COPROPORPHYRINOGENASE ACTIVITY IN EXTRACTS FROM RHODOPSEUDOMONAS SPHEROIDES

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An extract from Rhodopseudomonas spheroides, grown semi-anaerobically in the light, converts coproporphyrinogen to protoporphyrin when incubated anaerobically in the dark with Mg^{2+}, ATP and L-methionine. Extracts from R. spheroides grown under air or oxygen in the dark are inactive when assayed under these conditions. Extracts from organisms grown in the light or in the dark form protoporphyrin in the absence of ATP and methionine when incubated with coproporphyrinogen in the presence of oxygen. The anaerobic reaction is inhibited by 1,10-phenanthroline, whilst the aerobic reaction is not.

The enzymic conversion of coproporphyrinogen III to protoporphyrin IX by coproporphyrinogenase involves the oxidative decarboxylation of two of the propionic acid side chains to form vinyl groups. The enzyme from mammalian liver mitochondria has been shown to have an absolute requirement for molecular oxygen (Sano and Granick, 1961; Batlle, Benson and Rimington, 1965). Cytochromes and bacteriochlorophylls are formed by micro-organisms which grow under strictly anaerobic conditions. Thus it is apparent that these organisms must be able to convert coproporphyrinogen to protoporphyrin using a hydrogen acceptor other than oxygen. In spite of wide interest in such a reaction (cf. Lascelles, 1963) it was only recently that Ehteshamuddin (1968) reported briefly that an extract of a species of Pseudomonas exhibited coproporphyrinogenase activity under anaerobic conditions. Mori and Sano (1968), using extracts of Chromatium D, an anaerobic photosynthetic micro-organism, could only detect activity when assays were done in the presence of oxygen.

In this paper I wish to report that an extract from Rhodopseudomonas

spheroides, grown semi-anaerobically in the light, converts coproporphyrinogen to protoporphyrin when incubated anaerobically in the dark with Mg^{2+}, ATP and L-methionine.

METHODS. Rhodopseudomonas spheroides (National Collection of Industrial Bacteria No. 8253) was grown semi-anaerobically in the light, or by bubbling air or oxygen in the dark, in Medium S of Lascelles (1956) supplemented with $FeSO_4$ (10μM) and Mn Cl_2 (5μM). Cells were harvested by centrifuging towards the end of the exponential phase of growth, washed with cold 0.05M Tris-HCl buffer pH 8.0 saturated with N_2, and resuspended in the same buffer to a concentration of 50 to 80 mg. dry wt./ml. Suspensions were used immediately or kept frozen under N_2 at -20°. 2-Mercaptoethanol, to a final concentration of 1mM, was added to suspensions, and cell disruption was achieved by passage through a French Pressure Cell at 15000 lb/sq. in., or by sonication for 3 min at 0° (MSE Ultrasonic Power Unit 50W). The resulting suspensions were centrifuged at 25000 g for 7 min. The supernatant, designated "crude extract", was used immediately, or stored under N_2 at -20°.

Coproporphyrin III was prepared by illuminating a suspension of photosynthetically grown R. spheroides in Mixture I of Lascelles (1956) plus L-ethionine (1mM) (Gibson, Neuberger and Tait, 1962). The coproporphyrin, which accumulates in the culture medium, was purified as described by Neilands and Garibaldi (1960). [^{14}C]-coproporphyrin was prepared in the same way from illuminations done in the presence of [2^{14}C] glycine. Coproporphyrinogen was prepared immediately before use by treating a suspension of coproporphyrin in water (1 mg/ml) with Na BH_4 (cf. Nishida and Labbe, 1959).

Coproporphyrinogenase activity was measured as described in the legend to Table I and in the text. The reaction was stopped, and the porphyrins were extracted and measured, essentially as described by Sano and Granick (1961).

TABLE I

Coproporphyrinogenase activity under anaerobic conditions. To conical tubes, flushed with N_2, was added crude extract from semi-anaerobically grown cells as stated, 0.03 ml. of coproporphyrinogen (1mg/ml.), 10μmoles of $MgSO_4$, 2μmoles of ATP, 1μmole of L-methionine and water saturated with N_2 to 0.9 ml. N_2 was bubbled vigorously over the surface to mix the contents and the tubes were stoppered. Incubation was in the dark at 37° for the times stated. Reactions were stopped and coproporphyrin and protoporphyrin were isolated and determined as described in the Methods section.

