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Carbon Monoxide-Reacting Pigment from *Desulfotomaculum nigrificans* and Its Possible Relevance to Sulfite Reduction

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The separation of an autoxidizable brown pigment, P582, from *Desulfotomaculum nigrificans* is described. It reacted with $Na_2S_2O_4$ and was characterized by absorption maxima in the oxidized state at 392, 582, and 700 nm. In the presence of $Na_2S_2O_4$, P582 formed complexes with CO and, under alkaline conditions, pyridine. There was no reaction with cyanide. The molecular weight of P582 was approximately 145,000, and the purest preparations contained Fe, Zn, and acid-labile sulfide but not Cu, Mo, or Mn. Preparations of P582 catalyzed the reduced methyl viologen (MVH)-linked reduction of sulfite, hydroxylamine, and nitrite but not of sulfate, thiosulfate, or nitrate. Reduced pyridine nucleotides did not substitute for MVH. A major product of the MVH-sulfite reaction was sulfide. CO partially inhibited the enzymatic activities. Sulfite, hydroxylamine, and nitrite and CO caused changes in the spectrum of $Na_2S_2O_4$-reduced P582. Fe^{2+}-chelating reagents reacted with part of the Fe of P582 and caused partial losses of labile sulfide and enzymatic activity. The spectral and CO-reacting properties of P582 were, however, unaffected by chelating agents. The reaction between P582 and chelating agents was stimulated by reducing agents.

Desulfotomaculum nigrificans (9) is a thermophilic, sporeforming, anaerobic bacterium which obtains energy by coupling the oxidation of hydrogen and some organic compounds to the reduction of sulfate to sulfide. Organisms with this type of metabolism (the genera *Desulfotomaculum* and *Desulfovibrio*, reference 38) have been described as dissimilatory sulfate reducers to distinguish them from assimilatory sulfate reducers which transform only small amounts of sulfate for the synthesis of sulfur-containing cell constituents (36). An intermediate in both types of sulfate reduction appears to be at the oxidation level of sulfite which, in the case of dissimilatory organisms, arises from adenosine-5′-phosphosulfate (APS) through the action of APS-reductase (34).

In assimilatory organisms, the subsequent reduction of sulfite is catalyzed by sulfite reductase (hydrogen sulfide:NADP oxidoreductase, EC 1.8.1.2.) and several homogeneous preparations of this enzyme have been described (reference 42 and this paper). Sulfite metabolism by extracts of *Desulfovibrio* sp. has been studied by a number of workers (12, 17, 24, 40, and earlier references cited in 42). R. Haschke and L. L. Campbell (Bacteriol. Proc. p. 118, 1967; Fed. Proc. **27**: 390, 1968) reported that sulfite reductase from *Desulfovibrio vulgaris* is a low-molecular-weight, colorless protein containing a group with properties of a nucleotide. It reduces sulfite to sulfide in the presence of H_2, hydrogenase, and methyl viologen (MVH). Few studies have been made on sulfite reduction by *Desulfotomaculum* sp. The formation of dithionite, thiosulfate, and sulfide has been reported (B. Suh, W. Nakatsuka, and J. M. Akagi, Bacteriol. Proc. p. 133, 1968), and a thiosulfate reductase (33) has been demonstrated. It has been suggested (2) that a ferredoxin-like material is involved in sulfite reduction by *D. nigrificans*.

This paper describes the isolation of a CO-reacting pigment from *D. nigrificans* which may be related to sulfite reductase in this organism.

MATERIALS AND METHODS

Organism and growth conditions. *D. nigrificans* NTCC 8351 was kindly supplied by L. L. Campbell. It was grown in 20-liter carboys which were filled to the neck with the peptone-yeast extract—glucose-salts medium described by Postgate (35). Each carboy was inoculated with 1 liter of a 24-hr culture and incubated at 55 C for 18 to 24 hr. Yields of cells of approximately 150 mg (dry weight) per liter were obtained. Checks for contaminants were made by microscopic examination and by plating samples of the cultures on the same medium supplemented with $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$ (10 mg per liter) and 2% agar; aerobic and anaerobic incubations at 55 C were made. The bacteria were harvested and washed twice with 0.1 M potassium phosphate (*p*H 6.8); if not used immediately, they were stored at −20 C.

Preparation of extracts. The bacteria were suspended in 0.1 M potassium phosphate (*p*H 6.8) and passed twice through a French pressure cell at 20,000 psi. The extracts were centrifuged at 10,000 × *g* for 30 min, and the supernatant fluid was then centrifuged for 1 hr at 198,000 × *g*, in a Spinco model L2-50 ultracentrifuge. The precipitate (198,000 × *g* particles) was washed once with 0.1 M potassium phosphate (*p*H 6.8) and the supernatant (198,000 × *g* supernatant) was dialyzed against the same buffer.

Preparation of diethylaminoethyl (DEAE) cellulose. DEAE cellulose (Whatman DE11) was washed according to the precycling procedure described in the Whatman Technical Bulletin IE2.

Electrofocussing. Electrofocussing apparatus (model 8102, LKB-Produkter AB, Sweden) and ampholytes were used as recommended by the manufacturers.

Electrophoresis. Thin-layer electrophoresis was carried out at 5 C on Sepraphore III (Gelman Instrument Co.) by using the Gelman Deluxe Electrophoresis Chamber and 0.05 M tris(hydroxymethyl) aminomethane (Tris)-barbital buffer (Gelman High Resolution Buffer) at *p*H 8.8. A potential of 300 v (approximately 2 ma per 1-inch strip) was applied for 60 min. The Gradipore system supplied by Townson & Mercer (Dist.), Pty. Ltd., Australia, was used for electrophoresis in polyacrylamide gradients (29). A potential of 90 v was applied for 16 hr in the following buffer (23): Tris, 10.75 g; ethylenediaminetetraacetic acid (EDTA), 0.93 g; boric acid, 5.04 g; water to 1 liter.

Proteins were detected by staining the Sepraphore strips and polyacrylamide gels with Ponceau S in 5% trichloroacetic acid followed by destaining with 5% acetic acid. Polyacrylamide gels were destained electrolytically.

Molecular weight determinations. Molecular weights were determined by gel filtration on a calibrated column (39 by 2.5 cm) of Sephadex G200 (44). The eluant was 0.5 M KCl in 0.01 M Tris-hydrochloride (*p*H 8.04); 5-ml fractions were collected at a rate of 0.25 ml per min.

Reduced MVH-linked reductase activities. Sulfite, nitrite, and hydroxylamine reductase activities were determined by measuring the oxidation of the electron donor, MVH, under O_2-free N_2 in 1-cm path length absorption cells equipped with stopcocks. The apparatus and the methods used for generating MVH and purifying N_2 were previously described (Trudinger, Biochem. Anal., *in press*). The enzyme, 200 μmoles of potassium phosphate (*p*H 6.8), and 1 μmole of EDTA in a final volume of 2.7 ml were gassed with N_2 for 5 min. Aqueous MVH (0.4 to 0.6 ml at a concentration of 3.9 mM) was added to give a final absorbancy of 2.5 to 3.5 at 600 nm.

