Microbiology (2003), **149**, 1699–1709 DOI 10.1099/mic.0.26212-0

The sulfane sulfur of persulfides is the actual substrate of the sulfur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp.

Thore Rohwerder† and Wolfgang Sand

Department of Microbiology, Institute for General Botany, University of Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany

Correspondence
Thore Rohwerder
rohwerd@umb.ufz.de

Received 23 December 2002
Revised 5 March 2003
Accepted 11 March 2003

To identify the actual substrate of the glutathione-dependent sulfur dioxygenase (EC 1.13.11.18) elemental sulfur oxidation of the meso-acidophilic *Acidithiobacillus thiooxidans* strains DSM 504 and K6, *Acidithiobacillus ferrooxidans* strain R1 and *Acidiphilium acidophilum* DSM 700 was analysed. Extraordinarily high specific sulfur dioxygenase activities up to 460 nmol min^{-1} $(mg\ protein)^{-1}$ were found in crude extracts. All cell-free systems oxidized elemental sulfur only via glutathione persulfide (GSSH), a non-enzymic reaction product from glutathione (GSH) and elemental sulfur. Thus, GSH plays a catalytic role in elemental sulfur activation, but is not consumed during enzymic sulfane sulfur oxidation. Sulfite is the first product of sulfur dioxygenase activity; it further reacted non-enzymically to sulfate, thiosulfate or glutathione *S*-sulfonate ($GSSO_3^-$). Free sulfide was not oxidized by the sulfur dioxygenase. Persulfide as sulfur donor could not be replaced by other sulfane-sulfur-containing compounds (thiosulfate, polythionates, bisorganyl-polysulfanes or monoarylthiosulfonates). The oxidation of H_2S by the dioxygenase required GSSG, i.e. the disulfide of GSH, which reacted non-enzymically with sulfide to give GSSH prior to enzymic oxidation. On the basis of these results and previous findings a biochemical model for elemental sulfur and sulfide oxidation in *Acidithiobacillus* and *Acidiphilium* spp. is proposed.

INTRODUCTION

For the biological oxidation of elemental sulfur conclusive enzymic structures have not yet been identified. This oxidation is a bottleneck in the general sulfur cycle (Ehrlich, 2002) and is also an important reaction in biotechnological processes such as the biomining of base and precious metals (Rawlings, 2002; Rohwerder *et al.*, 2002) or the desulfurization of waste waters (Lens & Hulshof Pol, 2000). The most prominent bacteria which catalyse the oxidation of inorganic sulfur compounds under acidic conditions (pH 1–3) and ambient temperatures (up to 45 °C) are *Acidithiobacillus* and *Acidiphilium* spp. (Ehrlich, 2002; Harrison, 1984). A characteristic among representatives of both genera is the ability to grow chemolithoautotrophically with sulfide, elemental sulfur, thiosulfate or polythionates (Hiraishi *et al*, 1998; Kelly & Wood, 2000; Parker & Prisk, 1953; Pronk *et al.*, 1990). Among these bacteria are *Acidithiobacillus thiooxidans*, *Acidithiobacillus caldus* and *Acidiphilium acidophilum* which can thrive chemolithotrophically only by sulfur compound oxidation. In addition, *Acidithiobacillus ferrooxidans* is a species within this group which can also oxidize iron(II) ions.

$$1/8S_8 + O_2 + H_2O \rightarrow SO_3^{2-} + 2H^+ \quad (1)$$

$$1/8S_8 + 1/4O_2 + H_2O \rightarrow 1/2SO_3^{2-} + 1/2H_2S + H^+ \quad (2)$$

Diverse enzymic systems are considered to be involved in the aerobic oxidation of elemental sulfur to sulfite (equation 1). A 46 kDa sulfur dioxygenase (EC 1.13.11.18) for the meso-acidophilic *Atb. thiooxidans* and *Atb. ferrooxidans* (Suzuki 1965b; Silver & Lundgren, 1968; Chahal, 1986; Sugio *et al.*, 1987) was described consisting of two subunits of 21 and 26 kDa or two 23 kDa proteins and absolutely requiring low-molecular-mass thiol compounds for activity. Quite similar but thiol-independent systems were isolated from thermoacidophilic *Acidianus* spp. In *Acidianus brierleyi* a 560 kDa dioxygenase was found consisting of identical subunits of 35 kDa (Emmel *et al.*, 1986). In *Acidianus ambivalens* and *Acidianus* sp. S5, a 550 kDa enzyme with 40 kDa subunits catalyses the simultaneous disproportionation and oxygenation of elemental sulfur to sulfide and sulfite (equation 2) (Kletzin, 1989, 1992; He *et al.*, 2000). Details of the reaction centres of the dioxygenases and oxygenase-reductases have not been described (reviewed by

†Present address: Centre for Environmental Research Leipzig-Halle, Permoserstr. 15, D-04318 Leipzig, Germany.

Abbreviations: GSH, glutathione; GSSG, disulfide of glutathione; GSSH, glutathione persulfide; $GSSO_3^-$, glutathione *S*-sulfonate.

Suzuki, 1994). However, from spectroscopic analyses it could be concluded that typical cofactors of redox enzymes such as flavins and cytochromes are not involved. A common feature of the sulfur-oxidizing enzymes from acidophilic bacteria is the incorporation of molecular oxygen into sulfur atoms to form sulfite. By this reaction none of the 4 electrons transferred is released and no energy can be conserved for ATP synthesis. On the other hand, enzyme systems using electron acceptors other than oxygen might be involved in elemental sulfur oxidation feeding the respiratory chain with at least 4 electrons per sulfur atom (sulfite formation). In neutrophilic sulfur bacteria a reverse sulfite reductase was described which should be able to oxidize sulfide or elemental sulfur to sulfite (Schedel & Trüper, 1979; Pott & Dahl, 1998). Like dissimilatory sulfite reductases from sulfate-reducing bacteria this enzyme mainly consists of two subunits containing sirohaem and iron–sulfur clusters as cofactors (Hipp *et al.*, 1997). The reduction of sulfite to sulfide in the presence of reduced viologen dyes by the pure enzyme was proved experimentally. However, until now catalytic activity has not been demonstrated for the oxidative direction from sulfide or sulfur to sulfite. Only recently has the involvement of a second oxygen-independent system in elemental sulfur oxidation come under discussion. It has been demonstrated that the thiosulfate-oxidizing complex from the meso-neutrophilic *Paracoccus pantotrophus* (the Sox complex; reviewed by Friedrich *et al.*, 2001) is able to oxidize elemental sulfur by reducing cytochrome *c*. A molybdenum cofactor and several cytochromes are involved in this reaction. The experimentally observed stoichiometry (4 cytochrome molecules reduced per sulfur atom) also indicated sulfite as the reaction product, but no chemical analyses proving this assumption have been performed so far (Rother *et al.*, 2001).

