Properties and Role of Sulphite:cytochrome *c* Oxidoreductase Purified from *Thiobacillus versutus* (A2)

By WEI-PING LU† AND D. P. KELLY*

Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, UK

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Sulphite:cytochrome *c* oxidoreductase (sulphite dehydrogenase) was purified 2000-fold from *Thiobacillus versutus* (A2). The enzyme monomer had a molecular weight of 44 000 and a pI value of 4.5 ± 0.3 . Cytochrome c_{551} was intimately associated with the enzyme: separation of the two greatly decreased sulphite dehydrogenase activity, which was not restored by remixing them. The enzyme had a pH optimum around pH 8.0, exhibited a K_m of 14 μM for sulphite, and was inhibited noncompetitively by phosphate, with a K_i value of 12 mM. It was also inhibited by *p*-hydroxymercuribenzoate and cyanide. Its involvement in the oxidation of thiosulphate in *T. versutus* is discussed.

INTRODUCTION

We reported previously that the thiosulphate-oxidizing multi-enzyme system in *Thiobacillus versutus* consisted of five components (Lu & Kelly, 1983*d*, 1984), namely 'enzyme A', 'enzyme B', cytochrome c_{551} , cytochrome $c_{552.5}$ and sulphite:cytochrome *c* oxidoreductase. The system could effect the reduction of mammalian cytochrome *c* with thiosulphate and could oxidize thiosulphate completely to sulphate in the presence of an electron transfer chain to oxygen, such as mammalian cytochrome *c* and cytochrome oxidase or the membrane fraction from *T. versutus*. Sulphite:cytochrome *c* oxidoreductase was purified 660-fold (Lu & Kelly, 1983*d*) but the preparation still showed more than six bands after SDS-PAGE. The significance of the enzyme in the thiosulphate-oxidizing system was also still obscure (Lu & Kelly, 1983*d*).

The present paper describes the further purification and some properties of the sulphite:cytochrome *c* oxidoreductase as well as its role in the oxidation of thiosulphate. Some comparison is made with the enzyme reported from other thiobacilli.

METHODS

Enzyme nomenclature. Sulphite:cytochrome *c* oxidoreductase has been found in several thiobacilli but is commonly erroneously referred to as 'sulphite oxidase' (Kelly, 1982; Toghrol & Southerland, 1983). This trivial name would only be applicable to an enzyme coupling sulphite oxidation directly to oxygen consumption (International Union of Biochemistry, 1979). In the thiobacilli, including the present work on *T. versutus*, the enzyme is a sulphite:ferricytochrome oxidoreductase (EC 1.8.2.1, International Union of Biochemistry, 1979) and must therefore be referred to using the trivial name 'sulphite dehydrogenase'.

Culture of organism and the preparation and fractionation of crude extracts. *Thiobacillus versutus* (formerly *Thiobacillus* A2; Lu & Kelly, 1983*d*) was grown autotrophically at 30 °C in continuous chemostat culture (Lu & Kelly, 1983*b*) in a medium containing (g l⁻¹): Na₂S₂O₃ · 5H₂O, 50; MgSO₄ · 7H₂O, 0.1; NH₄Cl, 0.8; KH₂PO₄, 1.5; Na₂HPO₄ · 2H₂O, 7.9; trace metal solution, 10 ml. Cultures (5.2 l) were stirred at 1000 r.p.m. and gassed (320 ml min⁻¹) with 1% (v/v) CO₂ in air. Organisms were collected and harvested and cell-free extracts prepared as described previously (Lu & Kelly, 1983*a, b*). Purification of enzymes A and B and cytochromes c_{551} and $c_{552.5}$ is described elsewhere (Lu & Kelly, 1983*c, d*, 1984).

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

Abbreviation: DCPIP, 2,6-dichlorophenol-indophenol.

Sulphite:cytochrome c oxidoreductase. This was measured spectrophotometrically as described before (Lu & Kelly, 1983a). The reaction mixture contained, unless otherwise specified, cytochrome *c* (horse heart), 40 nmol; enzyme preparation, 0.5–50 µg protein; Na₂SO₃, 2 µmol in 5 mM-EDTA; Tris/HCl, pH 7.3, 40–45 µmol, in a final volume of 1 ml.

