Purification and Some Properties of Two Principal Enzymes of the Thiosulphate-oxidizing Multi-enzyme System from *Thiobacillus* A2

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(Received 5 May 1983; revised 9 August 1983)

A soluble thiosulphate-oxidizing multi-enzyme system, precipitated from a crude cell extract of *Thiobacillus* A2 with ammonium sulphate, has been resolved into four essential components by DEAE-Sepharose chromatography, gel filtration of Sephadex G-100 and G-200, hydrophobic interaction chromatography on phenyl-Sepharose and preparative isoelectric focusing. Oxidation of thiosulphate to sulphate coupled to the reduction of horse-heart cytochrome *c* as electron acceptor was catalysed by two colourless proteins (enzyme A: *M*, 16000; and enzyme B: *M*, 64000), cytochrome *c*$_{552}$ (*M*, 32000) and 'cytochrome *c*$_{554}$' (*M*, 300000). Enzymes A and B were purified 110- and 280-fold, respectively. Sulphite : cytochrome *c* oxidoreductase was also purified 660-fold. The mechanism of action of the system is discussed.

INTRODUCTION

The preparation of a cell extract from *Thiobacillus* A2, capable of the complete oxidation of thiosulphate to sulphate with the consumption of two mol oxygen for each thiosulphate oxidized, was described previously (Lu & Kelly, 1983 *a*). Complete oxidation of thiosulphate required both a membrane system and a soluble fraction. The soluble fraction coupling thiosulphate oxidation to cytochrome *c* reduction was separated into three major components (Lu & Kelly, 1983 *b*). The earlier work indicated the thiosulphate : cytochrome *c* oxidoreduction process to be effected by a soluble multi-enzyme system. Subsequently, the rhodanese present in the system was shown not to be required for thiosulphate oxidation (Lu & Kelly, 1983 c). Further analysis of this multi-enzyme system was undertaken with an initial objective of establishing the nature of the thiosulphate-cleaving enzyme, believed from earlier work to be a primary step in thiosulphate oxidation, (Suzuki, 1974; Kelly, 1982) and to seek the presence of a 'sulphane-sulphur oxidase' in addition to the sulphite oxidase already demonstrated (Lu & Kelly, 1983 b).

The present paper describes the purification and some properties of two principal enzymes from the enzyme system and the reconstitution of the thiosulphate-oxidizing activity with the two enzymes, two partially purified c-type cytochromes, mammalian cytochrome *c* and cytochrome oxidase. The involvement of thiosulphate cleavage and of sulphite oxidase in thiosulphate oxidation is discussed.

METHODS

Organisms and chemostat cultures. *Thiobacillus* A2, which has recently been designated as a new species, *Thiobacillus versutus* (Harrison, 1983), was grown in continuous culture as described previously (Lu & Kelly, 1983 a, b). Cell suspensions (80-100 mg dry wt ml$^{-1}$) collected and concentrated from the culture were stored at $-70$ °C. This storage had no effect on the thiosulphate-oxidizing activity of crude extracts prepared from the frozen cells.

Preparation of cell extract and A65% fraction, and resolution of the A65% fraction into three main fractions involved in thiosulphate oxidation. These procedures were as described previously (Lu & Kelly, 1983 b) and are summarized in Fig. 1. The c-type cytochromes in the 0-35 M-NaCl(II) and (II) fractions were found to have z bands at 551 and 552-5 nm, respectively, rather than at 552 nm, as was previously thought (Lu & Kelly, 1983 b). The previous observation was probably due to incomplete separation of the two cytochromes from each other.
Fig. 1. Fractionation of the A65% ammonium sulphate fraction into constituent enzymes and cytochromes.
Thiosulphate oxidation by Thiobacillus A2

Resolution of the A65% fraction. Enzymes A and B, sulphite:cytochrome c oxidoreductase and cytochrome c551, were resolved as summarized in Fig. 1.

Enzyme assays. Thiosulphate:cytochrome c oxidoreductase activity could only be observed using mixtures of the main fractions or of the further purified enzymes (Fig. 1; Lu & Kelly, 1983b,c). The 0-1 M-NaCl and 0-35 M-NaCl(I) fractions contained activities we shall refer to, respectively, as 'enzyme A' and 'enzyme B'. Both were required for thiosulphate-oxidizing activity. The 0-35 M-NaCl(I) fraction was resolved into two major components, one being enzyme B, the other being cytochrome c551, both of which were required for full activity.

Enzyme A activity was measured as described by Lu & Kelly (1983c), except that a smaller amount of enzyme A solution (0-01-0-1 mg) was used at the later stages of the purification. Enzyme B was assayed by essentially the same procedure as for enzyme A. The reaction mixture (1 ml in a 1 cm light-path cuvette) contained (μmol): Na2S2O3, 2; Tris buffer, pH 7-3, 45; cytochrome c (horse-heart type II), 0-07; A65% fraction (0-3 mg protein, as enzyme A); 0-35 M-NaCl(II) fraction (0-6 mg protein); G-200-I fraction (0-4 mg protein, containing cytochrome c551; see Results) and enzyme B solution (0-01-0.08 mg protein). Reaction at 30 °C was initiated by adding the enzyme B solution. Activity was expressed as cytochrome c reduction in terms of the protein added as enzyme B. The activities of enzyme A and enzyme B measured and calculated in this way are relative, as the amounts of enzyme B (for assay of enzyme A), enzyme A (for assay of enzyme B), cytochrome c551 and cytochrome c552.5 used in the assay will affect the activity. In order to obtain a linear relationship between amount of enzyme A assayed and the activity, a ten times or greater excess of enzyme B (in terms of protein) was used. Similarly, enzyme A was used in excess to assay enzyme B. Sulphite:cytochrome c oxidoreductase activity was measured as described before (Lu & Kelly, 1983a), except that less protein (4-50 μg) was used.

