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Purification and characterization of a periplasmic thiosulfate dehydrogenase from the obligately autotrophic *Thiobacillus* sp. W5

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Abstract A periplasmic thiosulfate dehydrogenase (EC 1.8.2.2) was purified to homogeneity from the neutrophilic, obligately chemolithoautotrophic *Thiobacillus* sp. W5. A five-step procedure resulted in an approximately 2,300-fold purification. The purified protein had a molecular mass of 120 ± 3 kDa, as determined by gel filtration. It is probably a tetramer containing two different subunits with molecular masses of 33 ± 1 kDa and 27 ± 0.5 kDa, as determined by SDS-PAGE. UV/visible spectroscopy revealed that the enzyme contained haem *c*; haem staining showed that both subunits contained haem *c*. A haem *c* content of 4 mol per mol of enzyme was calculated using the pyridine haemochrome test. The pH optimum of the enzyme was 5.5. At pH 7.5, the K_m and V_{max} were 120 ± 10 μ M and $1,160 \pm 30$ U mg^{-1} , respectively. The absence of 2-heptyl-4-hydroquinoline-*N*-oxide (HQNO) inhibition for the oxidation of thiosulfate by whole cells suggested that the electrons enter the respiratory chain at the level of cytochrome *c*. Comparison with thiosulfate dehydrogenases from other *Thiobacillus* species showed that the enzyme was structurally similar to the thiosulfate dehydrogenase of the acidophilic, facultatively chemolithoautotrophic *Thiobacillus acidophilus*, but not to the thiosulfate dehydrogenases published for the obligately chemolithoautotrophic *Thiobacillus tepidarius* and *Thiobacillus thioparus*.

Key words *Thiobacilli* · Chemolithoautotrophy · Thiosulfate dehydrogenase · Thiosulfate · Tetrathionate · Cytochrome *c* · Respiratory chain

Abbreviations *BV* Benzyl viologen · *DCPIP* 2,6-Dichloroindophenol · *HQNO* 2-Heptyl-4-

hydroquinoline-*N*-oxide · *NEM* *N*-ethylmaleimide · *PES* Phenazine ethosulfate · *PMS* Phenazine methosulfate

Introduction

Thiosulfate and other reduced sulfur compounds serve as energy sources in many chemolithotrophic bacteria (Kelly 1988). The thiobacilli belong to a heterogeneous group of bacteria known as the colorless sulfur bacteria and are specialists in oxidizing reduced sulfur compounds (Robertson and Kuenen 1992). With the introduction of molecular biological techniques in bacterial taxonomy, it has become evident that the genus *Thiobacillus* is phylogenetically very diverse (Lane et al. 1992). In line with the heterogeneity of the whole group of colorless sulfur bacteria, three different thiosulfate-oxidation pathways have thus far been proposed (Beffa et al. 1993). The first pathway is found in the facultatively chemolithoautotrophic *Thiobacillus versutus* (Lu and Kelly 1983a), now known as *Paracoccus versutus*, which oxidizes thiosulfate via a periplasmic, multi-enzyme system. This enzyme system consists of two colorless proteins (enzymes A and B), cytochrome $c_{552.5}$, and cytochrome c_{551} . *Thiobacillus novellus* (Oh and Suzuki 1977) uses the second pathway, oxidizing thiosulfate by means of a membrane-bound, multi-enzyme complex. This complex has been shown to include rhodanese, a sulfur-oxidizing enzyme, sulfite: cytochrome *c* oxidoreductase, and a cytochrome *c* oxidase. In both of these pathways, thiosulfate is oxidized stoichiometrically to sulfate by the multi-enzyme complexes. The third, and apparently most common pathway seems to occur mainly in the obligately autotrophic *Thiobacillus* species. The first step involves the oxidation of thiosulfate ($S_2O_3^{2-}$) to tetrathionate ($S_4O_6^{2-}$) by a thiosulfate dehydrogenase (EC 1.8.2.2; thiosulfate:cytochrome *c* oxidoreductase). All obligately chemolithoautotrophic sulfur oxidizers thus far studied appear to possess a thiosulfate dehydrogenase. However, this is not exclusive to the obligately autotrophic thiobacilli since the facultatively chemolitho-

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autotrophic *Thiobacillus acidophilus* also possesses a thiosulfate dehydrogenase (Meulenberg et al. 1993).

