Evidence for the Existence of a Sulphur Oxygenase in *Sulfolobus brierleyi*

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A sulphur-oxidizing enzyme was purified to homogeneity from cell-free extracts of mixotrophically grown cells of *Sulfolobus brierleyi*. The enzyme catalysed the oxidation of elemental sulphur to sulphite and had an M_r of 560 000. Only one type of subunit (M_r 35 000) could be detected. The pH and temperature optima for activity were 7.0 ± 0.5 and 65 °C respectively and an apparent K_m for sulphur was calculated to be 0.05 M. Enzyme assays with ^{18}O-enriched oxygen demonstrated directly that atmospheric oxygen was attached to sulphur. The first detectable reaction product was sulphite. These results provide evidence that sulphur oxidation in *S. brierleyi* is performed by an enzyme of the oxygenase type.

INTRODUCTION

Sulfolobus brierleyi belongs to the thermophilic archaebacteria. It grows at temperatures between 45 °C and 70 °C and at pH values down to 1·0 (Zillig *et al.*, 1980). Its favoured habitats are hot sulphur springs (Brierley & Brierley, 1973). *S. brierleyi* is able to derive energy from the oxidation of elemental sulphur to sulphate under mixotrophic and autotrophic growth conditions. Under mixotrophic conditions several organic substances are metabolized, whilst under autotrophic growth conditions carbon dioxide is fixed via a reductive carboxylic acid pathway (Kandler & Stetter, 1981). The mechanism of sulphur oxidation has not yet been elucidated.

In contrast, sulphur oxidation in mesophilic thiobacilli has been thoroughly investigated. According to Kelly (1982), in thiobacilli elemental sulphur is oxidized to sulphate via sulphite. For *Thiobacillus thioparus*, *T. thiooxidans* and *T. neapolitanus* it has been proposed that the oxidation of elemental sulphur is linked to an enzyme of the oxygenase type with molecular oxygen as electron acceptor (Suzuki, 1965*b*; Suzuki & Silver, 1966; Taylor, 1968). In the facultative anaerobe *T. denitrificans* (Schedel & Trüper, 1980) another pathway of sulphur oxidation is used. Molecular oxygen is not involved and sulphur is oxidized under anaerobic conditions by a sulphite reductase, which contains sirohaem as a prosthetic group.

In the present paper we show that, although *S. brierleyi* belongs to the archaebacteria, the oxidation of elemental sulphur in this organism is catalysed by an enzyme of the oxygenase type, which also appears to occur in some aerobic thiobacilli.

METHODS

Organism and growth conditions. A *Sulfolobus* strain isolated from hot acid springs in Yellowstone National Park, USA, was used. The cells of the isolate had an irregular polyhedral form with a diameter of about 1 μm. Best growth occurred between pH 1 and pH 2 and at temperatures from 65 °C to 70 °C. The G + C content of its DNA was 30 ± 1 mol%. On account of these properties and according to the classification of Zillig *et al.* (1980) the isolate was identified as *S. brierleyi*. It was grown mixotrophically in a nutrient solution according to Mackintosh

(1978) without Fe(II) and supplemented with 0·05% yeast extract and 1% (w/v) elemental sulphur. The growth temperature was 65 °C and the pH was adjusted to 1·8. Stock cultures were kept at 65 °C in 100 ml Erlenmeyer flasks that contained 40 ml growth medium. The stock cultures were transferred every 2 weeks. For biochemical experiments cells were grown in 1 l Erlenmeyer flasks that contained 400 ml medium. Cultures were incubated on a rotary waterbath shaker at 220 r.p.m. and a temperature of 65 °C. The cells were harvested at the end of the exponential growth phase.

