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Characterization of a Novel Thiosulfate-Forming Enzyme Isolated from *Desulfovibrio vulgaris*

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An enzyme that formed thiosulfate from bisulfite and trithionate was purified from extracts of *Desulfovibrio vulgaris*. This enzyme, designated as "thiosulfate-forming" enzyme, required the presence of both bisulfite and trithionate. Various ^{35}S-labeling studies showed that thiosulfate was formed from bisulfite and the inner sulfur atom of trithionate. This involved a nucleophilic attack by the bisulfite ion, resulting in the displacement of the two outer sulfonate groups of trithionate that recycled to participate as free bisulfite in subsequent reactions. This reaction required a reduction, presumably by a concerted mechanism with thiosulfate formation. The natural electron carrier cytochrome c_3 participated in this reductive formation of thiosulfate. This reaction was coupled to the bisulfite reductase-catalyzed reaction, which resulted in the reconstruction of a thiosulfate-forming pathway from bisulfite.

The biological reduction of inorganic sulfate has been divided into assimilatory- and dissimilatory-type processes. Assimilatory reduction is widespread in nature, occurring in both procaryotes and eucaryotes, and is primarily concerned with the incorporation of sulfur into cellular material. Dissimilatory reduction is apparently unique to the sulfate-reducing bacteria, in which inorganic sulfur compounds are used as terminal electron acceptors. Common to both the assimilatory and dissimilatory pathways is the initial activation of sulfate by adenosine triphosphate followed by the reduction of the respective activated form to sulfite (23, 29, 33). One mode of assimilatory reduction occurring in bacteria and yeast involves the direct reduction of sulfite to sulfide, without the formation of any detectable intermediates, by sulfite reductase (5, 21, 29, 35). In these organisms, another pathway may exist that uses bound intermediates. In oxygen-evolving photosynthetic organisms, sulfite reductase, although present, does not appear to be the enzyme employed; rather, a carrier-bound sulfite is reduced to carrier-bound sulfide by an enzyme called thiosulfonate reductase (27, 28, 32, 33).

In contrast, a series of complex reactions has been proposed for the dissimilatory pathway leading to the formation of sulfide. Ishimoto and Koyama (15) reported the presence of thiosulfate reductase activity in *Desulfovibrio*. Later investigators purified thiosulfate reductase from several sulfate-reducing bacteria (12, 13, 25), thus establishing the importance of thiosulfate in dissimilatory reduction. Suh and Akagi (31) observed the presence of a thiosulfate-forming system in *Desulfovibrio vulgaris*. This thiosulfate-forming system required two protein fractions, referred to as FII and FIII, and reduced bisulfite to thiosulfate. Kobayashi et al. (20) found that trithionate was formed by extracts of *D. vulgaris* and proposed a dissimilatory pathway with trithionate and thiosulfate as intermediates linking the reduction of (bi)sulfite to sulfide:

$$3\ (H)SO_3^- \xrightarrow{2e^-} S_3O_6^{2-} \xrightarrow[\searrow SO_3^{2-}]{2e^-} S_2O_3^{2-} \xrightarrow[\searrow SO_3^{2-}]{2e^-} S^{2-}$$

Subsequently, Lee and Peck (22) purified the green pigment desulfoviridin from *D. gigas* and identified it as the trithionate-forming enzyme bisulfite reductase. *Desulfotomaculum nigrificans* was shown to contain another type of trithionate-forming bisulfite reductase (2).

During our investigation of the thiosulfate-forming pathway of *D. vulgaris*, we succeeded in purifying an enzyme from the FII (31) fraction that formed thiosulfate from bisulfite plus trithionate. This protein was designated thiosulfate-forming enzyme (TF). This study was conducted to elucidate the mechanism of this catalysis and to determine the role of this enzyme in the thiosulfate-forming pathway of *D. vulgaris*.

MATERIALS AND METHODS

Organism. *D. vulgaris* NCIB 8303 was grown and harvested as previously described (3).

Enzyme assay conditions. Standard manometric techniques were used throughout this study, using Warburg flasks of approximately 8-ml capacity. The particulate hydrogenase of *D. vulgaris* was prepared as described earlier (31). Unless otherwise indicated, the standard assay mixture contained: potassium phosphate buffer (pH 6.0), 100 μmol; methyl viologen, 1.0 μmol; hydrogenase, 0.1 mg; and substrate(s) and enzyme in a total volume of 1.1 ml. The center well contained either 0.1 ml of 20% $CdCl_2$ for sulfide determination or 0.1 ml of 20% KOH for trapping acid-volatilized [^{35}S]bisulfite. Both the $CdCl_2$ and the KOH were absorbed on fluted filter paper. The gas phase was hydrogen, and the temperature was 30°C.