The first "tetrathionate-forming enzyme" has been reported by Trudinger (1961) in *Thiobacillus neapolitanus*. Partial purification of "thiosulfate-oxidizing" enzymes in *Thiobacillus ferrooxidans* (Silver and Lundgren 1968), *Thiobacillus thioparus* (Lyric and Suzuki 1970), and *Thiobacillus tepidarius* (Lu and Kelly 1988b) have followed. More recently, tetrathionate production by the obligate autotrophs *Thiobacillus thiooxidans* (Chan and Suzuki 1994) and *Thiobacillus caldus* (Hallberg et al. 1996) has also been reported. Despite the fact that the thiosulfate dehydrogenases from the different bacteria catalyze the same reaction, only a few structural features are shared by the enzymes that have been (partially) purified. This paper describes the purification to homogeneity of a thiosulfate dehydrogenase from the neutrophilic, obligately chemolithoautotrophic *Thiobacillus* sp. W5. This bacterium has been shown to be the dominant organism in a sulfur-producing reactor and appears to be a new species closely related to *T. neapolitanus* (J. M. Visser, L. A. Robertson, and J. G. Kuenen, unpublished results). The properties of the newly isolated thiosulfate dehydrogenase are presented and compared with the properties of the previously purified thiosulfate dehydrogenases of other *Thiobacillus* species.

Materials and methods

Organism and cultivation

Thiobacillus sp. W5 (LMD 94.73) was obtained from the Delft Culture Collection. The bacteria were grown under sulfide (50 mM) limitation in an Applicon chemostat with a working volume of 1.5 l at a dilution rate of 0.05 h⁻¹. The dissolved oxygen concentration was maintained at 50% by regulating the stirrer speed, while the temperature was set at 25°C. Air was supplied at a flow rate of 0.25 l min⁻¹. The pH was maintained at 7.5 by automatic titration with 1 M sodium carbonate. The chemostat was supplied with a 1:1 mixture of two media. Medium 1 contained a 100 mM sodium sulfide solution that had been kept anoxic by a gentle flow of nitrogen gas through the headspace of the reservoir. Medium 2 contained the following minerals (g l⁻¹): NH₄Cl (0.8), KH₂PO₄ (1.0), K₂HPO₄ (1.0), MgSO₄ × 7 H₂O (1.6) and 4 ml l⁻¹ of a trace element solution according to Vishniac and Santer (1957), except that it contained 2.2 g l⁻¹ ZnSO₄ × 4 H₂O. The mineral medium was made slightly more acid than required with 5.5 ml l⁻¹ sulfuric acid to ensure that sufficient carbonate was added by the pH control system to prevent carbon limitation. Anti-foam (1 ml) was added to 20 l medium 2. When *Thiobacillus* sp. W5 was cultivated under thiosulfate limitation, the medium was supplied in a single vessel. The thiosulfate medium contained 50 mM sodium thiosulfate and the following minerals (g l⁻¹): NH₄Cl (0.4), KH₂PO₄ (0.5), K₂HPO₄ (0.5), MgSO₄ × 7 H₂O (0.8), and 2 ml l⁻¹ of the Vishniac trace element solution.

Oxygen-uptake experiments

The maximum thiosulfate-dependent oxygen-uptake rate of whole cells, taken directly from chemostat cultures, was measured in a biological oxygen monitor. Oxygen consumption was measured polarographically at 25°C using a Clark-type oxygen electrode mounted in a thermostatically controlled cell that was closed ex-

cept for a small hole through which additions could be made. Thiosulfate (50 µM) was added to obtain maximum oxidation rates. Cells were preincubated for 5 min with 150 µM 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) or 200 µM antimycin A when the effect of these inhibitors on the thiosulfate oxidation was to be tested.

Dry weight determination

The dry weight of the culture was determined by filtering 50 ml of chemostat effluent through predried membranes (Gelman GA 8, pore diameter 0.45 µm). Filtered biomass was washed with demineralized water and dried in a microwave oven for 15 min at 180 W.

Enzyme assay

The method used to assay the thiosulfate-oxidizing enzyme was that described by Trudinger (1961). The standard reaction mixture (1 ml) for measuring thiosulfate dehydrogenase activity contained 25 mM Tris-HCl (pH 7.5), 1 mM K₃Fe(CN)₆, 1 mM Na₂S₂O₃, and enzyme. The measurements were performed at room temperature and started with the addition of thiosulfate. Reduction of ferricyanide was measured at 420 nm in a spectrophotometer (HP 8524A diode array spectrophotometer) using an extinction coefficient of 1.0 mM⁻¹ cm⁻¹. One unit of activity (U) is defined as 1 µmol ferricyanide reduced per min. For determination of the enzyme activity at lower pH values, Tris-HCl buffer was replaced by 50 mM phosphate buffer. The pH was adjusted by 0.1 M NaOH or 0.1 M H₂SO₄, and the pH was measured before and after determining the enzyme activity. For kinetic studies, various amounts of thiosulfate were added to the reaction mixture.

