Coproporphyrinogenase Activities in Extracts of *Rhodopseudomonas spheroides* and *Chromatium* Strain D

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1. The anaerobic coproporphyrinogenase activity in an extract of *Rhodopseudomonas spheroides* is inhibited by 1,10-phenanthroline, $\alpha\alpha'$ -bipyridyl, flavins, 2,4-dinitrophenol and 1,4-naphthaquinone. These compounds have no effect on the aerobic coproporphyrinogenase activity. 2. On removal of small-molecular-weight material from a crude extract, the anaerobic system becomes very unstable; it can be stabilized by adding succinate. Now nicotinamide nucleotides, in addition to Mg^{2+} , ATP and methionine, are required for protoporphyrin to be formed. 3. A mechanism for the anaerobic reaction is proposed, based on the cofactor requirements and the effect of inhibitors. 4. The enzyme responsible for aerobic activity has been partially purified and some of its properties are reported. 5. A crude extract of *Chromatium* strain D also exhibits coproporphyrinogenase activity under anaerobic conditions in the presence of *S*-adenosylmethionine or ATP plus methionine. The requirement for other cofactors is variable.

In a previous paper (Tait, 1969) it was reported that a crude extract of *Rhodopseudomonas spheroides* catalysed the conversion of coproporphyrinogen into protoporphyrinogen under both anaerobic and aerobic conditions. Anaerobically coproporphyrinogenase activity was observed only in the presence of Mg^{2+} , ATP and L-methionine or *S*-adenosylmethionine. In air the reaction required the addition of cyanide, which probably prevents the preferential use of oxygen by succinoxidase and NADH oxidase.

Apart from a brief report of anaerobic activity in an extract of a species of *Pseudomonas* (Ehteshamuddin, 1968), coproporphyrinogenases detected in other cells, such as mammalian liver mitochondria (Sano & Granick, 1961; Batlle *et al.*, 1965), tobacco-leaf mitochondria (Hsu & Miller, 1970), bacteria (Jacobs *et al.*, 1971) and even the photosynthetic anaerobe *Chromatium* strain D (Mori & Sano, 1968), have an absolute requirement for molecular oxygen. In the anaerobic reaction there must be some acceptor other than oxygen for the hydrogens removed during the oxidative decarboxylation of coproporphyrinogen to protoporphyrinogen. This paper reports the involvement of nicotinamide adenine dinucleotides and possibly also a flavoprotein and iron in the anaerobic reaction. It also reports some properties of the aerobic reaction in *R. spheroides* and the detection of anaerobic activity in extracts of *Chromatium* strain D. A preliminary account of some of these results has been published (Tait, 1970).

Experimental

Chemicals

[2- ^{14}C]Glycine and *S*-adenosyl-L-[*Me*- ^{14}C]methionine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; NAD^+ , NADH, $NADP^+$, NADPH and ATP were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; Sephadex G-25, G-50 and G-100 were from Pharmacia, Uppsala, Sweden; DEAE-cellulose, FMN and FAD were from Sigma (London) Chemical Co., Ltd., London S.W.6, U.K.; L-ethionine was from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Bacterial peptone, yeast extract and malt extract were purchased from Difco Laboratories, Detroit, Mich., U.S.A. and from Oxoid Ltd., London, U.K. Calcium phosphate gel was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. *S*-Adenosyl-L-methionine and *S*-adenosyl-L-ethionine were prepared enzymically by the method of Cantoni (1957), and *S*-adenosyl-L-homocysteine by the method of de la Haba & Cantoni (1959). *S*-Adenosyl-L-methionine was also purchased from C. F. Boehringer und Soehne G.m.b.H.

Protoporphyrin dimethyl ester was prepared from blood as described by Grinstein (1947). The free porphyrin was prepared by hydrolysis with 7M-HCl for 5h at room temperature in the dark.

Coproporphyrin III was prepared by illuminating

a suspension of photosynthetically grown *R. spheroides* in mixture I of Lascelles (1956), plus L-ethionine (1 mM) (Gibson *et al.*, 1962b). The coproporphyrin that accumulates in the culture medium was purified as described by Neilands & Garibaldi (1960). [^{14}C]-Coproporphyrin was prepared in the same way from illuminations done in the presence of [^{14}C]-glycine. Coproporphyrinogen was prepared immediately before use by treating a suspension of coproporphyrin in water with NaBH_4 (cf. Nishida & Labbe, 1959; Schwartz *et al.*, 1960). To 1 mg of coproporphyrin suspended in 1 ml of water 1 drop of 1.5M-HCl was added; N_2 was bubbled through the suspension and about 4 mg of NaBH_4 added. The flow of gas through the solution was maintained until the solution changed from red to colourless. The solution was then neutralized by careful addition of 1.5M-HCl until the solution could be pipetted into a 0.2 ml graduated pipette without the formation of gas bubbles in the pipette.

