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Some Properties of Thiosulfate-Oxidizing Enzyme from Marine Heterotroph 16B†

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Thiosulfate-oxidizing enzyme has been demonstrated in cell-free extracts of the marine, thiosulfate-oxidizing pseudomonad strain 16B. The enzyme, partially purified by ion-exchange chromatography and calcium phosphate gel treatment, catalyzed the oxidation of thiosulfate to tetrathionate with the concomitant reduction of ferricyanide. Native but not mammalian cytochrome *c* was also reduced by the enzyme in the presence of thiosulfate. The enzyme was located exclusively in the supernatant of ultracentrifuged cell extracts. The most purified enzyme preparation, like intact cells, exhibited a temperature optimum of 30 to 31°C. However, it exhibited no definite pH optimum. At pH 6.1 to 6.3 and 30°C, the K_m for thiosulfate was 1.57 mM. At lower temperatures, the apparent K_m for thiosulfate increased, but the apparent maximum velocity remained virtually unchanged. Thiosulfate oxidation in intact cells exhibited an increase in the pH optimum at lower temperatures. The thiosulfate-oxidizing enzyme of marine heterotroph 16B is compared with thiosulfate-oxidizing enzymes from other bacteria, and the effect of temperature on the relationship between pH and thiosulfate oxidation is discussed with reference to the natural habitat of the bacterium.

Thiosulfate-oxidizing enzyme (TSO) catalyzes the oxidation of thiosulfate to tetrathionate. This enzyme is common in the thiobacilli (7, 8, 10, 16) and has also been demonstrated in extracts of *Chromatium* sp. strain D (11). TSO in the thiobacilli is believed to function as the initial enzyme of an alternate pathway for thiosulfate oxidation (9, 17). The physiological importance of this so-called polythionate pathway is not clear, but TSO in *Thiobacillus thioparus* has been suggested to play a role in energy generation at high concentrations of thiosulfate (7).

In addition to chemolithotrophs and photolithotrophs, certain heterotrophic bacteria have long been known to oxidize inorganic sulfur compounds, thiosulfate in particular (12, 14). In fact, the heterotrophs may be more important than chemolithotrophs in the oxidative turnover of inorganic sulfur in soils (13, 27, 28). Thiosulfate-oxidizing heterotrophs have also been found in freshwater (19, 26) and in a variety of marine habitats (22, 23, 25). These bacteria likely represent several genera and species, but all oxidize thiosulfate to polythionates (12, 18, 22, 23). The physiological role of TSO in the soil isolates is still unclear, but the oxidation of thiosulfate to tetrathionate by some marine heterotrophs, including strain 16B, substantially increases their growth rate in media containing organic carbon (21). The generation of ATP during thiosulfate oxidation as well as increased incorporation and decreased respiration of organic carbon by cell suspensions of strain 16B has recently been suggested to account for the observed growth rate stimulation (20).

Little is known about the mechanism of thiosulfate oxidation in heterotrophic bacteria. Except for the demonstration of TSO activity in crude extracts of soil heterotroph A-50 (18), cell-free TSO has remained uninvestigated in these microorganisms. Thiosulfate oxidation in intact cells of strain 16B occurs most rapidly at pH 6.0 to 6.5 and 32 to 37°C (19), whereas the pH (7.7) and temperature (9°C) of the environment from which the bacterium was isolated are considerably different (1). In this communication, we report the isolation and partial purification of TSO from marine heterotroph 16B and the results of experiments to investigate pH and tem-

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perature effects on thiosulfate oxidation by intact cells and cell-free extracts.

MATERIALS AND METHODS

Bacteria, media, and culture conditions. Marine heterotroph 16B was originally isolated from the oxygen-sulfide interface of the Black Sea (22) and has been maintained by monthly transfer on TB medium (22) prepared with two-thirds-strength aged seawater or filtered (0.45-μm membrane) freshwater from Lake Arlington (Arlington, Tex.) supplemented with 2% (wt/vol) NaCl. TSO is a constitutive enzyme in strain 16B (21). Other details of the aerobic and anaerobic metabolism of this bacterium have been discussed elsewhere (19–25).