Enzyme purification

Cells (50 g wet weight) collected at 4°C from a sulfide-limited chemostat were centrifuged and resuspended in 200 ml 25 mM Tris-HCl (pH 7.5). Subsequently, the cell suspension was passed three times through a French pressure cell (American Instrument, Silver Spring, Md., USA) at 110 MPa. The broken cells were treated with DNase, after which whole cells and cell debris were removed by centrifuging at 10,000 × g for 30 min. The cell-free extract was then separated into membrane and soluble fractions by centrifuging at 200,000 × g for 8 h. Ammonium sulfate was added to the soluble fraction to give a concentration of 1.5 M. Precipitated protein was removed by centrifuging at 20,000 × g for 15 min. The supernatant was applied to a phenyl-Sepharose column (2.5 cm × 25 cm; Biorad) equilibrated with 25 mM Tris-HCl/1.5 M ammonium sulfate (pH 7.5). Unbound protein, DNA and RNA were removed by washing the column (3 ml min⁻¹) with the equilibration buffer until the absorbance returned to its initial level. Proteins were eluted by a linear gradient (480 ml) of 1.5–0 M ammonium sulfate in 25 mM Tris-HCl (pH 7.5). Active fractions were pooled and concentrated by ammonium sulfate precipitation. The protein was redissolved in 10 ml 25 mM Tris-HCl (pH 7.5), dialyzed against 25 mM Tris-HCl (pH 7.5), and loaded on a prepacked Mono Q 10/10 column (Pharmacia) that had been pre-equilibrated with 25 mM Tris-HCl (pH 7.5). Thiosulfate dehydrogenase activity was recovered as unbound protein. Elution with a 0–1 M NaCl gradient (45 ml) released only nonactive protein. Active fractions were further purified by gel filtration on a prepacked Superdex 200 HR 10/30 column (Pharmacia) pre-equilibrated with 25 mM Tris-HCl (pH 7.5)/0.2 M NaCl at 0.5 ml min⁻¹. The active fractions were concentrated (Centricon 10; Amicon), ammonium sulfate was added to a concentration of 1.5 M, and the mixture was applied to a prepacked phenyl-Superose 10/10 column (Pharmacia) pre-equilibrated with 25 mM Tris-HCl (pH 7.5)/1.5 M ammonium sulfate at 1 ml min⁻¹. Elution was performed on a 1.5–0 M ammonium sulfate gradient (45 ml) in 25 mM Tris-HCl (pH 7.5).

All purification steps were performed at 4°C. Active fractions were stored in liquid nitrogen.

Molecular mass determination

The apparent molecular mass of the native thiosulfate dehydrogenase was determined by gel filtration on a Superdex 200 HR 10/30 column. The column was calibrated with ferritin (440 kDa; Pharmacia), catalase (232 kDa; Pharmacia), aldolase (158 kDa; Sigma), conalbumin (86 kDa), and bovine serum albumin (67 kDa and 134 kDa; Sigma). Retention times were plotted as K_{av} against the logarithm of the molecular mass.

Protein electrophoresis

SDS-PAGE was performed at room temperature on vertical 18% polyacrylamide slab gels according to the method of Laemmli (1970) using Mini Protean equipment (Biorad). Gels were stained for protein with Coomassie Brilliant Blue G250 or with silver stain. A low molecular mass calibration kit (Pharmacia) was used to derive the molecular masses. Haem staining was performed with 3,3',5,5'-tetramethylbenzidine (Bartsch 1971). Prestained markers (Biorad) were used when gels were stained for haem.

UV/visible spectroscopy

Room-temperature and 77-K spectra of purified thiosulfate dehydrogenase in 25 mM Tris-HCl (pH 7.5) were recorded on an SLM Aminco DW-2000 spectrophotometer. The spectral band width was 1 nm, while the scanning speed was 2 nm s⁻¹. Thiosulfate-reduced spectra were obtained by adding thiosulfate to a final concentration of 100 µM. The pyridine haemochrome method (Berry and Trumpower 1987) was used to determine the cytochrome content, and the absorption ratio A_{280}/A_{205} (Van Iersel et al. 1985) of an oxidized spectrum was used for the determination of the protein content ($A_{280}^{0.1\%} = 34.14 \times A_{280}/A_{205} - 0.02$).

Substrate specificity and electron acceptors

Substrate specificity was investigated with the enzyme assay described above. Sodium thiosulfate (1 mM), potassium tetrathionate (1 mM), potassium trithionate (1 mM), potassium pentathionate (1 mM), and sodium sulfite (1 mM) were tested as substrates. In order to test alternative electron acceptors, ferricyanide was replaced in the standard assay by 0.2 mM Wurster's blue (measured at a wavelength of 600 nm, absorption coefficient 9 mM⁻¹ cm⁻¹), 0.2 mM benzyl viologen (BV; 600 nm, 14.7 mM⁻¹ cm⁻¹), 0.2 mM NAD⁺ (340 nm, 6.2 mM⁻¹ cm⁻¹), 0.1 mM dichlorophenol indophenol (DCPIP; 600 nm, 16.3 mM⁻¹ cm⁻¹), 0.1 mM DCPIP + 0.6 mM phenazine methosulfate (PMS), 0.1 mM DCPIP + 0.6 mM phenazine ethosulfate (PES), 0.1 mM horse heart cytochrome *c* (550 nm, 19.6 mM⁻¹ cm⁻¹), 0.1 mM horse heart cytochrome *c* + 0.6 mM PES, or 0.1 mM horse heart cytochrome *c* + 0.6 mM PMS.

