An AMP-independent Sulphite Oxidase from *Thiobacillus denitrificans*: Purification and Properties

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(Received 3 September 1973; revised 8 December 1973)

SUMMARY

A membrane-bound sulphite oxidase from *Thiobacillus denitrificans* uses nitrate, oxygen or ferricyanide as an electron acceptor and is not stimulated by AMP. Studies with inhibitors of oxygen uptake indicated that an electron-transport chain is involved during sulphite oxidation. The inhibition of oxygen uptake by nitrate was found to be of the non-competitive type. The enzyme, which was purified about 50-fold, used ferricyanide as an electron acceptor, but neither nitrate nor oxygen was utilized. Thiol-binding reagents strongly inhibited the enzyme and this was completely reversed by dithiothreitol. The optimum pH was 8.3 with a K_m of 0.5 mM for sulphite.

INTRODUCTION

There are two pathways for the oxidation of sulphite in the thiobacilli. One mechanism, proposed by Peck (1960; 1962), involves the formation of adenine 5'-sulphatophosphate (APS) from AMP and sulphite, catalysed by APS reductase. The APS is subsequently converted to ATP with a concomitant regeneration of AMP by the combined action of ADP-sulphurylase and adenylate kinase. The properties of purified APS reductases have been studied in *Thiobacillus denitrificans* (Bowen, Happold & Taylor, 1966) and *T. thioparus* (Lyric & Suzuki, 1970*b*).

The enzymic sulphite oxidation reported by other workers (London & Rittenberg, 1964; Charles & Suzuki, 1966*b*; Lyric & Suzuki, 1970*a*) does not require AMP and therefore involves a different mechanism. Charles & Suzuki (1966*b*) purified a sulphite oxidase from *Thiobacillus novellus* which required ferricyanide or cytochrome *c* as an electron acceptor. A similar enzyme was purified from *T. thioparus* (Lyric & Suzuki, 1970*a*). In contrast to these soluble enzymes, the sulphite oxidases from *T. thio-oxidans* (Adair, 1966; Kodama & Mori, 1968) and *T. concretivorus* (Moriarty & Nicholas, 1970) are bound to cell membranes.

We have shown that sulphite is oxidized either to APS in the soluble fraction or to sulphate in the membrane fraction of *Thiobacillus denitrificans* (Aminuddin & Nicholas, 1973). This paper presents data on the purification and properties of the membrane-bound sulphite oxidase.

METHODS

Culture and harvest of organism. *Thiobacillus denitrificans* was grown anaerobically as described by Adams, Warnes & Nicholas (1971). The culture was maintained at pH 7.0 by adding sterile 25% (w/v) K_2CO_3 to the cultures by a pH-stat unit to neutralize the nitrous acid produced. After 3 days' growth the cells were collected at 2 °C in a Sorvall

RC-2 refrigerated centrifuge fitted with a continuous-flow rotor. They were washed twice with either 50 mM-tris-HCl buffer (pH 7.5) or 50 mM-phosphate buffer (pH 7.0), both containing 0.2 mM-Na-EDTA.

Preparation of cell extracts. Washed bacteria (25 g wet wt/100 ml. buffer) suspended in either 50 mM-tris-HCl buffer (pH 7.5) or 50 mM-phosphate ($K_2HPO_4 \cdot KH_2PO_4$) buffer (pH 7.0), both containing 0.2 mM-EDTA, were passed twice through a French pressure cell at 20000 lb/in.², at 4 °C. The crude homogenate was centrifuged at 10000 g for 30 min and the supernatant fraction (S 10), hereafter referred to as the 'crude extract', was further centrifuged at 144000 g for 90 min, resulting in a supernatant, or 'soluble', fraction (S 144) and a particulate, or membrane, fraction (P 144). The latter was resuspended and dispersed in the appropriate buffer in a glass homogenizer. Both the S 144 and P 144 fractions were extensively dialysed against several changes of the appropriate buffer at 4 °C and the dialysed extracts were used.

Assay of sulphite oxidase. Sulphite oxidase activity was measured by the following methods.

Oxygen uptake. This was measured polarographically with a Beckman oxygen electrode (Model 39065) fitted with an adaptor box and a Beckman recorder. To 3.0 ml of 50 mM-tris-HCl buffer (pH 8.3, containing 0.2 mM-EDTA) in the reaction vessel equilibrated at 30 °C, was added by micro-syringe 0.1 ml each of 50 mM- Na_2SO_3 and cell extract (S 10, S 144 or P 144). The oxygen electrode was standardized by the method of Dixon and Kleppe (1965). Activity is expressed as nmol O_2 utilized/min/mg protein.

