Purification and properties of methyl mercaptan oxidase from *Thiobacillus thioparus* TK-m

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Methyl mercaptan (MM)-oxidase was purified to near homogeneity from *Thiobacillus thioparus* TK-m grown on dimethyl sulphide. The enzyme was a monomer with an M_r value of approximately 40 000. It oxidized MM stoichiometrically to formaldehyde, S^{2-} and H_2O_2 . The enzyme had a K_m of 31.3 μM for MM. It was also shown to oxidize ethyl mercaptan. MM-oxidase from *T. thioparus* was shown to have some different characteristics from MM-oxidase of *Hyphomicrobium* EG, such as a higher K_m , the absence of inhibition by S^{2-} and the inability to catalyse the oxidation of S^{2-} .

Introduction

Methyl sulphides such as dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and methyl mercaptan (MM) are among the major volatile organic sulphur compounds produced in nature (Andreae & Raemdonck, 1983; Bremner & Steele, 1978; Lovelock *et al.*, 1972; Nguyen *et al.*, 1978). Several strains of *Hyphomicrobium* (de Bont *et al.*, 1981; Suylen & Kuenen, 1986; Suylen *et al.*, 1986) and *Thiobacillus* (Kanagawa & Kelly, 1986; Kanagawa & Mikami, 1989; Sivela & Sundman, 1975; Smith & Kelly, 1988) have been shown to metabolize methyl sulphides.

De Bont *et al.* (1981) isolated a *Hyphomicrobium* sp. capable of growth on dimethyl sulphoxide (DMSO) and DMS. They suggested that DMSO metabolism involves an initial reduction of the substrate to DMS which is subsequently oxidized to formaldehyde and MM. MM is further oxidized to formaldehyde and S^{2-} . One of the key reactions in this pathway is the oxidation of MM. Suylen *et al.* (1987) purified MM-oxidase to near homogeneity from *Hyphomicrobium* grown on DMSO. This enzyme catalysed the oxidation of MM to formaldehyde and S^{2-} with the production of H_2O_2 . Ethyl mercaptan and S^{2-} were also oxidized by the enzyme, although S^{2-} was a non-competitive inhibitor for MM oxidation.

T. thioparus TK-m grew on MM, DMS, DMDS and H_2S and stoichiometrically oxidized them to SO_4^{2-}

(Kanagawa & Mikami, 1989). This strain was found to be very useful for removing those compounds from contaminated air (Tanji *et al.*, 1989). The objectives of this study were to purify and characterize MM-oxidase from *T. thioparus* TK-m.

Methods

Culture conditions. Cells of *T. thioparus* TK-m were obtained from a continuous culture column (60 × 570 mm) to which was added 100 ml of inoculum and 1100 ml of medium C (Kanagawa & Kelly, 1986) containing: 2 mM- $Na_2S_2O_3$, trace metal solution (4 ml l⁻¹) and NH_4Cl (1.8 g l⁻¹). The column was run for 3 d at 25 °C using $Na_2S_2O_3$ as a substrate, DMS gas was introduced into the column and then the concentration of DMS was increased as the cells grew. After 1 week, continuous culture ($D = 0.028$ h⁻¹) was begun and this was carried on for 25 d under aseptic conditions. At regular intervals the cells were harvested by centrifugation, washed with 26 mM-potassium phosphate buffer (pH 6.8) and stored at -80 °C.

Chemicals. Horseradish peroxidase type VI-A, reagents for protein determination and protein standards for M_r determination were all obtained from Sigma. DEAE-Sephacel was obtained from Pharmacia LKB and Bio-Gel P60 from Bio-Rad; SDS-PAGE gels were obtained from Daiichi Pure Chemicals (Tokyo, Japan). *N*-Ethyl maleimide (NEM), 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate and 3-(dimethylamino)benzoic acid were obtained from Wako Chemical Co. (Osaka, Japan). MM gas (2 ml and 5 ml l⁻¹ of nitrogen) and DMS gas (2 ml l⁻¹ of nitrogen) in 40 l gas tanks were obtained from Seitetsu Chemical Industries (Osaka, Japan). All other chemicals were of reagent grade.

Purification of MM-oxidase. Approximately 1.2 g (dry wt) of *T. thioparus* TK-m cells were washed with 50 ml 50 mM-Tris/HCl, pH 8.2, centrifuged, suspended in 30 ml 10 mM-Tris/HCl, pH 8.2, and sonicated five times for 30 s at full power (200 W) (UD-201; Tomy

Abbreviations: DMS, dimethyl sulphide; DMDS, dimethyl disulphide; DMSO, dimethyl sulphoxide; MM, methyl mercaptan; NEM, *N*-ethyl maleimide.

