Anaerobic and aerobic coproporphyrinogen III oxidases of *Rhodopseudomonas spheroides*

Mechanism and stereochemistry of vinyl group formation

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(Received 24 August 1982/Accepted 12 November 1982)

An improved method for the preparation of various species of porphobilinogen stereospecifically labelled with ^3H in the side chains (at C-6, C-7 and C-8) is described. These labelled samples were used to study the mechanism and stereochemistry of anaerobic as well as aerobic coproporphyrinogen III oxidase of light-grown *Rhodopseudomonas spheroides*. It was shown that both the oxidases catalyse the conversion of the propionate side chains of coproporphyrinogen III into the vinyl groups of protoporphyrinogen IX,

with the labilization of the pro-*S*-hydrogen atom at the β -position. These results are similar to those previously recorded for such conversions in animal and plant systems. In the light of the cumulative information available to date, mechanisms for the conversion,

are discussed and doubt is cast on the participation of hydroxylated intermediates in the process.

Coproporphyrinogen III oxidase (EC 1.3.3.3) is involved in the later stages of haem and chlorophyll biosynthesis, converting coproporphyrinogen III into protoporphyrinogen IX. In this process the two propionate side chains in rings A and B of coproporphyrinogen III (structure 2 in Scheme 3) are successively decarboxylated to the corresponding vinyl groups of protoporphyrinogen IX (for a review on porphyrin biosynthesis see Akhtar & Jordan, 1979). Preparations of the enzyme isolated from mammalian liver mitochondria possess an absolute requirement for molecular oxygen (Yoshinaga & Sano, 1980, and references cited therein). The participation of hydroxylated intermediates in the reaction has been suggested by the demonstration that β -hydroxy side chains of the type present in 3β -hydroxypropionic acid-8-propionic acid deuteroporphyrinogen (cf. structure 3, Scheme 3) are elaborated to vinyl groups by the bovine liver

coproporphyrinogen III oxidase (Yoshinaga & Sano, 1980).

In contrast with the enzymes from mammalian, avian and plant tissues, which exhibit a requirement for oxygen, micro-organisms growing under strictly anaerobic conditions must be able to convert coproporphyrinogen III into protoporphyrinogen IX using a hydride acceptor other than oxygen. Indeed, an anaerobic coproporphyrinogen III oxidase activity was first reported in a species of *Pseudomonas* by Ehteshamuddin (1968) and later demonstrated in *Rhodopseudomonas spheroides* along with an aerobic coproporphyrinogen III oxidase activity by Tait (1972).

The substrate stereochemistry and the status of side-chain hydrogen atoms in the coproporphyrinogen III oxidase-catalysed reaction has previously been studied with the enzymes from avian reticulocytes and plants (Zaman & Akhtar, 1976; Abboud & Akhtar, 1978; Battersby *et al.*, 1972, 1975; Battersby, 1978). These studies demonstrate that the β -hydrogen atom with the pro-*S*-con-

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

figuration (β -H $\cdot$ in Scheme 3, structure 2) is eliminated during the formation of the vinyl group and that the overall reaction proceeds via an antiperiplanar mechanism (see Scheme 1). In the present paper we extend these studies and describe a new method for the preparation of tritiated porphobilinogen from stereospecifically tritiated succinate. In this new method, succinate is converted chemically into succinoyl-CoA, and the succinoyl-CoA is then incorporated enzymically into porphobilinogen, whereas in previous methods labelled porphobilinogen was prepared from succinate in a less reliable linked enzyme system (Zaman & Akhtar, 1976). The labelled samples of porphobilinogen (Scheme 1) have been used to study the stereochemical courses for the anaerobic as well as aerobic coproporphyrinogen III oxidase-catalysed reactions. Mechanistic implications of these results are also discussed.

Experimental

Materials

[2,3- $^{14}\text{C}_2$]Succinic acid and $^3\text{H}_2\text{O}$ were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Dowex-1 (Cl^- form), 5-amino-laevulinic acid hydrochloride, disodium 2-oxoglutarate, isocitrate dehydrogenase (from pig heart) and Sigma Cell 20 plates were bought from Sigma Chemical Co., Poole, Dorset, U.K. Dicyclohexylcarbodi-imide was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. DEAE-Bio-Gel A was purchased from Bio-Rad Laboratories, St. Albans, Herts., U.K. All other laboratory reagents were purchased from BDH Chemicals, Poole, Dorset, U.K.

Preparation of 2-oxo[3- $^3\text{H}_2$]glutarate

2-Oxo[3- $^3\text{H}_2$]glutarate was prepared from unlabelled 2-oxoglutarate by an exchange reaction with $^3\text{H}_2\text{O}$ (5 Ci/ml) (Rose, 1960; Zaman & Akhtar, 1976) at elevated temperature. The specific radioactivity of the 2-oxo[3- $^3\text{H}_2$]glutarate was 23.68 $\mu\text{Ci}/\mu\text{mol}$.