In all cases, an activation reaction prior to oxidation is postulated, because elemental sulfur consists of a stable octasulfane ring system (S_8) that forms orthorhombic crystals with extremely poor water solubility (Steudel, 2000). This activation reaction could be an opening of the S_8 ring by nucleophilic reagents, resulting in the formation of linear inorganic or organic polysulfanes. In addition, the reduction of elemental sulfur to water-soluble sulfide is discussed. Both reactions could be carried out by the thiol groups of cysteine residues. Consequently, meso- and moderately thermophilic, acidophilic sulfur-oxidizing bacteria seem to have a pathway depending on low-molecular-mass thiols, namely glutathione (GSH), which is the thiol compound preferentially used for *in vitro* assays. This GSH-dependent activity has not only been detected in *Acidithiobacillus* spp., but also in *Sulfobacillus thermosulfidooxidans* (Krasil'nikova *et al.*, 1998). Although the known sulfur dioxygenases are very similar with respect to molecular size, substructure and lack of redox cofactors, the role of the essential thiol group is in dispute. The work of Suzuki (1965a, b) and Silver & Lundgren (1968) suggests a catalytic role for GSH, where organic polysulfanes are formed and sulfur is oxidized at the zero valence state to sulfite. In contrast, Sugio *et al.* (1987, 1989) described an enzyme system that probably used dissolved sulfide as substrate. This system would catalyse not a 4- but a 6-electron step to sulfite. Consequently, GSH would be consumed stoichiometrically in the course of sulfur oxidation due to the preceding non-enzymic reduction of elemental sulfur to sulfide.

In all studies so far, the sulfur chemistry and especially the fate of GSH has not been analysed, leaving the activation mechanism for elemental sulfur unresolved. Therefore, we reinvestigated the GSH-dependent sulfur-oxidizing system in meso-acidophilic leaching bacteria. Special analytical attention was given to the organic sulfur species that occur in the *in vitro* assay. Besides elemental sulfur, other sulfane compounds and H_2S were tested to elucidate the actual substrate used by the sulfur dioxygenase. For this purpose the strictly lithotrophic species *Atb. thiooxidans* and *Atb. ferrooxidans* were tested and cell-free extracts of the facultatively lithotrophic *Acp. acidophilum* and the obligately heterotrophic *Acidiphilium cryptum* were included.

METHODS

Bacterial strains, media and growth conditions. The bacterial strains used in this study were *Acidithiobacillus thiooxidans* strains DSM 504 and K6, *Acidithiobacillus ferrooxidans* strain R1, *Acidiphilium acidophilum* strain DSM 700, *Acidiphilium cryptum* strain DSM 2389 and *Leptospirillum ferrooxidans* strain DSM 2705. All strains were obtained from the DSMZ, except strains K6 and R1 which were isolates from a German sewage system (Milde *et al.*, 1983) and a Romanian copper mine (Sand *et al.*, 1992), respectively, and have been cultured in our own strain collection. Lithotrophic sulfur oxidizers were grown on a medium containing a salt solution as described by Mackintosh (1978) with the exception that 1 mM $(NH_4)_2SO_4$ was substituted by 2 mM NH_4Cl and that the initial pH was adjusted to 3·0 with HCl instead of sulfuric acid. As substrate, 5 g elemental sulfur powder per litre was added. In addition, a lithotrophic iron medium (Mackintosh, 1978) with 5 g iron(II) ions per litre instead of 10 g and a heterotrophic glucose-based medium (Harrison, 1981) were used for the growth of iron oxidizers and facultative or obligate heterotrophs, respectively. All strains were cultivated aerobically at 28 °C. For mass cultivation, aerated reactors with a working volume of up to 20 l were used.

Cell harvest and preparation of cell-free extracts. All steps for harvesting and disruption of cells were performed at $\leqslant 4$ °C. Cultures in the late exponential growth phase were concentrated by filtration (Ultrafilter; Fresenius). Excess elemental sulfur and iron precipitates were removed by centrifugation at 120 ***g*** for 5 min. Cell pellets were obtained by centrifugation at 10 000 ***g*** for 10 min and washed twice in a salt solution (107 mg NH_4Cl, 25·4 mg $MgCl_2.6H_2O$ and 147 mg $CaCl_2.2H_2O$ per litre, adjusted to pH 3·0 with HCl). The final cell pellet was suspended in a phosphate buffer (50 mM, pH 6·5) at densities of 25 mg cell protein ml^{-}. The disruption procedure was performed anaerobically under an artificial atmosphere of $N_2/CO_2/H_2$ (88 : 10 : 2, by vol.; in an anaerobic workstation; Du Scientific). An equivalent volume of glass beads (150–212 μm; Sigma) was added to this suspension, which was cooled by an ice-water bath and stirred at 1000 r.p.m. for 1 h. Afterwards, the liquid phase was decanted, filled up with the same volume of phosphate buffer (50 mM, pH 6·5) and centrifuged (20 min, 25 000 ***g***, twice) to remove intact cells and cell residues.

The supernatant, hereafter referred to as crude or cell-free extract, usually contained 1–2 mg protein ml^{-} and was either used directly for activity assays or was stored under an anaerobic atmosphere at −25 °C (less than 3 months).