Purification of bisulfite reductase. Bisulfite reductase was purified by a modification of the previously described procedure (8). The final bisulfite reductase preparation was passed through a Sephadex G-200 column (2.5 by 30 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.5. The green pigment (bisulfite reductase) eluting from this column was dialyzed against 10^{-3} M potassium phosphate buffer, pH 7.0, and concentrated by pressure filtration with an Amicon EC-20 unit and a PM30 filter. Portions (1 ml) of the enzyme were stored at −20°C and thawed once for each experiment. The major and minor bands, characteristic for pure bisulfite reductase from *D. vulgaris* (8, 16, 19, 22), were observed by discontinuous gel electrophoresis.

Purification of TF. All purification steps were carried out at 0 to 4°C. A 600-ml amount of crude extract, prepared as previously described (3), was fractionated by differential precipitation with ammonium sulfate. The fraction precipitating between 0.3 and 0.5 saturation was dissolved in water and dialyzed against 30 volumes of 10^{-3} M buffer, pH 7.0 (potassium phosphate buffer was used throughout the purification of TF). This fraction was applied to a diethylaminoethyl (DEAE)-cellulose column (5 by 30 cm) (chloride), and the column was washed with 1 liter of 0.01 M buffer, pH 7.5. The TF activity was eluted with 0.05 M buffer (pH 7.5), dialyzed against 10^{-3} M buffer (pH 7.0) until free of salts, and lyophilized. One-half of the lyophilized material was dissolved in a minimum amount of 0.02 M buffer, pH 7.5, and applied to a Sephadex G-100 column (4 by 40 cm) equilibrated with the same buffer. Three distinct yellowish bands migrated down the column. The middle band, containing all of the TF activity, was collected, dialyzed, and lyophilized. (This partially purified TF preparation was employed in the ^{35}S-labeling studies.) This fraction was dissolved in 0.02 M buffer, pH 7.5, and applied to a DEAE Bio-Gel A column (2.5 by 30 cm) equilibrated with the same buffer. A linear buffer gradient (0.01 to 0.15 M) was passed through the column at a rate of 25 ml/h for 16 h. The TF fraction, eluting as a shoulder just behind the second major protein peak, was dialyzed and lyophilized. The pooled lyophilized material from two DEAE Bio-Gel A columns was dissolved in a small amount of 0.02 M buffer, pH 7.5, and applied to a Sephadex G-100 column (2.5 by 30 cm) equilibrated with the same buffer. The TF fraction eluting from this column was dialyzed, concentrated by lyophilization, and applied to a second DEAE Bio-Gel A column (2.5 by 30 cm). A linear buffer gradient was passed through the column as described above, and the TF was collected, dialyzed, and lyophilized.

Electron carriers. The flavodoxin preparation was obtained by passing 1 liter of cell-free extract through a DEAE column (5 by 10 cm). After extensive washing with 0.05 M KCl in 0.075 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.0, the fraction containing flavodoxin was removed from the column with 0.5 M KCl in 0.15 Tris-hydrochloride buffer, pH 7.0. This material was diluted 10-fold and applied to another DEAE column (5 by 5 cm). The column was washed with 0.1 M KCl in 0.15 M Tris-hydrochloride buffer, pH 7.0, until the eluate was clear. Flavodoxin was eluted with 0.5 M KCl in 0.15 M Tris-hydrochloride buffer (pH 7.0), dialyzed free of salts, and lyophilized. The lyophilized flavodoxin was dissolved in a small amount of water and applied to a Sephadex G-100 column (4 by 40 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.5. The intense yellow band (flavodoxin) eluting from this column was dialyzed, lyophilized, and stored at −20°C. The absorption spectrum for flavodoxin exhibited peak maxima at 453, 370, and 275 nm, with a shoulder at 478 nm. These peaks disappeared when the flavodoxin was reduced with sodium dithionite. These spectral properties are in good agreement with those reported to be characteristic for flavodoxin (10, 18). Flavodoxin activity was confirmed by testing its ability to couple with the phosphoroclastic reaction in carrier-free extracts (1) of *D. vulgaris*. By analytical discontinuous gel electrophoresis, the flavodoxin preparation was observed to be a homogeneous protein. Cytochrome c_3 was obtained by modified methods of Horio and Kamen (14) as previously described (1).

