Partial Purification and Resolution of a Thiosulphate-oxidizing System from *Thiobacillus* A2

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A crude thiosulphate-oxidizing extract of *Thiobacillus* A2 has been resolved by ammonium sulphate fractionation and DEAE-Sepharose CL-6B chromatography into fractions containing several components necessary for thiosulphate oxidation and reduction of cytochrome *c*. These components include two distinct *c*-type cytochromes, sulphite : cytochrome *c* oxidoreductase, rhodanese and enzymes probably responsible for thiosulphate cleavage and sulphane-sulphur oxidation. The complete cytochrome *c*-dependent thiosulphate-oxidizing system could be reconstituted by mixing selected fractions from the column chromatography.

INTRODUCTION

Despite considerable study over many years, a complete understanding of the mechanism of thiosulphate oxidation to sulphate by thiobacilli has not yet been achieved (Kelly, 1982). In the preceding paper we reported complete thiosulphate oxidation and energy-coupling with high efficiency by crude cell-free preparations from *Thiobacillus* A2 (Lu & Kelly, 1983). The principal unresolved problems of the oxidation pathway are the mechanism of the initial 'activation' reaction of thiosulphate and the nature of the enzymes oxidizing the sulphane-sulphur atom of thiosulphate (and probably also of sulphur and sulphide) to sulphite. A number of hypothetical schemes have appeared (for general reviews, see Kelly, 1968, 1978, 1982; Suzuki, 1974; Aleem, 1975), but substantive evidence to confirm these hypotheses, including highly purified enzymes and the demonstration of thiosulphate oxidation by reconstituted enzyme systems *in vitro* are still lacking except for the terminal conversion of sulphite to sulphate. Our recent work (Lu & Kelly, 1983) demonstrated that the enzyme system for thiosulphate oxidation was completely soluble (i.e. not sedimented at 130 000 *g*) and that membrane material was required only to provide the cytochrome oxidase system for terminal electron transport. The present paper describes the partial purification, resolution and reconstitution of this soluble enzyme system into three major components.

METHODS

Organism and chemostat culture. *Thiobacillus* A2 was grown in continuous culture on a medium as described by Lu & Kelly (1983) except that 200 mM- $\text{Na}_2\text{S}_2\text{O}_3$ was used in order to maximize biomass production. A chemostat culture was established in a Gallenkamp glass fermenter vessel with a culture volume of 5.2 l, stirred (1000 r.p.m.) with a direct drive shaft stirrer module and aerated (320 ml min⁻¹) with air containing 1% (v/v) CO_2 . Temperature was maintained at 30 °C with an LH Engineering heater module and pH was maintained at pH 7.7 by automatic titration with 10 M-NaOH using an LH Engineering pH controller. Thiosulphate was completely consumed and was the growth-limiting nutrient, giving about 7 g dry wt of bacteria d⁻¹ at a dilution rate of 0.06 h⁻¹. The effluent from the chemostat was collected in a flask maintained at 2 °C and stored at 4 °C until centrifuged.

Preparation of cell-free extract. Bacteria were sedimented by centrifuging and the cell pellet suspended to about 80 mg dry wt ml⁻¹ in 55 mM-phosphate buffer (pH 7.5). Organisms were disrupted by sonication for 5 min at 30 s

intervals in a 100 ml beaker immersed in ice using a 3/4" (19 mm) probe and maximum power output from a MSE ultrasonic disintegrator. The treated cell suspension was centrifuged at 10000 *g* for 15 min and the supernatant centrifuged at 48000 *g* for 30 min to remove any unbroken cells and large membrane fragments. The resultant supernatant was designated the 'crude extract'. 'Membrane fraction' was prepared as described by Lu & Kelly (1983).

Enzyme assays. Thiosulphate and sulphite-oxidizing activities were measured polarographically as described by Lu & Kelly (1983) except that 'membrane fraction' (0.5 mg protein) and various amounts of cytochrome *c* (horse heart III) were added as indicated. Thiosulphate:cytochrome *c* oxidoreductase, sulphite:cytochrome *c* oxidoreductase, rhodanese and succinate-oxidizing activity were assayed as described by Lu & Kelly (1983). [³⁵S]Sulphate production from [³⁵S]thiosulphate was assayed as described by Kelly & Syrett (1966).