Cells were cultured at 22 ± 2°C in 18 liters of filter-sterilized (0.2-μm membrane) 5 mM thiosulfate–6 mM sodium lactate medium (24) adjusted to pH 7.0 with 2 N NaOH and contained in a 20-liter glass carboy. Inocula (2.5%, vol/vol) consisted of 30-h cultures grown aerobically at 22 ± 2°C in the same medium. The mass cultures were mixed with a magnetic stirrer and aerated with sterile air passed through a gas dispersion tube.

After incubation for 30 to 37 h, the cultures (final pH 7.5) were harvested with a continuous-flow centrifuge apparatus (KSB; Dupont-Sorvall Instruments, Inc.) operated at 25,000 × *g* and 5°C. The cultures were continuously aerated throughout the 2.5- to 3-h harvesting procedure. Packed cells were transferred from the continuous-flow centrifuge tubes into 250-ml bottles and washed twice by resuspending them in 250 ml of cold (4°C) 0.1 M sodium phosphate–2% (wt/vol) NaCl buffer (pH 7.5), followed by centrifugation at 10,000 × *g* for 20 min at 4°C. Washed cells for cell-free studies were taken up in 1 to 3 ml of the phosphate-NaCl buffer and stored at −20°C until used. Cells used in manometric experiments were resuspended in sterile artificial seawater (pH 8.0; Seven Seas Marine Mix; Utility Chemical Co.) to a concentration of 0.04 g (wet weight) of cells per ml and were stored for a maximum of 3 days at 4°C before use.

The average yield from 18-liter cultures was 14.1 ± 1.5 g (wet weight) of cells. Culture purity was checked just before harvesting by streaking a portion of the culture onto nutrient agar (Difco Laboratories) supplemented with 2% (wt/vol) NaCl.

Manometry. Manometric experiments with suspensions of intact cells were done with a Gilson differential respirometer (Gilson Medical Electronics) operated at a shaking rate of 120 strokes per min. The main wells of reaction flasks contained 3.0 ml of 0.25 M Tris–2% (wt/vol) NaCl or 0.25 M sodium phosphate–2% (wt/vol) NaCl (depending upon the experimental pH range) and 0.5 ml of 0.32 M $Na_2S_2O_3 \cdot 5H_2O$ prepared in artificial seawater. Artificial seawater replaced thiosulfate solution in endogenous controls. CO_2 was absorbed by 0.2 ml of 20% (wt/vol) KOH. Reactions were started by tipping in from the side arm 0.5 ml of cell suspension (described above) diluted 1:2 (vol/vol) in artificial seawater (0.56 mg of protein). Oxygen uptake rates determined in the presence of thiosulfate were corrected for endogenous respiration, and the corrected values were normalized at 0°C and 760 mmHg (ca. 101 kPa). The pH of complete reaction mixtures was determined at each experimental temperature.

Preparation of cell extracts. Frozen cell packs were thawed and brought to 10-ml volume with phosphate-NaCl buffer, and the mixture was passed once through a chilled French pressure cell (American Instrument Co.) operated at 20,000 lb/in^2. The extract was stirred at 22 ± 2°C with a few grains of DNase I (Sigma Chemical Co.) and then centrifuged for 30 min at 10,000 × *g* and 4°C to remove unbroken cells and debris. The supernatant, hereafter referred to as fraction SI, was used as a crude extract for some experiments or further purified as outlined below.

Purification procedure. Fraction SI was treated according to a modification of the procedure used to purify TSO from *T. thioparus* (7). SI was centrifuged for 1 h at 155,000 × *g* and 4°C with a Beckman L565 ultracentrifuge equipped with an SWTi rotor (Beckman Instruments). The pellet was resuspended in 0.5 M sodium phosphate, assayed for TSO activity as described below, and subsequently discarded. Seven- to 15-ml portions (101 to 422 mg of protein) of the ultracentrifuge supernatant, fraction SII, were layered onto a Plexiglas column (30 cm long by 2.5-cm diameter) packed with DEAE–Sephadex-A25 (Pharmacia Fine Chemicals, Inc.) equilibrated in 0.05 M sodium phosphate buffer (pH 7.5) at 4°C. Proteins were eluted from the column by a stepwise gradient of 0.05 to 0.25 M sodium phosphate buffer, pH 7.5. Eluant volumes were 50 ml. At each step of the gradient, buffer was removed from the top of the gel before more concentrated buffer was added. Fractions, 5 ml, were collected with a Buchler Fractomette 200 fraction collector (Buchler Instruments). Protein content of the fractions was estimated by measuring absorbance at 280 nm with a Beckman 25 split-beam spectrophotometer (Beckman Instruments). All 280-nm absorbance peaks were analyzed for TSO activity as described below.