Enzyme purification. Cells (1 g dry wt) were suspended in 10 ml of a mineral salts solution (Mackintosh, 1978) adjusted to pH 7·0 and disrupted in a French pressure cell at 20000 lbf in^{-2} (138 MPa). The crude extract was centrifuged at 25000 g and 4 °C for 1 h. The supernatant was layered on top of a linear 25–45% (w/v) sucrose gradient, which was centrifuged at 300000 g and 4 °C for 4 h. The fraction containing sulphur-oxidation activity was isolated by using a density gradient fractionator (model 185, Isco, Superior, USA) with the monitor UA 5 (Isco) at a wavelength of 280 nm. This fraction was further purified by non-denaturing PAGE (3–15%, w/v, polyacrylamide gradient). The composition of the gels was: 0·4 M-Tris/HCl, 0·25% sodium deoxycholate and an acrylamide:bisacrylamide ratio of 30:0·8. Electrophoresis was done for 12 h at 120 V.

After electrophoresis, the position of the enzyme on the gel was determined with a gel scanner at a wavelength of 280 nm. The gel area containing active enzyme was removed and transferred into a dialysis tube together with 2 ml 0·0025 M-Tris/0·5 M-glycine buffer adjusted to pH 8·4 and containing 0·25% sodium deoxycholate. The dialysis tube was fixed in the middle of a 20 × 10 × 5 cm horizontal gel chamber filled with the same buffer. The enzyme was eluted from the gel by an electric field of 15 V cm^{-1} for 3 h.

M_r *determination.* The subunit composition of the purified enzyme was determined by SDS-PAGE (3–15%, w/v, gradient). The composition of the gels was: 0·4 M-Tris/HCl, 0·1% SDS and an acrylamide:bisacrylamide ratio of 30:0·8. A low and a high M_r calibration kit (14000–94000 and 67000–660000, Pharmacia) were used for standards. Gels were stained and destained according to Weber & Osborn (1969).

Enzyme assays. Assays for the determination of sulphur-oxidation activity were done at 65 °C in test tubes shaken at 300 r.p.m. The reaction mixture contained 1 ml 70 mM-Tris/H$_2$SO$_4$ buffer adjusted to pH 7·0 with 0·01 g sulphur and 0·005% Tween 20 to disperse the sulphur powder. Sulphur was powdered by grinding with a mortar and pestle. The reaction was started by the addition of enzyme or cell fractions. The increase in sulphite concentration during assays was measured at intervals of 5 min; the incubation time was 1 h. The specific activity was expressed as nmol sulphite formed min^{-1} (mg protein)$^{-1}$. Reaction velocity was calculated by the linear increase in sulphite concentration.

Analytical procedures. Sulphite concentration was determined according to Grant (1947) and thiosulphate concentration according to Anonymous (1984). Sulphate concentration was determined turbidimetrically according to Dugan & Apel (1978).

Mass spectrometry. For mass spectrometric investigations enzyme assays were done in 10 ml Hungate tubes containing 1 ml of the reaction mixture and sealed with rubber stoppers. The atmosphere in the tubes was replaced with ^{18}O-enriched (98%) oxygen (Ventron). As controls, tubes with an ^{16}O atmosphere (Messer-Griesheim) were used. Incubation time was 1 h after addition of the enzyme. The sulphite formed was analysed by mass spectrometry after acid addition and conversion to sulphur dioxide. A Hewlett-Packard 5985 A instrument with a capillary gas chromatographic inlet was employed in the 'single ionmonitoring' mode. The gas chromatograph (Hewlett-Packard model 5840 A) was operated at 40 °C with splitless injection.

RESULTS

Purification of the sulphur-oxidizing enzyme

Our aim was to investigate the mechanisms of sulphur oxidation in *S. brierleyi*. The products from the oxidation of elemental sulphur were analysed for an increase in the concentration of sulphite, thiosulphate or sulphate. Cell-free extracts of *S. brierleyi* formed 1800 nmol sulphite and 50 nmol thiosulphate (mg protein)$^{-1}$ h^{-1}. No increase in the sulphate concentration was detectable. Hence the production of sulphite was taken as an indication of sulphur oxidation. In a first purification step, cell-free extracts of *S. brierleyi* were centrifuged at 25000 g for 1 h. The supernatant was further purified by density-gradient centrifugation. The absorption spectrum of samples from the gradient showed three peaks (Fig. 1). Fraction 6 (corresponding to peak A) had a faint green colour, whilst fractions 9 (B) and 10 (C) were colourless. Sulphur oxidation activity, which could only be measured in fraction 10, was further purified by PAGE.