Preparation of (2RS)-[2- $^3\text{H}_2$]succinate

A 5% (w/v) solution of H_2O_2 (6.6 ml) was added to 2-oxo[3- $^3\text{H}_2$]glutarate disodium salt (52 μmol ; 1.23 mCi) in 10 ml of 100 mM-triethanolamine/HCl buffer, pH 7.6, containing 0.6 mM-MgCl $_2$. After 30 min at 30°C, the reaction mixture was applied to a Dowex-1 (Cl^- form) column (2.5 cm $\times$ 10 cm) that was washed with an excess of water (50 ml) to remove the buffer and any remaining H_2O_2 . Succinic acid was eluted with 0.1 M-HCl (approx. 20 ml), and the solution was evaporated under reduced pressure [yield of (2RS)-[2- $^3\text{H}_2$]succinic acid was 43 μmol ; specific radioactivity 23.65 $\mu\text{Ci}/\mu\text{mol}$].

Preparation of (3R)-2-oxo[^3H]glutarate (Lienhard & Rose, 1964)

2-Oxo[3- $^3\text{H}_2$]glutarate disodium salt (60 μmol ; 1.42 mCi) was dissolved in 10 ml of 100 mM-triethanolamine/HCl buffer, pH 7.6, containing 0.6 mM-NADPH, 0.6 mM-MgCl $_2$ and isocitrate dehydrogenase [25 units ($\mu\text{mol}/\text{min}$)]. Samples (0.1 ml) were removed at timed intervals and applied to a Dowex-1 (Cl^- form) column (0.5 cm $\times$ 4 cm) that was washed with water (10 ml), and the radioactivity in the eluent was counted to determine free tritium in the medium. After 50% of the tritium in 2-oxo[3- $^3\text{H}_2$]glutarate had been released into the medium, the reaction was stopped by the addition of 5% H_2O_2 (6.6 ml). The (2R)-[^3H]succinate was purified by ion-exchange chromatography as described above (yield 51 μmol ; specific radioactivity 11.84 $\mu\text{Ci}/\mu\text{mol}$).

Preparation of (2S)-[2- ^3H]succinate

2-Oxoglutarate (68.5 μmol) was dissolved in 0.2 ml of 500 mM-triethanolamine/HCl buffer, pH 7.5, containing 30 mM-NADPH and isocitrate dehydrogenase (12 units). Then 5 Ci of $^3\text{H}_2\text{O}$ (1 ml) was added to the buffer solution to give a final volume of 2 ml. The mixture was incubated at 30°C in a sealed vial, and the reaction was terminated after 3 h by the addition of 1.5 ml of 30% (w/v) H_2O_2 . After 30 min, the (2S)-[2- ^3H]succinate was purified on a Dowex-1 (Cl^- form) column as above [yield of (2S)-[2- ^3H]succinate was 52 μmol ; specific radioactivity 7.32 $\mu\text{Ci}/\mu\text{mol}$].

Conversion of labelled succinates into succinic anhydride

Tritiated succinates were mixed with [2,3- $^{14}\text{C}_2$]-succinate ($^3\text{H}/^{14}\text{C}$ ratios as in Table 1), and the doubly labelled succinates (200 μmol) were dissolved in anhydrous acetone (5 ml). Dicyclohexylcarbodi-imide (200 μmol) was added, and the reaction mixture was gently stirred at room temperature for 30 min until all the carbodi-imide had reacted (as monitored by i.r. spectroscopy: $\bar{\nu} = 2100\text{ cm}^{-1}$). The conversion of succinic acid into succinic anhydride was completed by the further addition of dicyclohexylcarbodi-imide (20 μmol). Dicyclohexylurea was filtered off, and the acetone was removed from the filtrate under reduced pressure. The filtrate residue was extracted with anhydrous diethyl ether (3 $\times$ 10 ml), and the ether was evaporated from the extract under reduced pressure to give the doubly labelled succinic anhydride (specific radioactivities shown in Table 1). In a typical experiment 200 μmol of succinate gave 160–170 μmol of succinic anhydride.

Preparation of labelled succinoyl-CoA

Labelled succinoyl-CoA was freshly prepared by stirring the labelled succinic anhydride (1.0 mg) in a 1 ml solution containing NaHCO_3 (4.5 mg) and CoA (8 mg) (Simon & Shemin, 1953; Zaman *et al.*, 1973). The mixture was stirred at 0°C for 30 min, during which time samples (5 μl) were removed and mixed with 500 μl of 1 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). The lack of appearance of the yellow 5-thio-(2-nitrobenzoic acid) anion established that the CoA thiol had reacted quantitatively with the succinic anhydride to yield succinoyl-CoA.

Growth of bacteria

Rhodopseudomonas spheroides (N.C.I.B. 8253) was grown semi-anaerobically in the light, in Lascelles's medium S (Lascelles, 1956) supplemented with 0.2% (w/v) yeast extract and 2.5 mg of ferric citrate/l. The cells were grown at 26°C, harvested when in the exponential phase and washed with 0.1 M-potassium phosphate buffer, pH 7.9, that had previously been saturated with N_2 . The harvested cells were used fresh.