Fractions containing a high specific activity were pooled for further purification. The pooled material is hereafter referred to as DEAE eluate. A portion of the protein in the DEAE eluate was precipitated by decreasing the pH to 4.0, stirring slowly for 20 min at 4°C, and readjusting the pH to 7.5. Precipitated protein was removed by centrifugation of the mixture for 20 min at 20,000 × *g* and 4°C. The resultant supernatant, fraction SIII, was stirred for 20 min in an ice bath with calcium phosphate gel (15% solids; Sigma Chemical Co.) in a ratio of 1 mg of protein per 5 to 10 mg of gel. The gel was then removed from the mixture by centrifugation for 20 min at 20,000 × *g* and 4°C, and the supernatant, fraction SIV, was retained as the highest purified TSO preparation.

Determination of TSO activity. Thiosulfate oxidation was measured by spectrophotometric determination of the decrease in potassium ferricyanide absorbance at 420 nm with a Beckman 25 split-beam spectrophotometer or with a Beckman DU spectrophotometer fitted with a constant-temperature block and recorder. Unless indicated otherwise, complete reaction mixtures (3.0-ml total volume) contained 25 μmol of NaH_2PO_4, 25 μmol of Na_2HPO_4, 1.0 μmol of $K_3Fe(CN)_6$, and 1.0 μmol of $Na_2S_2O_3 \cdot 5H_2O$ per ml of appropriately diluted cell-free extract, and measurements were made at pH 7.5 and 30°C. Spectrophotometric references consisted of complete reaction mixtures with thiosulfate omitted.

Ferricyanide reduction catalyzed by TSO was linear for 30 to 60 s, depending upon the amount of extract used and the concentrations of ferricyanide and thiosulfate supplied. Reaction velocities were determined only from the linear portion of absorbance plots. One enzyme unit is defined as 1 μmol of ferricyanide reduced per min. Cytochrome spectra were determined with the Beckman spectrophotometer operated in the scanning mode.

Electrophoresis. Twenty- to 40-μl portions of fractions SI to SIV were applied to a 7.5% acrylamide slab gel prepared according to Davis (3). Sample wells were prepared by mixing 1.25 ml of 0.5 M Tris-hydrochloride buffer (pH 6.8), 0.75 ml of 30% (wt/vol) acrylamide, 5 μl of *N,N,N',N'*-tetramethylethylenediamine ammonium persulfate, and 3 ml of catalyst. Proteins were electrophoresed at 5°C and 8 mA for 1 h to allow the proteins to enter the gel and then at 15 mA until the tracking dye reached a point 1.5 cm from the bottom of the gel. The slab was then fixed in 12.5% (wt/vol) trichloroacetic acid for 30 min, stained in 0.05% (wt/vol) Coomassie blue for 30 min, and cleared in 10% (vol/vol) acetic acid (2).

Chemicals and chemical determinations. Thiosulfate, tetrathionate, and trithionate were determined by the method of Kelly et al. (5). Protein was measured by the procedure of Lowry et al. (6), with bovine serum albumin as the standard, and pH was determined with a Metrohm expanded-scale pH meter (Brinkmann Instruments, Inc.). $K_2S_4O_6$ and $K_2S_3O_6$ were prepared according to methods described by Roy and Trudinger (9). All commercial chemicals used were of reagent grade quality.