The absorption cells were immersed in a water bath at 50 C for 5 min and then placed in a thermostatted cell holder (at 50 C) in a Cary spectrophotometer. Neutral density filters were placed in the reference beam to compensate for the high absorbancy of the samples. Substrates (20 to 50 μliters) were injected through the stopcock after 5 to 10 min by which time temperature equilibration was reached. Oxidation of MVH was measured by the decrease in absorbancy at 600 nm. A molar absorption coefficient of 1.18×10^4 per cm (Trudinger, Anal. Biochem., *in press*) was used to determine the concentration of MVH.

Enzymatic formation of sulfide. Sulfide formed in enzymatic experiments was determined by a modification of the procedure of Ellis (10) which depends on the measurement of the red color produced by the adduct of sulfide and *N*-ethylmaleimide in alkaline solution. The reaction mixtures were brought to 30 C and 0.5 ml of 0.5 M *N*-ethylmaleimide in 50% ethanol was added. After 10 min at 30 C, 2 ml of 2 N Na_2CO_3 was added and the mixture was then incubated for a further 10 min at 30 C; the absorbancy at 520 nm was determined. Standard curves were constructed by adding known amounts of sulfide to the complete incubation mixture. Sulfide solutions were standardized by adding samples to 10 ml of 0.1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 0.1 M Tris-hydrochloride (*p*H 8); one molecule of sulfide and one molecule of DTNB react to give two molecules of thionitrobenzoate (25) which has a molar absorption coefficient at 412 nm of 13,600 per cm (11).

Acid-labile sulfide. The material (1 to 2 ml) to be analyzed was acidified with 5 ml of 5 N H_2SO_4 under a stream of O_2-free N_2 which was then passed through a gas-absorption tube containing 10 ml of 0.1 mM DTNB. After 10 min, the absorbancy of the DTNB solution at 412 nm was determined. Recoveries of 92 to 98% were obtained with standard solutions of Na_2S.

Formation of methylene blue from sulfide. The reaction of sulfide with *p*-aminodimethylaniline and Fe^{3+} to give methylene blue was carried out essentially by the method of Yoshimoto and Sato (46).

Assay of ferredoxin-like activity. Ferredoxin-like activity was assayed by stimulation of the pyruvate phosphoroclastic reaction catalyzed by "ferredoxin-free" extracts of *Clostridium butyricum* (isolated and kindly supplied by A. Rovira, C.S.I.R.O., Division of Soils, Adelaide, Australia). The organisms were grown overnight at 35 C in the medium of Hirsch and Grinsted (16) supplemented with 0.01% $FeCl_3 \cdot 6H_2O$. Extracts were prepared with the French pressure cell and were fractionated with 40% (w/v) 2-propanol

(31). The precipitate (ferredoxin-free extract) was used as the source of phosphoroclastic enzymes. Ferredoxin in the supernatant was partly purified by chromatography on DEAE cellulose by the method of Mortenson, Valentine, and Carnahan (30), except that the resin was washed exhaustively with 0.2 M potassium phosphate (*p*H 6.5) before the elution of ferredoxin. The phosphoroclastic reaction was assayed by the method of Mortenson et al. (31).

Pseudoperoxidase assay. Qualitative tests for pseudoperoxidase activity were made by observing the appearance of a blue color when the material to be assayed was added to a mixture of 5 ml of 0.1 M potassium acetate (*p*H 5), 0.5 ml of saturated aqueous benzidine, and 10 μliters of 30% H_2O_2.

Assay of metals. The material to be analyzed was digested with concentrated H_2SO_4 and 30% H_2O_2 as described by Yoshimoto and Sato (45). Fe was estimated with 1,10-phenanthroline (7) after neutralization of the sample with NH_4OH. Mo was determined by the thiocyanate method after extraction with carbon-tetrachloride and amyl alcohol (19). Zn, Cu, and Mn were assayed with the Unicam SP90 or Techtron AA-4 atomic absorption spectrophotometers.

Flavine analyses. The materials were boiled for 10 min in water to release flavines which were determined with the recording spectrofluorimeter described by Boardman and Thorne (8): 0.01 μM flavine mononucleotide could be detected by the method.

Spectrophotometry. Spectra were recorded with a Cary model 14 spectrophotometer equipped with 0 to 1, 1 to 2, and 0 to 0.1, 0.1 to 0.2 absorbancy slide wires. The Unicam SP500 series 2 spectrophotometer with program controller and thermostatted cell holder was used for studying the reactions of chelating agents and most other spectrophotometric assays. A 1-cm optical path was used throughout.

Protein estimation. The biuret method (26) was used with bovine serum albumin as the standard.

Ultrafiltration. The ultrafiltration apparatus and membranes were obtained from the Amicon Corp., Lexington, Mass.

Other materials. These were obtained from the following sources: crystalline bovine serum albumin, Sigma Chemical Co.; human gamma globulin, ovalbumin, myoglobin, mammalian cytochrome *c*, DTNB, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), Biogel P200, dithiothreitol, Calbiochem; MHV, 2,2'-dipyridyl, 1,10-phenanthroline, British Drug Houses; bathophenanthroline (4,7-diphenyl-1,10-phenanthroline), bathocuproin (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), Merck & Co., Inc.; Sephadex G200, DEAE Sephadex A25, Ficoll, Pharmacia; CO, Matheson; 2-thenoyltrifluoroacetone, Eastman Chemical Co. N-methylacethydroxamic acid was a gift from J. N. Phillips.

Solutions of Na_2SO_3 (A.R., By Products and Chemicals, Pty. Ltd., Australia) were standardized by titration with iodine.

RESULTS

Spectral properties of the 198,000 × g supernatant fraction. The absolute spectrum (Fig. 1) of the 198,000 × *g* supernatant fraction was characterized by absorption maxima at 581 and 383 nm. The difference spectrum, reduced ($Na_2S_2O_4$) minus oxidized (Fig. 2, bottom curve), showed peaks at 621 and 557 nm and a trough at 581 nm as well as a marked trough at 450 nm which was due, in large measure, to the presence of flavoprotein. The presence of a CO-reacting pigment was shown by the difference spectrum, reduced + CO minus reduced (Fig. 2, top curve). The pigment responsible for the CO reaction and for the absorption at 581 nm is referred to subsequently as P582 on the basis of results obtained with purified material (*see below*).

Separation of P582: step 1, $(NH_4)_2SO_4$ fractionation. Operations for each of the four steps were carried out at 2 to 5 C. The dark brown supernatant liquid (198,000 × *g*) from 4.4 g (dry weight) of bacteria (2.7 g of protein) was made 1 mM with respect to EDTA and brought to 40% saturation with $(NH_4)_2SO_4$. The precipitate, which contained much black metal sulfide, was removed by centrifuging. The supernatant was then brought to 90% saturation with $(NH_4)_2SO_4$. The precipitate, after centrifugation, was dissolved in a small volume of 0.01 M Tris-hydrochloride (*p*H 8.04) containing 0.1 M KCl and 1 mM EDTA and dialyzed overnight against 100 volumes of the same buffer.