Thiosulphate:cytochrome c oxidoreductase. Enzyme activity and assay of sulphite or thiosulphate oxidation using an oxygen electrode were as described previously (Lu & Kelly, 1983d, 1984).

Biochemical procedures. SDS-PAGE (including molecular weight determination), non-denaturing disc-PAGE, preparative PAGE and preparative isoelectric focusing in Sephadex IEF were described previously (Lu & Kelly, 1983d, 1984).

Purification of sulphite dehydrogenase. As shown previously (Lu & Kelly, 1983b; see Table 1), fractionation of the crude extract with ammonium sulphate concentrated most of the sulphite-oxidizing and thiosulphate-oxidizing activities into the A44% to A65% fractions. The total sulphite-oxidizing activity recovered in the A65% fraction was even higher than in the crude extract. This could indicate removal of inhibitors of enzyme activity present in the crude extract. The A65% fraction was resolved into five major fractions by chromatography on DEAE-Sephadex CL-6B (Lu & Kelly, 1983b), of which the 0.35 M-NaCl (I) fraction contained sulphite dehydrogenase as well as enzyme B and cytochrome *c*₅₅₁. Sulphite dehydrogenase in the 0.35 M-NaCl (I) fraction was separated from the bulk of the cytochrome *c*₅₅₁ and enzyme B by gel filtration on Sephadex G-200 (Lu & Kelly, 1983d) or on Sephadex G-100 (Lu & Kelly, 1984). Several batches of sulphite dehydrogenase (about 400 mg) from the previous step were combined, subjected to gel filtration on a Sephadex G-100 column of 3.2 × 81 cm, and then eluted with 50 mM-Tris/HCl buffer, pH 7.3 at 4 °C using a flow rate of 30 ml h⁻¹ (Fig. 1a). Active fractions were pooled and concentrated by (NH₄)₂SO₄ precipitation. The resulting preparation was called the G-100 fraction. This fraction (about 200 mg) was then loaded on to a column (2.6 × 7 cm) of Phenyl-Sepharose CL-4B equilibrated at 4 °C with 50 mM-Tris/HCl buffer, pH 7.3, containing (NH₄)₂SO₄ at 10% of saturation and eluted with a linear gradient of decreasing (NH₄)₂SO₄ concentration, which was produced from two 150 ml volumes of 50 mM-Tris/HCl buffer, pH 7.3, one of which contained (NH₄)₂SO₄ at 10% of saturation. Fig. 1(b) shows the elution profile. The active fractions were combined and concentrated by ultrafiltration under N₂-pressure through an Amicon PM 10 membrane. This preparation was termed the HIC fraction. The purification is summarized in Table 1.

Separation of a fluorescent component from the HIC fraction. Enzyme (5 mg HIC fraction) was denatured by boiling for 5 min with 5% (w/v) SDS. The resulting solution was chromatographed on a column of Sephadex G-25 (0.5 × 30 cm; bed volume, 24 ml) using 55 mM-phosphate, pH 8.3, containing 0.1% SDS. The apoprotein eluted in the fractions between 13–15 ml and a fluorescent material appeared in the 21–24 ml fractions. This procedure was based on that of Johnson *et al.* (1980). Excitation and emission spectra were obtained using a Perkin-Elmer LS-3 luminescence spectrometer.

Protein. This was determined by the Lowry method using BSA as a standard.

RESULTS

Assessment of purity of enzyme

Sulphite dehydrogenase was purified about 2000-fold in terms of specific activity with a recovery of 39% (Table 1). The purified enzyme was almost free of enzyme B activity. The purified enzyme of the HIC fraction comprised only about 0.05% of the total crude extract protein. This preparation gave one major band (>90% of the total protein) with several minor bands on SDS-PAGE, but showed multiple bands on non-denaturing gel. The major band on the SDS gel had an *R_F* value nearly identical to that of cytochrome *c*₅₅₁. This apparent inhomogeneity was investigated by attempting further purification of the HIC fraction using preparative isoelectric focusing on Sephadex. After running for 6 h using Pharmalyte, pH 4–6.5, three to four protein bands appeared on the gel plate in the region of 2.5–4.5 cm from the anode. Only the fraction at 3.5–4.0 cm contained sulphite dehydrogenase activity. The same active band appeared at 15–16 cm from the anode after focusing on Pharmalyte, pH 2.5–5. However, both the specific and total activity of this preparation had dropped considerably, to about 7% and 1.2% of the initial values, respectively. Addition of purified cytochrome *c*₅₅₁ to the reaction mixture enhanced the specific activity, but only by about 10–15%. Nevertheless, the preparation showed one major band on both non-denaturing gel and SDS gel.