RESULTS

TSO assay requirements and stoichiometry. Thiosulfate was required for enzymatic ferricyanide reduction. In a complete reaction mixture containing 1.3 mg of SI protein and incubated at 23 ± 2°C, 98% of the original ferricyanide was reduced after 10 min, whereas only 2% of the ferricyanide in a control mixture lacking SI protein was reduced. Addition of both fresh ferricyanide and thiosulfate to the complete reaction mixture was required to reinitiate the reaction. Under identical assay conditions, the initial velocity of ferricyanide reduction was proportional to SI protein concentration over a range of 0.9 to 7.8 mg of protein. Tetrathionate or trithionate added in place of thiosulfate failed to reduce ferricyanide. Sulfite reduced ferricyanide nonenzymatically, but the rate of ferricyanide reduction was not increased in the presence of SI protein. Although strain 16B is an obligately marine bacterium, NaCl does not seem to be required for TSO activity. Desalting of SI protein by dialysis against 0.05 M sodium phosphate (pH 7.5) for 8 h at 4°C did not affect rates of ferricyanide reduction.

Stoichiometry of the TSO assay was determined under experimental conditions identical to those described above, except that complete reaction mixtures contained 1.8 mg in place of 1.3 mg of SI protein. Controls consisting of reaction mixtures lacking either ferricyanide or thiosulfate permitted correction for ferricyanide color interference in the colorimetric analyses of thiosulfate, tetrathionate, and trithionate. In an experiment in which the reaction was run to completion (ferricyanide reduction ceased), thiosulfate completely disappeared and tetrathionate formed accounted for 84% of the thiosulfate consumed. Ferricyanide disappearance accounted for 69% of the thiosulfate oxidized and 83% of the tetrathionate formed. The differences between theoretical (equal to thiosulfate and twice tetrathionate) and actual ferricyanide formed may be explained in part by ferricyanide reduction occurring during the short time interval required for addition and mixing of ferricyanide to initiate the reaction and placing of the reaction cuvette into the spectrophotometer. In a second experiment in which 20% of the initial thiosulfate remained at termination of the reaction, small amounts (5% of thiosulfate lost) of trithionate were formed, presumably from alkaline hydrolysis of tetrathionate. Tetrathionate and trithionate sulfur accounted for 90% of the thiosulfate disappearance.

Purification. TSO activity could not be detected in the ultracentrifuge pellet but was retained in SII (Table 1), indicating that the enzyme is soluble. TSO activity was eluted from a DEAE column with the starting buffer and was detected as a band which corresponded with the first protein peak (Fig. 1). In five different purifications, TSO activity was always restricted to the first protein band (fractions 11 to 24). The data indicate that TSO did not absorb to the column. The enzyme (SIV) was routinely purified from 14- to 18-fold, with recoveries from 16 to 58%. Additional recovery of enzyme activity in fraction SIV was obtained by resuspending the centrifuged calcium phosphate gel in 10 ml of 0.1 M sodium phosphate buffer (pH 7.5), followed by centrifugation at 10,000 × *g* and 4°C for 20 min. The resulting supernatant had a specific activity similar to that of the original SIV and was pooled with this fraction.

The results of polyacrylamide gel electrophoresis revealed a substantial decrease in the number of protein bands in fractions SIII and SIV

TABLE 1. Typical purification of TSO

Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
SI	571.2	392.3	0.69	1.0	100.0
SII	422.4	357.1	0.85	1.2	91.0
SIII	12.8	87.3	6.82	9.9	22.3
SIV	5.5	62.6	11.38	16.5	16.0

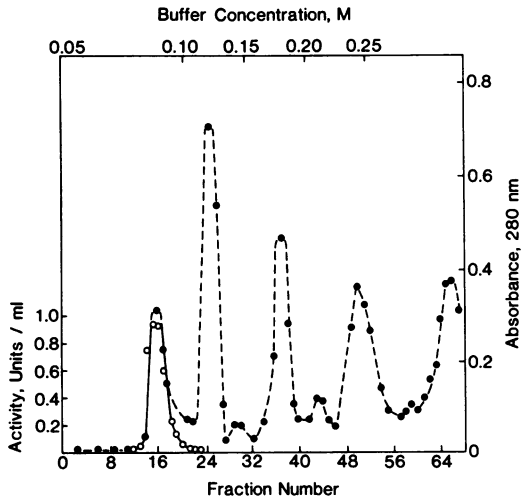

FIG. 1. Elution pattern of protein and TSO activity from DEAE-Sephadex column chromatography of fraction SII. Fraction volumes were 5 ml. Symbols: ●, protein (absorbance, 280 nm); ○, TSO activity (units per milliliter).