Step 2, chromatography on DEAE cellulose. After dialysis, the material precipitated between 40 and 90% saturation $(NH_4)_2SO_4$ (670 mg of protein) was clarified by centrifugation and applied to a column of DE11 (43 by 3.8 cm) preequilibrated with 0.1 M KCl-1 mM EDTA-0.01 M Tris-hydrochloride, *p*H 8.04. The column was washed sequentially with 1 liter each of 0.1, 0.2 and 0.5 M KCl in 0.01 M Tris-hydrochloride (*p*H 8.04) containing 1 mM EDTA; 13-ml fractions were collected. Each of the two weaker eluates contained a flavoprotein. A third flavoprotein

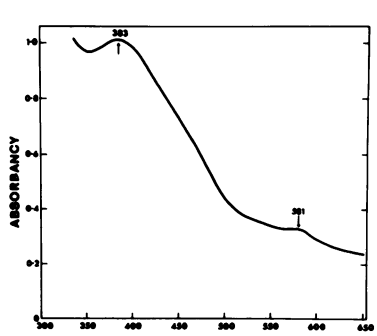

FIG. 1. *Absolute spectrum of 198,000 × g supernatant fraction of D. nigrificans extract (4.2 mg of protein per ml). The numbers are the absorption maxima in nanometers.*

was eluted by 0.5 M KCl just ahead of a greenish brown pigment. The tubes containing the latter material were combined (DE11 fraction). The ratio of absorbancy at 280 to 392 nm of this fraction was 6.3:1 and the yield was 390 mg of protein.

Step 3, first gel filtration. The DE11 fraction from step 2 was concentrated to 30 ml by dialysis against 40% Ficoll in 0.01 M Tris-hydrochloride (*p*H 8.04) containing 0.5 mM KCl, and 2-ml samples were applied to a column of Biogel P200 (37 by 2.5 cm). The column was eluted with 0.5 M KCl in 0.01 Tris-hydrochloride (*p*H 8.04), and 5-ml fractions were collected at a rate of about 0.5 ml per min. An example of the elution pattern is shown in Fig. 3. The tubes which had an absorbancy ratio, 280 to 392 nm, of 3 to 3.3 were combined (Biogel fraction 1); the overall yield was 65 mg of protein.

Step 4, second gel filtration. Portions of Biogel fraction 1 were concentrated to a small volume by ultrafiltration and again fractionated on Biogel P200 as in step 3. A single broad protein peak was eluted. The middle tubes (Biogel fraction 2) had an absorbancy ratio, 280 nm to 392 nm, of 2.74 and contained about 50% of the protein applied to the column.

Essentially similar results were obtained when Sephadex G200 was substituted for Biogel P200 in steps 3 and 4.

Spectral properties of P582. The absolute spectrum of the Biogel fraction 2 showed pronounced absorption maxima at 582, 392, and 280

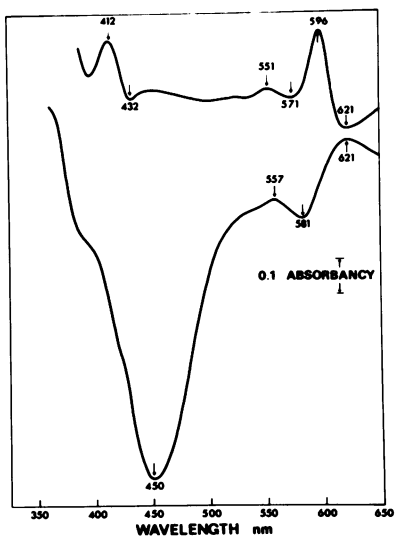

FIG. 2. *Difference spectra of 198,000 × g supernatant fraction from D. nigrificans (21 mg of protein per ml). Top curve, reduced ($Na_2S_2O_4$) + CO minus reduced. Bottom curve, reduced minus oxidized.*

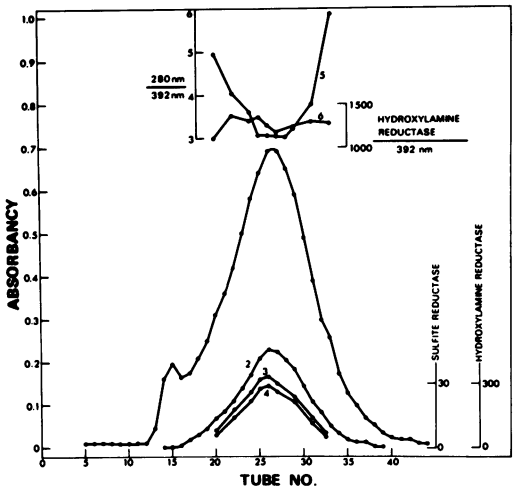

FIG. 3. *Elution of P582 and reductases from Biogel P200. DE11 fraction (13 mg) was eluted as described in the text. Curve 1, absorbancy at 280 nm; curve 2, absorbancy at 392 nm; curve 3, sulfite reductase, nanomoles of MVH oxidized per minute per 0.5 ml; curve 4, hydroxylamine reductase, nanomoles of MVH oxidized per min per 0.1 ml; curve 5, ratio of absorbancy at 280 nm to that at 392 nm; curve 6, ratio of hydroxylamine reductase activity to absorbancy at 392 nm.*

nm and a weaker maximum at 700 nm (Fig. 4, curve 1). On the addition of $Na_2S_2O_4$, the absorption maxima at 582 and 700 nm were considerably weakened and a new maximum at 610 nm appeared (Fig. 4, curve 2). About 10 min at room temperature was required for the spectrum to reach stability after the addition of $Na_2S_2O_4$ and the spectrum suggested that, even after this time, the absorption peak at 582 nm was not completely abolished (Fig. 4, insert). The original oxidized spectrum was regenerated by flushing the reduced pigment with 100% O_2 or by adding ferricyanide. The spectrum of the oxidized pigment was unaffected under anaerobic conditions by ascorbic acid, NADH, NADPH, sodium formaldehyde sulfoxylate, or dithiothreitol. A slight reaction (less than 10% of the spectral change caused by $Na_2S_2O_4$) was obtained with $NaBH_4$ under H_2.

Purity of Biogel fraction 2. The ratio of absorption at 582:392:280 nm of the Biogel fraction 2 was 0.27:1:2.74. This ratio was not altered by further chromatography of P582 on Sephadex G200, DEAE cellulose, and DEAE Sephadex A25, under a variety of conditions. Nevertheless, two colorless contaminating proteins separated from P582 during the early stages of electrofocussing. The three proteins migrated toward the anode when Biogel fraction 2 was applied at the midpoint of a *p*H 3 to 10 gradient. From the relative absorbancies at 280 nm, it was calculated

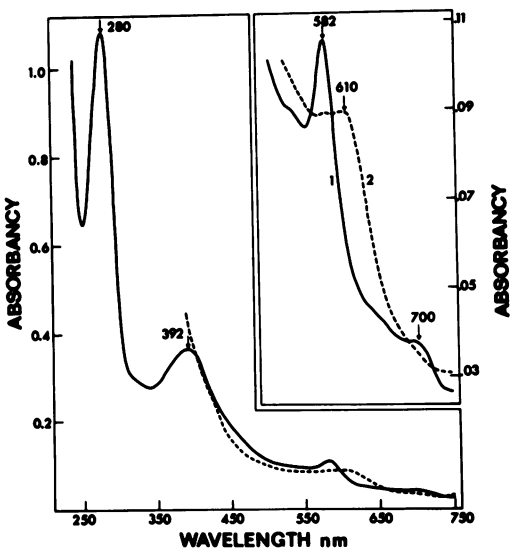

FIG. 4. *Absolute spectra of P582 (Biogel fraction 2, 1.3 mg of protein per ml in 0.01 M Tris-hydrochloride-0.5 M KCl, pH 8). Curve 1, oxidized; curve 2, reduced with $Na_2S_2O_4$. Insert, spectra from 500 to 750 nm in greater detail.*

that the contaminants represented 20 to 30% of the total protein. Considerable precipitation of P582 occurred at *p*H values below 6 and at low ionic strengths. This precluded the use of electrofocussing as a purification procedure.

