Characterization of Anaerobic Sulfite Reduction by *Salmonella typhimurium* and Purification of the Anaerobically Induced Sulfite Reductase

PATRICK C. HALLENBECK,¹ MARTA A. CLARK,² AND ERICKA L. BARRETT^{3*}

Département de Microbiologie et Immunologie, Université de Montréal, Montréal, Québec, Canada H3C 3J7,¹ and Department of Bacteriology² and Department of Food Science,³ University of California, Davis, California 95616

Received 30 September 1988/Accepted 5 March 1989

Mutants of *Salmonella typhimurium* that lack the biosynthetic sulfite reductase (*cysI* and *cysJ* mutants) retain the ability to reduce sulfite for growth under anaerobic conditions (E. L. Barrett and G. W. Chang, J. Gen. Microbiol., 115:513–516, 1979). Here we report studies of sulfite reduction by a *cysI* mutant of *S. typhimurium* and purification of the associated anaerobic sulfite reductase. Sulfite reduction for anaerobic growth did not require a reducing atmosphere but was prevented by an argon atmosphere contaminated with air (<0.33%). It was also prevented by the presence of 0.1 mM nitrate, which argues against a strictly biosynthetic role for anaerobic sulfite reduction. Anaerobic growth in liquid minimal medium, but not on agar, was found to require additions of trace amounts (10^{-7} M) of cysteine. Spontaneous mutants that grew under the argon contaminated with air also lost the requirement for 10^{-7} M cysteine for anaerobic growth in liquid. A role for sulfite reduction in anaerobic energy generation was contraindicated by the findings that sulfite reduction did not improve cell yields, and anaerobic sulfite reductase activity was greatest during the stationary phase of growth. Sulfite reductase was purified from the cytoplasmic fraction of the anaerobically grown *cysI* mutant and was purified 190-fold. The most effective donor in crude extracts was NADH. NADPH and methyl viologen were, respectively, 40 and 30% as effective as NADH. Oxygen reversibly inhibited the enzyme. Two high-molecular-weight proteins separated by gel filtration (M_r 360,000 and 490,000, respectively) were required for maximal activity with NADH. Indirect evidence, including in vitro complementation experiments with a *cysG* mutant extract, suggested that the 360,000- M_r component contains siroheme and is the terminal reductase. This component was further purified to near homogeneity and was found to consist of a single subunit of molecular weight 67,500. The anaerobic sulfite reductase showed some resemblance to the biosynthetic sulfite reductase, but apparently it has a unique, as yet unidentified function.

Although much is known about sulfite reduction by the sulfate-reducing bacteria (1, 23, 28), very little is known about the anaerobic production of H_2S from sulfite by members of the family *Enterobacteriaceae*. The ability to perform this reaction is routinely used in diagnostic laboratories to differentiate *Salmonella* species (H_2S positive) from *Escherichia coli* (H_2S negative). We have hypothesized that the enzymes involved may explain the observation that *cysI* and *cysJ* (biosynthetic sulfite reductase) mutants of *S. typhimurium* behave as prototrophs under anaerobic conditions (2), whereas *E. coli cysI* and *cysJ* mutants are auxotrophs under both aerobic and anaerobic conditions (E. L. Barrett, unpublished findings). Like *E. coli*, *S. typhimurium* mutants defective in *cysG*, which is required for synthesis of the siroheme prosthetic group (25), do not produce H_2S from sulfite and do not behave as prototrophs under anaerobic growth conditions. Therefore, we have hypothesized that H_2S production from sulfite by *S. typhimurium* involves a siroheme-containing anaerobic dissimilatory sulfite reductase that can, incidentally, provide reduced sulfur for biosynthesis during anaerobic growth.

S. typhimurium also produces H_2S from thiosulfate, a property that is much more widespread among members of the *Enterobacteriaceae* than is the ability to produce it from sulfite (3). Previous tests of H_2S production from sulfite as compared with its production from thiosulfate (8) demonstrated that these two processes proceed via different enzy-

matic pathways: a fermentable sugar is required for production of H_2S from sulfite, but glucose represses its production from thiosulfate, menaquinone mutants and molybdenum incorporation mutants produce H_2S from sulfite but not from thiosulfate, and siroheme mutants retain the ability to reduce thiosulfate to H_2S . Other qualitative tests (8) indicated that both pathways for H_2S accumulation are excluded from the cysteine biosynthetic regulon; they are not negatively regulated by cysteine, the corepressor of the cysteine biosynthetic enzymes (19), or by the absence of the *cysB* gene product, which is necessary for induction of the biosynthetic pathway (5, 16).

The role that anaerobic sulfite and thiosulfate reduction play in the anaerobic survival of *Salmonella* species has not yet been explored. To address this question, we are investigating the biochemistry, physiology, and genetics of H_2S production by *S. typhimurium*. Here we report an initial characterization of sulfite reduction by a *cysI* mutant and describe the purification and properties of its anaerobically induced sulfite reductase.

MATERIALS AND METHODS

Strains, media, and growth conditions. Wild-type strain LT2 was obtained from B. N. Ames. Strains EB218 ($\Delta cysI68$), EB230 (*cysJ299 araB9*), EB232 ($\Delta cysG458$), and EB245 (*cysB403*) were all from the *Salmonella* Genetic Stock Centre and provided by K. Sanderson.

