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Oxidation of Protoporphyrinogen in the Obligate Anaerobe *Desulfovibrio gigas*

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The anaerobic oxidation of protoporphyrinogen to protoporphyrin was demonstrated in extracts of *Desulfovibrio gigas*. Protoporphyrin formation occurred in the presence of nitrite, hydroxylamine, sulfite, thiosulfate, ATP plus sulfate, NAD^+, $NADP^+$, flavin adenine dinucleotide, flavin mononucleotide, fumarate, 2,6-dichlorophenol-indophenol, methyl viologen, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. With dialyzed cell extracts, highest activities were observed with sulfite, NAD^+, and $NADP^+$ as electron acceptors. The enzyme for protoporphyrinogen oxidation was localized in the membrane of *D. gigas* and displayed optimal activity at pH 7.3 and 28°C.

The oxidation of protoporphyrinogen to protoporphyrin, a late step in heme biosynthesis, is coupled to the reduction of molecular oxygen in mammalian liver and yeast mitochondria (25–27) and in most bacteria (13, 14). However, an oxygen-independent mechanism must be widely distributed, because many bacteria synthesize cytochromes and bacteriochlorophylls during anaerobic growth (2, 5, 15, 23, 24).

The anaerobic oxidation of protoporphyrinogen to protoporphyrin has been observed in extracts of *Escherichia coli*, wherein nitrate and fumarate were capable of replacing oxygen in the reaction (7, 8). It was further demonstrated that anaerobic heme synthesis in *E. coli* required a functional electron-transport system (9–11). The anaerobic synthesis of protoporphyrin has not been reported for obligate anaerobic bacteria. The oxidation of coproporphyrinogen III to protoporphyrin IX was reported for the obligate anaerobe, *Chromatium* D, if the reaction was conducted under aerobic conditions (20). Protoheme synthesis for *b*-type cytochromes has been reported for *Selenomonas ruminantium* and *Bacteroides succinogenes* when deuteroporphyrin IX was present in the growth medium (3). Anaerobic synthesis of protoporphyrin has been observed with intact cells of aerobically grown *Rhizobium japonicum* (16). The inability to demonstrate the synthesis of coproporphryin and protoporphyrin from δ-aminolevulinic acid (20) in extracts of *Desulfovibrio vulgaris* suggests that anaerobic sulfate-reducing bacteria synthesize heme by a unique pathway. This communication reports the anaerobic oxidation of protoporphyrinogen to protoporphyrin in *Desulfovibrio gigas* by an electron-transfer system which interfaces with numerous electron acceptors.

MATERIALS AND METHODS

Organisms and growth conditions. *D. gigas* (NCIB 9332) and *E. coli* K-12 (ATCC 14948) were used in this study. *D. gigas* was grown in the lactate-sulfate medium described previously (19), and *E. coli* was grown in a broth consisting of 0.5% peptone and 0.2% of either $NaNO_3$ or sodium fumarate. For the protoporphyrinogen oxidase assay by *E. coli* with nitrate or fumarate as the electron acceptor, the cells were grown anaerobically in peptone-nitrate or peptone-fumarate medium, respectively.

Preparation of cell extracts. Cells were harvested from rapidly growing cultures at 7,000 × *g* for 10 min and washed once with 0.05 M Tris hydrochloride (pH 7.3). After centrifugation, the cell pellet was suspended in a volume of 0.05 M Tris hydrochloride equal to the weight of the pellet and disrupted in a French pressure cell. *D. gigas* was disrupted at 4,000 lb/in^2, and *E. coli* was subjected to 10,000 lb/in^2. The cell extract was treated with a few crystals of DNase C (Sigma Chemical Co., St. Louis, Mo.) and clarified by sedimentation at 23,000 × *g* for 7 min. The supernatant of this cell extract was designated the crude extract. For some reactions, the crude extract from *D. gigas* was dialyzed against 0.001 M Tris-hydrochloride (pH 7.3) for 24 h at 4°C.

Membrane and soluble protein fractions were prepared from dialyzed extracts of *D. gigas* by ultracentrifugation at 140,000 × *g* for 2 h on buffered discontinuous sucrose gradients of 20 and 60% sucrose (1). The soluble fraction was collected from the top of the 20% sucrose layer, and the membrane fraction was collected from the interface of the 20 and 60% sucrose layers.