Organisms

Rhodopseudomonas spheroides (N.C.I.B. 8253) was maintained in stab-culture as described by Lascelles (1956). *Chromatium* strain D was obtained from Professor F. R. Whatley, Department of Botany, King's College, University of London, and *Micrococcus denitrificans* from Dr. J. G. Morris, Department of Biochemistry, University of Leicester.

Growth of organisms. *R. spheroides* was grown semi-anaerobically in the light at 32–34°C for 40–44 h in medium S of Lascelles (1956) supplemented with FeSO_4 (10 μM) and MnCl_2 (5 μM) in 3 litre flasks filled almost to the top. The illumination was supplied and the temperature was maintained by 60 W bulbs at appropriate distances from the flasks. After growth organisms were harvested by centrifuging at 4°C, washed with N_2 -saturated 0.05M-tris-HCl, pH 8.0 or 8.8, and resuspended in the same buffer to a concentration between 50 and 80 mg dry wt./ml. The suspension was either used immediately or kept frozen at –20°C until required.

Chromatium strain D was grown anaerobically in the light at 32–34°C in 1 litre glass-stoppered bottles completely filled with medium. The medium used was the inorganic one described by Morita *et al.* (1965), but instead of the addition of FeSO_4 , a portion (2 ml/litre) of the 'Trace Elements Solution' used by R. C. Fuller (Bose, 1963) was added. Organisms were grown for up to 7 days. After growth they were harvested by centrifuging at 4°C, washed twice with N_2 -saturated 0.05M-tris-HCl, pH 7.7, and resuspended in the same buffer to give a volume 0.5% of that of the original culture.

Micrococcus denitrificans was grown anaerobically with nitrate and aerobically in the complex medium described by Scholes & Smith (1968), and also in the

defined medium of Chang & Morris (1962). Growth was for 24 h at 23°C. After growth, organisms were harvested by centrifuging at 4°C, washed with N_2 -saturated 0.05M-tris-HCl, pH 8.0, and resuspended in the same buffer to give a volume of 0.5–1% of that of the original culture.

Determinations

The dry weight of *R. spheroides* was determined as described by Gibson *et al.* (1962a). Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceuticals Co. Ltd., Eastbourne, Sussex, U.K.) as standard. Fractions eluted from columns were monitored for protein by measuring the extinction at 280 nm.

Solutions containing radioactivity were plated on 6.25 cm² aluminium planchets, dried and counted at infinite thinness in a Nuclear-Chicago gas-flow counter with a Micromil end window and operated at the centre of the plateau.

Preparation of extracts

Concentrated suspensions of organisms, in some instances supplemented with 1 mM-mercaptoethanol, were disrupted under N_2 in 3 ml lots by irradiation in an MSE 50 W Ultrasonic Disintegrator operated at maximum output for 3 min. Suspensions were cooled by immersing the tube in crushed ice. Alternatively, suspensions of organisms were disrupted by passing them through a French pressure cell at 104000 kN/m² (15000 lb/in²). Suspensions obtained by both methods were centrifuged at 25000g for 7 min in stoppered tubes filled with N_2 and the supernatants, termed 'crude extract', were removed. Crude extracts, which contained 25–35 mg of protein/ml, were either used immediately or were kept at –20°C before use.

Assays for coproporphyrinogenase activities

Aerobic activity. This activity was assayed essentially as described by Sano & Granick (1961). The standard assay mixture contained, in a total volume of 1.0 ml, 100 μmol of phosphate buffer (KH_2PO_4 – NaOH), pH 7.5, 10 μmol of KCN, 10 μmol of sodium thioglycollate, 30 μl (approx. 30 nmol) of freshly prepared coproporphyrinogen and enzyme extract. Incubation was at 37°C, usually for 1 h in 125 mm × 15 mm glass test tubes, with occasional shaking. The reaction was stopped by adding 3 ml of ethyl acetate–acetic acid (3:1, v/v). After mixing, the tubes were centrifuged, the supernatant was removed and the pellet was washed with another 3 ml of ethyl acetate–acetic acid. To the combined extracts 5 ml of ether was added and the resulting solution was washed with 3 ml of water. The aqueous layer was discarded and the porphyrins were removed from the organic layer by extracting twice with 2 ml portions of 3M-HCl. To the combined acid extracts, 5 ml of ether

and 3 ml of saturated sodium acetate solution were added and the mixture was shaken. The ether layer, which contained all the porphyrins, was then extracted twice with 2 ml portions of 0.05 M-HCl and once with 2 ml of 0.15 M-HCl to remove all the coproporphyrin, and then twice with 2 ml portions of 1.5 M-HCl to remove the protoporphyrin. The amounts of coproporphyrin and protoporphyrin were determined by measuring the spectra of the acid solutions between 390 and 430 nm in a recording spectrophotometer (Unicam SP.700). The amounts of each were determined by using $\epsilon_{\text{m}} 489$ at 401 nm for coproporphyrin and $\epsilon_{\text{m}} 262$ at 408 nm for protoporphyrin. The two solutions always gave absorption maxima at the correct wavelengths for coproporphyrin and protoporphyrin respectively, showing that all the coproporphyrin, but none of the protoporphyrin, was extracted from ether with the two lower concentrations of HCl.