The mineral base used in minimal media was Vogel-Bonner medium E (29) formulated with 50 mM phosphate

* Corresponding author.

buffer (pH 6.6) and supplemented with 1.0 mM CaCl_2 , 1.0 μM each ZnCl_2 , FeSO_4 , $\text{Na}_2\text{Se}_2\text{O}_3$, and Na_2MoO_4 , and 0.1 μM each MnSO_4 , CuSO_4 , and $\text{Co}(\text{N}_3)_2$. Glucose-sulfite minimal medium consisted of the mineral base supplemented with 1% D-glucose and 1.0 mM Na_2SO_3 , with or without 1.5% agar. All incubations were at 37°C.

Anaerobic atmospheres were tested by using glucose-sulfite minimal plates incubated for 36 h in either a GasPak (BBL Microbiology Systems, Cockeysville, Md.) anaerobic jar (95% H_2 –5% CO_2) or a Torbal model AJ-3 anaerobic jar evacuated and filled with 95% N_2 –5% CO_2 , argon (specified as <0.33% air), or the same argon scrubbed free of oxygen by passage through a tower of BASF copper catalyst (Kontes Glass Co.). Unless otherwise noted, anaerobic growth conditions for physiological studies were achieved by completely filling screw-cap tubes. A_{650} was measured in a Spectronic 21 (Bausch & Lomb, Inc., Rochester, N.Y.). For enzyme purification, strain EB218 (2% inoculum) was grown in the mineral base supplemented with 0.03 mM cysteine, 0.5 mM Na_2SO_3 , 0.5 mM $\text{Na}_2\text{S}_2\text{O}_3$, 0.2% galactose, and 0.4% tryptone (Difco Laboratories, Detroit, Mich.). Incubation was in 20-liter glass carboys filled to the top.

Sulfite reductase assay. Sulfite reductase activity was assayed as sulfide production, using an adaptation of the previously described method (7, 12) for the colorimetric determination of sulfide through the formation of methylene blue. The complete assay procedure is given below. Unless otherwise noted, all assays were carried out at 37°C for 10 min in 5-ml serum-stoppered vials that had been made anaerobic by alternate evacuation and filling with O_2 -free argon. The reaction mixture for in vitro assays consisted of 50 mM degassed Tris buffer (pH 7.5) containing extract and 1 mM electron donor in a total volume of 0.5 ml. When NADH was the electron donor, the reaction mixture also contained, to regenerate NADH, 10 mM sodium malate and 18 U of malate dehydrogenase. The reaction was started by adding sodium sulfite (final concentration, 1 mM). The assay was stopped after 10 min by addition of 0.5 ml of 1% zinc acetate (made fresh daily by dilution of a 10% stock) and 0.025 ml of 12.5% NaOH. Color was developed by addition of 0.2 ml of 20 mM *N,N*-dimethyl-*p*-phenylenediamine in 6.5 N HCl, followed by 0.1 ml of 46 mM FeCl_3 in 1.2 N HCl. After 15 min, particulate matter was removed by centrifugation in a microfuge, 4 ml of water was added to the supernatant, and A_{670} was measured. Whole-cell assays were performed by using, in place of the reaction mixture described above, harvested cells resuspended at about 1/20 the original volume in anaerobic 50 mM phosphate buffer (pH 6.6). Electron donors were added at the following concentrations: sugars, 22 mM; sodium formate, 29 mM; hydrogen, 100% in gas phase of the reaction vial. Spheroplasts, prepared by the method of Osborn and Munson (24), were used in place of whole cells in some assays, as noted.

Thiosulfate reductase activity was measured in whole cells as described above except that sodium thiosulfate was substituted for sodium sulfite, and the zinc sulfide precipitate was collected by centrifugation in a microfuge for 2 min at top speed and suspended in 0.5 ml of water before the color-developing reagents were added.

Purification of sulfite reductase. (i) **Preparation of crude extract.** Two 20-liter cultures of strain EB218 (*cysI68*), grown as described above, were harvested after 18 h of growth by using a continuous-flow Sharples Super centrifuge and stored frozen at -70°C . About 100 g of cell paste was thawed in 100 ml of 50 mM Tris (pH 7.5)–100 mM NaCl–2 mg

of DNase and then sonicated on ice in a model 300 sonicator (Fisher Scientific Co., Pittsburgh, Pa.) equipped with a macrotip. Sonication was carried out with three cycles of power, on for 1 min and then off for 20 s. The supernatant from a subsequent low-speed centrifugation ($19,600 \times g$ for 12 min) was saved, and the pellet was resuspended in 50 ml of the original buffer, resonicated, and recentrifuged as described above. The combined supernatants (crude extract) were then centrifuged at $92,000 \times g$ for 1.5 h, and the supernatant was recovered.

(ii) **DEAE-Trisacryl chromatography.** The supernatant from the high-speed centrifugation was loaded on a column (2.5 by 14 cm) of DEAE-Trisacryl previously equilibrated with 50 mM Tris (pH 7.5)–100 mM NaCl, followed by 2 bed volumes of the same buffer. The column was then step eluted with 50 mM Tris (pH 7.5)–200 mM NaCl.

(iii) **Ammonium sulfate fractionation.** The 0.2 M NaCl eluate from the DEAE column was brought to 35% saturation of $(\text{NH}_4)_2\text{SO}_4$ by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ and, after 20 min of incubation at 0°C , centrifuged at $30,900 \times g$ for 15 min. The supernatant was then brought to 60% saturation with respect to $(\text{NH}_4)_2\text{SO}_4$ by addition of solid $(\text{NH}_4)_2\text{SO}_4$ and centrifuged as described above. The pellet was resuspended in a minimal volume of 50 mM Tris (pH 7.5)–100 mM NaCl.