Analytical determinations. Thiosulfate and trithionate were determined by the method of Kelly et al. (17) as previously described (4). Sulfide was analyzed by the method of Fogo and Popowski (11). Protein was estimated by the method of Lowry et al. (24), using bovine serum albumin as a standard. [^{35}S]bisulfite was volatilized as $^{35}SO_2$ by the addition of 0.1 ml of 20 N H_3PO_4. The KOH filter paper used to trap the $^{35}SO_2$ was placed in 10 ml of counting solution and incubated in the dark for 4 h prior to liquid scintillation counting. The isolation of [^{35}S]thiosulfate was accomplished by column chromatography on Dowex-1 and was degraded as previously described (8). Degradation of authentic sulfane and sulfonate-labeled thiosulfate, purchased from Amersham/Searle, confirmed the validity of the degradations.

To eliminate the possibility that a nonenzymatic exchange occurred among the various sulfur atoms of bisulfite, trithionate, and thiosulfate in the ^{35}S-labeling studies (26, 30), controls were run to determine whether nonenzymatic exchange occurred; no appreciable exchange was detected.

Trithionate was synthesized by a modification of the method of Roy and Trudinger (26), as described earlier (4). [^{35}S]sulfonate-labeled trithionate was

synthesized by supplementing the $K_2S_2O_5$ solution with $Na_2{}^{35}SO_3$ prior to the addition of the SCl_2. The accuracy of the synthesis was determined by degradation of the [^{35}S]trithionate by methods previously described (8); the inner sulfur atom was unlabeled. The [^{35}S]sulfonate-labeled trithionate was free of [^{35}S]sulfite as determined by acid volatilization experiments. Sodium bisulfite solutions were prepared fresh in 10^{-3} M disodium ethylenediaminetetraacetate before each assay. Oxidation of $Na_2{}^{35}SO_3$ to $Na_2{}^{35}SO_4$ was periodically monitored by methods previously described (8). $Na_2{}^{35}SO_3$ was purchased from New England Nuclear Corp. Liquid scintillation counting was performed with a Beckman LS-3150P spectrometer. All counts were corrected for quench and background. Molecular weight determinations were performed by gel filtration on Sephadex G-100 by the method of Whitaker (34).

RESULTS

Properties of TF. Purified TF was completely stable after repeated lyophilization or freezing and thawing. The enzyme was colorless, and a single protein band was observed after polyacrylamide discontinuous gel electrophoresis. The pH optimum for TF activity was 6.0 and, since at this pH the protonated form of sulfite predominates, we concluded that bisulfite was the preferred ionic species of substrate in the TF reaction. The molecular weight of TF activity was estimated to be 43,200.

Enzymatic formation of thiosulfate. Table 1 shows the products formed by the enzymes constituting the thiosulfate-forming system. When bisulfite reductase was incubated with bisulfite, trithionate was formed as the major product. When bisulfite reductase and TF were incubated with bisulfite, both trithionate and thiosulfate were detected, with thiosulfate being more predominant. This system constituted the thiosulfate-forming system as previously reported (8, 31). Although it appeared that TF functioned as a trithionate reductase, incubation of TF with either bisulfite or trithionate alone resulted in no activity. However, when TF was incubated with both bisulfite and trithionate together, thiosulfate was formed. This indicated that both of these compounds were substrates for TF activity.

Effect of substrate concentrations on thiosulfate formation. Table 2 shows the effect of bisulfite and trithionate concentrations on thiosulfate formation. When the concentration of trithionate was kept constant while that of bisulfite was varied, thiosulfate formation increased slightly with an increasing bisulfite concentration. The reciprocal experiment showed that increasing the trithionate concentration resulted in a concomitant increase in thiosulfate formation. Furthermore, the amount of thiosulfate was dependent upon trithionate concentration, and only a catalytic amount of bisulfite was necessary.

Degradation of [^{35}S]thiosulfate formed by TF. To determine the origin of the sulfur atoms in the thiosulfate molecule formed by TF, ^{35}S-labeled substrates were incorporated into reaction mixtures. The labeled thiosulfate formed in the reaction was isolated by Dowex-1 chromatography and chemically degraded to locate the radioactive atom. An analysis of the thiosulfate formed from [^{35}S]bisulfite and unlabeled trithionate showed that the sulfonate sulfur atom contained essentially all of the radioactivity, whereas the sulfane atom was unlabeled. The identical labeling pattern was observed when either [^{35}S]bisulfite- and [^{35}S]sulfonate-labeled trithionate or unlabeled bisulfite- and [^{35}S]sulfonate-labeled trithionate were substrates for the reaction (Table 3). From these

