Eur. J. Biochem. 243, 678–683 (1997)

Isolation of the tetrathionate hydrolase from *Thiobacillus acidophilus*

Govardus A. H. DE JONG, Wim HAZEU, Piet BOS and J. Gijs KUENEN

Kluyver Laboratory of Biotechnology, Department of Microbiology and Enzymology, Delft, The Netherlands

(Received 6 September 1996) – EJB 96 1326/4

An enzyme capable of hydrolysing tetrathionate was purified from cell-free extracts of *Thiobacillus acidophilus*. The purified enzyme converts tetrathionate into thiosulfate, sulfur and sulfate. In addition, pentathionate could also be converted by the same enzyme. Measurement of the enzyme activity during purification is based on the absorbance of the initial intermediates formed from tetrathionate in the ultraviolet region, which have not been identified. Enzyme activity could also be measured by the scattering of insoluble sulfur in the visible region. The purified enzyme has a pH optimum of 2.5 and a temperature optimum of 65°C. Enzyme activity is strongly stimulated by the presence of sulfate ions. The purified enzyme is a dimer with two identical subunits of 48 kDa. The ultraviolet–visible absorption spectra and denaturation experiments indicate the presence of an organic cofactor.

Keywords: tetrathionate hydrolase; *Thiobacillus acidophilus*; sulfur metabolism; polythionate.

Acidophilic thiobacilli are applied in the bacterial leaching of metals from ores. Insoluble sulfidic minerals, such as pyrite, chalcopyrite and or copper sulfide, are oxidized by these bacteria and the metals are dissolved. Acidophilic thiobacilli are capable of oxidizing several sulfur compounds at low pH values. The knowledge on the enzymology of inorganic sulfur oxidation by acidophilic *Thiobacillus* species is incomplete. This is partly caused by the chemical reactivity of these compounds at low pH. At this moment there is still doubt concerning the mechanism of sulfur chemistry, especially with respect to bacterial leaching [1].

Despite the problems of chemical reactivity, two enzymes have been isolated from *Thiobacillus acidophilus*, responsible for the conversion of trithionate into thiosulfate and sulfate [2] and thiosulfate into tetrathionate [3], respectively. These enzymes seem to be periplasmic as indicated by their low pH optimum of about 3. The oxidation of thiosulfate results in the formation of tetrathionate [3]. Preliminary experiments with cell free extracts of *T. acidophilus* have indicated that tetrathionate is hydrolysed into thiosulfate, sulfur and sulfate [4]. The enzyme involved in the hydrolysis of tetrathionate in *T. acidophilus* also appeared to have a pH optimum of 3, but seemed to be different from trithionate hydrolase, previously purified [4]. In contrast to *T. acidophilus*, *Thiobacillus thiooxidans* contains a tetrathionate-decomposing enzyme which converts tetrathionate to thiosulfate and sulfate, without the formation of sulfur [5]. Detailed analysis of the aqueous phase after incubation of *Thiobacillus ferrooxidans* with tetrathionate and pentathionate by chromatographic separation revealed the presence of polythionates of higher length [6]. The hydrolysis of tetrathionate is supposed to produce sulfane-monosulfonic acid ($HS_2SO_3^-$) as an intermediate, which is chemically further metabolized [6].

Correspondence to J. G. Kuenen, Kluyver Laboratory of Biotechnology, Department of Microbiology and Enzymology, Julianalaan 67, 2628 BC Delft, The Netherlands

Fax: +31 15 2782355.

Enzymes. Isocitric dehydrogenase (EC 1.1.1.42); alcohol dehydrogenase (EC 1.1.1.1).

Experiments to purify the tetrathionate hydrolase from *T. acidophilus* have not been successful, because of complete loss of activity during the purification procedure. This article describes the induction of tetrathionate metabolism in whole cells by changing growth conditions and the purification and characterization of the tetrathionate hydrolase from *T. acidophilus*.

MATERIALS AND METHODS

Chemicals. Sodium thiosulfate was obtained from Merck, sodium tetrathionate from Fluka sodium pentathionate was kindly supplied by Prof. Dr R. Steudel (Technical University Berlin). Isocitric dehydrogenase and baker's yeast alcohol dehydrogenase were obtained from Sigma.