Isoelectric focusing for a shorter time gave better recovery in terms of both total and specific activity, but the preparation exhibited multiple bands on non-denaturing gel and SDS gel, although the major band had the same *R_F* as that of the purer preparation.

Fig. 1. Purification of sulphite dehydrogenase. For details see Methods and Results. (a) Elution pattern on Sephadex G-100 of combined active preparations from Sephadex G-200 and Sephadex G-100 columns. The void volume was 280 ml. Δ , Protein (A_{280}); $\square$, c -type cytochrome (A_{416}); $\bullet$, sulphite dehydrogenase; the horizontal bar represents the pooled active fractions. Enzyme B activity, eluting from 370 ml to 400 ml, is not shown. (b) Elution profile on Phenyl-Sepharose CL-4B of the G-100 fraction using 50 mM-Tris/HCl, pH 7.3, containing a decreasing linear gradient of $(\text{NH}_4)_2\text{SO}_4$ initially of 10% of saturation (dashed line). Δ , Protein (A_{280}); $\square$, c -type cytochrome (A_{416}); $\bullet$, sulphite dehydrogenase; the horizontal bar represents the pooled active fractions. Enzyme B activity eluting after the sulphite dehydrogenase is not shown.

Using sulphite dehydrogenase purified by isoelectric focusing as a marker, the enzyme in the HIC fraction was isolated from a preparative polyacrylamide gel electrophoretogram. The specific activity of this preparation was higher than the isoelectric focusing preparation, but was still only about 60% of that of the initial sample, and only 7% of the initial activity was recovered. The preparation showed one major band on non-denaturing gel and on SDS gel, with the same R_F as that of the first isoelectric focusing preparation.

The molecular weight of the purified sulphite dehydrogenase was 44000, determined by PAGE. The preparative isoelectric focusing indicated that the pI value was 4.5 (± 0.3).

Table 1. *Purification of sulphite dehydrogenase*

Purification step	Total protein (mg)	Specific activity [$\mu\text{mol cytochrome } c \text{ reduced min}^{-1} (\text{mg protein})^{-1}$]	Total activity ($\mu\text{mol cytochrome } c \text{ reduced min}^{-1}$)	Purification (fold)	Yield (%)
Crude extract	52700			1	100
First ammonium sulphate fraction (A65%)	15200	0.22	3344	3.5*	100*
DEAE-Sephacrose CL-6B [0.35M-NaCl(I) fraction]	1500	2.1	3213	33	96
Sephadex G-100 and Sephadex G-200	380	4.9	1862	78	56
Sephadex G-100 (G-100 fraction)	220	6.2	1364	99	41
Phenyl-Sephacrose CL-4B (HIC fraction)	10.3	128	1318	2036	39

* Derived from previous results using the oxygen electrode procedure since the crude extract showed a low activity of sulphite:cytochrome *c* oxidoreductase, due in part to the presence of cytochrome oxidase.

Role of cytochrome c_{551} in sulphite dehydrogenase activity

Cytochrome c_{551} exhibited an R_F value almost identical on SDS gel to that of the sulphite dehydrogenase in both HIC and further purified fractions, but they seemed not to be the same protein as the isoelectric focusing fraction gave a single band on a non-denaturing gel, while cytochrome c_{551} showed its typical pattern of multiple bands (Lu & Kelly, 1984). The same pattern of multiple bands did, however, appear in the HIC fraction on the non-denaturing gel, which indicated the presence of a substantial amount of cytochrome c_{551} in the fraction. This was also indicated by the red colour of the fraction. These observations strongly suggested that the cytochrome c_{551} was required for sulphite dehydrogenase activity and was possibly a major and integral part of the enzyme itself, as removal of the cytochrome from the preparation in preparative isoelectric focusing and PAGE resulted in a dramatic decrease in enzyme activity. Activity could not be restored by mixing samples of the purified sulphite dehydrogenase with purified cytochrome c_{551} or by remixing the fractions separated by isoelectric focusing or PAGE.