One major and two minor protein components were also detected by electrophoresis of Biogel fraction 2 on Sepraphore III and polyacrylamide.

Molecular weight determination. The molecular weight of P582 was estimated to be approximately 145,000 (Fig. 5). The molecular weights of the reference proteins were those listed by Andrews (3): human globulin, 160,000; bovine serum albumin, 67,000; ovalbumin, 45,000; myoglobin, 17,800; cytochrome *c*, 12,400.

Components of P582. The purest preparation of P582 (Biogel fraction 2; ratio of 280 to 392 nm, 2.74) contained 54 μatoms of Fe, 7 μatoms of Zn, and 15 μmoles of acid-labile sulfide per g of protein. The amounts of Cu, Mn, Mo, and extractable flavine were less than 3, 1, and 0.5 μatoms and 0.02 μmole per g of protein, respectively.

Effect of CO on P582. CO had no effect on the spectrum of oxidized P582 but reacted with the pigment in the presence of $Na_2S_2O_4$ to give a complex with absorption maxima at 596 and 550 to 552 nm and a shoulder at 406 nm (Fig. 6, bottom trace). The peaks and troughs of the difference spectrum, $Na_2S_2O_4$ + CO minus $Na_2S_2O_4$ (Fig. 6, top trace) were similar to those of the corresponding difference spectrum of the crude 198,000 × *g* supernatant (*compare* Fig. 2, top curve). The troughs at 427 nm and 615 to 620 nm in the top trace of Fig. 6 were close to

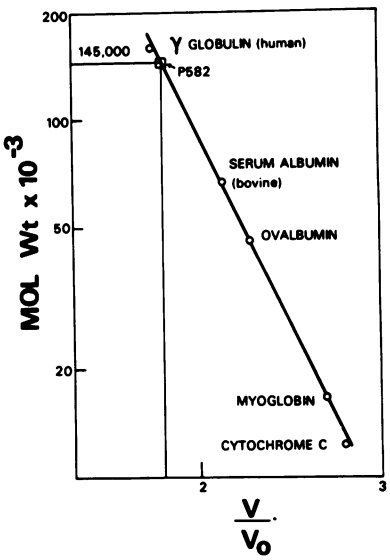

FIG. 5. *Molecular weight of P582 determined by gel filtration. V is the elution volume of the applied material; the void volume (V_0) was determined by using Blue Dextran 2000 (Pharmacia). Molecular weights of the reference proteins are given in the text.*

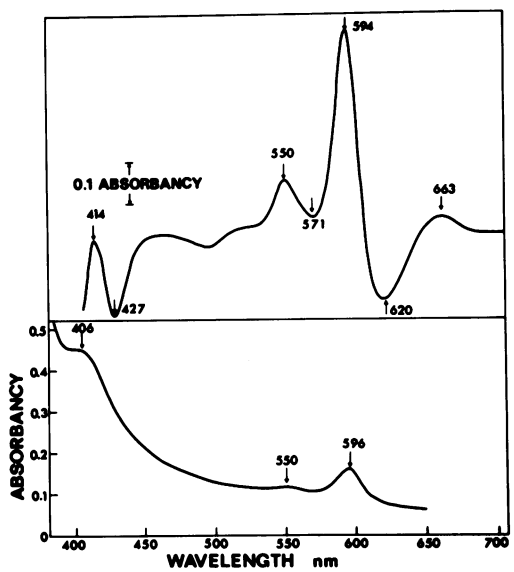

FIG. 6. *Spectra of the reduced CO-complex of P582 (Biogel fraction 2). Curves: top, difference spectrum reduced ($Na_2S_2O_4$) + CO minus reduced, 1.3 mg of protein per ml; bottom, absolute spectrum, 1.4 mg of protein per ml.*

in the oxidized minus reduced ($Na_2S_2O_4$) difference spectrum of P582 (Fig. 10, curve 4). The intensity of the 594 minus 620 nm absorption in the CO-difference spectrum relative to the 582 minus 650 nm absorption in the absolute spectrum was 1.07 for the Biogel fraction 2 compared with values of 0.9 to 1.1 for crude extracts. These results indicate that the CO reaction was a property of P582. No CO-complex was formed by oxidized P582 in the presence of dithiothreitol.

Effect of alkali, pyridine, cyanide, and sulfide on P582. The effects of alkali and pyridine on the spectrum of P582 are shown in Fig. 7. In 0.04 N NaOH, the spectrum was almost featureless except for minor absorption maxima in the regions of 405, 540, and 575 nm, and it was unaffected by the addition of $Na_2S_2O_4$. Similar results were obtained with NaOH concentrations up to 1 M. The addition of pyridine to P582 followed by 0.04 N NaOH and $Na_2S_2O_4$ gave a purplish-red solution with maxima at 560 and about 400 nm. The spectrum was not obtained when the alkali was added before the pyridine or when it was increased in strength to 0.1 N. The spectrum was abolished by excess ferricyanide. The alkaline spectrum of P582 was not changed by KCN in the presence or absence of $Na_2S_2O_4$.

Na_2S had no effect on the spectrum of P582 at *p*H 8; at *p*H 14, however, it reacted with the pigment to give an emerald green solution with absorption maxima at 430, 528, and 630 nm. The spectrum was almost identical to that given by Fe^{2+} in alkaline sulfide solutions (J. Ferguson, *personal communication*), indicating that Fe was released from P582 by this treatment.

Reaction of P582 with chelating agents. Oxidized P582 reacted over a period of several hours with the Fe^{2+}-chelating agents, 1,10-phenanthroline and bathophenanthroline (or its sulfonic acid derivative), to form the Fe^{2+}-complexes. The rate of reaction was markedly stimulated by reducing agents such as $Na_2S_2O_4$, NH_2OH, ascorbate, or dithiothreitol but was unaffected by Na_2SO_3 or KNO_2. An example with 1,10-phenanthroline is shown in Fig. 8A. Reduced P582 also reacted with 2,2′-dipyridyl but not with the Cu^+-complexing agent, bathocuproin (or its sulfonate). Neither oxidized nor reduced P582 reacted with the Fe^{3+}-chelating agents, *N*-methylacethydroxamic acid (1) and 2-thenoyltrifluoroacetone (22).