Anaerobic activity. The standard assay mixture contained, in a total volume of 0.9 ml, 10 μmol of MgSO_4 , 4 μmol of ATP (added as a solution at pH 8), 1 μmol of L-methionine, 30 μl (approx. 30 nmol) of freshly prepared coproporphyrinogen and enzyme extract. Differences from this standard assay mixture are described in the text at the appropriate place. Solutions of all the constituents, and the water used to make up the volume, had been previously saturated with N_2 . After making all the additions to 15 ml glass conical centrifuge tubes, N_2 was blown vigorously over the surface of the solutions, the tubes were tightly stoppered with rubber bungs, and they were incubated at 37°C for up to 3 h in the dark. Reaction was stopped and coproporphyrin and protoporphyrin were isolated and determined as described above. The only difference in procedure was that tubes were left 20–30 min in the light after addition of ethyl acetate–acetic acid mixture, to allow oxidation of the porphyrinogens to porphyrin.

The amounts of protoporphyrin extracted from incubations were corrected for the amounts extracted from non-incubated controls, or from controls incubated without cofactors (see Tait, 1969).

Results

Comparison of the properties of the anaerobic and aerobic activities in crude extracts of R. spheroides

Preliminary experiments. No buffer, except that present in the crude extract, is added to the anaerobic assay mixture described in the Experimental section (see also Tait, 1969). This is because large volumes, up to 0.6 ml, of crude extract, which are 0.05 M with respect to tris–HCl, are added to the assay, whose final volume is 0.9 ml. It was found that addition of extra tris–HCl buffer markedly inhibited the reaction. For example, in an anaerobic assay done as

described in the Experimental section, in which 25 μmol of tris–HCl, pH 8.0, was added as part of the crude extract (0.6 ml containing 13.7 mg of protein), 4.0 nmol of protoporphyrin was formed; on adding an extra 40 and 80 μmol of tris–HCl, pH 8.0, the amounts of protoporphyrin formed decreased to 3.4 and 2.1 nmol respectively.

Crude extracts prepared with and without mercaptoethanol were equally active in catalysing anaerobic coproporphyrinogenase activity. However, extra mercaptoethanol added to the assay was inhibitory. At final concentrations of 2 and 10 mM, it inhibited protoporphyrin formation by 18 and 65% respectively.

pH optima. Because of the inhibitory effect of excess of tris–HCl on the anaerobic reaction, the activity as a function of pH was measured as follows. Samples of organisms from the same culture were washed and suspended in 0.05 M-tris–HCl of different pH values, crude extracts were prepared, and their coproporphyrinogenase activities and their pH values were determined (Fig. 1). The pH optimum for the anaerobic activity, about pH 8.5, was markedly higher than that for the aerobic reaction, about pH 7.3. The pH optimum for the anaerobic reaction was about the same with either methionine or S-adenosylmethionine. It was subsequently found that the aerobic reaction was not inhibited by a high concentration of buffer and its pH optimum was found to be the same as above when it was assayed in the presence of 0.1 M-phosphate buffers of known pH values.

In the experiments described below, anaerobic and aerobic activities were determined with crude extracts prepared from cells suspended in 0.05 M-tris–HCl, pH 8.8 and pH 7.7 respectively; no extra buffer was added to either assay.

Effect of inhibitors. Low concentrations of 1,10-phenanthroline, $\alpha\alpha'$ -bipyridyl, riboflavin, FMN, FAD and 2,4-dinitrophenol and 1,4-naphthaquinone markedly inhibited the anaerobic reaction (Table 1), but even relatively high concentrations of these compounds had little or no effect on the aerobic reaction.

Location of activities. A crude extract was centrifuged at 105 000 g for 90 min; the supernatant, which was practically free of chromatophores, was removed and the pellet was resuspended in buffer. All the aerobic activity was found in the supernatant (Table 2); there was none in the pellet. Anaerobic activity was only detected when the supernatant and pellet were recombined. In another experiment, the supernatant was removed in fractions, starting from the top of the tube, and the pellet was also separated into two layers. Aerobic activity was confined to the supernatant fractions, although only 50% of the activity was recovered. When fractions were tested individually for anaerobic activity in the presence of

Fig. 1. *pH optima for anaerobic and aerobic coproporphyrinogenase activities*

A culture of *R. spheroides* was harvested in six lots. These were washed and resuspended in 0.05M-tris-HCl buffer, pH 7.0, 7.7, 8.0, 8.4, 8.8 or 9.2. Crude extracts were prepared from each, with pH values 7.0, 7.6, 7.9, 8.3, 8.55 and 8.8 respectively and the protein concentrations were all approx. 33mg of protein/ml. A portion (0.5ml) of each crude extract was used to assay for anaerobic activity (○) exactly as described in the Experimental section. A further portion (0.5ml) of each crude extract was also assayed for aerobic activity (●) in a total volume of 1.0ml containing coproporphyrinogen and 0.1 ml of 100mM-KCN. Incubation was for 2h. Porphyrins were extracted and measured as described in the Experimental section. The amount of 'protoporphyrin' extracted from non-incubated controls (cf. Tait, 1969) containing 0.5ml of crude extract and coproporphyrinogen was subtracted from all values.

