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Purification and Properties of Protoporphyrinogen Oxidase from an Anaerobic Bacterium, *Desulfovibrio gigas*

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Protoporphyrinogen oxidase has been solubilized from plasma membranes of *Desulfovibrio gigas*. The enzyme was purified to apparent homogeneity with single silver-stained protein bands on isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. This protoporphyrinogen oxidase has a molecular weight (M_r) of 148,000 and is composed of three dissimilar subunits of M_rs 12,000, 18,500, and 57,000, which are held together by sulfhydryl bonds. Unlike other protoporphyrinogen oxidases, which use molecular oxygen as an electron acceptor, this enzyme does not couple to oxygen. The protoporphyrinogen oxidase donates electrons to 2,6-dichlorophenol-indophenol but not to NAD^+, $NADP^+$, flavin adenine dinucleotide, or flavin mononucleotide. The natural physiological electron acceptor of the protoporphyrinogen oxidase from *D. gigas* is unknown. By using 2,6-dichlorophenol-indophenol as the electron acceptor, the K_m and V_{max} values for oxidation of protoporphyrinogen were determined to be 21 μM and 8.38 nmol/min per 70 μg of protein, respectively. The catalytic rate constant, K_{cat}, was calculated to be 17.7 mol of protoporphyrin formed per mole of enzyme per min of incubation, and the K_{cat}/K_m was 0.84. Energies of activation were calculated from Arrhenius plots with 7,429 cal (ca. 31,080 J)/mol per degree below 10°C and 1,455 cal (ca. 6,088, J)/mol per degree above 10°C. Optimum enzyme activity was at 23°C, and inhibition was observed with both *N*-ethylmaleimide and iodoacetamide.

In the final stages of heme synthesis, protoporphyrinogen (PGEN) is oxidized to protoporphyrin (PROTO) by PGEN oxidase. Molecular oxygen is required for PROTO formation from PGEN in animal, plant, and aerobic bacterial systems (23, 24, 35, 36). Several bacteria grown under anaerobic conditions appear to contain PGEN oxidase as evidenced by the isolation of heme proteins and bacteriochlorophylls from purple, nonsulfur bacteria (4, 34); green, sulfur bacteria (5, 13, 18) and heme proteins from the nonphotosynthetic *Vibrio* spp. (25), *Bacteroides* spp. (11, 12, 30), and numerous molecules in *Desulfovibrio* spp. contain hemes as reviewed by LeGall et al. (28). Species of *Rhizobium* are known to synthesize the porphyrin prosthetic group of leghemoglobin in reduced-oxygen environments (2, 8).

An oxygen-independent mechanism for PGEN oxidation has been observed in extracts of *Escherichia coli* when nitrate or fumarate served as the electron acceptor (19, 20). Recently we have described the coupling of PGEN oxidation to the reduction of physiological and artificial electron acceptors in membranes isolated from *Desulfovibrio gigas* (26). The PGEN oxidase is highly active in *D. gigas*, as evidenced by the observation that over 1% of cell protein in *D. gigas* is cytochrome (32) and multiheme cytochromes predominate (28).

PGEN oxidase has been solubilized from membranes of aerobically grown *Saccharomyces cerevisiae* by sonication in detergents, and the purified enzyme has a molecular weight of 180,000 (36). Attempts to solubilize the PGEN oxidase from *E. coli* and *Rhodopseudomonas sphaeroides* with Triton X-100 were of limited success (21). PGEN oxidase in eucaryotic cells functions as an oxygenase, whereas the enzyme from the anaerobe *D. gigas* does not react under physiological conditions with molecular oxygen but displays activities characteristic of a dehydrogenase (26); however, for simplicity the enzyme involved in formation of PROTO from PGEN in *D. gigas* is referred to here as an oxidase. In this paper, we report the solubilization, purification, and physical characteristics of the PGEN oxidase from *D. gigas*. This is the first report of an enzyme purified from obligate anaerobic bacteria which is involved in PGEN synthesis, and activity in the absence of oxygen would suggest that it reflects a type of enzyme distinct from that in the aerobic organisms.

MATERIALS AND METHODS

Cell growth and preparation of extracts. *D. gigas* NCIB 9332 was grown in a lactate-sulfate medium previously described (3). Cells were harvested by centrifugation at 7,000 × *g* and were washed once with 0.05 M Tris hydrochloride, pH 7.3. Cell pellets were suspended in a volume of 0.05 M Tris hydrochloride, pH 7.3, which was equal to the net weight of cells before being disrupted in a French pressure cell at 4,000 lb/in^2. The cell extract was treated with DNase C (Sigma Chemical Co., St. Louis, Mo.) before centrifugation at 23,000 × *g* for 7 min at 10°C. Membrane and soluble fractions were separated by ultracentrifugation at 140,000 × *g* for 2 h on a discontinuous sucrose gradient of 20 and 60% sucrose in a buffer containing 0.01 M Tris hydrochloride, pH 7.3, and 0.1 M $MgCl_2$ (3). 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.