Enzyme assays. Cell-free extracts of various meso-acidophilic sulfur-oxidizing bacteria were tested for sulfur dioxygenase activity according to the assay established by Suzuki (1965a) with minor modifications. All assays were performed aerobically at 30 °C with stirring at 300 r.p.m. The reaction mixtures contained 10 mM phosphate buffer at pH 6·5 and varying amounts of crude extracts, various substrates (see below) and GSH (individual values are given in the Results section). The assays were started by an addition of substrate or, if applied, by adding GSH. If needed, the pH was maintained at 6·5 by titration with 50 mM KOH or 50 mM HCl. To determine non-enzymic reactions, assays without protein, with 0·2 mg BSA ml^{-1}, or with heat-inactivated crude extracts (90 °C for 30 min) were used. Samples were collected at appropriate time intervals and analysed for sulfur species (see below). With the exception of elemental sulfur quantification, all samples were filtered (nylon filter, 0·2 μm) prior to sulfur analyses to remove suspended sulfur. Sulfur-oxidizing activity is expressed as the amount of sulfur atoms that oxidized from the zero valence state to at least the oxidation state of sulfite (i.e. +4).

Substrates tested in the enzyme assays. For measuring elemental sulfur oxidation a system of dispersed sulfur in water was developed. To 50 ml deionized water an equal volume of acetonic sulfur solution (saturated solution containing about 20 mM sulfur) was added, mixed and dialysed against 5 l deionized water (24 h, exchanging the water three times) to remove the acetone. The whitish dialysis product had a final volume of 150 ml and contained about 6 mM elemental sulfur. The sulfur formed droplets of 2–10 μm in diameter and was used for experiments within 10 h after preparation at a concentration of 4 mM.

When thiosulfate, tetrathionate and *p*-toluenethiosulfonate were tested as substrates for sulfur dioxygenase, stock solutions of 10 mM of the potassium or sodium salts in deionized water were prepared. Thiosulfate and tetrathionate were applied at 500–1000 μM and *p*-toluenethiosulfonate was added at 500–2000 μM. Experiments were performed with and without the addition of 200–500 μM GSH.

A mixture of the disulfide of GSH (GSSG) and higher homologues (analysed by HPLC; see below) was obtained by incubation of 500 mM elemental sulfur (powder) with 100 mM GSH at pH 7·5 (adjusted with KOH) under stirring and anaerobic conditions until the solution became lemon-coloured within 1–2 days. At this point the pH was lowered to 5·0 by addition of HCl. The resulting H_2S was removed by evacuation of the gas phase. Afterwards, the pH was adjusted to 6·5. The final solution contained GSSG and higher homologues up to the pentasulfane homologue (GS_5G), but no GSH was detectable. Tests were performed with a total of about 1·15 mM GS_nG species ($n=2$–5) and ±200 μM GSH.

H_2S was tested at concentrations of 180 μM. Stock solutions were prepared from the sodium salt at 50 mM in deionized water. Assays were performed with and without 500–1000 μM GSSG.

Analyses of sulfur compounds. For the analyses of sulfur speciation in the enzyme assays as well as for the verification of purity and concentration of stock solutions, all relevant sulfur species were quantified. Thiosulfate, polythionates, GSH, GSH-derivatives [GS_nG species with $n=2$–5; GSH *S*-sulfonate ($GSSO_3^-$)], *p*-toluenethiosulfonate and *p*-toluenesulfinate were analysed by ion pair chromatography. An HPLC system from Kontron/BIO-TEK Instruments was used, consisting of a pump (422), a gradient former (425), a guard column cartridge (PLRP-S 5 × 3 mm; Latek Labortechnik), a separation column (PLRP-S 100 Å, 8 μm, 150 × 4·6 mm; Latek Labortechnik), an autosampler (465), a diode array detector (440) and software 450-MT2/DAD. Chromatograms were recorded at 205, 215, 265 and 300 nm concomitantly with spectra from 190 to 320 nm.

Two different elution methods were used (methods 1 and 2). Method 1 was designed for separating GSH, GSH-derivatives and thiosulfate. An eluent consisting of an aqueous solution of 18 % acetonitrile, 2 mM tetrabutylammonium chloride and 2 mM acetate buffer at pH 4·0 was pumped isocratically at 1 ml min^{-1}. Thiosulfate was eluted as the last compound after about 17 min. Method 2 was gradient-based, designed to separate the above-mentioned sulfur compounds plus polythionates (tri-, tetra- and pentathionate), *p*-toluenesulfinate and *p*-toluenethiosulfonate. In contrast to method 1, the initial acetonitrile concentration of 18 % was increased linearly to 34 % within 11 min, then maintained for 4 min at this level, and finally decreased back to 18 % within 3 min. After 5 min of isocratic pumping the next sample could be injected. The pumping speed was 1 ml min^{-1}. Retention times were 12 min for thiosulfate and 14 and 15 min for tetra- and pentathionate, respectively. As the separation performance of GSSG and higher homologues was insufficient with the gradient method, these compounds were preferentially analysed by using method 1.

As calibration standards, aqueous solutions of commercially available potassium salts of thiosulfate and tetrathionate were applied. Tri- and pentathionate were synthesized according to Fehér (1975). Chromatograms recorded at 215 nm were used for quantification. Solutions of the free acids of GSH and GSSG were used for calibration and were quantified at 205 and 215 nm. Due to the lack of appropriate standards, higher homologues of GSSG could not be determined at molar concentrations. Therefore, only peak areas were recorded at 205, 215, 265 and 300 nm. Identification of higher bisorganylpolysulfanes (GS_nG) was achieved on account of their spectra and their specific retention characteristics in an isocratic chromatographic system as described by Göbel (1988) and Steudel & Kustos (1994). Independent of the HPLC system used for separation of homologous bisorganylpolysulfanes, a linear relationship is found between the number of sulfur atoms and the logarithm of the capacity factor. A calibration for $GSSO_3^-$ was achieved by monitoring the stoichiometric conversion of sulfite plus GSSG to $GSSO_3^-$ plus GSH (Steudel & Albertsen, 1992) under anaerobic conditions to prevent autoxidation of sulfite. For quantification, chromatograms recorded at 205 and 215 nm were analysed. Aqueous solutions of potassium or sodium salts of *p*-toluenesulfinate and *p*-toluenethiosulfonate were used as standards. Quantification was performed at 215 nm.