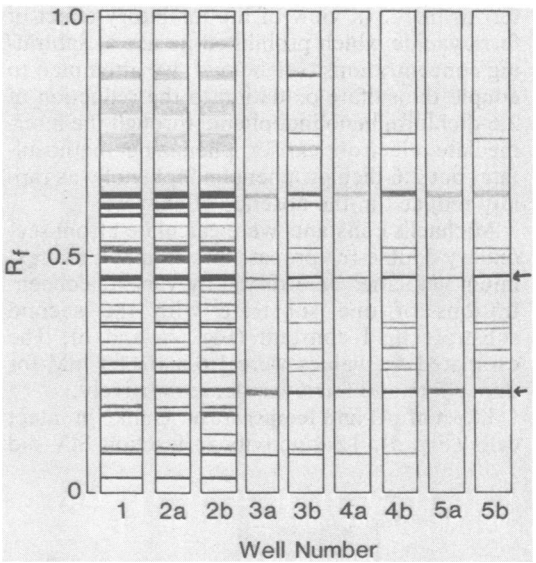

FIG. 2. Tracing of protein band patterns after electrophoresis on a polyacrylamide gel slab. Well numbers indicate protein fractions and amount of protein applied to the gel as follows: 1, SI protein, 350 μg; 2a and b, SII protein, 300 and 600 μg, respectively; 3a and b, DEAE eluate, 15 and 30 μg, respectively; 4a and b, SIII protein, 10 and 20 μg, respectively; 5a and b, SIV protein, 6 and 12 μg, respectively. Arrows indicate darkly staining bands associated with fraction SIV.

compared with SII (Fig. 2). However, two darkly stained and several weakly stained bands still remained in the most purified fraction. Attempts to demonstrate TSO activity of the separated proteins either directly on the gel slabs or by TSO assay of ground gel slices were unsuccessful.

Stability of TSO activity. Activity of both SI and SIV protein was completely destroyed by boiling for 1 min, and SI was inactivated by holding at 55°C for 30 min. SIV protein held at 4°C for 9 days retained 74% of its original activity, whereas frozen (−20°C) SIV retained 64% of the original activity. Virtually no activity of either SI or SIV protein was lost during storage at 4°C for 2 days.

Electron acceptors and Michaelis constants. Potassium ferricyanide is clearly not the in vivo electron acceptor for thiosulfate oxidation in strain 16B. TSO in extracts of *Thiobacillus neapolitanus* (16), *T. thioparus* (7), and soil heterotroph A-50 (18) couples to cytochrome *c*, but *Thiobacillus ferrooxidans* TSO does not (10). Fractions SI and SII from strain 16B contained a *c*-type cytochrome (absorbance maxima at 425, 522, and 522 nm) which was reduced by thiosulfate under the conditions of the ferricyanide assay but with ferricyanide omitted. A cytochrome having the same absorbance spectrum was also located in the ultracentrifuge pellet, but it was not reduced by thiosulfate, even when SII protein was added to the pellet preparation. Horse heart cytochrome *c* (type III; Sigma) was not reduced by SI protein in the presence of thiosulfate, and cytochromes were not enriched during purification of TSO. SIV protein did not contain detectable amounts of the *c*-type cytochrome.

Ferricyanide inhibited TSO at concentrations of >2 mM (Fig. 3). Up to 35 mM thiosulfate did not inhibit TSO activity measured at 2 mM initial

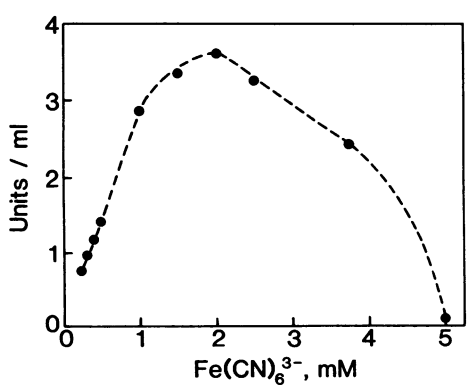

FIG. 3. Inhibitory effect of ferricyanide on TSO activity measured at pH 6.1 and 30°C. Reaction mixtures contained 7.3 mM thiosulfate and 1.1 mg of SIV protein.

ferricyanide. In view of the inhibitory effect of ferricyanide which prohibited its use at saturating concentrations (see below), we attempted to couple thiosulfate oxidation to the reduction of 2,6-dichlorophenolindophenol through the intermediate electron carrier phenazine methosulfate, but 2,6-dichlorophenolindophenol was rapidly reduced in the absence of enzyme.

