PURIFICATION OF THE ENZYME REDUCING BISULFITE TO TRITHIONATE FROM *DESULFOVIBRIO GIGAS* AND ITS IDENTIFICATION AS DESULFOVIRIDIN

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Received August 3, 1971

Summary: An enzyme which catalyzes the reductive formation of trithionate from sulfite is present in extracts of the sulfate reducing bacterium *Desulfovibrio gigas* and has been termed, bisulfite reductase. This activity has been purified and the enzyme identified as the green pigment, desulfoviridin, by gel electrophoresis and ultracentrifugation. The stoichiometry of the reduction of sulfite to trithionate is established and some physical properties of the enzyme reported

Early observations made with intact cells of *Desulfovibrio vulgaris* had indicated that thiosulfate, although reduced to sulfide, was not an intermediate in the reduction of sulfite with hydrogen (1). Suh and Akagi (3) have reported the accumulation of thiosulfate during the reduction of bisulfite by *D. vulgaris* and Kobayashi *et al* (2) have described the sequential accumulation of trithionate and thiosulfate during the reduction of bisulfite by partially fractionated extracts of this sulfate reducing bacterium. These observations plus the previously established ability of these bacteria to reduce thiosulfate (4) indicate the existence of a new pathway of sulfite reduction involving at least three enzymes.

Desulfoviridin, the green protein of taxonomic significance for the genus, *Desulfovibrio* (5), was described by both Postgate (6) and Ishimoto *et al* (7). Although it appears to be a porphyroprotein, the spectrum is not effected by reducing agents and reactions with various ligands have not been detected. This protein is found in high concentration in crude extracts, but its enzymatic function has remained obscure.

In this paper, we report the purification of an enzyme reducing bisulfite

*This work was supported by Grants AM 10879 from NIH and GB-4189 from NSF.

to trithionate from extracts of *D. gigas* and its identification as the green protein, desulfoviridin.

METHODS

D. gigas was grown at 37° in a lactate-sulfate medium and extracts prepared as described earlier (8,9). The cell-free extract (ca 45 mg/ml) was passed through a short DEAE-cellulose column (1.8 x 10 cm) to remove acidic proteins and diluted to 20 mg/ml. This "crude extract" was then fractionated with solid ammonium sulfate at 30, 55 and 80% saturation. After centrifugation, the precipitates were dissolved in a minimum volume of 0.01 M phosphate, pH 6.0, and dialyzed overnight against 4 liters of the same buffer. The 55-80% fraction was adsorbed on a column of DEAE-cellulose (3.0 x 25 cm) equilibrated with 0.01 M phosphate, pH 7.6. The column was washed with 50 ml of 0.01 M, 0.05 M and 0.1 M phosphate, pH 7.6, and the enzyme then eluted by means of linear phosphate gradient (pH 7.6) from 0.1 to 0.5 M (400 ml). The green fractions were combined and the protein, precipitating at 80% saturation with solid ammonium sulfate, dissolved in a minimum volume of 0.01 M phosphate, pH 6.0. The enzyme was then applied to a column of Sephadex, G-200, (2.5 x 100 cm) equilibrated with 0.05 M Tris-Cl buffer, pH 7.6, and eluted with the same buffer. The enzyme was stored at -20° and was stable for several months.

Bisulfite reductase activity was determined manometrically as hydrogen utilization by reaction mixtures containing crude hydrogenase from *Escherichia coli* (10), 3.0 mg; 0.14 M phosphate, pH 6.0; methyl viologen, 1.4 x 10^{-3}M; and bisulfite reductase. Protein was determined by the biuret method (11).

RESULTS

A summary of the results of a fractionation of desulfoviridin and bisulfite reductase are shown in Table 1. Desulfoviridin, as determined by its absorption at 628 nm, was purified about 14-fold but bisulfite reductase in terms of hydrogen consumption was only enriched to four-to five-fold. This

Table 1. Purification of Bisulfite Reductase and Desulfoviridin.

