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Metabolism of tetrathionate in *Thiobacillus acidophilus*

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Abstract: Cell-free extracts of *Thiobacillus acidophilus* catalysed the stoichiometric conversion of tetrathionate to thiosulphate, sulphur and two protons. The pH optimum of the enzyme activity was 3.0 and its temperature optimum 40°C. The enzyme was unstable at 30 and 40°C, at which its activity decreased to zero within 100 and 20 h, respectively. Enzyme activity was not affected by incubation for 1 week on ice or by freezing and thawing of the extract. The K_m for tetrathionate was 0.3 mM. Enzyme activity was stimulated by ammonium sulphate up to a concentration of 1 M. The results indicate that trithionate hydrolase cannot account for the observed conversion of tetrathionate.

Key words: Thiobacillus acidophilus; Acidophile; Sulphur metabolism; Polythionate hydrolysis; Tetrathionate metabolism; Trithionate hydrolase

Introduction

In a recent study [1] of sulphur metabolism by whole cells of *Thiobacillus acidophilus* we found that, under anaerobic conditions, tetrathionate ($S_4O_6^{2-}$) was stoichiometrically converted to thiosulphate, sulphur and sulphate. This reaction has also been demonstrated with anaerobic suspensions of the neutrophilic species *Thiobacillus neapolitanus* [2]. In the acidophile *Thiobacillus ferrooxidans*, transient formation of elemental sulphur has been observed during aerobic oxidation of tetrathionate [3]. In these experiments, formation of thiosulphate was not observed [3], possibly as a result of the high thiosulphate-oxidizing activity of the cells.

The aims of the present study were to demonstrate conversion of tetrathionate in cell-free extracts of *T. acidophilus*, to perform an initial characterization of the enzyme activity involved, and to develop a continuous enzyme activity assay.

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Materials and Methods

Organism and growth conditions

T. acidophilus DSM 700 was maintained as described previously [4] and grown in high cell

density mixotrophic chemostat cultures as reported before [1].

Preparation of cell-free extract

Cells (30 g dry weight) were collected on ice and centrifuged (10 min at 12000 × *g*). Biomass was suspended in 50 ml 2 M ammonium sulphate, pH 3.0, and passed through a French pressure cell (American Instrument Company, Silver Spring, MD) at 80 MPa. This extract was centrifuged for 30 min at 48000 × *g*. The supernatant was again centrifuged for 60 min at 48000 × *g*. The resulting clear supernatant was used as cell-free extract.

Analytical procedures

Tetrathionate and thiosulphate concentrations were determined by cyanolysis [5], modified for measurements at high ammonium sulphate concentrations [6]. Precipitated sulphur was extracted overnight in acetone and analysed by cyanolysis [5].

Continuous enzyme assays

Cell-free extract (20 mg protein) in 2 M ammonium sulphate, pH 3.0, was incubated in a mixed, thermostated (30°C) reaction chamber (5 ml). After equilibration, 1 mM tetrathionate was added to start the reaction. Acidification was registered with a pH electrode (electrode-type CMAWL 32570/6, Russel, Auchtermuchty, UK, combined with a Philips PW 9421 mV/pH meter) coupled to a recorder. Proton production was quantified by back-titration with 0.1 M NaOH after all tetrathionate had been metabolized. Trithionate hydrolase activity in cell-free extracts was determined as described previously [6].

Enzyme activity is expressed in U (mg protein)$^{-1}$. 1 U enzyme activity is defined as the amount of enzyme catalysing the production of 2 μmol H^+ min^{-1}. As the high ammonium sulphate concentration interfered with standard protein determination procedures, protein concentrations were determined from the total organic carbon content of extracts [6,7].

Purification of enzyme activity

Cell-free extract (40 ml) was diluted 15-fold with demineralized water, adjusted to pH 3.0 with H_2SO_4. Precipitated protein was centrifuged (10 min at 12000 × *g*), redissolved in 9 ml 0.4 M ammonium sulphate, pH 3.0, and centrifuged again (20 min at 48000 × *g*). The supernatant (10 ml) contained the enzyme activity.

Cation exchange chromatography was performed with a Pharmacia FPLC system as described previously [6]. Extract (2 ml) was loaded on the Mono-S column, equilibrated with 0.4 M ammonium sulphate, pH 3.0. The column was washed with equilibration buffer at a flow rate of 0.5 ml min^{-1} until A_{280} was below 0.005. Then a linear gradient (20 ml) of 0.4 to 1 M ammonium sulphate, pH 3.0, was applied to the column at the same flow rate.