Purification of Enzyme A. Protein precipitated from the combined 0-1 M- and 0-12 M-NaCl fractions by precipitation between 60-90% saturation with ammonium sulphate was recovered as described previously and referred to as the A90% fraction (Lu & Kelly, 1983c). This was stored at −20 °C. The A90% fraction (420 mg) was thawed and applied to the top of a 2-6 cm × 6-5 cm column of phenyl-Sepharose CL-4B equilibrated with 18 mM-phosphate buffer, pH 6-5 containing 2 mM-Na2S2O3 and (NH4)2SO4 at 17% saturation. After sample addition, elution was continued with one bed volume of equilibrating buffer, followed by a linear gradient of decreasing ammonium sulphate concentration and increasing ethylene glycol concentration, which was produced by constant-head mixing of 250 ml each of (NH4)2SO4 (17% saturation) and 50% (v/v) ethylene glycol, both in 18 mM-phosphate buffer, pH 6-5 containing 2 mM-Na2S2O3 at 4 °C and a flow rate of 30 ml h−1. The typical elution pattern is shown in Fig. 2. The active fractions were combined and then concentrated at 4 °C in a 50 ml Amicon ultra-filtration cell over an Amicon PM10 membrane under nitrogen at 0-7 bar (7 × 104 N m−2). In the presence of ethylene glycol about 30% of the enzyme passed through the membrane in the filtrate. The ultrafiltration had to be repeated three times to obtain 95% of the enzyme.

Gel filtration on Sephadex G-100. The concentrated enzyme A solution (120 mg) was loaded on the bottom of a 2-6 cm × 83 cm column of Sephadex G-100 equilibrated with 50 mM-Tris buffer, pH 7-3, containing 2 mM-Na2S2O3, and eluted upwards with the same buffer at 4 °C and a flow rate of 16 ml h−1. The elution pattern was as

Fig. 2. Elution pattern of the A90% fraction on phenyl-Sepharose CL-4B. For details see Methods.

○, Protein (A200); ○, enzyme A activity; ---- ammonium sulphate and ethylene glycol gradient.
Fig. 3. Elution pattern on Sephadex G-100 of the active fraction from phenyl-Sepharose CL-4B chromatography. For details see Methods. ●, Protein (A280); ○, enzyme A activity. The void volume was 160 ml.

seen in Fig. 3. The active fractions were pooled and concentrated by (NH₄)₂SO₄ as described before (Lu & Kelly, 1983c).

Preparative isoelectric focusing. The concentrated enzyme A solution (10–15 mg protein) was dialysed against 1% (w/v) glycine for 3 h at 4 °C and then loaded on to a flat bed Sephadex IEF gel containing 1/15 (v/v) Pharmalyte, pH 2.5–5. Preparation of gel and gel bed (115 × 240 × 2 mm), application of sample, focusing condition and recovery of separated proteins were essentially the same as described in the instruction manual from Pharmacia. An LKB Multiphor 2117 and constant power supply 2197 were used. Cold water (0 °C) was circulated through the cooling plate during focusing. A quick paper print technique for detection of focused proteins as described in the LKB manual was employed. A typical paper print is shown in Fig. 4(a). Recovered enzyme A was desalted by dialysis or gel filtration on Sephadex G-25, followed by dialysis against solid polyethylene glycol to concentrate the sample.

Possible modifications of the purification procedure. Thiosulphate was included in the elution buffers as an enzyme stabilizing agent, because purified enzyme A was subsequently found to be unstable in the absence of thiosulphate. The ultrafiltration steps could use membranes with lower molecular weight cut-off levels (e.g. YM5 instead of PM10) in order to avoid passage of the enzyme in the presence of ethylene glycol.

Purification of enzyme B. Resolution of 0-35 M-NaCl(I) fraction into two major fractions containing enzyme B and cytochrome c₅₅₁. The 0-35 M-NaCl(I) fraction (500 mg protein) was applied to the bottom of a column (3.2 × 85.5 cm) of Sephadex G-200 equilibrated with 50 mM-Tris buffer, pH 7.3 and eluted downwards with same buffer at 4 °C and a flow rate of 30 ml h⁻¹. Active fractions were pooled and then concentrated by ultrafiltration under nitrogen pressure (0.7 bar) through an Amicon PM10 membrane at 4 °C.

