Purification and partial characterization of thiosulphate dehydrogenase from *Thiobacillus acidophilus*

ROGIER MEULENBERG, JACK T. PRONK,* WIM HAZEU, JOHANNES P. VAN DIJKEN, JOHANNES FRANK, PIET BOS and J. GIJS KUENEN

Department of Microbiology and Enzymology, Kluyver Laboratory for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

(*Received 11 February 1993; revised 12 May 1993; accepted 27 May 1993*)

Thiosulphate dehydrogenase (EC 1.8.2.2; thiosulphate:acceptor oxidoreductase) was purified to apparent homogeneity from *Thiobacillus acidophilus* by a combination of ammonium sulphate precipitation, hydrophobic interaction chromatography, anion-exchange chromatography and gel filtration. The enzyme catalysed the oxidation of thiosulphate ($S_2O_3^{2-}$) to tetrathionate ($S_4O_6^{2-}$) with potassium ferricyanide as an artificial electron acceptor. The molecular mass of the native enzyme, as determined by gel filtration, was 102 ± 4·2 kDa. The enzyme contained two different subunits with a molecular mass of 24 ± 0·9 and 20 ± 1·0 kDa (SDS-PAGE), respectively. Both subunits contained c_{553}-type haem with absorption bands at 553, 524 and 416 nm. A 77 K spectrum of purified thiosulphate dehydrogenase revealed that the absorption at 553 nm is due to different haem groups. A cytochrome content of 5·3 mole *c*-type haem per mole of native enzyme was calculated. The pH optimum of the purified enzyme was 3. Apart from ferricyanide, Wurster's blue (the free radical of tetramethyl *p*-phenylenediamine) and horse heart cytochrome *c* could also serve as electron acceptors, though less effectively than ferricyanide. At pH 7·0, the K_m for thiosulphate was 0·54 mM. The K_m could not be determined at the pH optimum due to the chemical reactivity of thiosulphate at low pH values. Sulphite was a potent inhibitor of enzyme activity.

Introduction

Despite its economic and environmental importance, knowledge about the enzymology of inorganic sulphur oxidation by acidophilic *Thiobacillus* species is incomplete (for a review see Pronk *et al.*, 1990*a*). Studies with the obligate autotrophs *T. ferrooxidans* and *T. thiooxidans* (Sinha & Walden, 1966; Okuzumi & Kita, 1965) and the facultative autotroph *T. acidophilus* (Meulenberg *et al.*, 1992*a*) indicated that the initial step in the metabolism of thiosulphate ($S_2O_3^{2-}$) by these bacteria is its oxidation to tetrathionate ($S_4O_6^{2-}$). Thiosulphate dehydrogenase (EC 1.8.2.2), the enzyme catalysing this reaction, has been partially purified from *T. ferrooxidans* by Silver & Lundgren (1968). In the acidophilic thiobacilli, thiosulphate dehydrogenase is probably a periplasmic enzyme, exposed to low pH values (Pronk *et al.*, 1990*a*). However, no data are available on the catalytic activity of this enzyme at pH values below 4·5.

The aim of the present study was the purification and characterization of thiosulphate dehydrogenase from cell extracts of *T. acidophilus*, with special emphasis on product formation and enzyme characteristics at low pH.

Methods

Organism and growth conditions. T. acidophilus DSM 700 was maintained as described previously (Pronk *et al.*, 1990*b*). Mixotrophic chemostat cultures were used for production of biomass and were grown as described by Meulenberg *et al.* (1992*a*).

Enzyme assay. The standard reaction mixture (1 ml) for assays at neutral pH contained 25 mM-$(NH_4)_2SO_4$ (pH 7·0), 0·9 mM-$K_3Fe(CN)_6$ and cell extract. After 2 min equilibration at 30 °C, the reaction was started by the addition of 5 mM-$Na_2S_2O_3$. For determination of enzyme activity at lower pH values, the pH of the $(NH_4)_2SO_4$ buffer was adjusted with H_2SO_4 and the reaction was started with 0·2 mM-thiosulphate. Reduction of ferricyanide was measured at 420 nm in a spectrophotometer (Hitachi 100-60), using an extinction coefficient of $0{\cdot}9 \times 10^3$ M^{-1} cm^{-1}. Initial reaction rates were proportional to the amount of enzyme added. Activities were expressed as units (mg protein)$^{-1}$: 1 U is defined as the amount of enzyme catalysing the reduction of 1 μmol ferricyanide min^{-1}.

* Author for correspondence. Tel. 15 782387; fax 15 782355.