Purification of 5-aminolaevulinate synthase (EC 2.3.1.37) from Rps. spheroides

Freshly harvested cells (100 g wet weight) were resuspended in 200 ml of 0.1 M-potassium phosphate buffer, pH 6.9, containing 0.5 mM-dithioerythritol and 0.5 mM-EDTA. The cells were disrupted by sonication for 20 min (5 $\times$ 4 min bursts) with intermittent cooling and centrifuged at 18 000 g for 30 min. The supernatant from the low-speed centrifugation was centrifuged at 120 000 g for 3 h, and the clear supernatant was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation (0–35%, 35–45% and 45–60% saturation). The most active fraction (0–35% saturation) was dissolved in 25 ml of 10 mM-potassium phosphate buffer, pH 7.9, containing 0.5 mM-EDTA and dialysed overnight against the same buffer (2 $\times$ 5 ml). The dialysed enzyme was applied to a DEAE-Bio-Gel A column (2.5 cm $\times$ 5.0 cm) previously equilibrated with the same buffer. After the column had been washed with the application buffer, the enzyme was eluted with a linear gradient of KCl (0–150 mM) in the same buffer. The fractions containing the enzymic activity were pooled and precipitated with $(\text{NH}_4)_2\text{SO}_4$ (55% saturation) and centrifuged. The protein pellet was dissolved in 10 ml of 5 mM-potassium phosphate buffer, pH 6.8, containing 5% (v/v) glycerol and dialysed against the same buffer (5 litres) overnight. The dialysed protein was applied to a pre-equilibrated hydroxyapatite column (2.5 cm $\times$ 5 cm), and the column was washed with 200 ml of 40 mM-potassium phosphate buffer, pH 6.9, containing 5% (v/v) glycerol. The enzyme was eluted

with a linear gradient of potassium phosphate buffer, pH 6.9 (40–150 mM; 100 ml). The fractions containing the enzyme activity [approx. 25 units ($\mu\text{mol/h}$)] were stored frozen.

Enzyme activity was assayed as described previously (Zaman *et al.*, 1973).

Purification of bovine liver 5-aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24)

5-Aminolaevulinate dehydratase was purified from bovine liver by previously described methods (Seehra, 1980). The enzyme was stored as a 60%-satn.-(NH_4)₂SO₄ precipitate until required.

Conversion of succinoyl-CoA into porphobilinogen

The incubation mixture contained 50 μmol of Tris/HCl buffer, pH 6.8, 800 μmol of glycine, 1 μmol of pyridoxal 5'-phosphate, 350 units ($\mu\text{mol/h}$) of 5-aminolaevulinate dehydratase (in 1 ml of 5 mM-potassium phosphate buffer, pH 6.8) and 200 units of 5-aminolaevulinate synthase in a final volume of 7.5 ml. Freshly prepared succinoyl-CoA solution (2.5 ml) (see above) was added, and the mixture was incubated at 37°C under an atmosphere of N_2 until porphobilinogen formation was complete as monitored by reaction with *p*-dimethylaminobenzaldehyde (Mauzerall & Granick, 1956; Seehra & Jordan, 1980). Porphobilinogen was purified by applying the reaction mixture to a Dowex-1 X8 (acetate form) column (2 cm $\times$ 10 cm) that was first washed with water (approx. 40 ml) and then eluted with 20 ml of ice-cold aqueous 1 M-acetic acid. The solvent was removed by rotary evaporation under high vacuum [7 Pa (0.05 mmHg)] at 20–30°C, and porphobilinogen was further purified by t.l.c. on Sigma Cell 20 plates (0.45 mm thickness) developed in butan-1-ol/acetic acid/water (67:27:10, by vol.). Porphobilinogen was located by spraying a small area with *p*-dimethylaminobenzaldehyde in 80% (v/v) acetic acid containing HClO_4 (12%, v/v), eluted with dilute aqueous NH_3 and freeze-dried. In a typical experiment 25 μmol of labelled succinic anhydride yielded 7.8–8.1 μmol of labelled porphobilinogen.

Biosynthesis of porphyrins by cell-free extracts of Rps. spheroides

Harvested cells of *Rps. spheroides* were suspended in 0.1 M-potassium phosphate buffer, pH 7.6 (1 g wet wt. of cells/ml of buffer). The suspension was sonicated for 20 min in 5 min bursts, with cooling between each burst, and centrifuged at 34 000 g for 10 min. The supernatant was used for all incubations under anaerobic conditions, or was centrifuged at 140 000 g for 3 h for aerobic conditions (pellet discarded).

Anaerobic incubations. The incubation mixture

(Tait, 1972) contained 0.2 mmol of MgSO_4 , 0.8 mmol of ATP, 0.2 mmol of L-methionine, 0.6 mmol of NADH, 0.4 mmol of NADP^+ and cell-free extract (130 ml) in a final volume of 150 ml. Porphobilinogen (5 mg) was added and the mixture was incubated at 37°C in the dark under O_2 -free N_2 in sealed flasks. Samples (10 ml) were removed at timed intervals, and the porphyrin content was analysed by h.p.l.c. (see below), after esterification with BF_3 /methanol. For the optimal formation of protoporphyrin IX from porphobilinogen the incubation was terminated after 16 h. As a control the cell-free extract was also incubated in the presence of air and in the absence of MgSO_4 , L-methionine, ATP, NADH and NADP^+ .