Inhibitors

The effect of potential inhibitors was studied using the standard assay. The assay mixture containing the enzyme was preincubated with the inhibitors for 5 min at room temperature. The following inhibitors (concentration) were used: potassium trithionate (1 mM), potassium tetrathionate (1 mM), potassium pentathionate (1 mM), *N*-ethylmaleimide (NEM; 1 mM), potassium cyanide (1 mM), EDTA (1 mM), sodium sulfate (0.01–1 M), ammonium sulfate (0.01–1 M), sulfite (0.05–1 mM), and selenite (0.01–1 mM).

Separation of cells into subcellular fractions and analytical procedures

Whole cells were separated into periplasmic, cytoplasmic, and membrane fractions according to the procedure of Witholt (1976), except that the lysozyme (Sigma) incubation step was extended from 15 to 90 min.

Thiosulfate and tetrathionate were determined colorimetrically according to Kelly et al. (1969). Protein concentrations were determined as described by Bradford (1976).

Results

Thiosulfate oxidation by whole cells

Thiobacillus sp. W5 was cultivated in a chemostat under sulfide or thiosulfate limitation. In both cases, sulfate was the only end product, and the yields were comparable (5.2 g dry weight mol⁻¹ substrate). These yields are similar to those reported for *Thiobacillus thioparus*, *Thiobacillus versutus* and *Thiobacillus neapolitanus* (Kuenen 1979; Mason et al. 1987). Oxygen-uptake experiments with the sulfide- and thiosulfate-grown cells showed identical maximum oxidation capacities for thiosulfate of 1.8 ± 0.2 µmol O₂ (mg protein)⁻¹ min⁻¹. The stoichiometry of thiosulfate oxidation in whole cells revealed that 2 mol oxygen was consumed for 1 mol of thiosulfate, which shows that complete oxidation to sulfate occurred. Thiosulfate-dependent oxygen uptake exhibited a monophasic pattern, suggesting that there is no transient accumulation of intermediary sulfur compounds under the conditions imposed. The addition of 2-heptyl-4-hydroquinoline-*N*-oxide (HQNO) or antimycin A, both inhibitors of the electron flux through the *bc_L*-complex, did not result in a decrease in the oxidation capacity for thiosulfate.

Purification of thiosulfate dehydrogenase

Thiosulfate dehydrogenase was purified to homogeneity from the soluble fraction of *Thiobacillus* sp. W5 by the steps outlined in Table 1. The five-step purification procedure finally resulted in an approximately 2,300-fold purification with a recovery of 34%.

Table 1 Purification of thiosulfate dehydrogenase from *Thiobacillus* sp. W5

Purification step	Total protein (mg)	Total activity (U)	Recovery (%)	Specific activity (U/mg)	Purification (-fold)
Crude extract	1,312	651	100	0.5	1.0
Soluble extract	533	593	91	1.1	2.2
Phenyl Sepharose	48	527	81	11	22
Mono Q	5.3	403	62	76	152
Superdex 200	0.25	246	38	984	1,968
Phenyl Superose	0.19	218	34	1,149	2,298

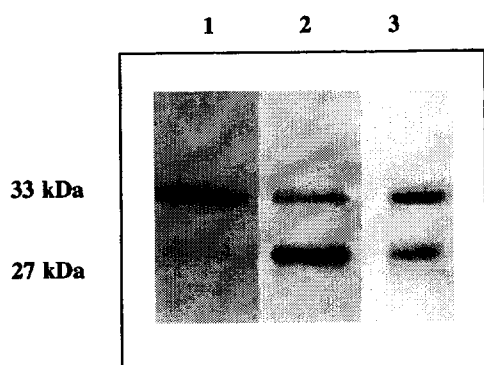


Fig. 1 Coomassie-, silver-, and haem-stained SDS-polyacrylamide gel after electrophoresis of thiosulfate dehydrogenase. All stainings were performed according to standard procedures. *Lane 1* Coomassie Brilliant Blue staining (20 μ g purified protein), *Lane 2* silver staining (5 μ g purified protein), *Lane 3* haem staining (5 μ g purified protein)