Michaelis constants were calculated from secondary double-reciprocal plots of apparent maximum velocities determined by varying concentrations of one substrate with the second substrate held constant (Fig. 4a and b). The estimated K_m values were 1.6 and 11.1 mM for thiosulfate and ferricyanide, respectively.

Effect of pH and temperature. Unlike in intact cells (Fig. 5), TSO activity in fraction SIV did not exhibit a definite pH optimum. Rather, activity measured at 30°C increased as the pH was decreased to 5.0, below which thiosulfate is unstable. This difference appears to be related to the use of ferricyanide as an electron acceptor. All cell-free TSO examined so far exhibited maximum activity at pH 5 or below when assayed with ferricyanide (7, 10, 11, 16, 18), whereas the pH optima of TSO activity measured with cytochrome *c* (7) or polarographically (18) were in the range of pH 6.0 to 6.8.

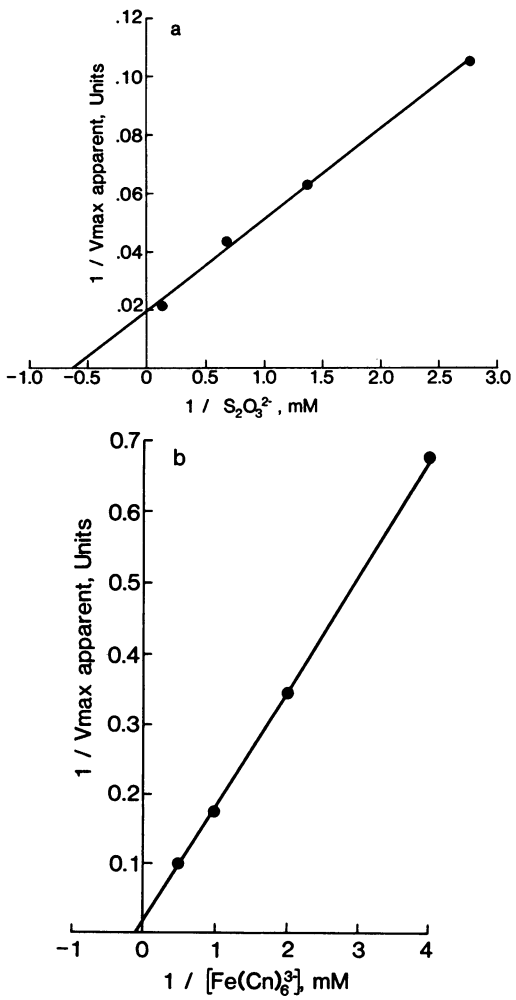

FIG. 4. Secondary plots used for determination of Michaelis constants for (a) thiosulfate and (b) ferricyanide. TSO activity was measured at pH 6.1 to 6.3 and 30°C with 1.1 mg of SIV protein.

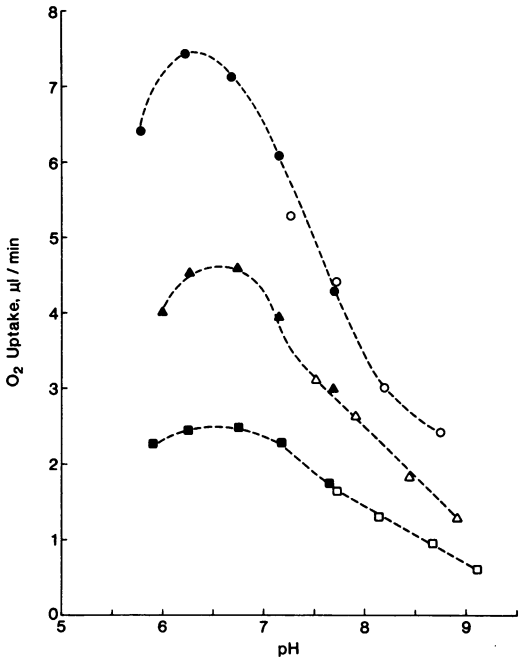

FIG. 5. Oxidation of thiosulfate measured as oxygen consumption by intact cells at 10, 20, and 30°C incubation temperatures. Symbols: ●, 30°C, phosphate; ○, Tris buffer; ▲, 20°C, phosphate; △, Tris buffer; ■, 10°C, phosphate; □, Tris buffer.

