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REDUCTION OF BISULFITE BY THE TRITHIONATE PATHWAY BY CELL EXTRACTS FROM DESULFOTOMACULUM NIGRIFICANS

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SUMMARY: Bisulfite was reduced to sulfide by cell extracts of Desulfotomaculum nigrificans. When trithionate was added to reaction mixtures reducing bisulfite, sulfide formation was inhibited with accumulation of thiosulfate. The thiosulfate reductase activity of cell extracts was found to be inhibited by trithionate. Trithionate alone was reduced to thiosulfate and purified bisulfite reductase (P582) was not affected by trithionate. It is concluded that the pathway for bisulfite reduction in Dt. nigrificans includes trithionate and thiosulfate as intermediate compounds.

Sulfate-reducing bacteria belonging to the genera Desulfotomaculum and Desulfovibrio reduce inorganic sulfate to sulfide by a pathway that involves bisulfite as one of the intermediate compounds. The pathway for bisulfite reduction to sulfide has not been established although two possibilities have been considered. One possibility is a six-elections reduction to sulfide catalyzed by a single enzyme, bisulfite reductase (1,2,3), while another view advocates the formation of intermediates such as trithionate and thiosulfate (4-11); the latter pathway would require at least three separate enzymes operating in tandem. This study presents evidence that supports the hypothesis that Desulfotomaculum nigrificans reduces bisulfite to sulfide by a dissimilatory pathway involving trithionate and thiosulfate as intermediates.

METHODS

Dt. nigrificans, ATCC 19998, was grown in lactate-sulfate medium and harvested as previously described (12). Cell extracts were prepared with a French pressure cell as reported earlier (12). Desulfovibrio vulgaris hydrogenase was prepared as described previously (4). All enzyme assays were conducted in 8 ml Warburg flasks using standard manometric techniques. Bisulfite reductase (P582) from Dt. nigrificans was purified as described earlier (13). Protein was measured according to Lowry et al. (14) using bovine serum albumin as a standard. Trithionate was synthesized as previously described (15). Hydrogen sulfide was estimated by developing the filter paper from the center well of Warburg flasks according to Fogo and Popowski (16). Samples for thiosulfate and/or trithionate were prepared by diluting the

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contents of reaction mixtures to 2.5 ml with water. One ml aliquots were removed for thiosulfate and trithionate analyses according to Kelly et al. (17).

RESULTS AND DISCUSSION

When crude extracts of Dt. nigrificans were incubated with bisulfite or thiosulfate, methyl viologen and hydrogenase under a hydrogen atmosphere, sulfide was rapidly formed (Table 1). With bisulfite serving as the substrate it is seen that thiosulfate in addition to sulfide was formed. When trithionate was incorporated into reaction mixtures containing either bisulfite or thiosulfate, sulfide formation was inhibited. Furthermore, trithionate alone was reduced to thiosulfate but not to sulfide. These data show that, at the concentrations used, trithionate was inhibitory to sulfide formation from bisulfite and thiosulfate, presumably at the thiosulfate reduction step. To determine how trithionate inhibited sulfide formation from bisulfite the effect of time on

Table 1.
Effect of trithionate ($S_3O_6^{2-}$) on bisulfite (HSO_3^-) and thiosulfate ($S_2O_3^{2-}$) reduction by cell extracts from Dt. nigrificans

System		µmoles H_2S	µmoles $S_2O_3^{2-}$
HSO_3^-			
complete		1.1	1.2
"	+ S_3O_6, 5mM	0	2.3
"	" , 2.5 mM	0	2.1
"	- HSO_3^- + $S_3O_6^{2-}$, 5mM	0	2.1
"	" " , 2.5mM	0	1.8
$S_2O_3^{2-}$			
complete		1.3	-
"	+ $S_3O_6^{2-}$, 5mM	0	-
"	" , 2.5mM	0.1	-

Complete system contained in µmoles: HSO_3^- or $S_2O_3^{2-}$, 10; methyl viologen, 1.0; potassium phosphate buffer, pH7.0, 100; hydrogenase, 1mg; cell extract, 6 mg in a total volume of 1.1 ml. Center well contained 0.1 ml of 20% $CdCl_2$ absorbed on fluted filter paper. Gas phase, H_2; temperature, 37°C; incubation time, 30 min.

Table 2.
Effect of time on trithionate inhibition of bisulfite reduction by Dt. nigrificans extracts

	μmoles					
	H_2S		$S_2O_3^{2-}$		$S_3O_6^{2-}$	
Time (min.)	$-S_3O_6^{2-}$	$+S_3O_6^{2-}$	$-S_3O_6^{2-}$	$+S_3O_6^{2-}$	$-S_3O_6^{2-}$	$+S_3O_6^{2-}$
0	0	0	0	0	0	5.8
15	0.4	0	1.4	2.8	0	3.4
30	0.7	0	1.4	2.8	0	3.4
45	1.1	0.1	1.6	2.8	0	0.4
60	1.4	0.2	1.4	2.8	0	0.4
90	1.5	0.3	1.4	2.8	0	0.4