Abbreviations: DCPIP, dichlorophenol indophenol; MTT, methylthiazol tetrazolium bromide, PES, phenazine ethosulphate; PMS, phenazine methosulphate; PQQ, pyrrolo-quinoline quinone.

Enzyme purification

To avoid protein precipitation, which occurred at pH 5 and below, enzyme purification and activity measurements after each purification step were performed at pH 7. Enzyme purification was performed at room temperature, except for the ammonium sulphate precipitation, which was carried out on ice. Fractions containing activity were frozen and stored in liquid nitrogen. Solutions for activity measurements and enzyme purification were prepared and stored in disposable plastic labware.

Cell disruption. Cells (10 g dry mass) were suspended in 100 ml of culture supernatant adjusted to pH 7. Cells were passed twice through a French pressure cell (Aminco) at 110 MPa and centrifuged (20 min at 48000 ***g***). The pellet was extracted three times with 60 ml of culture supernatant adjusted to pH 7·0. All extracts were pooled and again centrifuged (20 min at 48000 ***g***). The resulting supernatant was used for further purification steps.

Ammonium sulphate precipitation. $(NH_4)_2SO_4$ was added to the enzyme solution to a concentration of 3·0 M. Precipitated protein was centrifuged (40 min at 48000 ***g***), redissolved in 50 ml 25 mM-sodium citrate, pH 7·0, and again centrifuged (40 min at 48000 ***g***). The supernatant was used for further purification.

Hydrophobic interaction chromatography. Hydrophobic interaction chromatography was performed with a phenyl-sepharose column (HiLoad XK 26, HR 26/10, Pharmacia) combined with a Pharmacia FPLC system (described by Meulenberg *et al.*, 1992*b*). After addition of $(NH_4)_2SO_4$ to a concentration of 1·5 M and centrifugation (30 min at 48000 ***g***), enzyme solution was loaded on the column, equilibrated with 25 mM-sodium citrate + 1·5 M-$(NH_4)_2SO_4$ (pH 7·0). The column was eluted with equilibration buffer at a flow rate of 3·0 ml min^{-1} until the A_{280} of the eluent was below 0·2. A linear gradient (560 ml) of 1·5 to 0 M-$(NH_4)_2SO_4$ in 25 mM-sodium citrate (pH 7·0) was then applied at the same flow rate. Fractions containing enzyme activity were pooled, concentrated and desalted to a final $(NH_4)_2SO_4$ concentration of 25 mM (Centriprep-30, Amicon).

Anion-exchange chromatography. Anion-exchange FPLC was performed with a Mono-Q column (HR 5/5, Pharmacia), equilibrated with 25 mM-sodium citrate (pH 7·0). After loading the enzyme solution on the column, the latter was washed with equilibration buffer at a flow rate of 0·5 ml min^{-1} until the A_{280} of the eluent was below 0·0005. A linear gradient (20 ml) of 0 to 1·0 M-NaCl in 25 mM-sodium citrate (pH 7·0) was then applied at the same flow rate. Fractions containing enzyme activity were stored in liquid nitrogen.

Gel filtration. Final purification was performed by gel filtration on a Superose 6 column (HR 10/30, Pharmacia) combined with an HPLC system consisting of a Hitachi L-6210 intelligent pump and a Waters 991 photodiode-array detector. The column was equilibrated with 0·5 M-$(NH_4)_2SO_4$ (pH 7·0) at a flow rate of 0·5 ml min^{-1}. Elution of protein was followed at 280 nm and spectra (190–800 nm) were recorded at 6 s intervals. Active fractions from successive injections were pooled, concentrated (Centricon-30, Amicon) and stored in liquid nitrogen.

PAGE. Analytical SDS-PAGE (PhastSystem, Pharmacia) was performed at room temperature according to Laemmli (1970), using an 8–25% (w/v) gradient acrylamide resolving gel and a 4·5% (w/v) acrylamide stacking gel. Purified thiosulphate dehydrogenase (30 ng) was boiled for 5 min in a buffer containing 62·5 mM-Tris, 50 mM-dithiothreitol, 50 mM-β-mercaptoethanol, 10%, w/v, glycerol, 2·5%, w/v, SDS and 0·002% bromophenol blue, pH 6·8. Electrophoresis was carried out with 30 ng of purified enzyme. Protein was stained with the Bio-Rad silver staining kit. Haem-containing proteins were visualized with tetramethylbenzidine (Thomas *et al.*, 1976).