Ammonium sulphate fractionation. Crude extract was fractionated at 4 °C by the addition of serially increasing amounts of a saturated (4 M) ammonium sulphate solution. In each step the precipitated protein was centrifuged at 17000 *g* for 10 min, then dissolved in a small volume of phosphate buffer (25 mM, pH 6.5 or 55 mM, pH 7.5) and dialysed against the same buffer for 5 h. The supernatant at each step was brought to a higher percentage saturation with saturated ammonium sulphate. The fraction precipitating between 44 and 65% of saturation with ammonium sulphate was called fraction A65%.

Gel filtration. Experimental details are provided in the legends to the figures.

DEAE-Sephacryl CL-6B chromatography. The A65% fraction (25 ml, 1550 mg protein) was applied to a column (2.6 × 16.5 cm) of DEAE-Sephacryl 6B, equilibrated at 2 °C with 25 mM-phosphate buffer, pH 6.5, containing 2 mM-thiosulphate and eluted stepwise with a total of 1600 ml of the same buffer containing NaCl at 0 M, 0.1 M, 0.2 M, 0.3 M, 0.35 M and 0.4 M. Fractions (18 ml) collected at 2 °C using a flow rate of 105 ml h⁻¹ were monitored for protein, *A*₂₈₀, *c*-type cytochrome (by absorbance at 416 nm), and sulphite:cytochrome *c* oxidoreductase. Fractions containing distinct peaks of protein or cytochrome were combined and brought to 63% saturation with ammonium sulphate. The protein precipitated was collected by centrifugation and dissolved in a small volume of 55 mM-phosphate buffer, pH 7.4.

Analytical procedures. Thiosulphate was estimated colorimetrically as described by Kelly *et al.* (1969). Protein was measured by the Lowry procedure, using crystalline bovine serum albumin as standard.

Materials. Sepharose 4B, Sephacryl 300 Superfine and DEAE-Sephacryl CL-6B were from Pharmacia. Cytochrome *c* (horse heart III), cytochrome oxidase (bovine heart), rhodanese (liver) and catalase (liver) were from Sigma.

RESULTS AND DISCUSSION

Ammonium sulphate fractionation of the crude extract

The thiosulphate- and sulphite-oxidizing activities were recovered in the fractions above 44% of saturation with ammonium sulphate (Table 1). The specific activity of thiosulphate oxidation was increased about fourfold in the A61% fraction compared with that in the crude extract, and total activity recovered was about 91%. Fractions A21% and A39% contained cell membrane, because (a) these fractions contained nearly all the succinate-oxidizing activity, which was previously shown to be membrane-bound, and (b) they could also replace the requirement for the 'membrane fraction' for electron transfer to oxygen (Table 1). The rate of thiosulphate oxidation in fraction A61% was stimulated about tenfold by the addition of horse heart cytochrome *c*. This contrasted with the crude extract, to which addition of cytochrome *c* had no effect (Table 1). The stimulation could have been due to the loss of a certain amount of specific *c*-type cytochrome during the fractionation, even though the content of *c*-type cytochrome increased from about 1.4 nmol (mg protein)⁻¹ in the crude extract to about 2 nmol (mg protein)⁻¹ in fraction A61%. This possibility was supported by the fact that addition of fractions A21% and A39% to the reaction mixture instead of the membrane fraction (prepared as described by Lu & Kelly, 1983) showed higher thiosulphate-oxidizing activity in the absence of added horse heart cytochrome *c*. The thiosulphate:cytochrome *c* oxidoreductase activity of fraction A65% showed a *K_m* value of about 200 μM for horse heart cytochrome *c*. The fact that a multiplicity of enzymes is involved in this system means, however, that this value might not be highly significant. The A65% fraction oxidized thiosulphate completely to sulphate. This was demonstrated by the consumption of two moles of oxygen for each thiosulphate oxidized and the demonstration, using thiosulphate labelled with ³⁵S in either atom, that both the sulphane- and sulphone-sulphur were oxidized to sulphate (Table 2).

Table 1. Ammonium sulphate fractionation of the crude cell-free extract prepared by sonication of *Thiobacillus A2*

Fraction*	Thiosulphate-oxidizing activity					Recovery (%)	Sulphite-oxidizing activity			Succinate-oxidizing activity [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]
	Total protein (mg)	Specific activity [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]			Total activity recovered (nmol O ₂ min ⁻¹)		Cytochrome <i>c</i> added (μM):			
		0	40	70			0 70 100 140			
Crude extract	660	12.0	12.0	12.0	7920	100	1	2.5	25	
A21%	105	0	0	0	—	—	0	2.5	51	
A39%	192	0	0	0	—	—	0	2.6	41	
A44%	60	2.5	6.8	9.1	546	7	0	4.2	1	
A61%	140	4.4	35.0	47.0	6580	83	2.2	12	0	
		(13)†	(34)†				(4.1)†			
A65%	10	0	8.5		85	1	—	—	—	

* A21% etc., refer to protein precipitated at 21%, etc., of saturation with ammonium sulphate. Enzymes were assayed as described in Methods except that 10 mM-Tris/HCl, pH 7.4, was used for assay of sulphite or succinate oxidation.