Mg²⁺, ATP, *S*-adenosylmethionine, NADH (0.3 μmol) and NADP⁺ (0.2 μmol), none had more than a trace of activity, but when supernatant fractions were tested along with either of the pellet fractions high activity was found.

Further studies on the anaerobic reaction

Time and enzyme curves with crude extract. In the early experiments all incubations were for 90min or

longer with relatively high concentrations of crude extract. The effect of varying the amount of crude extract is shown in Fig. 2. In the absence of cofactors other than ATP, methionine and Mg²⁺, there was little or no formation of protoporphyrin with less than 7–10mg of protein/assay. With greater amounts of protein there was activity which increased as more protein was added. Since succinate and NADH had been found earlier to stimulate activity in some experiments on high concentrations of crude extract, they were tested in the presence of different amounts of extract. In their presence plots of activity versus protein were almost linear, even at low protein concentration. The rate of formation of protoporphyrin was rapid in the first 30min (Fig. 3); it then slowed down and no more protoporphyrin was formed after 60min. Similar results were obtained in assays done in the presence of succinate or NADH.

Effect of removing small molecules from the crude extract. To investigate the roles of succinate and NADH further, the crude extract was freed from small molecules by passage through columns of Sephadex G-25 or G-50. Crude extract (3ml; 25–35mg of protein/ml) was applied to a column (15cm × 1cm) equilibrated with N₂-saturated 0.05M-tris-HCl, pH 8.8. The column was eluted with the same buffer and 4–6ml of eluate was collected, collection being started when the red chromatophores came off the column. This filtration, which was done at 4°C, was complete in 5–10min. At first very variable results were obtained even when the 'Sephadex extract' was assayed immediately in the standard assay mixture plus nicotinamide nucleotides, and usually the recovery of activity was low. Addition of mercaptoethanol to the extract or to the assay had little effect on the results. Addition of succinate to a concentration of 10–20mM to the fraction immediately after elution from the Sephadex column gave an extract that was fully active when assayed in the presence of one or more nicotinamide nucleotides (Table 3), although it had little or no activity in their absence. A number of experiments were done to try to establish which of the nicotinamide nucleotides were active. It was found that any one alone produced some activity, but that the highest activity, equal to that of the crude extract, was obtained in the presence of both NADH (0.33mM) and NADP⁺ (0.22mM).

With a 'Sephadex extract' the specific activity per mg of protein was almost constant over a wide range of protein concentration (Fig. 4a) and the rate of protoporphyrin production was constant for about 60min (Fig. 4b).

Role of S-adenosylmethionine. With Sephadex extracts maximum activity was obtained with about 0.2mM-*S*-adenosylmethionine (Fig. 5). This activity was generally slightly lower than that obtained with the optimum concentration of L-methionine,

Table 1. *Effect of inhibitors on anaerobic and aerobic coproporphyrinogenase activities*

Anaerobic activity was assayed as described in the Experimental section in the presence of MgSO_4 , ATP and methionine, with crude extract (16mg of protein) prepared from organisms suspended in 0.05M-tris-HCl, pH8.8. Aerobic activity was assayed as described in Fig. 1 in the presence of KCN with crude extract (14mg of protein) prepared from organisms suspended in 0.05M-tris-HCl, pH7.7; no additional buffer was present in the assay. Incubation was for 2h at 37°C. Porphyrins were isolated and measured as described in the Experimental section. Activities in the presence of inhibitors are expressed as percentages of those without inhibitor.

Additions to assays	Concn.	Activity (% of controls)	
		Anaerobic	Aerobic
1,10-Phenanthroline	0.056mM	71	143
	0.17 mM	10	139
	0.56 mM	0	133
$\alpha\alpha'$ -Bipyridyl	1.5 mM	23	115
Riboflavin	30 μM	3	—*
FMN	30 μM	6	92
FAD	30 μM	2	—
1,4-Naphthaquinone	15 μM	39	98
2,4-Dinitrophenol	0.3 mM	24	—
	1.0 mM	5	93

* Not tested.

Table 2. *Distribution of coproporphyrinogenase activities in subcellular fractions of R. spheroides*

A crude extract (22.6mg of protein/ml) of *R. spheroides* prepared from cells suspended in 0.05M-tris-HCl, pH8.8–1mM-mercaptoethanol was centrifuged for 90min at 105000g. The clear supernatant (18.4mg of protein/ml) was removed and the pellet was suspended in 0.05M-tris-HCl, pH8.8–1mM-mercaptoethanol to give a volume one-half of that of the original crude extract (20.2mg of protein/ml). Anaerobic activity was assayed in the presence of MgSO_4 , ATP and methionine and aerobic activity in the presence of KCN.