Molecular mass. The apparent molecular mass of native enzyme was determined by gel filtration. The Superose 6 column was calibrated with α-chymotrypsin from bovine pancreas (25 kDa, Sigma), ovalbumin (43 kDa, Pharmacia), bovine serum albumin (67 kDa and 134 kDa, Sigma), catalase (232 kDa, Pharmacia), ferritine (440 kDa, Pharmacia) and thyroglobulin (669 kDa, Pharmacia). Retention was plotted as K_{av} [$=(V_e-V_0)\cdot(V_t-V_0)^{-1}$; V_e, retention volume of calibration proteins; V_0, void volume of the column; V_t, column volume] against the logarithm of the molecular mass. The molecular mass of denatured enzyme was determined by SDS-PAGE, calibrated with the Pharmacia low molecular mass electrophoresis calibration kit. For determination of molecular masses after haem-staining, a kit of pre-stained markers (Pharmacia) was used. Mobility of marker proteins relative to the electrophoresis front was plotted versus the logarithm of the molecular mass.

High resolution absorption spectra. Spectra of purified thiosulphate dehydrogenase were recorded on an Aminco DW-2000 (room temperature) and a DW-2 (77K) UV-Vis dual wavelength spectrophotometer (SLM Instruments).

Analytical procedures. Thiosulphate and tetrathionate concentrations were determined as described by Sörbo (1957). After cyanolysis, samples were centrifuged (10 min at 13000 ***g***) and absorption at 460 nm was measured. Protein concentrations were determined according to the method of Bradford (1976). For identification of the cytochrome type and content, the pyridine haemochromogen method (Rieske, 1967) was used, with horse heart cytochrome *c* and a *b*-type cytochrome from *Thiosphaera pantotropha* as references. For calculation of the cytochrome content of native thiosulphate dehydrogenase, an extinction coefficient at 550 nm of $31{\cdot}18\times10^3$ M^{-1} cm^{-1} of pyridine haemochrome was used (Bartsch, 1971).

Discontinuous enzyme assay. Enzyme solution was incubated in a well-mixed, thermostated (30 °C) reaction chamber (15 ml) containing 25 mM-$(NH_4)_2SO_4$ (pH 3·0), 1 mM-$K_3Fe(CN)_6$ and 0·5 mM-$Na_2S_2O_3$. Samples were taken at appropriate time intervals to determine thiosulphate and tetrathionate concentrations.

pH optimum, stability and kinetic constants. The pH optimum for enzyme activity was determined with the standard assay mixture, using 0·2 mM-thiosulphate. The pH of the assay buffer was adjusted with either H_2SO_4 or KOH. The actual pH of the assay mixture was measured before addition of thiosulphate and did not change significantly during the reaction. The stability of thiosulphate dehydrogenase as a function of pH was studied at 0 and 30 °C. Purified enzyme was diluted in 25 mM-$(NH_4)_2SO_4$ (pH 1·0–7·0) and incubated on ice or at 30 °C. At appropriate intervals, enzyme activity was assayed at pH 3·0 and at 30 °C. Kinetic constants were determined at pH 7·0 and at 30 °C. K_m and V_{max} were calculated by a non-linear least squares fit of the data according to the Michaelis–Menten model (software: Fig P 4.1, Biosoft).

Substrate specificity and inhibitors. Substrate specificity was studied at pH 3·0 and at pH 7·0. Sodium sulphite (0·5 mM), sodium trithionate (5 mM) and potassium tetrathionate (5 mM) were tested as substrates. Sodium sulphide could not be tested since it reacted chemically with ferricyanide. The following alternative electron acceptors were tested at pH 7·0 (wavelength and absorption coefficients are given in brackets): 0·05 mM-dichlorophenol indophenol (DCPIP; 600 nm; 16·3 mM^{-1} cm^{-1}), also tested in combination with 0·6 mM-phenazine methosulphate (PMS) or 0·6 mM-phenazine ethosulphate (PES), 0·1 mM-Wurster's blue (the free radical of tetramethyl *p*-phenylenediamine; Michaelis & Gramick, 1943) (600 nm; 9 mM^{-1} cm^{-1}), 0·05 mM-horse heart cytochrome *c* (550 nm; 19·6 mM^{-1} cm^{-1}), 0·06 mM-cytochrome c_{550} from *Thiobacillus versutus* (550 nm; 24 mM^{-1} cm^{-1}), 0·1 mM-methylthiazol tetrazolium bromide (MTT; 580 nm; 13·0 mM^{-1} cm^{-1}), 0·2 mM-benzyl viologen (600 nm; 14·7 mM^{-1} cm^{-1}), 0·2 mM-methyl viologen (600 nm; 11·3 mM^{-1} cm^{-1}), 0·01 mM-methylene blue