TABLE 1. *Product formation by the thiosulfate-forming system*[a]

System	Product (μmol)		
	$S_3O_6^{2-}$	$S_2O_3^{2-}$	S^{2-}
Bisulfite reductase + HSO_3^-	2.37	0	0.23
Bisulfite reductase + TF + HSO_3^-	0.27	3.68	0.20
Bisulfite reductase + $S_3O_6^{2-}$		0	0.10
TF + $S_3O_6^{2-}$		0	0
TF + HSO_3^-	0	0	0
TF + HSO_3^- + $S_3O_6^{2-}$		5.53	0
Boiled TF[b] + HSO_3^- + $S_3O_6^{2-}$		0	0

[a] Standard assay conditions. Bisulfite reductase concentration, 1.19 mg; TF concentration, 0.39 mg; $NaHSO_3$ concentration, 10.0 μmol; $K_2S_3O_6$ concentration, 11.0 μmol; incubation time, 60 min.

[b] TF placed in boiling water bath for 10 min.

TABLE 2. *Effect of substrate concentration on thiosulfate formation by TF*[a]

Substrate (μmol)		Thiosulfate formed (μmol)
$S_3O_6^{2-}$	HSO_3^-	
11.0	0.5	4.1
11.0	1	5.0
11.0	2	5.7
11.0	4	6.3
11.0	10	7.6
1.2	10	1.4
2.3	10	2.4
3.4	10	3.2
11.0	0	0
0	10	0

[a] TF concentration, 0.41 mg; incubation time, 100 min. Standard assay conditions employed.

TABLE 3. *Degradation of thiosulfate formed from ^{35}S-labeled substrates*[a]

Expt	Thiosulfate (cpm)		cpm degraded	Recovery (%)
	S	SO_3		
A	4,140	84,860	92,700	96
	7,610	100,650	115,875	93
Avg distribution (%)	6	94		
B	15,970	246,530	295,000	89
	10,910	145,810	158,400	99
Avg distribution (%)	6.4	93.6		
C	2,900	96,140	105,000	94
	1,390	57,540	58,500	101
Avg distribution (%)	2.7	97.3		

[a] Standard assay conditions. In all experiments, the TF concentration was 2.0 mg and that of $NaHSO_3$ was 5.0 μmol. Experiment A, $NaH^{35}SO_3$ specific activity, 4×10^5 cpm; unlabeled $K_2S_3O_6$ concentration, 7.0 μmol; time, 60 min. Experiment B, $NaH^{35}SO_3$ specific activity, 6×10^5 cpm; [^{35}S]sulfonate-labeled $K_2S_3O_6$, 10 μmol containing 1.25×10^5 cpm/μmol; time, 70 min. Experiment C, unlabeled $NaHSO_3$; [^{35}S]sulfonate-labeled $K_2S_3O_6$, 10 μmol containing 1.25×10^5 cpm/μmol; time, 70 min.

results we concluded that the sulfane atom of thiosulfate originated from the inner (sulfane) sulfur atom of trithionate, whereas the sulfonate sulfur atom of thiosulfate arose from free bisulfite and the sulfonate group(s) on the trithionate molecule.

Recycling of the sulfonate groups of trithionate as bisulfite. The above studies suggested that the sulfonate sulfur atom of the product, thiosulfate, originated from free bisulfite and the two sulfonate groups of trithionate. This was visualized by a bisulfite ion making a nucleophilic displacement attack on the inner sulfane atom of trithionate, releasing both sulfonate groups as free bisulfite ions. A concomitant reduction of one of the leaving sulfonate groups presumably occurred during this reaction. To test this hypothesis, [^{35}S]sulfonate-labeled trithionate and unlabeled bisulfite were incubated in the presence of TF. At various time intervals the reaction was stopped by tipping in 0.1 ml of 20 N H_3PO_4 from one side arm into the main compartment. Any free bisulfite ions were volatilized and trapped by KOH in the center well. Table 4 shows that the radioactivity became increasingly acid volatile with time. This was interpreted to mean that the two outer [^{35}S]-sulfonate groups were released as free bisulfite ions from the trithionate molecule.

