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Purification and partial characterization of a thermostable trithionate hydrolase from the acidophilic sulphur oxidizer *Thiobacillus acidophilus*

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Cell-free extracts of *Thiobacillus acidophilus* catalysed the quantitative conversion of trithionate ($S_3O_6^{2-}$) to thiosulphate and sulphate. A continuous assay for quantification of experimental results was based on the difference in absorbance between trithionate and thiosulphate at 220 nm.

Trithionate hydrolase was purified to near homogeneity from cell-free extracts of *T. acidophilus*. The molecular masses of the native enzyme and the subunit were 99 kDa (gel filtration) and 34 kDa (SDS/PAGE). The purified enzyme has a pH optimum of 3.5–4.5 and a temperature optimum of 70°C. Enzyme activity was stimulated by sulphate. The stimulation of the enzyme activity by sulphate was half maximal at a concentration of 0.23 M. The K_m for trithionate is 70 μM at 30°C and 270 μM at 70°C. Enzyme activity was lost after 36 days at 0°C, 27 days at 70°C; but after 97 days at 30°C, 40% of the initial activity was still present. The enzyme activity was inhibited by mercury chloride, *N*-ethylmaleimide, thiosulphate and tetrathionate. Tetrathionate $S_4O_6^{2-}$ was not hydrolysed by trithionate hydrolase.

Oxidation of inorganic sulphur compounds by acidophilic bacteria is a key microbial leaching process (Hazeu et al., 1988) and the biological desulphurization of coal (Bos et al., 1988; Klein et al., 1988), and causes the environmental problem known as acid mine drainage (Lundgren et al., 1972). Attempts to unravel the pathways involved in acidophilic sulphur oxidation are hindered by the high reactivity of many of the compounds involved (Roy and Trudinger, 1970). Therefore, in spite of the economic and environmental implications of these processes, knowledge of the enzymology of inorganic sulphur oxidation in acid environments is incomplete (for a review see Pronk et al., 1990b).

Many of the organisms involved in the above-mentioned processes (including the acidophile *Thiobacillus ferrooxidans*, an extensively used acidophilic model organism; Ingledew, 1982), are obligate autotrophs. Since biomass yields of these organisms are low, it is difficult to obtain the substantial amounts of biomass required for enzyme-purification studies. To circumvent this problem, we decided to use a facultatively autotrophic model organism in our studies of acidophilic sulphur oxidation.

Thiobacillus acidophilus is an acidophilic, facultatively autotrophic, sulphur-oxidizing bacterium with optimum growth at approximately pH 3. Carbon sources supporting heterotrophic growth include a number of monosaccharides, tricarboxylic-acid-cycle intermediates and some amino acids (Guay and Silver, 1975; Pronk et al., 1990a). Growth substrates for autotrophic growth include formate (Pronk et al., 1990d), elemental sulphur (Guay and Silver, 1975), tetrathionate (Norris et al., 1986), thiosulphate ($S_2O_3^{2-}$) and trithionate (Mason et al., 1987).

In natural environments, trithionate can be formed during dissimilatory sulphite reduction by sulphate-reducing bacteria (Fitz and Cypionka, 1990). Furthermore, formation of trithionate as an intermediate during tetrathionate oxidation has been reported for *T. ferrooxidans* (Sinha and Walden, 1966) and *Thiobacillus thiooxidans* (Okuzumi, 1965, 1966a). Trithionate can also be formed chemically by a reaction between tetrathionate and sulphite (Trudinger, 1964a).

Hydrolysis, yielding thiosulphate and sulphate, has been described as the initial step of trithionate metabolism in intact cells of the obligately autotrophic neutrophiles *Thiobacillus neapolitanus* (Trudinger, 1964b) and *Thiobacillus tepidarius* (Lu and Kelly, 1988), and in the facultatively autotrophic acidophile *T. acidophilus* (Meulenberg et al., 1992). Initial reduction of trithionate to thiosulphate and sulphite, as occurs in one of the proposed mechanisms for dissimilatory sulphite reduction (Fitz and Cypionka, 1990), was suggested for the obligately autotrophic acidophile *T. thiooxidans* (Okuzumi, 1966b).

Lu and Kelly (1988) partially purified a trithionate hydrolase from the neutrophile *T. tepidarius*. The activity was assayed in a coupled system in which cytochrome *c* was reduced by purified thiosulphate dehydrogenase with the oxidation of thiosulphate formed during trithionate hydrolysis (Lu and Kelly, 1988).

The aim of the present study was the purification and characterization of trithionate hydrolase from cell-free extracts of *T. acidophilus*.

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Enzymes. Trithionate hydrolase (EC 3.12.1.1); thiosulphate dehydrogenase (EC 1.8.2.2); chymotrypsinogen (EC 3.4.21.1); lactate dehydrogenase (EC 1.1.1.27).

MATERIALS AND METHODS

Organism and growth conditions

T. acidophilus DSM 700 was obtained from the *Deutsche Sammlung von Mikroorganismen* as a liquid culture grown on glucose and maintained as described previously (Pronk et al., 1990c).