Aerobic incubation. The supernatant from the ultracentrifugation was used directly for determining the stereochemical course in protoporphyrinogen IX biosynthesis, under aerobic conditions. In a typical experiment, porphobilinogen (5 mg) in 0.1 M-potassium phosphate buffer, pH 7.6 (5 ml), containing $100\ \mu\text{M}$ -dithioerythritol was added to 75 ml of the extract. The reaction mixture was incubated under an atmosphere of air (with continuous gentle swirling) at 37°C for 16 h. The reaction was stopped by the addition of benzoquinone, and porphyrins were extracted as described above. The control experiment was performed anaerobically.

Analytical procedures for monitoring the formation of porphyrins

T.l.c. Samples (1 ml) from incubations were treated with benzoquinone (6 mg/ml in methanol) to oxidize porphyrinogens to porphyrins. The sample was freeze-dried, and $200\ \mu\text{l}$ of BF_3 /methanol (Sigma Chemical Co.) was added. Reaction was allowed to proceed for 30 min at 60°C . After this time 1 ml of water was added, and the porphyrin esters were extracted into $500\ \mu\text{l}$ of chloroform. The chloroform layer was washed with saturated NaHCO_3 (with care), and the porphyrins were finally washed with water. The chloroform layer was dried over anhydrous Na_2SO_4 and was finally evaporated to dryness. The porphyrins were purified by t.l.c. on silica gel G with benzene/ethyl acetate/ethanol (75:23:2, by vol.) (or 80:18:2, by vol., for optimal resolution of uroporphyrin) and in the presence of standard (see below). The porphyrins were detected by u.v. light: uroporphyrin III octamethyl ester (R_F 0.2), coproporphyrin III tetramethyl ester (R_F 0.48), harderoporphyrin trimethyl ester (R_F 0.55) and protoporphyrin IX dimethyl ester (R_F 0.7). The bands were eluted from the silica and subjected to h.p.l.c. (see below). Standard porphyrin esters were purchased from Porphyrin Products (Logan, UT, U.S.A.) or from Sigma Chemical Co.

H.p.l.c. The samples were processed as above

except that 10 ml samples were used. After esterification, the porphyrin esters were purified by h.p.l.c. with light petroleum (b.p. $60\text{--}80^\circ\text{C}$)/ethyl acetate (13:7, v/v) as the developing solvent at a speed of 1.5 ml/min [$3.5\text{--}4.1\ \text{MPa}$ ($500\text{--}600\ \text{lbf/in}^2$)] and with a μ -Porasil column ($300\ \text{mm} \times 4\ \text{mm}$) (Waters Associates). The porphyrin esters were eluted in the order of increasing polarity (Evans *et al.*, 1976): protoporphyrin IX dimethyl ester (5 min), harderoporphyrin ester (6 min), coproporphyrin III tetramethyl ester (7 min) and uroporphyrin octamethyl ester (17 min). The esters were collected in scintillation vials, and after evaporation of solvent were assessed for radioactivity as described previously (Zaman & Akhtar, 1976).

Separation, purification and degradation of protoporphyrin IX

The bulk of the incubation mixture was processed for obtaining protoporphyrin IX by using a modification (Seehra & Jordan, 1980) of the method described by Tait (1972). The methods for the degradation of protoporphyrin IX were the same as previously used by Zaman & Akhtar (1976). These involve first the saturation of two vinylic double bonds to give mesoporphyrin IX and then oxidation of the latter with $\text{CrO}_3/\text{H}_2\text{SO}_4$ to give ethylmethylmaleimide (structure 5 in Scheme 3) and haematinic acid (structure 6 in Scheme 3).

Results

Synthesis of stereospecifically labelled species of porphobilinogen (Scheme 1)

(3*RS*)-2-Oxo[3- ^3H]glutarate was prepared by exchange of the C-3 hydrogen atoms of 2-oxoglutarate with $^3\text{H}_2\text{O}$ (Rose, 1960). Incubation of (3*RS*)-2-oxo[3- ^3H]glutarate with isocitrate dehydrogenase resulted in the exchange of the pro-S-hydrogen atom with the medium, yielding (3*R*)-2-oxo[3- ^3H]glutarate (Englard & Hanson, 1969; Lienhard & Rose, 1964). The converse sequence, in which unlabelled 2-oxoglutarate was incubated with isocitrate dehydrogenase in the presence of $^3\text{H}_2\text{O}$, gave (3*S*)-2-oxo[3- ^3H]glutarate. All these samples of tritiated 2-oxoglutarate were converted into succinic acid by using H_2O_2 . Previous work in this laboratory (Abboud & Akhtar, 1978) had shown the presence of an exchange activity in partially purified preparations of 5-aminolaevulinate synthase that catalysed the exchange of CoA between succinate and succinoyl-CoA and was used for the preparation of labelled succinoyl-CoA. However, the partially purified preparation of 5-aminolaevulinate synthetase available for this work did not possess this exchange activity, and thus it was necessary to convert the succinates into succinoyl-CoA chemically. The ^3H -labelled succinates were admixed

Scheme 1. Structures of stereospecifically labelled species of porphobilinogen

It should be noted that the tritium atoms at C-2 of succinate would label C-2 and C-3 of 5-aminolaevulinate and therefore C-6, C-8 and C-9 of porphobilinogen.

