*Journal of General Microbiology* (1988), **134**, 877–885. *Printed in Great Britain* 

# Cellular Location and Partial Purification of the 'Thiosulphate-oxidizing Enzyme' and 'Trithionate Hydrolyase' from *Thiobacillus tepidarius*

By WEI-PING LU† AND DON P. KELLY*

*Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK*

(*Received 4 August 1987; revised 27 November 1987*)

The enzyme oxidizing thiosulphate to tetrathionate ('tetrathionate synthase') has been purified: the native enzyme has a $M_r$ of 138000 and subunit $M_r$ of 45000, contains no haem and shows $K_m$ values for thiosulphate of 4 and 110 μM respectively with cytochrome *c* or ferricyanide as electron acceptor. Most of the enzyme was recovered from the periplasm of the cell. Evidence is also presented for a trithionate hydrolyase, catalysing the hydrolytic cleavage of trithionate to thiosulphate and sulphate. This activity was partially purified and assayed in a coupled system in which cytochrome *c* reduction by the purified tetrathionate synthase was measured. Tetrathionate oxidation by cell-free preparations could not be obtained, but a sulphite dehydrogenase (with ferricyanide as electron acceptor) was demonstrated and sulphite-dependent reduction of cytochrome *c* in *T. tepidarius* membrane preparations occurred. The latter was inhibited by HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide), indicating the involvement of cytochrome *b*. Sulphite oxidation by APS reductase (adenylylsulphate reductase) could not be detected, which was consistent with the earlier demonstration of complete inhibition of ATP synthesis in intact organisms by FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). A scheme is presented which is consistent with all the whole organism and enzyme data available to date.

## INTRODUCTION

In the previous paper (Lu & Kelly, 1988) we demonstrated that the oxidation of thiosulphate by *Thiobacillus tepidarius* appeared to involve the formation of tetrathionate as an intermediate. The probable location of the tetrathionate-forming enzyme was in the periplasm of the cells, the tetrathionate thus formed being subsequently transported across the cytoplasmic membrane prior to its complete oxidation to sulphate. The sensitivity of tetrathionate and sulphite oxidation to FCCP and HQNO suggested that both were metabolized on the cytoplasmic side of the membrane, and that their oxidation involved electron transport from cytochrome *b* to cytochrome *c*. The intermediate sensitivity of trithionate oxidation to inhibitors, relative to thiosulphate and tetrathionate, and the sulphate-related loss of trithionate-oxidizing activity from washed bacteria, suggested that trithionate might also undergo initial metabolism in the periplasm, possibly involving thiosulphate and tetrathionate as oxidation intermediates. We have now sought to identify enzymic functions in extracts of *T. tepidarius* that could confirm the observations made with intact organisms.

## METHODS

*Organism and culture conditions.* These were described in the previous paper (Lu & Kelly, 1988).

*Preparation of cell-free extracts.* Organisms (60 mg dry wt $ml^{-1}$) harvested from chemostat culture were stored at −25 °C, then thawed and passed twice through a French pressure cell at 140 MPa. DNAase (30 μg $ml^{-1}$) and

† Present address: Biology Department, Fudan University, Shanghai, People's Republic of China.

*Abbreviations*: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; PMS, phenazine methosulphate; PES, phenazine ethosulphate; INT, *p*-iodonitrotetrazolium violet; DCPIP, phenolindo-2,6-dichlorophenol; APS reductase, adenylylsulphate reductase.

$MgCl_2$ (10 mM) were added and the extract was centrifuged at 10000 *g* for 20 min. The supernatant extract was further centrifuged at 45000 *g* for 30 min. This gave a supernatant liquid described as the 'crude extract' and a pellet which was referred to as the 'membrane fraction'.

*Preparation of periplasmic, cytoplasmic and membrane fractions.* Organisms (80 mg dry wt $ml^{-1}$) collected from a thiosulphate-limited chemostat were centrifuged, washed and resuspended in a solution containing 0·5 M-sucrose, 0·05 M-potassium phosphate (pH 7·0), 0·05 M-Tris/HCl (pH 7·0) and 5 mM-EDTA. The suspension (5 ml) was mixed with 1 ml 1·5% (w/v) lysozyme and incubated at 30 °C for 2 h. This procedure produced wall-less spheroplasts. After centrifuging at 10000 *g* for 18 min the supernatant solution was removed as the periplasmic fraction. The pellet was resuspended in 2 ml 0·1 M-potassium phosphate, pH 7·0, 0·5 mg DNAase added, and respun at 10000 *g* for 15 min. The pellet was discarded as unlysed spheroplasts and large debris, and the supernatant liquid respun at 45000 *g* for 30 min. The pellet was saved as the membrane fraction and the supernatant solution as the cytoplasmic fraction. It was recognized that some membrane fragments would remain in the cytoplasmic fraction, but this gentle preparation procedure minimized membrane disruption into very small fragments. The distribution of the total protein recovered was periplasm, 27%; cytoplasm, 53%; membrane, 20%.