Hydrophobic interaction chromatography. Concentrated enzyme B solution (60 mg) was brought to about 15% saturation with (NH₄)₂SO₄, loaded on to the top of a 2·6 × 6·5 cm column of phenyl-Sepharose CL-4B equilibrated with 50 mM-Tris buffer, pH 6·5, containing 10% saturation with (NH₄)₂SO₄. After sample application, the column was eluted downwards with one bed volume of the same buffer at 4 °C and a flow rate of 30 ml h⁻¹, followed by a linear gradient of decreasing ammonium sulphate concentration, which was produced from two 150 ml volumes of 50 mM-Tris buffer, pH 7·3, one of which contained (NH₄)₂SO₄ (10% saturation). The active fractions were combined and concentrated by ultrafiltration as stated above.

Preparative isoelectric focusing. The procedure was the same as described for purification of enzyme A (Fig. 4b).

Assay of stoicheiometry of thiosulphate oxidation by the reconstituted system. This was done in a Clark oxygen electrode cell essentially as described by Lu & Kelly (1983a). Reaction mixture (final volume 1 ml) contained: purified enzyme A (0–15 mg), pure enzyme B (0–2 mg), cytochrome c₅₅₁ fraction (G-200-I fraction, 0–5 mg), cytochrome c₃₅₂₋₃ (0-35 M-NaCl(II) fraction, 0-5 mg), horse-heart cytochrome c (2 mg), bovine-heart cytochrome oxidase (5 units), Tris buffer, pH 7·3 (40 μmol) and Na₂S₂O₃ (50 or 100 nmol precisely). Oxygen concentration in the experimental conditions was calibrated using the method described by Robinson & Cooper (1970).

PAGE. SDS-PAGE was carried out to monitor the protein purification and to determine molecular weight using the method of Laemmli ((1970). Acrylamide (12% w/v) was used in the resolving gel. Protein samples were incubated at 60 °C for 10 min in Tris buffer, pH 6·5, containing 3% (v/v) 2-mercaptoethanol and 1% (w/v) SDS before loading and were electrophoresed at a constant 40 mA for 5 to 6 h at 4 °C. The gels were stained overnight in a mixture of 30% (v/v) methanol, 5% (v/v) acetic acid in distilled water containing 0·2% (w/v) Coomassie brilliant blue G and destained in the same solution without the dye. Standard marker proteins (M, in parentheses)
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Fig. 4. Paper prints of flat-bed isoelectric focusing gels of purified enzymes A and B. (a) Isoelectric focusing of active material (enzyme A) from Sephadex G-100 chromatography (see Methods). Sample (12 mg protein) was loaded and focused for 6 h at 12 W, constant power, 1500 V maximum. The gel was pre-run for 1 h. (b) Isoelectric focusing of the enzyme B fraction (HIC-I1) from phenyl-Sepharose CL-4B chromatography (see Methods and Results). Sample (15 mg protein) was loaded and focused for 6 h at 30 W, constant power, 2000 V maximum. The gel was pre-run for 1 h.

Purity of the samples was also examined by discontinuous PAGE under nondenaturing conditions essentially as described by Davis (1964). Acrylamide (8%, w/v) was used in the resolving gel. The electrophoresis was performed at a constant 30 mA for 4 h at 4 °C. The staining procedure was the same as for the SDS-gel.

**Determination of the molecular weight of enzyme B by gel filtration.** A method based on Andrews (1965) was used with bovine serum albumin (mol. wt 67000), egg albumin (mol. wt 43000), bovine pancreas chymotrypsinogen A (mol. wt 25000) and bovine ribonuclease A (mol. wt 13700) as marker proteins. Enzyme B (4 mg protein) and marker proteins (5 mg protein each) were run separately on a 2.6 cm × 84 cm column of Sephadex G-100 with 50 mM-Tris buffer, pH 7.3 at 4 °C. The positions of the marker proteins and enzyme B were determined by measuring absorbance at 280 nm and enzyme activity.
Determination of isoelectric point. The isoelectric points of the purified proteins were measured by flat bed electro-focusing in polyacrylamide gels using Pharmalyte, pH 2.5-5 and an LKB Multiphor 2 11 17 and constant power supply 2197. Preparation of gel (100 x 50 x 1 mm), application of samples, running conditions and staining of gel were based on the instruction for pl calibration kits from Pharmacia. The pH gradient profile across the IEF gel was calibrated by using a low pl calibration kit.

Reaction of purified enzymes with $^{35}$S$_2$O$_3^{2-}$. Reaction mixtures (0.5 ml) in Tris/HCl, pH 7.3, contained either Na$_2$S$_2$O$_3$ or Na$_2$S-$^{35}$SO$_3$ (2 µmol at 2-10 x $10^6$ d.p.m. µmol$^{-1}$) and other components as indicated in Results. After incubation at 30°C, samples were analysed by paper chromatography using a butanol/pyridine/acetic acid/water (20:30:6:24, by vol.) solvent with and without treatment with iodine to convert residual thiosulphate to tetrathionate (Kelly & Syrett, 1966). Chromatograms were assayed for $^{35}$S by cutting strips into segments and counting in scintillation vials filled with 0.5% (w/v) butyl PBD in toluene.

Protein estimation. Protein was determined by the Lowry method using bovine serum albumin as a standard.