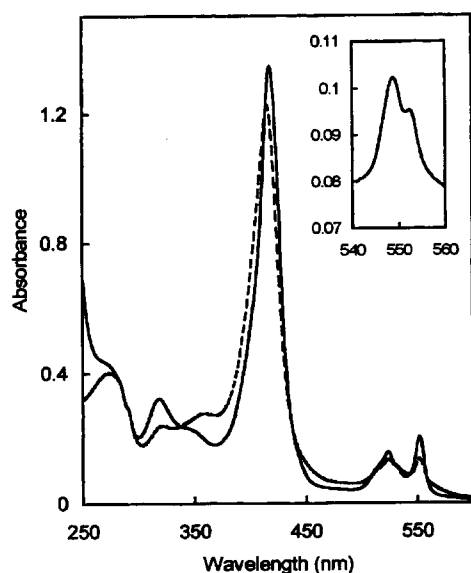


Fig. 2 UV/visible absorption spectrum of purified thiosulfate dehydrogenase (0.2 mg ml⁻¹). The absorption spectrum of thiosulfate dehydrogenase is shown from 250 to 600 nm and was recorded with an Aminco DW2000 spectrophotometer at room temperature. The air-oxidized spectrum is represented by the *dotted line*, while the thiosulfate-reduced spectrum is represented by the *solid line*. The *insert* shows the spectrum of the (-band of dithionite-reduced thiosulfate dehydrogenase recorded at 77 K

Characterization

The native thiosulfate dehydrogenase was applied to a Superdex 200 gel filtration column in order to determine the apparent molecular mass. The enzyme eluted at a volume corresponding to a molecular mass of 120 ± 3 kDa. SDS-PAGE analysis of the purified protein showed two protein bands of 33 ± 1 and 27 ± 0.5 kDa. It was observed that the Coomassie-stained bands did not have the same intensities, the larger subunit being more intensely stained

than the smaller subunit (Fig. 1A). Silver staining, however, showed the reverse pattern (Fig. 1B). These staining patterns indicate that the smaller subunit might have an acidic nature because Coomassie Brilliant Blue tends to be attracted by positively charged groups (Scopes 1982). Haem staining after denaturing SDS-PAGE of the enzyme revealed that both subunits contained haem *c* (Fig. 1C).

The UV/visible absorption spectrum of the thiosulfate-reduced enzyme gave maxima at 278, 419 (γ -band), 524 (β -band), and 552 nm (α -band) (Fig. 2), indicating the presence of *c*₅₅₂ haem. The addition of dithionite caused a small increase in reduction. The absorption spectrum of the air-oxidized enzyme still showed the presence of a significant peak at 552 nm. This indicates that the haem *c* was still partially reduced under the conditions imposed. Absorption spectra recorded at 77 K showed that the α -band was split with absorption maxima at 549.0 and 552.5 nm (Fig. 2, insert). The absorption maximum of the pyridine haemochrome complex was used to determine the haem concentration; the concentration of protein was determined from the absorption ratio A_{280}/A_{205} . From these data, it was calculated that 1 mol of thiosulfate dehydrogenase contained 3.9 ± 0.1 mol haem *c*.

Stoichiometry of thiosulfate oxidation

The end product of thiosulfate oxidation was tetrathionate. Cyanolysis revealed the stoichiometric conversion of 1.01 ± 0.03 mM thiosulfate into 0.48 ± 0.02 mM tetrathionate. In the absence of thiosulfate dehydrogenase or ferricyanide, no tetrathionate formation was measured. Thiosulfate was not formed after the addition of excess of tetrathionate to a mixture of the enzyme and ferrocyanide. This indicates that the reaction is practically irreversible under these conditions.

Kinetic properties

The kinetic properties of the thiosulfate dehydrogenase were determined at pH 7.5 and 25°C. Thiosulfate oxidation followed Michaelis-Menten kinetics. From the Lineweaver-Burk plot, an apparent V_{\max} value of 1160 ± 30 U (mg protein)⁻¹ was calculated. Under these conditions, a thiosulfate turnover rate of 2,300 s⁻¹ was obtained. The apparent K_m was 120 ± 10 μ M. The thiosulfate dehydrogenase made up only 0.04% of the total cell protein content. On the basis of its specific activity with ferricyanide as an electron acceptor, the capacity of the enzyme was almost high enough to account for the thiosulfate oxidation rate of whole cells.

pH optimum

The pH optimum for the enzyme was found to be 5.5, where activity was approximately 2.5 and 5 times higher than at pH 7.0 and pH 9.0, respectively. Activities at pH

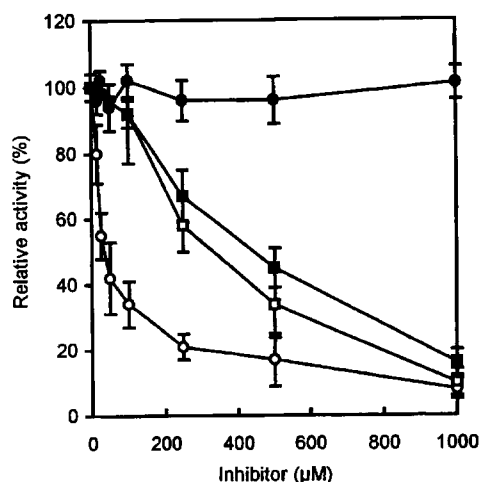


Fig. 3 Effect of sulfite and selenite on the activity of thiosulfate oxidation by the enzyme and whole cells. The influence of sulfite (SO_3^{2-} ; □, ■) and selenite (SeO_3^{2-} ; ○, ●) on the activity of thiosulfate dehydrogenase (●, ■) and thiosulfate-oxidation capacity of whole cells (○, □) was measured in the standard enzyme assay and biological oxygen monitor, respectively. The sample mixtures were preincubated for 2 min with the inhibitors. Maximum specific enzyme activity and specific thiosulfate oxidation capacity of whole cells were 1150 U mg^{-1} and $1.8 \mu\text{mol O}_2 (\text{mg protein})^{-1} \text{ min}^{-1}$, respectively