High cell density (5 g dry mass) mixotrophic chemostat cultures of *T. acidophilus* were used for production of biomass for enzyme-purification studies. These cultures were grown as described by Meulenberg et al. (1992).

The purity of the chemostat cultures was checked by phase-contrast microscopy and immunofluorescence as described by Pronk et al. (1990c).

The dry mass of cell suspensions was determined with dried nitrocellulose filters as described previously (Pronk et al., 1990c).

Discontinuous assay for activity measurements

A cell-free extract in 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0, was placed in a well-mixed, thermostatted (30 °C) reaction chamber (10 ml). After addition of 1 mM trithionate, 0.5-ml samples were taken after the desired time intervals and immediately mixed with 0.1 ml 125 mM potassium cyanide for determination of thiosulphate, according to the method of Sorbö (1957), modified by Kelly et al. (1969). After addition of 0.1 ml 75 mM copper chloride, 1.0 ml 300 mM ferric nitrate in 3 M nitric acid was added to form the orange ferric thiocyanate complex. The amount of ferric nitrate used here was tenfold higher than that used by Kelly et al. (1969) in order to achieve a satisfactory development of colour, since this is impaired by the high ammonium sulphate concentration necessary to keep the enzyme in solution. When smaller volumes of ferric nitrate solution were used, development of colour was incomplete at these high ammonium sulphate concentrations.

Continuous assay for activity measurements

The standard reaction mixture (1 ml in a quartz cuvette) for the determination of trithionate hydrolase activity contained 25 mM potassium phosphate, pH 3.0, 1 M ammonium sulphate and 1 mM sodium trithionate. The reaction was started by the addition of purified enzyme or cell-free extract and the increase in A_{220} due to formation of thiosulphate was recorded with a Hitachi double-beam spectrophotometer (model 100–60) at 30 °C. An assay mixture without trithionate and cell-free extract was used as the reference. Initial reaction rates were linearly proportional to the amount of enzyme added.

For quantification of experimental results a molar absorption coefficient of $3.13 \times 10^3\ M^{-1} \cdot cm^{-1}$ for thiosulphate formation was used (see also Results). 1 U enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmol thiosulphate/min.

Determination of protein concentration

Due to the high ammonium sulphate concentrations in the enzyme extracts, the protein determination methods of Lowry (1951) and Bradford (1976) could not be used. Therefore protein concentration was measured by determination of the total-organic-carbon content of extracts as described by Rouwenhorst et al. (1991). Protein concentration in (partly) purified fractions can be calculated by assuming that proteins have an average carbon content of 0.53 g/g (Rouwenhorst et al., 1991).

Enzyme-purification procedure

All purification steps were carried out at room temperature. Enzyme solutions were frozen and stored in liquid nitrogen between purification steps.

Cell disruption

Bacterial cells (13 g dry mass) were collected on ice from a chemostat culture of *T. acidophilus* and centrifuged (10 min at 12 000 × *g*). The biomass was washed and resuspended in 80 ml of a buffer containing 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 2.5. This cell suspension was passed three times through a French pressure cell (American Instrument Company, Silver Spring, Maryland, USA) at 110 MPa and then centrifuged for 20 min at 48 000 × *g* to remove the bulk of cell debris. The supernatant was centrifuged for 120 min at 131 000 × *g*. The resulting clear supernatant (the crude cell-free extract) was used for further purification steps.

Selective precipitation

Crude cell-free extract (24.0 ml) was diluted 12-fold with 25 mM potassium phosphate, pH 3.0. Precipitated protein was centrifuged (20 min at 48 000 × *g*), redissolved in 6.0 ml 25 mM potassium phosphate containing 0.4 M ammonium sulphate, pH 3.0, and centrifuged again (20 min at 48 000 × *g*). The supernatant (5.8 ml) contained the enzyme activity.

Cation-exchange chromatography

Cation-exchange chromatography was performed with a Mono-S column (HR 5/5, Pharmacia) combined with a Pharmacia FPLC system, consisting of two P-500 pumps, a GP-250 gradient programmer, an UV-M spectrophotometer, a V-7 injection unit, a LKB 2112 fraction collector (Redirac) and a BD 40 recorder (Kipp and Zonen).

The extract (2.0 ml) was loaded onto the Mono-S column, previously equilibrated with 25 mM potassium phosphate, 0.4 M ammonium sulphate, pH 3.0. The column was washed with equilibration buffer at a flow rate of 0.5 ml/min until A_{280} was below 0.005. A linear gradient (20 ml) of 0.4–1.0 M ammonium sulphate in 25 mM potassium phosphate, pH 3.0, was then applied to the column at the same flow rate. Active fractions, eluting at 0.58–0.76 M ammonium sulphate, were pooled and concentrated by ultrafiltration (Centricon-30, Amicon) to 1.3 ml.