(650 nm; 89 mM^{-1} cm^{-1}), 0·2 mM-NAD^+ (340 nm; 6·22 mM^{-1} cm^{-1}) and 0·2 mM-$NADP^+$ (340 nm; 6·22 mM^{-1} cm^{-1}). Molecular oxygen (0·236 mM), also in combination with 0·3 mM-PMS/catalase or 1·0 mM-pyrrolo-quinoline quinone (PQQ)/catalase was tested using a biological oxygen monitor. If necessary, the observed reaction rates were corrected for the low chemical reaction rates occurring in the absence of enzyme. The effect of potential inhibitors was studied by pre-incubation for 2 min at 30 °C, after which activity was measured at pH 3·0.

Chemicals. Sodium trithionate was prepared as described by Wood & Kelly (1986). Cytochrome c_{550} from *T. versutus* and Wurster's blue were kind gifts from Ton Mulder and Barend Groen (Delft University of Technology). All other chemicals were of analytical grade and were obtained from commercial sources.

Results

Enzyme activity in cell-free extracts

Previous inhibitor studies suggested that oxidation of thiosulphate to tetrathionate by *Thiobacillus acidophilus* occurs in the periplasm and, consequently, that the enzyme catalysing this reaction is exposed to low pH values (Meulenberg *et al.*, 1992*a*). However, when cell extracts were prepared in acidic (pH 3·0) buffers, no thiosulphate dehydrogenase activity was found in the supernatant after centrifugation. At this low pH, the enzyme apparently co-precipitated with cytoplasmic enzymes; sonication of the pellet of cell debris and precipitated proteins released small amounts of enzyme activity (results not shown). In contrast, cell extracts prepared at pH 7·0 reproducibly oxidized thiosulphate to tetrathionate at neutral pH with stoichiometric reduction of ferricyanide.

Enzyme purification

The purification of thiosulphate dehydrogenase from *T. acidophilus* is summarized in Table 1. Before anion exchange, the enzyme solution had to be desalted. However, at ammonium sulphate concentrations below 100 mM, the enzyme activity partially precipitated, which resulted in a low recovery after anion-exchange chromatography (Table 1). Since anion-exchange chromatography resulted in a substantial purification, this low recovery was accepted. When low-ionic-strength buffers were used for gel filtration, the enzyme activity was not adequately separated from contaminating proteins. However, when 0·5 M-ammonium sulphate was used as the elution buffer, thiosulphate dehydrogenase eluted as a single peak ahead of other proteins.

To check the purity of the final enzyme preparation, native gel electrophoresis was performed. However, for unknown reasons the enzyme did not migrate through the acrylamide gel. Some indication of the purity of the enzyme can be derived from the fact that, after a thousandfold purification, it produced only one single peak after gel filtration. The purity of that peak was assessed with the purity algorithm (Gorenstein *et al.*, 1993) included in the Millenium 2010 Chromatography Manager, which compares all the spectra (from 250–600 nm) taken in the peak. No impurities were detected in the central 95% of the peak.

Physical properties

SDS-PAGE of purified and denatured thiosulphate dehydrogenase revealed two different protein bands with molecular masses of $24 \pm 0{\cdot}9$ and $20 \pm 1{\cdot}0$ kDa, respectively (not shown). From gel filtration, a molecular mass of $102 \pm 4{\cdot}2$ kDa was calculated for the native enzyme. The absorption spectrum of purified thiosulphate dehydrogenase showed absorption maxima at 278, 416 (Soret-band), 524 (β-band) and 553 nm (α-band) (Fig. 1), implying the presence of a c_{553}-type haem. The absorption maximum at 550 nm of the reduced pyridine haemochrome, extracted from thiosulphate dehydrogenase, confirmed that the enzyme contains a *c*-type cytochrome (data not shown) (Rieske, 1967). From this absorption maximum a cytochrome content of 5·3 moles of haem per mole of native enzyme could be calculated (Bartsch, 1971). Haem-staining of the two protein bands after denaturing SDS-PAGE indicated that both subunits

Table 1. *Purification of thiosulphate dehydrogenase from T. acidophilus*

Activities were determined using the standard enzyme assay.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Purification (-fold)
Cell-free extract	1045	5025	4·8	100	1·0
Ammonium sulphate precipitation	633	3779	6·0	75	1·3
Hydrophobic interaction	19	2570	135	51	28
Anion exchange	0·58	543	936	11	195
Gel filtration	0·08	394	4925	8	1026