In the presence of $Na_2S_2O_4$, 26% of the Fe of Biogel fraction 2 (14 μmoles of Fe per g of protein) reacted with 1,10-phenanthroline as determined from the increase in absorbancy at 510 nm. The reaction of phenanthroline with P582 also resulted in a loss of acid-labile sulfide equiv-

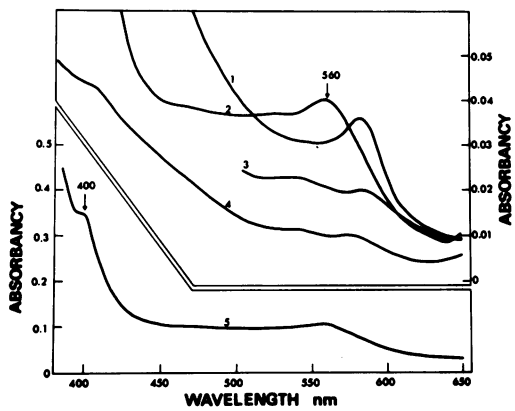

FIG. 7. *Effect of alkali and pyridine on P582 (Biogel fraction 1, 0.7 mg of protein per ml in 0.1 M potassium phosphate, pH 6.8). Curve 1, untreated pigment; curve 2, 0.1 ml of pyridine was added to 1 ml of P582 followed by 40 μliters of N NaOH and a few crystals of $Na_2S_2O_4$; curve 3, a slight excess of $K_3Fe(CN)_6$ was added to mixture 2; curve 4, 1 ml of P582 plus 40 μliters of M NaOH; curve 5, as curve 2 except P582 concentration 2.0 mg per ml.*

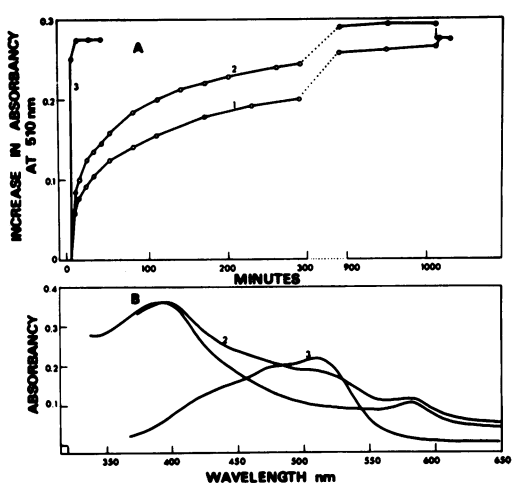

FIG. 8. *Reaction of 1,10-phenanthroline with P582. (A) P582 (Biogel fraction 2, 1.3 mg of protein per ml) was incubated with 1,10-phenanthroline (10 mM) at 30 C in 0.01 M Tris-hydrochloride (pH 8). Curve 1, change in absorbancy at 510 nm, $Na_2S_2O_4$ was added at the time indicated by the arrow; curve 2, as curve 1 except that $Na_2S_2O_4$ was added before phenanthroline; curve 3, as curve 1 except that 0.1 mM DTNB was added together with 1,10-phenanthroline. (B) Curve 1, spectrum of oxidized P582; curve 2, spectrum after 2 hr of reaction with 1,10-phenanthroline; curve 3, spectrum of 20 μM Fe^{2+}-1,10-phenanthroline in 0.01 M Tris-hydrochloride (pH 8).*

TABLE 1. *Effect of 1,10-phenanthroline on the acid-labile sulfide content of P582*[a]

Sulfide content	Amt
	nmoles/ml
Initial acid-labile sulfide	38
Final acid-labile sulfide	28
Fe chelated	22.5[b]
Ratio of sulfide removed to Fe chelated	0.45

[a] Three milliliters of P582 (Biogel fraction 2, 2.6 mg of protein per ml) was mixed with 10 μmoles of 1,10-phenanthroline and left at 4 C for 16 hr. Acid-labile sulfide was determined as described in the text. The Fe chelated was calculated from the change in absorbancy at 510 nm.

[b] Sixty-four per cent of the Fe reacting with phenanthroline in the presence of $Na_2S_2O_4$.

alent to about 50% of the Fe removed on a mole per atom basis (Table 1).

The spectrum of oxidized P582 after 2 hr of reaction with 1,10-phenanthroline is shown in Fig. 8B, curve 2. Apart from an increase in absorbancy in the region of 400 to 550 nm due to the Fe^{2+} complex there was no significant change in the spectrum. The difference spectrum, reduced + CO minus reduced, was also unaffected by the presence of 1,10-phenanthroline. Similarly the oxidized, reduced, and reduced + CO spectra of P582, after treatment with $Na_2S_2O_4$ and 1,10-phenanthroline or bathophenanthroline sulfonate and removal of the chelating agents by dialysis, were similar in all respects to those of the untreated pigment.

The rate of reaction between oxidized P582 and 1,10-phenanthroline was stimulated, particularly in the early stages, by DTNB (Fig. 8A, curve 3); an increasing absorbancy at 412 nm indicated that thionitrobenzoate was formed. Under the conditions described in Fig. 8A, DTNB alone reacted instantaneously with oxidized P582; the absorbancy at 412 nm increased by 0.04 (equivalent to 3 nmoles of thionitrobenzoate per ml) and thereafter remained constant for at least 90 min. When corrected for this value and for the absorption due to Fe^{2+}-1,10-phenanthroline, the increase in absorbancy at 412 nm in the experiment described in Fig. 8A (curve 3) paralleled that at 510 nm for the first 80 min; 0.93 mole of thionitrobenzoate was formed per atom of Fe chelated. It was not possible to determine the stoichiometry at the later stages of the reaction owing to the instability of thionitrobenzoate.

Enzymatic activities of fractionated extracts: MVH oxidation. Biogel fraction 2 catalyzed the anaerobic oxidation of MVH in the presence of Na_2SO_3, NH_2OH, and KNO_2 but not of Na_2SO_4, $Na_2S_2O_3$, or KNO_3. An example with sulfite as the electron acceptor is shown in Fig. 9. The reaction was approximately linear with time between absorbancies at 600 nm of 4.5 and 2 (0.38 to 0.17 μM MVH); thereafter, it was apparently first order with respect to MVH (Fig. 9, curve 3). The kinetics of the KNO_2-MVH and NH_2OH-MVH reactions were similar but the rates were approximately 10 and 40 times, respectively, those of the Na_2SO_3-MVH reaction. The linear rates of absorbancies above 2 were used as measures of the enzymatic activities. Biogel fraction 2 also stimulated the bleaching of MVH by $Na_2S_2O_4$. The rate increased with time particularly towards the end of the reaction. This was possibly due to a decrease in the rate of chemical back reduction of MVH by $Na_2S_2O_4$ as concentration of the latter fell.

All enzymatic activities were inactivated within 3 min at 100 C.