Conditions same as in Table 1. Bisulfite concentration, 10 μmoles; trithionate concentration when incorporated, 6 μmoles.

bisulfite reduction in the presence and absence of trithionate was examined. Table 2 shows that crude extracts reduced bisulfite alone to sulfide and thiosulfate. In the presence of trithionate, thiosulfate accumulated and sulfide formation was drastically reduced. The disappearance of trithionate from the reaction mixture concomitant with the accumulation of thiosulfate indicated that trithionate was reduced to thiosulfate. An identical experiment with thiosulfate and trithionate showed thiosulfate concentration to increase with time in the reaction mixtures without any formation of sulfide. Only when the trithionate concentration decreased to a point where it was no longer detectable did sulfide formation become evident. The effect of trithionate concentration on thiosulfate reduction is shown in Table 3. Under the conditions employed, 2 mM trithionate completely inhibited thiosulfate reduction while 1 mM trithionate only resulted in 8% inhibition.

The reduction of bisulfite to trithionate has been reported to be catalyzed by a bisulfite reductase (P582) in Dt. nigrificans (3,7). To determine if trithionate affected P582 activity, the effect of trithionate concentration on bisulfite reduction by P582 was tested. Table 4 shows that P582 reduced bisulfite mainly to trithionate with a smaller amount of sulfide formed. Virtually no activity

Table 3.
Effect of trithionate concentration
on thiosulfate reduction

$S_3O_6^{2-}$ conc. (mM)	H_2S (µmoles)	% inhibition
0	1.1	0
2.0	0	100
1.0	0.9	8
0.5	1.1	0

Conditions same as in Table 1. Thiosulfate concentration 5 µmoles.

was observed with trithionate alone and when trithionate plus bisulfite were incubated together with P582, no inhibition of bisulfite reduction occurred. These results demonstrated that trithionate did not affect P582 activity. Another view for bisulfite reductase catalysis is that this enzyme reduces bisulfite directly to sulfide and that trithionate is formed nonenzymatically (8,9,10). This hypothesis does not consider the occurrence of a pathway that involves trithionate and thiosulfate as intermediates. If bisulfite reductase is responsible for reducing bisulfite to sulfide directly as the principal mechanism, this enzyme should reduce bisulfite to sulfide as rapidly or even faster than crude extracts containing this enzyme. Table 5 shows that crude extracts reduced bisulfite to sulfide more quickly and to a greater extent than P582. Assuming

Table 4.
Effect of trithionate on bisulfite reductase
(P582) activity of <u>Dt. nigrificans</u>

	µmoles		
System	$S_3O_6^{2-}$	$S_2O_3^{2-}$	H_2S
complete + HSO_3^-	1.7	0.1	0.8
" + $S_3O_6^{2-}$	3.7	0	0.2
" + HSO_3^- + $S_3O_6^{2-}$	4.9	0.1	1.0

Complete system contained in µmoles: potassium phosphate buffer, pH7.0, 100; methyl viologen, 1.0; hydrogenase, 1mg; bisulfite reductase, 1.1 mg. Concentration of bisulfite, 10 µmoles; trithionate, 4.0 µmoles. Time, 30 min.

Table 5.
Sulfide formation from bisulfite
by P582 and crude extracts

Time (min.)	H_2S(μmoles) crude extract	P582
15	0.5	0
30	0.8	0.1
45	1.1	0.2

Conditions same as for Table 1. Concentration of crude extract was 5mg; P582, 1.1 mg.

that P582 constitutes approximately 5 to 10 percent of crude extracts, the experiment described in Table 5 was conducted with P582 at a protein concentration which was 22 percent of the crude extract. If P582 is responsible for reducing bisulfite to sulfide, it should be expected that this purified enzyme will form sulfide faster than crude extracts.

From this study, several lines of evidence provided strong support for bisulfite being reduced to sulfide through the dissimilatory (trithionate) pathway. These are (i) inhibition of sulfide formation from bisulfite by trithionate with accumulation of thiosulfate (ii) trithionate did not inhibit P582 activity, i.e., bisulfite was still reduced to trithionate and sulfide (iii) trithionate itself was reduced to thiosulfate by extracts, and (iv) crude extracts were more efficient in reducing bisulfite to sulfide than P582. The data indicate that in the dissimilatory pathway bisulfite is initially reduced to trithionate by bisulfite reductase. The trithionate is rapidly converted to thiosulfate by a trithionate reductase system. This prevents any accumulation of trithionate which would inhibit the subsequent reaction, thiosulfate reduction. For this reason, it is difficult to detect and/or isolate trithionate from reaction mixtures reducing bisulfite to sulfide. The terminal step, reduction of thiosulfate, is catalyzed by thiosulfate reductase. This enzyme in Dt. nigrificans is apparently quite sensitive to trithionate. It concluded that Dt. nigrificans reduces bisulfite to sulfide by the dissimilatory route (3,11-17) which includes trithionate and thiosulfate as intermediates.

It was interesting to note that extracts from D. vulgaris were not affected by trithionate during bisulfite and thiosulfate reduction processes.

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