*Assay of enzymes.* '*Thiosulphate-oxidizing enzyme*'. An enzyme oxidizing thiosulphate to tetrathionate, and using ferricyanide or cytochrome *c* as an electron acceptor, has been assayed in several thiobacilli and heterotrophs and partially purified in some cases (Trudinger, 1961, 1967; Kelly, 1966; Silver & Lundgren, 1968; Lyric & Suzuki, 1970*a*, *b*, *c*; Schook & Berk, 1979; Kurek, 1985; Mason & Kelly, 1988). *In vivo* this enzyme is a thiosulphate:cytochrome *c* oxidoreductase (tetrathionate-synthesizing) but *in vitro* it is commonly assayed by measurement of ferricyanide reduction. We propose that the trivial name tetrathionate synthase is more appropriate and precise than 'thiosulphate-oxidizing enzyme', and shall refer to it in this way in this paper.

*Tetrathionate synthase assay.* Activity was measured at 30 °C using ferricyanide or horse heart cytochrome *c* as electron acceptor. In the former, the reaction mixture (1 ml) contained 0·1 M-potassium hydrogen phthalate buffer, pH 5·5; 0·95 μmol ferricyanide; 30 μmol $Na_2S_2O_3$; and 2–50 μg protein. In the latter, the reaction mixture (1 ml) contained 0·1 M-potassium phosphate, pH 7·0; 1 μmol $Na_2S_2O_3$; 0·15 mg cytochrome *c*; and 0·05–0·5 mg protein.

*Trithionate hydrolyase assay.* Evidence is presented in Results that there is an enzyme in *T. tepidarius* catalysing the hydrolytic release of thiosulphate from trithionate. This was assayed in cell free extracts by using the cytochrome *c* tetrathionate synthase assay mixture in which $Na_2S_3O_6$ (0·2 μmol) was used instead of $Na_2S_2O_3$. Trithionate solutions were prepared in 0·1 M-potassium phosphate, pH 7·0, and used within 5 h to avoid complications resulting from chemical hydrolysis of trithionate solutions.

*Sulphite dehydrogenase and APS reductase assays.* Sulphite-dependent reduction of ferricyanide was measured in reaction mixtures (3 ml) containing (mM) Tris/HCl or Tris/$H_2SO_4$, pH 7·4, 8·0 or 8·4 (50); potassium ferricyanide (3); $Na_2SO_3$ (3); and 1–3 mg protein. Reaction was initiated by adding enzyme preparation or sulphite (50 μl of 0·18 M in 0·1 M-Tris/HCl, pH 7·8, containing 10 mM-EDTA) and decrease in absorbance at 420 nm was recorded on Pye-Unicam SP1700 or SP2-800 spectrophotometers. A molar extinction coefficient of $10^3$ $l^{-1}$ $mol^{-1}$ $cm^{-1}$ was used to calculate ferricyanide reduction. In order to test for the presence of APS reductase (Peck *et al.*, 1965; Bowen *et al.*, 1966; Lyric & Suzuki, 1970*d*), the above reaction mixture was supplemented with AMP (0·75 mM) either before or after the addition of sulphite or enzyme preparation.

*Purification of tetrathionate synthase.* (*a*) *Ion exchange chromatography.* Crude extract (200–500 mg protein) was applied to a column (2·6 × 7 cm) of DEAE-Sepharose CL-6B, equilibrated with 25 mM-potassium phosphate buffer, pH 6·5. The column was eluted, at 4 °C and a flow rate of 100 ml $h^{-1}$, with 20 ml of the same buffer, followed by a linear gradient of increasing NaCl concentration, produced by mixing the outflow of two reservoirs containing 25 mM-phosphate, one of which also contained 0·5 M-NaCl. The active fractions were contained in the 40–60 ml elution volume. These fractions were pooled and protein was recovered by salting out with ammonium sulphate (5 g per 10 ml).

(*b*) *Gel filtration.* The concentrated fraction from (*a*) was loaded to the bottom of a column (3·2 × 90 cm) of Sephadex G-100, equilibrated with 50 mM-potassium phosphate buffer, pH 7·0, at 4 °C, and eluted upwards with the same buffer at a flow rate of 30 ml $h^{-1}$. The active fractions (310–350 ml) were pooled and concentrated as above.

(*c*) *Hydrophobic interaction chromatography.* The concentrated fraction from (*b*) was loaded on a column (0·8 × 2·5 cm) of phenyl-Sepharose CL-4B, equilibrated with 25 mM-potassium phosphate buffer, pH 7·0, containing $(NH_4)_2SO_4$ (100 g $l^{-1}$). The column was eluted with 10 ml of the same buffer at a flow rate of 30 ml $h^{-1}$, followed by a linear gradient of decreasing $(NH_4)_2SO_4$ concentration produced from mixing two reservoirs of buffer, one containing $(NH_4)_2SO_4$ at 100 g $l^{-1}$. The active fractions (see Fig. 1) were pooled and concentrated as before. The concentrated fraction is referred to as HIC(I) in the Results.