Gel filtration

Final purification was achieved by gel filtration on a Superose-12 column (HR 10/30, Pharmacia) using the FPLC system described above. The column was equilibrated with 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0, at a flow rate of 1.0 ml/min, and after injection of the extract (0.1 ml), the activity eluted after 14.1 min. Active fractions were concentrated by ultrafiltration.

Polyacrylamide-gel electrophoresis

Analytical SDS/PAGE was performed at room temperature according to the method of Laemmli (1970), using a 12%

acrylamide resolving gel and a 3% acrylamide stacking gel. Samples were boiled for 5 min in a buffer containing 25 mM potassium phosphate, 62.5 mM Tris, 1.0 M ammonium sulphate, 50 mM dithiothreitol, 50 mM 2-mercaptoethanol, 10% glycerol, 2.5% SDS and 0.002% bromophenol blue, pH 3.3. Electrophoresis was carried out overnight with 1.5–5.5 μg protein/lane and at a constant potential difference of 40 V. Gels were stained with the Bio-Rad silver-staining kit.

Molecular mass determination

The apparent molecular mass of the native enzyme was determined by FPLC gel filtration on a Superose-12 column (HR 10/30, Pharmacia), and eluted in 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0, at a flow rate of 0.5 ml/min. The choice of calibration proteins was limited to proteins soluble in this buffer. The Superose-12 column was calibrated with ribonuclease from bovine pancreas (13.7 kDa; Boehringer), α-chymotrypsin from bovine pancreas (25 kDa; Sigma), ovalbumin (43 kDa; Pharmacia) and bovine serum albumin (67 kDa; Sigma). The void volume of both columns was determined with blue dextran (±2000 kDa, Pharmacia). Elution times were plotted as $K_{av} = (V_e - V_0)/(V_t - V_0)$ against log(molecular mass).

The molecular mass of the denatured enzyme was determined by SDS/PAGE, calibrated with the Pharmacia low-molecular-mass electrophoresis-calibration kit, containing phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa). Before electrophoresis, the molecular mass markers were treated as described above. The mobility of the marker proteins relative to the electrophoresis front was plotted against log (molecular mass).

Determination of pH optimum, optimum temperature and kinetic constants

The pH optimum was determined with the standard assay buffer and with a potassium phosphate/sodium citrate buffer. The pH was varied over the range 1.0–7.6 by adjusting the pH of the assay buffer with either 1 M H_2SO_4 or 1 M KOH.

The temperature optimum was studied under standard continuous assay conditions. The temperature was varied over the range 30–90 °C, and the actual temperature was measured in the cuvettes.

Kinetic constants were determined at 30 °C and 70 °C under standard assay conditions. K_m and V_{max} were calculated using non-linear regression following Michaelis-Menten kinetics (software: FIG. P 4.1, Biosoft).

Thermostability

Enzyme thermostability was tested by incubating the protein in sealed plastic tubes at 0 °C, 30 °C and 70 °C. Enzyme activity was assayed under standard conditions at different time intervals.

Effect of ammonium sulphate concentration and inhibitors on enzyme activity

The effect of the ammonium sulphate concentration (0.005–2 M) on enzyme activity was studied under standard assay conditions. Next to ammonium sulphate, also ammonium chloride, sodium sulphate and sodium chloride were tested as potential stimulators of enzyme activity. The enzyme was incubated for 30 min at 30 °C with the different salt concentrations before its activity was determined in the same concentration.

The effect of different potential enzyme inhibitors or stimulators and other additives was examined under standard assay conditions. The enzyme was incubated for 10 min at 30 °C with the different additives before activity was determined. The following additives were used: 1.0 mM EDTA, 0.1–0.5 mM diethyl dithiocarbamide, 1.0 mM sodium azide, 1.0 mM nickel chloride, 1.0 mM cobalt chloride, 1.0 mM zinc sulphate, 1.0 mM magnesium sulphate, 1.0 mM calcium chloride, 1.0 mM sodium sulphite, 0.1 mM mercury chloride, 0.05–0.25 mM *N*-ethylmaleimide, 0.25–1.0 mM thiosulphate and 0.125–0.5 mM tetrathionate.

Chemicals

Sodium trithionate was prepared as described by Wood and Kelly (1986). All other chemicals were analytical grade and obtained from commercial sources.

RESULTS

Development of a continuous assay for enzyme activity measurements

Cell-free extracts of mixotrophically grown *T. acidophilus* catalysed the quantitative conversion of trithionate to thiosulphate (reaction 1 below; Fig. 1). As cell-free extracts of *T. acidophilus* were prepared in a buffer containing 25 mM potassium phosphate and 1.0 M ammonium sulphate, sulphate, the other product of this hydrolysis in *T. neapolitanus* (Trudinger, 1964b), could not be determined.