Fraction	Wt. of protein (mg)	Protoporphyrin formed (nmol/2h)	
		Anaerobic	Aerobic
Crude extract	9.0	2.08	1.68
Supernatant	7.4	0.25	2.13
Pellet	4.0	0.00	0.25
Supernatant + pellet	11.4	1.65	1.65

which is 0.5mM (Tait, 1969). The formation of protoporphyrin was markedly inhibited by *S*-adenosyl-homocysteine and by *S*-adenosylethionine (Fig. 6).

There was no incorporation of radioactivity into any of the porphyrins isolated from anaerobic incubations done with coproporphyrinogen and *S*-adenosyl-[$\text{Me-}^{14}\text{C}$]methionine. Some of the radioactivity was incorporated into lipid, but the amount was the same in incubations lacking coproporphyrinogen.

It was thought that the enzyme or some other macromolecule in the crude extract might have to

be methylated before the system for making protoporphyrin became active. A crude extract was incubated anaerobically with all the cofactors but without coproporphyrinogen, and then passed through Sephadex G-25 to remove the cofactors. The resulting extract only formed protoporphyrin when reincubated with all the cofactors, including *S*-adenosyl-methionine. Thus so far there is no evidence that the conversion of coproporphyrinogen into protoporphyrin requires preliminary methylation of the enzyme or of some other macromolecule.

Attempts to purify the activities

Aerobic activity. When aerobic activity was assayed in the standard way with a high-speed supernatant, the specific activity/mg of protein was constant with

Fig. 2. Effects of succinate and nicotinamide nucleotides on anaerobic coproporphyrinogenase activity

Crude extract prepared from cells suspended in 0.05M-tris-HCl, pH8.8, was assayed for anaerobic activity exactly as described in the Experimental section: ○, ●, without further additions (○, ●: two separate experiments); □, with sodium succinate (10 μmol); ■, with NADH (0.3 μmol) plus NADP⁺ (0.2 μmol). Incubation was for 90 min at 37°C.

up to 9.8mg of protein/assay, and the rate of synthesis of protoporphyrinogen was constant for up to 2h.

The enzyme, which was reasonably stable, was partially purified by successive ammonium sulphate precipitation, chromatography on DEAE-cellulose, chromatography on Sephadex G-100, treatment with

Fig. 3. Formation of protoporphyrin as a function of time

Crude extracts, prepared from cells suspended in 0.05M-tris-HCl, pH8.8, were assayed for anaerobic activity exactly as described in the Experimental section. Assays contained 13.2mg of protein (○) or 15.9mg of protein (●).

Table 3. Effect on anaerobic activity of removal of small molecules from crude extract

A portion (2.5ml) of crude extract in 0.05M-tris-HCl, pH8.8-1mM-mercaptoethanol was passed through a 15cm×1cm column of Sephadex G-25 equilibrated with 0.05M-tris-HCl, pH8.8, at 4°C. Collection of eluate was started as soon as chromatophores were eluted, and 3ml was collected. 1M-sodium succinate (0.075 ml) was added and the extract used immediately to assay for anaerobic activity in the presence of MgSO₄, ATP, methionine and nicotinamide nucleotides as indicated.

Extract	Nicotinamide nucleotides	Protoporphyrin formed (nmol/90min per mg of protein)
Crude extract	None	0.470
	NADH (0.3 μmol) + NADP ⁺ (0.2 μmol)	0.528
'Sephadex extract'	None	0.013
	NAD ⁺ (0.5 μmol)	0.198
	NADP ⁺ (0.5 μmol)	0.224
	NADH (0.5 μmol)	0.204
	NADPH (0.5 μmol)	0.372
	NADPH (0.3 μmol) + NAD ⁺ (0.2 μmol)	0.495
	NADH (0.3 μmol) + NADP ⁺ (0.2 μmol)	0.630

Fig. 4. Anaerobic coproporphyrinogenase activity in Sephadex G-25-treated extracts

Crude extract, prepared from cells suspended in 0.05M-tris-HCl, pH 8.8, was passed through a column of Sephadex G-25 and the high-molecular-weight fraction was collected (see the text and Table 3). Succinate was added to a concentration of 20mM. Anaerobic coproporphyrinogenase activity was assayed as described in the Experimental section; also present in the assays were NADH (0.3 μ mol) and NADP⁺ (0.2 μ mol). Where the amount of extract was varied (a), the time of incubation was 90 min. Where the time of incubation was varied (b), 6.0 mg of protein was present.