values below pH 4.0 could not be measured because of the instability of thiosulfate at these values (Roy and Trudinger 1970). It seems unlikely, however, that the *in vitro* pH optimum of the enzyme reflects its physiological optimum since the optimum growth pH of *Thiobacillus* sp. W5 is 7.0.

Substrate specificity and electron acceptors

Reduction of ferricyanide was not observed when tetrathionate, trithionate, pentathionate, or sulfite replaced thiosulfate as the substrate in the thiosulfate dehydrogenase enzyme assays. Sulfide could not be tested because it chemically reduced ferricyanide in the absence of enzyme. Thiosulfate appears to be the sole substrate for the enzyme. None of the other electron acceptors tested (i.e., Wurster's blue, BV, NAD⁺, DCPIP, DCPIP + PMS, DCPIP + PES, horse heart cytochrome *c*, horse heart cytochrome *c* + PES, or horse heart cytochrome *c* + PMS) were reduced by the enzyme in the presence of thiosulfate.

Inhibitors

Polythionates (e.g., trithionate, tetrathionate, and pentathionate) did not inhibit thiosulfate dehydrogenase activity. Addition of the sulfhydryl reagent NEM, the chelating agent EDTA, or potassium cyanide, a potent inhibitor of cytochrome *c* oxidases, also did not inhibit enzyme ac-

Table 2 Whole cells were separated into periplasmic, cytoplasmic, and membrane fractions using lysozyme and centrifugation. Distribution of the protein and activity of the thiosulfate dehydrogenase among the fractions is given as a percentage of the total protein (200 mg) and total activity (109 U), respectively

Fractions	Protein (%)	Activity (%)	Specific activity (U/mg)
Cytoplasmic	29	18	0.7
Membrane	58	9	0.2
Periplasmic	13	73	5.5

tivity. Sodium sulfate and ammonium sulfate did not inhibit at concentrations of up to 1 M. Sulfite was the only compound tested that significantly affected the enzyme, inhibiting it at concentrations above 50 μM , with a K_i of approximately 0.3 mM (Fig. 3). The sulfite inhibition of thiosulfate oxidation by whole cells was very similar to the inhibition pattern of the enzyme. Sulfite resembles the sulfonate part of thiosulfate and might, therefore, block the active site of the enzyme. However, it was observed that selenite, a sulfite analogue, did not inhibit thiosulfate oxidation by the pure enzyme although it did inhibit the oxidation of thiosulfate by whole cells (Fig. 3). The K_i for selenite was 30 μM , which is a factor 10 times lower than the K_i for sulfite. The effect of selenite is unclear. It is possible that it blocks access to the thiosulfate dehydrogenase *vivo*, or has an inhibiting effect on the respiratory chain itself.

Cellular location of the enzyme

Whole cells were separated into cytoplasmic, periplasmic, and membrane fractions (Table 2). The distribution of total protein in the cytoplasmic, periplasmic, and membrane fractions was 29, 13 and 58%, respectively. The largest portion (74%) of the total thiosulfate dehydrogenase activity was recovered from the periplasmic fraction. The specific activity of the periplasmic fraction was, therefore, 8 times higher than that of the cytoplasmic fraction and 32 times higher than that of the membrane fraction. The distribution of the activity over the cell fractions was similar to the distribution observed in *Thiobacillus tepidarius* (Lu and Kelly 1988b).

Discussion

A periplasmic thiosulfate dehydrogenase from *Thiobacillus* sp. W5 was purified to homogeneity. The native enzyme has a molecular mass that is in the same range as the thiosulfate dehydrogenases from other *Thiobacillus* species (Table 3). SDS-PAGE showed the presence of two different types of subunit. On the basis of the molecular masses of the subunits and of the native enzyme, an $\alpha_2\beta_2$ -tetramer composition is most likely. The tetramer composition appears to be similar to the proposed composition of the

Table 3 Comparison of some structural properties of purified thiosulfate dehydrogenases from *Thiobacillus* species

Species	Mol. mass of native enzyme (kDa)	Conformation	Mol. mass of subunits (kDa)	Prosthetic group	References
<i>Thiobacillus thioparus</i> ^a	115	Monomer	115	Nonhaem iron	Lyric and Suzuki (1970)
<i>Thiobacillus tepidarius</i> ^a	138	Trimer (α_3)	45	None	Lu and Kelly (1988a)
<i>Thiobacillus acidophilus</i>	102	Tetramer ($\alpha_2\beta_2$)	20 and 24	Cytochrome c_{553}	Meulenberg et al. (1993)
<i>Thiobacillus</i> sp. W5	120	Tetramer ($\alpha_2\beta_2$)	27 and 33	Cytochrome c_{552}	This study