(iv) **Sephacryl S-300 gel filtration.** The 35 to 60% ammonium sulfate fraction was chromatographed on a column (1.6 by 66 cm) of Sephacryl S-300 equilibrated and developed (0.3 ml/min) with 50 mM Tris (pH 7.5)–100 mM NaCl.

(v) **Gradient DEAE-Trisacryl chromatography.** The active fractions from the Sephacryl S-300 column were absorbed onto a column (1.5 by 39 cm) of DEAE-Trisacryl previously equilibrated with 50 mM Tris (pH 7.5)–100 mM NaCl, and the column was developed with a linear gradient consisting of 100 ml of equilibration buffer and 100 ml of 50 mM Tris (pH 7.5)–300 mM NaCl.

Additional analytical methods. Molecular weights of the purified proteins were determined by reference to a calibration curve for the Sephacryl S-300 column constructed by using blue dextran, thyroglobin (669 kilodaltons), ferritin (440 kilodaltons), catalase (232 kilodaltons), and albumin (67 kilodaltons) as molecular size standards. Gel chromatography on a column (1.6 by 66 cm) of Bio-Gel A-5M (Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted with 50 mM Tris (pH 7.5)–100 mM NaCl was used for examination of activity found in the void volume of the Sephacryl S-300 column. Before chromatography on the Bio-Gel A-5M column, the enzyme was concentrated by ultrafiltration (XM-50 membrane). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli and Favre (20) in 1.5-mm slab gels with a gradient (5 to 15%) of acrylamide. Analytical and semi-preparative nondenaturing gel electrophoresis was carried out in 1.5-mm slab gels with 5% acrylamide, using an *N,N*-bisacrylamide-Tris-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer system (14). Protein was determined by the Coomassie blue binding assay (6).

RESULTS

Factors affecting anaerobic prototrophy of *cysI* mutants. The anaerobic prototrophy of *cysI*, *cysJ*, and *cysB* mutants was first observed under a hydrogen-carbon dioxide atmosphere (2). To determine whether a reducing atmosphere was required for sulfite reduction by these mutants, we tested representatives of each class (strains EB218, EB230,

and EB245, respectively) for growth on glucose-sulfite minimal medium plates under nitrogen-carbon dioxide and under argon, both commercial-grade argon with a small amount (<0.33%) of air contamination and oxygen-free argon. All three strains grew as prototrophs under H_2 - CO_2 , N_2 - CO_2 , and oxygen-free argon but required cysteine for growth under the argon contaminated with air. These results indicated that the lack of oxygen was more important than the presence of hydrogen for activity of the anaerobic sulfite reductase facilitating prototrophic growth of the *cysI* and *cysJ* mutants.

In the earlier study (2), we noted that the anaerobic prototrophy of the *cysI* and *cysJ* mutants was readily demonstrated on plates but occurred in liquid only when the inoculum was a fresh culture grown on complex medium. Experiments directed toward identifying the specific factor required in liquid revealed that trace amounts (10^{-7} to 10^{-6} M) of cysteine facilitated growth of the *cysI* mutant, strain EB218, in anaerobic liquid medium. In contrast, a concentration greater than 10^{-4} M was required for the same amount of growth under aerobic conditions. The latter concentration was also required for both anaerobic and aerobic growth of the *Cys*⁻ mutants affected in *cys* genes other than *cysI*, *cysJ*, and *cysB* (2). Presumably, the previously reported rapid anaerobic prototrophic growth of strain EB218 on agar but not in liquid was due to trace amounts of sulfur in agar.

Stable spontaneous mutants of strain EB218 that no longer required the trace amounts of cysteine for anaerobic growth frequently appeared in anaerobic liquid minimal medium cultures. Such mutants also appeared on minimal plates incubated under the argon contaminated with air. The mutants appearing in both situations exhibited the same phenotype: aerobic cysteine auxotrophy, anaerobic prototrophy without need for trace amounts of cysteine, and ability to grow prototrophically under the argon contaminated with air. The sulfite reductase activity assayed in one was no greater than that of the parent strain. These results argue against a leaky *Cys*⁻ phenotype as the explanation for the requirement for trace amounts of cysteine in anaerobic liquid media by strain EB218. Instead, they suggest that the need for reduced sulfur is associated with a genetically suppressible oxygen-sensitive expression or oxygen-sensitive activity of the anaerobic sulfite reductase.

Anaerobic sulfite reduction as a possible anaerobic respiratory system. A role for the anaerobic sulfite reduction pathway in cysteine biosynthesis is difficult to reconcile with the positive effect of cysteine on initiation of anaerobic growth with sulfite by strain EB218 noted above and the ability of this strain to produce H_2S from sulfite in complex media. We therefore explored an alternative role for sulfite reduction in anaerobic energy generation. First, we examined the effect of sulfite (2 mM) on the anaerobic growth of the *cysI* mutant (EB218) in the presence of limiting carbon (Fig. 1). This concentration of sulfite was selected because concentrations in minimal medium of higher than 5 mM were toxic (data not shown). The sulfite was converted almost stoichiometrically to sulfide without affecting growth yield (Fig. 1). Similar results (not shown) were obtained for the wild-type strain, LT2.