Solubilization of PGEN oxidase. Membrane fractions from *D. gigas* which contained PGEN oxidase were suspended in 0.05 M Tris hydrochloride, pH 7.3, to a protein concentration of 40 mg/ml. Individual detergent solutions were added to separate portions of the suspended membrane fraction to give a final detergent concentration of 0.25% and a final protein concentration of 30 mg/ml. After being gently mixed for 30 min at 4°C, the sample was centrifuged at 130,000 × *g*

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for 1 h. Supernatant and pelleted fractions were examined for PGEN oxidase activity, and detergent efficiency was described in terms of enzyme activity solubilized per milligram of solubilized protein. The detergents were dissolved in 0.05 M Tris hydrochloride, pH 7.3, and included the following: Triton X-100 (J. T. Baker Chemical Co., Phillipsburg, N.J.), Tween 80 (Sigma), 3-[(3-chloramido)-dimethylamino]-1-propane-sulfonate (CHAPS; Pierce Chemical Co., Rockford, Ill.), Brij 35 (Calbiochem-Behring, San Diego, Calif.), Zwittergent 3-14 (Calbiochem), Genapol X-080 (Calbiochem), and octyl-D-glucopyranoside (Calbiochem).

Measurement of PGEN oxidase. The assay of PGEN oxidase was as previously described (26). The 1-ml reaction solution contained 50 μmol of Tris hydrochloride, pH 7.3; 2 μmol of L-cysteine; 50 μmol of PGEN; 10 μmol of 2,6-dichlorophenol indophenol (DCIP); and dialyzed cell extract. PGEN was prepared by reduction of PROTO with sodium amalgam as established by Jacobs and Jacobs (22). Before the addition of PGEN, the reaction mixture was placed in 1.3-ml cuvettes sealed with serum stoppers and purified N_2 was bubbled through the solution for 10 min to establish anaerobic conditions. PGEN was injected with a gastight syringe to initiate the reaction, which was followed by spectral measurements with a Varian model 219 spectrophotometer (Varian Associates, Inc., Palo Alto, Calif.). Special care was taken to protect the substrate from light, and the cuvette was placed in the spectrophotometer path only once at the end of the assay time (10). Enzyme activity was expressed as nanomoles of PROTO produced using the nanomolar extinction coefficients of 2.64×10^{-3} at 633 nm, 2.28×10^{-3} at 578 nm, and 3.36×10^{-3} at 540 nm (22).

Parameters of activity were examined to assist characterization of the PGEN oxidase enzyme. To determine the effect of temperature, PROTO formation was measured in purified enzyme preparations over a range from 1 to 60°C and activation energies were determined from linear regression analysis of Arrhenius plots of the temperature data. The pH optimum of the reaction was determined by using 0.05 M Tris hydrochloride or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). Simple kinetic analysis of the purified enzyme was performed over a range of PGEN concentrations from 0 to 100 μM with DCIP as the electron acceptor. Kinetic parameters were determined by regression analysis using general linear models of the SAS procedure (SAS Institute, Inc., Cavy, N.Y.) of kinetic data by the methods reviewed by Cleland (6). The catalytic rate constant, K_{cat}, was calculated from the V_{max} by using the molecular weight of the enzyme. The effect of sulfhydryl inhibitors on the enzyme was assessed by using 0.5 and 5 mM final concentrations of iodoacetamide, *para*-hydroxy mercuribenzoate, and *N*-ethylmaleimide (9).

Numerous controls were run on the PGEN oxidase assay to ensure that we were following an enzymatic process. These controls included examination of PROTO formation in the presence of electron acceptors under the conditions of the assays without enzyme added or using enzyme which had been denatured by being heated at 60°C for 1 h. Also, auto-oxidation of the PGEN was monitored to ensure that activity was not attributed to nonenzymatic processes.