Organism and growth conditions. *T. acidophilus* DSM 700 was maintained as described previously [7]. High-cell-density chemostat cultures of *T. acidophilus* were grown on mixtures of glucose (100 mM) and thiosulfate (50 mM) at a dilution rate of 0.05 h^{-1} as reported [8]. Autotrophic chemostat cultures were grown on thiosulfate (50 mM) at a dilution rate of 0.02 h^{-1}, in a mineral medium five-times diluted compared with that used for mixotrophic chemostat cultures. For the purification of the tetrathionate hydrolase, autotrophic chemostat cultures were grown in a 15-l fermenter. Bacterial cells from a chemostat culture were collected at 4°C and centrifuged (10 min at 12000×*g*).

Analytical procedures. Tetrathionate, pentathionate and trithionate concentrations were determined by cyanolysis [9], modified for measurements at high ammonium sulfate concentrations [2]. Precipitated sulfur was extracted overnight in acetone and analysed by cyanolysis [10]. Though differentiation between tetrathionate and thiosulfate with cyanolysis is well established [9], it is unclear at present time if intermediates formed in the reaction from tetrathionate are detected by this measurement. Analysis for the presence of molybdenum in the purified protein was performed according to the procedure by Cardenas and Mortenson [11].

Continuous enzyme assays. A continuous activity assay was used for measuring activities of fractions during purification. Activity of the tetrathionate-hydrolysing enzyme was mea-

sured with 1 mM tetrathionate or pentathionate in 1 M ammonium sulfate, pH 3. Activities during purification were measured routinely by an increase in absorbance at 290 nm, which is due to unidentified intermediates formed from tetrathionate. At this wavelength, tetrathionate has almost no absorbance, making it easy to measure formation of intermediates. Anaerobic enzyme assays were carried out in stoppered cuvettes after flushing with nitrogen gas for 5 min.

Discontinuous enzyme assays. The continuous enzyme assay was very suited for measuring activity during purification, however, the activity measured could not be used to calculate the specific activity of the purified protein and for protein fractions after each separation step, because the increase in absorbance at 290 nm is not linearly correlated with the decrease in tetrathionate consumption. For this purpose an assay had to be used which measures the concentration of tetrathionate during conversion by the tetrathionate hydrolase. Tetrathionate (1 mM) was added to 2 ml of a 1 M ammonium sulfate, pH 3, in a temperature-regulated (30°C) reaction chamber. After addition of protein, 0.2-ml samples were taken at desired time intervals and immediately mixed with 5 µl 1 M NaOH and put on ice to stop the reaction. Tetrathionate concentrations were determined by cyanolysis. 1 U is defined as the activity that hydrolysis 1 µmol tetrathionate/min.

Enzyme-purification procedure. Bacterial cells (19 g wet cells) were collected on ice from an autotrophically grown chemostat culture of *T. acidophilus* and centrifuged (10 min at 12000×*g*). The biomass was washed and resuspended in 40 ml of a buffer containing 25 mM potassium phosphate and 2.0 M ammonium sulfate, pH 3, and disrupted in a French pressure cell at 110 MPa. The suspension was centrifuged for 20 min at 48000×*g* to remove the bulk of cell debris. The supernatant was centrifuged for 120 min at 100000×*g*. The resulting clear supernatant (45 ml) was applied to a phenyl-Sepharose column (5 cm×3 cm) equilibrated in buffer containing 25 mM potassium phosphate and 2.0 M ammonium sulfate, pH 3. A gradient from 2.0 M to 0 M ammonium sulfate in 25 mM potassium phosphate, pH 3, was applied at a flow rate of 3 ml/min during 2 h. Pooled active fractions were dialysed for 24 h against a buffer containing 25 mM potassium phosphate and 0.4 M ammonium sulfate, pH 3, and the dialysate applied to a Mono-S column (Pharmacia, HR 16/10) previously equilibrated with a buffer containing 25 mM potassium phosphate and 0.4 M ammonium sulfate, pH 3. A gradient from 0.4 M to 1 M ammonium sulfate in 25 mM potassium phosphate, pH 3, was applied at a flow rate of 1.5 ml/min over 1 h. Pooled active fractions were diluted with an equal volume of 3.0 M ammonium sulfate, pH 3, and applied to a phenyl-Superose column (Pharmacia, 10/10) equilibrated in buffer containing 25 mM potassium phosphate and 2.0 M ammonium sulfate, pH 3. A gradient from 2.0 M to 0 M ammonium sulfate in 25 mM potassium phosphate, pH 3 was applied at a flow rate of 1.0 ml/min during 40 min and the eluting active fractions stored at −80°C.