Chemicals. Cytochrome c (horse heart III), cytochrome oxidase (bovine heart), catalase (bovine liver), egg albumin, chymotrypsinogen A, ribonuclease A, molecular weight marker kits (MW-SDS-70L), NADH were obtained from Sigma. Sephadex G-25, G-100, G-200, phenyl-Sepharose CL-4B, Sephadex IEF, Pharmalyte (pH 2-5-5) and low pl calibration kits were obtained from Pharmacia.

RESULTS

Purification of enzyme A

Enzyme A was purified some 100-fold by the procedures described (Fig. 1; Table 1). The progress of the separation, monitored by SDS-PAGE is shown in Fig. 5.

Purity and some properties of enzyme A

As shown in Fig. 5 enzyme A, after the final purification, still appeared as one major band with a molecular weight of 16200 and another minor band of 14600. The minor protein was about 15% of the major protein as measured by scanning the gel at 600 nm. The two bands had similar densities in the crude extract. On the basis of the 100-fold increase in specific activity and its concentration, the major band in the purified preparation is presumed to be enzyme A. The enzyme comprised about 0.8% (w/w) of the crude extract. Because both proteins had very similar molecular weights, nearly the same pl values, determined by isoelectric focusing in polyacrylamide gel, and more or less the same hydrophobic properties, it is very difficult to separate them by the techniques used so far. The isoelectric point of enzyme A is about 4.2. The enzyme lost about 50% and 80% of its activity at 20°C after 6 h and 20 h, respectively, and 30% and 50% at 4°C after 1 d and 2 d, respectively.

Purification of enzyme B

The 0.35 M-NaCl(1) fraction from DEAE-Sepharose-CL-6B chromatography was separated into two major protein peaks by gel filtration on Sephadex G-200 (Fig. 6). Three fractions were collected: fraction I (called G-200-I) contained cytochrome c$_{551}$; fraction II (G-200-II), contained some sulphite:cytochrome c oxidoreductase; and fraction III (G-200-III) contained enzyme B and most of the sulphite:cytochrome c oxidoreductase activity (Table 2).

Enzyme B and sulphite:cytochrome c oxidoreductase in the G-200-III fraction were further separated into two fractions by phenyl-Sepharose CL-4B chromatography (Fig. 7 and Table 3). Most sulphite:cytochrome c oxidoreductase activity was recovered in the first fraction (called HIC-I). Most of the enzyme B activity was in the second fraction (called HIC-II), but the specific activity was not increased and the total activity recovered (yield) was low. The specific activity of enzyme B was, however, increased about 40% by including a small amount of the HIC-I fraction, to give a ratio in the reaction mixture of HIC-I: HIC-II protein equivalent to that in the separated fractions. Yield was also restored to about 95% of that originally present.

Enzyme B in the HIC-II fraction was finally purified by preparative isoelectric focusing in Sephadex IEF. Two major protein bands were obtained on a paper print of the gel (Fig. 4b). Both of them contained enzyme B activity (Table 4). The SDS-PAGE disclosed that they were the same protein, although the protein from the bottom band had less enzyme activity and one or
Fig. 5. SDS-polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme A. (1) Marker proteins (55 µg); (2) crude extract (60 µg); (3) A65% fraction (60 µg); (4) 0.1 M and 0.12 M fraction (60 µg); (5) A90% fraction (60 µg); (6) pooled active fractions (30 µg) from phenyl-Sepharose CL-4B column; (7) pooled active fractions (30 µg) from Sephadex G-100; (8, 9) purified enzyme A after preparative isoelectric focusing (30 and 20 µg, respectively); (10) marker proteins (45 µg). For details see Methods and Results. Molecular weights of markers and direction of migration are indicated.

Table 1. Summary of the purification of enzyme A

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific activity [µmol cytochrome c reduced min⁻¹ (mg protein)⁻¹]</th>
<th>Total activity [µmol cytochrome c reduced min⁻¹]</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>545</td>
<td>49100</td>
<td>—</td>
<td>—</td>
<td>1*</td>
<td>100*</td>
</tr>
<tr>
<td>First ammonium sulphate fractionation (A65%)</td>
<td>251</td>
<td>12000</td>
<td>—</td>
<td>—</td>
<td>4*</td>
<td>91*</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>74</td>
<td>2192</td>
<td>0.25</td>
<td>543</td>
<td>18</td>
<td>74</td>
</tr>
<tr>
<td>Second ammonium sulphate fractionation (A90%)</td>
<td>34</td>
<td>946</td>
<td>0.54</td>
<td>511</td>
<td>39</td>
<td>70</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B</td>
<td>16</td>
<td>409</td>
<td>1.16</td>
<td>474</td>
<td>80</td>
<td>65</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>14</td>
<td>302</td>
<td>1.5</td>
<td>453</td>
<td>110</td>
<td>62</td>
</tr>
<tr>
<td>Isoelectric focusing in Sephadex IEF</td>
<td>—</td>
<td>140</td>
<td>1.4</td>
<td>196</td>
<td>102</td>
<td>27</td>
</tr>
</tbody>
</table>

* These figures were derived from results measured by the oxygen electrode method (Lu & Kelly, 1983b).
Fig. 6. Elution profile of the 0.35 M-NaCl(I) fraction on Sephadex G-200 (see text for detail). ∙, Protein ($A_{280}$); ○, cytochrome $c_{551}$ ($A_{416}$); □, enzyme B activity; △, sulphite:cytochrome $c$ oxidoreductase. The void volume was 265 ml.