The electrophoretic pattern of fraction 10 showed one major protein band (ii) and three minor protein bands (i), (iii) and (iv) (Fig. 2a). The different protein bands were eluted from the gel and assayed for sulphur-oxidizing activity. Only protein band (ii) was able to oxidize elemental sulphur. During purification, the specific activity increased from 30 U (mg protein)$^{-1}$ (U =
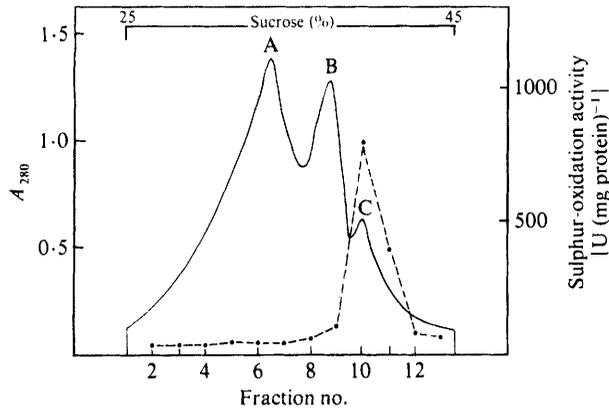
Fig. 1. Distribution of sulphur-oxidation activity in a linear sucrose gradient. A cell-free extract was centrifuged at 25000 *g* for 1 h and the resultant supernatant layered on to a 25–45% (w/v) sucrose gradient. After centrifugation at 300000 *g* for 4 h, fractions (1 ml) were taken from the gradient and A_{280} (——) and sulphur-oxidation activity (----) measured for each. One unit (U) of activity is 1 nmol sulphite formed min^{-1}.
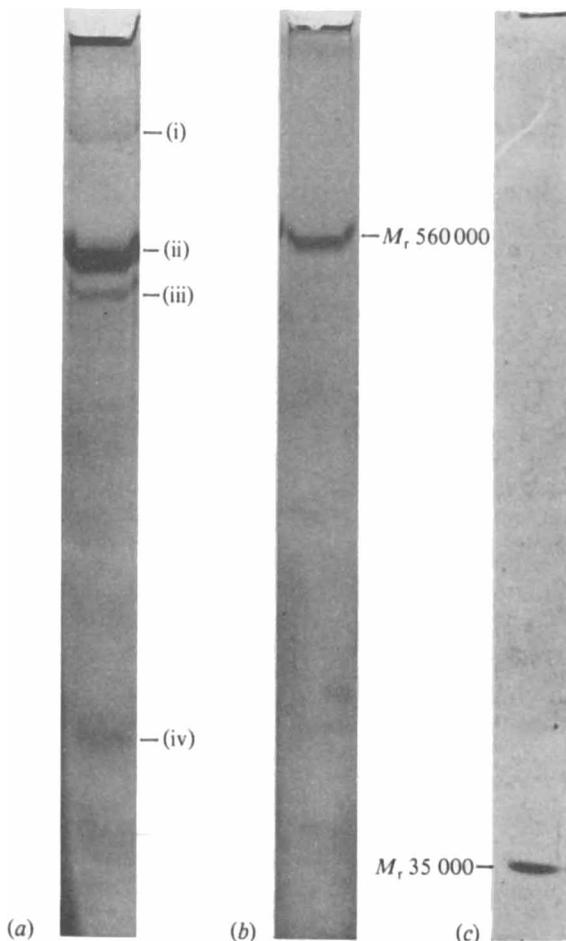