The aggregative property of the cytochrome c_{551} caused great difficulty in the purification of the cytochrome (Lu & Kelly, 1984), which might also have accounted for the problems encountered in the purification of sulphite dehydrogenase. The HIC fraction also appeared to contain a certain amount of enzyme B as judged by the R_F values on the SDS gel. Possibly enzyme B also has some role in the activity and structure of the sulphite dehydrogenase, similar to that of cytochrome c_{551} .

Some properties of sulphite dehydrogenase (HIC fraction, unless otherwise specified)

The pH optimum for sulphite dehydrogenase activity, measured spectrophotometrically in 50 mM-Tris buffer, was around pH 8. Activity was identical in 10 mM or 50 mM-Tris, pH 8.0, but was about 10% lower at pH 7.3 and 8.5. The absorption spectra of the oxidized and reduced sulphite dehydrogenase were like those of cytochrome c_{551} , with reduced α , β and γ bands at 551, 525 and 417 nm, respectively (Lu & Kelly, 1984). The 'sulphite oxidase' purified from *Thiobacillus novellus* by Toghrol & Southerland (1983) showed α , β and γ at 550, 522 and 415 nm, and appears from their spectra to contain about four times as much cytochrome or haem as the enzyme from *T. versutus*. The sulphite dehydrogenase also contained a fluorescent material with maxima at 285 and 375 nm (excitation spectrum) and 465 nm (emission spectrum). These spectra were at least superficially similar to those of sulphite 'oxidase' from other sources (Johnson *et al.*, 1980) including that of *T. novellus* (Toghrol & Southerland, 1983), which exhibited excitation maxima at about 280 and 375 nm and an emission maximum of 450 nm. The apoprotein of the sulphite dehydrogenase after removal of the fluorescent component (see Methods) did not exhibit significant fluorescence spectra. Using the same SDS extraction

Fig. 2. Effect of phosphate on sulphite dehydrogenase activity. (a) Double-reciprocal plot of velocity against sulphite concentration with variable amounts of sodium phosphate (pH 7.8). $\circ$, No phosphate; Δ , 5 mM-phosphate; $\square$, 10 mM-phosphate. (b) Reciprocal plot of velocity against phosphate concentration for sulphite at 0.01 mM ($\bullet$), 0.02 mM ($\circ$), 0.1 mM ($\blacksquare$), and 1.0 mM ($\square$). The tests were conducted as described in the Methods except that 40 μmol Tris/HCl (pH 8), 80 nmol horse-heart cytochrome *c* and 0.6 μg enzyme were used. Activity of sulphite dehydrogenase was measured as μmol cytochrome *c* reduced min^{-1} (mg protein^{-1}).

procedure, fluorescent materials were isolated from cytochrome c_{551} and enzyme B. Both showed the same emission spectrum as the material from the sulphite dehydrogenase and that from the cytochrome showed the same excitation spectrum. The apoproteins from cytochrome c_{551} and enzyme B also gave fluorescence spectra, with the same emission maximum (465 nm) as the fluorescent compounds but with different maxima for their excitation spectra (W-P. Lu and D. P. Kelly, unpublished results).

The sulphite dehydrogenase exhibited a high affinity for sulphite, showing an apparent K_m of 14 μM , calculated from a Lineweaver-Burk plot. From the same plot, a V_{max} of 192 μmol cytochrome *c* reduced min^{-1} (mg protein^{-1}) was obtained (Fig. 2a).

The activity of the purified sulphite dehydrogenase was inhibited about 95% by phosphate buffer (55 mM, pH 8.0) as found previously with the crude extract (Lu & Kelly, 1983a). Inhibition by phosphate was found to be noncompetitive (Fig. 2a) and the K_i , as determined from the intercept replot, was about 12 mM (Fig. 2b). Enzyme activity was also inhibited by *p*-hydroxymercuribenzoate and cyanide. Inhibition was dependent on the length of incubation with the inhibitors prior to addition of sulphite. Incubation for 1, 5 and 15 min with *p*-hydroxymercuribenzoate (1 mM) gave 30%, 40% and 75% inhibition, and incubation for 0.1, 2, 6 and 10 min with KCN (2 mM) showed 15%, 50%, 70% and 100% inhibition, respectively. KCN

Table 2. *Oxidation of sulphite by the purified dehydrogenase in the presence of electron carriers*

The activity was measured in the oxygen electrode as described in Methods using the quantities of the components indicated. Membrane particles were prepared as described before (Lu & Kelly, 1983a). Greater amounts of the membrane particles or horse-heart cytochrome *c* produced no further effect on activity.