We also examined the expression of sulfite reductase activity during anaerobic growth (Fig. 2). If anaerobic sulfite reduction served to produce metabolic energy, one would expect to find greatest activities during exponential growth. However, the maximal specific activity of sulfite reductase was obtained after the cells had reached stationary phase.

FIG. 1. Effect of sulfite on yields of EB218 grown anaerobically with limiting galactose. Cultures were grown anaerobically to stationary phase in galactose-containing minimal medium in which equimolar $MgCl_2$ replaced $MgSO_4$ and which contained Na_2SO_3 (2 mM) or cysteine (0.3 mM) as indicated: □, ■, sulfite; △, ●, sulfite and cysteine. Open symbols, Yields (optical density at 650 nm [OD_{650}]); closed symbols, sulfide (millimolar) produced.

To determine whether anaerobic sulfite reduction involved a molybdoenzyme as do the anaerobic reductions of nitrate, trimethylamine oxide, and thiosulfate (10, 27), chlorate-resistant mutants of strain EB218 were examined. All were found to retain anaerobic prototrophy as well as the ability to produce H_2S from sulfite in complex media. However, one similarity with certain anaerobic respiratory systems was identified. Dissimilatory reductases for several anaerobic electron acceptors have been shown to be negatively regulated by electron acceptors of greater energy potential, particularly nitrate (15). Similarly, 0.1 mM nitrate in the anaerobic glucose-sulfite minimal medium caused the *cysI* mutant to behave as a cysteine auxotroph. Nitrate in the medium did not affect the prototrophy of the wild-type strain, LT2. Nitrite (0.1 mM) in the medium also converted strain EB218 to an anaerobic auxotroph without affecting wild-type prototrophy.

FIG. 2. Anaerobic sulfite reductase as a function of growth phase. EB218 was grown anaerobically in minimal medium containing 0.2% galactose and 1 mM Na_2SO_3 . Symbols: ○, optical density at 650 nm (OD_{650}); ●, sulfite reductase activity, assayed with 11 mM galactose as the electron donor.

TABLE 1. Reduction of sulfite and thiosulfate by whole cells

Electron donor ^a	Sp act ^b (sulfide) produced from:			
	Sulfite		Thiosulfate	
	EB218	EB232	EB218	EB232
None	0.8	0.9	1.1	1.7
Galactose	2.6	0.2	8.4	10.5
Formate	2.0	0.8	4.5	6.5
Hydrogen	1.8	0.8	4.0	3.4

^a Concentrations are given in Materials and Methods.^b Expressed as nanomoles of sulfide produced per minute per milligram (dry weight). Cells were grown to stationary phase in medium used for sulfite reductase purification except that the cysteine concentration was 0.3 mM in cultures of strain EB232.

Anaerobic sulfite reductase activity in the *cysI* mutant. Whole cells of strain EB218 were assayed for sulfite reduction with galactose, formate, and hydrogen as electron donors (Table 1). All three compounds were found capable of driving the *in vivo* reduction of sulfite to sulfide at rates two- to threefold greater than the apparent endogenous rate. That this reaction was catalyzed by a sulfite reductase distinct from thiosulfate reductase was indicated by the results obtained by using a *cysG* (siroheme) mutant, strain EB232, and thiosulfate as the electron acceptor (Table 1). Strain EB232, which is a cysteine auxotroph under both anaerobic and aerobic conditions, was unable to reduce sulfite but exhibited activities with thiosulfate as high as those of strain EB218.

Sulfite reductase (assayed with galactose as the electron donor) was quantitatively recovered in the spheroplast fraction of strain EB218 grown anaerobically in minimal medium containing 0.5 mM sulfite, which indicated that it is not a periplasmic enzyme. A number of sugars and fermentation intermediates, including glucose, galactose, glycerol, formate, and pyruvate, were capable of driving the reduction of sulfite to sulfide by spheroplast preparations. The apparent K_m for sulfite in spheroplast preparations was determined to be 0.17 mM.

Several compounds were tested for the ability to support sulfite reduction by crude extracts of strain EB218, among which NADH was found to be the most effective electron donor (Table 2). Formate did not reduce sulfite in the crude extracts. Among the artificial dyes used, only dithionite-reduced methyl viologen supported sulfite reductase activ-

TABLE 2. Electron donors for sulfite reductase in crude extracts

Electron donor ^a	Relative sulfite reductase activity ^b
NADH.....	100
NADPH.....	40
Methyl viologen	30
PMS.....	5.5
Formate.....	5.1
DCPIP.....	4.6
PES.....	4.2
None.....	5.1

^a All donors were used at a concentration of 1 mM; the methyl viologen assay also contained 10 mM dithionite; PMS, DCPIP, and PES assays also contained 10 mM ascorbate. Abbreviations: PMS, *N*-methylphenazonium methosulfate; DCPIP, 2,6-dichlorophenol indophenol; PES, *N*-ethylphenazonium methosulfate.^b Assayed as nanomoles of sulfide produced per minute per 0.1 ml of crude extract of cells grown in purification medium and expressed relative to the value obtained with NADH.TABLE 3. Purification of anaerobic sulfite reductase from strain EB218 (*cysI68*)^a