With Na_2SO_3 and NH_2OH, the rate of MVH oxidation was a linear function of enzyme concentration over the ranges of about 0.1 to 1 mg of protein and 0.01 to 0.1 mg of protein, respectively, per 3.1 ml of reaction mixture. The KNO_2-MVH reaction was not studied further since a high rate of nonenzymatic bleaching of MVH by KNO_2 made accurate analyses difficult. Moreover, with cruder preparations of enzyme

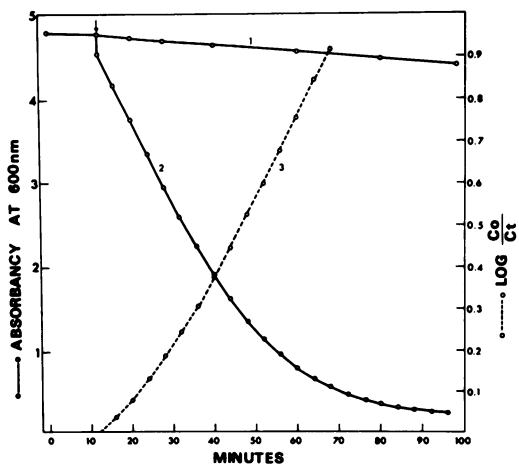

FIG. 9. *Reoxidation of MVH by Na_2SO_3. The reaction was carried out as described in the text. The reaction mixture contained P582 (Biogel fraction 2, 0.7 mg of protein), and 5 μmoles of Na_2SO_3 was added at the time indicated by the arrow. Curve 1, minus P582; curve 2, complete system; curve 3, first-order plot of curve 2, Co and Ct are the concentrations of MVH at zero time (after addition of Na_2SO_3) and time t, respectively.*

TABLE 2. *Production of sulfide from sulfite*[a]

Preparation	Protein	System	Initial MVH[b]	Incubation	MVH reoxidized	Sulfide produced	Ratio of MVH to sulfide
	mg		*μmoles*	*min*	*μmoles*	*μmoles*	
DE11 fraction	1.07	Complete	1.35	86	1.32	0.22	6.0
		Minus Na_2SO_3	1.13	86	0.18	0.035	
		Minus MVH		86		0.01	
		Minus enzyme	0.93	86	0.12	0.00	
Biogel fraction 2	0.65	Complete	1.14	68	0.85	0.079	10.9
	0.65	Minus Na_2SO_3	0.92	68	0.004	0.01	
	0.65	Complete	1.75	135	1.69	0.13	13.0
	1.30	Complete	1.28	52	0.75	0.065	11.5

[a] The complete system contained: 150 μmoles of potassium phosphate (pH 6.8), enzyme, 1 μmole of ethylenediaminetetraacetate, 5 μmoles of Na_2SO_3, and methyl viologen (MVH) in a volume of 3.5 ml.
[b] Determined from the absorbancy at 600 nm after addition of substrate.

(e.g., crude extracts and the DE11 fraction), there was an unexplained, almost instantaneous bleaching of MVH about 1 min after the addition of KNO_2 (*see* reference 6).

Products of sulfite reduction. The main product of the MVH-Na_2SO_3 reaction appeared to be sulfide (Table 2). It reacted not only with *N*-ethylmaleimide but also with *p*-aminomethylaniline and Fe^{3+} to form methylene blue; it was lost when the reaction mixture was acidified and flushed with O_2-free N_2. With the DE11 fraction, the stoichiometry approached the theoretical value of 6 moles of MVH oxidized per mole of sulfide produced according to equation 1. With the purer Biogel fractions, however,

$$6MVH + SO_3^{2-} \rightarrow 3H_2O + 6MV + S^{2-} \quad (1)$$

only about half of the expected sulfide was recovered (Table 2).

Cellular location and purification of reductases. The sulfite and hydroxylamine reductase activities were localized in the supernatant fraction from centrifugation of crude extracts of *D. nigrificans* at 198,000 × *g* (Table 3). To minimize a high endogenous activity of the supernatant fraction, which interfered with the assay of sulfite reductase, the protein was precipitated with 95% saturation $(NH_4)_2SO_4$, redissolved in buffer, and dialyzed. Less than 5% of the protein and hydroxylamine reductase activity was lost during this treatment. The reductase activities at different stages in the purification of P582 are shown in Table 4. The overall purification of hydroxylamine reductase was approximately threefold, and there was a close correlation between the activities of the different preparations and their P582 content as measured by the difference in absorbancy between 582 and 650 nm.

TABLE 3. *Distribution of methyl viologen (MVH)-linked reductases in extracts of D. nigrificans*[a]

Material	Total activity (units)[b]				
	Endogenous	Plus NH_2OH	Plus Na_2SO_3	Values in column 3 minus those in column 2	Values in column 4 minus those in column 2
Crude extract	330	1,500	—[c]	1,170	
Supernatant, 198,000 × *g*[d]	30	1,450	75	1,420	45
Particles, 198,000 × *g* (washed once)	41	60	42	19	1

[a] An extract of 6 g (dry weight) of bacteria was fractionated as described in the text.
[b] One unit of activity is defined as the amount of enzyme which oxidizes 1 μmole of MVH per min.
[c] No reliable rate could be determined because of high endogenous activity.
[d] Proteins were precipitated from the supernatant by 95% saturated $(NH_4)_2SO_4$ and dialyzed after redissolving in buffer.

Similar results were obtained for sulfite reductase activity when based on the total activity of the 198,000 × *g* supernatant fraction in the presence of Na_2SO_3. Much of the endogenous activity was apparently involved with the reduction of sulfur compounds since sulfide was a major product. Thus the total activity of the 198,000 × *g* supernatant was probably more representative of sulfite reductase activity in this fraction than the value corrected for endogenous oxidation of MVH.

The elution patterns of sulfite and hydroxylamine reductase activities from Biogel P200 closely followed that of the material absorbing at 392 nm (Fig. 3).

Pseudoperoxidase. Biogel fraction 2 (0.13 mg

TABLE 4. *Reductase activities at different stages in the purification of P582*[a]

Material	Endogenous		Sulfite		Hydroxylamine	
	A	B	A	B	A	B
Supernatant, 198,000 × *g*[b]	0.5	7.5	0.97	15	37	560
DE11 fraction	0	0	0.89	21	36	850
Biogel fraction 2	0	0	1.00	42	36	1,500

[a] Rates are expressed (A) as micromoles of MVH oxidized per minute per milliliter of enzyme with an absorbancy at 582 minus 650 nm of 1.0 and (B) as nanomoles of MVH oxidized per minute per milligram of protein.

[b] After precipitation with $(NH_4)_2SO_4$ (*see* Table 3).

of protein) caused an almost instantaneous oxidation of benzidine at room temperature in the pseudoperoxidase test above.

Phosphoroclastic reaction. Biogel fraction 2 (0.35 mg of protein per ml) failed to stimulate acetyl phosphate formation from pyruvate by ferredoxin-free extracts of *C. butyricum*. Under the same conditions, 0.4 μmole of MHV or 0.15 mg of the ferredoxin preparation per ml caused a fivefold stimulation.

NADH and NADPH-linked reactions. No significant reaction between NADH or NADPH and oxygen, sulfite, nitrite or hydroxylamine was catalyzed by the Biogel fractions.

Effect of pH on sulfite reductase activity. Detailed studies on the effect of *p*H on the enzymatic activities were not undertaken. In phosphate buffers, the following rates of sulfite reduction relative to that at *p*H 6.8 were found: *p*H 6.0, 1.6; *p*H 6.4, 2.7; *p*H 7.5, 0.6; *p*H 8.0, less than 0.1. At the more acid *p*H values, however, there was an instantaneous reaction between Na_2SO_3 and MVH which caused a fall in the absorbancy at 600 nm of about 0.8 at *p*H 6.4 and more than 2.0 at *p*H 6.0 on the addition of 2 μmoles of Na_2SO_3. For this reason, *p*H 6.8 was selected for the routine assay of the enzyme. The nature of the nonenzymatic reaction between Na_2SO_3 and MVH was not determined.