Composition of reaction mixture (mg protein)				Rate of oxidation [$\mu\text{mol O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$]
Sulphite dehydrogenase*	Horse-heart cytochrome <i>c</i>	Bovine-heart cytochrome oxidase	<i>Thiobacillus</i> membrane particles	
0.1	—	—	0.2	0
0.1	0.5	—	0.2	0.78
0.1	0.5	0.05	—	0.052
0.005	0.5	—	0.2	8.27

—, Component omitted.

* The G-100 fraction was used except in the last case, where the HIC fraction was used.

(4 mM) gave 35% and 100% inhibition after incubation for 0.1 and 4 min. Incubation for 15 min in the presence of both *p*-hydroxymercuribenzoate (1 mM) and glutathione (1 mM) gave 51% inhibition.

Table 2 shows the oxidation of sulphite by the purified sulphite dehydrogenase in the presence of electron chain systems coupling to oxygen uptake. Not only membrane particles or cytochrome oxidase but also *c*-type cytochrome were necessary for this enzyme activity. The membrane particles from *T. versutus* were more effective than bovine-heart cytochrome oxidase. In these conditions, sulphite was completely oxidized to sulphate by the sulphite dehydrogenase showing an $\text{O}_2/\text{SO}_3^{2-}$ ratio of 0.5, consistent with the equation:

The purified sulphite dehydrogenase was very stable: about 20% of the activity was lost on storage at 4 °C for one month, about 35% was lost after one week at 20 °C and none was lost after several months at -20 °C.

Involvement of sulphite dehydrogenase in the oxidation of thiosulphate

Earlier work (Lu & Kelly, 1983d) demonstrated that complete oxidation of thiosulphate to sulphate could be catalysed by a four-component multi-enzyme system lacking added sulphite dehydrogenase. The role of the dehydrogenase was uncertain, as thiosulphate-oxidizing activity was stimulated by 30–40% in enzyme B-limited assays by the addition of small amounts of the HIC (I) fraction (containing sulphite dehydrogenase; Lu & Kelly, 1983d). This has now been confirmed more comprehensively: thiosulphate-oxidizing activity was higher in the multi-enzyme system containing sulphite dehydrogenase or supplemented with the enzyme than that without the enzyme (Table 3). Generally the sulphite dehydrogenase increased the specific activity of the systems about 20–25%. The greater stimulation reported earlier presumably reflected the low concentration of enzyme B employed and further indicates a functional connection between enzyme B and sulphite dehydrogenase.

It was further demonstrated that when the rate of thiosulphate oxidation declined (after 20–25 min; Table 3, experiments 6 and 7), the addition of sulphite dehydrogenase restored the rate at least to that observed initially.

The subsequent decrease in oxidation rate (experiment 7) obviously resulted from loss of activity of components during long-term incubation. Table 3 also shows that membrane particles from *T. versutus* were more effective as an electron transfer system than mammalian cytochrome oxidase.

Although the four components (enzyme A, enzyme B, cytochrome c_{551} and cytochrome $c_{552.5}$) free of sulphite dehydrogenase were not able to reduce horse-heart cytochrome *c* with sulphite, they did show a capacity to oxidize sulphite in the oxygen electrode (Table 3). Moreover, the oxidation of thiosulphate to sulphate by the multi-enzyme system free of sulphite dehydrogenase

Table 3. *The effect of added sulphite dehydrogenase on the oxidation of thiosulphate by the reconstituted multi-enzyme system*

The experiments were done in an oxygen electrode cell essentially as stated in Methods, except that various amounts of the components were used as indicated. The activity was calculated in terms of total protein added as the four components. The components in the different sets came from several separate stocks of organisms and purification batches, so the absolute activities varied among experiments, for example, as in 5 and 7.