Preparation of protoporphyrinogen. A stock solution of 500 μM protoporphyrin was prepared by dissolving 1.4 mg of protoporphyrin (Porphyrin Products, Logan, Utah) in 5 ml of 0.1 N KOH containing 20% ethanol (vol/vol). The stock solution was diluted to 200 μM for experimental use with the KOH-ethanol solvent. Protoporphyrinogen was prepared by the procedure of Jacobs and Jacobs (12) by reducing the 200 μM protoporphyrin solution with sodium amalgam for 2 min in tubes fitted with serum stoppers and flushed with purified nitrogen gas. After reduction, the protoporphyrinogen solution was passed through a glass fiber membrane filter (Millipore Corp., Bedford, Mass.) and adjusted to pH 7.3 with 40% (wt/vol) H_3PO_4. The entire procedure for preparation of protoporphyrinogen was performed in the dark. Protoporphyrinogen was stored in a darkened, sealed serum bottle under nitrogen gas and prepared fresh on a weekly basis.

Sodium amalgam was prepared by adding 0.09 g of sodium metal (J. T. Baker Chemical Co., Phillipsburg, N.J.) in pieces (2 mm^3) to 3 g of mercury with vigorous agitation under a nitrogen atmosphere. When the sodium was com-

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pletely added and the amalgam had cooled and solidified, it was ground with mortar and pestle to form fine granules. The fine amalgam granules were then stored in sealed tubes under nitrogen gas.

Measurement of protoporphyrinogen oxidation. The 1.0-ml reaction mixture contained 50 mM Tris-hydrochloride (pH 7.3), 2 mM EDTA adjusted to pH 7.3, 5 mM cysteine, 50 μM protoporphyrinogen, 10 mM fumarate, and 20 mg of cell extract. Before the addition of protoporphyrinogen, the reaction mixture was placed in 1.3-ml cuvettes and sealed with serum stoppers; purified nitrogen gas was bubbled through the solution for 10 min to establish anaerobic conditions. Protoporphyrinogen was injected with a gas-tight syringe to initiate the reaction. The cuvettes were inverted several times, and incubation was at 28°C in a water bath.

Spectra were rapidly scanned (2 nm/s) from 450 to 700 nm with a Varian Cary 214 spectrophotometer at the specified times. The appearance of the four-banded porphyrin spectrum was used to determine the conversion of protoporphyrinogen to protoporphyrin. Absorbance values were determined by drawing a line between the troughs on either side of the alpha, beta, and gamma peaks at 633, 578, and 540 nm and measuring the absorbance from line to peak at each wavelength. Absorbance values were then converted to concentrations of protoporphyrin by using the following nanomolar extinction coefficients (12): $E_{633} = 2.64 \times 10^{-3}$, $E_{578} = 2.28 \times 10^{-3}$, and $E_{540} = 3.36 \times 10^{-3}$. Enzyme activities are expressed as nanomoles of protoporphyrin formed per milligram of protein in the reaction. All experimental tests were conducted in triplicate, and average values are presented.

Parameters of enzyme activity and related procedures. Protoporphyrinogen oxidation by *D. gigas* extracts was examined under various conditions when 10 mM fumarate was the electron acceptor and the incubation period was 10 min. To determine temperature effects, protoporphyrinogen oxidation was measured with Tris-hydrochloride buffer (pH 7.3) over a range of 1 to 50°C. The pH optimum for the reaction was determined by using bis-Tris-propane (1,3-bis[Tris]propane) buffer adjusted to specified values with HCl. To demonstrate dependency of the formation of protoporphyrin on the cell extract, the reaction was examined over a range of 1.75 to 15 mg of protein per reaction.

Electron acceptors were substituted for fumarate to provide a 10 mM final concentration and were freshly prepared solutions of sodium nitrate, sodium nitrite, hydroxylamine, sodium sulfite, sodium thiosulfate, potassium trithionate, and sodium sulfate. In the sulfate-containing reaction, ATP (10 mM) was added to generate adenosine phosphosulfate, which is an intermediate in the sulfate-reduction pathway. Final concentrations of NAD^+, $NADP^+$, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) were 1 mM, and DCIP (2,6-dichlorophenol-indophenol), methyl viologen, menadione, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were at final concentrations of 2.5 μM.