Chemicals

Sodium trithionate was prepared as described by Wood and Kelly [8]. All other chemicals were analytical grade and obtained from commercial sources.

Results

Tetrathionate metabolism by cell-free extracts

Preliminary experiments indicated that tetrathionate ($S_4O_6^{2-}$) was converted by cell-free extracts of *T. acidophilus*. As previously found for trithionate ($S_3O_6^{2-}$) hydrolase from this organism [6], high salt concentrations were needed to release enzyme activity during cell disruption. Highest specific enzyme activities were obtained when cell-free extracts were prepared in 2 M ammonium sulphate, pH 3.0.

Incubation of cell-free extract with tetrathionate led to the stoichiometric formation of thiosulphate and sulphur (Fig. 1). In addition, two moles of protons were produced per mole of tetrathionate (Fig. 1). Although sulphate could not be measured due to the high ammonium sulphate concentration in the assay mixture, these observations indicate that tetrathionate is metabolized according to: $S_4O_6^{2-} + H_2O \rightarrow S + S_2O_3^{2-} + SO_4^{2-} + 2H^+$, as has been reported for incubation of tetrathionate in anaerobic cell suspensions of *T. acidophilus* [1]. The production of protons enabled a continuous assay for activity measure-

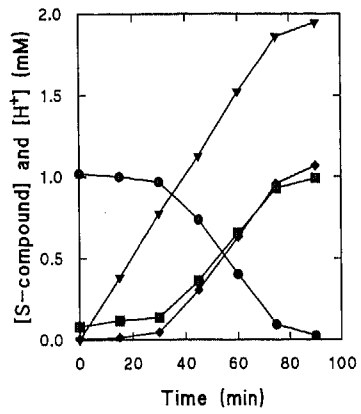

Fig. 1. Reaction stoichiometry of tetrathionate conversion. Cell-free extract (13 mg protein) was incubated in 2 M ammonium sulphate in a mixed, thermostated (30°C) reaction chamber (18 ml). After addition of 1 mM tetrathionate, 1.2-ml samples were taken at appropriate time intervals, immediately mixed with 40 μl 1 M KOH and centrifuged. The concentrations of tetrathionate and thiosulphate in the supernatant were determined by cyanolysis. The pellet was washed with 2 M ammonium sulphate, pH 3.0, recentrifuged and its sulphur content was determined after extraction with acetone. Proton production was registered with a pH electrode. (●) tetrathionate; (■) thiosulphate; (♦) sulphur and (▼) H^+.

ments using a sensitive pH electrode (see Materials and Methods). These cell-free extracts did not oxidize thiosulphate.

Proton production started immediately after the addition of tetrathionate to cell-free extracts. However, the consumption of tetrathionate and the formation of elemental sulphur and thiosulphate consistently showed a lag phase of several minutes (Fig. 1). A possible explanation is that the density of the growing sulphur particles is initially too low to be precipitated by centrifugation. Thus, the sulphur formed might still be in the supernatant after centrifugation and a delay in the increase of sulphur concentration is found. Cyanolysis, to analyse tetrathionate and thiosulphate, will then also analyse the small amounts of sulphur still present in the supernatant. In that case, since thiosulphate concentration is found by the difference between the concentrations of thiosulphate + tetrathionate and tetrathionate [5], the net result will indeed be an apparent delay in the decrease of tetrathionate concentration and the increase of thiosulphate concentration. Attempts to facilitate sulphur precipitation by adding 0.2 mM finely dispersed sulphur (using a 10 mM stock solution in acetone) did not change the pattern of product formation (data not shown). This might imply that sulphur introduced in the assay in this way did not provide adequate nuclei for sulphur precipitation. Unfortunately, the presence of high ammonium sulphate concentrations, the presence of particulate sulphur and the high protein concentrations in the assays precluded the use of more sophisticated methods (in particular HPLC) for the analysis of thiosulphate and tetrathionate.