Step	Total activity (U) ^b	Yield (%)	Total protein (mg)	Sp act (U/mg)	Fold purification
Crude extract	43,800	100	13,272	3.3	1
High-speed supernatant	32,640	75	4,675	5.1	1.6
DEAE-Trisacryl (0.2 M)	28,500	65	967	30	8
Ammonium sulfate (35–60%)	2,870	7	467	6.2	2
Gel filtration (Sephacryl S-300) ^c	11,360	26	129	88	27
Gradient DEAE-Trisacryl ^d	11,951	27	19	629	190

^a Data are for a typical preparation. As discussed in Results, the values for total and specific activities for the first three steps varied from preparation to preparation because of limitations of the assay.^b Units of activity are equivalent to nanomoles of sulfide produced per minute with NADH as the electron donor.^c Activity was determined by using 50 μ l of each fraction assayed in the presence of 100 μ l of a partially purified (through high-speed centrifugation) preparation of strain EB232 (*cysG458*). Specific activity is expressed in terms of milligrams of protein in the fraction itself.^d Activity was determined as described in footnote c except that 200 μ l of each fraction was combined with 100 μ l of the preparation from strain EB232.

ity, and at a rate only 30% of that supported by NADH. Under the conditions used here, approximately 1 mM NADH was required for maximal sulfite reductase activity in the presence of malate dehydrogenase and sodium malate to regenerate NADH. Activity was negligible in extracts maintained under an atmospheric concentration of oxygen, but the apparent inhibition by oxygen was fully reversible. Thus, anaerobic sulfite reductase could be purified under aerobic conditions.

Purification of anaerobic sulfite reductase. Anaerobic sulfite reductase was purified to near homogeneity (Table 3 and Fig. 3) from anaerobically grown cultures of the *cysI* deletion mutant, strain EB218, as described in Materials and Methods. Strain EB218 was used rather than the wild type to avoid potential confusion between the anaerobic enzyme and the biosynthetic sulfite reductase. Sulfite reductase was quantitatively recovered in the supernatant of a high-speed

FIG. 3. SDS-PAGE analysis of sulfite reductase purification (5 to 15% gradient acrylamide). Lanes: 1 and 8, M_r markers phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400); 2, crude extract (100 μ g); 3, high-speed supernatant (100 μ g); 4, 0.2 M NaCl DEAE-Trisacryl eluate (41 μ g); 5, S-300 pool (40 μ g); 6, gradient DEAE-Trisacryl pool (37 μ g); 7, eluted preparative gel electrophoresis fraction obtained after SDS-PAGE of active fraction from DEAE chromatography (6 μ g).

FIG. 4. Sephacryl S-300 chromatogram and activity profile of anaerobic sulfite reductase. Sephacryl S-300 chromatography was performed as described in Materials and Methods; 50- μ l portions of each fraction were assayed. Symbols: $\bullet$, no additions; $\circ$, assay in the presence of 100 μ l of a partially purified (through high-speed centrifugation) preparation of strain EB232 grown in the purification medium; $\square$, assay in the presence of 50 μ l of fraction 23.

centrifugation, which indicated that it is a soluble rather than a membrane-bound enzyme. The activity was bound by DEAE at 100 mM NaCl and eluted by 200 mM NaCl. After DEAE chromatography, sulfite reduction exhibited an absolute dependence on NADH; dithionite-reduced methyl viologen no longer served as an alternative electron donor.

Fractional precipitation with ammonium sulfate, which was used to concentrate activity after DEAE chromatography, resulted in a decrease in activity. However, the poor recovery after ammonium sulfate precipitation was only apparent, as activity increased in the next step. The apparent loss may have been due to a limitation in the assay associated with high protein concentration. In fact, although the enzyme assay enabled us to monitor enzyme activity through all purification steps, it was difficult to quantitate total enzyme activity in the early steps because the activity was nonlinear with enzyme concentration as a result of its apparent inhibition at high protein concentrations.

Gel chromatography on Sephacryl S-300 (Fig. 4) brought about a physical change in the sulfite reductase. Little or no activity was recovered in any single fraction outside the void volume (Fig. 4, fractions 5 to 9), but significant activity was obtained when early fractions (fractions 15 to 19) were combined with later fractions (fractions 19 to 27). This result suggested that the sulfite reductase activity involved two protein components separated by the gel chromatography. Their apparent molecular weights, as determined by using a standard calibration curve constructed for the Sephacryl S-300 column, were 363,000 (protein from fraction 23) and 490,000 (protein from fraction 17). Fraction 23, but not fraction 17, was also found to complement a partially purified extract of the *cysG* (siroheme-deficient) mutant EB232. The latter result suggested that the protein in fraction 23 contained siroheme and thus represented the active site of sulfite reduction, i.e., the terminal reductase.

The nature of the variable amount of sulfite reductase activity in the very early (void volume) fractions was further investigated. Gel chromatography (Bio-Gel A-5M) of the void volume fractions after concentration by ultrafiltration was found to separate the activity into the same two complementary components described above. This result indi-

FIG. 5. Gradient DEAE-Trisacryl chromatogram and activity profile of anaerobic sulfite reductase (360,000-dalton component from the Sephacryl S-300 column). Chromatography performed as described in Materials and Methods; 200- μ l portions of the indicated fractions were assayed. Symbols: —, continuous absorbance at 280 nm obtained with a UV detector; $\bullet$, enzyme activity without additions; $\circ$, enzyme activity in the presence of 100 μ l of a partially purified extract of strain EB232 (through high-speed centrifugation).

cated that the activity in the void volume of the Sephacryl S-300 chromatography was due to coelution of specific aggregates of the two components rather than the presence of polydisperse aggregates consisting of both components.