Controls for the enzymatic oxidation of protoporphyrinogen involved chemical oxidation by electron acceptors and by the cell extracts. Protoporphyrin formation was monitored for each electron acceptor by incubation of the anaerobic reaction under experimental conditions, except that no cell extract was present. Additionally, chemical activity of the cell fraction was assessed by heating the cell extract for 1 h at 55°C and substituting this enzyme-inactivated fraction for the cell extract in the reaction. Protein was determined by the biuret method (4) with bovine serum albumin as the standard. Potassium trithionate was prepared by the procedure of Palmer (22).

RESULTS

Protoporphyrin production. By established procedures, the oxidation of protoporphyrinogen to protoporphyrin was observed in cell extracts of *D. gigas*. With fumarate as the electron acceptor, the activity of protoporphyrinogen oxidation in 100 min of incubation with *D. gigas* extracts was 0.37 nmol of protoporphyrin produced per mg of protein, which was approximately twice as much as in extracts from *E. coli*, which produced 0.16 nmol of protoporphyrin per mg of protein. Protoporphyrin production by *D. gigas* with nitrite as the electron acceptor in 100 min was 0.54 nmol/mg of protein, whereas production by *E. coli* in the presence of nitrate was 0.26 nmol/mg. *E. coli* does not use nitrite and *D. gigas* does not use nitrate as an electron acceptor. The oxidation of protoporphyrinogen by *D. gigas* extracts was markedly reproducible and stable when fumarate was the electron acceptor. No chemical oxidation of protoporphyrinogen was observed with heat-treated cell extracts under the conditions of the anaerobic assay.

Protoporphyrin formation in *D. gigas* extracts was detected in the presence of several different electron acceptors (Table 1). The greatest activity was observed with sulfite, NAD^+, $NADP^+$, FAD, and FMN. Trithionate, the product of bisulfite reduction in *Desulfovibrio* sp. (17), did not

TABLE 1. Effect of various electron acceptors on protoporphyrin formation by dialyzed extracts of *D. gigas* under anaerobic conditions

Electron acceptor[a]	Protoporphyrin produced (nmol) per mg of protein[b]	
	20 min	60 min
Nitrogen compounds		
NO_2^-	0.716	0.269
NH_2OH	0.181	0.337
Sulfur compounds		
Adenosine phosphosulfate	0.199	0.325
SO_3^{2-}	0.393	1.878
$S_2O_3^{2-}$	0.086	0.010
$S_3O_6^{2-}$	0	0
Organic compounds		
Fumarate	0.179	0.578
FAD	0.409	0.960
FMN	0.410	0.960
NAD^+	0.821	1.529
$NADP^+$	0.720	1.341
Artificial acceptors		
DCIP	0.237	Not tested
MTT	0.166	Not tested
Methyl viologen	0.019	0.634
Menadione	0	0

[a] Fumarate, nitrogen, and sulfur compounds were present at 10 mM, flavins and pyridine nucleotides were present at 1 mM, and artificial acceptors were present at 2.5 μM.

[b] Each reaction contained 11 mg of bacterial protein, and values for activity were corrected for background. Comparable levels of protoporphyrinogen oxidation in these reactions with heat-treated cell extract and nitrite at 20 and 60 min were 0.008 and 0.016 nmol/mg of protein, respectively. With electron acceptors other than nitrite, oxidation of protoporphyrinogen of the controls did not exceed 10% of the values obtained with nitrite. All values listed here were subtracted from chemical controls.

TABLE 2. Effect of inhibitors on electron transport from protoporphyrinogen to fumarate in *D. gigas* membrane fraction

Addition[a] (mg/mg of protein)	Protoporphyrin produced (nmol) per mg of protein in 10 min	Inhibition (%)
None	0.136	
Rotenone		
4	0.046	66
0.4	0.054	60
HQNO		
1.65×10^{-2}	0.134	2
1.65×10^{-3}	0.135	1

[a] Ten minutes before the protoporphyrinogen was added to the reaction, rotenone and HQNO were introduced, by anaerobic procedures, into the reaction mixture. Each reaction contained 9 mg of membrane protein.

function as an acceptor of electrons for the protoporphyrinogen oxidase system. Artificial electron acceptors of DCIP, MTT, and methyl viologen also coupled to protoporphyrinogen oxidation at micromolar concentrations, whereas at millimolar levels, no oxidation of protoporphyrinogen was observed. Menadione was not an appropriate electron acceptor in this reaction. The decrease in the amount of protoporphyrin present at 60 min as compared with 20 min with nitrite and thiosulfate may be attributed to chemical or enzymatic utilization of protoporphyrin. For this reason, the activity of protoporphyrinogen oxidase was based on incubation periods of 20 min or less.