Molecular-mass determination. The apparent molecular mass was determined by gel filtration on a Pharmacia Superdex 200 column equilibrated with buffer containing 25 mM potassium phosphate and 1.0 M ammonium sulfate, pH 3, as described [2]. Subunit molecular masses were determined under denaturing conditions by SDS/PAGE according to Laemmli [12] using a 10% polyacrylamide gel and Mini Protean equipment (Bio-Rad). Enzyme samples were denatured by incubation for 5 min at 100°C in 2% SDS and 10% 2-mercaptoethanol. A low-molecular-mass calibration kit (Pharmacia) was used to derive the molecular masses. Gels were stained for protein with Coomassie brilliant blue G250.

Measurement of substrate-dependent oxygen consumption. Respiration rates of cell suspensions were assayed polarographically in a Biological oxygen monitor with a Clarck type oxygen electrode (Yellow Springs Instruments, Inc.) at 30°C. Assays were performed with cell suspensions (0.25 g dry mass/l taken directly from substrate-limited chemostat cultures. These suspensions were centrifuged (10 min at 12000×*g*) and the biomass was washed and resuspended in mineral medium. Calculations were made on the basis of an oxygen concentration of 236 µM in air saturated water at this temperature. The values have been corrected for endogenous respiration rates. The maximal substrate dependent oxygen uptake rate (V_{max}) was calculated from a reciprocal plot according to Hanes [13].

Determination of pH optimum, optimum temperature and kinetic constants. The pH optimum was determined using the continuous activity assay with 1 mM tetrathionate in 1 M ammonium sulfate at 30°C. The pH was varied between 1.0 and 7.0 by adjusting the pH of the assay buffer with either 1 M H_2SO_4 or 1 M NaOH. At low pH values, HSO_4^-/SO_4^{2-} will act as a buffer, at higher pH values the buffer capacity decreases, however, measurement of the pH after the assay showed no substantial difference with the initial pH value. The temperature optimum was determined using the continuous activity assay with 1 mM tetrathionate in 1 M ammonium sulfate, pH 3. The temperature was varied between 20 and 85°C and the actual temperature was measured in the cuvette.

Spectroscopy. Ultraviolet−visible absorption spectra of the purified enzyme and isolated cofactor were measured on a HP 8524A diode array spectrometer at 20°C. Calculation of the specific absorption coefficient of the purified enzyme was performed using the ratio between the absorbance at 205 and 280 nm as described [14].

Cofactor analysis by reverse-phase analysis. 50 µl of purified enzyme (0.5 mg) in 1 M ammonium sulfate, pH 3, was denatured by the addition of 1 ml methanol. Precipitated salt and denatured enzyme were removed by centrifugation (10 min, 25000×*g*). The resulting supernatant was evaporated with a vacuum pump and the remaining residue was dissolved in 100 µl 10% methanol in 10 mM potassium phosphate, pH 6.5. The product was analysed for the presence of NAD, NADP, NADH and NADPH on a Novapack C_{18} reverse-phase column (Waters) with photodiode array detector. Products were eluted using a 10% methanol in 10 mM potassium phosphate, pH 6.5, at a flow rate of 0.8 ml/min. As references NAD, NADP, NADH and NADPH were used.

Cofactor analysis by enzymatic assay. The cofactor was isolated as described above and dissolved in 100 µl water. This suspension was used in an activity assay with an NADP-dependent isocitric dehydrogenase and an NAD-dependent baker's yeast alcohol dehydrogenase using the combination of phenazine methosulfate (0.12 mM) and 2,6-dichloroindophenol (0.16 mM) as electron acceptors.