*Determination of the $M_r$ of tetrathionate synthase.* The $M_r$ of the undenatured enzyme was determined by gel filtration on a column of Sephadex G-100, as described above, using bovine serum albumin ($M_r$ 67000), lactate dehydrogenase ($M_r$ 120000) and alcohol dehydrogenase ($M_r$ 150000) as standards. Subunit $M_r$ was measured by SDS-PAGE (Weber & Osborn, 1969) as described previously for enzymes from *T. versutus* (Lu & Kelly, 1983) using protein standards of $M_r$ 14·2, 20, 24, 29, 36, 45 and 66 kDa. Gels containing the purified tetrathionate

Table 1. *Purification of tetrathionate synthase from T. tepidarius*

| Procedure | Total protein (mg) | Total activity* | Recovery (%) | Specific activity† | Purification factor |
|---|---|---|---|---|---|
| Crude extract | 316 | 2528 | 100 | 8 | 1 |
| Ion exchange chromatography | 180 | 2160 | 85 | 12 | 1·5 |
| Gel filtration (Sephadex G-100) | 30 | 1550 | 62 | 52 | 6·5 |
| Hydrophobic interaction chromatography [HIC(I)] | 1·8 | 569 | 22 | 316 | 39·5 |

* μmol ferricyanide reduced $min^{-1}$.
† μmol ferricyanide reduced $min^{-1}$ (mg protein)$^{-1}$.

synthase were stained with Coomassie Blue to detect protein bands, or with the haem-staining procedure of Thomas *et al.* (1976) to seek haem in the purified enzyme.

*Analytical procedures.* Thiosulphate and polythionates were estimated cyanolytically (Kelly *et al.*, 1969). Protein was estimated by the Lowry procedure, using bovine serum albumin as standard.

*Chemicals.* $Na_2S_3O_6$ was synthesized (Wood & Kelly, 1986); $K_2S_4O_6$ was from Fluka; FCCP was from Aldrich; HQNO, cytochrome *c* (horse heart III), NADH, phenazine methosulphate (PMS), *p*-iodonitrotetrazolium violet (INT) and phenazine ethosulphate (PES) were from Sigma; phenolindo-2,6-dichlorophenol (DCPIP) was from BDH. Reagents and buffers were from BDH, Fisons and Sigma.

## RESULTS

### *Distribution of tetrathionate synthase in T. tepidarius and purification of the enzyme*

Thiosulphate-dependent ferricyanide reduction by tetrathionate synthase was catalysed by all three subcellular fractions (see Methods) but at least 73% of the enzyme activity was recovered in the periplasmic fraction, with a specific activity of 9·6 μmol ferricyanide reduced $min^{-1}$ (mg protein)$^{-1}$, compared with values of 1·0 and 2·0 for the cytoplasmic and membrane fractions respectively. There was stoichiometric production of tetrathionate from thiosulphate by the crude extract, in the presence of excess thiosulphate, according to the equation

$$2S_2O_3^{2-} + 2Fe(CN)_6^{3-} \rightarrow S_4O_6^{2-} + 2Fe(CN)_6^{4-}$$

The stoichiometry of ferricyanide reduction by the crude extract was $0{\cdot}97 \pm 0{\cdot}03$ μmol ferricyanide reduced per mol of thiosulphate added (mean ± SE of 10 determinations).

Using sequential ion exchange chromatography, gel filtration and hydrophobic interaction chromatography (see Methods), a 40-fold purification was achieved (Table 1). The greatest increase in specific activity accompanied the hydrophobic interaction chromatography [HIC(I); Fig. 1], although only about 20% of the original activity was finally recovered. The low recovery was due in part to lability of the activity during purification and on storage at −20 °C.

### *Evidence for a trithionate hydrolyase in T. tepidarius*

When a suspension of thiosulphate-grown organisms (4 mg dry wt $ml^{-1}$ in 0·1 M-potassium phosphate buffer, pH 7·0) was incubated anaerobically under nitrogen at 40 °C and supplemented with 33 mM-$Na_2SO_4$ and 1·5 mM-$Na_2S_3O_6$ for 15 min, 0·8 mM-thiosulphate was formed, the remainder of the added trithionate being recovered unchanged. This indicated a minimum rate for trithionate conversion to thiosulphate by intact organisms of 13 nmol $min^{-1}$ (mg dry wt)$^{-1}$. Insignificant thiosulphate production occurred in the absence of organisms. Little thiosulphate could be detected when a crude extract (1 mg protein $ml^{-1}$) was incubated in 25 mM-HEPES/NaOH buffer, pH 7·0, with 5 mM-trithionate. Significant activity was, however,

Table 2. *Copurification of trithionate hydrolyase with tetrathionate synthase*

Activities were measured using the cytochrome *c* assay for tetrathionate synthase (see Methods) but with 0·2 mM-trithionate as substrate.

| Procedure | Total protein (mg) | Trithionate-dependent cytochrome *c* reduction: Total activity* | Trithionate-dependent cytochrome *c* reduction: Specific activity† | Recovery | Purification factor |
|---|---|---|---|---|---|
| Crude extract | 316 | 1896‡ | 6‡ | 100‡ | 1 |
| Ion exchange chromatography | 180 | 2160 | 12 | 114 | 2 |
| Gel filtration (Sephadex G-100) | 30 | 600 | 20 | 32 | 3·3 |
| Hydrophobic interaction chromatography [HIC(I) + HIC(II)] | 1·8 + 1·1§ | 426 | 147 | 20 | 24·5 |

* nmol cytochrome *c* reduced $min^{-1}$.