The effect of electron-transport inhibitors on the protoporphyrinogen-fumarate couple in *D. gigas* is presented in Table 2. The sensitivity to rotenone but not to hydroxyquinoline-*N*-oxide (HQNO) suggests the functioning of a flavoprotein, but not a quinone, in the transfer of electrons from protoporphyrinogen to fumarate.

Parameters of enzyme activity. The optimal temperature for oxidation of protoporphyrinogen by *D. gigas* with fumarate as the electron acceptor was 28°C (Fig. 1). At 1°C, the rate of protoporphyrin production was 35% that at the optimum temperature. Above 28°C, enzyme activity was diminished, with complete inactivation at 50°C. The calculated Q_{10} of the reaction was 1.3.

The pH optimum for the protoporphyrinogen-fumarate couple was 7.3, and activity was difficult to detect outside the pH range of 6.5 to 7.8. At pH 7.0 and 7.5, the rate of protoporphyrin formation was only 30% of optimal activity. No buffer effect was observed, because bis-Tris-propane-hydrochloride or Tris-hydrochloride buffer at pH 7.3 produced identical activity.

Protoporphyrin formation in dialyzed extracts with fumarate as the electron acceptor was proportional to the protein concentration in the reaction mixture. For routine assays wherein high activity in a short incubation period was required, 20 mg of protein in crude extract was appropriate. Protoporphyrin formation in membranes with fumarate was also dependent on protein concentration, and 10 mg of membrane protein was used in 10-min incubations.

Various electron acceptors supported oxidation of protoporphyrinogen with *D. gigas* extracts (Fig. 2). Production of protoporphyrin with NAD^+ or $NADP^+$ was rapid and linear for the first minutes of the reaction, and activity continued to increase slowly thereafter. Protoporphyrin formation increased with incubation time when FAD and FMN were the electron acceptors. In contrast, oxidation of protoporphyrinogen with fumarate as the electron acceptor did not continue after 7.5 min.

The enzyme system for oxidation of protoporphyrinogen was in the membrane of *D. gigas* (Fig. 3). Furthermore, the addition of soluble protein to membrane fractions did not affect the production of protoporphyrin with nitrite or fumarate as the electron acceptor. This suggests that electron-transferring components for coupled reactions of protoporphyrinogen-fumarate or protoporphyrinogen-nitrite were also localized in the membrane.

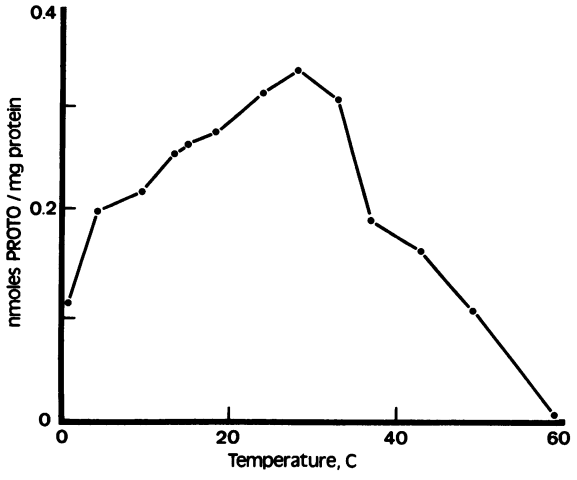

FIG. 1. Protoporphyrin formation in dialyzed extracts of *D. gigas* at different incubation temperatures. Points reflect activity with 10 mM fumarate as the electron acceptor, 10 mg of protein in each reaction, and incubation for 10 min.