^a Partially purified

Table 4 Catalytic characteristics of thiosulfate-oxidizing enzymes in different *Thiobacillus* species (*n.i.* not investigated)

Species	V_{max} ^a [U (mg protein) ⁻¹]	K_m (mM)	pH optimum ^a	Other artificial electron acceptors ^a	Sulfite inhibition (μ M)	References
<i>Thiobacillus tepidarius</i>	320	0.1	n.i.	Cytochrome <i>c</i>	n.i.	Lu and Kelly (1988a)
<i>Thiobacillus thioparus</i>	170	0.1	< 4.5	Cytochrome <i>c</i>	5	Lyric and Suzuki (1970)
<i>Thiobacillus ferrooxidans</i>	350	0.9	5	None	n.i.	Silver and Lundgren (1968)
<i>Thiobacillus neapolitanus</i>	100	1.0	< 4.5	Native cytochrome $c_{553.5}$	n.i.	Trudinger (1961)
<i>Thiobacillus acidophilus</i>	4,900	0.5	3	Cytochrome <i>c</i> Wurster's blue Cytochrome c_{550} ^d	3	Meulenberg et al. (1993)
<i>Thiobacillus</i> sp. W5	1,210	0.1	5.5	None	300	This study

^a V_{max} and pH optima were determined at pH 7.0 with ferricyanide as the electron acceptor

^b All tested thiosulfate-oxidizing enzymes were able to use ferricyanide as an electron acceptor; it is, therefore, not mentioned in the table

^c The sulfite concentration is given at which the activity is 50% of its maximum value

^d *Thiobacillus versutus* cytochrome c_{550}

Thiobacillus acidophilus enzyme, but differs from the *T. tepidarius* and *Thiobacillus thioparus* enzymes. The split α -peak, revealed by UV/visible absorption spectroscopy at 77 K, could indicate that haem *c* is present in two different protein environments. With the additional observations that both types of subunit contained haem *c* and that 1 mol of enzyme contained 4 mol of haem *c*, it seems likely that each subunit contains a single haem. The summary of the structural properties in Table 3 clearly shows major differences between the thiosulfate dehydrogenases of *Thiobacillus* sp. W5 and those of *T. thioparus* and *T. tepidarius*. The strongest similarity was to those of *T. acidophilus*, although the apparent molecular masses of the native enzyme and the subunits were different. In addition, the 77-K spectra in the 545- to 560-nm region gave different patterns (Fig. 2; Meulenberg et al. 1993). Further confirmation that the *T. acidophilus* and *Thiobacillus* sp. W5 enzymes are not identical can be found in Table 4. It can be seen that significant differences were observed for the pH optima, sulfite inhibition, kinetic properties, and ability to donate electrons to artificial electron acceptors.

The presence of haem *c* in the thiosulfate dehydrogenase of *Thiobacillus* sp. W5 suggests that electrons from the thiosulfate oxidation are fed into the respiratory chain at the level of cytochrome *c*. The inability of HQNO and antimycin A to inhibit the oxidation of thiosulfate by whole cells supports this. However, it cannot be excluded that the inhibitors are unable to penetrate the cell. Since HQNO and antimycin A have been shown to be effective

against the oxidation of reduced sulfur compounds by whole cells in a wide variety of *Thiobacillus* species (Lu and Kelly 1988a; Beffa et al. 1993; Chan and Suzuki 1994; Hallberg et al. 1996), this, however, seems unlikely. The coupling of electrons from thiosulfate oxidation at the level of cytochrome *c* is similar to the proposed energy coupling in *Thiobacillus neapolitanus* (Drozd 1976), *Thiobacillus novellus* (Leefelt and Matin 1980) and *Thiobacillus versutus* (Lu and Kelly 1983b). The fact that the growth yields of these thiobacilli are comparable also suggests a similar efficiency for energy conservation.

The strong structural similarity between the thiosulfate dehydrogenase of the neutrophilic, obligately autotrophic *Thiobacillus* sp. W5 and the acidophilic, facultatively autotrophic *T. acidophilus* was unexpected because the two species are not closely related taxonomically. *Thiobacillus* sp. W5 is positioned at the borderline between the beta and gamma subgroups of the Proteobacteria, while *T. acidophilus* is placed in the alpha-1 subgroup. It has even been proposed that *T. acidophilus* should be renamed as a species of the genus *Acidiphilium* (Lane et al. 1992). Comparison of the thiosulfate dehydrogenases from several *Thiobacillus* species has shown at least three different types of enzyme. Clearly the heterogeneity within the genus *Thiobacillus* is also reflected by the diversity in the structure of thiosulfate dehydrogenases. Convergent, rather than divergent evolution probably lies at the heart of these differences.