During incubation of trithionate in anaerobic cell suspensions of *T. acidophilus*, some tetrathionate was also formed (Meulenberg et al., 1992), but hydrolysis of trithionate by cell-free extracts occurred without tetrathionate formation (Fig. 1). Optimal conditions for cell-free extract preparation and activity assays included the use of a buffer with a high osmolarity and a low pH (pH 3). Initial experiments were performed by measuring the rate of thiosulphate production, using the cyanolysis method (Sorbö, 1957; Kelly et al., 1969). However, determination of trithionate hydrolase activity by this method is very laborious and time consuming (Fig. 1). Lu and Kelly (1988) assayed trithionate hydrolase from *T. tepidarius* in a coupled system in which the thiosulphate formed in reaction 1 was used to reduce cytochrome *c*, catalysed by purified thiosulphate dehydrogenase (reaction 2):

$$2\,S_3O_6^{2-} + 2\,H_2O \rightarrow 2\,S_2O_3^{2-} + 2\,SO_4^{2-} + 4\,H^+; \quad (1)$$

$$2\,S_2O_3^{2-} + 2\,\text{cyt}\ c_{ox} \rightarrow S_4O_6^{2-} + 2\,\text{cyt}\ c_{red}; \quad (2)$$

cyt, cytochrome, ox, oxidised; red, reduced.

This method, as already stated by Lu and Kelly (1988), requires large amounts of purified thiosulphate dehydrogenase, and the presence of at least two different proteins in the assay mixtures which may interact with each other. We therefore examined the absorption spectra (190–300 nm) of trithionate and thiosulphate. Trithionate has an absorption maximum at 205 nm and thiosulphate at 215 nm (Fig. 2A, B). The difference spectrum (Fig. 2C) shows a maximum at 220 nm. Thus, in principle, the hydrolysis of trithionate to thiosulphate can be followed by monitoring the absorbance at 220 nm.

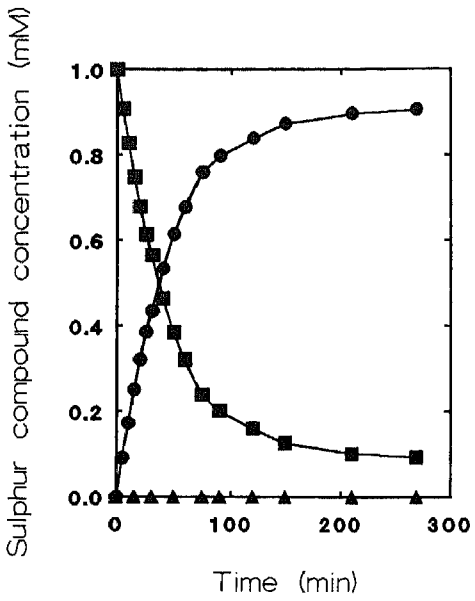

Fig. 1. Product formation. Formation of thiosulphate during incubation of 1 mM trithionate with cell-free extract containing trithionate hydrolase activity at 30°C. The buffer contained 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0. Thiosulphate and tetrathionate were analysed by cyanolysis (Sorbö, 1957; Kelly et al., 1969) and trithionate concentration was calculated by subtracting thiosulphate and tetrathionate concentrations from the initial trithionate concentration. (■) Trithionate; (●) thiosulphate; (▲) tetrathionate.

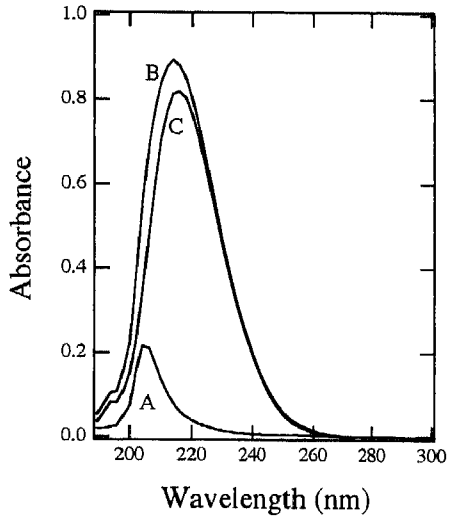

Fig. 2. Absorption spectra of trithionate (A) and thiosulphate (B). Thiosulphate (0.25 mM) and trithionate (0.25 mM) were dissolved in 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0. Buffer without sulphur compound was used as a reference. Spectrum C is the difference spectrum.

For quantification of experimental results, the molar absorption coefficients of trithionate and thiosulphate were determined. The molar absorption coefficients for trithionate and thiosulphate at 220 nm were determined to be 0.15 $\times 10^3$ $M^{-1} \cdot cm^{-1}$ and 3.28×10^3 $M^{-1} \cdot cm^{-1}$, respectively, and were not significantly affected by temperature, pH and ammonium sulphate concentration. The specific molar absorption coefficient for hydrolysis of trithionate to thiosulphate is therefore 3.13×10^3 $M^{-1} \cdot cm^{-1}$. Enzyme activity calculated in this way agreed within 90% with the activity found with the discontinuous cyanolysis assay, and increased linearly with the amount of enzyme used in the assay.

Table 1. Purification of trithionate hydrolase from *T. acidophilus*.