Stoichiometric relationship between H_2 utilized and thiosulfate formed. Table 5 shows the stoichiometry of thiosulfate formation and molecular H_2 utilization. An equimolar amount of hydrogen was consumed for every mole of thiosulfate formed. This was evidence that a direct relationship existed between hydrogen utilization and thiosulfate formation. If the recycling hypothesis is valid, the reaction should proceed according to the following equation:

$$\overset{*}{HSO_3^-} + O_3S\text{-}\overset{*}{S}\text{-}SO_3^{2-} \xrightarrow{2e^-} O_3\overset{*}{S}\text{-}\overset{*}{S}^{2-} + HSO_3^- + SO_3^{2-}$$

This equation shows that for every bisulfite consumed in the reaction, forming thiosulfate, two more (bi)sulfite ions are generated. A kinetic experiment was conducted to obtain additional evidence for the recycling hypothesis. Figure 1 shows the rates of hydrogen consumption when 11 μmol of trithionate was incubated with varying amounts of bisulfite. At high concentrations of both bisulfite (10 μmol) and trithionate, the rate of hydrogen utilization was linear with time. When the bisulfite concentration was decreased by 10- and 20-fold, there was a concomitant decrease in the initial velocity of the reaction. As the reaction proceeded, the

TABLE 4. *Effect of time on bisulfite population*[a]

Time (min)	Bisulfite (cpm)
20	67,220
40	120,316
60	155,176
80	185,340
Control[b]	6,436

[a] TF concentration, 2.5 mg; $K_2S_3O_6$ concentration, 10.0 μmol, 7.5×10^4 cpm/μmol (sulfonate labeled); $NaHSO_3$ concentration, 5.0 μmol. KOH trap for acid-volatilized bisulfite. Standard assay conditions.
[b] Eighty minutes without TF.

TABLE 5. *Stoichiometry of thiosulfate formation and H_2 uptake with TF*[a]

Time (min)	Thiosulfate formed (μmol) (a)	H_2 utilized (μmol) (b)	Ratio of a/b
30	2.63	2.8	0.94
60	4.87	4.4	1.11
90	7.24	7.1	1.02
120	9.46	9.1	1.04

[a] TF concentration, 0.41 mg; $K_2S_3O_6$ concentration, 11.0 μmol; $NaHSO_3$ concentration, 10.0 μmol. Standard assay conditions.

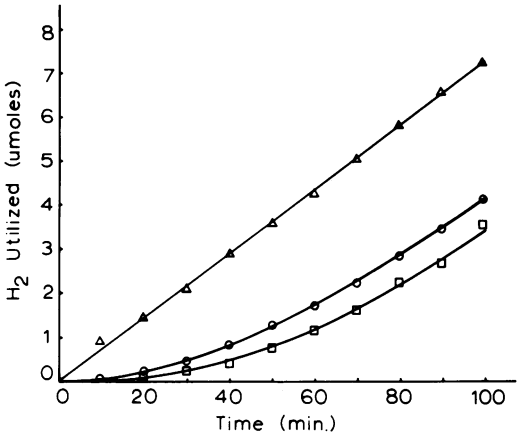

FIG. 1. *Kinetics of H_2 consumption in TF reaction. TF concentration, 0.41 mg; $K_2S_3O_6$ concentration, 11.0 μmol. $NaHSO_3$ concentration (symbols): △, 10.0 μmol; ○, 1.0 μmol; □, 0.5 μmol.*

rate of hydrogen utilization gradually increased until it paralleled that exhibited by the reaction containing high concentrations of both substrates. These results are in agreement with our hypothesis that bisulfite ions are generated from trithionate during thiosulfate formation. For every 1 mol of thiosulfate formed, 2 mol of bisulfite is generated. After a suitable reaction time, the concentration of bisulfite increases in the reaction mixture to the point where the maximum velocity is reached.

Reconstruction of the thiosulfate-forming system by use of natural electron carriers. Cytochrome c_3 was capable of interposing electrons between hydrogenase and TF, forming thiosulfate (Table 6). Flavodoxin did not function in this reaction, presumably because it did not interact with hydrogenase. When both carriers were incorporated, no further increase in thiosulfate was noted. In the bisulfite reductase system, i.e., the formation of trithionate from bisulfite, it was seen that both cytochrome c_3 and flavodoxin were required. We concluded that flavodoxin participates in the bisulfite reductase assay, whereas cytochrome c_3 participates in the formation of thiosulfate in the TF-catalyzed reaction. Figure 2 illustrates our version of the mechanism for TF catalysis, and Fig. 3 summarizes the roles of flavodoxin and cytochrome c_3 in the reduction of bisulfite to thiosulfate by the thiosulfate-forming system.

