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Characterization of a Trithionate Reductase System from *Desulfovibrio vulgaris*

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A trithionate reductase system was isolated and purified from extracts of *Desulfovibrio vulgaris*. This system reduced trithionate to thiosulfate and consisted of two proteins. One was bisulfite reductase, an enzyme that reduces bisulfite to trithionate, and the second component was designated TR-1. Both enzymes were required to reduce trithionate to thiosulfate. Flavodoxin and cytochrome c_3 from *D. vulgaris* were tested for their ability to function as electron carriers during trithionate reduction. When molecular hydrogen was the source of electrons for the reduction, both flavodoxin and cytochrome c_3 were required. In contrast, when the pyruvate phosphoroclastic system was the reductant, flavodoxin alone participated as the electron carrier. The results indicate that flavodoxin, but not cytochrome c_3, interacted with the trithionate reductase system. The cytochrome in the hydrogenase-linked assay functioned as an electron carrier between hydrogenase and flavodoxin.

Sulfate-reducing bacteria such as *Desulfovibrio vulgaris* reduce inorganic sulfate to sulfide by a dissimilatory process involving bisulfite as one of the intermediates (17). The following steps leading to sulfide formation are not completely understood. Kobayashi et al. (11, 12) described the sequential formation of trithionate and thiosulfate as intermediates during bisulfite reduction. They proposed that bisulfite was reduced to sulfide by the following pathway:

$$n\mathrm{HSO_3^-} \xrightarrow{2e^-} \underset{\text{trithionate}}{\mathrm{O_3S{-}S{-}SO_3^{2-}}} \xrightarrow[-\mathrm{SO_3^{2-}}]{2e^-}$$
bisulfite

$$\mathrm{S{-}SO_3^{2-}} \xrightarrow[-\mathrm{SO_3^{2-}}]{2e^-} \mathrm{HS^-}$$
thiosulfate sulfide

Support for this trithionate pathway came from Suh and Akagi (21) when they isolated thiosulfate from reaction mixtures reducing bisulfite. Furthermore, Lee and Peck (14) isolated and identified the green pigment, desulfoviridin, as an enzyme that reduced bisulfite to trithionate. They named this enzyme bisulfite reductase. The overall process involving the sequential reduction of bisulfite to trithionate to thiosulfate to sulfide would require the enzymes bisulfite reductase, trithionate reductase, and thiosulfate reductase, respectively. Of these, bisulfite reductase and thiosulfate reductase have been purified from sulfate-reducing bacteria (2, 5–9, 11, 12, 14, 15, 19, 22), but a trithionate reductase has not been isolated. Drake and Akagi (4) reported on the existence of a thiosulfate-forming enzyme in *D. vulgaris* which formed thiosulfate from bisulfite plus trithionate. Whether this enzyme actually participates in the dissimilatory reduction remains to be seen. If the trithionate pathway is used by sulfate-reducing bacteria, an enzyme that reduces trithionate to thiosulfate must be present in these organisms. This study presents evidence for the existence of an enzyme system that is capable of reducing trithionate to thiosulfate.

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MATERIALS AND METHODS

Organisms. *D. vulgaris* NCIB 8303 was grown and harvested as previously described (3). Cell extracts were obtained as described earlier (21).

Enzyme assays. The standard assay for trithionate reductase activity was performed in 8-ml Warburg flasks by using standard manometric techniques. The particulate hydrogenase of *D. vulgaris* was prepared as described previously (21). Unless otherwise indicated, the standard reaction mixture contained 100 μmol of potassium phosphate buffer (pH 7.0), 1.0 μmol of methyl viologen, 5.0 μmol of $K_2S_3O_6$, 0.1 mg of hydrogenase, and enzyme in a total volume of 1.0 ml. The center well contained 0.1 ml of a 20% $CdCl_2$ solution saturated on fluted filter paper; the gas phase was H_2, and the temperature was 37°C. To analyze for thiosulfate, we transferred the flask contents to test tubes and developed them as described by Kelly et al. (10). The phosphoroclastic system and flavodoxin were prepared as described by Drake and Akagi (5). Cytochrome c_3 was prepared as previously described (5). Sirochlorohydrin was extracted with acetone hydrochloride as described by Murphy and Siegel (18). The phosphoroclastic reaction was coupled to the trithionate reductase system as described previously (5) for the standard reaction mixture.