† nmol cytochrome *c* reduced $min^{-1}$ (mg protein)$^{-1}$.

‡ These values were underestimates because of the presence of cytochrome oxidase activity in the membrane fragments remaining in the crude extract.

§ see Fig. 1 and Table 4.

Table 3. *Reconstitution of trithionate-dependent cytochrome c reduction*

The activities shown are typical of several tests in which the HIC(I) and HIC(II) fractions were combined to assay trithionate-dependent cytochrome *c* reduction. ND, Not determined.

| Fraction* | Cytochrome *c* reduction [nmol $min^{-1}$ (mg protein)$^{-1}$]: Trithionate | Thiosulphate |
|---|---|---|
| HIC(I) | 19 | 440 |
| HIC(II) | 12 | 20 |
| HIC(I + II) | 147† | ND |

* The assay procedure is given in Methods. Amounts of protein used were (mg): HIC(I), 0·056; HIC(II), 0·068.

† Specific activity calculated on total protein (0·124 mg) in the assay mixture.

found when the extract (at 4 mg protein $ml^{-1}$) was incubated at 40 °C with 5 mM-trithionate in 33 mM-Tris/$H_2SO_4$ buffer, pH 7·8, or 18·5 mM-potassium phosphate, pH 8·0, supplemented with 30 mM-$Na_2SO_4$. Under these conditions thiosulphate formation proceeded linearly for at least 40 min at rates of 8–14 nmol trithionate hydrolysed $min^{-1}$ (mg protein)$^{-1}$.

Conditions required for optimum expression of trithionate hydrolyase activity have not been determined. The relative insensitivity of the assay procedure used also meant that an indirect means of determining activity during partial purification was desirable. This was achieved by coupling the hydrolysis of trithionate to measurement of thiosulphate-dependent cytochrome *c* reduction by tetrathionate synthase, as described below.

Using horse heart cytochrome *c*, instead of ferricyanide, as electron acceptor, crude extracts catalysed cytochrome *c* reduction with either thiosulphate or trithionate as substrate. The trithionate-dependent reductase activity copurified with tetrathionate synthase activity (Table 2). It was clearly due to a trithionate hydrolyase producing thiosulphate, rather than to a 'trithionate : cytochrome *c* reductase' because cytochrome reduction by trithionate was only catalysed effectively when the purified tetrathionate synthase fraction [HIC(I)] was mixed with a second HIC fraction [HIC(II), Fig. 1; Table 3] that contained very little tetrathionate synthase. Table 3 demonstrates the reconstitution of the trithionate-dependent cytochrome *c*-reducing activity. The interpretation of these data is that the HIC(II) fraction contains a

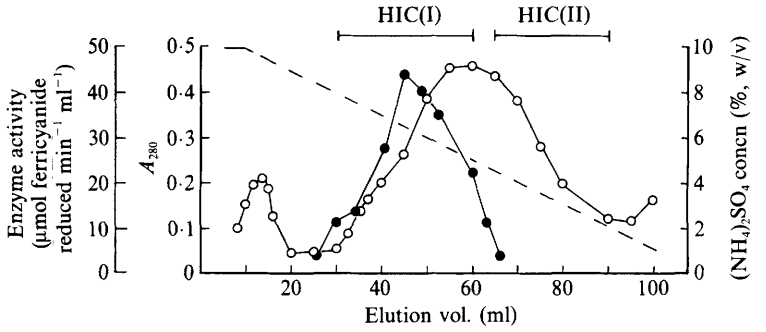


Fig. 1. Purification of tetrathionate synthase by hydrophobic interaction chromatography. The active fractions from ion exchange chromatography followed by gel filtration were eluted from a column of phenyl-Sepharose CL-4B as described in Methods and in Table 2. The fractions labelled HIC(I) contained the tetrathionate synthase. The fractions labelled HIC(II) contained trithionate hydrolyase (see text). Absorbance at 280 nm (○) shows the protein elution profile; enzyme activity (●) eluted mainly between $(NH_4)_2SO_4$ concentrations (broken line) of 8 and 4% (w/v).

trithionate hydrolyase, producing thiosulphate that acts as substrate for the tetrathionate synthase in the HIC(I) fraction. The specific activity given in the Table for HIC(I + II) was calculated for the total protein added from both fractions. This clearly underestimates the activity of the presumptive trithionate hydrolyase. If the rate of trithionate-dependent cytochrome *c* reduction was limited by the rate of trithionate hydrolysis, rather than by the activity of tetrathionate synthase, then the specific activity of trithionate hydrolyase [based only on the HIC(II) protein] could be as high as 268 nmol trithionate hydrolysed $min^{-1}$ [mg HIC(II) protein]$^{-1}$. Direct measurement of trithionate hydrolysis by the HIC(II) fraction has not been attempted as a sufficiently sensitive specific analytical method for detection of thiosulphate formation was not available.