† Activities obtained when A21% or A39% fractions were added (1 mg protein ml⁻¹) instead of the *Thiobacillus* membrane fraction (see Methods).

Table 2. Stoichiometry of thiosulphate oxidation by fraction A65% from *Thiobacillus A2*

Experimental conditions were as described in Methods, using 7 mg A65% fraction protein ml⁻¹, 70 μM-horse heart cytochrome *c* and 4 units cytochrome oxidase ml⁻¹.

Na ₂ S ₂ O ₃ added (nmol)	O ₂ consumed (nmol)	O ₂ /Na ₂ S ₂ O ₃
50	98	1.96
100	185	1.85

Fig. 1. Gel filtration of the A65% fraction from *Thiobacillus* A2 on Sepharose CL-4B. The sample (92 mg protein) was applied to a column of Sepharose CL-4B (2.2 × 78 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, and eluted with the same buffer. Fractions (4.8 ml) were collected at a flow rate of 19.5 ml h⁻¹ at 4 °C and analysed for protein, A_{280} (○), A_{552} (▲) and activity of thiosulphate-dependent cytochrome *c* reduction (●) as described in Methods. The column was calibrated using commercial lactate dehydrogenase (LDH, M_r 140 000; 45 mg), bovine serum albumin (BSA, M_r 66 000; 10 mg) and horse heart cytochrome *c* (C, M_r 12 384; 5 mg), chromatographed under identical conditions and eluted in the fractions indicated by the arrows. A_{280} in the first 175 ml of eluate was due to nucleic acid, as insignificant protein eluted in these fractions. Protein and A_{280} elution profiles were coincident between 175–250 ml of eluate.

Gel filtration of the thiosulphate-oxidizing system precipitated by ammonium sulphate between 44 and 65% of saturation (A65%)

Passage of fraction A65% down a column of Sepharose CL-4B or Sephacryl-300 Superfine yielded similar elution patterns (Figs 1 and 2) with one main peak of protein (A_{280}) with which a peak of *c*-type cytochrome coincided. The peak of thiosulphate:cytochrome *c* oxidoreductase activity, however, eluted in fractions slightly behind this main peak (Figs 1 and 2). These results indicate that little resolution of the complex mixture of enzymes necessary for thiosulphate oxidation by the A65% fraction had been achieved, although the complex was partly resolved from the bulk protein of the preparation.

Resolution of the thiosulphate-oxidizing system into three main fractions by DEAE-Sepharose CL-6B chromatography

Chromatography of fraction A65% on a column of DEAE-Sepharose CL-6B yielded seven distinct peaks with absorbance at 280 nm or 416 nm (Fig. 3). The first and last of these (0 M and 0.4 M-NaCl) contained little protein when analysed by the Lowry procedure and were not further studied. The 0.1 M-NaCl fraction contained no detectable cytochrome, but *c*-type cytochrome (A_{416} and characteristic 550–552 nm absorbance) was present in the other four peak fractions. The fractions with 0.1 M and 0.2 M-NaCl had a greenish colour. The 0.35 M-NaCl eluate was resolved into two parts: the first (0.35 M-I) contained all the sulphite:cytochrome *c* oxidoreductase activity and the second (0.35 M-II) mainly consisted of *c*-type cytochrome.

None of the fractions alone showed thiosulphate:cytochrome *c* oxidoreductase activity (Table 3). This activity was, however, recovered when the 0.1 M and 0.35 M-I NaCl fractions were mixed. The 0.2 M-NaCl could replace the 0.1 M-NaCl fraction, but gave lower activity. Activity of the 0.35 M-I fraction with either the 0.1 M or the 0.2 M-NaCl fraction was stimulated about fivefold by the addition of the 0.35 M-II NaCl fraction. Boiling the 0.35 M-II fraction destroyed the stimulatory activity. These results demonstrate that a number of components were necessary for the expression of thiosulphate:cytochrome *c* oxidoreductase activity. Thiosulphate-oxidizing activity could also be measured polarographically using reconstituted mixtures

Fig. 2. Gel filtration of the A65% fraction from *Thiobacillus* A2 on Sephacryl S-300 Superfine. The sample (1 ml containing 65 mg protein and 5 μl glycerol) was applied to a column (1.6 $\times$ 86 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7.2, and eluted with the same buffer. Fractions (2.2 ml) were collected at 7.8 ml h^{-1} at 4 $^{\circ}\text{C}$. Measurements were made of A_{280} (○), A_{552} (▲) and thiosulphate-dependent cytochrome *c* reduction (●).