RESULTS

Enzyme induction. *T. acidophilus* is routinely grown mixotrophically on glucose and thiosulfate in a 1-l chemostat to produce large cell yields [8]. Cells grown in this way have a very low oxidation rate for tetrathionate (4 nmol $O_2 \cdot min^{-1} \cdot$ mg dry $cells^{-1}$) and an oxidation rate for thiosulfate of 48 nmol $O_2 \cdot min^{-1} \cdot$ mg dry $cells^{-1}$. Cell-free extracts prepared from these cells proved to be unsuited for purification of the tetrathionate hydrolase. Autotrophic growth on thiosulfate increased the rate of tetrathionate oxidation by a factor of 20 (88 nmol $O_2 \cdot min^{-1} \cdot$ mg dry $cells^{-1}$), whilst the oxidation of thiosulfate was

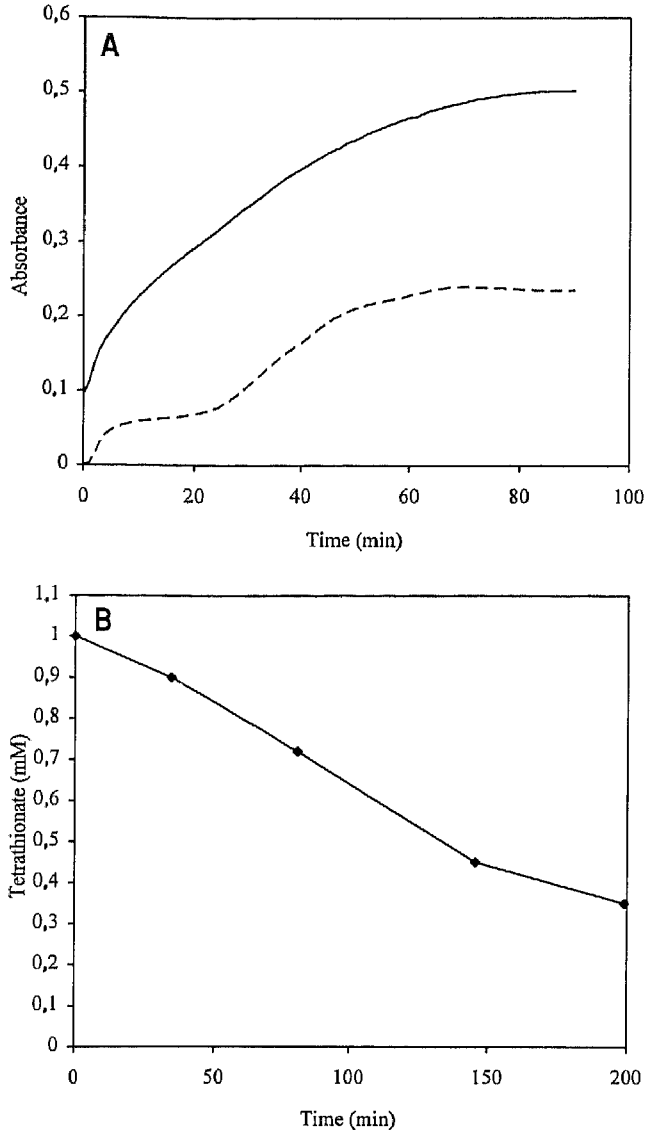

Fig. 1. Activity measurement of the tetrathionate hydrolase of *T. acidophilus*. (A) 2.5 μg tetrathionate hydrolase was added to a solution of 1 mM tetrathionate in 1 M ammonium sulfate, pH 3, at 30°C and the increase in absorbance at 290 nm (——) and 430 nm (– – –) was followed. (B) Decrease in the concentration tetrathionate by incubation with tetrathionate hydrolase. Purified enzyme (0.5 μg) was incubated in 1 ml 1.0 M ammonium sulfate pH 3 in a mixed temperature-regulated (30°C) reaction chamber. After addition of 1 mM tetrathionate, 0.2 ml samples were taken at appropriate time intervals, immediately mixed with 12 μl 1 M KOH and centrifuged. The concentration of tetrathionate in the supernatant was detected by cyanolysis.

increased only by a factor of five (370 nmol $O_2 \cdot min^{-1} \cdot$ mg dry cells^{-1}).