Fraction	Specific Activity of Bisulfite Reductase (a) (μmoles H_2/min/mg)	Concentration of Desulfoviridin (b) (units[1]/mg)	Ratio $^a/_b$ X 10^2
Crude	0.146 (0.049)[2]	1.9	7.7 (2.6)[3]
0-30% $(NH_4)_2SO_4$	0.044	0.4	11
30-55% $(NH_4)_2SO_4$	0.059	0.59	10
55-80% $(NH_4)_2SO_4$	0.278	5.8	4.8
DEAE Column	0.438	15.5	2.8
Sephadex G-200	0.632	27.5	2.3

[1]-one unit equals 0.1 O.D. at 628 nm.

[2]-Initial specific activity divided by three to correct for complete reduction to sulfide.

[3]-Calculated employing corrected specific activity for bisulfite reductase.

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discrepancy is only apparent as hydrogen oxidation through the DEAE step is three times greater than that required for the reduction of bisulfite to trithionate. This is due to the reduction of trithionate to sulfide by the other enzymes in the pathway. After reducing the specific activity of the crude extract by a factor of three, a specific activity of 0.049 for bisulfite reductase is obtained and an overall purification identical with that obtained for desulfoviridin. The ratio of the specific activity of hydrogen utilization to desulfoviridin is 7.7 in the crude extract but, after removal of the other reductive enzymes, it decreases to relatively constant values ranging from 2.3-2.8. Employing the calculated value for reductase activity in the crude extract, this ratio was 2.6 which suggests that bisulfite reductase is the major and limiting enzyme concerned with sulfite reduction. The yield of both bisulfite reductase and desulfoviridin varied from 10-20%.

In spite of the low value for enrichment in the fractionation, 13- 14 fold, the protein sediments as a single colored symmetrical peak in the ultracentrifuge with a $S_{20,s}$ value of 10.2 (12). At pH 6.0 and a protein concentration of 6.0 mg/ml, no other components were observed. After disc electrophoresis (13) of the purified protein at pH 8.3 and 8.9, two closely migrating green bands were observed but the faster moving band contained most of the protein. These were the only components in the gel that stained with buffalo black. The green bands catalyzed *in situ* the sulfite-dependent oxidation of reduced methyl viologen when the gels were incubated anaerobically with the appropriate standard reaction mixtures (17). Hydrogenase activity as evidenced by a sulfite-independent oxidation of the reduced dye was not observed. From sedimentation equilibrium experiments (15), the molecular weight was determined to be 200,000 which was in good agreement with that calculated from the sedimentation velocity and the partial specific volume (0.734) derived from amino acid analysis (12).

In the presence of reduced methyl viologen, the purified protein reduces sulfite solely to trithionate and the stoichiometry of the reaction is shown in Table 2. ^{35}S-sulfite (22 μmoles) was incubated under hydrogen with purified hydrogenase from *D. vulgaris* (16) and methyl viologen in the presence and absence of bisulfite reductase. The reduction was allowed to go to completion and the reaction mixtures analyzed by paper (2) and column chromatography (17).

Table 2. Stoichiometry of Bisulfite Reduction to Trithionate

Reaction Mixture	Sulfite Added	Hydrogen Utilized	Radioactivity in Products (CPM)			Recovery of S^{35}
	(μmoles)	(μmoles)	SO_3^{-2}	SO_4^{-2}	$S_3O_6^{-2}$	(%)
Complete[1]	22	8.5	0	1.9×10^6	5.4×10^6	73
Enzyme	22	0.0	5.2×10^6	1.4×10^6	0	66

[1]The reaction mixture (2.2 ml) contained in μmoles: phosphate buffer, 200 (pH 6.0); methyl viologen, 3; $Na^{35}{}_2SO_3$, 22 (1×10^7 c.p.m.); and bovine serum albumin, 1.0 mg; purified hydrogenase from *D. vulgaris* (16), 6 mg; bisulfite reductase, 1.68 mg. Incubation was at 37° for 35 min.

Trithionate was the only product detected and its formation was accompanied by the utilization of one μmole of hydrogen per 3 μmoles of sulfite. The recovery of radioactivity was about 70% in both the control and complete system. The data indicate that bisulfite reductase at its optimum pH of 6.0 catalyzes the following reaction:

$$2\ MVH + 3\ HSO_3^{-1} \rightarrow S_3O_6^{-2} + 2\ MV + 2\ H_2O + OH^{-1}$$

As suggested by Suh and Akagi (3), bisulfite is probably the actual ionic species of sulfite functioning as substrate and it is on this basis that the enzyme has been called bisulfite reductase. This distinguishes it from the assimilatory sulfite reductase which directly reduces sulfite to sulfide without the formation of intermediates (18). The actual reaction is also supported by the observation that both sulfur atoms of thiosulfate are quantitatively derived from ^{35}S-bisulfite in extracts of *D. vulgaris* (19) in contrast to results obtained with thiosulfate formation in other organisms (20).