Effect of CO on reductase activities. The effects of CO on reductase activities were determined by using the standard assay procedure except that the absorption cells were flushed with 100% CO in place of N_2. Hydroxylamine, sulfite, and nitrite reductases were inhibited by 85 to 90%, 30 to 40%, and 30 to 35%, respectively. The enzymatic bleaching of MVH by $Na_2S_2O_4$ was also inhibited 90 to 100% by CO.

Effect of chelating agents on reductase activities. The hydroxylamine and sulfite reductase activities of 1,10-phenanthroline-treated Biogel fraction 2 were about 30% those of the untreated pigment (Table 5). Treatment of Biogel fraction 2 with the Cu^+-reagent, bathocuproin sulfonate, lowered the enzymatic activities by about 25%.

Reactions of Na_2SO_3, NH_2OH, and KNO_2 with P582. Na_2SO_3, NH_2OH, and KNO_2 had no effect on the spectrum of oxidized P582, but changed that of the $Na_2S_2O_4$-reduced pigment (Fig. 10). The peaks at 580 to 581 nm and 450 nm and the trough at 615 nm in the reduced + NH_2OH (or Na_2SO_3) minus reduced difference spectra (Fig. 10, curves 2 and 3) suggested that some reoxidation of P582 had occurred. The shapes of these spectra, however, were not identical in all respects with that of the oxidized (air) minus $Na_2S_2O_4$-reduced spectrum (Fig. 10, curve 4). A large excess of Na_2SO_3 was needed to cause the spectral changes and its effect was slight; it was, however, reproducible. KNO_2 formed a complex (possibly an NO-complex) with an absorption spectrum (Fig. 10, curve 1) similar to that of the $Na_2S_2O_4$-reduced CO-complex (Fig. 6, top trace).

P582 content of D. nigrificans. Supernatant fractions (198,000 × *g*) of *D. nigrificans* had an absorbancy at 582 minus 650 nm of 0.02 per mg protein per ml compared with 0.046 for the purest preparation of P582 (ratio 280 to 392 nm = 2.74). Assuming 70% purity for the latter (*see above*), this indicates that P582 accounted

TABLE 5. *Effect of 1,10-phenanthroline and bathocuproin sulfonate on reductase activities*[a]

Materials	Sulfite reductase activity[b]		Hydroxylamine reductase activity[b]	
		%		%
Untreated	0.89	100	36	100
1,10-Phenanthroline	0.29	33	10	28
Bathocuproin sulfonate	0.65	73	27	75

[a] DE11 fraction (4.3 mg of protein) was mixed in a final volume of 3 ml with 100 μmoles of potassium phosphate (*p*H 7), a few crystals of $Na_2S_2O_4$, and 10 μmoles of 1,10-phenanthroline or bathocuproin sulfonate. After 30 min at room temperature, the mixtures were dialyzed overnight against 0.1 M potassium phosphate (*p*H 7), centrifuged, and then tested for reductase activity.

[b] To allow for dilutions and losses of precipitated material, the activities are expressed as micromoles of methyl viologen oxidized per minute per 1.0 ml of enzyme with an absorbancy at 582 minus 650 nm of 1.0.

for about 30% of the protein of the 198,000 × *g* supernatant or 10 to 15% of the dry weight of the cell.

Reduction of P582 in crude extracts. A number of compounds were tested for their ability to reduce P582 in crude, dialyzed extracts. Based on the difference in absorbancy between 620 and 581 nm in reduced minus oxidized difference spectra, the following values for the reduction of P582, relative to the reduction by $Na_2S_2O_4$, were obtained: ascorbate, 10%; lactate, 10%; pyruvate, 70%; and NADH, 20%. None of these compounds reduced the purified pigment.

Distribution of CO-reacting pigments. *D. nigrificans* grown on pyruvate in a "sulfate-free" medium (37) had about the same content of P582 as organisms grown in the presence of sulfate. CO-reacting pigments with spectral properties similar to those of P582 were detected in *Desulfotomaculum orientis* (NCIB 8382) and two locally isolated, sporeforming, sulfate-reducing bacteria; they appeared to be absent from *Desulfovibrio vulgaris* strain Wandle (NCIB 8305) and three locally isolated, nonsporing, sulfate-reducing bacteria.

DISCUSSION

Three lines of evidence suggest that the reductase activities reported in this paper may be properties of P582. (i) There was a close correlation between the sulfite and hydroxylamine activities and the absorption at 582 nm of materials at different stages of purification (Table 4), and the elution patterns of P582 and the reductases from Biogel P200 were similar (Fig. 3). (ii) CO, which appeared to react with reduced P582, inhibited MVH oxidation in the presence of KNO_2, NH_2OH, Na_2SO_3, or $Na_2S_2O_4$, although not to equal extents. (iii) Na_2SO_3, NH_2OH, and KNO_2 reacted with $Na_2S_2O_4$-reduced P582. The resulting spectral changes (Fig. 10), however, were not consistent with a simple reoxidation of the pigment by these compounds. The marginal effect of Na_2SO_3 on the spectrum of $Na_2S_2O_4$-reduced P582, could be explained by the fact that $Na_2S_2O_4$ solutions contain appreciable amounts of sulfite. In view of these considerations, it is possible that the effect of $Na_2S_2O_4$ on the spectrum of P582 was not due solely to reduction of the pigment.

A further indication of a relationship between P582 and the reductase activities was a marked similarity between the spectrum of oxidized P582 and those of sulfite reductases from assimilatory sulfate-reducing organisms (Table 6). All are characterized by absorption maxima in the regions of 582 to 589 nm and 380 to 404 nm.

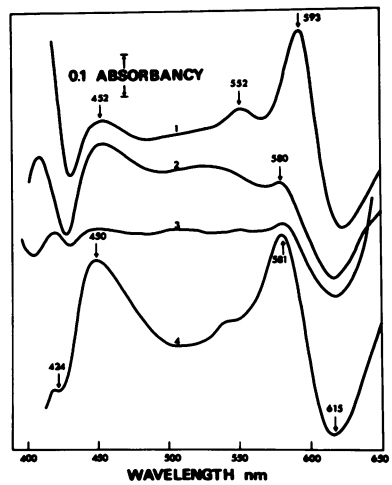

FIG. 10. *Difference spectra of reduced ($Na_2S_2O_4$) P582 + substrates minus reduced. Curve 1, DE11 fraction, 1.4 mg of protein per ml in 0.033 M potassium phosphate (pH 6.8) plus 1 mg of KNO_2; curve 2, as curve 1 except 1 mg of neutralized $NH_2OH \cdot HCl$ was used in place of KNO_2; curve 3, as curve 1 except 10 mg of Na_2SO_3 was used in place of KNO_3; curve 4, Biogel fraction 2, 1.3 mg of protein per ml, oxidized minus reduced ($Na_2S_2O_4$).*

Like P582, yeast (39) and spinach (5, 6) sulfite reductases react with CO. The spectrum obtained by reducing the spinach enzyme with $NaBH_4$ under CO in the presence of Na_2SO_3 (6) has a peak at 607 nm, a minor peak at about 560 nm, and a shoulder in the region of 404 to 419 nm. Besides a general shift towards longer wavelengths, this spectrum resembles that of the reduced CO-complex of P582. The corresponding maxima of the latter are at 596, 550 to 552, and 406 nm.