Fig. 5. Anaerobic coproporphyrinogenase activity in the presence of different amounts of S-adenosylmethionine

Crude extract, prepared from cells suspended in 0.05M-tris-HCl, pH 8.8, was passed through a column of Sephadex G-25 and the high-molecular-weight fraction was collected (see the text and Table 3). Anaerobic coproporphyrinogenase activity was determined as follows. Assays, volume 0.9 ml, contained extract (0.6 ml), MgSO₄ (10 μ mol), ATP (4 μ mol), coproporphyrinogen (30 μ l), NADH (0.3 μ mol), NADP⁺ (0.2 μ mol) and S-adenosylmethionine as stated. Incubation was at 37°C for 90 min under N₂. In the experiment with commercial S-adenosylmethionine (●) 9.1 mg of protein was present in the assay and, with methionine (1 μ mol) instead of S-adenosylmethionine, 6.7 nmol of protoporphyrin was formed. In the experiment with S-adenosylmethionine prepared in this laboratory (○), 7.7 mg of protein was present in the assay; with methionine, 4.4 nmol of protoporphyrin was formed.

calcium phosphate gel and chromatography again on Sephadex G-100. A 60-fold purification and a 14% recovery of activity were obtained.

The K_m value for coproporphyrinogen was found to be approx. 30–50 μ M with enzyme purified on DEAE-cellulose; this is similar to values reported for other coproporphyrinogenase (cf. Batlle *et al.*, 1965; Hsu & Miller, 1970). This value is only approximate because it is difficult to be sure of the exact amount of coproporphyrinogen added to each assay, and also because some of the coproporphyrinogen

Fig. 6. Inhibition of anaerobic coproporphyrinogenase activity by *S*-adenosylhomocysteine and *S*-adenosylethionine

Crude extract was passed through a column of Sephadex G-25 to remove small molecules. The resulting extract, after addition of succinate, was assayed for anaerobic coproporphyrinogenase activity as follows. The assay, volume 0.9ml, contained extract (0.6ml; 7.0mg of protein), MgSO_4 ($10\mu\text{mol}$), ATP ($4\mu\text{mol}$), *S*-adenosylmethionine (180nmol) and coproporphyrinogen ($30\mu\text{l}$). *S*-Adenosylhomocysteine (●) or *S*-adenosylethionine (■) was added as shown. Incubation was at 37°C for 90min.

is probably oxidized to coproporphyrin during the course of the assay. From the elution volumes of the enzyme and of proteins of known molecular weight on Sephadex G-100, the enzyme has been found to have a molecular weight of around 44000; this is smaller than the value of 80000 reported for the mammalian enzyme (Batlle *et al.*, 1965).

Further purification was not attempted because it was thought that the purification already achieved was sufficient to show that the aerobic activity, like those from other sources (cf. Batlle *et al.*, 1965; Hsu & Miller, 1970), is catalysed by what appears to be a single protein that does not appear to require cofactors.

Anaerobic activity. In contrast with the aerobic activity, the anaerobic activity appears to require at least two proteins, one in the supernatant and one in the pellet obtained by centrifuging a crude extract at high speed (cf. Table 2). The supernatant was fractionated in a number of ways and the fractions

Fig. 7. Anaerobic coproporphyrinogenase activity with a crude extract of *Chromatium* strain D

Crude extract was prepared from cells suspended in 0.05M-tris-HCl, pH7.7–1mM-mercaptoethanol. Anaerobic activity was determined as follows. Assays, volume 1.0ml, contained MgSO_4 ($10\mu\text{mol}$), coproporphyrinogen ($30\mu\text{l}$) and crude extract (0.6ml; 11.2mg of protein). Incubation was at 37°C under N_2 . Other additions to the assay were: ■, none; ○, *S*-adenosylmethionine (200nmol); ●, *S*-adenosylmethionine (200nmol) and ATP ($4\mu\text{mol}$); ▲, *S*-adenosylmethionine (200nmol), NADH (300nmol) and NADP^+ (200nmol); ▲, *S*-adenosylmethionine (200nmol), ATP ($4\mu\text{mol}$), NADH (300nmol) and NADP^+ (200nmol).

were assayed for anaerobic activity in the presence of the pellet and all the cofactors which have been used previously. However, little or no activity could be detected with any of the fractions, even ones which were known to contain the enzyme that catalyses aerobic coproporphyrinogenase activity.

Anaerobic activity in extracts of *Chromatium* strain D

Mori & Sano (1968) detected oxygen-dependent coproporphyrinogenase activity in extracts of the photosynthetic anaerobe *Chromatium* strain D, but they were unable to find activity under anaerobic assay conditions. It was thus of interest to test extracts of this organism with assay conditions that had proved successful with *R. sphaeroides*. A suspension of *Chromatium* strain D in 0.05M-tris-HCl, pH7.7–1mM-mercaptoethanol was disrupted by sonicating under N_2 and a crude extract was prepared by centrifuging. The crude extract contained 23mg

of protein/ml. On incubating 0.6ml of this extract under anaerobic conditions in the standard assay mixture, containing Mg^{2+} , ATP and methionine plus NADH ($0.3\mu\text{mol}$) and $NADP^+$ ($0.2\mu\text{mol}$), 1.27nmol of protoporphyrin was formed in 2h. When the assay was repeated with the addition of $100\mu\text{mol}$ of tris-HCl, pH 7.7, only 0.32nmol of protoporphyrin was formed. Protoporphyrin was not synthesized in the absence of cofactors. In contrast with extracts of *R. spheroides* (Tait, 1969), those from *Chromatium* strain D contained no endogenous 'protoporphyrin'. The effect of various cofactors on the rate and extent of protoporphyrin formation is shown in Fig. 7. There is activity with *S*-adenosyl methionine alone, but synthesis stops after 60min and the amount of protoporphyrin recovered is less after 90min incubation. The fate of the protoporphyrin that disappears is not known. With *S*-adenosylmethionine, NADH and $NADP^+$ the shape of the curve is the same as with *S*-adenosylmethionine alone. However, when ATP is present the initial rate of protoporphyrin synthesis is slower than when it is absent, although protoporphyrin is still being synthesized at 90min.