Elemental sulfur was analysed by reversed-phase chromatography followed by UV-detection as described by Schippers & Jørgensen (2001). One volume of sample was diluted with 5 vols of ethanol and injected directly. In the case of low concentrations of elemental sulfur, samples were extracted with n-octane prior to dilution with ethanol.

Sulfite and sulfate were quantified by ion-exchange chromatography and conductivity detection as described by Schippers & Jørgensen (2001). A Dionex DX 500 system with a guard column AG9-SC/4 mm and a separation column AS9-SC/4 mm was applied. Sulfite was fixed by methanal (Weiß, 1991). Standards of sulfite and sulfate were made of aqueous stock solutions of potassium or sodium salts. In addition to ion pair chromatography, thiosulfate was also determined together with sulfite and sulfate. Thus, two independent chromatographic methods were applied to quantify this sulfur compound.

Dissolved sulfide was determined photometrically by the methylene blue method (Anonymous, 1984). Samples were fixed with zinc acetate and stored frozen or analysed immediately.

Other analytical procedures. Protein concentration was determined by the method of Bradford (1976) as modified by Spector (1978). The pH was measured potentiometrically.

Statistical analyses. Experimental data such as concentrations of sulfur compounds or enzymic activities are given as mean values of at least five independent experiments with error bars representing the standard deviation. Linear regressions were performed with the least squares method using the standard deviation of the individual values as weight. The significance of linear correlation was tested by *t*-test analyses.

RESULTS AND DISCUSSION

Survey for sulfur dioxygenase activity among *Acidithiobacillus* and *Acidiphilium* strains

In agreement with the assumption that meso-acidophilic sulfur oxidizers possess a sulfur dioxygenase, all extracts obtained from the lithotrophic strains K6, DSM 504, R1 and DSM 700 showed GSH-dependent sulfur-oxidizing activity (Table 1). The lowest specific activity of 17 nmol min^{-1} (mg protein)$^{-1}$ was observed using *Atb. thiooxidans* strain K6. This value is equal to results of earlier examinations with strains of *Atb. thiooxidans* and *Atb. ferrooxidans* (reviewed by Suzuki, 1994). In contrast, extracts of *Atb. thiooxidans* DSM 504 exhibited two- to three-times increased rates than previously published, and with *Atb. ferrooxidans* R1 even higher sulfur oxidation activities of up to 80 nmol min^{-1} mg^{-1} were obtained. Moreover, with up to 460 nmol min^{-1} mg^{-1} an extraordinarily high dioxygenase activity was present in cell-free extracts of the facultatively chemolithotrophic strain *Acp. acidophilum* DSM 700. Extracts of sulfur- or iron-grown cells of *Atb. ferrooxidans* R1 exhibited equal activity values. In the case of *Acp. acidophilum* DSM 700, activities for sulfur-grown cells were three times higher than for heterotrophically grown ones. Obviously, the sulfur dioxygenase is constitutive in these strains. This makes sense because both species thrive in biotopes where reduced sulfur compounds and alternative substrates concomitantly occur (Harrison, 1984). Unexpectedly, extracts of glucose-grown *Acp. cryptum* DSM 2389 exhibited a low but significant activity for sulfur dioxygenase (Table 1). This strain is not known to be able to grow chemolithoautotrophically on reduced sulfur species. However, evidence exists that strain DSM 2389 and other obligately heterotrophic *Acidiphilium* spp. can oxidize elemental sulfur in the presence of organic substrates (Harrison, 1983; Hallberg *et al.*, 2001). Obviously, this ability resulted in the detection of sulfur dioxygenase activity in this work.

No activity for the sulfur-oxidizing enzyme was detected in cell-free extracts of the acidophilic, iron(II) oxidizer *L. ferrooxidans* DSM 2705, which served as a negative control. This finding is in contrast to previous data, as it has been reported that strain DSM 2705, among other strains of *L. ferrooxidans*, exhibited sulfur dioxygenase activity (Sugio *et al.*, 1992, 1994). *L. ferrooxidans* is absolutely dependent on iron(II) oxidation, and the oxidation of inorganic sulfur compounds by whole cells or enzyme preparations of this species have never been observed by other investigators (reviewed by Hippe, 2000). Consequently, the finding of a sulfur-oxidizing enzyme activity in *L. ferrooxidans* strains is questionable. As we obtained higher specific activities in crude extracts from *Acidithiobacillus* spp. than have previously been published it can be concluded that our preparation method is superior with respect to enzyme stability and assay sensitivity. However, we were not able to detect sulfur dioxygenase activity in *L. ferrooxidans*. The contradictions cannot yet be explained, but probably the previous studies with dioxygenase-positive preparations (Sugio *et al.*, 1992, 1994) were erroneous due to the use of cultures contaminated with sulfur oxidizers. As mass culturing in these studies were performed successively with various strains of *Acidithiobacillus* and *Leptospirillum*, this explanation is the most likely. In addition, it was also claimed that intact cells of the dioxygenase-positive *L. ferrooxidans* strains were able to oxidize elemental sulfur

Table 1. Specific activities and K_m values for GSH of the sulfur dioxygenase in crude extracts of different strains of meso-acidophilic bacteria

Strain	Substrate for cell growth*	Activity† (nmol min^{-1} mg^{-1})	K_m for GSH (mM)
Atb. ferrooxidans R1	Elemental sulfur	63 ± 23	140 ± 20
	Iron(II) ions	58 ± 10	120 ± 10
Atb. thiooxidans DSM 504	Elemental sulfur	40 ± 13	160 ± 20
Atb. thiooxidans K6	Elemental sulfur	25 ± 8·3	190 ± 10
Acp. acidophilum DSM 700	Elemental sulfur	373 ± 90	230 ± 20
	Glucose	130 ± 42	240 ± 10
Acp. cryptum DSM 2389	Glucose	5·0 ± 1·7	NM‡
L. ferrooxidans DSM 2705	Iron(II) ions	ND‡	NA‡

*Strains were subcultured for at least 2 years on the indicated substrates.

†Specific activity is expressed as the amount of sulfur atoms oxidized to the valence state of sulfite within 1 min by 1 mg protein.