Purification step	Total protein	Total activity	Specific activity	Yield	Purification
	mg	U	U/mg	%	-fold
Cell-free extract	304.0	932	3.1	100	1.0
Selective precipitation	45.0	418	9.3	45	3.0
Cation exchange	4.4	210	47.7	23	15.4
Gel filtration	1.2	149	124.2	16	40.1

Purification and physical properties

Trithionate hydrolase was purified to near homogeneity from cell-free extracts of *T. acidophilus*, grown on glucose and thiosulphate in mixotrophic chemostat cultures. The results of the purification procedure are summarized in Table 1.

High specific enzyme activities were obtained when cell-free extracts were prepared in 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0. This was the optimum pH for preparation of cell-free extracts with respect to specific trithionate hydrolase activity.

As described in Materials and Methods, protein concentration was determined by total-organic-carbon analysis. However, in addition to protein, cell-free extracts are likely to contain other organic compounds, such as lipids, nucleic acids and carbohydrates (Rouwenhorst et al., 1991). Therefore, the protein concentration in the initial cell-free extracts may be somewhat overestimated. However, we assume that such other organic compounds were also removed during purification of trithionate hydrolase.

Upon dilution of the cell-free extract with 25 mM potassium phosphate, pH 3.0, the enzyme activity precipitated and the centrifuged protein could be resuspended in a buffer containing 25 mM potassium phosphate and 0.4 M ammonium sulphate, pH 3.0. This fractionation procedure resulted in a threefold purification and a recovery of 45% (Table 1). This ammonium sulphate concentration (0.4 M) was the highest concentration at which enzyme activity still could be bound to the cation exchanger (Mono-S). Purification and recovery after cation-exchange chromatography were 15-fold and 23%, respectively (Table 1). As a final purification step, gel filtration on Superose 12, yielded an overall purification and recovery of 40-fold and 16%, respectively (Table 1).

Denaturation and SDS/PAGE of the extract after gel filtration yielded a major band at 34 kDa and a very faint band at 41 kDa (Figs 3 and 4B). Using gel filtration on Superose 12, a molecular mass of 99 kDa could be derived for the native enzyme by comparing its elution behaviour with that of marker proteins (Fig. 4A). This suggests that the native trithionate hydrolase contains three identical subunits.

The absorption spectrum of trithionate hydrolase is characterized by a single maximum at 280 nm and no absorbance above 300 nm (data not shown).

Catalytic properties of trithionate hydrolase

All characterization experiments with trithionate hydrolase were performed with the purest fraction.

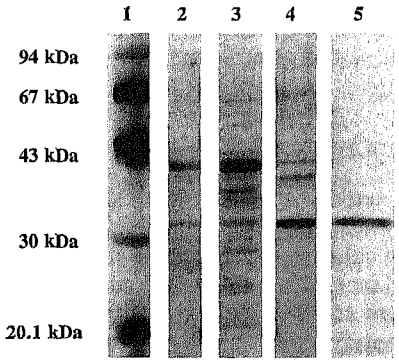

Fig. 3. SDS/PAGE of extracts from purification of trithionate hydrolase. Electrophoresis was carried out according to Laemmli (1970) on a 12% acrylamide gel. The protein containing extracts were boiled for 5 min before application to the gel. The gel was stained with the Bio-Rad silver-staining kit. Lane 1: marker proteins; phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa). Lane 2: cell-free extract (5.6 μg). Lane 3: after selective precipitation (5.6 μg). Lane 4: after cation-exchange chromatography (1.4 μg). Lane 5: after gel filtration (1.4 μg). All quantities of protein were derived from total-organic-carbon analysis.

Effect of pH, temperature and ammonium sulphate concentration on enzyme activity

Maximum enzyme activity was found at pH 3.5–4.5 in the standard assay. Above and below this pH, enzyme activity decreased to zero at pH 1 and 7.5. With a potassium phosphate/sodium citrate buffer the pH optimum was found in the same region.

The enzyme activity as a function of the assay temperature exhibited a maximum at about 70°C (Fig. 5). The enzyme activity at this temperature was about five times higher than the activity at 30°C, the optimum temperature for growth of *T. acidophilus* (Guay and Silver, 1975). Above 70°C, the activity decreased rapidly to 20% of optimum activity at 90°C (Fig. 5). Thus, enzyme activities at 30°C and 90°C were comparable.

The enzyme activity was maximal at an ammonium sulphate concentration of 1.4–2.0 M. When the ammonium sulphate concentration in the assay decreased to below 1.0 M, the enzyme activity rapidly decreased (Fig. 6). Enzyme activity was stimulated to the same extent by an equal concentration of sodium sulphate, while sodium chloride and ammonium chloride, at concentrations with the same ionic strength, totally inhibited enzyme activity. Apparently, the stimulation of the trithionate hydrolase activity is due to the high concentration of sulphate. The K_s for sulphate was calculated by non-linear regression as (Fersht, 1985b)

$$[E_{act}] = [E_0] \cdot [SO_4^{2-}]/([SO_4^{2-}] + K_s) \quad (1)$$

E_0, total enzyme concentration; E_{act}, concentration of active enzyme and was 0.23 M.