The stoichiometry of cytochrome *c* reduction and trithionate or thiosulphate oxidation was estimated using the partially purified tetrathionate synthase [HIC(I); 0·025 mg protein] with and without the trithionate hydrolyase fraction [HIC(II); 0·07 mg total protein], respectively. Using two different quantities each of thiosulphate and trithionate the mean molar stoichiometry of cytochrome *c* reduced to thiosulphate or trithionate added was 1·01 ± 0·04 (mean of four determinations ± SE). This was consistent with cytochrome reduction being due exclusively to the tetrathionate synthase, with the trithionate-dependent reduction resulting from oxidation of thiosulphate generated by the trithionate hydrolyase.

### *Some properties of tetrathionate synthase*

The $M_r$ of the native enzyme, determined by gel filtration (see Methods) was 138000. SDS-PAGE (Lu & Kelly, 1983) of the purified enzyme [HIC(I) fraction] gave a subunit $M_r$ of 45000, indicative of three subunits of equal $M_r$ in the native enzyme. The purity of the enzyme in the HIC(I) fraction was estimated at about 70–80% by SDS-PAGE. The enzyme band in this fraction contained no haem that could be detected by haem-staining of the gel (Thomas *et al.*, 1976). After freezing at −20 °C and thawing, 20–30% of the activity of the HIC(I) fraction was lost.

The $K_m$ value for thiosulphate depended on whether cytochrome *c* or ferricyanide was used as the electron acceptor. Using appropriate ranges of concentration of thiosulphate (0·1–10 mM with ferricyanide; 5–200 μM with cytochrome *c*), the $K_m$ values with cytochrome *c* or ferricyanide were determined from Lineweaver–Burk plots to be 4 and 110 μM thiosulphate respectively.

In common with tetrathionate synthase enzymes found in some other bacteria (Barrett & Clark, 1987), the reaction catalysed by the enzyme was reversible; some reoxidation of reduced cytochrome *c* was observed qualitatively when tetrathionate replaced thiosulphate in the assay procedure.

*Effect of sulphate on the partially purified trithionate hydrolyase*

It was noted in assaying thiosulphate production from trithionate by frozen-and-thawed crude extracts that activity was considerably greater in the presence of sulphate. This was confirmed using the HIC(II) fraction in the coupled assay using purified tetrathionate synthase. The activity of a preparation after 2–3 d at 4 °C (or frozen storage) was 38 nmol cytochrome *c* reduced $min^{-1}$ (mg protein)$^{-1}$. This was increased to 120 in the presence of 20 mM-sulphate. This is consistent with the earlier observation of restoration of trithionate-oxidizing ability to whole organisms by the addition of sulphate (Lu & Kelly, 1988; preceding paper).

Although the trithionate hydrolyase appeared to have been purified about 28-fold in the HIC(II) fraction, its further physiochemical characterization has not yet been achieved.

*Sulphite oxidation by crude extracts of T. tepidarius*

Ferricyanide reduction by cell-free extracts supplemented with sulphite in the presence or absence of AMP was assayed at pH values of pH 7·3, 7·6, 8·0 and 8·4. Sulphite-dependent ferricyanide reduction, indicative of sulphite dehydrogenase (EC 1.8.2.1; Lu & Kelly, 1984), was similar at all pH values tested, and showed a mean activity of $77 \pm 7$ nmol ferricyanide reduced $min^{-1}$ (mg protein)$^{-1}$, for 13 determinations.

Addition of AMP had no effect on the rate of ferricyanide reduction, identical rates being seen in assays without AMP or when supplemented with AMP prior to sulphite addition, or after adding AMP during sulphite oxidation. There was thus no evidence of sulphite oxidation by an AMP-dependent APS reductase.

*Reduction of T. tepidarius cytochromes by thiosulphate or sulphite catalysed by the membrane fraction*

When the isolated membrane fraction of *T. tepidarius* was incubated at 30 °C with thiosulphate, sulphite or NADH, reduction of the endogenous cytochrome *c* was observed. HQNO inhibited the sulphite- and NADH-dependent cytochrome reduction, but did not affect that seen with thiosulphate.

*Tetrathionate metabolism by extracts of T. tepidarius*

The ability to oxidize tetrathionate was very labile in whole organisms: after freezing and thawing once, or breaking freshly harvested organisms by sonication or with the French pressure cell, 99% of the fresh cell rate of oxidation was lost. Crude extracts and the membrane fraction oxidized thiosulphate rapidly [typically 35 nmol $O_2$ $min^{-1}$ (mg protein)$^{-1}$], but tetrathionate oxidation proceeded only at 1–2 nmol $O_2$ $min^{-1}$ (mg crude extract or membrane protein)$^{-1}$. Using assay mixtures (1 ml) containing crude extracts or membrane-free soluble fractions in Tris, phosphate, glycylglycine or HEPES buffers at pH 6·5–8·0 and at 30–40 °C, no significant oxidation could be observed using PMS, PES, INT, DCPIP, ferricyanide or cytochrome *c* as electron acceptors.