Purification of PGEN oxidase. Fractions of detergent-solubilized enzyme were applied to a column (1.5 by 23 cm) of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.05 M Tris hydrochloride, pH 7.3, containing 0.25% Tween 80. Elution was with the same buffer used to equilibrate the column, and 1-ml fractions were collected by using an Isco model 1220 fraction collector (Isco, Lincoln, Nebr.). Fractions containing PGEN oxidase were pooled and applied to a Sephadex G-150 column, 1.5 by 24 cm, which was equilibrated with the 0.05 M Tris hydrochloride, pH 7.3, buffer containing 0.25% Tween 80. The enzyme was eluted with the equilibrating buffer, and 1-ml fractions were collected. Fractions containing the peak of PGEN oxidase activity were pooled and dialyzed at 4°C overnight against two changes of 0.001 M Tris hydrochloride, pH 7.3.

Polyacrylamide gel electrophoresis. Native gels of 7.5 and 12% polyacrylamide were prepared in tubes (5 by 75 mm) by the procedure of Cooper (7). Protein fractions containing PGEN oxidase were loaded on the top of gels with 0.05% bromphenol blue or methyl green as tracking dyes. Alkaline running buffer was Tris-glycine, pH 8.9, and acidic running buffer was acetic acid-glycine, pH 3.6. Electrophoresis was at a constant current of 2 mA per gel and 75 V until the tracking dye had migrated to 5 mm from the bottom of the gel. Gels were removed and placed in isopropyl-glacial acetic acid-water (40:10:50 [vol/vol/vol]) overnight and stained for 2 h in 7% acetic acid containing 0.25% Coomassie blue R-250.

Sodium dodecyl sulfate (SDS)-polyacrylamide gels were 7.5% acrylamide preelectrophoresed in Tris hydrochloride and 4% SDS adjusted to pH 8 (27). The purified PGEN oxidase was prepared for electrophoresis by mixing the enzyme fraction with an equal volume of 2× SDS running buffer containing 20% glycerol and 0.002% bromphenol blue and boiled for 3 min. Electrophoresis was at a constant voltage of 200 V until the tracking dye reached the end of the gel.

To discern the subunit structure of the PGEN oxidase, the enzyme was prepared under reducing conditions by boiling in 0.125 M Tris hydrochloride, pH 6.8, containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromphenol blue. This mixture was applied to discontinuous 12% polyacrylamide-SDS gels and electrophoresed as described above. The molecular weight markers used were prepared for electrophoresis by the above treatment; standards were myosin heavy chain, 200,000; phosphorylase *b*, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; and cytochrome *c*, 12,400 (Bethesda Research Laboratories, Gaithersburg, Md.). The molecular weight of each protein was plotted as a fraction of the distance migrated with respect to the migration of the tracking dye (37).

An assay for PGEN oxidase was run on fractions from Sephadex G-150 electrophoresed on 7.5% native polyacrylamide gels. The gel was placed in a test tube (13 by 125 mm) which contained 4 ml of assay solution, which contained 200 μmol of PGEN, 10 μmol of nitroblue tetrazolium, and 80 μmol of HEPES, pH 7.3. The gel was incubated in the reaction mixture at 30°C until the band of reduced tetrazolium was apparent, which required 10 to 15 min, and then the reaction was terminated by replacing the assay solution with distilled water.

Isoelectric focusing. The procedure for isoelectric focusing was modified from that described in the report by O'Farrell (33). The polyacrylamide gels for isoelectric focusing contained 5% acrylamide, 0.33% bisacrylamide, 48% urea, 4.3% ampholytes (pH 3.5 to 10), and 1% Nonidet P-40. The gels were polymerized in 2-mm-diameter glass tubes by using sodium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED). The gels were preelectrophoresed at constant voltage according to the following schedule: 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. Portions

TABLE 1. Solubilization of PGEN oxidase from membranes of *D. gigas*

Addition to the membranes[a]	Protein solubilized (mg)	PROTO formed (nmol)	Sp act[b]
Ionic detergent			
CHAPS	4.8	2.1	0.9
Zwittergent 3-14	3.6	1.2	0.7
Nonionic detergent			
Brij 25	4.8	2.0	0.8
Genapol X-080	4.0	2.1	0.9
Mega-10	5.6	2.5	0.9
Octyl-D-glucoside	5.6	3.1	1.1
Triton X-100	5.8	1.8	0.6
Tween 80	6.0	3.0	1.0

[a] Final concentration of each detergent was 0.25%.
[b] Specific activities are nanomoles of PROTO formed per milligram of protein per 10 min where the reaction contained 2.5 mM DCIP and 50 mM PGEN with incubation at 25°C for 10 min.

of PGEN oxidase, in dialysis buffer, were then applied to the cathode end of each gel and overlaid with 10 ml of 9 M urea containing 2% ampholytes. Electrophoresis was done for 16 h at a constant voltage of 400 V. Before the end of the electrophoresis, the voltage was increased to 800 V for 1 h. Gels were removed from tubes by rimming with a 24-gauge needle. One set of duplicate tubes was silver stained to demonstrate protein, and the other set was used to measure pH in the gel. The upper chamber buffer, cathode, was 20 mM NaOH, degassed by vacuum overnight. The lower anode chamber buffer was 20 mM phosphoric acid.