Table 1. Preparation of stereospecifically labelled samples of porphobilinogen from labelled succinates
Tritiated succinates were mixed with [2,3-¹⁴C]succinic acid and the mixture was converted into succinic anhydride. The latter, via succinoyl-CoA, were converted into porphobilinogen by using a coupled enzyme system consisting of 5-aminolaevulinate synthase and 5-aminolaevulinate dehydratase (details are given in the Experimental section).

Labelled succinate precursor (d.p.m. of ³ H/μmol/d.p.m. of ¹⁴ C/μmol)	³ H/ ¹⁴ C ratio		
	Succinate	Succinic anhydride	Porphobilinogen
(2RS)-[¹⁴ C ₂ ,2- ³ H ₂]Succinate $\frac{6.65 \times 10^6}{3.01 \times 10^5}$	22.09	22.10	16.58 (structure 1a)
(2R)-[¹⁴ C ₂ ,2- ³ H ₂]Succinate $\frac{2.37 \times 10^7}{1.009 \times 10^6}$	23.50	23.46	17.60 (structure 1b)
(2S)-[¹⁴ C ₂ ,2- ³ H ₂]Succinate $\frac{1.39 \times 10^6}{6.07 \times 10^4}$	22.90	22.97	17.20 (structure 1c)

Scheme 2. Conversion of porphobilinogen into porphyrins in *Rps. spheroides*

Reactions are catalysed by: (a) porphobilinogen deaminase plus co-synthetase; (b) porphyrinogen carboxy-lyase; (c) anaerobic or aerobic coproporphyrinogen III oxidase; (d) protoporphyrinogen IX oxidase.

with [2,3-¹⁴C₂]succinate, and the samples were then converted into succinic anhydride with complete retention of the tritium label (Table 1). The latter were used to produce succinoyl-CoA, which on incubation with a coupled enzyme system from *Rps. spheroides*, containing 5-aminolaevulinate synthase and bovine liver 5-aminolaevulinate dehydratase, gave porphobilinogen in 60–65% yield. The close agreement of the ³H/¹⁴C ratio in the samples of porphobilinogen to the predicted values suggested that the overall transformation had occurred without labilization of the ³H (Table 1).

Conversion of porphobilinogen into porphyrins

Our projected approach for studying the mechanistic details of anaerobic and aerobic coproporphyrinogen III oxidase of *Rps. spheroides* was based on the use of stereospecifically labelled species of porphobilinogen (Scheme 1). The cell-free extracts of light-grown *Rps. spheroides* contain the three enzymes necessary for the conversion of porphobilinogen into coproporphyrinogen III, as well as the

two types of oxidases that may utilize coproporphyrinogen III, produced *in situ*, as the substrate to furnish protoporphyrinogen IX (Scheme 2). The functional separation of the anaerobic coproporphyrinogen III oxidase from its aerobic counterpart was originally achieved by Tait (1972). We first describe experiments in which the enzymological findings of Tait (1972) have been combined with the use of porphobilinogen as the substrate to establish the two types of oxidase activities.

Anaerobic coproporphyrinogen III oxidase

Incubation of porphobilinogen with the cell-free extract of light-grown *Rps. spheroides* under strictly anaerobic conditions followed by analyses of the products (after oxidation and methylation) by h.p.l.c. showed that within 20 min all the porphobilinogen was consumed to furnish uroporphyrinogen III. (The major tetrapyrroles produced during the enzymic incubation and involved in the elaboration of the side chains are uroporphyrinogen III, coproporphyrinogen III, protoporphyrinogen IX

and protoporphyrin IX. The porphyrinogens are isolated and identified after their oxidation to porphyrins and conversion into methyl esters. Within the context of the present work, the porphyrinogens, the porphyrins derived therefrom and their corresponding methyl esters, may be considered as interchangeable compounds.) In such experiments small amounts of (less than 5%) uroporphyrinogen I, arising from the non-enzymic rearrangement of pre-uroporphyrinogen, were also detected. Conversion of uroporphyrinogen III into coproporphyrinogen III was next observed, and this stage was complete within 1 h. Under the preceding anaerobic conditions no further metabolism of coproporphyrinogen III into protoporphyrin IX occurred even after 8 h. However, as previously described by Tait (1972), supplementation of the cell-free extracts with Mg^{2+} , L-methionine, ATP, NADH and $NADP^+$ (quantities as described in the Experimental section) additively increased the yield of protoporphyrin IX (results not shown, but see Tait, 1972). In the presence of all the cofactors, coproporphyrinogen III, produced from porphobilinogen, was converted into protoporphyrin IX in estimated 80% overall yield after 6 h.