## DISCUSSION

These data provide direct enzymological evidence for a mechanism of sulphur compound oxidation in *T. tepidarius* that involves the periplasmic hydrolysis of trithionate to thiosulphate and oxidation of thiosulphate to tetrathionate by a cytochrome *c*-dependent oxidoreductase enzyme. The subsequent oxidation of tetrathionate (probably involving sulphite as an intermediate) appears to require an intact membrane-cytoplasm system and electron transport via ubiquinone/cytochrome *b*.

The trithionate hydrolyase, for which direct and indirect evidence is presented, appears to catalyse the reaction

$$S_3O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + 2H^+$$

This stoichiometry is the one shown by Trudinger (1964*a*) for the hydrolysis of trithionate by intact cells of *T. neapolitanus*. As no reductant substrate was present in our assays, and added

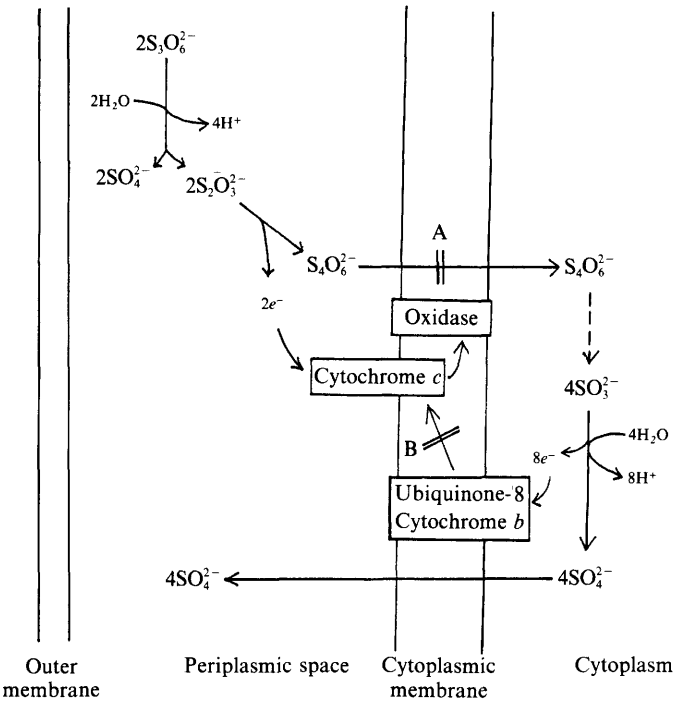


Fig. 2. Pathway for the oxidation of trithionate, thiosulphate and tetrathionate by *T. tepidarius*, as deduced from whole organism and cell free extract and enzyme studies. The sites of inhibition of oxidation by FCCP (A) and HQNO (B) are indicated. For clarity of presentation the oxygen reduction reactions at the oxidase site have been omitted.

trithionate was stoichiometrically recovered as thiosulphate and unaltered trithionate, the alternative possibility that trithionate was reductively cleaved to thiosulphate and sulphite (Okuzumi, 1966*a*) can be discounted. The equivalence of the growth yields on thiosulphate and trithionate (Wood & Kelly, 1986) is also consistent with the operation *in vivo* of such a trithionate hydrolyase.

The oxidation of thiosulphate to tetrathionate has been shown unequivocally to be catalysed by a thiosulphate:cytochrome *c* oxidoreductase (tetrathionate synthase) that is located in the periplasm of the bacteria. This enzyme was of similar $M_r$ (138000) to those purified from *T. thioparus* (115000; Lyric & Suzuki, 1970*b*) and marine heterotroph strain 16B (132000; Whited & Tuttle, 1983). As with the *T. thioparus* enzyme (Lyric & Suzuki, 1970*b*, *c*), the $K_m$ for thiosulphate for the tetrathionate synthase was much lower with horse heart cytochrome *c* as the electron acceptor than with ferricyanide. Specific activity was, however, much greater with ferricyanide than with the mammalian cytochrome [e.g. 700-fold for the HIC(I) fraction, Tables 1 and 3], as was also reported for the *T. thioparus* and *T. ferroxidans* enzymes (Silver & Lundgren, 1968; Lyric & Suzuki, 1970*b*). Comparisons with *T. neapolitanus* (Trudinger, 1961), marine heterotroph 16B (Tuttle *et al.*, 1983) and soil heterotroph A50 (Trudinger, 1967) shows that the enzyme from these was unable to couple thiosulphate oxidation to mammalian cytochrome *c* reduction, although it could catalyse reduction of native endogenous cytochrome *c*. *In vivo* it is therefore probable that thiosulphate oxidation to tetrathionate proceeds very rapidly and with a very low $K_m$, as indicated by the intact organism study (Lu & Kelly, 1988, preceding paper). In common with some other tetrathionate synthases (Barrett & Clark, 1987), the enzyme from *T. tepidarius* may exhibit some degree of reversibility, but this was not investigated further.