Silver staining. SDS-polyacrylamide gels were fixed for three consecutive 30-min periods in 50% methanol–10% acetic acid, 5% methanol–7% acetic acid, and 10% gluteraldehyde, respectively (31). Gels were then rinsed in several changes of distilled water for 2 h and then soaked for 30 min in dithiothreitol (5 μg/ml). The dithiothreitol was poured off, and the gels were soaked in 0.1% silver nitrate for 30 min. After silver nitrate exposure, gels were rinsed rapidly in a small volume of water and two washes of 3% sodium carbonate–0.0014% formaldehyde. The gels were then developed in 3% sodium carbonate–0.0014% formaldehyde until the desired level of staining was achieved. Development was stopped by adding citric acid to a final concentration of 0.43 M, with gentle agitation for 10 min. The gels were then soaked in distilled water for 30 min, followed by soaking in 0.03% sodium carbonate to prevent bleaching. We performed all steps using acid-washed glassware while wearing gloves, and all solutions were filtered through 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.).

Molecular weight measurements. Purified PGEN oxidase was applied to a column (1.5 by 125 cm) of Sephacryl S-200 (Pharmacia) equilibrated with 0.05 M Tris hydrochloride, pH 7.3, containing 0.25% Tween 80. Elution was with the equilibration buffer solution, and 1-ml fractions were collected, analyzed for protein, and measured for PGEN oxidase activity. The Sephacryl S-200 column was standardized with the following molecular weight standards prepared in the eluting buffer: catalase (M_r 232,000), alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), and cytochrome *c* (M_r 12,500).

Other measurements. Protein was measured by the biuret method (15) or the procedure of Lowry et al. (29) with bovine serum albumin fraction V as the standard.

RESULTS

Solubilization of PGEN oxidase. The release of PGEN oxidase from membranes of *D. gigas* was effected by ionic and nonionic detergents (Table 1). The greatest enzyme solubilization (over 85%) was observed with octyl-D-glucopyranoside and Tween 80. The reproducibility in solubilizing PGEN oxidase was high, and about 10% variation occurred with different cell membrane preparations. Enzyme activity was unaffected by conducting membrane-bound enzyme assays in the presence of detergents listed in Table 1. All subsequent experiments used Tween 80 as the solubilizing agent for PGEN oxidase.

Purification of PGEN oxidation. The steps in manipulation of PGEN oxidase which resulted in a 318-fold purification are presented in Table 2. Because PGEN oxidase is membrane bound, the separation of membranes from the soluble proteins in the cell extract produces a twofold purification. The solubilized enzyme was passed over a Sephadex G-50 column, and the enzyme was eluted in the void volume. Subsequent passage over a Sephadex G-150 column resulted in a fraction containing the purified enzyme. The fraction containing the detergent treatment of the membrane produced considerable variability in PGEN oxidase activity. In this detergent extract, the purification yield from one membrane fraction to the next would range from 2 to 40. As seen in Table 2 and in other purification attempts, a marked increase in enzyme yield occurred with the fraction from Sephadex G-50. We have not investigated the cause of this apparent increase of enzyme activity but suggest that the detergent solubilizes something from the membrane which interferes with the PGEN oxidase assay.

Evaluation of enzyme purity was by polyacrylamide gel electrophoresis. A single protein band was apparent in tubes of 7.5 and 12% polyacrylamide gel by using running buffers at both pHs 8.9 and 3.6. The assay for PGEN oxidase in the 7.5% polyacrylamide gel revealed enzyme activity at the same region where the single protein band occurred (Fig. 1).