Crude extracts that had been freed from small molecules by passing them through Sephadex G-25 also formed protoporphyrin when *S*-adenosylmethionine was the only cofactor added; there was no activity with methionine alone, although there was with methionine plus ATP. Succinate had to be added to the extract or to the assay for activity to be demonstrated. In some experiments the activity with *S*-adenosylmethionine alone was low and it was markedly stimulated by adding NADH and $NADP^+$.

The supernatant prepared by centrifuging a crude extract at $105000g$ for 1h formed protoporphyrin under anaerobic conditions, and re-addition of the pellet only stimulated activity slightly. As with the

other extracts, *S*-adenosylmethionine was the only essential cofactor, although activity varied from experiment to experiment as did the requirement for nicotinamide nucleotides.

The reasons for the poor reproducibility of the assays with these extracts could not be established. Indeed, extracts from some batches of cells gave virtually no activity. It was thought that the age of the cells might be a factor. However, experiments with extracts prepared from cells grown for 3, 5 and 7 days gave similar results. With some batches of cells, extracts prepared without adding mercaptoethanol had higher activity than those with mercaptoethanol and, in later experiments, mercaptoethanol was omitted. However, variable results were still obtained.

As with *R. spheroides*, attempts to purify the enzyme further only resulted in loss of activity.

Experiments with extracts from *Micrococcus denitrificans*

M. denitrificans is a non-photosynthetic organism that forms haem when grown under anaerobic conditions (cf. Porra & Lascelles, 1965). This organism is known to have an oxygen-dependent coproporphyrinogenase (Jacobs *et al.*, 1971). No clear-cut indications of anaerobic activity were obtained when assays were done in the presence of Mg^{2+} , ATP, methionine or *S*-adenosylmethionine and nicotinamide nucleotides.

Discussion

Possible mechanism of the anaerobic reaction

Two mechanisms have been proposed for the conversion of coproporphyrinogen into protoporphyrinogen. Granick & Sano (1961) suggested that

Scheme 1. Proposed mechanisms for conversion of coproporphyrinogen into protoporphyrinogen

a hydride ion is removed from the β -carbon atom of the propionate side chain with simultaneous decarboxylation to yield a vinyl group (Scheme 1a). Sano (1966) thought that this suggestion did not explain the requirement for oxygen in the reaction and proposed another mechanism. This involves sequential oxidation to form a β -hydroxypropionate side chain, dehydration to form an acrylic side chain and decarboxylation to form a vinyl side chain (Scheme 1b); alternatively, the last two steps may occur by a concerted mechanism. Consistent with Scheme 1(b), Sano (1966) synthesized 2,4-bis-(β -hydroxypropionic acid) deuteroporphyrin and found that on reduction to the porphyrinogen it was converted into protoporphyrinogen by the mitochondrial coproporphyrinogenase in the absence of oxygen. He also pointed out that this porphyrinogen was unstable in neutral and acid solution in the absence of enzyme and that one of the products was protoporphyrinogen.

The finding of an acrylic porphyrin in meconium (Smith *et al.*, 1969) and a hydroxylated porphyrin in urine and faeces of patients with cutaneous hepatic porphyria (Elder & Chapman, 1970) would fit in with Scheme 1(b). However, it is not yet known whether these compounds are intermediates in protoporphyrin synthesis.

Although both schemes outlined above may be equally probable for the oxygen-dependent activity in mitochondria and in bacteria, Scheme 1(b) would seem to be ruled out as the mechanism of the anaerobic reaction in extracts of *R. spheroides* and *Chromatium* strain D. In *R. spheroides* the eventual acceptor of the hydrogen released on oxidation of coproporphyrinogen appears to be a nicotinamide adenine dinucleotide. That other factors play a role in the reaction is suggested by the experiments with inhibitors. The very marked inhibition by low concentrations of flavins suggests that a flavoprotein is involved; the inhibition by both 1,10-phenanthroline and $\alpha\alpha'$ -bipyridyl suggests that a metal, possibly iron, is involved, either as part of an iron-flavoprotein or in the form of some other non-haem protein; the requirement for ATP, and the inhibitory effect of 2,4-dinitrophenol, both suggest that a substantial energy change is involved. On the basis of these results it is suggested that the hydrogen removed from the coproporphyrinogen is accepted by a component of the electron-transport chain, perhaps a non-haem iron-protein, and that it is then transferred first to a flavoprotein and then to NAD(P)⁺ in an energy-requiring step. Energy-dependent reverse electron transport of this sort has been shown to occur in mitochondria and in autotrophic bacteria (cf. Sewell & Aleem, 1969) and also in the oxidation of thiosulphate by *R. palustris* (Knobloch *et al.*, 1971). Further work is necessary to show whether or not this suggested mechanism is correct.