Another feature of the anaerobic oxidase activity described by Tait (1972) was also confirmed. The

centrifugation of the cell-free extracts of *Rps. spheroides* at 140000 g for 3 h gave a supernatant and a pellet. The supernatant under anaerobic conditions, with or without added cofactors, displayed no coproporphyrinogen III oxidase activity. The supplementation of the supernatant with the cofactors as well as the pellet resulted in the restoration of the anaerobic oxidase activity.

Aerobic coproporphyrinogen III oxidase

More importantly, however, the 140000 g supernatant in air efficiently converted coproporphyrinogen III, produced *in situ* from porphobilinogen, into protoporphyrin IX through the participation of the aerobic coproporphyrinogen III oxidase. Examination of Table 2 reveals that in the cell-free extracts of light-grown *Rps. spheroides* the anaerobic/aerobic oxidase activities are approximately in the ratio of 4:1 (compare Expts. *b* and *c* in Table 2).

With the availability of the two systems for the metabolism of porphobilinogen under conditions in which several of the intermediates of the porphyrin-biosynthetic pathway could be accumulated and also protoporphyrin IX produced in relatively large amounts, the stage was set for performing the stereochemical experiments with the variously labelled species of porphobilinogen.

Substrate stereochemistry of the reactions catalysed by coproporphyrinogen III oxidases

Anaerobic oxidase. A doubly labelled sample, (RS)-[$^{14}C_4, ^3H_6$]porphobilinogen (structure 1a in Scheme 1), was anaerobically incubated with the cell-free system of *Rps. spheroides* in the presence of various cofactors for conversion into protoporphyrin IX via the intermediates of Scheme 2. H.p.l.c. analysis of aliquots removed during the incubation showed that the conversion had a similar profile to that described in the preceding subsection. The results in Table 3 show that the $^3H/^{14}C$ ratios of uroporphyrin III and coproporphyrin III (purified by h.p.l.c. as porphyrin esters) was identical with that of the starting (RS)-[$^{14}C_4, ^3H_6$]porphobilinogen (structure 1a in Scheme 1). The data also show that there was a slight decrease in the $^3H/^{14}C$ ratio of protoporphyrin IX compared with that of coproporphyrin III (from 16.6 to 15.2), which suggested that out of 24 tritium atoms in coproporphyrin III two had been lost in the formation of protoporphyrin IX. This conclusion was confirmed by the degradation of protoporphyrin IX to ethylmethylmaleimide (structure 5 in Scheme 3) and haematinic acid (structure 6 in Scheme 3), when the tritium composition of the latter was unchanged from that of porphobilinogen (Table 3); however, ethylmethylmaleimide had a diminished tritium content corresponding to the loss of one tritium atom out of

Table 2. Biosynthesis of porphyrins by cell-free systems from *Rps. spheroides*

Labelled porphobilinogen was incubated either with the cell-free homogenates prepared from light-grown *Rps. spheroides* or the supernatant obtained after the centrifugation of the homogenate at 140000 g for 3 h. The yield is based on the incorporation (via the pathway of Scheme 2) of the precursor into protoporphyrin IX, which was isolated and purified as its dimethyl ester. The conditions of incubation and other details regarding the quantity and nature of cofactors are described in the Experimental section.

Condition of incubation	Type of activity operational	Yield of protoporphyrin IX from porphobilinogen in 6 h (%)
Cell-free homogenate		
(a) N_2	None	Not detectable
(b) N_2 + cofactors	Anaerobic	80 (90 after 16 h)
(c) O_2	Aerobic	21.3
(d) O_2 + cofactors	Aerobic + anaerobic	34.1
Supernatant from centrifugation at 140000 g		
(e) N_2	None	Not detectable
(f) N_2 + cofactors	None	Not detectable
(g) O_2	Aerobic	24.6
(h) O_2 + cofactors	Aerobic	23.1

Table 3. Biosynthesis of porphyrins from stereospecifically labelled samples of porphobilinogen by using cell-free systems from *Rps. spheroides*

Doubly labelled samples of variously tritiated porphobilinogens (structures 1a, 1b and 1c in Scheme 1) were diluted with 4.5 mg of unlabelled porphobilinogen and incubated in the anaerobic system or in the aerobic system as described in the Experimental section. The incubations were terminated after 20 min, 40 min, 90 min and 16 h, and the biosynthetic products were aromatized, methylated and purified by t.l.c. or h.p.l.c. The yields of porphyrins were similar to those found in the experiments of Table 2. The $^3\text{H}/^{14}\text{C}$ atomic ratio for porphobilinogen is known from the design of the experiment. For other compounds the atomic ratios are calculated from the experimentally determined $^3\text{H}/^{14}\text{C}$ ratios by assuming a theoretical distribution of ^{14}C in the compound. Abbreviations: (RS)-PBG = (6RS,8RS,9RS)-[4,6,8,9- $^{14}\text{C}_4$,6,8,9- $^3\text{H}_6$]porphobilinogen; (R)-PBG = (6R,8R,9R)-[4,6,8,9- $^{14}\text{C}_4$,6,8,9- $^3\text{H}_3$]porphobilinogen; (S)-PBG = (6S,8S,9S)-[4,6,8,9- $^{14}\text{C}_4$,6,8,9- $^3\text{H}_3$]porphobilinogen.