Molecular weight calculation and gel characteristics. From the Sephacryl S-200 chromatography, the molecular weight of PGEN oxidase was calculated to be 148,000 (Fig. 2). This enzyme was dissociated with mercaptoethanol to produce three distinct proteins separated by SDS-polyacrylamide electrophoresis with M_rs of 12,000, 18,500, and 57,000 (Fig. 3A). Shown in Fig. 3B is an SDS-polyacrylamide gel with a single protein band when the enzyme was not reduced with mercaptoethanol. A single protein band was seen in the isoelectric focusing gel, and migration of the PGEN oxidase

TABLE 2. Summary of the purification of PGEN oxidase from *D. gigas*

Procedure	Vol (ml)	U/ml[a]	Total U	Protein (mg/ml)	Sp act	Yield (%)	Purification
Dialyzed extract	3.2	76	246	24.75	3	100	
Membrane fraction	3.0	34	102	6.00	6	41	2
Detergent extract	3.3	12	40	1.60	8	16	2.6
Sephadex G-50	1.2	313	376	0.90	348	153	116
Sephadex G-150	1.5	171	257	0.18	950	104	318

[a] One unit of activity is a nanomole of PROTO formed per milligram of protein per 10 min. Specific activity is defined as units of PGEN oxidase activity per milligram of protein per 10 min of incubation.

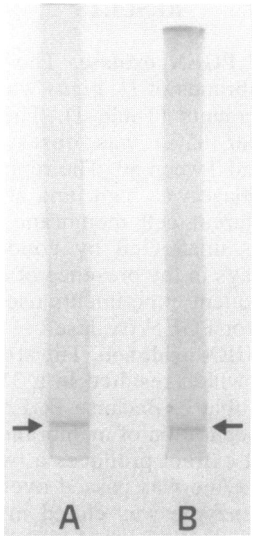

FIG. 1. Polyacrylamide gel electrophoresis of PGEN oxidase. Twenty micrograms of enzyme purified from *D. gigas* was added to each 7.5% polyacrylamide gel. Running buffer was Tris-glycine, pH 8.9. Gel A was stained with Coomassie blue; gel B was incubated in a buffer solution containing PGEN and nitroblue tetrazolium.

indicated an isoelectric point of pH 5.71. The presence of single protein bands in both the isoelectric focusing gel and nonreducing SDS-acrylamide gel provides further evidence of the purity of the PGEN oxidase.

Purified PGEN oxidase readily reduces DCIP and slowly reduces methylviologen but does not reduce NAD^+, $NADP^+$, flavin adenine dinucleotide, or flavin mononucleotide. Sulfate-reducing bacteria have many dehydrogenases which are non-pyridine nucleotide linked (28, 32), and it appears that this is another such enzyme. In a reaction in which 0.41 μmol of PROTO was enzymatically produced for every milligram of protein in a 10-min reaction with DCIP, only 0.06 μmol was produced if DCIP was deleted and incubation was aerobic. PROTO produced in aerobic reactions was comparable to the 0.008 to 0.01 μmol produced in reactions with heat-denatured enzyme or aerobic reactions run without enzyme.

Characteristics of purified enzyme. Activity of PGEN oxidase with DCIP was examined during a 50-min incubation period, and the rate of enzyme activity was linear from 0 to 12 min (Fig. 4). Data in Fig. 4 indicate reduction of DCIP by PGEN oxidase. Corrections for decolorization of DCIP were made by using control reactions which contained all reagents but were without enzyme. The stoichiometry of the reaction of 10 min was calculated to be 5.95 nmol of DCIP reduced for every nanomole of PGEN oxidized. This figure is the only expression of enzyme activity in the report which is made on the basis of DCIP reduction. All other enzyme activities resulted from direct measurement of PROTO produced.

An evaluation of enzyme parameters revealed maximum production of PROTO over a range of pH 7 to 11 (Fig. 5). Identical results were obtained with Tris hydrochloride and HEPES buffers. Controls for these reactions included incubation of DCIP and PGEN at various pH levels to correct for instability of electron donor and acceptor.

The activity of the purified enzyme was markedly influenced by temperature, with optimal activity at 23°C (Fig. 6). A transformation of data in Fig. 6 to an Arrhenius plot (Fig. 7) showed an apparent change in enzyme activity at 10°C with linear activities above and below 10°C. Energies of