The presumed siroheme-containing component was further purified. Sephacryl S-300 fractions 20 to 26 (Fig. 4) were pooled and concentrated by ultrafiltration (data not shown). After concentration, these fractions were found to exhibit low levels of NADH-dependent sulfite reduction in the absence of the *cysG* mutant extract. This intrinsic activity supports the contention that the protein undergoing purification represented a terminal reductase which, however, required an additional component for full activity. The concentrated fractions were purified to near homogeneity by DEAE-Trisacryl chromatography with gradient elution of 100 to 300 mM NaCl (Fig. 5). The purified protein did not require the *cysG* mutant extract for activity, although greater specific activities were still obtained when this extract was present. SDS-PAGE showed that the major protein in the active DEAE-Trisacryl fraction was at least 95% pure and had an apparent molecular weight of 67,000 (Fig. 3).

That this 67,000- M_r subunit represented the anaerobic sulfite reductase was demonstrated as follows. The active fraction from DEAE-Trisacryl chromatography was subjected to semipreparative nondenaturing gel electrophoresis, and the major protein component was determined by Coomassie blue staining. Elution and assay of protein of the same R_f from a nonstained lane of the gel demonstrated that the protein had sulfite reductase activity (23% yield). The active protein was then eluted and subjected to SDS-PAGE. The results showed unequivocally that the protein with sulfite reductase activity consisted of a 67,500- M_r subunit (Fig. 3, lane 7).

FIG. 6. Anaerobic sulfite reductase protein in a *cysG* mutant. Fractions were subjected to nondenaturing gel electrophoresis in 5% acrylamide, and protein was stained with Coomassie blue. Lanes: 1, purified enzyme from strain EB218 (from gradient DEAE column); 2 and 3, partially purified protein (high-speed supernatant fraction) from strains EB218 (lane 2) and EB232 (lane 3).

Anaerobic sulfite reductase in the *cysG* mutant. To test whether the *cysG* (siroheme) mutant EB232 synthesized enzyme protein, we compared the soluble fractions (after high-speed centrifugation) of extracts of EB232 and EB218 with purified anaerobic sulfite reductase by using nondenaturing gel electrophoresis (Fig. 6). Because of the relatively high molecular weight of anaerobic sulfite reductase, it was easily located even in only partially purified extracts. Both strains appeared to contain anaerobic sulfite reductase protein, which suggested that that siroheme is not required for synthesis of anaerobic sulfite reductase and moreover that its presence may not be required for association of the subunits.

DISCUSSION

The results of this study have shown that the sulfite-reducing system facilitating the anaerobic prototrophy of *cysI*, *cysJ*, and *cysB* mutants of *S. typhimurium* requires (i) the absence of oxygen, (ii) the presence of 10^{-7} M cysteine or an unidentified compound (presumably a reducing agent) that is present in agar, and (iii) the absence of nitrate and nitrite. The first requirement is consistent with a physiological role for this system in sulfur assimilation under anaerobic conditions. In such a model for sulfur assimilation, the previously characterized cysteine biosynthetic pathway might be the aerobic pathway, with the sulfite reduction step being replaced, under anaerobic conditions, by the anaerobic sulfite reductase under investigation. However, because ii and iii were requirements of the *cysI*, *cysJ*, and *cysB* mutants but not for prototrophy of the wild type, the model would have to include the designation "auxiliary" for this novel anaerobic sulfite reductase. The difficulty in rationalizing the advantage of such an auxiliary system to anaerobic growth is then compounded by the nature of requirement iii. Inhibition by nitrite might be explained by competition for the active site of a sulfite reductase (17), but the effect of nitrate is without precedent in the regulation of anabolism.

Nitrate control is more consistent with a role for the anaerobic sulfite reductase in anaerobic energy metabolism. Nitrate in the growth medium is known to repress less favorable anaerobic energy-generating systems (15). We had hypothesized that sulfite reduction performed by *cysI* and

cysJ mutants was the activity of a dissimilatory enzyme responsible for H_2S production from sulfite in complex media. In fact, detectable H_2S production from sulfite also requires a low oxidation-reduction potential, as indicated by the location of the zone of blackening in H_2S detection tubes (8) and the absence of nitrate (8) and nitrite (Barrett, unpublished findings). Furthermore, H_2S production from sulfite is more vigorous in complex media than in defined solid media lacking cysteine (M. A. Clark and E. L. Barrett, unpublished findings). The hypothesis predicts that sulfite reduction can contribute to anaerobic energy generation. The prediction was tested in this study and was not borne out: the nearly stoichiometric reduction of sulfite to sulfide by *S. typhimurium* did not affect growth yield. Although sulfite is a poor electron acceptor compared with nitrate and trimethylamine oxide (28), one would expect it to offer some contribution to yields obtained with a very limiting carbon source if its reduction were part of an anaerobic respiration. That sulfite reductase activity was greatest in stationary phase and was carried out by a soluble cytoplasmic enzyme also points to a role outside of the electron transport associated with energy generation during anaerobic growth.