Fig. 3. Fractionation of the A65% fraction from *Thiobacillus* A2 on DEAE-Sephacrose CL-6B. The chromatography was performed as described in Methods with stepwise elution with increasing concentration of NaCl in Tris/HCl buffer. NaCl concentration is indicated (---) for each step. Fractions were assayed for A_{280} (○), A_{416} (●) and sulphite:cytochrome *c* oxidoreductase (□).

of these fractions. Oxygen uptake was observed when a mixture of the 0.1 M, 0.35 M-I and 0.35 M-II fractions were supplied with thiosulphate in the presence of horse heart cytochrome *c* and either bovine cytochrome oxidase or *Thiobacillus* A2 'membrane fraction'. Oxygen was

Table 3. *Reconstitution of thiosulphate : cytochrome c oxidoreductase activity using fractions from the A65% fraction of Thiobacillus A2 separated by DEAE-Sephadex CL-6B chromatography*

Amount of protein per assay (mg)					Cytochrome <i>c</i> reduced (nmol min ⁻¹)	Specific activity† [nmol cytochrome <i>c</i> reduced min ⁻¹ (mg protein) ⁻¹]
Fraction(s) assayed*:						
0.1 M	0.2 M	0.3 M	0.35 M (I)	0.35 M (II)		
1.64					0	0
	2.4				0	0
		2.7			0	0
			1.04		0	0
				0.6	0	0
1.64			1.04		5.3	2.0
1.64			1.04	0.3	25.0	10.0
1.64				0.3	0	0
			1.04	0.3	0	0
	2.4		1.04		1.8	0.5
	2.4		1.04	0.3	13.0	5.8
		2.7	1.04		0	0
1.64		2.7	1.04		4.5	0.8
1.64	2.4		1.04		5.3	0.7

* The fractions are designated by the molarity of NaCl with which they were eluted.

† Enzyme activity was measured as described in Methods, using 70 μ M-horse heart cytochrome *c*, 90 mM-Tris/HCl, pH 7.4, and amounts of protein as indicated. Specific activity is expressed relative to the total protein added in the various fractions.

consumed at about 9 nmol min⁻¹ (mg protein)⁻¹, calculated only for the protein added in the three NaCl fractions. This oxidation rate would require cytochrome *c* reduction and reoxidation at a rate of 36 nmol min⁻¹ (mg protein)⁻¹, or rather more than was observed spectrophotometrically (Table 3). The reaction mixture contained only 70 μ M-cytochrome *c* (or only one-third of that for the K_m value), so oxidation could have been stimulated when complete electron transfer to oxygen was possible.

Separation of two cytochrome c components

Two distinct cytochrome *c* spectra were observed in the different NaCl fractions (Table 4). One was that of the predominant cytochrome c_{552} , which was present largely in the oxidized form in fractions 0.35 M-I and 0.35 M-II, and was rapidly reduced by sulphite when the 0.35 M-I fraction was present. The other was cytochrome c_{550} , which was mainly present in the 0.2 M-NaCl fraction. This was always recovered in the reduced form, presumably because of the presence of thiosulphate as an enzyme protector in the eluate. The existence of cytochrome c_{550} in the crude extract and A65% fraction was masked by the intense absorbance of cytochrome c_{552} . Two types of cytochrome *c* (550 and 551 nm maxima) were also purified from *T. novellus* (Yamanaka *et al.*, 1971, 1981).

Distribution of enzyme activities among the fractions from DEAE-Sephadex CL-6B chromatography

Thiosulphate : cytochrome *c* oxidoreductase activity in mixtures of the 0.35 M-I, 0.35 M-II and either the 0.1 M or 0.2 M-NaCl fractions (Table 3) always showed a lag and acceleration phase of about 2 min if the 0.1 M or 0.2 M-NaCl fraction was the component added to initiate the assay. This implied that these two fractions contained an enzyme or enzymes required for the initial attack on thiosulphate, and that this activity was initially rate-limiting. It is likely (Kelly, 1982) that this initial step is the cleavage of thiosulphate to sulphite and a sulphane-sulphur moiety. Enzymes implicated as possible cleavage enzymes include rhodanese (Silver & Kelly, 1976; Kelly, 1982).