Fig. 2. (*a*) Electrophoretic pattern of fraction 10 (Fig. 1) in a 3–15% (w/v) polyacrylamide gradient gel run under non-denaturing conditions; (*b*) pattern of the active extract of protein band (ii) under the same conditions as (*a*); and (*c*) pattern of protein band (ii) on an SDS-polyacrylamide (3–15%) gradient gel.
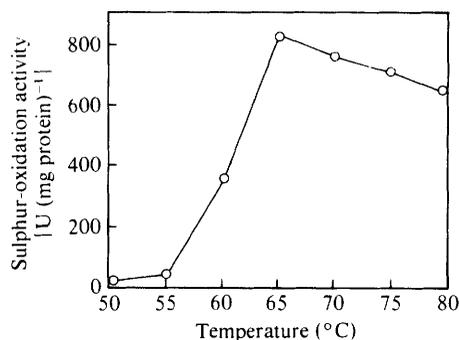
Fig. 3. Temperature optimum curve for sulphur-oxidation activity.

Table 1. *Scheme for the purification of the sulphur-oxidizing enzyme of S. brierleyi*

One unit of activity (U) is equal to 1 nmol sulphite produced min^{-1}. Results are from a representative experiment.

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Activity yield (%)	Purification (fold)
Cell-free extract	460	14000	30	100	0
Supernatant (25000 *g*)	100	9000	90	64	3
Fraction 10*	5	4200	840	30	28
Protein band (ii) after PAGE	1	900	910	7	31

* From the density-gradient centrifugation (Fig. 1).

nmol sulphite formed min^{-1}) in cell-free extracts of *S. brierleyi* to 910 U (mg protein)$^{-1}$ in the highly purified preparation (Table 1).

Properties of the purified enzyme

To elucidate the size, homogeneity and structure of the sulphur-oxidizing enzyme system, non-denaturing PAGE was done on the extract of protein band (ii) (Fig. 2). Under these conditions one single band corresponding to an M_r of 560000 was visible (Fig. 2*b*). In order to demonstrate the number and M_r of the subunits of the enzyme system, extracts of protein band (ii) were treated with SDS and then analysed by SDS-PAGE. Under these conditions, one protein band was visible, of apparent M_r 35000 (Fig. 2*c*).

In a further experiment the temperature optimum for activity was determined. Sulphite formation was demonstrable above temperatures of 55 °C; the optimum for activity was 65 °C, whilst higher temperatures were inhibitory (Fig. 3). The pH optimum was 7·0. An apparent K_m of 0·05 M for elemental sulphur was calculated from enzyme assays done with different quantities of substrate.

Mechanism of sulphur oxidation

The conversion of elemental sulphur to sulphite is an oxidative process. In an enzymic oxidation reaction the oxygen bound in the end-product may originate from atmospheric oxygen as well as from the oxygen bound in water. In order to identify the origin of the oxygen in the sulphite molecule, enzyme assays were done in an ^{18}O-enriched oxygen atmosphere. The sulphite formed during assay was converted to sulphur dioxide and analysed by mass spectrometry for incorporation of ^{18}O. The samples were analysed for the relative amounts of molecules with ^{16}O and ^{18}O atoms, i.e. M_r 64 corresponded to $^{32}S^{16}O^{16}O$, M_r 66 to $^{32}S^{18}O^{16}O$ and M_r 68 to $^{32}S^{18}O^{18}O$ (without regard to minor and constant contributions of the natural ^{34}S isotope) (Table 2). Enzyme assays contained on average 0·1 mg sulphite ml^{-1} at the beginning of the incubation (t_0). The sulphite concentration increased to an average of 0·4 mg sulphite ml^{-1}

Table 2. *Reaction of ^{18}O-enriched atmospheric oxygen with sulphur by the purified oxygenase enzyme of S. brierleyi*

The sulphite formed during assay was converted to sulphur dioxide and analysed by mass spectroscopy. Masses 64 = $^{32}S^{16}O^{16}O$, 66 = $^{32}S^{18}O^{16}O$ and 68 = $^{32}S^{18}O^{18}O$. The constant contribution of natural ^{34}S isotope was ignored. Values are the means ($\pm$SD) from six determinations. t_0-t_1 = 1 h.