Enzyme activities in crude cell-free extracts were typically around 0.03 U (mg protein)$^{-1}$. Although low, this activity is sufficient to account for the observed rate of tetrathionate metabolism in chemostat cultures (0.01 U (mg protein)$^{-1}$ when grown on a mixture of 100 mM glucose and 50 mM thiosulphate at a dilution rate of 0.04 h^{-1}). The cell-free extracts used to measure tetrathionate conversion also contained high activities of trithionate hydrolase. This enzyme, which recently has been purified from *T. acidophilus* [6], catalyses the hydrolysis of trithionate ($S_3O_6^{2-}$) to thiosulphate and sulphate. The activities of trithionate hydrolase were approximately 70-fold higher than those of the tetrathionate-metabolizing enzyme system present in the extracts. We have previously reported that tetrathionate was not a substrate for purified trithionate hydrolase [6]. However, in view of the relatively low activities of tetrathionate conversion, we could not exclude the possibility that tetrathionate-hydrolysing activity of trithionate hydrolase may have been overlooked. Therefore, experiments aimed at the further characterization of the tetrathionate-metabolizing activity were also performed with trithionate as a substrate.

Effects of pH and temperature

Maximum rates of tetrathionate-dependent proton production were measured in 1 M ammonium sulphate at pH 3.0. At pH 2.0, the activity was still nearly 50% of its optimum, while at pH 6.0 the activity decreased to zero. The pH optimum for trithionate hydrolysis by the same extracts was slightly lower than that for tetrathion-

Table 1

Fate of tetrathionate-metabolizing activity during purification of trithionate hydrolase *T. acidophilus*

After each purification step the activity of the extract with trithionate ($S_3O_6^{2-}$) and tetrathionate ($S_4O_6^{2-}$) was determined.

	Activity (U)		Recovery (%)		Activity ratio
	$S_3O_6^{2-}$	$S_4O_6^{2-}$	$S_3O_6^{2-}$	$S_4O_6^{2-}$	$S_3O_6^{2-}/S_4O_6^{2-}$
Cell-free extract	3324	48	100	100	69
Selective precipitation	1119	4	34	8	280
Cation exchange chromatography	601	0	18	0	∞

ate conversion. However, the pH range over which activity could be measured was broader with trithionate as a substrate.

The stability of the tetrathionate-metabolizing activity was strongly dependent on the incubation temperature. After incubation for 5 min at 100°C, all activity was lost. No significant loss of activity was found after 1 week incubation at 0°C. At 30 and 40°C, enzyme activity decreased to zero within 100 and 20 h, respectively. In contrast, trithionate hydrolase activity remained constant for over 1 week at all three incubation temperatures (data not shown). In fact, extracts containing trithionate hydrolase still retain 50% of their initial activity after incubation at 30°C for over 1 year (unpublished data). Enzyme activities with both substrates were not significantly affected by freezing at −25, −80 or −196°C (liquid nitrogen) and thawing.

Kinetic parameters and inhibitors

The apparent substrate saturation constant K_m for tetrathionate-dependent proton production was determined at pH 3.0 and 30°C. Under these conditions, an apparent K_m for tetrathionate of 0.30 ± 0.05 mM was found. This implies that the activity observed in standard assays, containing 1 mM substrate, was only 75% of the maximum activity. Since thiosulphate, one of the products, is very unstable at low pH [9], the substrate concentration was not increased.

Trithionate hydrolysis and conversion of tetrathionate by cell-free extracts were stimulated by ammonium sulphate in the assay mixture. For both activities, the optimum was found at 1 M ammonium sulphate (Fig. 2). At suboptimal ammonium sulphate concentrations, the relative activity with tetrathionate decreased faster than with trithionate (Fig. 2). A requirement for sulphate anions has also been observed for purified trithionate hydrolase [6].

Tetrathionate conversion was inhibited completely by 0.05 mM $HgCl_2$, while trithionate hydrolysis was inhibited by only 25%. Thiosulphate, a product of both conversions, inhibited trithionate hydrolysis by 40% at a concentration of 0.1 mM. To attain the same degree of inhibition with tetrathionate as a substrate, a ten-fold higher concentration of thiosulphate had to be added. Both activities were insensitive to azide (1 mM) and the sulfhydryl reagent *N*-ethylmaleimide (0.05 mM).

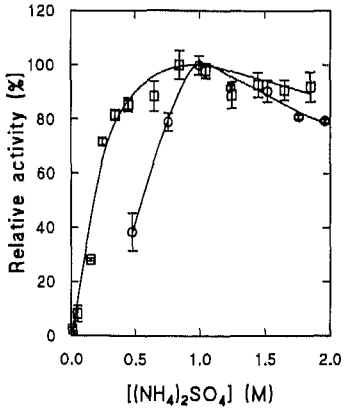

Fig. 2. Effect of ammonium sulphate concentration on trithionate (□) and tetrathionate conversion (○). Stimulation of trithionate and tetrathionate conversion rates by ammonium sulphate was determined at 30°C and pH 3.0. Cell-free extract was pre-incubated for 30 min before 1 mM substrate was added to start the reaction. Relative activities are given with 100% activity in the optimum. Error bars indicate the standard deviation ($n = 2$).