Table 4. Separation of two types of cytochrome *c* during DEAE-Sepharose CL-6B chromatography of the A65% fraction from *Thiobacillus A2*

Fraction assayed	Absorption maxima* in fractions (nm)			Concentration† [nmol (mg protein) ⁻¹]
	α	β	γ	
0 M-NaCl	550		415(412)	ND
0.2 M-NaCl	550	522	416	0.4
0.35 M-NaCl (I)	552	522	418(410)	3.0
0.35 M-NaCl (II)	552	522	418(410)	9.0

* Reduced cytochrome absorbance maxima were determined after reduction with dithionite. Oxidized peaks for the γ bands are given in parenthesis.

† Cytochrome *c* concentration in the fractions was calculated as the reduced *minus* oxidized absorbance using a millimolar extinction coefficient of 28, and expressed relative to the total protein content of the fraction. ND, Not determined.

Table 5. Rhodanese activity in the fractions from DEAE-Sepharose CL-6B chromatography of the A65% fraction

Rhodanese activity was assayed as described in Methods.

Fraction assayed	Thiocyanate formed [nmol min ⁻¹ (mg protein) ⁻¹]
A65%	270
0.1 M-NaCl	420
0.2 M-NaCl	130
0.3 M-NaCl	100
0.35 M-NaCl (I)	0
0.35 M-NaCl (II)	0
Catalase (bovine liver)	1500
Rhodanese (bovine liver)	1600

Table 6. Separation of sulphite : cytochrome *c* oxidoreductase during chromatography of the A65% fraction on DEAE-Sepharose CL-6B

Experimental conditions were as described in Methods and in Fig. 2, except that 35 μ M-horse heart cytochrome *c*, 90 mM-Tris/HCl buffer, pH 7.4, and the indicated amounts of protein were used in the assay.

Fraction assayed	Amount of protein in assay (mg)	Specific activity [nmol cytochrome <i>c</i> reduced min ⁻¹ (mg protein) ⁻¹]
A65%	0.30	110
0.2 M-NaCl	0.24	4
0.3 M-NaCl	1.65	2
0.35 M-NaCl (I)	0.10	1200
0.35 M-NaCl (II)	0.15	7
0.35 M-NaCl (I + II)	0.1 + 0.05	1500

Rhodanese was present at high activity in the 0.1 M-NaCl fraction and to a lesser extent in the 0.2 M fraction (Table 5). Addition of liver rhodanese instead of the 0.1 M-NaCl fraction in the assay mixture with the 0.35 M-I and 0.35 M-II fractions (as in Table 3) did not, however, give any thiosulphate : cytochrome *c* oxidoreductase activity. Pure catalase also exhibited rhodanese activity (Table 5), but it could not replace the 0.1 M-NaCl fraction either. The cleaving enzyme in this fraction is thus either not rhodanese, or the activity requires enzymes or cofactors other

Fig. 4. Summary of the fractionation procedure employed with the cell-free thiosulphate-oxidizing system from *Thiobacillus A2*.

than rhodanese which are provided by the 0.1 M-NaCl fraction. The location of the enzyme or enzymes catalysing the oxidation of sulphane-sulphur to sulphite has not yet been established, but the complete absence of absorbance in the 590 nm region in the 0.1 M and 0.2 M-NaCl fractions suggests that they did not contain a sirohaem sulphite reductase of the kind found in *T. denitrificans*, that might *in vivo* act as a sulphane-oxidase (Schedel & Trüper, 1979; Kelly, 1982).

The specific activity of sulphite:cytochrome *c* oxidoreductase was increased about tenfold after chromatography and was recovered in the 0.35 M-I fraction (Table 6). Activity was further enhanced by addition of the 0.35 M-II fraction (Table 6). The latter stimulation may reflect improved catalysis of the reaction by the additional *Thiobacillus* cytochrome *c*.

Conclusions

This is the first time that a cell-free thiosulphate-oxidizing system from a thiobacillus has been resolved into distinct fractions and then reconstituted from those fractions. The fractionation so far achieved is summarized in Fig. 4. These fractions are, however, still relatively crude and the total number and nature of the enzymes involved is not yet known. The system catalysing the oxidation of sulphane-sulphur to sulphite has clearly survived the fractionation procedure so far, and it is hoped that further purification of the 0.1 M and 0.2 M-NaCl fractions from DEAE-Sepharose chromatography may enable unequivocal identification of the enzymes and kind of

reactions involved in the initial metabolism of thiosulphate, and the oxidation of sulphane-sulphur.

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