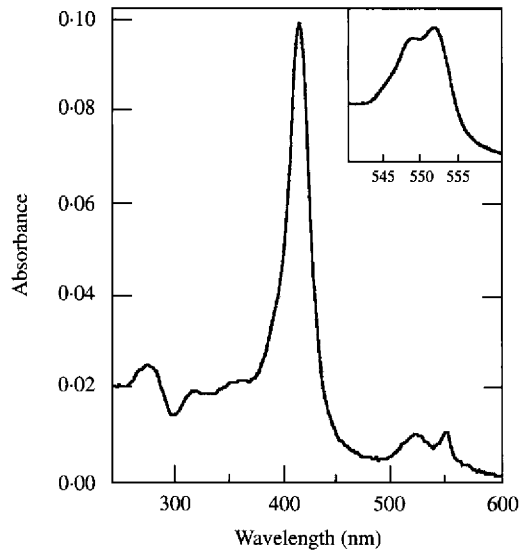

Fig. 1. Absorption spectrum of purified thiosulphate dehydrogenase. The spectrum was recorded from the eluent of gel filtration. The protein concentration in the peak fraction was 11 μg ml^{-1}, as determined from the absorption ratio A_{280}/A_{205} (Van Iersel *et al.*, 1985). Inset: 77 K spectrum of the absorption peak at 553 nm.

contained haem. A 77 K spectrum of purified thiosulphate dehydrogenase revealed a splitting of the α-band, indicating that the native enzyme contains two different haem groups (Fig. 1).

Table 2. *Electron acceptor specificity of thiosulphate dehydrogenase*

Activities were determined using the standard enzyme assay.

Electron acceptor	Concn (mM)	Relative activity (%)
Ferricyanide	0·9	100
Cytochrome *c* (horse heart)	0·05	12·1
Wurster's blue	0·10	12·1
Cytochrome c_{550} (*Thiobacillus versutus*)	0·06	2·5
DCPIP/PMS	0·05/0·6	1·8
DCPIP/PES	0·05/0·6	1·6

Catalytic properties

Stoichiometry. Incubation at pH 3·0 of purified thiosulphate dehydrogenase with 0·5 mM-thiosulphate and ferricyanide resulted in a stoichiometric formation of tetrathionate (Fig. 2). Tetrathionate formation did not occur in the absence of enzyme.

Effect of pH on enzyme activity and stability. The enzyme exhibited maximum activity at pH 3·0 (Fig. 3), where activity was approximately 30-fold higher than at pH 7·0. Below pH 2, the enzyme activity rapidly decreased. The enzyme activity was not very stable. The

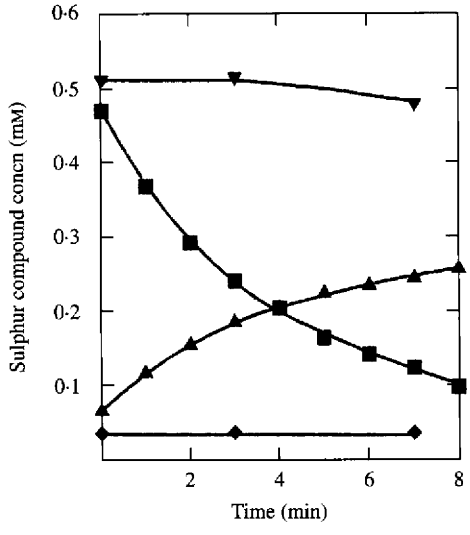

Fig. 2

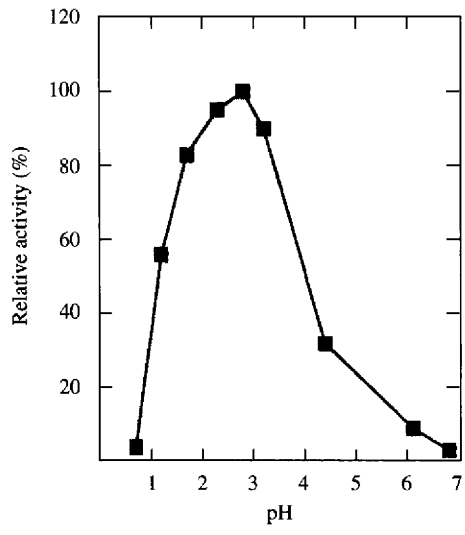

Fig. 3

Fig. 2. Reaction stoichiometry at pH 3·0. Formation of tetrathionate during incubation of 0·5 mM-thiosulphate with purified enzyme at pH 3·0 and 30 °C. Thiosulphate in the presence (■) and absence (▼) of enzyme. Tetrathionate in the presence (▲) and absence (◆) of enzyme. The small amount of tetrathionate found in the absence of enzyme is due to slow cyanolysis of thiosulphate in the absence of cupric ions.