Nitrate reduction. The reaction mixture contained 5.0 μ mol Na_2SO_3 and 0.1 ml enzyme in a final volume of 2.0 ml, made up with 50 mM-tris-HCl (pH 8.3) containing 0.2 mM-EDTA. The reaction was initiated by adding the electron donor, the mixture incubated for 10 min at 30 °C and the reaction terminated with the nitrite reagents (Hewitt & Nicholas, 1968). After a further 15 min the extinction of the solution was measured at 540 nm. Enzyme activity was expressed as nmol nitrite formed in 10 min/mg protein.

Ferricyanide reduction. A modification of the APS reductase assay described by Peck, Deacon & Davidson (1966) was employed. The reaction mixture contained 10 μ mol Na_2SO_3 , 5 μ mol $K_3Fe(CN)_6$, and enzyme in a final volume of 3.0 ml made up with 50 mM-HCl (pH 8.3) containing 0.2 mM-Na-EDTA. The reaction, carried out in quartz cuvettes (1 cm), was initiated by adding sulphite to the sample cuvette. The reference cuvette contained all components except sulphite. Changes in extinction at 420 nm were followed in a Unicam SP-800 recording spectrophotometer equilibrated at 30 °C. Activity was expressed as μ mol ferricyanide reduced/h/mg protein.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Chemicals. Standard analytical reagent chemicals were dispensed in double-distilled water. Solutions of sodium sulphite were prepared fresh daily. AMP, bovine serum albumin, cytochrome *c* (horse heart), dithiothreitol and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) were obtained from Sigma; sodium diethyldithiocarbamate (DIECA) from Merck, Darmstadt, Germany; bathocuproin from AG, Buchs, Switzerland; mepacrine from Imperial Chemical Industries; DEAE-cellulose from Whatman Co.; and Sepharose-6B from Pharmacia, Uppsala, Sweden.

Table 1. *Distribution of sulphite-oxidizing enzyme systems in cell-free extracts*

Method	Specific activity		S 10	S 144	P 144
Oxygen uptake	nmol O ₂ utilized/h/mg protein	+ AMP	755	162	990
		– AMP	900	162	975
Fe(CN) ₆ ³⁻ reduction	μmol Fe(CN) ₆ ³⁻ reduced/h/mg protein	+ AMP	63	85	12.2
		– AMP	8	3	13.5
NO ₃ ⁻ reduction	μmol NO ₃ ⁻ produced/h/mg protein	+ AMP	0.3	0.02	0.8
		– AMP	0.2	0.01	0.8

The assays were conducted as described in Methods. The specific activities were determined in the presence and absence of AMP (5 μmol).

Table 2. *Effect of inhibitors on oxygen uptake during sulphite oxidation by P 144 fraction*

Inhibitor	Final concentration (mM)	Inhibition (%)
<i>p</i> CMB	0.1	69
	1.0	97
Iodoacetamide	1.0	79
KCN	0.1	90
Azide	1.0	77
DIECA	1.0	0
<i>o</i> -phenanthroline	1.0	5
Bathocuproin	1.0	0
Amytal	1.0	20
HOQNO	0.1	78
Arsenite	1.0	74
Nitrate	0.1	23
	1.0	43
	5.0	55
Nitrite	1.0	0
NH ₂ OH	1.0	0

Oxygen uptake was determined by the oxygen electrode at 30 °C. The inhibitor was incubated for 5 min prior to addition of sulphite. The specific activity of the control reaction (without inhibitor) was 21 nmol oxygen utilized/min/mg protein.

RESULTS

Sulphite oxidation by cell extracts

Crude extracts (S 10) of *Thiobacillus denitrificans* oxidized sulphite, as measured by nitrate or ferricyanide reduction or by oxygen uptake (Table 1). In the pellet fraction (P 144) the activity of the enzyme was not significantly affected by AMP. In the supernatant fraction (S 144), however, enzyme activity measured by reduction of ferricyanide increased with the addition of AMP. Sulphite oxidase activity measured in the absence of AMP by all three methods was much greater in the P 144 than the S 144 fraction, suggesting an AMP-independent enzyme associated with cell particles.

