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 138 000 and subunit $M_r$ of 45 000, 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 $-25^\circ$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.
MgCl₂ (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 washed 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 referred to as the 'membrane fraction'.

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, 1970a, 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; 0.05 mM ferricyanide; 30 μM Na₂S₃O₆; and 2–50 μg protein. In the latter, the reaction mixture (1 ml) contained 0.1 M-potassium phosphate, pH 7.0; 1 μmol Na₂S₂O₃; 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 Na₂S₃O₆ (0.2 μmol) was used instead of Na₂S₂O₃. Tetrathionate 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/H₂SO₄, pH 7.4, 8.0 or 8.4 (50); potassium ferricyanide (3); Na₂S₂O₃ (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 10³ 1⁻¹ mol⁻¹ cm⁻¹ was applied to a column (2.6 x 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).

**Gel filtration.** The concentrated fraction from (a) was loaded to the bottom of a column (3.2 x 90 cm) of Sephadex G-100, equilibrated with 50 mM-potassium phosphate buffer, pH 7.0, 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.

**Hydrophobic interaction chromatography.** The concentrated fraction from (b) was loaded on a column (0.8 x 2.5 cm) of phenyl-Sepharose CL-4B, equilibrated with 25 mM-potassium phosphate buffer, pH 7.0, containing (NH₄)₂SO₄ (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 (NH₄)₂SO₄ concentration produced from mixing two reservoirs of buffer, one containing (NH₄)₂SO₄ 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 Mₘ of tetrathionate synthase.** The Mₘ of the undenatured enzyme was determined by gel filtration on a column of Sephadex G-100, as described above, using bovine serum albumin (Mₘ, 67000), lactate dehydrogenase (Mₘ, 120000) and alcohol dehydrogenase (Mₘ, 150000) as standards. Subunit Mₘ was measured by SDS-PAGE (Weber & Osborn, 1967) as described previously for enzymes from *T. versutus* (Lu & Kelly, 1983) using protein standards of Mₘ, 14-2, 20, 24, 29, 36, 45 and 66 kDa. Gels containing the purified tetrathionate
Enzymes of *Thiobacillus tepidarius*

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

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity</th>
<th>Recovery (%)</th>
<th>Specific activity†</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>316</td>
<td>2528</td>
<td>100</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>180</td>
<td>2160</td>
<td>85</td>
<td>12</td>
<td>1.5</td>
</tr>
<tr>
<td>Gel filtration (Sephadex G-100)</td>
<td>30</td>
<td>1550</td>
<td>62</td>
<td>52</td>
<td>6.5</td>
</tr>
<tr>
<td>Hydrophobic interaction chromatography [HIC(I)]</td>
<td>1.8</td>
<td>569</td>
<td>22</td>
<td>316</td>
<td>39.5</td>
</tr>
</tbody>
</table>

* μmol ferricyanide reduced min⁻¹.
† μmol ferricyanide reduced min⁻¹ (mg protein)⁻¹.

Enzymes of *Thiobacillus tepidarius* 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. Na₂S₃O₆ was synthesized (Wood & Kelly, 1986); K₂S₄O₆ 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

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

The stoichiometry of ferricyanide reduction by the crude extract was 0.97 ± 0.03 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 hydrolase 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-Na₂SO₄ and 1.5 mM-Na₂S₃O₆ 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.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Trithionate-dependent cytochrome c reduction</th>
<th>Recovery</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total activity*</td>
<td>Specific activity†</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>316</td>
<td>1896‡</td>
<td>6‡</td>
<td>100‡</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>180</td>
<td>2160</td>
<td>12</td>
<td>114</td>
</tr>
<tr>
<td>Gel filtration (Sephadex G-100)</td>
<td>30</td>
<td>600</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>Hydrophobic interaction chromatography [HIC(I) + HIC(II)]</td>
<td>1.8 + 1.1§</td>
<td>426</td>
<td>147</td>
<td>20</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Trithionate</th>
<th>Thiosulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIC(I)</td>
<td>19</td>
<td>440</td>
</tr>
<tr>
<td>HIC(II)</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>HIC(I + II)</td>
<td>147‡</td>
<td>ND</td>
</tr>
</tbody>
</table>

* 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/H₂SO₄ buffer, pH 7.8, or 18.5 mM-potassium phosphate, pH 8.0, supplemented with 30 mM-Na₂SO₄. 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(II)] 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
Enzymes of *Thiobacillus tepidarius*

881

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 of 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*₅ of the native enzyme, determined by gel filtration (see Methods) was 138 000. SDS-PAGE (Lu & Kelly, 1983) of the purified enzyme [HIC(I) fraction] gave a subunit *M*₅ of 45 000, indicative of three subunits of equal *M*₅ 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 ± 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 O₂ min⁻¹ (mg protein)⁻¹], but tetrathionate oxidation proceeded only at 1-2 nmol O₂ 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

\[ \text{S}_2\text{O}_8^{2-} + \text{H}_2\text{O} \rightarrow \text{S}_2\text{O}_3^{2-} + \text{SO}_4^{2-} + 2\text{H}^+ \]

This stoichiometry is the one shown by Trudinger (1964a) for the hydrolysis of trithionate by intact cells of T. neapolitanus. As no reductant substrate was present in our assays, and added
Enzymes of *Thiobacillus tepidarius* 883

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, 1966a) 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* (138000) to those purified from *T. thioparus* (115000; Lyric & Suzuki, 1970b) and marine heterotroph strain 16B (132000; Whited & Tuttle, 1983). As with the *T. thioparus* enzyme (Lyric & Suzuki, 1970b, c), the *K* 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(1) fraction, Tables 1 and 3], as was also reported for the *T. thioparus* and *T. ferroxidans* enzymes (Silver & Lundgren, 1968; Lyric & Suzuki, 1970b). 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* 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, 1964a, b) or by dismutation to other polythionates (Okuzumi, 1965, 1966b). 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 tetrahionate by extracts of *T. thioparuse* 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. thioparuse* (Peck et al., 1965), although the enzyme from other strains of thiobacilli appeared less labile (Bowen et al., 1966; Lyric & Suzuki, 1970d). 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*; Hazeu 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


Enzymes of Thiobacillus tepidarius