Purification of enzymes. All purification steps were carried out at 0 to 4°C and, unless otherwise indicated, potassium phosphate buffer (PB) was at pH 7.0. Bisulfite reductase (desulfoviridin) was purified as described earlier (5). The protein fraction designated TR-1 was purified from crude extracts that were centrifuged at 55,000 × *g* for 2 h to remove particulate matter. The soluble fraction (210 ml) was heated quickly to 60°C in a boiling water bath and held at that temperature for 60 s. It was cooled rapidly in an ice bath and centrifuged at 27,000 × *g* for 20 min to remove denatured proteins. The supernatant fraction, containing 26 mg of protein per ml, was applied to a DEAE-cellulose column (2.6 by 15 cm) equilibrated against 1 mM PB. The column was washed successively with 400 ml of 0.01 M PB, 200 ml of 0.1 M PB, 50 ml of 0.3 M PB, and 200 ml of 0.5 M PB. The

TABLE 1. Summary of TR-1 purification

Fraction[a]	Vol (ml)	Protein (mg/ml)	Sp act[b]	Total units[c]	% Recovery	Fold increase
Crude	210	610.0	0.14	1,793	100	
USS	150	35.6	0.28	1,495	83	2
HES	110	25.9	0.42	1,196	67	3
DEAE 1	120	8.4	0.58	585	33	4.1
G-25	45	18.7	0.55	463	26	3.9
DEAE 2	100	2.8	1.28	358	20	9
G-100	42	0.34	3.0	43	2.4	21.4
DEAE 3	30	0.15	8.5	38	2.1	60.7

[a] USS, Untreated Spinco supernatant; HES, heated extract supernatant.
[b] Specific activity is expressed as μmol of thiosulfate formed per 30 min.
[c] Units per mg of protein were determined by the hydrogenase standard assay.

brown fraction that eluted with 0.1 M PB was concentrated with an Amicon EC-20 unit (Amicon Corp., Lexington, Mass.) with a PM-30 filter. The concentrated material was passed through a Sephadex G-25 gel filtration column (4 by 25 cm) equilibrated against water and then passed through an Amberlite CG-50 column (2.5 by 3 cm) (Rohm & Haas Co., Philadelphia, Pa.) to remove residual cytochrome c_3. The unabsorbed fraction was applied to a second DEAE column (2.6 by 7 cm) equilibrated against 1 mM PB. The column was washed with 100 ml of 0.04 M PB, and an amber fraction was eluted with 0.07 M PB. This fraction was concentrated by ultrafiltration with a PM-30 filter and applied to a Sephadex G-100 column (1.6 by 100 cm) equilibrated against 0.01 M PB. When the same buffer was passed through the column, two major yellow bands and a faint yellow band between the two were observed. The middle faint yellow band, which contained trithionate-reducing activity, was concentrated and applied to a third DEAE-cellulose column (1.6 by 3 cm). The column was washed with 30 ml of 0.02 M PB, and the enzyme was removed with 0.05 M PB. This enzyme was concentrated by ultrafiltration and stored at −20°C. The purification of TR-1 is summarized in Table 1. From 100 g of cell paste, the yield of TR-1 was 4.5 mg. The criterion used for the purity of TR-1 was the observation of a single band after polyacrylamide gel electrophoresis.

Analytical determinations. Trithionate and thiosulfate were analyzed as described by Kelly et al. (10). Protein was estimated as described by Lowry et al. (16) with bovine serum albumin as a standard. Trithionate was synthesized as described earlier (4). Sodium bisulfite solutions were prepared fresh in 1 mM EDTA before use. Analytical polyacrylamide gel electrophoresis was performed as described by Buchler Instruments (Div., Nuclear-Chicago Corp., Fort Lee, N.J.), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (13). Molecular weight approximations by Sephadex chromatography were obtained as described by Whitaker (23).