There was a concomitant uptake of O₂ by cell-free extracts during sulphite oxidation. Thus, in the crude extract and in the P 144 fraction there was a gradual uptake of oxygen reaching a maximum after 8 min. The S 144 fraction, however, had only 15% of this activity. There was very little O₂ uptake on adding either sulphite or cell extract (S 10 or P 144) and the rate was dependent on the relative amounts of reductant and enzyme. AMP did not affect oxygen uptake in any of the fractions.

Fig. 1. Effect of nitrate on oxygen uptake by a P 144 fraction at various concentrations of sulphite. The inset shows the increase of oxygen uptake with increasing concentrations of sulphite at various concentrations of nitrate. In the main diagram the same results are presented as double-reciprocal plots. Curve A: reaction mixture contained 3 mg protein of P 144 in 50 mM-tris-HCl (pH 8.3) containing 0.2 mM-Na-EDTA. Curve B: reaction mixture as in A except that P 144 was incubated with 0.1 mM- NaNO_3 for 5 min prior to adding SO_3^{2-} . Curves C and D: as for B, except that 1 mM- and 5 mM- NaNO_3 respectively were used.

The effect of inhibitors on oxygen uptake during sulphite oxidation by the P 144 fraction is shown in Table 2. Thiol-binding reagents, cyanide and azide were potent inhibitors, which suggests a requirement for sulphhydryl groups and the presence of a terminal oxidase. Inhibitors of the electron-transfer chain such as amytal, HOQNO and arsenite were also effective but DIECA, *o*-phenanthroline and bathocuproin had no effect. The inhibition by nitrate was found to be of the non-competitive type (Fig. 1). Nitrite and hydroxylamine, did not inhibit O_2 uptake.

Purification of sulphite oxidase

Crude extracts and particulate fractions (fractions I and II respectively) were prepared in 50 mM-phosphate buffer (pH 7.0) containing 0.2 mM Na-EDTA. The pellet fraction was washed and resuspended and its protein concentration adjusted to 30 mg/ml. Sodium deoxycholate (0.2 mg/mg protein) was added to the suspension after the pH was adjusted to 8.0 with 0.1 M-NaOH. After incubating at 30 °C for 30 min the extract was centrifuged at 105000 *g* for 30 min and the supernatant fraction (fraction III) dialysed extensively against the same buffer to remove residual deoxycholate. Fraction III was then loaded onto a Sepharose-6B column (50 × 2 cm) which had been equilibrated with 50 mM-phosphate buffer (pH 7.0) containing 0.2 mM-Na-EDTA, and this buffer was also used to elute the enzyme (Fig. 2). The enzyme was eluted in the void volume. The active fractions of the

Fig. 2. Elution of sulphite oxidase from Sepharose-6B. The solubilized pellet (fraction III) was loaded onto a Sepharose-6B column and eluted with 50-mM-phosphate buffer (pH 7.0) containing 0.2 mM-Na-EDTA. ○, E_{280} (1 cm cell); △, enzyme activity.

Fig. 3. Elution of sulphite oxidase from a DEAE-cellulose column. Fraction IV obtained by Sepharose gel-filtration chromatography was loaded on to a DEAE-cellulose column and eluted with a linear gradient 0.05 to 0.3 M- $\text{KH}_2\text{PO}_4:\text{K}_2\text{HPO}_4$ buffer (pH 7.0). ○, E_{280} ; △, enzyme activity.

Fig. 4. Effect of pH on sulphite oxidation. (a) Influence of pH on O₂ uptake in P 144 fraction. (b) Influence of pH on ferricyanide reduction in purified extract. ●, 50 mM-tris-HCl buffer; ○, 50 mM-borate-phosphate; △, 50 mM-phosphate buffer.

Table 3. Purification of sulphite oxidase

Fraction	Total activity (μmol Fe(CN) ₆ ³⁻ reduced/h)	Specific activity (μmol Fe(CN) ₆ ³⁻ reduced/h/mg protein)	Recovery	Relative purity
I. 10000 g supernatant of cell homogenate prepared in a French pressure cell	18230	3.8	100	1
II. 144000 g pellet fraction of I	16760	6.5	92	1.7
III. II solubilized with deoxycholate	11570	19.3	64	5.7
IV. III eluted from Sepharose-6B column	9190	40	50	10.6
V. IV eluted from DEAE-cellulose column	530	88.2	29	23
VI. 35–50% (NH ₄) ₂ SO ₄ precipitate of V	200	183	11	48