Seiko Co., Tokyo, Japan). The cell suspension was made up to 60 ml with 10 mM-Tris/HCl, pH 8.2, calcium chloride was added to a final concentration of 2.0 mM and the cell suspension was centrifuged at 20000 *g* for 40 min.

The crude extract was made up to 100 ml in 10 mM-Tris/HCl, pH 8.2, containing a final concentration of 40 mM-KCl. The extract was then eluted through a column (20 × 2.5 cm) containing DEAE-Sephacel. The column was then washed with 100 ml of the elution buffer. The eluate plus washing buffer were combined and eluted through a second DEAE-Sephacel column of the same dimensions. The second column was also washed with 100 ml of the elution buffer. The eluate plus washing buffer were combined and concentrated tenfold using an Amicon ultrafiltration unit with a PM10 membrane filter. The extract was made up to 200 ml with 10 mM-Tris/HCl, pH 8.2, and concentrated tenfold. This procedure was repeated and 10 ml of partially purified extract were obtained; 7 ml of this extract were applied to a gel (Bio-Gel P60) filtration column (100 × 2.6 cm) and eluted with 50 mM-Tris/HCl, pH 8.2, containing 100 mM-KCl at a flow rate of 8.0 ml h⁻¹.

M_r and purity check. The *M_r* of the enzyme was determined by gel permeation chromatography (Bio-Gel P60 gel) and FPLC. FPLC was done using 50 mM-Tris/HCl buffer, pH 8.2, containing 200 mM-KCl at a flow rate of 0.5 ml min⁻¹ and a Pharmacia Superose-12 column. The subunit *M_r* was determined by SDS-PAGE (Laemmli, 1970). Protein was determined by the Lowry procedure.

Enzyme assays. The substrate was prepared by first bubbling nitrogen gas through 10 mM-potassium phosphate buffer, pH 7.5, in a sealed vial for 0.5 h. Then MM (5 ml l⁻¹ in nitrogen) was bubbled through the same buffer for 3.0 h. The MM concentration was determined using a total organic carbon analyser (TOC-500; Shimadzu). MM concentrations between 1.3 and 1.5 mM were consistently obtained by this method. Enzyme assays were done in duplicate by determination of formaldehyde, the initial product of MM oxidation. A 30 s assay time was used, the reaction was stopped by addition of an equal volume of 1.3% perchloric acid and formaldehyde was determined colorimetrically (Nash, 1953). All enzyme assays were done in 50 mM-Tris/HCl, pH 8.2, at 25 °C using 30 µg enzyme ml⁻¹ with an initial substrate concentration of 75 µM. The activity of MM-oxidase towards several other substrates was also measured using an oxygen electrode (Oxygraph-8; Central Kagaku Co., Tokyo, Japan). The assay was done at 30 °C in a 2.8 ml cell containing 40 µg of enzyme and 540 nmol MM dissolved in 50 mM-Tris/HCl, pH 8.2. The reaction was initiated by addition of enzyme. The effect of pH on MM-oxidase activity was determined using 25 mM-citric acid/sodium phosphate buffer over the pH range 3.0 to 8.0 and 25 mM-glycine/sodium hydroxide buffer over the range 8.6 to 10.6.

Stoichiometry of MM oxidation. Serum vials (68.8 ± 0.6 ml vol.) were soaked in 0.1 M-HCl for 2 h, washed with distilled water, dried at 105 °C and then the inside of each vial was coated with phosphoric acid using 150 mM-H₃PO₄ in acetone. To each vial 3.0 ml 50 mM-Tris/HCl containing 0.2 mM-EDTA, pH 8.2, was added and the vial was sealed with a Teflon-coated rubber stopper which reduced the vial volume by

0.4 ml. To the sealed vials 239 nmol MM was added by replacing 3.0 ml of head-space gas with 3.0 ml MM gas (2 ml l⁻¹ of nitrogen) and the vials were shaken at 25 °C for more than 10 min. The reaction was initiated by injecting 50 µl of enzyme solution dissolved in 50 mM-Tris/HCl containing 0.1 mM-EDTA, pH 8.2, and the vials were shaken at 25 °C. The reaction was terminated by the injection of 0.1 ml 2.0 M-H₂SO₄. MM and H₂S in the head-space gas were determined by gas chromatography (Shimadzu GC-14A) (Kanagawa & Mikami, 1989). The total amount of MM and H₂S in the vial was estimated by comparing the experimental data with concentrations obtained by adding various amounts of MM and H₂S to the vials followed by the addition of H₂SO₄. Samples (2 ml) of the liquid in the vials were transferred to test-tubes and formaldehyde was determined colorimetrically (Nash, 1953).