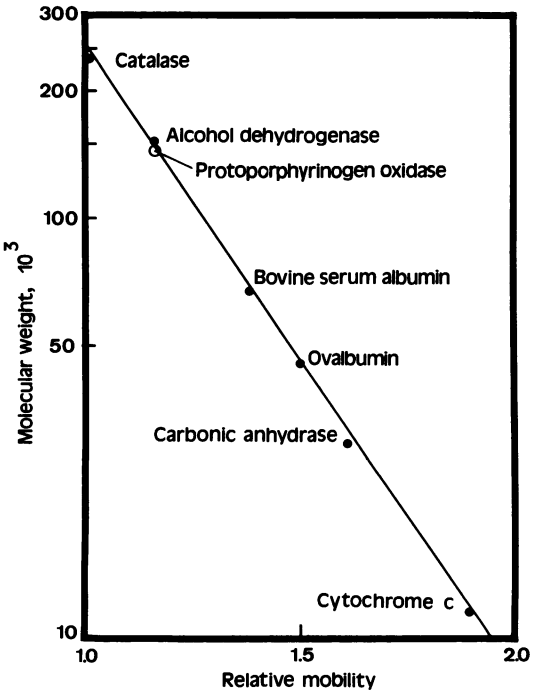

FIG. 2. Determination of molecular weight of PGEN oxidase from *D. gigas* by gel filtration. Standard proteins were as follows: cytochrome *c* (M_r 12,500), carbonic anhydrase (M_r 29,000), ovalbumin (M_r 45,000), bovine serum albumin (M_r 67,000), alcohol dehydrogenase (M_r 150,000), and catalase (M_r 232,000).

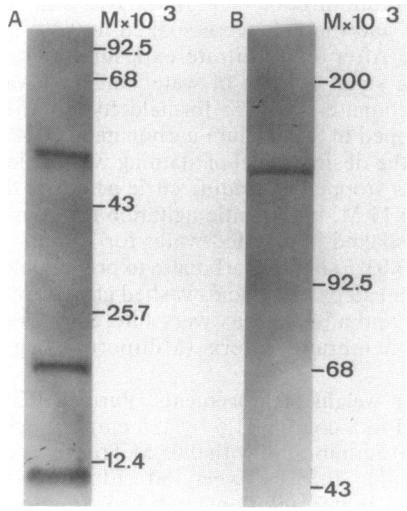

FIG. 3. SDS-polyacrylamide gel electrophoresis of PGEN oxidase. (A) Purified PGEN oxidase was dissociated into subunits with SDS and 2-mercaptoethanol before electrophoresis in discontinuous 12% polyacrylamide-SDS gels with a running buffer of Tris hydrochloride and 4% SDS adjusted to pH 8.0. (B) PGEN oxidase was heated in a 4% SDS solution before application was made to the 7.5% polyacrylamide-SDS gel. Running buffer contained 4% SDS and Tris hydrochloride, pH 8.0. Each gel was loaded with 5 μg of purified PGEN oxidase, and visualization of proteins was by silver staining technique. Positions for migration of marker proteins are indicated along the gels. M, Molecular weight.

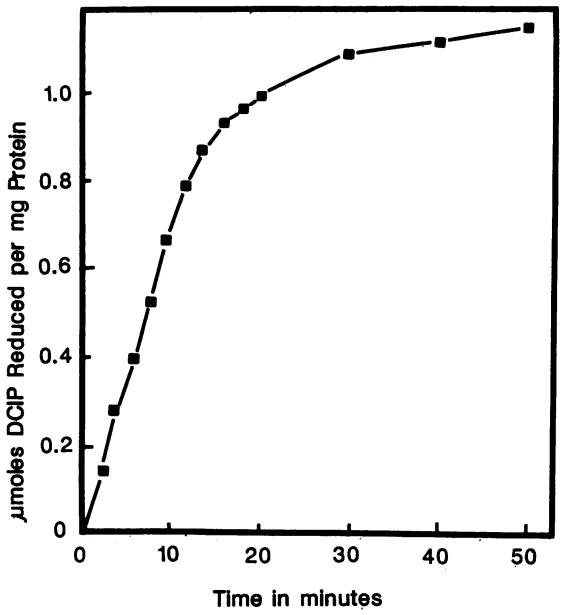

FIG. 4. Reduction of DCIP by PGEN oxidase. The reaction contained 0.4 mg of protein, 60 mM PGEN, and 2.5 mM DCIP. The time of assay was as indicated in the figure.

activation were calculated to be 7,429 cal (ca. 31,080 J)/mol per degree below 10°C and 1,455 cal (ca. 6,088 J)/mol per degree above 10°C.

Sulfhydryl-blocking agents had a marked effect on PGEN oxidase activity. The presence of iodoacetamide and *N*-ethylmaleimide at 0.5 mM greatly reduced the rate of protoporphyrin formation, and at 5.0 mM levels even greater levels of inhibition were observed (Table 3). In control reactions not containing PGEN oxidase, iodoacetamide and *N*-ethylmaleimide at 0.5 mM did not modify the spectral properties of PGEN. *p*-Hydroxymercuribenzoate at 0.5 mM reacted with PGEN, as determined from spectral evaluations of controls, making it impossible to evaluate the effect of *p*-hydroxymercuribenzoate on PGEN oxidase.