Labelled porphobilinogen	$^3\text{H}/^{14}\text{C}$ ratio ($^3\text{H}/^{14}\text{C}$ atomic ratios in parentheses)					
	Porpho-bilinogen	Uro-porphyrin III	Copro-porphyrin III	Proto-porphyrin IX	Haematinic acid (6)	Ethylmethyl-maleimide (5)
(a) Anaerobic system						
(RS)-PBG (cf. structure 1a)	16.60 (6:4)	16.50 (23.9:16)	16.60 (24:16)	15.20 (22:16)	16.50 (5.96:4)	13.90 (5:4)
(R)-PBG (cf. structure 1b)	17.60 (3:4)	17.55 (11.96:16)	17.60 (12:16)	17.60 (12:16)	17.50 (2.98:4)	17.60 (3:4)
(S)-PBG (cf. structure 1c)	17.20 (3:4)	17.25 (12:16)	17.20 (12:16)	14.30 (9.97:16)	17.15 (2.99:4)	11.40 (1.98:4)
(b) Aerobic system						
(RS)-PBG (cf. structure 1a)	16.60 (6:4)		16.50 (23.85:16)	15.30 (22.10:16)	16.60 (6:4)	14.40 (5.05:6)
(R)-PBG (cf. structure 1b)	17.60 (3:4)		17.55 (11.96:16)	17.65 (12.03:16)	17.60 (3:4)	17.55 (2.99:4)
(S)-PBG (cf. structure 1c)	17.20 (3:4)		17.20 (12:16)	14.20 (9.90:16)	17.25 (3:4)	11.2 (1.95:4)

Scheme 3. Loss of the pro-S-hydrogen atom from the β -carbon atom in the conversion of coproporphyrinogen III (structure 2) into protoporphyrin IX (structure 4) via protoporphyrinogen IX (not shown)

$\text{H}^\bullet$, pro-S-hydrogen atom; H^Δ , pro-R-hydrogen atom. The orientation of hydrogen atoms at the α -carbon atom of the vinyl groups in structure (4) is based on the work of Battersby *et al.* (1975). The analysis in the present work involved the degradation of structure (4) to structure (5) plus structure (6), the first step of which was the reduction of the two vinyl double bonds.

every six present in the precursor, porphobilinogen. In experiments performed with (*R*)-[$^{14}\text{C}_4, ^3\text{H}_3$]porphobilinogen (structure 1*b* in Scheme 1) the tritium content of the biosynthetic protoporphyrin IX was found to be identical with that of the starting material. Degradation of the protoporphyrin IX gave ethylmethylmaleimide and haematinic acid with $^3\text{H}/^{14}\text{C}$ ratios that were also identical with that of the (*R*)-[$^{14}\text{C}_4, ^3\text{H}_3$]porphobilinogen (structure 1*b* in Scheme 1), showing that the hydrogen atoms occupying the pro-*R*-orientation in the side-chain methylene positions of porphobilinogen, and hence of the metabolic intermediates derived therefrom, are not disturbed in vinyl group formation. Additional confirmation was obtained by repeating the above experiment with the enantiomeric species, (*S*)-[$^{14}\text{C}_4, ^3\text{H}_3$]porphobilinogen (structure 1*c* in Scheme 1). The relative $^3\text{H}/^{14}\text{C}$ ratios of protoporphyrin IX and coproporphyrin III were in accordance with the overall loss of one ^3H atom in the formation of each vinyl group, and the data also established that, whereas haematinic acid had all the three tritium atoms originally present in porphobilinogen, ethylmethylmaleimide had only two (Table 3).

Aerobic oxidase. A comparison of the results obtained for the aerobic incubation (Expt. *b* in Table 3) with those obtained from the anaerobic enzyme (Expt. *a* in Table 3) shows that the reaction catalysed by aerobic and anaerobic coproporphyrinogen III oxidases from *Rps. spheroides* proceed with the same stereochemistry, in which the pro-*S*-hydrogen atom located at the β -carbon atoms of the propionate side chains of coproporphyrinogen III are removed (structure 2 $\rightarrow$ structure 4, Scheme 3).