On the other hand, the fact that NADH was an effective electron donor for sulfite reduction indicates that sulfite reduction, as estimated by the degree of H_2S production in diagnostic media, can consume significant amounts of reducing power generated by the cell under anaerobic conditions. Interestingly, maximal activity in stationary phase is also a feature of anaerobic thiosulfate reductase (8) and the secondary nitrate reductase (Barrett, unpublished findings), and neither activity appears capable of supporting growth with a nonfermentable carbon source (4; R. Bradford and E. Barrett, unpublished findings). Perhaps the sulfite and thiosulfate reductions as well as nitrate reduction by the secondary nitrate reductase all play a unique role under anaerobiosis in, for example, cell maintenance or survival under nongrowing conditions.

Anaerobic sulfite reductase activity was demonstrated in the *cysI* deletion mutant EB218 by using an assay in which sulfide was quantitatively recovered and determined. Endogenous activities, representing sulfide found in the absence of added sulfite, were detected even in the *cysG* mutant, strain EB232. Perhaps this sulfide was the product of cysteine desulfhydrase activity (9, 13). The same assay, but with thiosulfate instead of sulfite, was also found suitable for the assay of thiosulfate reductase.

Here we report the purification and initial characterization of the anaerobic sulfite reductase that facilitates the growth of *S. typhimurium* in the absence of the biosynthetic sulfite reductase. Like the latter enzyme, the anaerobic sulfite reductase requires a functional *cysG* gene (hence, presumably siroheme) for activity, as does the production of H_2S from sulfite in diagnostic media (3). Both reductases are synthesized in a siroheme mutant (L. M. Siegel and H. Kamin, Fed. Proc. 30:1261, 1971; this paper). Furthermore, both enzymes occur naturally as high-molecular-weight complexes, but they differ with respect to the stability of the complex. The NADPH-specific assimilatory enzyme exists in solution as a 670,000- M_r hemoflavoprotein consisting of two subunits in an $\alpha_8\beta_4$ arrangement (25). This complex can be dissociated with 5 M urea to yield a flavoprotein subunit (α_8) and monomeric hemoprotein β subunits (M_r 54,600) which contain a siroheme and an Fe_4S_4 cluster (18, 21, 22, 25). The isolated hemoprotein, the product of the *cysI* gene, is capable of carrying out reduction of sulfite with reduced methyl viologen as the electron donor, whereas the flavo-

protein component, the product of the *cysJ* gene, is required for NADPH-dependent activity (11, 25, 26).

The anaerobic sulfite reductase, in contrast, appears to exist in solution as two components, both of which are required for maximal NADH-driven sulfite reduction. Their apparent molecular weights, as determined by reference to a standard calibration curve constructed for the Sephacryl S-300 column, were 363,000 and 490,000, respectively. This type of molecular weight determination assumes that the molecules have shapes similar to those of the marker proteins; therefore, additional experiments are required to obtain a more precise molecular weight estimate. Under the conditions reported here, we have obtained no evidence for stable complex formation between these two components. (The sulfite reduction activity detected in the void volume of the Sephacryl S-300 column appeared to be due to the coelution of specific aggregates of the two components, since they were separated on subsequent chromatography on Bio-Gel A-5M.) The predominant form of the terminal reductase consistently obtained during different purifications was the 360,000-molecular-weight component. In SDS-PAGE, this protein formed a single band of M_r about 67,000, which indicated that the purified protein is a homo-oligomer of four to six subunits. A detailed analysis of the protein which, like the *cysG* mutant extract, complemented the activity of the purified protein awaits its further purification. Although it is possible that this complementing factor was the *cysJ* product, the requirement for NADPH by the *cysJ* product (25, 26) would necessitate the additional participation of an NADH-NADPH transhydrogenase in Sephacryl S-300 fractions containing one or the other component.

A number of differences between the terminal reductase purified here and the assimilatory reductase suggest that they may differ in enzymological and molecular (i.e., active-site configuration) properties. Oxygen reversibly inhibited the anaerobic reductase but has not been reported to affect the assimilatory reductase. NADH, but not methyl viologen, reduced the anaerobic enzyme, whereas methyl viologen has been shown to be an effective reductant of the hemoprotein of assimilatory sulfite reductase (26). On the other hand, the structural similarities between these enzymes tempts the speculation that they may be related through ancestral gene duplication. This question can be answered after cloning and sequencing of the respective genes. The *cysI* and *cysJ* genes have recently been cloned (J. Ostrowski and N. M. Kredich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H-183, p. 170), and cloning of the anaerobic sulfite reductase is now in progress.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI-22865 from the National Institutes of Health and by funds from the California Agricultural Experiment Station.

We thank Nicholas Kredich for critical reading of the manuscript, Jane Tang for contributions to our experimental planning, and Richard Bradford for technical artistry.