Failure to observe tetrathionate oxidation by our crude extracts could indicate that a labile system involving the cytoplasmic membrane is involved. Alternatively, there is the possibility that the further metabolism of tetrathionate, either on or inside the cytoplasmic membrane,

requires its cleavage to smaller units, such as sulphur and sulphite, possibly by a hydrolysis analogous to that seen for trithionate (Trudinger, 1964*a*, *b*) or by dismutation to other polythionates (Okuzumi, 1965, 1966*b*). There is evidence for free sulphur as an intermediate in reduced sulphur compound oxidation by thiobacilli (Kelly, 1982; Steudel *et al.*, 1987; Hazeu *et al.*, 1988), but this has not been further tested in *T. tepidarius*. Oxidation of tetrathionate by extracts of *T. thioparus* and *T. thiooxidans* was reported (London & Rittenberg, 1964) but the mechanism was apparently never resolved.

Finally, crude extracts of *T. tepidarius* have now been shown to contain an AMP-independent sulphite dehydrogenase at an activity comparable to that of *T. denitrificans* (Aminuddin & Nicholas, 1974). A point of particular interest is the apparent absence of AMP-stimulated sulphite oxidation by crude extracts. This suggests that APS reductase may not be present in *T. tepidarius*: this enzyme, when present, is usually at considerably higher specific activity than sulphite dehydrogenase (Aminuddin & Nicholas, 1974; Stille & Trüper, 1984). Failure to demonstrate APS reductase could be due to extreme lability as reported for *T. thioparus* (Peck *et al.*, 1965), although the enzyme from other strains of thiobacilli appeared less labile (Bowen *et al.*, 1966; Lyric & Suzuki, 1970*d*). It is present with sulphite dehydrogenase in some thiobacilli, but absent from others (Hempfling *et al.*, 1967; Lu & Kelly, 1984). The implication from the preceding paper Lu & Kelly, 1988) that sulphite oxidation by *T. tepidarius* occurs within the cytoplasmic membrane would be consistent either with a labile cytoplasmic APS reductase (Hooper & DiSpirito, 1985) or more probably indicates a cytochrome-*b*-linked enzyme (as in *T. thiooxidans*; Tano *et al.*, 1982) that requires sulphite oxidation at the inner surface of the cytoplasmic membrane. This view is consistent with the observed inhibition by HQNO of membrane-catalysed cytochrome *c* reduction with sulphite as substrate.

A scheme for sulphur-compound oxidation as indicated by the results of this and the preceding paper is shown in Fig. 2. Direct evidence for the components of the electron transport pathway during sulphur compound oxidation will be presented elsewhere.

We wish to acknowledge financial support from the Science Funds of the Chinese Academy of Sciences, the International Foundation for Science (Sweden), the President's Fund of the Society for General Microbiology, and Fudan University, Shanghai.

## REFERENCES

AMINUDDIN, M. & NICHOLAS, D. J. D. (1974). An AMP-independent sulphite oxidase from *Thiobacillus denitrificans*: purification and properties. *Journal of General Microbiology* **82**, 103–113.

BARRETT, E. L. & CLARK, M. A. (1987). Tetrathionate reduction and production of hydrogen sulfide from thiosulfate. *Microbiological Reviews* **51**, 192–205.

BOWEN, T. J., HAPPOLD, F. C. & TAYLOR, B. F. (1966). Studies on adenosine-5′-phosphosulfate reductase from *Thiobacillus denitrificans*. *Biochimica et biophysica acta* **118**, 566–576.

HAZEU, W., STEUDEL, R., BATENBURG-VAN DE VEGTE, W. H., BOS, P. & KUENEN, J. G. (1988). Elemental sulphur as an intermediate in the oxidation of reduced sulphur compounds by *Thiobacillus ferrooxidans*: localization and characterization. In *Biohydrometallurgy 1987*. Edited by P. R. Norris & D. P. Kelly. (In the Press.)

HEMPFLING, W. P., TRUDINGER, P. A. & VISHNIAC, W. (1967). Purification and some properties of sulfite oxidase from *Thiobacillus neopolitanus*. *Archiv für Mikrobiologie* **59**, 149–157.

HOOPER, A. B. & DISPIRITO, A. A. (1985). In bacteria which grow on simple reductants, generation of a protein gradient involves extracytoplasmic oxidation of substrate. *Microbiological Reviews* **49**, 140–157.

KELLY, D. P. (1966). Fluoroacetate toxicity in *Thiobacillus neapolitanus* and its relevance to the problem of obligate chemoautotrophy. *Archiv für Mikrobiologie* **61**, 59–76.

KELLY, D. P. (1982). Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. *Philosophical Transaction of the Royal Society* **B298**, 499–528.

KELLY, D. P., CHAMBERS, L. A. & TRUDINGER, P. A. (1969). Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulphate and tetrathionate. *Analytical Chemistry* **41**, 898–901.

KUREK, E. (1985). Properties of an enzymatic complex active in sulfite and thiosulfate oxidation by *Rhodotorula* sp. *Archives of Microbiology* **143**, 277–282.

LONDON, J. & RITTENBERG, S. C. (1964). Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. *Proceedings of the National Academy of Sciences of the United States of America* **52**, 1183–1190.

LU, W.-P. & KELLY, D. P. (1983). Purification and some properties of two principal enzymes of the thiosulphate-oxidizing multienzyme system from *Thiobacillus* A2. *Journal of General Microbiology* **129**, 3549–3564.