Kinetic constants

The kinetic parameters of trithionate hydrolase were determined at both 30°C and 70°C (at pH 3.0). The apparent K_m for trithionate was 70 μM at 30°C and 270 μM at 70°C. When the specificity constant of an enzyme for a substrate is defined as V_{max}/K_m (Fersht, 1985a), the calculated specificity constants of trithionate hydrolase for trithionate at 30°C and 70°C are roughly equal, in spite of the much higher maximum activity at the latter temperature (Fig. 5).

High concentrations of trithionate inhibited enzyme activity (substrate inhibition). At concentrations of 6 mM and 9 mM, the enzyme activity was inhibited by 50% and 80%, respectively.

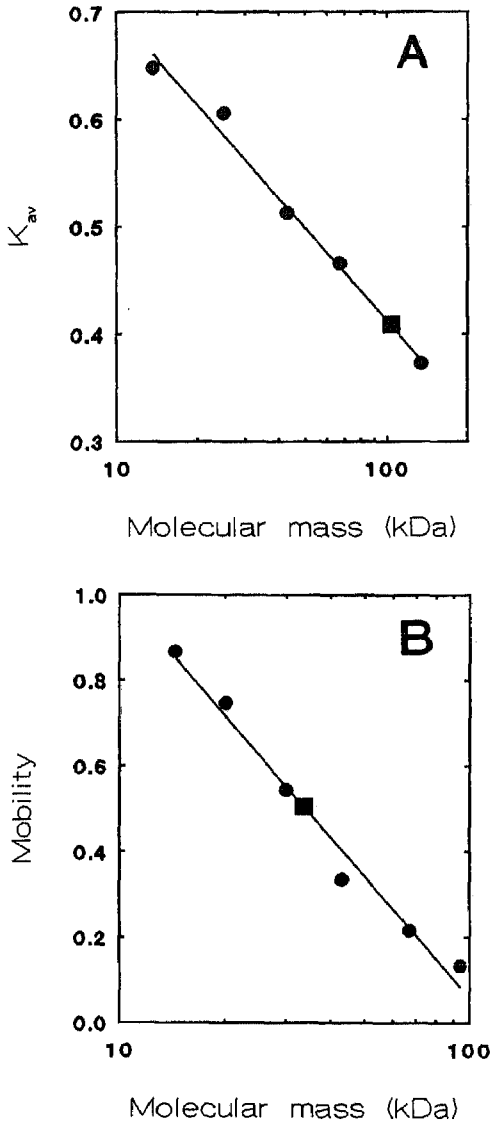

Fig. 4. Molecular mass determination of native trithionate hydrolase and subunits. (A) The molecular mass of the native enzyme (■) was determined by gel filtration on Superose 12. The Superose-12 column was calibrated using a buffer containing 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0, and the calibration proteins (●): ribonuclease from bovine pancreas (13.7 kDa; Boehringer), α-chymotrypsin from bovine pancreas (25 kDa and 50 kDa; Sigma), ovalbumin (43 kDa and 86 kDa; Pharmacia) and bovine serum albumin (67 kDa and 134 kDa; Sigma). The void volume of the column was determined with blue dextran (±2000 kDa; Pharmacia). Elution times were plotted as $K_{av} = (V_e - V_0)/(V_t - V_0)$) against log(molecular mass). (B) The molecular mass of the denatured enzyme (■) was determined by SDS/PAGE. Marker proteins (●) were as described in legend to Fig. 3. Penetration depths of marker proteins were divided by penetration depth of the electrophoresis front, and the data (mobility) were plotted against log(molecular mass).

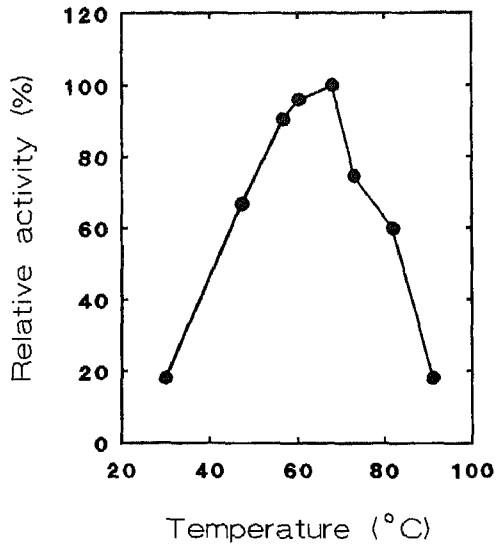

Fig. 5. Effect of temperature on trithionate hydrolase activity. The experiments were performed in a buffer containing 25 mM potassium phosphate and 1.0 M ammonium sulphate, pH 3.0. The temperature was controlled by measuring the actual temperature in the cuvette.