RESULTS

Crude extracts of *D. vulgaris* contained trithionate reductase activity as evidenced by thiosulfate and sulfide formation under standard assay conditions. After the first DEAE chromatography step (Table 1), the individual protein fractions eluting with their respective buffer concentrations were negative for trithionate reductase activity. Testing different combinations of the eluted fractions, we found that trithionate reductase activity was restored when the green 0.3 M PB fraction and the 0.1 M PB fraction were both present in the reaction mixture. The exact composition of the trithionate reductase activity was ultimately determined to be bisulfite reductase (desulfoviridin) and a factor that we designated TR-1. Both enzymes were required to reduce trithionate to thiosulfate.

TABLE 2. Requirement for bisulfite reductase and TR-1 for trithionate reduction

System[a]	μmol of $S_2O_3^{2-}$
TR-1 plus bisulfite reductase	0.80
Boiled TR-1 plus bisulfite reductase	0.3
TR-1 plus boiled bisulfite reductase	0.1
TR-1 alone	0.1
Bisulfite reductase alone	0.1

[a] Standard assay conditions were used. Bisulfite reductase, 2 mg/ml; TR-1, 0.25 mg/ml; time, 60 min.

General properties of TR-1. Solutions of TR-1 remained stable during storage at −20°C for at least 2 months, whereas repeated freezing and thawing or storage at 4°C resulted in a progressive decrease in activity during this time. The purified enzyme was stable to lyophilization, but dialysis for 24 h at 4°C under any condition caused a considerable loss of activity. Purified TR-1 was colorless and exhibited no unusual absorption peaks in the visible or UV regions of the spectrum. The molecular weight of TR-1 was estimated to be 30,000.

Two-component trithionate reductase system. Under the conditions of the standard assay, both bisulfite reductase and TR-1 were required for trithionate reduction. The native forms of both enzymes were necessary, since boiling either fraction resulted in a considerable loss of trithionate reductase activity (Table 2). In addition, sirochlorohydrin (18) was inactive when it was substituted for bisulfite reductase in the assay.

Inhibition of trithionate reduction by sulfhydryl reagents. Several sulfhydryl reagents were tested to determine if sulfhydryl groups were involved in catalyzing trithionate reduction. The inhibitors used were *p*-hydroxymercuribenzoate, iodoacetate, *N*-ethylmaleimide, silver nitrate, and mercurous chloride. At a concentration of 1 mM, all of the reagents inhibited the reaction, ranging from 17 (*N*-ethylmaleimide) to 94% (iodoacetate) inhibition. When these reagents were tested with bisulfite reductase reducing bisulfite to trithionate, no inhibition occurred; this indicated that TR-1 was affected by the sulfhydryl reagents. Reversal of inhibition by compounds such as cysteine was not possible, since sulfhydryl compounds react spontaneously with trithionate.

TABLE 3. Effect of flavodoxin and cytochrome c_3 on trithionate reduction[a]

Electron carrier	Electron source	$S_2O_3^{2-}$ formed (μmol)
Methyl viologen	H_2	1.29
Flavodoxin	H_2	0.06
Cytochrome c_3	H_2	0.21
Flavodoxin plus cytochrome c_3	H_2	0.87
Methyl viologen	Pyruvate	0.68
Flavodoxin	Pyruvate	0.83
Cytochrome c_3	Pyruvate	0.20
Flavodoxin plus cytochrome c_3	Pyruvate	0.47

[a] Assay conditions for the hydrogenase and phosphoroclastic systems were as described in the text. Time, 30 min.