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References

- Bartsch RG (1971) Cytochromes: bacterial. In: Pietro AS (ed) *Methods in enzymology*, vol 23. Academic Press, New York, pp 344–363
- Beffa T, Fischer C, Aragno M (1993) Growth and respiratory oxidation of reduced sulfur compounds by intact cells of *Thiobacillus versutus* grown on thiosulfate. *Curr Microbiol* 26:323–326
- Berry EA, Trumpower BL (1987) Simultaneous determination of hemes a,b, and c from pyridine hemochrome spectra. *Anal Biochem* 161:1–15
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Chan CW, Suzuki I (1994) Thiosulfate oxidation by sulfur-grown *Thiobacillus thiooxidans* cells, cell-free extracts, and thiosulfate-oxidizing enzyme. *Can J Microbiol* 40:816–822
- Drozd JW (1976) Energy conservation in *Thiobacillus neapolitanus* C: sulphide and sulphite oxidation. *J Gen Microbiol* 98:309–312
- Hallberg KB, Dopson M, Lindström EB (1996) Reduced sulfur compound oxidation by *Thiobacillus caldus*. *J Bacteriol* 178:6–11
- Kelly DP (1988) Physiology and biochemistry of unicellular sulfur bacteria. In: Schlegel HG and Bowien B (eds) *Autotrophic bacteria*. Science Tech, Madison, pp 93–218
- Kelly DP, Cambers LA, Trudinger PA (1969) Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. *Anal Chem* 41:899–901
- Kuenen JG (1979) Growth yields and “maintenance energy requirement” in *Thiobacillus* species under energy limitation. *Arch Microbiol* 122:183–188
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680–685
- Lane DJ, Harrison AP, Stahl D, Pace B, Giovannoni SJ, Olsen GJ, Pace R (1992) Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J Bacteriol* 174:269–278
- Leefelt RH, Matin A (1980) Growth and physiology of *Thiobacillus novellus* under nutrient-limited mixotrophic conditions. *J Bacteriol* 142:645–650
- Lu W-P, Kelly DP (1983a) Partial purification of two principal enzymes of the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus* A2. *J Gen Microbiol* 129:3549–3564
- Lu W-P, Kelly DP (1983b) Thiosulfate oxidation, electron transport and phosphorylation in cell-free systems from *Thiobacillus* A2. *J Gen Microbiol* 129:1673–1681
- Lu W-P, Kelly DP (1988a) Kinetic and energetic aspects of inorganic sulphur compound oxidation by *Thiobacillus tepidarius*. *J Gen Microbiol* 134:865–876
- Lu W-P, Kelly DP (1988b) Cellular location and partial purification of the “thiosulfate-oxidizing enzyme” and “trithionate hydrolase” from *Thiobacillus tepidarius*. *J Gen Microbiol* 134:877–885
- Lyric RM, Suzuki I (1970) Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. *Can J Biochem* 48:355–363
- Mason J, Kelly DP, Wood AP (1987) Chemolithotrophic and autotrophic growth of *Thermotrix thiopara* and some Thiobacilli on thiosulphate and polythionates, and a reassessment of the growth yields of *Thx. thiopara* in chemostat culture. *J Gen Microbiol* 133:1249–1256
- Meulenbergh R, Pronk JT, Hazewé W, Van Dijken JP, Frank J, Bos P, Kuenen JG (1993) Purification and partial characterization of thiosulphate dehydrogenase from *Thiobacillus acidophilus*. 139:2033–2039
- Oh JK, Suzuki I (1977) Resolution of a membrane-associated thiosulfate-oxidizing complex of *Thiobacillus novellus*. *J Gen Microbiol* 99:413–423
- Robertson LA, Kuenen JG (1992) The colorless sulfur bacteria. In: Balows A, Trüper HG, Dworkin M, Harder H, Schleifer KH (eds) *The prokaryotes*, vol 1. Springer, Berlin Heidelberg New York, pp 385–413
- Roy AB, Trudinger PA (1970) *The biochemistry of inorganic compounds of sulphur*. Cambridge University Press, Cambridge
- Scopes RK (1982) *Protein purification: principles and practice*. Springer Berlin Heidelberg New York
- Silver M, Lundgren DG (1968) The thiosulfate-oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). *Can J Biochem* 46:1215–1220
- Trudinger PA (1961) Thiosulfate oxidation and cytochromes in *Thiobacillus* X. *Biochem J* 78:680–686
- Van Iersel J, Frank J, Duine JA (1985) Determination of absorption coefficients of purified proteins by conventional ultraviolet spectrophotometry and chromatography combined with multi-wavelength detection. *Anal Biochem* 151:196–204
- Vishniac W, Santer M (1957) The *Thiobacilli*. *Bacteriol Rev* 21:195–213
- Witholt B, Boekhout M, Brock M, Kingma J, Van Heerikhuizen H, De Leij L (1976) An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. *Anal Biochem* 74:160–170