The absorption spectrum of the purified enzyme is shown in Fig. 1, curve 1. It exhibits maxima at 628, 580, 408 and 390 nm and closely resembles the absorption spectrum of desulfoviridin (6). The addition of either sulfite, ascorbate, dithionite or borohydride alone has no effect on the absorption spectrum of the protein; however, spectral changes were observed in the presence of both sulfite and borohydride. The sequential addition of borohydride and sulfite has no effect on the maxima at longer wavelengths but a general 10-20% bleaching of the absorption spectrum is observed from 340-415 nm. When the order of additions is reversed i.e. sulfite followed by borohydride, a new large absorption peak appears at 310 nm in addition to the previously described changes. These spectral changes suggest that the green chromophore is in some fashion involved in the reduction of bisulfite to trithionate and might possibly reflect formation of an enzyme-substrate complex.

The structure of the green chromophore has not yet been determined but, as suggested by Postgate (6) on the basis of its absorption spectrum, it may be a dihydropophyrin or d-type heme. The chromophore is released from the

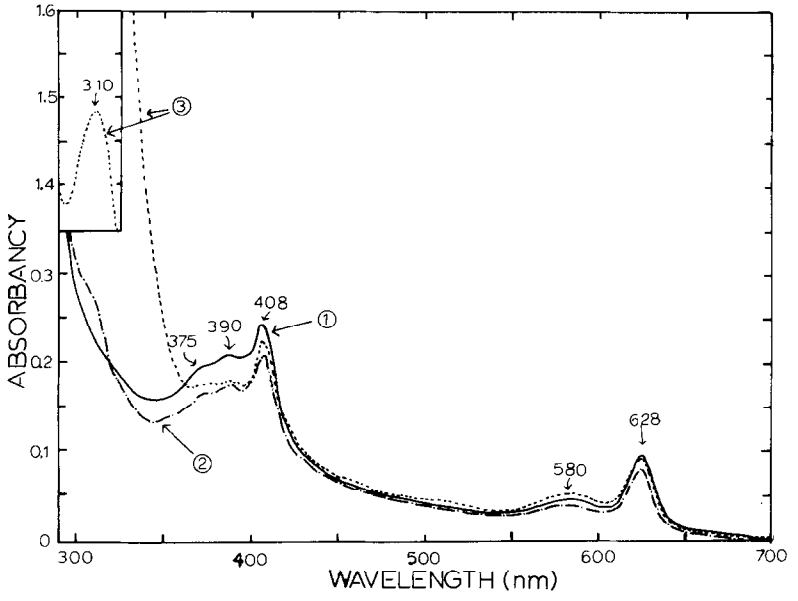

Figure 1. The effect of sulfite and borohydride on the absorption spectrum of bisulfite reductase (Desulfoviridin).

1. Enzyme, plus SO_3^{-2} or enzyme plus $NaBH_4$.
2. Enzyme - followed by the sequential addition of $NaBH_4$ and sulfite.
3. Enzyme followed by the sequential addition of sulfite and $NaBH_4$. Each cuvette contained in 1 ml of 0.1 M phosphate buffer, pH 6.0, 0.3 mg of bisulfite reductase and where indicated $NaBH_4$, 1 mg and Na_2SO_3, 60 µmoles.

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protein by acid, alkali or heat treatment, but it was not possible to detect the formation of a pyridine hemochromogen (6).

The purification of bisulfite reductase and the intermediate formation of trithionate and thiosulfate during the reduction of bisulfite indicate conclusively that a pathway for sulfite reduction exists in the sulfate reducing bacteria which differs from that found in most other organisms. However, results from a number of laboratories (21, 22, 23) suggest that the trithionate pathway may not be the only pathway for sulfite reduction in this group of organisms.

Acknowledgement: We wish to thank Dr. Jean LeGall for many helpful discussions and suggestions during the course of these investigations.

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