	Relative mass proportions (%) at:					
	t_0			t_1		
	64	66	68	64	66	68
Enzyme assays with ^{16}O	82 $\pm$ 3	11 $\pm$ 1	7 $\pm$ 2	84 $\pm$ 2	10 $\pm$ 1	6 $\pm$ 1
Enzyme assays with ^{18}O	85 $\pm$ 5	10 $\pm$ 1	5 $\pm$ 1	73 $\pm$ 5	16 $\pm$ 2	11 $\pm$ 1
Average sulphite concn in all enzyme assays	100 $\pm$ 5 µg ml^{-1}			400 $\pm$ 20 µg ml^{-1}		

towards the end of the test due to enzymic sulphur oxidation. In control tubes containing ^{16}O, the ratio of the masses 64, 66 and 68 remained almost constant during incubation. In contrast, in tubes with an ^{18}O-enriched oxygen atmosphere, the proportion of molecules with masses 66 and 68 increased relative to those with mass 64.

DISCUSSION

In the sulphur-oxidizing system of the archaebacterium *S. brierleyi*, only one enzyme (M_r 560000) could be detected by PAGE; it appeared to be composed of equal subunits of M_r 35000. The isolated enzyme first oxidized elemental sulphur to sulphite, which is also the case in the aerobic thiobacilli. The low amount of thiosulphate observed probably resulted from a chemical reaction between sulphite and elemental sulphur.

The apparent K_m for elemental sulphur was calculated to be 50 mM, which seems to indicate a low substrate affinity for the sulphur-oxidizing enzyme of *S. brierleyi*. But as the limiting factor for enzymic activity is the surface area and not the quantity of the sulphur (Laishley *et al.*, 1986), this apparent K_m seems to be grossly overestimated. To avoid the difficulties caused by the insolubility of elemental sulphur, the rate of sulphite formation was taken as a basis in calculations of enzymic activity. We found a specific activity of 30 U (mg protein)$^{-1}$ in cell-free extracts of *S. brierleyi* compared with 1 and 0·3 U (mg protein)$^{-1}$ in cell-free extracts of *T. thiooxidans* and *T. thioparus* respectively (Suzuki, 1965a; Suzuki & Silver, 1966). This 30–100-fold higher sulphur-oxidation activity might be explained by the high growth temperature of *Sulfolobus* compared to thiobacilli, although it may be that the amount of sulphur-oxidizing enzyme in *S. brierleyi* is higher than that in thiobacilli.

The observed increase of sulphur dioxide molecules with M_r values of 66 and 68 during incubation with ^{18}O indicated a direct combination of atmospheric oxygen with the sulphur molecule in the *S. brierleyi* system. The relatively small recovery of ^{18}O in sulphur dioxide is probably due to loss of ^{18}O bound in sulphite as $H_2^{18}O$ by the conversion of sulphite to sulphur dioxide and water under acid conditions. It appears that sulphur oxidation by *S. brierleyi* functions by similar mechanisms to those demonstrated for aerobic thiobacilli (Hooper & Dispirito, 1985). In the oxidation of elemental sulphur to sulphite by an oxygenase-type enzyme, energy coupling is not possible (Kelly, 1982), so the question as to how *S. brierleyi* derives energy by sulphur oxidation remains unanswered. Further investigations are under way to elucidate this problem by investigating the mechanisms of sulphite oxidation in *S. brierleyi*.

This article is based on a doctoral study by T.E. in the Faculty of Biology, University of Hamburg.

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