Continuous activity assay. Activity of the tetrathionate hydrolase was measured on a ultraviolet–visible spectrophotometer at several wavelengths. Blank experiments without enzyme showed no increase in absorbance indicating that tetrathionate is stable at pH 3. The products initially formed from tetrathionate and pentathionate absorb at higher wavelengths than the original substrates, though at this moment it is not clear what products are formed. The endproducts of the reaction have been identified as thiosulfate, sulfur and most probably sulfate [5]. Fig. 1A shows the activity measurements of tetrathionate hydrolase at 290 nm and 430 nm. 290 nm was randomly chosen as a standard wavelength and proved to be easily usable without to much background absorbance of tetrathionate. The increase in absorbance at 290 nm is not linear, probably because the intermediates formed are subsequently further modified because of their chemical instability. Detailed ultraviolet–visible analysis of product formation by measuring all wavelengths between 200 nm and 600 nm during activity measurements showed increase and subsequent decrease in the absorbance in the ultraviolet region and increase in the visible region, without any clear peaks. Increase of absorbance at 430 nm is supposed to be caused by the production of intermediate sulfur, which because of its low solubility produces turbidity. The absorbance increase at 430 nm, however, shows that the reaction mechanism is more complicated than exclusively the production of sulfur, with the formation of several intermediates and sulfur simultaneously.

Table 1. Purification of tetrathionate hydrolase from *T. acidophilus*. 19 g cell paste was used for the purification scheme. Activities were measured by the discontinuous enzyme assay.

Purification step	Total protein	Specific activity	Yield	Purification factor
	mg	U/mg	%	-fold
Cell-free extract	225	0.48	100	1
Phenyl-Sepharose	8	5.0	37	10.4
Mono-S	4.5	7.2	30	15.0
Phenyl Superose	3.5	8.0	26	16.7

Discontinuous activity assay. Fig. 1B shows the decrease in concentration of tetrathionate during incubation with tetrathionate hydrolase. The decrease in the concentration of tetrathionate for the first 2 h is not linear, probably because initial intermediates are also measured by cyanolysis. For calculation of the specific activities, the highest linear slope is used. Determination of the amount of sulfur, thiosulfate and protons produced with purified enzyme under both aerobic and anaerobic conditions, led to the reaction scheme: $S_4O_6^{2-} + H_2O \rightarrow S + S_2O_3^{2-} + SO_4^{2-} + 2H^+$, also previously determined with incubations of tetrathionate with cell-free extracts of *T. acidophilus* [4]. Formation of sulfate is predicted from the reaction stoichiometry, however, the high amount of sulfate ions necessary to prevent tetrathionate hydrolase precipitation, does not allow the detection of additional formation of sulfate in the assay. Analysis of the end products after incubation of pentathionate with tetrathionate hydrolase showed the same products, but only the amount of sulfur produced was doubled: $S_5O_6^{2-} + H_2O \rightarrow 2S + S_2O_3^{2-} + SO_4^{2-} + 2H^+$. The rate of pentathionate hydrolysis was about three times as fast as the hydrolysis of tetrathionate.

Purification and physical properties. A representative cultivation of *T. acidophilus* at 30°C on 50 mM thiosulfate produces 0.5 g cell paste/l (wet mass). The purification scheme for the isolation of the tetrathionate hydrolase from such cells is given in Table 1. Active fractions after phenyl-Superose appeared to be homogeneous, as judged by peak purity criteria on gel filtration on Superdex 200 as well as by SDS/PAGE. Determination of the native molecular mass by gel filtration gave a value of 100 kDa, subunit mass determination by SDS/PAGE gave a value of 48 kDa. Preparation of cell-free extract at neutral pH showed no tetrathionate hydrolase activity, indicating that the enzyme is not stable at this pH.