Fig. 3. Effect of pH on thiosulphate dehydrogenase activity. Activities were determined at pH 3·0 with 0·2 mM-thiosulphate in the enzyme assay.

Table 3. *Inhibition of thiosulphate dehydrogenase at pH 3·0*

Activities were determined at pH 3·0 with 0·2 mM-thiosulphate in the enzyme assay.

Inhibitor	Concn (mM)	Relative activity (%)
None	–	100
Na_2SO_3	0·005	27
$K_2S_4O_6$	5	64
$Na_2S_3O_6$	0·05	47
Na_2SeO_3	0·5	61
Na_2SeO_4	5	49
$NaHAsO_4$	0·5	74
$NaAsO_2$	0·5	88
p-Chloromercuribenzoate	0·1	68
NaN_3	0·5	37

effect of pH on the stability of enzyme activity was studied at 0 and 30 °C. At both temperatures, the decrease in enzyme activity was not affected by the pH at which the enzyme was incubated (pH 1·0–7·0). At 30 °C, the enzyme activity decreased to 50% in 2 h. At 0 °C, 80% and 40% of the initial thiosulphate dehydrogenase activity was present after 2 and 7 h, respectively. Enzyme frozen in liquid nitrogen was stable for at least 3 months.

Kinetic constants. At pH 7·0, a K_m for thiosulphate of 0·54 mM was calculated. From the specific activity of the purified enzyme at pH 7·0, a turnover number of 8500 s^{-1} could be calculated. The K_m for thiosulphate at the pH optimum could not be determined because of the chemical reactivity of thiosulphate at millimolar concentrations at pH 3 (Roy & Trudinger, 1970). Enzyme activity at this pH increased linearly with increasing thiosulphate concentration up to 0·2 mM (results not shown). Although this indicates that the enzyme activities, measured with 0·2 mM-thiosulphate were suboptimal, they were over 30-fold higher than the activities observed at pH 7·0 with saturating substrate concentrations (Fig. 3). The maximum turnover number at pH 3·0 may therefore be at least 250000 s^{-1}.

Substrate and electron acceptor specificity. Thiosulphate-dehydrogenase-dependent reduction of ferricyanide at pH 3·0 or pH 7·0 was not observed with sulphite (0·5 mM), trithionate (5 mM) or tetrathionate (5 mM). At pH 7·0, several potential electron acceptors were tested. The enzyme activities with the different electron acceptors were not determined as a function of electron acceptor concentration. Therefore, they can be compared only qualitatively (Table 2). At higher concentrations of Wurster's blue and DCPIP, chemical interactions in the standard assay mixture prevented accurate activity measurements. Although activities were lower than with ferricyanide, horse heart cytochrome *c* and Wurster's blue supported substantial activity. PMS and PES (assayed in the presence of DCPIP) and *T. versutus* cytochrome c_{550} supported low, but significant enzyme activities (Table 2). No activity was detected with molecular oxygen, NAD^+, $NADP^+$, PQQ, methyl- or benzyl viologen, methylene blue or MTT.

Inhibitor studies. The effect of potential inhibitors of enzyme activity was tested at pH 3·0. At micromolar concentrations, sulphite was a potent inhibitor (Table 3). Inhibition of enzyme activity by sulphite did not follow saturation kinetics, but was linear with the sulphite concentration (data not shown). The sulphite-analogue selenite also inhibited enzyme activity (Table 3). Other compounds which negatively affected enzyme activity included the polythionates tetrathionate and trithionate, selenate, azide and relatively high concentrations of the