DISCUSSION

The results obtained from this study show that bisulfite can be reduced to thiosulfate through a pathway involving trithionate as an intermediate compound. Kobayashi et al. (20) proposed that bisulfite was reduced to sulfide by the sequence bisulfite to trithionate to thiosulfate to sulfide. This study shows that the reduction of trithionate to thiosulfate occurs by a mechanism consisting of a nucleophilic attack by bisulfite on the sulfane atom of trithionate, forming thiosulfate and releasing two bisulfite ions (Fig. 2). Cytochrome c_3 participates in this reaction by transferring electrons from hydrogenase to one of the leaving sulfonate groups, probably in a concerted fashion with thiosulfate formation. This reaction is postulated to be analogous to the cyanolysis of trithionate by a cyanide ion. In this reaction, cyanide combines with the trithionate sulfane atom, releasing two sulfonate groups. One leaves as sulfite,

TABLE 6. *Native electron carriers for the thiosulfate-forming system*[a]

Enzyme	Carrier	Substrate	Product (μmol)			H_2 utilized (μmol)
			$S_3O_6^{2-}$	$S_2O_3^{2-}$	S^{2-}	
TF	Cytochrome c_3	$HSO_3^- + S_3O_6^{2-}$		5.46	0	5.99
TF	Flavodoxin	$HSO_3^- + S_3O_6^{2-}$		0	0	0
TF	Cytochrome c_3 + flavodoxin	$HSO_3^- + S_3O_6^{2-}$		5.66	0	5.50
Bisulfite reductase	Cytochrome c_3	HSO_3^-	0	0	0	0
Bisulfite reductase	Flavodoxin	HSO_3^-	0	0	0	0
Bisulfite reductase	Cytochrome c_3 + flavodoxin	HSO_3^-	2.04	0	0.02	1.67
Complete[b]	Cytochrome c_3	HSO_3^-	0	0	0.02	0.49
Complete	Flavodoxin	HSO_3^-	0.13	0	0	0.15
Complete	Cytochrome c_3 + flavodoxin	HSO_3^-	0.66	2.10	0.02	3.88
Complete	Methyl viologen	HSO_3^-	0.20	4.60	0.28	9.04

[a] Bisulfite reductase concentration, 1.16 mg; TF concentration, 0.39 mg; cytochrome c_3 concentration, 2.1 mg; flavodoxin concentration, 2.6 mg; $NaHSO_3$ concentration, 10.0 μmol; $K_2S_3O_6$ concentration, 11.0 μmol; incubation time, 90 min.

[b] Bisulfite reductase plus TF.

whereas the other leaving group is sulfate. If an analogy is drawn from this, one could assume that one of the leaving sulfonate groups is reduced to sulfite in the TF-catalyzed reaction.

Employing natural electron carriers, we have succeeded in reconstructing the thiosulfate-forming pathway of *D. vulgaris* (Fig. 3). Both flavodoxin and cytochrome c_3 were necessary for the reduction of bisulfite to thiosulfate. Cytochrome c_3 functions at the TF level, whereas flavodoxin acts during trithionate formation from bisulfite. In this reaction, cytochrome c_3 transfers electrons from hydrogenase to flavodoxin. Trithionate does accumulate, but is immediately utilized by the TF reaction, forming thiosulfate. Thiosulfate reductase, which was shown to require cytochrome c_3 (6, 13), completes the pathway, forming sulfide. Earlier work by Suh and Akagi (31) showed that ferredoxin and cytochrome c_3 were both required for thiosulfate formation from bisulfite. Since in this study flavodoxin was used, we conclude that either flavodoxin or ferredoxin can participate in the bisulfite reductase-catalyzed reaction.

Recently, several investigators have questioned the validity of a dissimilatory pathway. Kobayashi et al. (19) suggested that bisulfite reductase reduced (bi)sulfite to sulfide and that trithionate and thiosulfate formation involved the nonenzymatic reaction of sulfite with the sulfoxylate and elemental sulfur precursors of sulfide. Chambers and Trudinger (7) further questioned the importance of trithionate and thiosulfate as intermediates in dissimilatory sulfate reduction. These investigators implied that assimilatory and dissimilatory sulfite reduction may closely parallel one another.