The ultraviolet–visible absorption spectrum of the tetrathionate hydrolase is characterized by a peak at 280 nm and a broad shoulder at 340 nm (Fig. 2). This indicates the presence of an

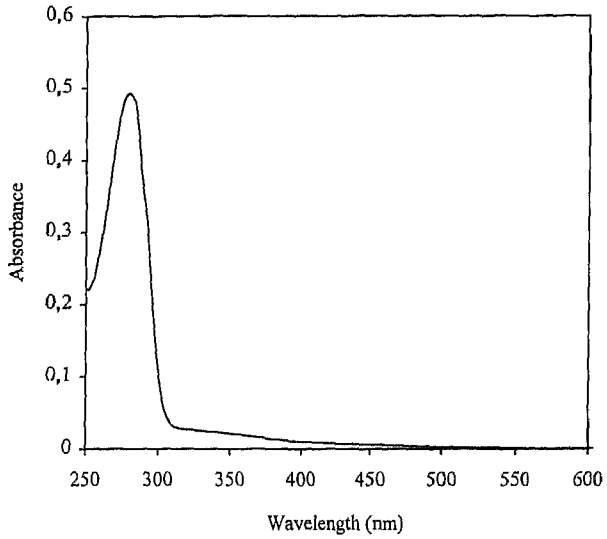

Fig. 2. Ultraviolet visible spectrum of tetrathionate hydrolase. A sample of 2.5 μM tetrathionate hydrolase in 1 M ammonium sulfate, pH 3, was measured.

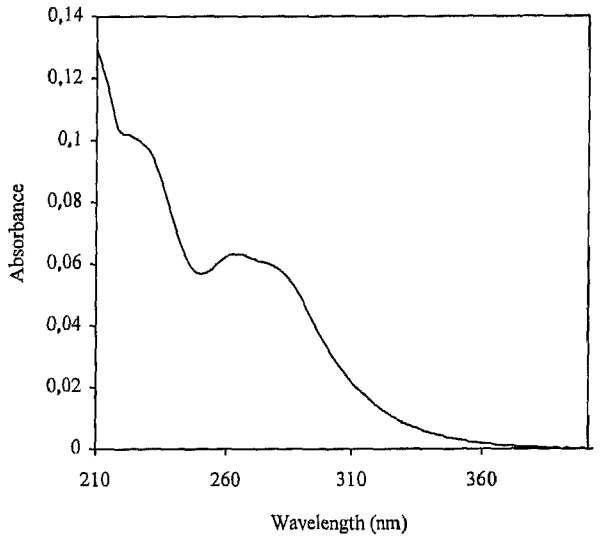

Fig. 3. Ultraviolet visible spectrum of the purified cofactor of the tetrathionate hydrolase. 50 μl of purified enzyme (0.5 mg) in 1 M ammonium sulfate, pH 3, was denatured by the addition of 1 ml methanol. Precipitated salt and denatured enzyme were removed by centrifugation (10 min, 25000×*g*). The spectrum of the cofactor in methanol was recorded.

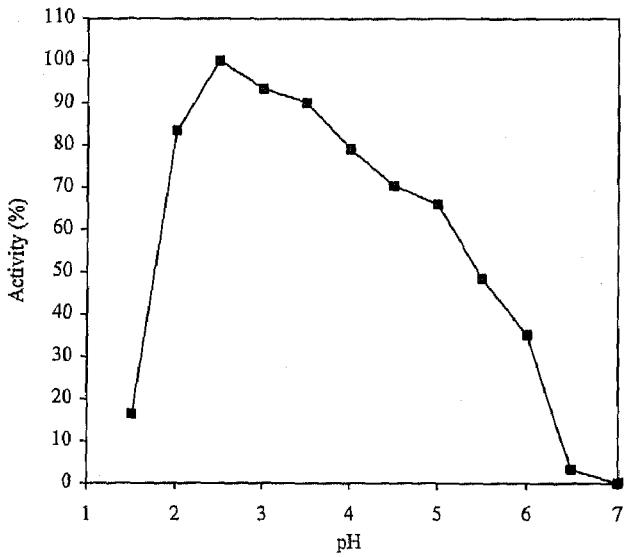

Fig. 4. Effect of pH on the hydrolysis of tetrathionate by tetrathionate hydrolase. Activities were determined by measuring the increase in absorbance at 290 nm in a buffer containing 1 M $(NH_4)_2SO_4$ and 25 mM potassium phosphate, adjusted to the required pH with H_2SO_4 or NaOH. Relative activities are given as a percentage of the maximum activity.