Flavodoxin and cytochrome c_3 as electron carriers. The endogenous electron carriers flavodoxin and cytochrome c_3 were purified from crude extracts and tested for their ability to participate in trithionate reduction. The sources of electrons used for this purpose were molecular hydrogen and pyruvate. When hydrogen was used as the electron source, both cytochrome c_3 and flavodoxin were required for trithionate reduction (Table 3). In contrast, when pyruvate was the electron donor, flavodoxin alone was sufficient for the reduction. Previous work from this laboratory showed that the flow of electrons from hydrogen to flavodoxin or ferredoxin required cytochrome c_3, which transferred electrons from hydrogenase to flavodoxin or ferredoxin (4). Cytochrome c_3 can interact with hydrogenase, whereas flavodoxin or ferredoxin is not very effective in coupling with hydrogenase (4). From pyruvate, the flow of electrons is from pyruvate dehydrogenase to flavodoxin or ferredoxin to cytochrome c_3 to hydrogenase (4). Because the phosphoroclastic system contains hydrogenase activity, the decreased amount of thiosulfate formed in the presence of flavodoxin and cytochrome c_3 was presumably due to some of the electrons being directed to hydrogenase (Table 3). These results showed that flavodoxin was the electron carrier participating during trithionate reduction. This was interesting, since we observed that an enzyme which we trivially named "thiosulfate-forming" enzyme (4) reduced trithionate to thiosulfate with cytochrome c_3 participating as the electron carrier.

DISCUSSION

For *D. vulgaris* to reduce bisulfite to sulfide by the trithionate pathway, it must be capable of carrying out the reaction sequence bisulfite to trithionate to thiosulfate to sulfide. The first reaction, catalyzed by bisulfite reductase, has been studied extensively with purified preparations of the enzyme (2, 5, 8, 9, 11, 12, 14, 15, 22). The reduction of thiosulfate to sulfide by thiosulfate reductase represents the terminal step in the overall process of sulfate reduction. This enzyme has been purified from sulfate-reducing bacteria and characterized to some extent (6, 7, 19). This study presents evidence that extracts of *D. vulgaris* contain an enzyme system that reduces trithionate to thiosulfate. This activity, together with the bisulfite and thiosulfate reductases, seemingly satisfies the requirement for the three enzymes making up the trithionate pathway. It should be noted that the trithionate reductase system is not the only one that can reduce trithionate to thiosulfate. An enzyme designated as a thiosulfate-forming enzyme was reported earlier to form thiosulfate from trithionate plus bisulfite (4). The presence of a thiosulfate-forming enzyme in *D. vulgaris* was confirmed; however, it was not detected in extracts of *Desulfovibrio gigas*, suggesting that it is not uniformly distributed among sulfate-reducing bacteria (20).

The effect of sulfhydryl reagents on trithionate reduction demonstrated that a sulfhydryl group(s) is involved in the catalysis. The susceptible fraction was presumably TR-1, since bisulfite reductase was not affected in its ability to reduce bisulfite to trithionate. It seems reasonable to assume that the mechanism of trithionate reduction involves a nucleophilic displacement reaction—the formation of a sulfur-sulfur bond between a TR-1 sulfhydryl group and the bivalent sulfur in trithionate concomitant with the release of one of the sulfonate groups as sulfite. This results in the formation of a thiosulfonate–TR-1 complex which is eventually reduced to thiosulfate plus TR-1. No evidence for this is available, and the description above is purely speculative. The role of bisulfite reductase in this reaction is not understood. One possibility would be that this enzyme orients the trithionate molecule in such a way that TR-1 can interact with the substrate. During the bisulfite-reducing process, perhaps bisulfite reductase reduces bisulfite to form trithionate, and, while the trithionate is still located at the active site of bisulfite reductase, TR-1 reduces it to thiosulfate. This would explain the apparent lack of trithionate accumulation in reaction mixtures reducing bisulfite. To determine whether a complex between bisulfite reductase and TR-1 occurred in solution, the two enzymes were incubated together for a short time, and the mixture was analyzed by analytical polyacrylamide gel electrophoresis. No new protein fraction was observed other than those fractions corresponding to bisulfite reductase and TR-1.

The demonstration of a trithionate reductase system consisting of bisulfite reductase and TR-1 provides evidence for the existence of the trithionate pathway during dissimilatory bisulfite reduction. This two-component system may be the most efficient way for the cell to metabolize an intermediate such as trithionate. This compound was an inhibitor of thiosulfate reductase when its concentration became excessive (1).

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