Table 2. Resolution of the 0.35 M-NaCl(I) fraction from DEAE-Sepharose CL-6B chromatography into two major components by gel filtration on Sephadex G-200 (see Fig. 6)

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein (mg)</th>
<th>Cytochrome $c_{551}$ [nmol (mg protein)$^{-1}$]</th>
<th>Specific (μmol cytochrome $c$ min$^{-1}$ (mg protein)$^{-1}$)</th>
<th>Total (μmol cytochrome $c$ min$^{-1}$)</th>
<th>Specific (μmol cytochrome $c$ min$^{-1}$ (mg protein)$^{-1}$)</th>
<th>Total (μmol cytochrome $c$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample [0.35 M-NaCl(I)]</td>
<td>500</td>
<td>4</td>
<td>0.3</td>
<td>150</td>
<td>0.86</td>
<td>428</td>
</tr>
<tr>
<td>Fraction I</td>
<td>134</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>Fraction II</td>
<td>50</td>
<td>0.5</td>
<td>0.03</td>
<td>1.6</td>
<td>0.56</td>
<td>28</td>
</tr>
<tr>
<td>Fraction III</td>
<td>103</td>
<td>0</td>
<td>1.43</td>
<td>147</td>
<td>2.97</td>
<td>308</td>
</tr>
</tbody>
</table>

* Enzyme B and sulphite:cytochrome $c$ oxidoreductase activities were measured as described in Methods. Omitting Fraction I (i.e. cytochrome $c_{551}$) from the reaction mixture reduced the specific activity by half.

two minor contaminants. Enzyme B accounted for at least 90% of the total protein in the HIC-II fraction and the double-banding observed was probably due merely to overloading.

The purification of enzyme B is summarized in Table 4 and Fig. 1. The final product represents some 280-fold purification over the crude extract with an overall recovery of about 50%. The enzyme comprised about 0.6% (w/w) of the crude extract protein.

Purity and molecular weight of enzyme B

The purified enzyme appeared as a single sharp band after SDS-PAGE and discontinuous PAGE (Fig. 8a and b, respectively). However, the enzyme band shifted from the position equivalent to a molecular weight of 63000 (±2000) to that of half this molecular weight on the SDS polyacrylamide gel, indicating the enzyme to consist of two subunits with a molecular weight of 32000 (±2000). Determination of molecular weight on Sephadex G-100 also confirmed that the native enzyme possessed a molecular weight of about 64000. Gel
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![Graph](image_url)

Fig. 7. Elution profile of the further resolution of the G-200-III fraction on phenyl-Sepharose CL-4B (see Fig. 6 and text for detail). ●, Protein $A_{280}$; ○, enzyme B; ▲, sulphite:cytochrome c oxidoreductase; ---, ammonium sulphate concentration gradient.

Table 3. Separation of Enzyme B and sulphite:cytochrome c oxidoreductase by hydrophobic interaction chromatography of fraction G-200-III (see Fig. 6) on phenyl-Sepharose-4B (see Fig. 7)

<table>
<thead>
<tr>
<th>Fractions assayed (G-200-III)</th>
<th>Protein (mg)</th>
<th>Enzyme B activity*</th>
<th>Sulphite:cytochrome c oxidoreductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific [μmol cytochrome c min$^{-1}$ (mg protein)$^{-1}$]</td>
<td>Specific [μmol cytochrome c min$^{-1}$ (mg protein)$^{-1}$]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total [μmol cytochrome c min$^{-1}$]</td>
<td>Total [μmol cytochrome c min$^{-1}$]</td>
</tr>
<tr>
<td>Sample</td>
<td>103</td>
<td>1.43</td>
<td>147</td>
</tr>
<tr>
<td>Fraction I</td>
<td>12</td>
<td>0.59</td>
<td>3.5</td>
</tr>
<tr>
<td>Fraction II</td>
<td>68</td>
<td>1.43</td>
<td>97</td>
</tr>
<tr>
<td>Fraction I + II†</td>
<td>68</td>
<td>2.05</td>
<td>139</td>
</tr>
</tbody>
</table>

* Enzyme B and sulphite:cytochrome c oxidoreductase activities were measured as described in the Methods.† Assayed using 16 μg Fraction II protein supplemented with 3 μg Fraction I protein in terms of the sum of the protein samples added.

electrophoresis of the crude extract and A65% fraction showed a band at the position expected for enzyme B ($M_r$ about 63000; Fig. 8a), but most of enzyme B in the 0-35 m-NaCl(I) fraction moved down to the subunit position. Since all of the samples were prepared in the same way before being loaded on the gel, it is unclear why the enzyme behaved so differently. Treatment with SDS at 100 °C did not cleave the 63000 $M_r$ enzyme into smaller units. Boiling the purified enzyme from IEF (Fig. 8a, lane 6) resulted in most of the protein running as $M_r$ 63000, although the milder treatment gave the smaller unit seen in Fig. 8(a). This behaviour is anomalous and cannot be further explained at present.