‡NM, Not measured; ND, not detectable; NA, not applicable.

(Sugio *et al.*, 1992). However, two of these strains were provided by our group (strains P3A and BKM-b-1339), but in routine tests to check for contaminants our laboratory has been unable to reproduce this observation.

All K_m values for GSH with sulfur dioxygenase ranged between 110 and 250 μM (Table 1). These values were 10–20 times lower than those published before (reviewed by Suzuki, 1994). As discussed below, elemental sulfur and GSH are not substrates for the sulfur dioxygenase, but a reaction product of these two substances is oxidized by the enzyme system. Thus, previous studies have most likely been performed under sulfur-limiting conditions, resulting in erroneously high K_m values for GSH.

Identification of the actual substrate of sulfur oxidation: the role of GSH

The mechanism for elemental sulfur oxidation in cell-free extracts from all sulfur oxidizers tested, irrespective of the growth substrate and the specific activities, was identical with respect to substrate usage and product formation. Under the assay conditions and in parallel with the enzymic activity a complex set of non-enzymic reactions between GSH and elemental sulfur took place. It turned out that part of these reactions was necessary for enzymic sulfur oxidation to be active. As already discussed by Suzuki (1965a, b), the non-enzymic reaction proceeds via monoorganylpolysulfane compounds (GS_nH, $n>1$) by a nucleophilic attack of GSH on the S_8 ring (equation 3). The GS_nH species react with each other or with excess GSH to form GSSG or its higher homologous bisorganylpolysulfanes (GS_nG, $n>2$) and H_2S (equation 4).

$$S_8 + GSH \rightarrow (GS_9H) \rightarrow GS_nH + (9-n)/8S_8 \quad (3)$$

$$GS_xH + GS_yH \rightarrow GS_{x+y-1}G + H_2S \quad x>1, y \geq 1 \quad (4)$$

Consequently, the reduction of elemental sulfur to sulfide and the formation of GSSG as well as higher GS_nG species were observed in our experiments. To elucidate which of these sulfur compounds were actually used as the substrate of the sulfur dioxygenase (GS_nH, GS_nG or H_2S) the relationship between enzymic activity and non-enzymic sulfur reduction (equation 3 plus 4) was examined.

Generally, the concentration of GSH declined according to second order kinetics as described by equation 5 (Fig. 1a, b).

$$\frac{d[GSH]}{dt} = -k_G[GSH]^2 \quad (5)$$

The rate constant, k_G, was highest with the controls containing only heat-inactivated cell extracts, BSA or no protein at all. It decreased with increasing enzymic activity

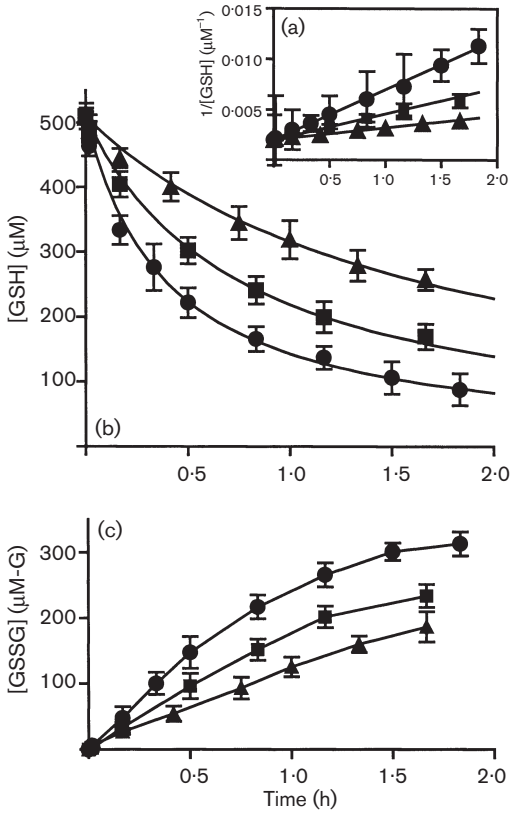

Fig. 1. Consumption of GSH (a, b) and formation of GSSG (c) in enzyme assays containing dispersed sulfur and 500 μM GSH at various absolute sulfur dioxygenase activities: no activity (circles), 5 μM (squares), and 10 μM (triangles) oxidized sulfur atoms min^{-1} (in all cases, the abiotic background activity was 0·12 μM oxidized sulfur atoms min^{-1}). The GSH decrease showed second order kinetics according to equation (5) and reciprocal plots against time (a) gave a highly significant linear correlation (significance level = 0·0001). Solid lines in (b) were calculated with the rate constants (k_G) derived from the linear regressions shown in (a). For comparison to (b) all concentration values in (c) are referred to the number of GSH residues per molecule (μM-G).

for sulfur oxidation (Fig. 1a, b) and the main oxidation product of GSH was GSSG, which accumulated in the assays (Fig. 1c). Besides the disulfide, GSH was also oxidized to higher GS_nG species, of which the tri-, tetra- and pentasulfane could be identified (Fig. 2). Generally, the concentrations of the higher polysulfanes were highest within the first 20 to 30 min of the experiments. Afterwards, a slow decrease in the concentrations of these compounds occurred (Fig. 2a). The rates of formation of GS_nG accompanied the consumption kinetics of GSH and, thus, also decreased with increasing sulfur dioxygenase activity (Fig. 2b). There are two possible explanations for these results. (1) The sulfur dioxygenase uses the sulfane sulfur of GS_nH species ($n>1$) as substrate. In this case, non-enzymic sulfur reduction (equation 3 plus 4) will decrease because