This study clearly shows the importance of both trithionate and thiosulfate in dissimilatory reduction. Although not a trithionate reductase as proposed by Kobayashi et al. (20), TF represents the first reported case where a purified protein has been shown to utilize trithionate in the formation of thiosulfate. We have also shown that the reduction of these inorganic sulfur atoms is mediated by natural electron carriers. We conclude from these findings that a dissimilatory pathway is operative

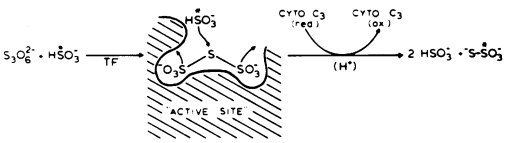

FIG. 2. *Proposed mechanism for TF in D. vulgaris.*

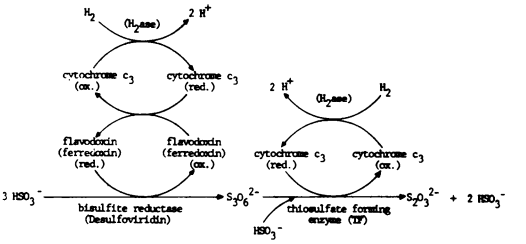

FIG. 3. *Thiosulfate-forming pathway in D. vulgaris.*

and that trithionate and thiosulfate are intermediates connecting the reduction of bisulfite to sulfide.

It is now apparent that several modes of dissimilatory reduction may be possible in the sulfate-reducing bacteria. Since trithionate reductase activity has been observed in this laboratory with crude extracts (unpublished data), one possible pathway would be as proposed by Kobayashi et al. (20). Another pathway may involve bisulfite reductase II (9). Unlike assimilatory sulfite reductases that form only sulfide (5, 21, 23, 35), bisulfite reductase II forms both sulfide and thiosulfate. Thiosulfate could subsequently be reduced to sulfide by thiosulfate reductase (12, 13, 25).

This study shows that another mechanism for thiosulfate formation exists in these microorganisms. It is possible that a strict regulatory process may be operating in these organisms, and the pathway utilized for the metabolism of various sulfur species may be dictated by certain environmental conditions in which the bacteria find themselves.

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LITERATURE CITED

1. **Akagi, J. M.** 1967. Electron carriers for the phosphoroclastic reaction of *Desulfovibrio desulfuricans*. J. Biol. Chem. 242:2478–2483.
2. **Akagi, J. M., and V. Adams.** 1973. Isolation of a bisulfite reductase activity from *Desulfotomaculum nigrificans* and its identification as the carbon monoxide-binding pigment P582. J. Bacteriol. 116:392–396.
3. **Akagi, J. M., and L. L. Campbell.** 1962. Studies on thermophilic sulfate-reducing bacteria. III. Adenosine triphosphate-sulfurylase of *Clostridium nigrificans* and *Desulfovibrio desulfuricans*. J. Bacteriol. 84:1194–1201.
4. **Akagi, J. M., M. Chan, and V. Adams.** 1974. Observations on the bisulfite reductase (P582) isolated from *Desulfotomaculum nigrificans*. J. Bacteriol. 120:240–244.