Some other properties of enzyme B

The enzyme had a pI value of about 4.25 and had an absorption spectrum only in the UV region, with a sharp maximum at 280 nm and a broad absorbance below 250 nm. The enzyme was more stable than enzyme A. It lost about 30% and 50% activity at 20 °C after 1 d and 2 d, respectively, and 4% and 20% at 4 °C after 1 d and 2 d, respectively. Storage at −20 °C for at least three months, with freezing and thawing had no significant effect on the enzyme activity.
Fig. 8. (a) SDS-polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme B. (1) Crude extract (60 µg); (2) A65% fraction (60 µg); (3) 0-35 M-NaCl(I) fraction (60 µg); (4) G-200-III fraction (see Fig. 6) (30 µg); (5) HIC-II fraction (see Fig. 8) (30 µg); (6) pure enzyme B from preparative isoelectric focusing (20 µg); (7) marker proteins (45 µg). (b) Discontinuous polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme B. (1) 0-35 M-NaCl(I) fraction (60 µg); (2) G-200-III fraction (see Fig. 6) (25 µg); (3) HIC-II fraction (see Fig. 7) (25 µg); (4) pure enzyme B from preparative isoelectric focusing (20 µg). For details see Methods and Results.
Table 4. Summary of the purification of Enzyme B

Enzyme B was assayed as described in Methods.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Specific activity [µmol cytochrome c reduced min⁻¹ (mg protein)⁻¹]</th>
<th>Total activity (µmol cytochrome c reduced min⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>49100</td>
<td>--</td>
<td>--</td>
<td>1*</td>
<td>100*</td>
</tr>
<tr>
<td>1st ammonium sulphate fraction (A65%)</td>
<td>12000</td>
<td>--</td>
<td>--</td>
<td>4*</td>
<td>91*</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B [0-35 m-NaCl(II) fraction]</td>
<td>1155</td>
<td>0.30</td>
<td>346</td>
<td>34</td>
<td>75</td>
</tr>
<tr>
<td>Sephadex G-200 (G-200-III) (see Fig. 6)</td>
<td>240</td>
<td>1.43</td>
<td>343</td>
<td>162</td>
<td>73</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B (HIC-II) (see Fig. 7)</td>
<td>158</td>
<td>2.05†</td>
<td>323</td>
<td>231</td>
<td>69</td>
</tr>
<tr>
<td>Preparative iso-electric focusing (I)‡</td>
<td>67</td>
<td>2.5†</td>
<td>167</td>
<td>280</td>
<td>53</td>
</tr>
<tr>
<td>Preparative iso-electric focusing (II)‡</td>
<td>81</td>
<td>0.9†</td>
<td>73</td>
<td>101</td>
<td>53</td>
</tr>
</tbody>
</table>

* Figures derived from results obtained using the oxygen electrode procedure (Lu & Kelly, 1983b).
† For these assays some of the HIC-I fraction (3 µg protein) was added to the reaction mixture. For details see Table 3 and the text.
‡ I and II are the upper and lower bands respectively of Fig. 4b.

Table 5. Purification of sulphite : cytochrome c oxidoreductase

Sulphite : cytochrome c oxidoreductase was assayed as described in Methods.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Specific activity [µmol cytochrome c reduced min⁻¹ (mg protein)⁻¹]</th>
<th>Total activity (µmol cytochrome c reduced min⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>--</td>
<td>0.04</td>
<td>--</td>
<td>1*</td>
<td>--</td>
</tr>
<tr>
<td>1st ammonium sulphate fraction (A65%)</td>
<td>12000</td>
<td>0.22</td>
<td>2640</td>
<td>5.6*</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>1155</td>
<td>1.10</td>
<td>1270</td>
<td>28</td>
<td>48</td>
</tr>
<tr>
<td>Sephadex G-200 (G-200-III) (see Fig. 6)</td>
<td>240</td>
<td>2.97</td>
<td>713</td>
<td>93</td>
<td>27</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B (fraction 1, see Fig. 7)</td>
<td>28</td>
<td>21.10</td>
<td>591</td>
<td>660</td>
<td>22</td>
</tr>
</tbody>
</table>

* Figures derived from results obtained using the oxygen electrode procedure (Lu & Kelly, 1983b).

Sulphite : cytochrome c oxidoreductase and the stimulation of enzyme B activity by the HIC-I fraction

The specific activity of sulphite : cytochrome c oxidoreductase was increased over 600 times during purification even though the recovery was rather low (Table 5). However, the final product (HIC-I fraction) gave more than six protein bands on SDS polyacrylamide gel (Fig. 9), in which the top major band was most probably enzyme B and the rest had more or less the same density.

The HIC-I fraction stimulated enzyme B specific activity in the HIC-II fraction (Table 3) and pure enzyme B (Table 4) by 20–40%. Fractions from previous steps were also stimulated. The stimulation could not simply be accounted for by the presence of the small amount of enzyme B in the HIC-I fraction.
Fig. 9. SDS-polyacrylamide gel electrophoresis of fractions in the separation of sulphite oxidoreductase and cytochrome cSs1.