Expt.	Cell Extract	Time of Incubation (hours)	Omissions from assay	Coproporphyrin	Protoporphyrin Total	Protoporphyrin Synthesised
				(μmmoles)		
1	18.1	0	None	14.9	2.02	-
	18.1	1	None	12.0	4.65	2.63
	18.1	2	None	10.5	6.00	3.98
	18.1	3	None	11.4	5.30	3.28
	18.1	3	Methionine	14.3	2.95	0.93
	18.1	3	Methionine, ATP, $MgSO_4$	16.0	2.52	0.50
2	17.6	3	Methionine, ATP, $MgSO_4$	13.6	1.52	-
	8.8	3	None	13.6	2.29	1.53
	17.6	3	None	11.8	4.40	2.88
	26.4	3	None	11.1	5.80	3.52

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

RESULTS. When a crude extract of anaerobically grown R. spheroides is incubated under N_2 in the dark with coproporphyrinogen, L-methionine, ATP and $MgSO_4$, protoporphyrin is formed (Table I). The extract used contains small amounts of magnesium protoporphyrin and its monomethyl ester which both assay as protoporphyrin in this procedure. This accounts for the presence of protoporphyrin in the non-incubated control. The rate of production of protoporphyrin is linear for about 2 hours, and

it is approximately linearly proportional to the amount of enzyme used up to 20 mg. protein per assay (Table I). There is little or no formation of protoporphyrin if ATP, or Mg^{2+}, or methionine are omitted (Figure I). The optimum concentration of $MgSO_4$ is about 10mM, of ATP 2mM and of L-methionine 0.5mM (Figure I). Ca Cl_2 (2.5 mM) is almost as effective as $MgSO_4$ in allowing protoporphyrin formation, but $MnSO_4$ (1mM) is not effective. S-Adenosylmethionine cannot replace ATP and methionine, but protoporphyrin is formed if it is used instead of methionine. A number of amino acids cannot replace methionine: those tested were L-serine, L-alanine, DL-homocysteine thiolactone, L-cysteine, L-ethionine. L-Ethionine partly inhibits protoporphyrin formation in the presence of

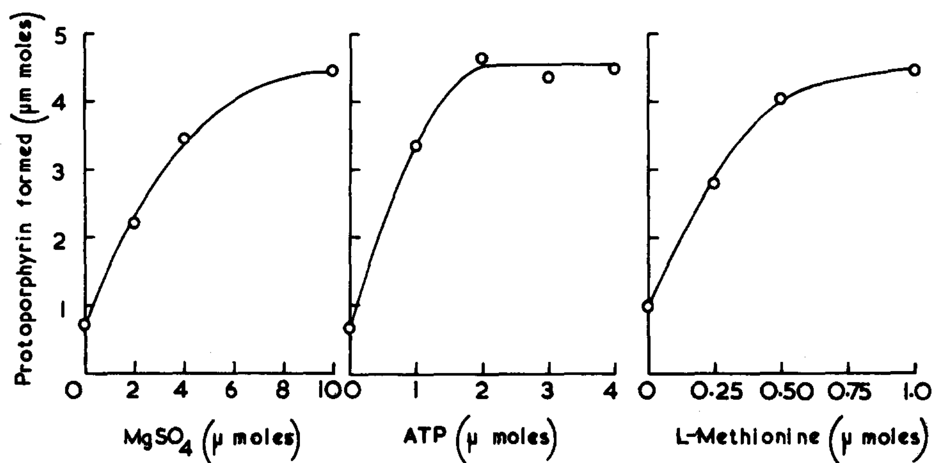

Fig.1. Effect on protoporphyrin formation of varying the concentrations of $MgSO_4$, ATP and L-methionine. To conical tubes flushed with N_2 were added 0.5 ml. of crude extract from semi-anaerobically grown cells (28.8 mg.protein/ml), 0.03 ml. of coproporphyrinogen (1 mg/ml), $MgSO_4$, ATP, L-methionine and water, saturated with N_2, to 0.9 ml. Unless stated otherwise 10 µmoles of $MgSO_4$, 4 µmoles of ATP and 1 µmole of L-methionine were added. N_2 was bubbled vigorously over the surface to mix the contents; the tubes were stoppered and incubated at 37° in the dark for 2 hr. The reaction was stopped and the protoporphyrin was isolated and determined as described in the Methods section. The amount of protoporphyrin extracted from a non-incubated control containing crude extract and coproporphyrinogen has been substracted from all values.

methionine. The optimum rate of protoporphyrin formation is obtained with about 25 µmmoles of coproporphyrinogen per assay. It was initially thought that traces of oxygen in the reagent solutions or in the cell extract might function as hydrogen acceptor. However, it was found, using an oxygen electrode, that the reaction mixture became completely anaerobic in about 2 minutes. It is probable that the crude extract contains sufficient endogenous substrates to remove all the oxygen.