To estimate H₂O₂ production the sealed serum vial containing the complete reaction mixture was shaken at 25 °C for 20 min to complete MM oxidation and 0.6 ml of the liquid was transferred to a test-tube. The pH was adjusted to 7 by the addition of 12 µl 1 M-HCl and H₂O₂ was determined colorimetrically by the procedure of Ngo & Lenhoff (1980).

Inhibition of MM-oxidase. Various inhibitors were pre-incubated for 20 min with MM-oxidase. MM-oxidase activity was then assayed (by measurement of formaldehyde production) using 60 µg of enzyme in a 2.0 ml reaction mixture at a temperature of 25 °C, a substrate concentration of 75 µM and a time duration of 2.0 min.

Induction of MM-oxidase. *T. thioparus* TK-m was grown at 25 °C in a glass column (100 × 600 mm) to which was added 100 ml of inoculum and 2.5 l of medium C containing 0.8 mM-Na₂S₂O₃. The pH of the culture was maintained at 6.7 by addition of 0.4 M-K₂CO₃. When 0.4 M-K₂CO₃ was added, twice the volume of 0.2 M-Na₂S₂O₃ was also added simultaneously. After 4 d addition of Na₂S₂O₃ was stopped, the culture was made up to 3.1 l by addition of distilled water and then DMS gas (2 ml l⁻¹ of nitrogen) was added at a flow rate of 20 ml min⁻¹. The total volume of 0.2 M-Na₂S₂O₃ added was 200 ml. At various times 100 ml of the culture was removed and centrifuged. The harvested cells were washed once and suspended in 3 ml 50 mM-Tris/HCl, pH 8.2. The crude extracts of the cells were sonicated, prepared for SDS-PAGE and the MM-oxidase activity was measured.

Results and Discussion

Purification and physical characteristics of MM-oxidase

A typical purification procedure is shown in Table 1. The crude extract showed a lower total activity than the eluate of the DEAE-Sephacel column, probably due to the presence of inhibitors contained in the crude extract. MM-oxidase activity readily elutes through the DEAE-Sephacel column at pH 8.2, which results in an effective

Table 1. *Purification of methyl mercaptan oxidase*

	Vol. (ml)	Total units (µmol min ⁻¹)	Total protein (mg)	Specific activity [µmol min ⁻¹ (mg protein) ⁻¹]	Purification ratio	Yield (%)
Crude extract	60	33.3	794	0.042	1	100
DEAE-Sephacel	10	41.8	39.8	1.05	25	126
Gel filtration	21	21.6	17.0	1.27	30	65

Fig. 1. SDS-PAGE of extracts of *T. thioparus* TK-m. ST, M_r standards. Lanes 1–3 show the purification steps. Lane 1, crude extract; lane 2, DEAE-Sephacel; lane 3, gel filtration. Lanes 4–9 show the induction of MM-oxidase by changing the substrate from $\text{Na}_2\text{S}_2\text{O}_3$ to DMS. Lane 4, cells grown on $\text{Na}_2\text{S}_2\text{O}_3$; lane 5, 2.5 h after changing to DMS; lane 6, 5.0 h; lane 7, 7.5 h; lane 8, 20 h; lane 9, 44 h.

and convenient initial purification step that also provides a high yield of the enzyme. One major difference between the enzymes obtained from the two different sources is that *T. thioparus*/MM-oxidase did not bind to DEAE-Sephacel whereas the enzyme from *Hyphomicrobium* did (Suylen *et al.*, 1987). This suggests that there are significant differences in the surface charges of the two enzymes. The enzyme co-eluted with carbonic anhydrase on the Bio-Gel P60 column indicating an M_r value of approximately 29000. This result was also confirmed by FPLC. On SDS-PAGE the enzyme proved to be nearly homogeneous with a subunit M_r of approximately 40000 (Fig. 1). The higher M_r value obtained by SDS-PAGE compared to those obtained by FPLC and gel filtration is unusual. The low surface charge of the enzyme from *T. thioparus* may be due to it having a hydrophobic surface which results in hydrophobic interactions during chromatography which would increase the retention time of the enzyme. In spite of these conflicting results it appears that the enzyme is a monomer. The M_r value of 40000 for MM-oxidase from *T. thioparus* is in the same range as the value of 40–50000 reported for MM-oxidase from *Hyphomicrobium* EG (Suylen *et al.*, 1987). Maximum enzyme activity was observed between the pH values of 8.0 and 9.6. The K_m of MM-oxidase at pH 8.2 was calculated to be $31.3 \mu\text{M}$ and substrate inhibition of the enzyme at high MM concentrations was observed. The K_m value of $31.3 \mu\text{M}$ for MM-oxidase from *T. thioparus* is significantly higher than the value of $10 \mu\text{M}$ reported for

the enzyme from *Hyphomicrobium* EG. However, the enzyme from both sources appears to be inhibited by high substrate concentrations.