Table 4. *Properties of thiosulphate dehydrogenases from Thiobacillus species*

Organism	Mol. mass (kDa)	K_m* (mM)	Observed V_{max}† [U (mg protein)$^{-1}$]	Prosthetic group	Artificial electron acceptors‡	Reference
T. neapolitanus	NI	1	3100 (pH 4·5)[a] 100 (pH 7)	NI	$K_3Fe(CN)_6$, Cytochrome $c_{553\cdot5}$[a]	Trudinger (1961)
T. thioparus	115	0·1	220 (pH 4·5)[a] 170 (pH 7)	Non-haem iron	$K_3Fe(CN)_6$, Cytochrome c[b]	Lyric & Suzuki (1970)
T. tepidarius	138	0·1	315 (pH 7)	No haem detected	$K_3Fe(CN)_6$, Cytochrome c[b]	Lu & Kelly (1988)
T. ferrooxidans	NI	0·9	2250 (pH 5)[b] 350 (pH 7)	NI	$K_3Fe(CN)_6$[c]	Silver & Lundgren (1968)
T. acidophilus	102	0·5	> 150000 (pH 3)[b,c] 4900 (pH 7)	Cytochrome c_{553}	$K_3Fe(CN)_6$, Wurster's blue, Cytochrome c[b], Cytochrome c_{550}[d]	This study

* K_m for thiosulphate with $K_3Fe(CN)_6$ as electron acceptor.

† $K_3Fe(CN)_6$ as electron acceptor: *a*, lowest pH value used; *b*, pH optimum; *c*, non-saturating substrate concentration.

‡ *a*, Native cytochrome $c_{553\cdot5}$; *b*, horse heart cytochrome *c*; *c*, no activity with horse heart cytochrome *c* as electron acceptor; *d*, cytochrome c_{550} from *T. versutus*.

NI, Not investigated.

sulphydryl-binding agents sodium arsenate, sodium arsenite and *p*-chloromercuribenzoate. No inhibition was observed with the chelating agents citrate and EDTA, nor with *N*-ethylmaleimide.

Discussion

Thiosulphate dehydrogenase activity has been studied in a number of acidophilic and neutrophilic thiobacilli (Table 4). This paper represents the first successful attempt to purify this enzyme to apparent homogeneity. From a comparison of structural and catalytic properties of thiosulphate dehydrogenases from various *Thiobacillus* species (Table 4), it appears that there are very few conserved features. This observation is compatible with the genetic heterogeneity of the genus *Thiobacillus*, as evident from 16S RNA sequence data (Lane *et al.*, 1992).

The absorption spectrum of the purified thiosulphate dehydrogenase described here clearly indicates the presence of a haem moiety (Fig. 1). In contrast, no haem could be detected in partially purified thiosulphate dehydrogenases from *T. tepidarius* (Lu & Kelly, 1988) and *T. thioparus* (Lyric & Suzuki, 1970). Instead, in the latter organism, involvement of non-haem iron was suggested. A tetrameric conformation, containing two moles of each subunit per mole of enzyme, represents the best match for the molecular mass of the native thiosulphate dehydrogenase (102 kDa) and that of the two subunits (24 and 20 kDa). This is consistent with the observations that both subunits contain haem and that the calculated cytochrome content was 5·3 moles of haem per mole of native enzyme. The low yields of purified protein prevented us from investigating the presence of other redox-centres.

As was shown for thiosulphate dehydrogenase from *T. thioparus* (Lyric & Suzuki, 1970), sulphite was a very potent inhibitor of enzyme activity (Table 3). Inhibition of enzyme activity was linear with sulphite concentration. Thiosulphate oxidation by intact cells of *T. acidophilus* is only inhibited by sulphite concentrations above 0·2 mM. At lower concentrations, thiosulphate and sulphite are simultaneously oxidized to sulphate (results not shown).

The low pH optimum of thiosulphate dehydrogenase from *T. acidophilus* (Fig. 3) is consistent with its supposed localization in the periplasm (Meulenberg *et al.*, 1992*a*; Pronk *et al.*, 1990*a*). The ability of the purified enzyme to use horse heart cytochrome *c* and a bacterial *c*-type cytochrome as electron acceptor at pH 7·0 (Table 2) implies that the physiological electron acceptor may be a *c*-type cytochrome. Coupling of thiosulphate oxidation to the respiratory chain at the level of cytochrome *c* would be compatible with a periplasmic localization and with the observed growth efficiencies in mixotrophic chemostat cultures (Pronk *et al.*, 1990*c*). It is not yet clear which cytochrome *c* is involved as analysis of cytochrome spectra of intact cells of *T. acidophilus* has revealed the presence of at least five different *c*-type cytochromes (J. T. Pronk & J. E. van Wielink, unpublished).

The authors are grateful to Cornel Verduyn for many stimulating discussions. We thank Marten Hazelaar for recording the 77 K spectra, Barend Groen for help with the photodiode array detector and Ton Mulder for providing us with a sample of purified cytochrome c_{550} from *T. versutus* and for help with the gel electrophoresis. Marijke Luttik is acknowledged for preparation of Fig. 1.