Role of *S*-adenosylmethionine

The role of *S*-adenosylmethionine in this reaction is difficult to explain since it does not seem to be acting as a methylating agent. It is presumed that *S*-adenosylmethionine is the actual cofactor and that methionine is only active after being converted enzymically into *S*-adenosylmethionine. It may be that the *S*-adenosylmethionine attaches to the enzyme in such a way that the positively charged sulphur atom is close to the active site, where it could then facilitate the transfer of a hydride ion from the substrate to the acceptor (cf. Scheme 1a). A number of enzyme reactions have been described recently where *S*-adenosylmethionine plays a role other than as a methyl donor. Chirpich *et al.* (1970) purified the lysine 2,3-aminomutase from *Clostridium* SB4 and showed that maximum activity was obtained when 3.7 mol of *S*-adenosylmethionine/mol of enzyme were added to the assay, and that *S*-adenosylhomocysteine was a competitive inhibitor. They suggest that *S*-adenosylmethionine acts as an allosteric effector. Knappe *et al.* (1969) found a requirement for *S*-adenosylmethionine in the pyruvate-formate lyase of *Escherichia coli*; it seems to be involved in the activation of an inactive enzyme to the active lyase. Some DNA-restriction enzymes require ATP and *S*-adenosylmethionine (cf. Roulland-Dussoix & Boyer, 1969) and here also their roles are unknown.

The requirement for *S*-adenosylmethionine explains a number of findings we have made previously with *R. spheroides* (Gibson *et al.*, 1962b). Illumination of these organisms in the presence of ethionine or threonine inhibits bacteriochlorophyll synthesis and causes the excretion of large amounts of coproporphyrin. We originally thought that lack of methionine and hence of *S*-adenosylmethionine caused by threonine, or competition by *S*-adenosylethionine, blocked the *S*-adenosylmethionine-dependent methylation of magnesium protoporphyrin, one of the later steps in bacteriochlorophyll synthesis (Gibson *et al.*, 1962c, 1963). However, it seemed a little surprising that coproporphyrin and not protoporphyrin or magnesium protoporphyrin accumulated under these conditions. Involvement of *S*-adenosylmethionine in the conversion of coproporphyrinogen into protoporphyrin would explain why coproporphyrin is excreted under conditions of methionine deficiency. Indeed, *S*-adenosylmethionine seems to play a very important role in bacteriochlorophyll synthesis in *R. spheroides*. In addition to being involved in the methylation of magnesium protoporphyrin and in the anaerobic coproporphyrinogenase reaction, it plays some part in controlling the insertion of magnesium into protoporphyrin. Gorchein (1972) has recently shown that when *R. spheroides* is incubated with protoporphyrin, magnesium is only inserted in the presence of methionine or *S*-adenosylmethionine. This require-

ment is explained by the finding that magnesium protoporphyrin monomethyl ester and not magnesium protoporphyrin is the product, but it is not yet known why the magnesium protoporphyrin itself cannot be formed in the absence of methionine.

Iron deficiency in *R. spheroides* (Lascelles, 1956) causes cells to excrete coproporphyrin under anaerobic conditions but not under aerobic conditions of growth. These findings are also consistent with those made here; namely that iron is involved in the anaerobic but not in the oxygen-dependent conversion of coproporphyrinogen into protoporphyrin.

The inability of myself and others (cf. Jacobs *et al.*, 1971) to detect anaerobic activity in extracts of non-photosynthetic anaerobic bacteria may mean that the assay conditions found to be successful for *R. spheroides* and *Chromatium* strain D are peculiar to these photosynthetic organisms where *S*-adenosylmethionine is known to play other important roles in bacteriochlorophyll synthesis. In other organisms, some other positively charged compound may be required instead of *S*-adenosylmethionine. The inability of Mori & Sano (1968) to detect anaerobic activity in *Chromatium* strain D may be due partly to their not using the correct cofactors, but also partly to the way in which they prepare coproporphyrinogen. Most workers (cf. Sano, 1966) prepare coproporphyrinogen by reducing a solution of coproporphyrin with sodium amalgam. This produces a very alkaline solution, which has to be neutralized with much acid. In view of the sensitivity of the anaerobic reaction to excess of tris-HCl, it may be that the high concentration of salt added to the assay along with the coproporphyrinogen may inhibit the reaction. In the experiments reported here the final concentration of borate in the assay, produced from the sodium borohydride, is about 1 mM. Although the effect of higher concentrations of borate has not been tested, it is possible that if the ratio of borohydride to coproporphyrin during the reduction had been higher, activity might not have been detected.

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