LITERATURE CITED

1. Badziong, W., and R. K. Thauer. 1978. Growth yields and growth rates of *Desulfovibrio vulgaris* (Marburg) growing on hydrogen plus sulfate and hydrogen plus thiosulfate as the sole energy sources. *Arch. Microbiol.* **117**:209–214.
2. Barrett, E. L., and G. W. Chang. 1979. Cysteine auxotrophs of *Salmonella typhimurium* which grow without cysteine in a hydrogen/carbon dioxide atmosphere. *J. Gen. Microbiol.* **115**:513–516.
3. Barrett, E. L., and M. A. Clark. 1987. Tetrathionate reduction and production of hydrogen sulfide from thiosulfate. *Microbiol. Rev.* **51**:192–205.
4. Barrett, E. L., and D. L. Riggs. 1982. Evidence for a second nitrate reductase activity that is distinct from the respiratory enzyme in *Salmonella typhimurium*. *J. Bacteriol.* **150**:563–571.
5. Borum, P. R., and K. J. Monty. 1976. Regulatory mutants and the control of cysteine biosynthetic enzymes in *Salmonella typhimurium*. *J. Bacteriol.* **125**:94–101.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
7. Chen, J.-S., and L. E. Mortenson. 1977. Inhibition of methylene blue formation during the determination of acid-labile sulfide of iron-sulfide protein samples containing dithionite. *Anal. Biochem.* **79**:157–165.
8. Clark, M. A., and E. L. Barrett. 1987. The *phs* gene and hydrogen sulfide production by *Salmonella typhimurium*. *J. Bacteriol.* **169**:2391–2397.
9. Collins, J. M., A. Wallenstein, and K. J. Monty. 1973. Regulatory features of the cysteine desulfhydrase of *Salmonella typhimurium*. *Biochim. Biophys. Acta* **313**:156–162.
10. Davidson, A. E., H. E. Fukumoto, C. E. Jackson, E. L. Barrett, and G. W. Chang. 1979. Mutants of *Salmonella typhimurium* defective in the reduction of trimethylamine oxide. *FEMS Lett.* **6**:417–420.
11. Faeder, E. J., P. S. Davis, and L. M. Siegel. 1974. NADPH-sulfite reductase of enterobacteria. V. Studies with the *Escherichia coli* hemoflavoprotein depleted of flavin mononucleotide: distinct roles for the flavin adenine dinucleotide and flavin mononucleotide prosthetic groups in catalysis. *J. Biol. Chem.* **249**:1599–1609.
12. Fogo, J. K., and M. Popowsky. 1949. Spectrophotometric determination of hydrogen sulfide. *Anal. Chem.* **21**:732–734.
13. Guarneros, G., and M. V. Ortega. 1970. Cysteine desulfhydrase activities of *Salmonella typhimurium* and *Escherichia coli*. *Biochim. Biophys. Acta* **198**:132–142.
14. Hallenbeck, P. C., E. R. Vimr, F. Yu, B. Bassler, and F. A. Troy. 1987. Purification and properties of a bacteriophage induced endo-N-acetylneuramidase specific for poly- α -2,8 sialosyl carbohydrate units. *J. Biol. Chem.* **262**:3553–3561.
15. Iuchi, S., and E. C. C. Lin. 1987. The *narL* gene product activates the nitrate reductase operon and represses fumarate reductase and trimethylamine-N-oxide reductase operons. *Proc. Natl. Acad. Sci. USA* **84**:3901–3905.
16. Jagura, G., and D. Hulanicka. 1978. Analysis of merodiploids of the *cysB* region of *Salmonella typhimurium*. *Mol. Gen. Genet.* **165**:31–38.
17. Janick, P. A., D. C. Rueger, R. J. Krueger, M. J. Barber, and L. M. Siegel. 1983. Characterization of complexes between *Escherichia coli* sulfite reductase hemoprotein subunit and its substrates sulfite and nitrite. *Biochemistry* **22**:396–408.
18. Janick, P. A., and L. M. Siegel. 1982. Electron paramagnetic resonance and optical spectroscopic evidence for interaction between siroheme and Fe_4S_4 prosthetic groups in *Escherichia coli* sulfite reductase hemoprotein subunit. *Biochemistry* **21**:3538–3547.
19. Kredich, N. M. 1983. Regulation of cysteine biosynthesis in *Escherichia coli* and *Salmonella typhimurium*, p. 115–132. In K. M. Herrmann and R. L. Somerville (ed.), *Amino acids: biosynthesis and genetic regulation*. Addison Wesley Publishing Co., London.
20. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* **80**:575–599.
21. McRee, D. E., D. C. Richardson, J. S. Richardson, and L. M. Siegel. 1986. The heme and Fe_4S_4 cluster in the crystallographic structure of *Escherichia coli* sulfite reductase. *J. Biol. Chem.* **261**:10277–10281.
22. Murphy, M. J., L. M. Siegel, and H. Kamin. 1973. Reduced NADP-sulfite reductase of enterobacteria. II. Identification of a

- new class of heme prosthetic group: an iron-tetrahydroporphyrin (isobacteriochlorin type) with eight carboxylic acid groups. *J. Biol. Chem.* **248**:2801–2814.
23. **Nethe-Jaenchen, R., and R. K. Thauer.** 1984. Growth yields and saturation constant of *Desulfovibrio vulgaris* in chemostat culture. *Arch. Microbiol.* **137**:236–240.
24. **Osborn, M. T., and R. Munson.** 1974. Separation of inner (cytoplasmic) and outer membranes of gram-negative bacteria. *Methods Enzymol.* **31**:642–653.
25. **Siegel, L. M., and P. S. Davis.** 1974. NADPH-sulfite reductase of enterobacteria. IV. Subunit structure and dissociation into hemoprotein and flavoprotein components. *J. Biol. Chem.* **249**:1587–1598.
26. **Siegel, L. M., P. S. Davis, and H. Kamin.** 1974. NADPH-sulfite reductase of enterobacteria. I. The *Escherichia coli* hemoflavoprotein: catalytic parameters and the sequence of electron flow. *J. Biol. Chem.* **249**:1572–1586.
27. **Stouthamer, A. H.** 1969. A genetical and biochemical study of chlorate resistant mutants of *Salmonella typhimurium*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **35**:505–521.
28. **Thauer, R. K., K. Hungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**:100–180.
29. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **218**:97–106.