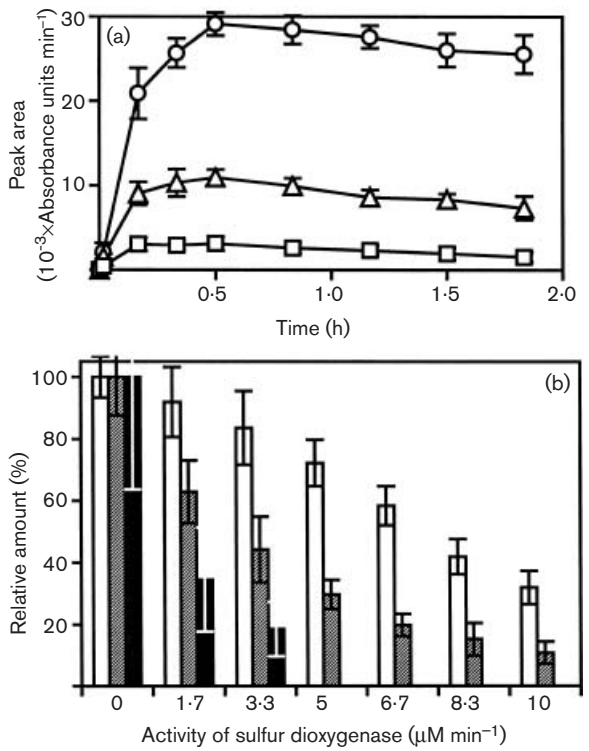

Fig. 2. Formation of higher homologues of GSSG in enzyme assays containing dispersed sulfur and 500 μM GSH. (a) Formation of trisulfane (circles), tetrasulfane (triangles) and pentasulfane (squares) in assays without enzymic activity. (b) Relative formation of trisulfane (white bars), tetrasulfane (shaded bars) and pentasulfane (black bars) after 30 min incubation with absolute dioxygenase activities ranging from 0 to 10 μM oxidized sulfur atoms min^{-1}. All values are displayed as a percentage of the amount measured in assays without enzymic activity.

less GS_nH molecules are available to the sulfide-producing half reaction (equation 4). (2) The sulfane sulfur of higher GS_nG species ($n>2$) is oxidized enzymically, and, consequently, the concentration of higher bisorganylpolysulfanes will be reduced. Whether both GS_nH and GS_nG serve as substrates or only one of these compounds is used cannot be concluded from the presented data. Therefore, a mixture of GSSG and higher homologues (GS_nG, $n=3$–5) was tested separately as a substrate of sulfur dioxygenase (in the absence of GSH and elemental sulfur). In these experiments, higher GS_nG species were not degraded by the enzyme (data not shown). With GSH addition, however, the sulfane sulfur of the bisorganylpolysulfanes was readily oxidized. These findings confirm monoorganylpolysulfanes as one possible substrate of sulfur dioxygenase because in the presence of GSH these polysulfane species will be formed from higher GS_nG according to equation (6).

$$GS_nG + GSH \rightarrow GS_xH + GS_yG \quad n>2, x>1, y>1 \qquad (6)$$

The same mechanism must have been active in the GS_nG mixture tested positively by Suzuki (1965a). Certainly, this mixture contained sufficient amounts of GSH to form GS_nH species which were oxidized by the enzyme preparation.

Because GS_nH species were identified as substrates of sulfur dioxygenase, free sulfide can be ruled out as its exclusive substrate, in contrast to a previous proposal (Sugio *et al.* 1989). Furthermore, this finding was confirmed on the basis of the observed stoichiometry between GSH consumption and sulfur oxidation (Fig. 1b). For example, an enzymic oxidation rate of 5 μM min^{-1} was accompanied by the consumption of 280 μM GSH within the first hour. With this thiol amount only 140 μM sulfur atoms could be reduced to sulfide (equations 3 and 4), whereas a total of 300 μM was really oxidized. However, because free sulfide regularly occurred in the assays it could be a substrate of sulfur dioxygenase, besides GS_nH species. To test this possibility, the fate of free sulfide was examined. Sulfide production can be calculated from the GSH decrease by integrating equation 5. Interestingly, the measured sulfide concentration did not follow the calculated curves, but decreased in the course of the experiments (Fig. 3). To understand this decrease, control experiments without elemental sulfur were done. Instead, H_2S was added to the assays (data not shown). From the results of these experiments it was deduced that under the assay conditions (with or without active enzyme preparations) 99 % of the dissolved sulfide escaped unoxidized into the gas space as H_2S with first order kinetics according to equation 7 and with a rate constant (k_S) = 2·34 ± 0·06 h^{-1}.

$$\frac{d[H_2S]}{dt} = -k_S[H_2S] \qquad (7)$$

By combining equations 5 and 7, i. e. the production and the removal of H_2S, respectively, it is possible to formulate a differential term (equation 8), where $[GSH]_0$ represents the initial concentration of GSH and t is the experimental time. As proven by comparison of the calculated with the measured concentrations of dissolved sulfide, this equation is a good approximation for the observed sulfide values (Fig. 3).

$$\frac{d[H_2S]}{dt} = \frac{k_G}{2}\left(\frac{[GSH]_0}{[GSH]_0 k_G t + 1}\right)^2 - k_S[H_2S] \qquad (8)$$

Consequently, the gap between sulfide production and actual sulfide concentration is not related to oxidation processes. In other words, sulfide is not a substrate of sulfur dioxygenase, but the sulfane sulfur of GS_nH species ($n>1$) is oxidized by the enzyme. The higher polysulfanes of the GS_nH type with $n>2$ are extremely unstable and rapidly decompose to sulfur and short-chain, linear polysulfanes (equation 3). However, the persulfide ($n=2$) seems to be stable enough to be oxidized by the enzyme (equation 9) or

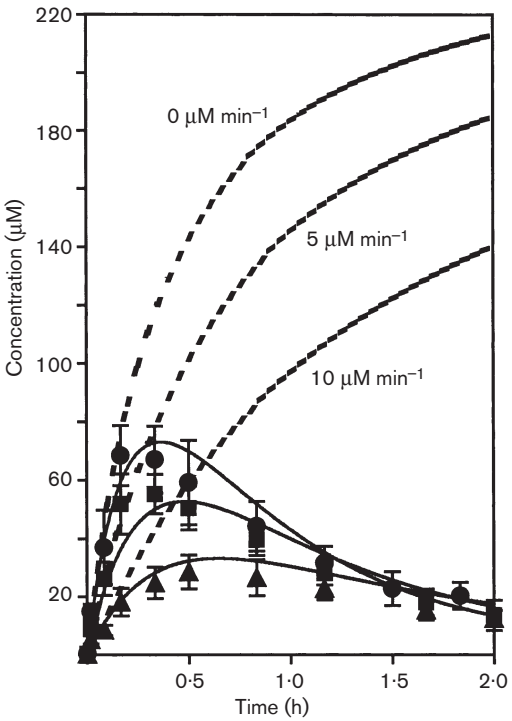

Fig. 3. Formation of dissolved sulfide in enzyme assays containing dispersed sulfur and 500 μM GSH. Sulfide concentrations measured at various absolute dioxygenase activities are shown: no activity (circles), 5 μM (squares) and 10 μM (triangles) oxidized sulfur atoms min^{-1}. Dashed lines represent sulfide concentrations predicted according to equation (5) for enzymic activities as indicated. Continuous lines give sulfide concentrations calculated according to equation (8).