Substrate saturation occurs with PGEN oxidase and kinetic analysis by an Eadie plot (Fig. 8) enabled us to calculate an apparent K_m of 21 μM PGEN with a V_{max} of 8.38

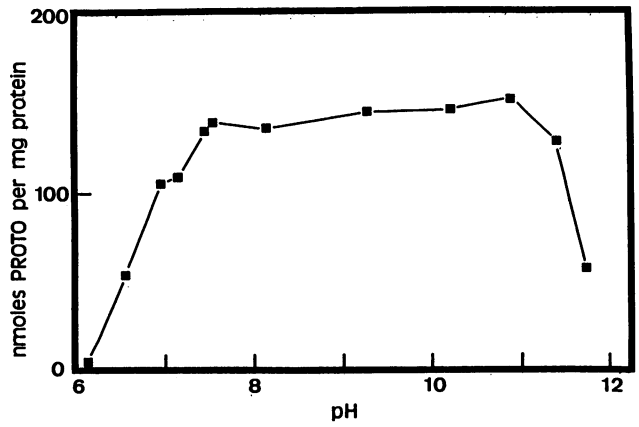

FIG. 5. Activity of PGEN oxidase influenced by pH of assay. Each reaction contained 0.6 mg of protein, 60 mM PGEN, and 2.5 mM DCIP. The time of assay was 10 min.

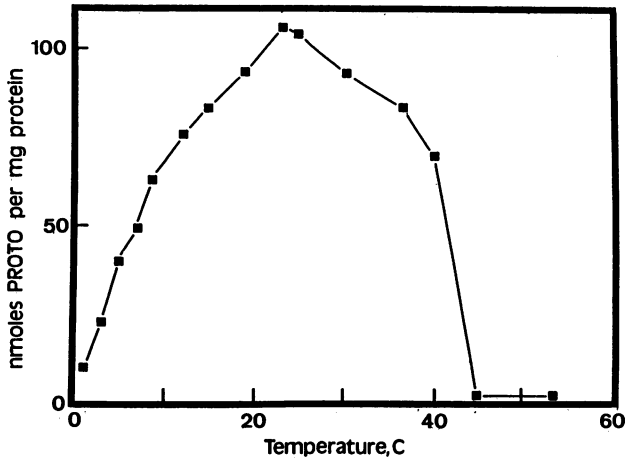

FIG. 6. Activity of PGEN oxidase influenced by temperature of incubation. Points reflect activity with 0.6 mg of enzyme protein, 70 mM PGEN, and 2.5 mM DCIP with the reaction incubated at pH 7.3 for 10 min.

nmol/min per 70 μg of protein. Calculation of enzyme activities from Lineweaver-Burk plots (not shown) produced an apparent K_m of 21 μM PGEN with a V_{max} of 8.41 nmol/min per 70 μg of protein. By using 148,000 as the molecular weight (M_r) for PGEN oxidase and V_{max} from the Eadie plot, the turnover number or K_{cat} was calculated to be 17.7 mol of PROTO formed per mol of enzyme per min of incubation. The K_{cat}/K_m for the PGEN oxidase was determined to be 0.84.

DISCUSSION

PGEN oxidase has been solubilized from the plasma membrane of *D. gigas* by a variety of detergents. Small nonionic detergents were the most effective; however, their efficiency was only twice that of the least effective agent. The high efficiency of octyl-D-glycoside and Mega-10 is most

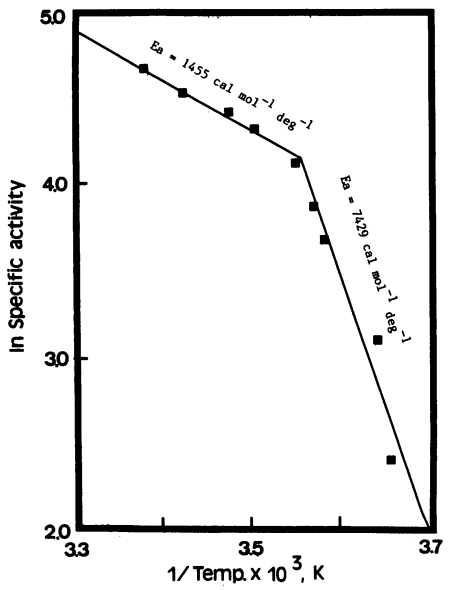

FIG. 7. Arrhenius plot of the data from Fig. 6. Specific activity refers to nanomoles of PROTO produced per milligram of PGEN oxidase protein in 10 min. All points below 23°C are plotted. Ea, Activation energy.