Experiment no.	Reaction mixture components (mg protein)						Supplementary addition of sulphite : cytochrome <i>c</i> oxidoreductase	Rate of oxidation [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]
	Enzyme A	Enzyme B	Cytochrome <i>c</i> _{552.5}	Cytochrome <i>c</i> ₅₅₁	Horse-heart cytochrome <i>c</i>	Cytochrome oxidase (<i>aa</i> ₃)		
1	0.22	0.15	0.07	0.2	1	1	—	3
2	0.22	0.15	0.07	0.2	1	1	—	12
3	0.22	0.15	0.07	0.25†	1	1	—	18
4	0.2	0.1	0.07	0.2	1	—	—	4
5	0.2	0.1	0.07	0.2	1	—	—	53 (Initial) 68 (After addition)
6	0.4	0.2	0.09	0.3	1	1	3 µg	15 (Initial) 8 (After 30 min)
7	0.22	0.1	0.07	0.28	0.5	—	3 µg	17 (After 10 min) 23 (Initial) 7 (After 30 min) 18 (After addition)

* The membrane particles were prepared from *Thiobacillus A2* as described before (Lu & Kelly, 1983*a*).

† The cytochrome *c*₅₅₁ preparation (G-100 fraction) contained sulphite dehydrogenase.

was complete, with an $\text{O}_2/\text{S}_2\text{O}_3^{2-}$ ratio of 2.0, as reported before (Lu & Kelly, 1984). The explanations for these observations might be (a) free sulphite or sulphonate-sulphur were slowly oxidized chemically in the experimental conditions in the oxygen electrode, although the reaction mixture containing horse-heart cytochrome *c*, cytochrome oxidase or the membrane particles showed no sulphite-oxidizing activity at all; and (b) sulphonate-sulphur was oxidized by the thiosulphate-oxidizing multi-enzyme system without appearing in the system as free sulphite.

Demonstration that free sulphite was not formed during the oxidation of thiosulphate by the multi-enzyme system

2,6-Dichlorophenol-indophenol (DCPIP) was spontaneously reduced by sulphite: addition of 50 nmol Na_2SO_3 to a solution (1 ml) of 60 nmol DCPIP in 50 mM-Tris/HCl buffer, pH 7.3, exhibited an initial rate of 30 nmol DCPIP reduced min^{-1} (ΔA_{605} at 30 °C). However, there was no DCPIP reduction by the multi-enzyme system containing enzyme A, enzyme B, cytochrome c_{551} , cytochrome $c_{552.5}$ and sulphite dehydrogenase (either with or without a small amount of horse-heart cytochrome *c*) supplemented with $\text{Na}_2\text{S}_2\text{O}_3$ instead of Na_2SO_3 under the same experimental conditions. The same reaction mixture showed a rapid reduction of horse-heart cytochrome *c* by $\text{Na}_2\text{S}_2\text{O}_3$. This result provides direct evidence that no free sulphite was released during the oxidation of thiosulphate by the multi-enzyme system. It also revealed that the multi-enzyme system could not use DCPIP as an electron acceptor as an alternative to horse-heart cytochrome *c* though the dye had a E'_0 of 0.193 V.

DISCUSSION

AMP-independent and soluble sulphite 'oxidases' have been found and partially purified from several thiobacilli (Aminuddin & Nicholas, 1974; Yamanaka *et al.*, 1981). Before 1983 the highest purification achieved was 163-fold and none of the preparations was claimed to be highly purified. It is, therefore, difficult to make a proper comparison with the present study in which a 2000-fold purification was achieved. However, the sulphite dehydrogenase of *T. novellus* (Yamanaka *et al.* 1981) did show an interesting similarity to its counterpart in *T. versutus* in that the enzyme was tightly associated with cytochrome c_{551} and the separation of the two resulted in complete loss of the enzyme activity. Furthermore the great difficulty encountered generally in attempts to purify sulphite dehydrogenase reflect a resemblance to the enzyme in the thiosulphate-oxidizing system from *T. versutus*. Recently Toghrol & Southerland (1983) have reported a 206-fold purification to homogeneity of the enzyme from *T. novellus*. They provided evidence that the enzyme is a molybdohaemoprotein, in contrast to an earlier report that no haem was present (Charles & Suzuki, 1966), but did not indicate whether the haem might have been a *c*-type cytochrome that could be separated from the enzyme. A comparison of protein patterns for the purified *T. novellus* and *T. versutus* enzymes by non-denaturing PAGE would be of considerable interest. It is noteworthy that the results of Toghrol & Southerland (1983) indicated that sulphite dehydrogenase constituted about 0.45% of the crude extract protein of *T. novellus*: a value some 10 times greater than in *T. versutus*. This discrepancy could reflect real differences in concentration in the two bacteria or could result because of under-estimation of enzyme originally present in the crude extract (in line with our demonstration of 'unmasking' of activity in the A65% fraction). It could also reflect a greater concentration of cytochrome c_{551} being co-purified with the sulphite dehydrogenase.