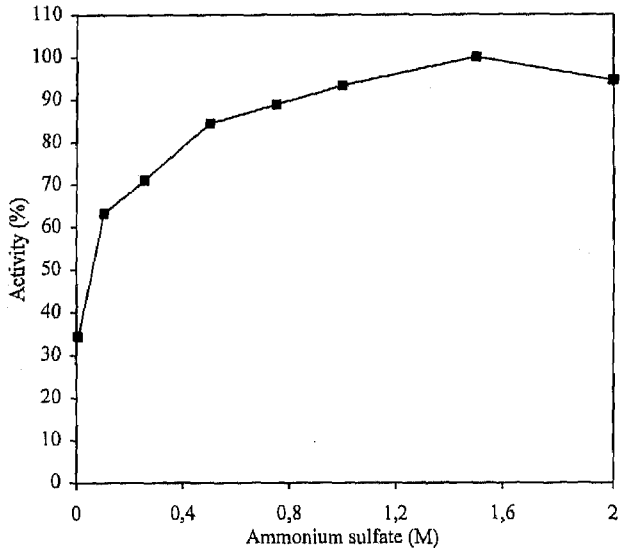

Fig. 5. Effect of ammonium sulfate concentration on the tetrathionate hydrolysis. Experiments were carried out in a buffer containing 25 mM potassium phosphate, with varying concentrations ammonium sulfate, at pH 3 and 30°C. Purified protein was incubated for 30 min before substrate (1 mM) was added. Activities were measured as increase in the absorbance at 290 nm. Relative activities are given as a percentage of the maximum activity.

organic cofactor very similar to NAD or NADP, however, assays with an NADP-dependent citrate dehydrogenase and an NAD-dependent alcohol dehydrogenase, showed no activity indicating that the cofactor of the tetrathionate hydrolase is not NAD or NADP. The absorption coefficient of a solution of 0.1 % purified tetrathionate hydrolase at 280 nm is 1.9, as determined by the method described [14]. No molybdenum was observed in the purified protein.

Cofactor analysis by reverse-phase analysis. Addition of methanol to a solution of tetrathionate hydrolase immediately showed the precipitation of salt and protein. The resulting clear supernatant after centrifugation showed a product with maximal extinction at 260 nm (Fig. 3). The supernatant was dried and dissolved in the elution buffer and analysed on a reverse-phase column. The chromatogram showed several peaks of which none coincided with NAD, NADP, NADH or NADPH, as judged from the spectra and from retention times (data not shown). A large part of the product was not detected by reverse-phase analysis, as it was strongly bound to the column used under the conditions described.

Effects of pH, temperature and substrate concentrations on enzyme activity. The effect of pH was tested to investigate the

maximum rate of tetrathionate hydrolysis. The maximum activity occurred at pH 2.5 (Fig. 4). At pH 1.0 and pH 7.0 the activity decreased to zero. The optimum temperature of tetrathionate hydrolysis was investigated with purified enzyme. The activity exponentially increased from 15°C to 65°C. Above 65°C the activity gradually decreased.

The kinetic parameters of tetrathionate hydrolase were determined at pH 3 and 30°C. The enzyme followed Michaelis Menten kinetics, with a V_{max} of 8 μmol · min^{-1} · mg $protein^{-1}$, the apparent K_m for tetrathionate was 0.3 M at a concentration of 1.0 M ammonium sulfate.

Effect of sulfate on tetrathionate hydrolysis. The presence of sulfate ions strongly enhances tetrathionate hydrolysis (Fig. 5). Similar effects to those observed with ammonium sulfate are observed with sodium sulfate, but not with ammonium chloride. Maximal activity is obtained between 1 M and 2 M ammonium sulfate. Stability of the enzyme during purification strongly relies on the presence of ammonium sulfate. Almost all activity is lost after overnight dialysis against 0.4 M ammonium sulfate, because of coprecipitation of the enzyme with other proteins. This phenomenon was no longer observed for completely purified tetrathionate hydrolase.