(1) Marker proteins (45 µg); (2) fraction HIC-I (see Fig. 7) (30 µg); (3) fraction G-200-I (see Fig. 6) (25 µg); (4) 0.35 M-NaCl(II) fraction (40 µg).

Reconstitution of thiosulphate-oxidizing activity with the purified components

Thiosulphate was completely oxidized to sulphate by the reconstituted system with a consumption of 1.95 ± 0.05 mol oxygen for each thiosulphate added (four determinations). The reaction was negligible in the absence of any one of enzyme A, enzyme B, or the cytochrome cSs1 and cytochrome c552.5 fractions.

Attempt to demonstrate thiosulphate cleavage by the purified enzyme(s)

Incubation of partially purified enzyme A (0.35 mg), enzyme B (1.5 mg), or mixtures of enzyme A, enzyme B and the cytochrome cSs1 and cytochrome c552.5 fractions with thiosulphate labelled in the inner or outer sulphur atom with 35S, in the absence of electron acceptors for as long as 45 min at 30 °C demonstrated no significant formation of sulphite or sulphate. In other words the S–S bond of thiosulphate was not split by any one of the enzymes or the enzyme system under these experimental conditions. However, the experiment did show that thiosulphate appeared to be associated with enzyme B (HIC-II fraction) with a molar ratio of about two thiosulphate : one protein.
Fig. 10. Schematic representation of the components of the enzyme:cytochrome complex catalysing thiosulphate oxidation by *Thiobacillus* A2. The sequence of electron transfer among the cytochrome c components is unresolved, cytochromes c₅₅₁ and c₅₅₂₅ being essential for thiosulphate oxidation, with the soluble cytochrome c₅₅₀ (Mᵣ, 15000) possibly linking electron transport from the complex to membrane-bound cytochrome c₅₅₂. Approximate relative molecular masses of the cytochromes are indicated by their areas in the scheme (W. P. Lu & D. P. Kelly, unpublished data), with enzyme B (Mᵣ, 64000) and enzyme A (Mᵣ, 16000) being expressed relative to each other as 0.6% (w/w) and 0.8% (w/w) of the crude extract protein, respectively. Cytochrome c₅₅₂₅ (Mᵣ, 32000) contains two subunits (Mᵣ, 16000 each) and 'cytochrome c₅₅₁' is a very large protein (Mᵣ, 300000) probably containing six subunits. Relative molecular masses have not been determined by us for the membrane cytochrome c₅₅₂ or uu₃, so they are assumed to be similar to literature values for animal cytochromes (Mᵣ, 12000 and 130000, respectively) for the purpose of diagrammatic representation.

### Table 6. A typical assay of thiosulphate:cytochrome c oxidoreduction activity with the four highly purified components

Enzyme activity was measured spectrophotometrically as described in Methods except that the components were added to the reaction mixture in the order and the amount listed below, following the addition of horse-heart cytochrome c and thiosulphate. Activity was calculated in terms of the total protein of the components added. The detailed purification and characterization of cytochrome c₅₅₂₅ and 'cytochrome c₅₅₁' are to be published elsewhere.

<table>
<thead>
<tr>
<th>Addition (mg protein)</th>
<th>Cytochrome c₅₅₁ reduction [nmol reduced min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme A</td>
<td>Enzyme B</td>
</tr>
<tr>
<td>0.1</td>
<td>0.08</td>
</tr>
<tr>
<td>0.1</td>
<td>0.08</td>
</tr>
<tr>
<td>0.1</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**Cytochrome c₅₅₁ and cytochrome c₅₅₂₅**

Thiosulphate oxidation and horse-heart cytochrome c reduction by the reconstituted enzyme system was negligible if the cytochrome c₅₅₁ and c₅₅₂₅ fractions were omitted from the reaction mixture and only commenced when one of them was added. Subsequent addition of the other cytochrome fraction increased the activity further. The apparent *Kₘ* for thiosulphate in the reconstituted thiosulphate:cytochrome c oxidoreductase system was probably less than 2 μM. The apparent *Kₘ* value for horse-heart cytochrome c was lower in the reconstituted system.
than in the A65% fraction (200 \mu M). The causes seemed related to the concentration of cytochrome c_{551} and cytochrome c_{552-5}, since there was more of them in the reconstituted system. However, the two cytochromes contained in the G-200-I fraction and 0-35 M-II fraction were still quite crude, as shown in Fig. 9. Subsequent purification (unpublished data) of cytochromes c_{551} and c_{552-5} enabled proof of their essential role in the thiosulphate-oxidizing complex (Table 6).

**DISCUSSION**

The conclusions to be drawn from our results to date indicate that enzymes thought to have some role in thiosulphate oxidation by thiobacilli (Oh & Suzuki, 1977; Kelly, 1982) do not seem to have central functions in *Thiobacillus* A2. These include rhodanese, which, although very active in *Thiobacillus* A2 (Silver & Kelly, 1976; Wood & Kelly, 1981; Lu & Kelly, 1983a), is not required for complete oxidation of thiosulphate by extracts (Lu & Kelly, 1983c), while the ‘thiosulphate-oxidizing enzyme’ (Trudinger, 1961), adenyl sulphate reductase and the sulphur-oxidizing oxygenase enzyme (Suzuki, 1965; Charles & Suzuki, 1966a; Suzuki & Silver, 1966) are either absent from *Thiobacillus* A2 or present only at low levels (Kelly & Tuovinen, 1975; Silver & Kelly, 1976). So far we have also failed to demonstrate a thiosulphate-cleaving enzyme (Peck, 1960; Kelly, 1982) or a major role for free sulphite as a substrate for sulphite oxidase in the system. It is generally believed that free sulphite is the penultimate intermediate in sulphate formation (Charles & Suzuki, 1966b; Kelly, 1982) and sulphite oxidase activity is present in all thiobacilli examined.