There are also other similarities between the sulphite dehydrogenase of *T. versutus*, *T. novellus* and *T. thioparus* (Charles & Suzuki, 1966; Lyric & Suzuki, 1970). The M_r of our preparation (44000) is very similar to that reported (41000) for the homogeneous preparation from *T. novellus* (Toghrol & Southerland, 1983). The enzymes from the three organisms showed a high affinity for sulphite, with K_m values of 14 μM , 20–40 μM and 40 μM , respectively. All of them were inhibited by phosphate and the K_i for *T. thioparus* (10 μM) was similar to that for *T. versutus*, although the latter was non-competitive and the former, uncompetitive. The pH optimum was around pH 8 for the three enzymes. They were also sensitive to sulphydryl inhibitors and could couple to either mammalian cytochrome *c* or ferricyanide as electron acceptors.

Even following the high degree of purification and characterization achieved, it is still difficult to describe precisely how the enzyme functions *in vivo*. In contrast to the other four major components of the thiosulphate-oxidizing multi-enzyme system (enzymes A and B, cytochromes c_{551} and $c_{552.5}$), each of which comprises 1–2% of the crude extract protein, sulphite dehydrogenase is less than 0.05% of the protein and its extraordinarily close association with cytochrome c_{551} may indicate the sulphite dehydrogenase protein to be integrated *in vivo* into the cytochrome itself. This would imply that sulphite dehydrogenase was an integral part of the complex, being closely associated with enzyme B and cytochrome c_{551} , rather than functioning as a discrete enzyme. Possible further evidence for close association of sulphite dehydrogenase, cytochrome c_{551} and enzyme B was the observation that all three seemed to contain a similar fluorescent component of unknown function. This may well be the molybdenum cofactor present in other sulphite 'oxidase' systems (Toghrol & Southerland, 1983; Southerland & Toghrol, 1983). The *in vivo* function, and true substrate, of the enzyme are thus in question, especially as free sulphite seemed not to be an intermediate in the conversion of thiosulphate to sulphate by the multi-enzyme complex. Sulphite oxidation might be a gratuitous function of the isolated enzyme, which in the complex *in vivo* could oxidize enzyme-bound intermediates analogous to sulphite or sulphonate residues. This probability goes some way to explaining the importance of sulphite dehydrogenase and its stimulation of thiosulphate oxidation in the multi-enzyme system. The process of thiosulphate oxidation would thus seem to depend on the sequential oxidation of sulphane-sulphur to the sulphonate level, and retention of the sulphonate moiety of thiosulphate in this form, all by means of multi-enzyme-complex-bound intermediates. The sulphonate-sulphur complex is indicated by the data not to be in equilibrium with free sulphite, which is consequently not a substrate for the multi-enzyme complex in the absence of 'free' sulphite dehydrogenase. Although the mechanism is still uncertain, and obviously depends on understanding the general mechanism of oxidation of thiosulphate by the whole multi-enzyme system, the evidence so far suggests that there are no individually separable steps in the oxidation of thiosulphate in the sense that free intermediates seem not to exist and the individual functions of the enzymes and cytochromes are at present impossible to define. The situation might be similar to the reduction of nitrogen by the nitrogenase system, where no free partially-reduced intermediates are found and the only one detected was an enzyme-bound dinitrogen hydride which could be cleaved from the enzyme by acid treatment to yield hydrazine. Free hydrazine was, however, a very poor substrate for the nitrogenase (Postgate, 1982) in contrast to the *Thiobacillus* system in which the presence of sulphite dehydrogenase with high activity towards free sulphite occurs.

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