen deaminase (Louie et al., 1992) have highlighted the direct interaction of several conserved arginine side chains with the acetic acid and propionate acid side chains of porphobilinogen in the catalytic cleft of this enzyme. Since uroporphyrinogens contain

Figure 8 H.p.l.c. separation of heptacarboxylic acid porphyrins produced by the incubation of uroporphyrinogen decarboxylase with uroporphyrinogen III during the first 30 s

Samples of the incubation (500 μ l) were separated by means of h.p.l.c. using a C_{18} reverse-phase column, as described in the Materials and methods section. The porphyrins were detected by means of a fluorescence detector. The peaks are assigned as follows: A, uroporphyrin III; B, 7C; C, 7D; and D, 7A + 7B.

eight negatively charged groups it is highly likely that several arginine residues may be involved (see also Figure 4).

Decarboxylation of uroporphyrinogen III was inhibited by the presence of both Hg^{2+} and Cu^{2+} , providing further evidence that a cysteine residue is important for activity. An interesting observation was that Mn^{2+} gave a 1.25-fold stimulation of activity, although there was no evidence for the requirement of metal ions, as judged by the lack of inhibition with EDTA.

Determination of the order of decarboxylation of uroporphyrinogen III

Whether the four decarboxylations involved in the transformation of uroporphyrinogen III into coproporphyrinogen III occur by a random or sequential route has been a matter of some controversy for several years (Akhtar, 1991). Previously, analysis of the accumulated decarboxylation products from patients suffering from porphyria cutanea tarda (Smith et al., 1980) and rats poisoned with hexachlorobenzene (San Martin de Viale et al., 1970; Jackson et al., 1976) suggested that an ordered mechanism was in operation in which the D ring was initially decarboxylated followed by the A, B and C rings. The intermediates found to accumulate were all of the D-ring isomer (Jackson et al., 1976), such as 7IIID in Figure 1, which have been shown to act as excellent substrates for the enzyme. In contrast, studies with the human erythrocyte decarboxylase suggested that the decarboxylation follows a random route (Luo and Lim, 1990).

To determine whether the decarboxylation route is ordered or random with the *R. sphaeroides* uroporphyrinogen decarboxylase, the production of enzymic products from uroporphyrinogen III was followed at early times during the reaction so that heptacarboxylic acid porphyrinogen intermediates could be analysed. Incubation of the purified enzyme with uroporphyrinogen III for only 30 s, followed by h.p.l.c., indicated

the formation of all four isomers of the heptacarboxylic acid porphyrinogens, with no isomer predominating significantly (Figure 8). This suggests that, under the conditions used, a random decarboxylation route is operative with the *R. sphaeroides* enzyme and that no ring preference is exhibited (Jones, 1992).

There are several possible explanations for these results: first, the decarboxylases from different sources may follow different reaction courses; secondly, the decarboxylases from patients with porphyria cutanea tarda, or rats poisoned with hexachlorobenzene, may possess modified decarboxylases that have altered substrate specificities (Billi de Catabbi et al., 1991); thirdly the differences in the results may be as a result of variations in the enzyme:substrate ratio during the assay. There is substantial evidence that changes in this ratio can affect the decarboxylation route (de Verneuil et al., 1983a; Kawanishi et al., 1983; Lash, 1991; Luo and Lim, 1993). Under the conditions used in our study, the high substrate:enzyme ratio may reduce the selectivity of the enzyme for the individual rings of the uroporphyrinogen III and could result in a random route of decarboxylation. *In vivo*, the lower level of substrate may favour a greater ring selectivity leading to an ordered decarboxylation route. It has not been excluded that under these conditions the intermediates may remain in the vicinity of the catalytic site and may be partially channelled, hence accounting for an ordered, clockwise decarboxylation route.

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