to react non-enzymically with other sulfur species. The bisorganyltrisulfane, for example, which was the predominant higher species of GS_nG detected in this study (Fig. 2a), is formed in a reaction of two molecules of GSSH (equation 10).

$$GSSH + O_2 + H_2O \rightarrow GSH + SO_3^{2-} + 2H^+ \qquad (9)$$

$$2GSSH \rightarrow GS_3G + H_2S \qquad (10)$$

Unfortunately, it is not possible to test the GSH persulfide or higher GS_nH species directly due to their extreme reactivity in polar solutions. Nevertheless, we got the ultimate proof for their involvement in the reaction mechanism of sulfur dioxygenase by investigating enzymic sulfur oxidation under GSH-limiting conditions. By continually increasing the amount of active enzyme, it was found that for a given GSH concentration a maximal sulfur oxidation rate was achieved at which no GSH consumption was observed. In other words, under these conditions GSH played a purely catalytic role and left the reaction absolutely unchanged (equation 3 plus 9). For example, at an elemental sulfur concentration of 4 mM and with 100 μM GSH a maximal sulfur dioxygenase activity of about 5·8 μM min^{-1} could be obtained (Fig. 4a). Generally, both the non-enzymic sulfur reduction (equation 4) and enzymic sulfur oxidation (equation 9) compete for the same sulfur compound, the GS_nH species ($n>1$). Consequently, at excess enzyme concentrations the complete GS_nH pool serves as substrate and non-enzymic sulfur reduction does not occur (Fig. 4a). At this point, the rate of GS_nH formation is equal to the rate of sulfur oxidation, because the dioxygenase cannot oxidize more GS_nH than was formed by the non-enzymic reaction of GSH with sulfur (equation 3). According to equation 3 the GS_nH formation rate is directly proportional to GSH concentration, provided that sufficient amounts of elemental sulfur are present. Consequently, a linear relationship between GSH concentration and the maximal enzymic

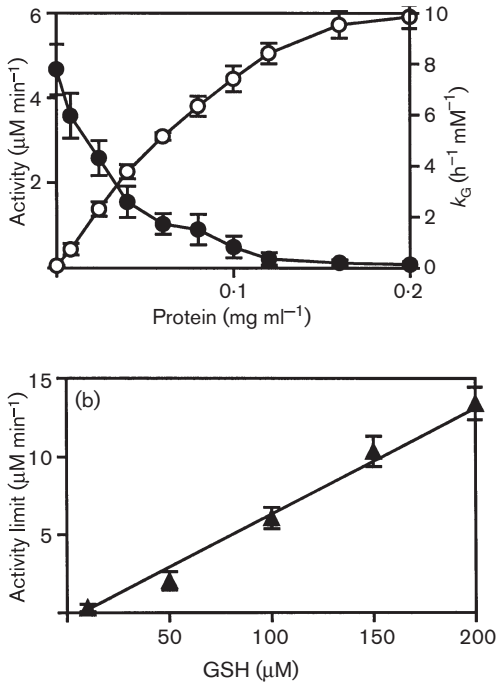

Fig. 4. Enzymic oxidation and non-enzymic reduction of dispersed elemental sulfur under GSH-limiting conditions. (a) Dioxygenase activities (open circles) and rates of GSH consumption (filled circles) at initial concentrations of 100 μM GSH and various amounts of active cell extract with a specific activity of 56 nmol min^{-1} (mg protein)$^{-1}$. GSH consumption is expressed as values of the rate constant k_G of equation (5). Dioxygenase activities could not be increased beyond 5·8 μM min^{-1}. At this maximal activity no GSH consumption occurred. (b) Linear correlation between various maximal dioxygenase activities and initial GSH concentrations ranging from 10 to 200 μM (significance level = 0·0005). Activity limits were determined as demonstrated in (a) for 100 μM GSH. Generally, at these maximal dioxygenase activities no GSH oxidation was observed.

activity (i.e. the activity at which no GSH consumption occurred) was found (Fig. 4b).

Identification of the reaction product of sulfur oxidation

Thiosulfate was the dominant reaction product (99 %) of enzymic sulfur oxidation under standard assay conditions. In addition, minor amounts of sulfate and $GSSO_3^-$ were detected (Fig. 5a). With the applied IC technique, sulfite could not be detected beyond traces of $\leqslant 1\,\mu M$ (data not shown). As previously proposed by Suzuki & Silver (1966), thiosulfate formed from sulfite in a non-enzymic reaction with excess sulfur. Sulfate and $GSSO_3^-$ may have resulted from auto-oxidation of sulfite and a non-enzymic reaction of sulfite with GSSG (Steudel & Albertsen, 1992; equation 11), respectively.

$$GSSG + SO_3^{2-} + H^+ \rightarrow GSSO_3^- + GSH \qquad (11)$$

By using comparable assay conditions (Suzuki & Silver, 1966; Silver & Lundgren, 1968), it has been possible to detect sulfite in significant amounts prior to its reaction with elemental sulfur or other electrophilic compounds by addition of methanal to the assay. A similar effect was achieved with GSSG in our studies. At least a part of the sulfite reacted with this disulfide to form $GSSO_3^-$, which we were able to detect regularly (Fig. 5a). Consequently, $GSSO_3^-$ formation was enhanced by the addition of GSSG to the assays. At an enzymic sulfur-oxidizing activity of 5 μM min^{-1} and with the addition of 250 μM GSSG the formation of the oxidation product, $GSSO_3^-$, was enhanced to about 10 % compared to less than 1 % formed without the addition of GSSG (Fig. 5b). Due to the rapid oxidation of GSH by elemental sulfur and the subsequent formation of GSSG (Fig. 1c), it can be concluded that the accumulation of the $GSSO_3^-$ is a general characteristic of activity assays involving the sulfur dioxygenase. However, in none of the previous studies has this compound been tested. In summary, our findings support sulfite as the first oxidation product of enzymic elemental sulfur oxidation (equations 1 and 9).