Stoichiometry

MM was converted to H_2S stoichiometrically (Table 2). The reaction was completed within 20 min. The production of formaldehyde coincided with the production of H_2S and H_2O_2 was also detected in the solution. No oxidation of S^{2-} by MM-oxidase was observed during the stoichiometry experiment (Table 2). The recovery of formaldehyde was 71–78% and that of the H_2O_2 was 60% if the enzyme catalysed the following reaction:

Table 2. Stoichiometry of MM oxidation
MM (239 nmol) was added to 3.0 ml of reaction mixture.

Time (min)	MM (nmol)	H_2S (nmol)	Formaldehyde (nmol)
0	231	0	0
2	177	51	44
4	123	115	88
6	89	153	117
8	76	166	126
10	57	194	142
20	0	223	183

Substrate specificity of MM-oxidase

Oxygen consumption occurred in the presence of both MM and ethyl mercaptan but no activity was observed when either methylamine or methanol were used as substrates. Oxygen consumption equivalent to 90% of the added MM occurred after the enzyme had been incubated with the substrate for 10 min. The consumption of oxygen concurrent with the oxidation of MM to S^{2-} , formaldehyde and H_2O_2 and the stoichiometry of the reaction are consistent with the behaviour of MM-oxidase. Oxygen uptake experiments indicate that MM-oxidase from *T. thioparus* is specific for mercaptans with no activity towards amines or alcohols which has also been observed for the enzyme from *Hyphomicrobium* sp. H_2S , which is also a substrate for the enzyme from *Hyphomicrobium* sp. was not oxidized by MM-oxidase from *T. thioparus*.

Effect of inhibitors on MM-oxidase activity

Methylamine and S^{2-} had no effect on MM-oxidase activity (Table 3). However, mercaptoethanol, ethyl mercaptan, cyanide and ammonium salts were shown to inhibit the enzyme. MM-oxidase was also inhibited by NEM, but with the exception of Cu^{2+} , metal ions had no effect on the enzyme. The differences in the magnitude of inhibition by various inhibitors of MM-oxidase from the two sources implies that there may be differences in the structure of the active site between the two enzymes. Another difference between the enzyme from the two different sources is the lack of inhibition by one of the main products, S^{2-} . The lack of inhibition by S^{2-} is also

Table 3. Sensitivity of MM-oxidase from *T. thioparus* towards various known enzyme inhibitors

In the absence of any inhibitor, MM-oxidase activity was 23 nmol MM oxidized $ml^{-1} min^{-1}$.

Compound	Inhibition	Concn (mM)	Inhibition (%)
Mercaptoethanol	+	0.5	53
Ethyl mercaptan	+	1.7	75
Methylamine	—	1.0	—
DMS	—	1.0	—
CH_3OH	—	100.0	—
S^{2-}	—	1.0	—
$Na_2S_2O_3$	—	5.0	—
$(NH_4)_2SO_4$	+	10	53
NH_4Cl	+	10	53
K_2SO_4	—	50	—
KCN	+	0.5	58
EDTA	—	1.0	—
NEM	+	0.1	64
$CuSO_4$	+	0.1	55
$CdCl_2$	—	1.0	—
$ZnSO_4$	—	1.0	—
$NiSO_4$	—	1.0	—

consistent with the fact that the enzyme from *T. thioparus* completely oxidizes MM whereas approximately 50% of the added substrate is oxidized by the enzyme from *Hyphomicrobium* sp. The inhibition of enzyme activity by NEM is consistent with the presence of an -SH group at the active site; however the lack of inhibition by most of the metal ions tested is not consistent with the involvement of an -SH group in catalysis. NEM has also been shown to react with α -amino functional groups (Plapp, 1982). Thus, our studies also have not yet provided definitive evidence for the prosthetic groups of MM-oxidase.

Induction of MM-oxidase activity

The crude extract obtained from cells grown on $Na_2S_2O_3$ had little MM-oxidase activity. When the substrate was changed from $Na_2S_2O_3$ to DMS, the MM-oxidase activity increased 1.6-, 2.4-, 3.9-, 9.4- and 12-fold after 2.5, 5.0, 7.5, 20 and 44 h respectively. The corresponding protein band on SDS-PAGE increased in size after the addition of DMS (Fig. 1). The MM-oxidase protein band after 20 h induction was almost the same size as the band for DMS-grown cells. Thus MM-oxidase activity is inducible.

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