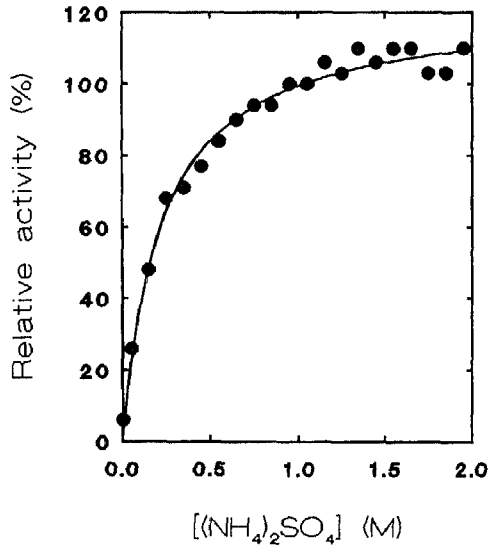

Fig. 6. Effect of ammonium sulphate concentration on trithionate hydrolase activity. The experiments were performed in a buffer containing 25 mM potassium phosphate, pH 3.0, at 30 °C. Enzyme was incubated for 30 min at 30 °C with different ammonium sulphate concentrations before its activity was determined. K_s was calculated using non-linear regression.

Stability

The thermostability of trithionate hydrolase at 30 °C was higher than at 0 °C and 70 °C. All activity was lost after 36 days at 0 °C and after 27 days at 70 °C. After 97 days at 30 °C, 40% of the initial activity was still present. The purified enzyme could withstand at least five freeze (−20 °C)/thaw (25 °C) cycles without any loss of activity.

The decrease in enzyme activity at 0 °C was neither due to a dissociation of the native enzyme into monomers, nor to a denaturation of the native enzyme, as was shown with gel filtration on Superose 12 after incubation of purified trithionate hydrolase for 6 days and 21 days on ice, respectively (data not shown). The total amount of protein (recorded as A_{280}) did not decrease and no monomer peak could be detected with gel filtration.

Inhibition and stimulation

Enzyme activity was not significantly affected by 1 mM EDTA, 0.5 mM diethyl dicarbamide, 1 mM sodium azide, 1 mM nickel chloride, 1 mM cobalt chloride, 1 mM zinc sulphate, 1 mM magnesium sulphate, 1 mM calcium chloride nor 1 mM sodium sulphite. Sulfhydryl-binding agents inhibited enzyme activity very effectively. Mercury chloride (0.1 mM) inhibited enzyme activity totally and 0.25 mM *N*-ethylmaleimide reduced the activity to 10% of the control. The sulphur compounds thiosulphate and tetrathionate both inhibited the activity of trithionate hydrolase. Thiosulphate (1 mM) inhibited the activity by more than 90% (product inhibition) and tetrathionate (0.5 mM), which is not hydrolysed by trithionate hydrolase, for 85%.

DISCUSSION

Purification and properties of trithionate hydrolase

By using high salt concentrations in the extraction buffer and during the subsequent purification procedure, pure trithionate hydrolase could be obtained in a satisfactory yield. Lu and Kelly (1988) partly purified, but not characterized, trithionate hydrolase from cell-free extracts of the neutrophile *T. tepidarius*. Although these authors did not use high salt concentrations for preparation of cell-free extracts, they also reported a stimulation of the trithionate hydrolase activity by more than 300% upon addition of 20 mM sulphate.

To measure trithionate hydrolase activity, Lu and Kelly (1988) used a coupled system in which the formed thiosulphate was oxidized to tetrathionate by a purified thiosulphate dehydrogenase and cytochrome *c*. However, this assay underestimated the activity of the trithionate hydrolase (Lu and Kelly, 1988), but direct measurement of trithionate hydrolysis has not been attempted as a sufficiently sensitive specific analytical method for the detection of thiosulphate was not available (Lu and Kelly, 1988). By monitoring the absorption difference between trithionate and thiosulphate at 220 nm (Fig. 2), we were able to develop a very sensitive continuous assay for trithionate-hydrolase-activity measurements and, hence, were able to purify the enzyme.

Native trithionate hydrolase has a molecular mass of 99 kDa (Fig. 4A). The subunit molecular mass was determined to be 34 kDa (Fig. 4B), suggesting that the native enzyme contains three identical subunits. Although even-numbered oligomers are much more common than trimers and the very rare pentamers (heptamers have so far not been described), there are some well-defined cases of odd-numbered oligomers (Friedrich, 1984).

Purified trithionate hydrolase showed optimum activity at pH 3.5–4.5. Intact cells of *T. acidophilus* oxidize trithionate optimally at pH 3 (Meulenberg et al., 1992). Oxidation of trithionate by intact cells is not significantly affected by uncouplers, suggesting that the process takes place in the periplasm (Meulenberg et al., 1992). The low pH optimum for the purified trithionate hydrolase is another indication that the initial hydrolysis of trithionate occurs in the periplasm.

A temperature optimum of 70 °C for purified enzymes is not unusual for proteins from (extreme) thermophiles (Brock, 1978; Gupta, 1991), but has not often been described for

proteins from a mesophile like *T. acidophilus* (Fig. 5). However, since trithionate hydrolase is most probably located in the periplasmic space and therefore confronted with a low pH during growth, it is possible that the structural requirements imposed on the enzyme by the necessity to withstand a very low pH may also have resulted in an increased thermostability.