Fate of tetrathionate-metabolizing activity during purification of trithionate hydrolase

The purification procedure for trithionate hydrolase [6] was also used for partial purification of tetrathionate-metabolizing enzyme. Trithionate hydrolase activity in cell-free extracts was approximately 70-fold higher than the rate of tetrathionate conversion (Table 1). Precipitation of protein by dilution of the extract and subsequent resuspension of the pellet in 0.4 M ammonium sulphate, yielded a four-fold higher recovery for trithionate hydrolase than for the tetrathionate-metabolizing enzyme activity (Table 1). Cation exchange chromatography resulted in a complete loss of tetrathionate-metabolizing activity, while trithionate hydrolase was further purified. As it was our objective at this point to show that conversion of trithionate and tetrathionate was not catalysed by the same enzyme, no further efforts were made to purify the tetrathionate-metabolizing enzyme.

Discussion

The data presented in this paper demonstrate that tetrathionate metabolism in *T. acidophilus* is initiated by a hydrolytic cleavage removing one of the sulphone moieties of the molecule, and the cleavage of the S-S bond between the two sulphane sulphur atoms. The stability, the pH range and the stimulatory effect of ammonium sulphate on this enzyme differ from that of the recently purified trithionate hydrolase from the same organism [6]. Also the observation that tetrathionate-metabolizing activity was lost during purification of trithionate hydrolase indicates that the latter enzyme cannot account for the conversion of tetrathionate.

The products of tetrathionate conversion by cell-free extracts of *T. acidophilus* (sulphur, thiosulphate and sulphate) are strikingly similar to the products of cyanolysis, in which a nucleophilic attack of CN^- results in the decomposition of tetrathionate into thiocyanate, thiosulphate and sulphate. It is tempting to speculate that enzyme-catalysed tetrathionate conversion may occur via a similar mechanism, for instance by a nucleophilic attack of an enzyme-bound sulfhydryl group. Such a mechanism could involve the formation of enzyme-bound polysulphide chains, which ultimately split off elemental sulphur. However, other mechanisms, for instance involving the initial formation of disulphane monosulphonate ($S_3O_3^{2-}$) [10], or the initial dismutation of tetrathionate to trithionate and pentathionate [11] might ultimately yield the same products as those found after a one-step reaction analogous to cyanolysis. Purification of the involved enzyme(s) is required to elucidate the molecular mechanism of tetrathionate metabolism in *T. acidophilus*.

The low pH optimum of the tetrathionate-metabolising activity indicates that it is located extracytoplasmically, probably in the periplasm. In this respect it resembles two other enzymes involved in inorganic sulphur metabolism of *T. acidophilus*, thiosulphate dehydrogenase (Meulenberg et al., unpublished) and trithionate hydrolase [6].

Tetrathionate is a key intermediate in the oxidation of the sulphur oxyanions thiosulphate and trithionate (Fig. 3) [1]. Of the 14 redox equivalents that can be derived from the complete oxidation of tetrathionate to sulphate, two are transferred to the electron transport chain as a result

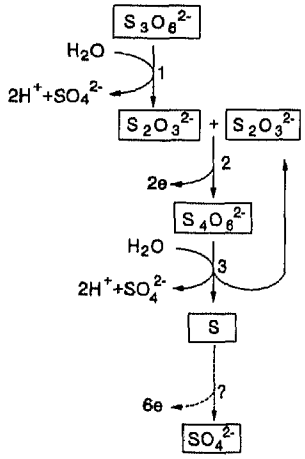

Fig. 3. Pathway of the oxidation of sulphur oxyanions in *T. acidophilus*. Enzymes involved: 1, Trithionate hydrolase; 2, thiosulphate dehydrogenase; 3, tetrathionate-metabolizing enzyme.

of the reoxidation of thiosulphate to tetrathionate [1]. The remaining 12 redox equivalents are transferred to the other reduced intermediate of tetrathionate conversion, sulphur (Fig. 3). Sulphur is also an initial product of tetrathionate degradation in cell-free extracts of *T. ferrooxidans* (Hazeu, unpublished observation). It is clear from Fig. 3 that the lack of insight in the molecular mechanisms involved in the oxidation of intermediary sulphur is the bottle-neck in our present understanding of sulphur compound oxidation in acidophilic thiobacilli.

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