LU, W.-P. & KELLY, D. P. (1984). Properties and role of sulphite:cytochrome *c* oxidoreductase purified from *Thiobacillus versutus* (A2). *Journal of General Microbiology* **130**, 1683–1692.

LU, W.-P. & KELLY, D. P. (1988). Kinetic and energetic aspects of inorganic sulphur compound oxidation by *Thiobacillus tepidarius*. *Journal of General Microbiology* **134**, 865–876.

LYRIC, R. M. & SUZUKI, I. (1970*a*). Kinetic studies of sulfite:cytochrome *c* oxidoreductase, thiosulfate-oxidizing enzyme, and adenosine-5′-phosphosulfate reductase from *Thiobacillus thioparus*. *Canadian Journal of Biochemistry* **48**, 594–603.

LYRIC, R. M. & SUZUKI, I. (1970*b*). Enzymes involved in the metabolism of thiosulphate by *Thiobacillus thioparus*. III. Properties of thiosulphate-oxidizing enzyme and proposed pathway of thiosulphate oxidation. *Canadian Journal of Biochemistry* **48**, 355–363.

LYRIC, R. M. & SUZUKI, I. (1970*c*). Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. I. Survey of enzymes and properties of sulfite:cytochrome *c* oxidoreductase. *Canadian Journal of Biochemistry* **48**, 334–343.

LYRIC, R. M. & SUZUKI, I. (1970*d*). Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. II. Properties of adenosine-5′-phosphosulfate reductase. *Canadian Journal of Biochemistry* **48**, 344–354.

MASON, J. & KELLY, D. P. (1988). Thiosulphate oxidation by obligately heterotrophic bacteria. *Microbial Ecology* **15** (in the Press).

OKUZUMI, M. (1965). Studies on the biochemistry of the thiobacilli. VII. Metabolism of tetrathionate by *T. thiooxidans*. *Agricultural and Biological Chemistry* **29**, 1069–1073.

OKUZUMI, M. (1966*a*). Studies on the biochemistry of the thiobacilli. IX. Reduction of trithionate by *T. thiooxidans*. *Agricultural and Biological Chemistry* **30**, 713–716.

OKUZUMI, M. (1966*b*). Studies on the biochemistry of the thiobacilli. VIII. Dismutation of tetrathionate by *T. thiooxidans*. *Agricultural and Biological Chemistry* **30**, 313–318.

PECK, H. D., DEACON, T. E. & DAVIDSON, J. T. (1965). Studies on adenosine-5′-phosphosulfate reductase from *Desulfovibrio desulfuricans* and *Thiobacillus thioparus*. *Biochimica et biophysica acta* **96**, 429–446.

SCHOOK, L. B. & BERK, R. S. (1979). Partial purification and characterization of thiosulphate oxidase from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **140**, 306–308.

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

STEUDEL, R., HOLDT, G., GÖBEL, T. & HAZEU, W. (1987). Chromatographic separation of higher polythionates $S_nO_6^{2-}$ (n = 3...22) and their detection in cultures of *Thiobacillus ferrooxidans*; molecular composition of bacterial sulfur secretions. *Angewandte Chemie* **26**, 151–153.

STILLE, W. & TRÜPER, H. G. (1984). Adenylylsulfate reductase in some new sulfate-reducing bacteria. *Archives of Microbiology* **137**, 145–150.

TANO, T., ITO, T., TAKESUE, H., SUGIO, T. & IMAI, K. (1982). b-Type cytochrome, an electron carrier in the sulfite-oxidation system of *Thiobacillus thiooxidans*. *Journal of Fermentation Technology* **60**, 181–187.

THOMAS, P. E., RYAN, D. & LEVIN, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecylsulfate polyacrylamide gels. *Analytical Biochemistry* **75**, 168–176.

TRUDINGER, P. A. (1961). Thiosulphate oxidation and cytochromes in *Thiobacillus* X. 2. Thiosulfate-oxidizing enzyme. *Biochemical Journal* **78**, 680–686.

TRUDINGER, P. A. (1964*a*). The metabolism of trithionate by *Thiobacillus* X. *Australian Journal of Biological Sciences* **17**, 459–468.

TRUDINGER, P. A. (1964*b*). Products of anaerobic metabolism of tetrathionate by *Thiobacillus* X. *Australian Journal of Biological Sciences* **17**, 446–458.

TRUDINGER, P. A. (1967). Metabolism of thiosulfate and tetrathionate by heterotrophic bacteria from soil. *Journal of Bacteriology* **93**, 550–559.

TUTTLE, J. H., SCHWARTZ, J. H. & WHITED, G. M. (1983). Some properties of thiosulfate-oxidizing enzyme from marine heterotroph 16B. *Applied and Environmental Microbiology* **46**, 438–445.

WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.

WHITED, G. M. & TUTTLE, J. H. (1983). Separation and distribution of thiosulfate-oxidizing enzyme, tetrathionate reductase, and thiosulfate reductase in extracts of marine heterotroph strain 16B. *Journal of Bacteriology* **156**, 600–610.

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.