High concentrations of ammonium sulphate (up to 1.4 M, Fig. 6) in the assay buffer stimulated the activity of the purified trithionate hydrolase. Stimulation of enzyme activity and stability by high salt concentrations is well-known in halophiles (Jaenicke and Závodszky, 1990; Jaenicke, 1991; Muriana and Relimpio, 1991). This stabilization by various salts follows the Hofmeister (lyotropic) series and seems to be, at least partially, due to the increase of hydrophobic interactions in the proteins (Muriana and Relimpio, 1991). The strength of hydrophobic interactions increases with increasing temperatures (Roe, 1989). At the optimum temperature (70 °C) the maximum stimulation of trithionate hydrolase by ammonium sulphate is indeed reached at much lower concentrations (0.1 – 0.2 M; data not shown).

For trithionate hydrolase, a high ammonium sulphate concentration is needed for two reasons. The first reason is that at a low ionic strength the enzyme precipitates. Other salts, however, like sodium chloride and potassium phosphate, could also be used to achieve a high ionic strength and keep the enzyme in solution. The second reason is that trithionate hydrolase needs a high sulphate concentration for its activity. This stimulation of enzyme activity was also observed using sodium sulphate, but not when sodium chloride or ammonium chloride were used. It seemed, therefore, a specific sulphate effect. Thus, ammonium sulphate is, for both reasons, a convenient salt to use.

In the case of trithionate hydrolase, where, in addition to a high ammonium sulphate concentration, a low pH (i.e. protonation of certain amino acids) is also necessary for enzyme activity, perhaps also charge shielding by ammonium sulphate, preventing electrostatic repulsion inside the protein (Jaenicke, 1991), plays a role in the solubilization of the enzyme. Indeed, at higher pH (i.e. less protonation of amino acids) lower concentrations of ammonium sulphate were needed to keep the trithionate hydrolase in solution (data not shown), but the enzyme activities were much lower.

Stability of trithionate hydrolase was recorded at three different temperatures. Surprisingly, stability at 0 °C was lower than at 30 °C. Cold inactivation of proteins has also been described for myoglobin (Privalov et al., 1986), chymotrypsinogen and lactate dehydrogenase (Franks, 1987). Privalov et al. (1986) suggest that this cold denaturation can be explained by the decrease in hydrophobic interactions upon cooling and, as described above, hydrophobic forces may be important for this enzyme to keep its native structure. Heat denaturation of protein can then be explained by the fact that, at a rise in temperature, dissipative forces increase faster than hydrophobic interactions. Therefore, the native protein is stable in a limited temperature range where hydrophobic interactions are strong enough to compete with dissipative forces for maintaining a native structure of the protein (Privalov et al., 1986). Franks (1987) found for lactate dehydrogenase a dissociation of tetrameric into dimeric structure upon cooling. With the monomeric chymotrypsinogen, denaturation because of chain unfolding upon cooling was suggested (Franks, 1987).

For trithionate hydrolase the decrease in enzyme activity at 0 °C was not because of the dissociation of the native structure into its monomers or because of denaturation of the native enzyme. Whether this inactivation of trithionate hydrolase at 0 °C is due to a change in the native conformation of the enzyme remains to be seen.

Hydrolytic cleavage in the metabolism of polythionates

Hydrolysis as the first step in the metabolism of trithionate has been described for a variety of thiobacilli. In *T. neapolitanus* (Trudinger, 1964b), *T. thiooxidans* (Okuzumi, 1966a), *T. ferrooxidans* (Sinha and Walden, 1966) and *T. tepidarius* (Lu and Kelly, 1988), the first step in trithionate metabolism was reported to be a hydrolytic cleavage, yielding thiosulphate and sulphate.

Steudel et al. (1987) proposed an initial hydrolytic cleavage in tetrathionate and pentathionate metabolism in the acidophile *T. ferrooxidans*, analogous to the hydrolysis of trithionate:

$$S_4O_6^{2-} + H_2O \rightarrow S_3O_3^{2-} + SO_4^{2-} + 2\,H^+. \qquad (3)$$

Hydrolysis of tetrathionate is then followed by spontaneous chain elongation of sulphane monosulphonic acids yielding hydrophilic sulphur and sulphite (Steudel et al., 1987; Hazeu et al., 1988). Incubation of tetrathionate in anaerobic cell suspensions of *T. acidophilus* yielded a stoichiometric conversion of tetrathionate to thiosulphate, sulphur and sulphate, but sulphite could not be detected (Meulenberg et al., 1992). Therefore our present hypothesis is that thiosulphate, sulphur and sulphate are produced in a single step during tetrathionate hydrolysis (Meulenberg et al., 1992).

We recently observed the hydrolysis of tetrathionate to thiosulphate, sulphur and sulphate in cell-free extracts of *T. acidophilus* (Meulenberg, R., unpublished results), and further research is now focused on the purification and characterization of this tetrathionate hydrolase.

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