DISCUSSION

Tetrathionate seems to play an important role in the metabolism of sulfur compounds in acidophilic thiobacilli. Previous attempts to purify tetrathionate hydrolase from *T. acidophilus* did not succeed because of loss of activity during purification [4]. This was partly caused by the insensitivity of the activity measurement, which was based on measuring proton production. Experiments described here show that a change from mixotrophic growth to autotrophic growth enhances the tetrathionate-dependent oxygen consumption by a factor of 20. Therefore the enzyme was purified from autotrophically grown cells. Trithionate could not be converted by the tetrathionate hydrolase as already observed [4]. Experiments here show that pentathionate is a substrate for tetrathionate hydrolase.

For activity measurements in protein fractions the empirical continuous spectrophotometric test proved to be suitable and relatively fast compared with the discontinuous measurement of proton production [4]. More detailed analysis of the activity assay in the ultraviolet–visible region showed formation of several intermediates, which are probably modified chemically. These analysis showed that differences exist between the initial products formed from tetrathionate by tetrathionate hydrolase from *T. acidophilus* and a similar enzyme recently purified from *T. ferrooxidans*. Initial products formed from tetrathionate by tetrathionate hydrolase from *T. ferrooxidans* only show absorbance in the ultraviolet region, whereas the initially products from the *T. acidophilus* enzyme show also substantial absorbance in the visible region. This shows that the mechanism of tetrathionate hydrolysis and probably the initial splitting of tetrathionate can be performed in different ways, though the end products are the same.

The optimum pH of 2.5 and the instability of the enzyme at neutral pH are an indication that the tetrathionate hydrolase is located in the periplasm as was already suggested previously [4]. In contrast, activity towards sulfite in cell-free extracts of *T. acidophilus* seems to have an optimum of pH 8, and is therefore probably cytoplasmic (data not shown). The location of the metabolism of elemental sulfur is still unknown, though the results presented here show that intermediary sulfur is formed in the periplasm. Whether it is transported to the cytoplasm for further degradation is still unclear. When the amount of thiosulfate fed to the fermenter was higher than the capacity of the cells to convert sulfur, the sulfur was excreted by the bacterium and could be seen under the light microscope as a globule attached to the cell. The optimum temperature for tetrathionate hydrolysis is very high compared with the optimal growth conditions, but is very similar to the optimal temperature of the trithionate hydrolase [2].

The ultraviolet–visible spectrum showed an absorbance similar to that found for nicotinoprotein [NAD(P) containing] alcohol/aldehyde oxidoreductases [15]. However, the ratio between the absorbance at 280 nm and 350 nm is about 1:9 in the case of nicotinoprotein [NAD(P) containing] alcohol/aldehyde oxidoreductases and the ratio for tetrathionate hydrolase is 1:23. This could be caused by a difference in molecular coefficient at 280 nm. Analysis of the cofactor by denaturation and reverse-phase chromatography showed no similarity to NAD or NADP. The function of this cofactor is still unclear. The ratio between the absorbance at 280 nm and 350 nm in all purified tetrathionate hydrolase preparations was the same, suggesting that the compound is always in the same ratio bound to the enzyme, which strongly indicates that unspecifically binding of any unknown substance is not the case. However, at this stage it cannot be excluded that the absorbance above 300 nm is caused by an attached substance and not by a bound cofactor. Although the net result of the process of degradation of tetrathionate is a hydrolysis reaction, it is clear at the same time that the production of sulfate may represent a redistribution of electrons over the different products of the reaction.

We thank Prof. Dr R. Steudel (Department of Inorganic and Analytical Chemistry, University of Technology, Berlin, Germany) for stimulating discussions and providing us with sodium pentathionate. This project was supported by the EC Programme, Biotechnology of Extremophiles, BIO2-CT93-0274 (DG12 SSMA).

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