We have, however, obtained a cell-free system catalysing the complete oxidation of thiosulphate, for which at least two novel colourless enzymes and several cytochromes are required. These two enzymes have now been highly purified (Fig. 2), although we are as yet unable to ascribe specific individual functions to them. To our knowledge, these two enzymes differ from any enzymes so far found in sulphur-oxidizing bacteria, although a relative lack of detailed enzymological data in other published work makes proper comparison difficult.

The present work confirms the view (Lu & Kelly, 1983b) that the thiosulphate :cytochrome c oxidoreduction system in *Thiobacillus* A2 is a soluble multi-enzyme complex. The complete oxidation of thiosulphate by the reconstituted purified components proves that no small cofactor molecules are needed. The relatively easy separation of the components suggests their association *in vivo* to be weak. Many multi-enzyme systems show much stronger association, although some (e.g. tryptophan synthetase) are easily separated into subunits, with a considerable decrease in overall activity (Miles, 1979). Dissociation of the complex could help explain why thiosulphate-oxidizing activity in the crude extract from *Thiobacillus* A2 was about 100-fold less than in intact cells (Kula et al., 1982; Lu & Kelly, 1983a). Thus, rapid and stoichiometric oxidation of thiosulphate *in vivo* depends on the integrity of the well-organized multi-enzyme complex and its association with the membrane system. Any disturbance of the structure, such as caused by disruption of the cell could thus dramatically affect oxidative ability. In fact, one advantage of a multi-enzyme complex is that it provides a very short transit time for passage of intermediates from one enzyme to another. This could be particularly important if unstable compounds such as sulphite and other reduced sulphur species are produced as intermediates in thiosulphate oxidation. Such intermediates might always be enzyme-bound in the multi-enzyme system. For thiosulphate to be oxidized to sulphate, three main processes must occur. These are cleaving of the S-S bond, the oxidation of the sulphane-sulphur group to sulphate, and the oxidation of the sulphone-group to sulphate. Cleavage has come to be regarded as the primary step (Kelly, 1982), followed by oxidation of the sulphur (or sulphide) and sulphite formed thereby. Our reconstituted system seems to contain two colourless enzyme proteins and two essential c-type cytochromes (of which ‘cytochrome c_{551}’ may in fact be a third enzyme of thiosulphate oxidation), which together can effect all three essential processes. We have so far failed to show a thiosulphate-cleaving function using purified enzymes or the enzyme system in the absence of the electron transport system. This might mean that cleavage only proceeds at the rate of overall oxidation, so that in the absence of electron
Thiosulphate oxidation binding to an enzyme can occur, but cleavage either does not occur or is so slight as not to be detectable. This could be because the products of cleavage are not released in the free state, but have to be transferred to acceptor-enzyme components of the multi-enzyme system and further cleavage ceases as oxidation of the transferred groups cannot occur. Alternatively, oxidation of sulphone-sulphur to sulphite might occur on the thiosulphate-binding enzyme with cleavage occurring only when an enzyme-bound intermediate analogous to dithionate (\(\text{O}_3\text{S}-\text{S}-\text{SO}_3^{-}\)) has been formed. At present it is not possible to decide on the exact nature of the partial reactions of sulphate formation or even the exact timing of the sulphur-sulphur bond cleavage. Certainly, however, thiosulphate cleavage is not a simple primary reaction of the rhodanese type.

In current work we are attempting to evaluate the midpoint potentials of the various c-type cytochromes, which should enable an assessment of the sequence of their involvement as electron transport carriers.

Figure 10 gives a schematic representation of the probable interrelations of the multi-enzyme complex and the membrane system in effecting thiosulphate oxidation.

The sulphite:cytochrome c oxidoreductase in *Thiobacillus* A2 compares with that in *T. novellus* (Yamanaka et al., 1981), in that cytochrome 551 appears to be the electron acceptor for sulphite oxidation and separation of the cytochrome from the enzyme considerably reduced its activity (unpublished observations). Nevertheless, the significance of the sulphite:cytochrome c oxidoreductase in *Thiobacillus* A2 is still obscure. One phenomenon of potential significance to understanding the system was the observed stimulation of enzyme B activity by the fraction containing the sulphite:cytochrome c oxidoreductase activity. A more critical analysis of the HIC-II fraction is essential before further conclusions can be drawn.

This work was made possible by financial support from The Government of the People's Republic of China, The British Council and the Committee of Vice Chancellors and Principals. We are grateful to Dr Mark Woodland for advice and the use of some items of equipment, and to Drs Ann Wood and Ben Swoboda for discussions.

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