TABLE 3. Effect of sulfhydryl-blocking agents on purified PGEN oxidase

Addition to the reaction[a]	Inhibitor (mM)	Sp act[b]	% Inhibition
None		110	
Iodoacetamide	0.5	64	42
	5.0	47	57
N-Ethylmalemide	0.5	27	76
	5.0	19	83

[a] The reactions contained 2.5 mM DCIP, 70 g of purified enzyme, and 60 mM PGEN. Reactions were run at pH 7.3 and 25°C for 10 min.

[b] Specific activity is defined in Table 1, footnote *b*. Results presented are mean values of three replicates for each inhibitor concentration.

certainly due to their stable, nonionic nature. The remaining nonionic detergents, primarily ethylene oxide adducts, may undergo peroxidation, leading to the formation of ketones and carboxylic acid residues (1). These carbonyl compounds and other decomposition products may interact with proteins, as do the amphoteric detergents, without producing full denaturation (14, 17). The solubilized PGEN oxidase was relatively stable to a variety of laboratory activities which included aerobic column chromatography, dialysis, and electrophoresis. Whereas anaerobic conditions were required to prevent substrate oxidation, it was apparent that the PGEN oxidase was not affected by exposure to oxygen.

A distinct optimum temperature was displayed at 23°C for the enzyme from *D. gigas*, whereas a broad temperature range from 4 to 30°C was reported for the yeast PGEN oxidase. The two activation energies observed determined from the Arrhenius plot observed with PGEN oxidase from *D. gigas* are interesting and may be explained in several ways. If the K_m of the reaction does not change with temperature, then the rate parameter plotted in Fig. 7 directly reflects K_{cat}, since the enzyme was always saturated with excess PGEN. The change in Eadie coordinates is then indicative of a multistep catalytic process, the steps having different Q_{10}s (9). Since K_m's were not determined for each temperature, the two activation energies may simply reflect different Q_{10}s of binding and catalysis. However, the most basic situation suggests that enzyme denaturation is insignificant below 10°C but is manifest above 10°C as a dramatically lower activation energy. Lipid-protein interactions would be important for this membrane-associated enzyme; however, the influence of lipids on PGEN oxidase activity has not been investigated.

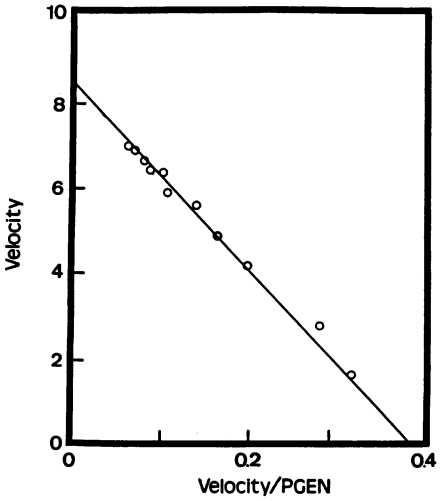

FIG. 8. Eadie plot of initial velocity data for PGEN oxidase at variable levels of PGEN. Points reflect activity with 2.5 mM DCIP and 70 μg of purified enzyme protein with incubation for 1 min. Units of velocity are nanomoles of PROTO formed per milligram of protein per minute.

The PGEN oxidase from *D. gigas* is inhibited by *N*-ethylmaleimide and iodoacetamide, which is important since Granick and Mauzerall (16) have suggested that thiol-containing amino acid residues of the PGEN oxidase mediate substrate binding by interacting with the vinyl groups of PGEN. A comparison of the kinetic parameters of the *D. gigas* and yeast enzymes (36) indicates that the yeast enzyme is more efficient. The yeast enzyme has a K_m of 4.8 and a V_{max} of 25 nmol of PGEN oxidized per min per mg of protein, whereas the enzyme from *D. gigas* has a K_m of 21 and a V_{max} of 8.5 nmol of PGEN oxidized per min per mg of protein.

The M_r 148,000 PGEN oxidase from *D. gigas* was found to be composed of three proteins of M_rs 12,000, 18,500, and 57,000. Mercaptoethanol dissociation of the enzyme suggests that the proteins are held together by disulfide bridges. The most straightforward combination for the assembly of the protein subunits would be a hexamer containing two of each protein subunit of an M_r 12,000, an M_r 18,500, and an M_r 57,000. By close-fit analysis, the enzyme may reveal the M_r 148,000 size and not the M_r 176,000, which is the sum of all individual subunits.

The ease of purification and the stability of the PGEN oxidase from *D. gigas* would make this enzyme useful for further research on late steps of heme synthesis in bacteria. With respect to the *Desulfovibrio* spp., it will be important to establish the electron carrier for the PGEN oxidase and the composition of the electron carriers in the plasma membranes.

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