eluate (10 ml each) were pooled and concentrated by ultrafiltration under N₂ using a PM-10 membrane and ultrafiltration cell model 52 (Amicon, Massachusetts U.S.A.), thus obtaining fraction IV. A DEAE-32 cellulose column (3.2 × 14 cm) was equilibrated with 50 mM-phosphate buffer (pH 7.0) containing 0.2 mM-Na-EDTA. Fraction IV was then loaded onto the column and was developed with a linear phosphate gradient (0.05 to 0.3 M, pH 7.0) and 5.0 ml fractions collected. A representative elution pattern is shown in Fig 3. The activity appeared in effluent fractions when the phosphate concentration was around 0.18 M. The active fractions were pooled and dialysed extensively against 50 mM-phosphate buffer (pH 7.0) containing 0.2 mM-Na-EDTA. The dialysed fraction (fraction V) was then fractionated further with ammonium sulphate. The precipitate which formed between 35 and 50% saturation was collected, resuspended and dialysed against the same buffer.

A summary of the purification procedure is given in Table 3. An overall purification of about 50-fold was achieved.

Fig. 5. Effect of sulphite concentration on the purified enzyme. The inset shows the data from the main diagram plotted according to the method of Lineweaver-Burk.

Table 4. *Effect of various sulphydryl inhibitors on enzyme activity*

Compounds added	Final concentration (mM)	Inhibition (%)
<i>N</i> -ethylmaleimide	0.1	12
	1.0	34
<i>p</i> CMB	0.1	90
	1.0	100
Iodoacetamide	0.1	43
	1.0	75
<i>p</i> CMB plus dithiothreitol	1.0	0

The reaction was carried out, under standard conditions described in the text, using the ferricyanide method. The specific activity of the control reaction (without inhibitor) was 127 μmol ferricyanide reduced/h/mg protein.

Some properties of the purified enzyme

The purified sulphite oxidase did not utilize nitrate or oxygen as an electron acceptor. During the purification process, however, fractions III (solubilized pellet) and IV (Sephadex-6B eluate) retained activity with these two electron acceptors, although the specific activity was lower than that of crude extracts. The activity of the purified enzyme with either mammalian or yeast cytochrome *c* was only about 10% of that observed with ferricyanide even when the assay was conducted at pH 9.5 (Lyric & Suzuki, 1970a).

The relationship between activity and enzyme concentration was linear up to a protein concentration of 90 μg . AMP had no effect on the enzyme. Unlike the sulphite oxidases from *Thiobacillus novellus* (Charles & Suzuki, 1966b) and *T. thioparus* (Lyric & Suzuki,

Table 5. Comparison of APS reductase and sulphite oxidase from *Thiobacillus denitrificans*

Properties	APS reductase	SO ₃ ²⁻ oxidase
Distribution	Soluble	Particulate
Product	APS	Sulphate
Specific activity (μ mol ferricyanide reduced/hr/mg)	498	183
pH optimum	7.2	8.3
K_m for sulphite	1.5 mM	0.5 mM
Electron acceptors	O ₂ , Fe(CN) ₆ ³⁻	Fe(CN) ₆ ³⁻
Sulphydryl inhibitors	Not inhibited	Strongly inhibited

Data for APS reductase from Bowen *et al.* (1965).

1970a), and in agreement with the enzyme from *T. neapolitanus* (Hempfling, Trudinger & Vishniac, 1967), the enzyme from *T. denitrificans* was not inhibited by tris-HCl or phosphate buffers (pH 8.3) at concentrations of up to 75 mM.

The pH optimum of the enzyme reaction was around 8.3 (Fig. 4). This was observed both with the purified enzyme using the ferricyanide assay and with the P 144 fraction assayed by O₂ uptake. Adams *et al.* (1971) found that the optimal pH for sulphite oxidation linked to nitrate reduction was 8.5.

A plot of the effect of various sulphite concentrations on enzyme activity exhibits a typical substrate-concentration curve (Fig. 5). The K_m for sulphite was found to be 0.5 mM for the purified enzyme using the ferricyanide assay and 0.4 mM for the P 144 fraction by the O₂ uptake method.

A starch-gel electrophoretogram of the purified enzyme indicates one major, fast-moving protein band and two slow-moving minor bands. The absolute spectrum of the purified enzyme indicates a major peak at 280 nm and small peaks at 410 and 550 nm associated with traces of cytochrome *c*.

As shown in Table 4, thiol-binding reagents are potent inhibitors of the enzyme. However, the effect may be completely reversed by dithiothreitol.