Discussion

During the biosynthesis of porphyrins and chlorophylls, the propionate side chains of rings A and B of coproporphyrinogen III (structure 2) are sequentially decarboxylated (Cavaleiro *et al.*, 1974) to the vinyl groups in protoporphyrin IX (structure 4) (for a review see Akhtar & Jordan, 1979; Bogorad, 1976; Battersby & McDonald, 1975). In animals and plants the decarboxylation reaction has an absolute requirement for molecular oxygen (Yoshinaga & Sano, 1980) and the overall reaction proceeds with the loss of the pro-*S*-hydrogen atom from each propionate side chain (Zaman & Akhtar, 1976; Abboud & Akhtar, 1978; Battersby, 1978). Anaerobic bacteria must have an alternative mechanism for the conversion of coproporphyrinogen III into protoporphyrin IX that is not dependent on molecular oxygen. The photosynthetic bacterium *Rps. spheroides* possesses the ability to grow anaerobically in the presence of suitable carbon sources for ATP production or anaerobically in the

photosynthetic mode of growth. Under both modes of growth the bacterium synthesizes protoporphyrin IX for incorporation into cytochromes; however, in the photosynthetic mode most of the protoporphyrin IX produced is directed to the synthesis of chlorophylls. The demonstration by Tait (1972) that cell-free extracts of *Rps. spheroides* possess anaerobic and aerobic coproporphyrinogen III oxidases with characteristic enzymological requirements prompted us to compare the stereochemical courses for the two enzyme systems.

The stereochemistry of vinyl group formation promoted by both the aerobic and anaerobic coproporphyrinogen III oxidases was studied by biosynthesizing protoporphyrin IX from porphobilinogen stereospecifically labelled with tritium in the side chains. The results (Table 3) show that in the conversion of porphobilinogen into coproporphyrinogen III the $^3\text{H}/^{14}\text{C}$ ratio does not change, showing that the decarboxylation of the acetate side chains of uroporphyrinogen III takes place with the retention of the tritium atoms present at the α -carbon atom of the acetate side chains, an observation in agreement with the results obtained with the chicken reticulocyte system (Barnard & Akhtar, 1979; also see Battersby *et al.*, 1981). However, the conversion of the propionate side chains of coproporphyrinogen III into the vinyl groups of protoporphyrin IX results in the loss of the pro-*S*-hydrogen atom from each propionate side chain. Thus the results obtained with the two types of coproporphyrinogen III oxidases from *Rps. spheroides* are identical with those reported for the chicken reticulocyte and plant cell systems (see above).

Mechanistic consideration

The loss of only one hydrogen atom during the formation of the vinyl group eliminates the participation of acrylic or β -oxo acid intermediates in the process, although the results cannot distinguish between the following equally plausible mechanisms (Scheme 4).

Mechanism 1 assumes that the reaction proceeds via an oxygen-dependent hydroxylation reaction in which the first step, hydroxylation, provides the energetic requirements for a decarboxylation-elimination process, giving rise to the vinyl group. Support for this type of mechanism comes from the demonstration that purified bovine heart mitochondrial coproporphyrinogen III oxidase catalyses the decarboxylation of the β -hydroxypropionic acid side chains (as in the structure of the type 3) to vinyl groups (structure 4) (Yoshinaga & Sano, 1980). The Mechanism 2 originally proposed by Granick & Sano (1961) necessitates the removal of a hydride ion from the β -carbon with a simultaneous decarboxylation resulting in the formation of a double

Mechanism 1

Mechanism 2

Mechanism 3

Scheme 4. Possible mechanisms for vinyl group formation

bond. In Mechanism 3, a variant of the Mechanism 2, the electrons of the pyrrolic nitrogen atom provide the driving force for the loss of the hydride ion from the β -carbon atom, and the resulting enimine intermediate aids the subsequent decarboxylation.

Our results show that the aerobic coproporphyrinogen III oxidase activity in extracts of *Rps. spheroides* can be demonstrated only in the presence of oxygen. This feature is in accordance with the basic tenets of a hydroxylation mechanism (Mechanism 1), even though a reduced nicotinamide nucleotide did not appear to be required for the full expression of the activity of the aerobic oxidase. In view of the crude nature of our preparation, however, no firm conclusion regarding the role of a reducing agent in the aerobic-oxidase-catalysed reaction is justified. Attention is, though, drawn to the previous work by Yoshinaga & Sano (1980), in which it was shown that a reductant was not required for the activity of a highly purified preparation of coproporphyrinogen III oxidase from bovine liver. Furthermore, the enzyme did not possess any of the characteristics normally associated with a hydroxylation system. Notwithstanding this fact, the authors reporting this observation favoured the participation of hydroxylated intermediates for the coproporphyrinogen III oxidase-catalysed reaction, and conjectured that, in

this case, the hydroxylation reaction may occur through a novel mechanism (Yoshinaga & Sano, 1980; also see Jackson *et al.*, 1980). In our view such a conclusion is premature at this stage, and the burden of evidence available to date does not allow an unambiguous choice between the three mechanisms shown in Scheme 4. In fact, if we were to be guided by conventional biochemical wisdom, then the mechanism operating via a hydroxylated species would appear to be the least likely of the three alternatives.

The anaerobic coproporphyrinogen III oxidase activity in the cell-free extracts could only be demonstrated by the addition of a complex mixture of cofactors. Although the cofactor requirement does not lend itself to an unambiguous mechanistic interpretation, a point to note is that the broad principle underlying Mechanisms 2 and 3 is applicable to both types of oxidases.

The Science and Engineering Research Council provided support for this research.

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