Although the purity of P582 is uncertain, the results to date indicate that *D. nigrificans* may contain an unusually large amount of the pigmented protein. This might account for the low overall purification factor for the reductase activities (approximately threefold over the 198,000 × *g* supernatant). Nevertheless, in view of the inhomogeneity of the purest preparations of the pigment, the assignment of the reductase activities to P582 must be regarded as tentative. At least in the case of spinach (6), the nitrite reductase and most of the hydroxylamine reductase activities can be separated from sulfite reductase.

The reduction of P582 in crude extracts of *D. nigrificans* by NADH and substrates such as lactate and pyruvate provides further evidence for the role of P582 in electron transport in this organism. The failure of the substrates to react

TABLE 6. *Comparison of sulfite reductases from various sources*

Source	*S. cerevisiae*	*Aspergillus nidulans*	*E. coli*	Spinach	*Allium odorum*	*D. nigrificans*
Absorption maxima (oxidized, nm)[a]	386,455,*490*, 587,*710*	384,*453*, *483*,585	385,587	*385*,404, 589	380,*410*, *490*,586	392,582, *700*
Molecular weight	350,000	N[b]	700,000	83–85,000	N	145,000
Nonheme iron	+	N	+	+	N	+
Acid-labile sulfide	+	N	+	N	N	+
Flavines	+	+	+	−	N	?
Hydroxylamine reductase	+	+	+	+	+	+
Nitrite reductase	+	N	+	−	−	+
Electron donors						
Methyl viologen	+	+	N	+	+	+
NADPH	+	−[c]	+	−	N	−
References	32,45,46	47	10,20,21	4,5,6	41	

[a] Numbers in italics refer to shoulders and minor peaks.
[b] Not recorded.
[c] Low reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome *c* reductase activity.

with purified P582 was presumably due to loss of the primary dehydrogenases.

A notable difference between P582 and sulfite reductases of *Aspergillus nidulans*, *Saccharomyces cerevisiae*, and *Escherichia coli* was the lack of a readily extractable flavine component. The absence of a pronounced flavine absorption peak in the absolute spectrum (Fig. 4) suggests that P582 may resemble the flavine-deficient forms of sulfite reductase from mutants of *S. cerevisiae* which catalyze MVH-linked but not NADPH-linked sulfite reduction (46). The preparations of P582 may not have been free of flavine, however, since there was a maximum at 450 nm in the oxidized minus reduced ($Na_2S_2O_4$) difference spectrum (Fig. 10, curve 4).

It has been suggested that some properties of the sulfite reductases from spinach (5, 6), *E. coli* (20), and *S. cerevisiae* (39) indicate the presence of a hemelike moiety in these enzymes. On the other hand, Yoshimoto and Sato (45) claimed that yeast sulfite reductase is not a hemoprotein since no heme could be detected by the pyridine hemochromogen test. Nevertheless, they pointed out that some of the spectral properties of the native and alkali-treated enzyme would be consistent with the presence of an *a*-type heme. The evidence for heme in P582 is inconclusive. P582 formed an oxidizable and reducible alkaline pyridine complex under certain conditions, but the spectra of the latter were unlike those of pyridine complexes of the common naturally occurring hemes. The spectrum of the $Na_2S_2O_4$-reduced CO-complex of P582 differs markedly from those of CO-complexes of *a*-type cytochromes in the ratio of γ to α peaks. The virtual abolition of spectral characteristics of P582 by alkali and the lack of formation of a cyanide complex were also atypical of hemoproteins; in these respects, P582 differed from *S. cerevisiae* (45) and spinach (4) sulfite reductases. Attempts to extract a hemelike component from P582 have thus far been unsuccessful.

The purest preparations of P582 also resembled sulfite reductases from assimilatory sulfate-reducing organisms in containing Fe and acid-labile sulfide (Table 6). The stoichiometry, however, was about 3.5 atoms of Fe per mole of labile sulfide whereas a value of 1:1 is generally characteristic of Fe-S proteins (14). Nevertheless, two forms of Fe appeared to be present in the Biogel fraction 2, one of which could react with Fe^{2+}-chelating agents such as 1,10-phenanthroline. The stoichiometry of the chelatable Fe and acid-labile sulfide approached 1:1.

Approximately 1 mole of acid-labile sulfide per 2 atoms of Fe was removed by treatment of oxidized Biogel fraction 2 with 1,10-phenanthroline. Further, about 1 mole of thionitrobenzoate per atom of chelated Fe appeared when oxidized Biogel fraction 2 was treated with both 1,10-phenanthroline and DTNB. This indicates that the chelatable Fe, labile sulfide, and DTNB-reactable groups were structurally related. The situation is somewhat analogous to that with ferredoxins. Labile sulfide is released by treatment of both plant (13) and clostridial (27) ferredoxins with Fe^{2+}-chelating agents and a relationship between chelatable Fe and DTNB-reactable groups in the latter pigments has been shown (28).

The oxidation state of the chelatable Fe in the

Biogel fraction 2 is uncertain. Reducing compounds markedly stimulated the rate, but not the extent, of the reaction between Biogel fraction 2 and Fe^{2+}-chelating reagents. Fe^{3+}-chelating reagents were not active. The effect of reducing agents, therefore, was probably not one of simple reduction of Fe^{3+}.

The chelatable Fe appeared not to be responsible for the spectral and CO-binding properties of P582 since these properties were unaffected by treatment of P582 with 1,10-phenanthroline. Removal of the chelatable Fe was, however, accompanied by a decrease of the enzymatic activities. Whether this decrease was, in fact, due to the removal of Fe rather than another element (e.g., sulfur) or to some other effect on the enzyme remains to be determined.

Its high molecular weight and spectral properties distinguish P582 from bacterial ferredoxins (43). P582 was also inactive in the pyruvate-phosphoroclastic reaction catalyzed by *C. butyricum*. In the latter respect, P582 appeared to differ from the brown pigment fraction isolated by Akagi (2) from extracts of *D. nigrificans* which could replace clostridial ferredoxin in the phosphoroclastic reaction in *C. pasteurianum*.

A major product of sulfite reduction by fractionated extracts of *D. nigrificans* appeared to be sulfide, but with the purer enzyme preparations the stoichiometry between MVH oxidized and sulfide produced indicated that another product(s) was also formed. It has been reported that thiosulfate (12, 24, 40), trithionate (24), and dithionite (B. Suh, W. Nakatsuka and J. M. Akagi, Bacteriol. Proc., p. 133, 1968) are produced from sulfite by extracts of *Desulfovibrio* or *Desulfotomaculum*. Disulfur monoxide and elemental sulfur may be formed in small amounts by *Desulfovibrio desulfuricans* growing on sulfate (18). Whether these compounds are true intermediates in the sulfite-sulfide reaction, or whether they are formed by secondary reactions, is uncertain.

From the brief survey of dissimilatory sulfate-reducing bacteria, it would appear that P582, or similar CO-reacting pigments, may be characteristic of the sporing genus *Desulfotomaculum*. It was absent from several strains of *Desulfovibrio* which instead contain low-potential, soluble, *c*-type cytochromes which are absent from species of *Desulfotomaculum* (9). This distinction may not be absolute since H. E. Jones (*personal communication*) has recently isolated a nonsporulating sulfate-reducing bacterium which appears to contain both a soluble *c*-type cytochrome and a CO-reacting pigment.

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