5. **Asada, K., G. Tamura, and R. S. Bandurski.** 1969. Methyl viologen-linked sulfite reductase from spinach leaves. J. Biol. Chem. 244:4904-4915.
6. **Bruschi, M., C. E. Hatchikian, L. A. Golovleva, and J. LeGall.** 1977. Purification and characterization of cytochrome c_3, ferredoxin, and rubredoxin isolated from *Desulfovibrio desulfuricans* Norway. J. Bacteriol. 129:30-38.
7. **Chambers, L. A., and P. A. Trudinger.** 1975. Are thiosulfate and trithionate intermediates in dissimilatory sulfate reduction? J. Bacteriol. 123:36-40.
8. **Drake, H. L., and J. M. Akagi.** 1976. Product analysis of bisulfite reductase activity isolated from *Desulfovibrio vulgaris*. J. Bacteriol. 126:733-738.
9. **Drake, H. L., and J. M. Akagi.** 1976. Purification of a unique bisulfite-reducing enzyme from *Desulfovibrio vulgaris*. Biochem. Biophys. Res. Commun. 71:1214-1219.
10. **Dubourdieu, M., and J. LeGall.** 1970. Chemical study of two flavodoxins extracted from sulfate reducing bacteria. Biochem. Biophys. Res. Commun. 38:965-972.
11. **Fogo, J. K., and M. Popowski.** 1949. Spectrophotometric determination of hydrogen sulfide. Anal. Chem. 21:732-734.
12. **Haschke, R. H., and L. L. Campbell.** 1971. Thiosulfate reductase of *Desulfovibrio vulgaris*. J. Bacteriol. 106:603-607.
13. **Hatchikian, E. C.** 1975. Purification and properties of thiosulfate reductase from *Desulfovibrio gigas*. Arch. Microbiol. 105:249-256.
14. **Horio, T., and M. D. Kamen.** 1961. Preparation and properties of three pure crystalline bacterial haem proteins. Biochim. Biophys. Acta 48:266-286.
15. **Ishimoto, M., and J. Koyama.** 1957. Biochemical studies on sulfate-reducing bacteria. VI. Separation of hydrogenase and thiosulfate reductase and partial purification of cytochrome and green pigment. J. Biochem. (Tokyo) 44:233-242.
16. **Jones, H. E., and G. W. Skyring.** 1974. Reduction of sulfite to sulfide catalyzed by desulfoviridin from *Desulfovibrio gigas*. Aust. J. Biol. Sci. 27:7-14.
17. **Kelly, D. P., L. A. Chambers, and P. A. Trudinger.** 1969. Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal. Chem. 41:898-901.
18. **Knight, E., and R. W. F. Hardy.** 1966. Isolation and characteristics of flavodoxin from nitrogen-fixing *Clostridium pasteurianum*. J. Biol. Chem. 241:2752-2756.
19. **Kobayashi, K., Y. Seki, and M. Ishimoto.** 1974. Biochemical studies on sulfate reducing bacteria. XIII. Sulfite reductase from *Desulfovibrio vulgaris*. Mechanism of trithionate, thiosulfate, and sulfide formation and enzymatic properties. J. Biochem. (Tokyo) 75:519-529.
20. **Kobayashi, K., S. Tachibana, and M. Ishimoto.** 1969. Intermediary formation of trithionate in sulfite reduction by a sulfate-reducing bacterium. J. Biochem. 65:155-157.
21. **Lee, L. P., J. LeGall, and H. D. Peck.** 1973. Isolation of assimilatory- and dissimilatory-type sulfite reductases from *Desulfovibrio vulgaris*. J. Bacteriol. 115: 529-542.
22. **Lee, J. P., and H. D. Peck.** 1971. Purification of the enzyme reducing bisulfite to trithionate from *Desulfovibrio gigas* and its identification as desulfoviridin. Biochem. Biophys. Res. Commun. 45:583-589.
23. **LeGall, J., and J. R. Postgate.** 1973. The physiology of sulfate-reducing bacteria. Adv. Microb. Physiol. 10:81-133.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
25. **Nakatsukasa, W., and J. M. Akagi.** 1969. Thiosulfate reductase isolated from *Desulfotomaculum nigrificans*. J. Bacteriol. 98:429-433.
26. **Roy, A. B., and P. A. Trudinger.** 1970. The biochemistry of inorganic compounds of sulfur. Cambridge University Press, London.
27. **Schmidt, A.** 1973. Sulfate reduction in a cell-free system of *Chlorella*. The ferredoxin dependent reduction of a protein-bound intermediate by a thiosulfonate reductase. Arch. Microbiol. 93:29-52.
28. **Schmidt, A., W. R. Abrams, and J. A. Schiff.** 1974. Reduction of adenosine 5'-phosphosulfate to cysteine in extracts from *Chlorella* and mutants blocked for sulfate reduction. Eur. J. Biochem. 47:423-434.
29. **Siegel, L. M.** 1975. Biochemistry of the sulfur cycle, p. 217-286. *In* D. M. Greenberg (ed.), Metabolic pathways. Metabolism of sulfur compounds, vol. 7. Academic Press Inc., New York.
30. **Stranks, D. R., and R. G. Wilkins.** 1957. Isotopic tracer investigations of mechanisms and structure in inorganic chemistry. Chem. Rev. 57:743-866.
31. **Suh, B., and J. M. Akagi.** 1969. Formation of thiosulfate from sulfite by *Desulfovibrio vulgaris*. J. Bacteriol. 99:210-215.
32. **Tsang, M. L. S., and J. A. Schiff.** 1976. Studies of sulfate utilization by algae. 17. Reactions of the adenosine 5'-phosphosulfate (APS) sulfotransferase from *Chorella* and studies of model reactions which explain the diversity of side products with thiols. Plant Cell Physiol. 17:1209-1220.
33. **Tsang, M. L. S., and J. A. Schiff.** 1976. Sulfate-reducing pathway in *Escherichia coli* involving bound intermediates. J. Bacteriol. 125:923-933.
34. **Whitaker, J. R.** 1963. Determination of molecular weight of proteins by gel filtration on Sephadex. Anal. Chem. 35:1950-1953.
35. **Yoshimoto, A., and R. Sato.** 1970. Studies on yeast sulfite reductase. III. Further characterization. Biochim. Biophys. Acta 220:190-205.