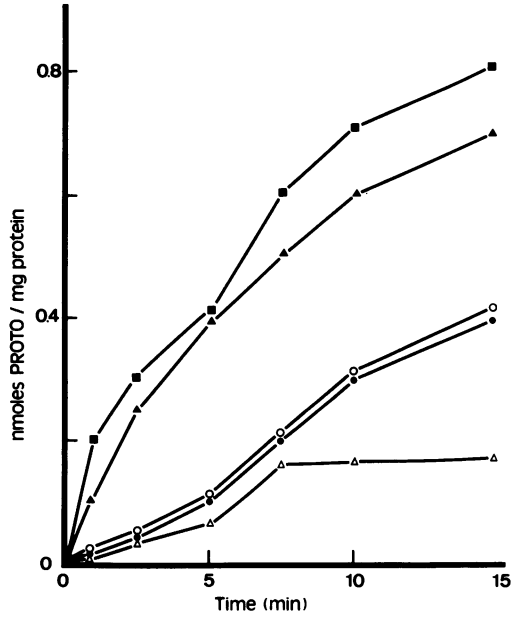

FIG. 2. Formation of protoporphyrin with dialyzed extracts from *D. gigas*. Final concentrations of NAD^+ (■), $NADP^+$ (▲), FAD (○), and FMN (●) were 1 mM, and the fumarate (△) concentration was 10 mM. Each reaction contained 14 mg of protein of the cell extract.

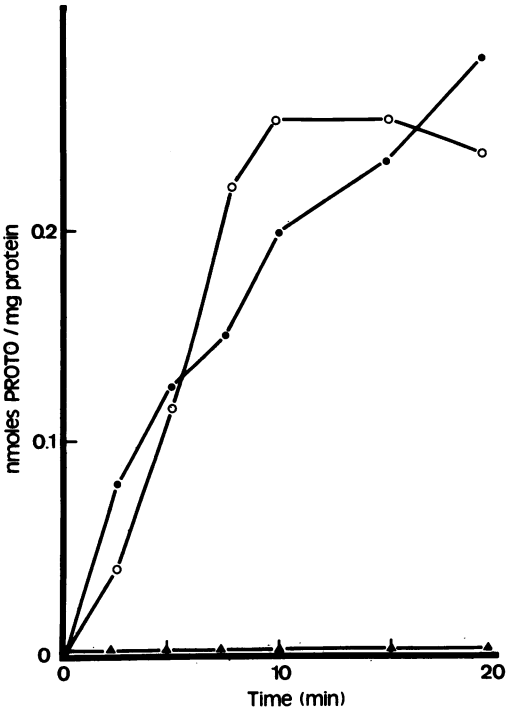

FIG. 3. Protoporphyrin formation with DCIP as the electron acceptor. Reactions contained dialyzed extract (○; 15 mg of protein), membrane fraction (●; 4 mg of protein), or soluble fraction (▲; 11 mg of protein) from *D. gigas*.

DISCUSSION

The physiological findings described here demonstrate the anaerobic oxidation of protoporphyrinogen to protoporphyrin in *D. gigas*. In contrast to the oxygen-dependent protoporphyrinogen oxidase of mitochondrial systems (25, 26), the electron-transfer activity displayed by *D. gigas* in protoporphyrin production is through an anaerobic electron-transfer process. In *D. gigas*, the protoporphyrinogen oxidase interfaces with respiratory systems composed of fumarate and nitrite reductases as well as intermediates of dissimilatory sulfate reduction. Additionally, flavins and pyridine nucleotides afford the appropriate couple for protoporphyrinogen oxidation in *D. gigas*.

The localization of the protoporphyrinogen oxidase in the membrane of *D. gigas* is indicated by our demonstration that the protoporphyrinogen-fumarate- and protoporphyrinogen-nitrite-coupled reactions require only a membrane fraction. Fumarate and nitrite reductases are known to be localized in the plasma membrane of *D. gigas* (1, 6). The protoporphyrinogen-sulfite couple requires both soluble and membrane fractions, because the sulfite reductase is soluble in *D. gigas* (17). Membrane localization of the enzyme for oxidation of protoporphyrinogen in *D. gigas* is consistent with reports wherein aerobic organisms (25, 26) or anaerobic *E. coli* (10, 11) have been examined.

The inhibition by rotenone and lack of sensitivity to HQNO suggest that the flow of electrons from protoporphyrinogen to fumarate in *D. gigas* is flavoprotein mediated but not dependent on a quinone. In contrast, the electron flow from H_2 to fumarate is through a quinone (6). The multiplicity of electron carriers and the absence of available specific inhibitors for electron flow in *D. gigas* (18, 21) make it difficult to accurately position protoporphyrinogen oxidase in the electron-transport chain.

This is the first demonstration of protoporphyrin synthesis in obligate anaerobic chemolithotrophic bacteria. The system by which protoporphyrinogen is converted to protoporphyrin in *D. gigas* may be similar to that in anaerobically grown *E. coli*. It will be important to determine whether protoporphyrinogen oxidase is widely distributed in the anaerobic bacteria.

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