When SIV TSO activity was measured at pH 6.25 in reaction mixtures containing 7.3 mM thiosulfate and 2.0 mM ferricyanide over a temperature range of 10 to 40°C, the reaction velocity increased almost linearly up to 31°C but remained constant at higher temperatures up to 40°C. This is in agreement with the temperature optimum for TSO activity in intact cells (19). At 10°C, the rate of thiosulfate oxidation was about 60% of the rate at 28°C, the optimum growth temperature of the bacterium (22).

The finding of substantial cell-free TSO activity at low temperature led to further investigation of the effect of the relationship of temperature and pH on TSO. The effect of temperature on TSO activity of SIV protein was examined kinetically at constant pH (Table 2; Fig. 6). The

TABLE 2. Effect of temperature on apparent V_{max} and K_m values for thiosulfate with SIV TSO[a]

Temp (°C)	K_m, apparent, $S_2O_3^{2-}$ (mM)	V_{max}, apparent (U/mg)
10	2.55	9.6
15	1.79	9.0
20	1.02	9.2
25	0.47	8.8
30	0.44	9.8

[a] Data are calculated from linear regression analysis of double-reciprocal plots shown in Fig. 6.

apparent K_m for thiosulfate increased about sixfold as the temperature was decreased from 30 to 10°C (Table 2). However, the apparent maximum velocity, i.e., the potential reaction rate which could be attained if ferricyanide were not limiting, remained virtually unchanged with decreasing temperature. This unusual result suggests that decreases in enzyme activity with decreasing temperature could be reversed by increasing the thiosulfate concentration. Such a conclusion should be viewed with caution, however, because reaction rates approaching kinetically derived V_{max} values could not be reached experimentally due to ferricyanide inhibition as discussed above and because ferricyanide is not the native electron acceptor for thiosulfate oxidation. Measurable reaction rates at 10°C were again about 60% of the rates at 30°C. In contrast to cell-free extracts, maximum rates of thiosulfate oxidation by intact cells measured at temperatures of 10, 20, and 30°C exhibited a Q_{10} of approximately 2 (Fig. 5). However, the pH optimum shifted to higher values at lower temperatures, and the loss of activity at pH higher than optimum was decreased.

DISCUSSION

Apart from studies on TSO in crude extracts of soil heterotroph A-50 (18), the present investigation is the only report of cell-free TSO from a heterotrophic bacterium. Therefore, a comparison of properties of TSO from strain 16B with properties of TSO enzymes from other bacteria is of interest.

Purification of 16B TSO (14- to 18-fold) was much lower than purifications of 152-, 250-, and 100-fold reported for TSO from *T. thioparus* (7), *T. ferrooxidans* (10), and *Chromatium* sp. strain D (11), respectively. Almost identical procedures were used to purify the enzymes from 16B and *T. thioparus*. The large difference in obtainable enzyme purity, the inability of 16B TSO to couple to mammalian cytochrome *c*, and the observation that 16B TSO was not retained by a DEAE column in the same buffer regime (0.05 M phosphate) in which *T. thioparus* TSO was adsorbed (7) suggest that 16B TSO is different from that of *T. thioparus*. Ammonium sulfate fractionation was used to purify TSO from *T. neapolitanus* (15) and *Chromatium* sp. strain D (11). Our attempts to precipitate fraction SII TSO activity with ammonium sulfate not only failed to increase enzyme purity, but decreased the yield to <4%. This suggests that 16B TSO is also different from TSO of *T. neapolitanus* and *Chromatium* sp. strain D. Cell-free TSO from heterotroph A-50 (18) was not purified farther than our fraction SII. However, 16B TSO as well as TSO from *T. thioparus* (7) and *T. neapolitanus* (15) are soluble, whereas 70 to 75% of the TSO activity in extracts of soil heterotroph A-50 were found in the particulate fraction sedimenting between 12,000 and 144,000 × *g* (18). This and the observations that TSO in strain A-50 couples