The protoporphyrin formed has spectra identical with that of authentic protoporphyrin in both dilute HCl and in ether. When [^{14}C] coproporphyrinogen (27 counts/min/µmmole) was used as substrate there was radioactivity in the protoporphyrin extracted from incubations done with ATP, methionine and $MgSO_4$, but none in the protoporphyrin fraction from incubations done without these factors. The specific radioactivity of the total protoporphyrin was 20 counts/min/µmmole. When a correction was made for protoporphyrin extracted from the cell extract the specific radioactivity was 30 counts/min/µmmole. Paper chromatography of the protoporphyrin fraction on paper in 2,6-lutidine; 0.05N NH_4OH (10:7 v/v) showed that there were two porphyrin spots corresponding to protoporphyrin and protoporphyrin monomethyl ester. Only the former was radioactive; the latter is therefore probably derived from magnesium protoporphyrin monomethyl ester present in the cell extract.

Extracts prepared from poorly pigmented or unpigmented R. spheroides grown in air or oxygen in the dark did not form protoporphyrin under anaerobic conditions in the presence of $MgSO_4$, ATP and methionine. When extracts from R. spheroides, grown anaerobically in the light, or in air or oxygen in the dark, were incubated with coproporphyrinogen in open tubes in the presence of 10mM KCN, to prevent oxygen utilisation via the cytochrome system, protoporphyrin was formed. With all three extracts about 0.2 µmmoles of protoporphyrin were formed per mg. of protein in 2 hours, a value close to that obtained in assays done under anaerobic

conditions in the presence of $MgSO_4$, ATP and methionine (cf. Table I).

1,10-Phenanthroline at a concentration of 0.56mM completely inhibited protoporphyrin formation under anaerobic conditions and even at 56μM it inhibited by 30%. Those concentrations of 1,10-phenanthroline did not inhibit the reaction in the presence of oxygen, indeed they slightly stimulated activity.

A crude extract of photosynthetically grown cells was centrifuged at 100,000g for 90 min. The clear almost colourless supernatant was removed and the deeply pigmented pellet was resuspended. When assayed for coproporphyrinogenase activity in open tubes in the presence of KCN the supernatant, but not the pellet, was active. Under anaerobic conditions with $MgSO_4$, ATP and methionine, neither the supernatant nor the pellet formed protoporphyrin, but full activity was restored when supernatant and pellet were assayed together.

DISCUSSION. The results show that an extract of photosynthetically grown *R. spheroides* forms protoporphyrin from coproporphyrinogen under strictly anaerobic conditions if supplemented with $MgSO_4$, ATP and methionine. In addition the extract can also perform the reaction with oxygen as hydrogen acceptor. Extracts from cells grown in air or oxygen can form protoporphyrin only in the presence of oxygen. In extracts from photosynthetically grown cells it appears that the hydrogen acceptor, which is used instead of oxygen, is synthesised in the presence of $MgSO_4$, ATP and methionine during the assay. The inhibitory effect of 1,10-phenanthroline suggests that ferrous iron may also be involved in the production of the hydrogen acceptor.

It is known that *R. spheroides* growing semi-anaerobically in the light or illuminated under non-growing conditions excretes large amounts of coproporphyrin when ethionine or threonine are added (Gibson et al., 1962), or when the medium is deficient in sulphate (Lessie and Sistrom, 1964). It was proposed (Gibson, Matthew,

Neuberger & Tait, 1961) that coproporphyrin excretion was a consequence of interference with the biosynthesis or metabolic function of methionine and hence of a failure to form magnesium protoporphyrin monomethyl ester. While this explanation may still be partly correct the present finding that methionine is required for formation of protoporphyrin under anaerobic conditions would seem to be more important in explaining why coproporphyrin is excreted in "methionine deficiency".

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