References

BARTSCH, R. G. (1971). Cytochromes: bacterial. *Methods in Enzymology* **23**, 344–363.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

GORENSTEIN, M. V., LI, J. B. & CHAPMAN, D. (1993). *Abstract no. 974*, Pittsburgh Conference, Atlanta, USA.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LANE, D. J., HARRISON, A. P., JR, STAHL, D., PACE, B., GIOVANNONI, S. J., OLSEN, G. J. & PACE, N. R. (1992). Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *Journal of Bacteriology* **174**, 269–278.

LU, W.-P. & KELLY, D. P. (1988). Cellular location and partial purification of the 'thiosulphate-oxidizing enzyme' and 'trithionate hydrolase' from *Thiobacillus tepidarius*. *Journal of General Microbiology* **134**, 877–885.

LYRIC, R. M. & 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. *Canadian Journal of Biochemistry* **48**, 355–363.

MEULENBERG, R., PRONK, J. T., HAZEU, W., BOS, P. & KUENEN, J. G. (1992*a*). Oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus*. *Archives of Microbiology* **157**, 161–168.

MEULENBERG, R., PRONK, J. T., FRANK, J., HAZEU, W., BOS, P. & KUENEN, J. G. (1992*b*). Purification and partial characterization of a thermostable trithionate hydrolase from the acidophilic sulphur oxidizer *Thiobacillus acidophilus*. *European Journal of Biochemistry* **209**, 367–374.

MICHAELIS, L. & GRAMICK, S. (1943). The polymerization of the free radicals of the Wurster dye type: the dimeric resonance band. *Journal of the American Chemical Society* **65**, 1747–1755.

OKUZUMI, M. & KITA, Y. (1965). Studies on biochemistry of the thiobacilli. VI. Oxidation of thiosulphate to tetrathionate by *T. thiooxidans*. *Agricultural and Biological Chemistry* **29**, 1063–1068.

PRONK, J. T., MEULENBERG, R., HAZEU, W., BOS, P. & KUENEN, J. G. (1990*a*). Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli. *FEMS Microbiology Reviews* **75**, 293–306.

PRONK, J. T., MEESTERS, P. J. W., VAN DIJKEN, J. P., BOS, P. & KUENEN, J. G. (1990*b*). Heterotrophic growth of *Thiobacillus acidophilus* in batch and chemostat cultures. *Archives of Microbiology* **153**, 392–398.

PRONK, J. T., MEULENBERG, R., VAN DEN BERG, D. J. C., BATENBURG-VAN DER VEGTE, W., BOS, P. & KUENEN, J. G. (1990*c*). Mixotrophic and autotrophic growth of *Thiobacillus acidophilus* on glucose and thiosulfate. *Applied and Environmental Microbiology* **56**, 3395–3401.

RIESKE, J. S. (1967). The quantitative determination of mitochondrial hemoproteins. *Methods in Enzymology*, **10**, 488–493.

ROY, A. B. & TRUDINGER, P. A. (1970). *The Biochemistry of Inorganic Compounds of Sulphur*. Cambridge: University Press.

SILVER, M. & LUNDGREN, D. G. (1968). The thiosulfate-oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). *Canadian Journal of Biochemistry* **46**, 1215–1220.

SINHA, D. B. & WALDEN, C. C. (1966). Formation of polythionates and their interrelationships during oxidation of thiosulphate by *T. ferrooxidans*. *Canadian Journal of Microbiology* **12**, 1041–1054.

SÖRBO, B. (1957). A colorimetric method for the determination of thiosulphate. *Biochimica et Biophysica Acta* **23**, 412–416.

THOMAS, P. E., RYAN, D. & LEVIN, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P450 on sodium dodecyl sulfate polyacrylamide gels. *Analytical Biochemistry* **75**, 168–176.

TRUDINGER, P. A. (1961). Thiosulphate oxidation and cytochromes in *Thiobacillus* X. *Biochemical Journal* **78**, 680–686.

VAN IERSEL, J., FRANK JZN, J. & DUINE, J. A. (1985). Determination of absorption coefficients of purified proteins by conventional ultraviolet spectrophotometry and chromatography combined with multiwavelength detection. *Analytical Biochemistry* **151**, 196–204.

WOOD, A. P. & KELLY, D. P. (1986). Chemolithotrophic metabolism of the newly-isolated moderately thermophilic, obligately autotrophic *Thiobacillus tepidarius*. *Archives of Microbiology* **144**, 71–77.