DISCUSSION

Both AMP-dependent and AMP-independent sulphite-oxidizing enzymes are present in *Thiobacillus denitrificans*. They are located in different parts of the cell; thus sulphite oxidase is associated with membrane fractions, whereas APS reductase is found in the more soluble fractions. A comparison of the properties of purified sulphite oxidase with those of purified APS reductase from *T. denitrificans* is presented in Table 5.

The particulate sulphite oxidase is linked to an electron-transport chain with either oxygen or nitrate as a terminal electron acceptor. Evidence for this is provided in our studies with inhibitors of electron transport, which agree well with the results of Adams *et al.* (1971). The inhibition of oxygen uptake by nitrate further indicates that the two terminal electron acceptors are associated with the same electron-transport chain. The cytochrome components involved in sulphite oxidation will be discussed in a subsequent paper (Aminuddin & Nicholas, 1974).

The strong inhibition of the purified enzyme by thiol-binding reagents and the complete reversal by dithiothreitol suggest the involvement of -SH groups. The properties of sulphite oxidase from *T. denitrificans* are compared with those from other thiobacilli in Table 6.

Table 6. *AMP-Independent sulphite oxidizing enzymes from various thiobacilli*

Source	Distribution	Effect of AMP	Electron acceptors	Degree of purification	pH optimum	κ_m for SO_3^{2-}	Reference
<i>Thiobacillus thio-parus</i>	Soluble	Inhibited by 21 % (cytochrome <i>c</i> coupled)	Cytochrome <i>c</i> $\text{Fe}(\text{CN})_6^{3-}$	163-fold (cytochrome <i>c</i> coupled)	8.0 (cytochrome <i>c</i> coupled)	88 μM	Lyric & Suzuki (1970 <i>a</i>)
<i>T. thio-oxidans</i>	Particulate	—	O_2	Not purified	6.9 (O_2 uptake)	—	London & Rittenberg (1964); Adair (1968); Kodama & Mori (1968)
<i>T. novellus</i>	Soluble	Inhibited by 25 % (cytochrome <i>c</i> coupled)	O_2 $\text{Fe}(\text{CN})_6^{3-}$ Cytochrome <i>c</i>	74-fold (cytochrome <i>c</i> coupled)	8.0	20 μM 40 μM (cytochrome <i>c</i> coupled)	Charles & Suzuki (1966 <i>a, b</i>)
<i>T. neapolitanus</i>	Soluble	Stimulated by 80 %	O_2	100-fold (O_2 uptake)	—	—	Hempfling <i>et al.</i> (1967)
<i>T. concretionivorus</i>	Particulate	Not affected	O_2 $\text{Fe}(\text{CN})_6^{3-}$	100-fold	7.0	1 mM 0.3 mM (O_2 uptake)	Moriarty (1969)
<i>T. denitrificans</i>	Particulate	Not affected	O_2 $\text{Fe}(\text{CN})_6^{3-}$ NO_3^-	48-fold	8.3	0.5 mM 0.4 mM (O_2 uptake)	Present study

Activity determined by the ferricyanide method, unless otherwise stated.

Trudinger (1967; 1969) proposed a mechanism involving the formation of an enzyme-sulphite complex which could react either with water to produce sulphate or with AMP to form APS. He further suggested that sulphite oxidase is a modified APS reductase which has lost the ability to react with AMP. Some evidence for this idea was provided by Hempfling *et al.* (1967), who purified sulphite oxidase from *Thiobacillus neapolitanus* and found that the relative activities with and without AMP remained fairly constant through the purification procedure. These authors concluded that the same enzyme was responsible for both the AMP-dependent and AMP-independent activities. However, our results and those of Lyric & Suzuki (1970*a, b*) suggest unequivocally that APS reductase and sulphite oxidase are distinct enzymes.

The physiological significance of two enzymes for utilizing sulphite is not clear. Lyric & Suzuki (1970*c*) considered that two enzymes operating at different pH optima (7.5 and 9.5) would be more effective in oxidizing sulphite in *Thiobacillus thio-parus*. In *T. denitrificans* the association of sulphite oxidase with membrane fractions may be of particular significance, since the enzyme is closely associated with a sulphide oxidase, a thiosulphate-cleaving enzyme and the denitrifying enzymes nitrate and nitrite reductase, all of which are membrane bound.

One of us (M.A.) is grateful to the University of Agriculture, Malaysia, for financial assistance.

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