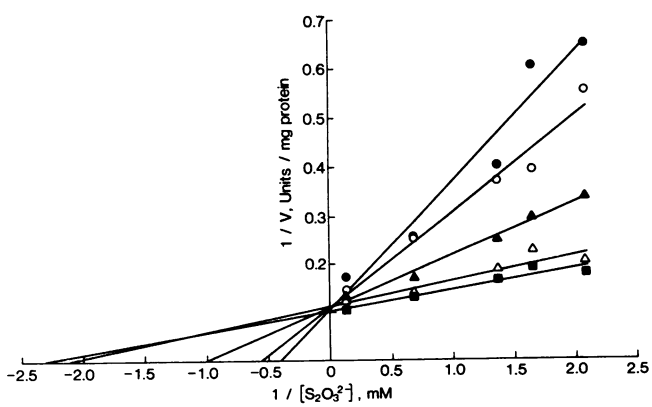

FIG. 6. Double-reciprocal plots of SIV protein TSO activity versus thiosulfate concentration at different temperatures. Reaction mixtures contained 0.05 M sodium phosphate buffer (pH 6.3), 2.0 mM ferricyanide, and 0.55 to 1.1 mg of SIV protein. Rate data are normalized (units per milligram of protein) to facilitate comparison. Symbols: ●, 10°C; ○, 15°C; ▲, 20°C; △, 25°C; ■, 30°C.

to both endogenous and mammalian cytochrome *c* whereas 16B TSO couples only to endogenous cytochrome suggest that the two enzymes are different. Insufficient information is presently available to determine whether any differences exist between *T. ferrooxidans* and 16B TSO.

Previously reported K_m values for thiosulfate, all based upon the ferricyanide assay system, range from a low value of 100 μM for *T. thioparus* enzyme at pH 6.0 (7) to a high of 1.5 mM for *Chromatium* sp. strain D enzyme (11). The thiosulfate K_m found for fraction SIV TSO (1.6 mM) is comparable to this range and to thiosulfate oxidation by intact cells (19). However, all of the values determined with cell extracts may be erroneously high due to the use of ferricyanide as an artificial electron acceptor. Further kinetic studies need to be done with more highly purified TSO from 16B and with the native cytochrome *c* as the electron acceptor.

The ability of thiosulfate to stimulate the growth rate of marine heterotroph 16B in pH-controlled batch cultures at 24 ± 2°C is greatest at pH 6.5, but diminishes as the pH is increased to 8.0 (21). These results correlate well with the pH optimum for thiosulfate oxidation in intact cells (21) and with the sparing effect of thiosulfate oxidation on organic carbon utilization (20), both of which were determined at 25°C. However, strain 16B was originally isolated from the oxygen-sulfide interface of the Black Sea at 200-m depth (23) where the pH was 7.7 and the temperature was about 9°C (1). On the basis of pH alone, it would therefore appear that thiosulfate oxidation by heterotrophic bacteria such as 16B has little significance for in situ growth, except at conditions of high concentrations of thiosulfate relative to organic carbon (20, 21). On the other hand, the findings that the apparent maximum velocity for SIV TSO is hardly affected by temperature (Table 2) and that the pH optimum for thiosulfate oxidation by intact cells is shifted upward at low temperatures indicate that the ability of marine heterotrophic bacteria to oxidize thiosulfate may be more important at environmental conditions than under optimal laboratory conditions. The influence of temperature on the relationship between TSO velocity and pH is not unique. The pH optima of a variety of enzymes from poikilothermic animals have been found to increase as temperature decreases (4). We suggest that the ability of a wide variety of heterotrophic marine bacteria to oxidize thiosulfate and perhaps other inorganic substrates, e.g., manganese, may be related to environmental stress conditions such as low temperature and low nutrient concentrations, which are characteristic of the deep-sea habitat. This possibility needs to be investigated more fully.

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