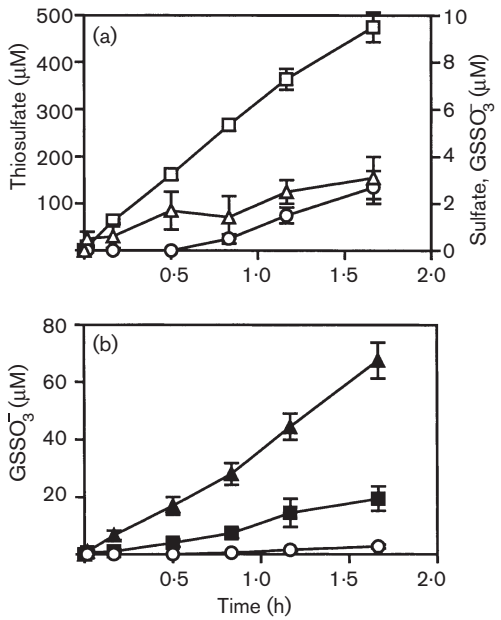

Fig. 5. Oxidation of elemental sulfur by sulfur dioxygenase in crude extracts with absolute dioxygenase activities of 5 μM oxidized sulfur atoms min^{-1}. (a) Speciation of oxidation products in assays containing dispersed elemental sulfur, 500 μM GSH and cell-free extracts. Besides thiosulfate (open squares), minor amounts of sulfate (open triangles) and $GSSO_3^-$ (open circles) were formed. (b) Enhanced formation of $GSSO_3^-$ by increased initial concentrations of GSSG: no addition of GSSG (open circles), 100 μM GSSG added (filled squares), 250 μM GSSG added (filled triangles).

Oxidation of alternative sulfane compounds

To test the substrate specificity of sulfur dioxygenase, the alternative sulfane-sulfur-containing compounds thiosulfate, tetrathionate and *p*-toluenethiosulfonate were tested (data not shown). In the absence of GSH none of these substances was degraded by the sulfur-oxidizing enzyme. As a result, we conclude that the enzyme was unable to mobilize the sulfane sulfur from any of these alternative substrates and can only oxidize organic persulfide species (RSSH) such as glutathione persulfide (GSSH).

Oxidation of H_2S

As stated above, dissolved sulfide was oxidized neither non-enzymically nor enzymically, but escaped into the gas phase during incubation. However, low enzymic oxidation activities were detected in the presence of high amounts of GSSG (data not shown). For example, with active cell-free extracts, 1000 μM GSSG and 180 μM H_2S, initial absolute enzymic activities of 0·4–0·8 μM min^{-1} were obtained. Besides negligible amounts of thiosulfate, $GSSO_3^-$ was the main product of this oxidation activity. Concomitant to the formation of $GSSO_3^-$, GSH was produced at a ratio of 1 : 2·7. This stoichiometry indicates the following reaction mechanism. GSSG non-enzymically oxidizes H_2S to form the persulfide of GSH according to equation (12). The sulfane sulfur is oxidized by the dioxygenase to sulfite (equation 9) followed by a reaction of sulfite with excess GSSG to $GSSO_3^-$ (equation 11). In summary, for 1 mol $GSSO_3^-$, 3 mol GSH are formed (equation 13), which matches quite well with the measured ratio.

$$GSSG + H_2S \rightarrow GSSH + GSH \qquad (12)$$

$$2GSSG + H_2S + O_2 + H_2O \rightarrow 3GSH + GSSO_3^- + H^+ \qquad (13)$$

These results shed new light on the work of Sugio *et al.*

(1989), declaring sulfide to be the actual substrate for the sulfur dioxygenase. Their enzyme preparation, isolated from *Atb. ferrooxidans* AP19-3, was described to be able to oxidize sulfide to sulfite, however, only in the presence of 8 mM GSH. Because such highly concentrated and aerobically prepared GSH solutions certainly also contain GSSG due to autoxidation, it is almost certain that the persulfide was formed (equation 12), which then could serve as substrate for the sulfur dioxygenase. Therefore, the proposed direct oxidation mechanism for H_2S under these conditions is questionable.

Proposal of a general scheme for meso-acidophilic sulfur oxidation

Summarizing the data on the sulfur chemistry of the GSH-dependent sulfur dioxygenase of this work, evidence was obtained that exclusively the sulfane sulfur of monoorganylpolysulfanes (RS_nH, $n>1$), predominantly persulfides ($n=2$), but not H_2S, is oxidized to sulfite. This finding is valid for species of the genera *Acidithiobacillus* and *Acidiphilium*, irrespective of the growth substrate. Collecting all data available in the literature, we now are able to propose a general scheme for elemental sulfur oxidation in these Gram-negative species (Fig. 6). Extracellular elemental sulfur is mobilized by thiol groups of special outer-membrane proteins and transported into the periplasmic space as persulfide sulfur. Candidates for the not yet identified thiol-bearing membrane proteins are the sulfide-binding protein isolated from *Atb. ferrooxidans* AP 19-3 (Sugio *et al.*, 1991) and several outer-membrane proteins which have been associated with sulfur oxidation in strains of *Atb. ferrooxidans* (Buonfiglio *et al.*, 1993, 1999; Ohmura *et al.*, 1996). The persulfide sulfur is oxidized by periplasmic sulfur dioxygenase to sulfite, which is further oxidized to sulfate by a sulfite : acceptor oxidoreductase. This enzyme most probably uses cytochromes as electron acceptors (Vestal & Lundgren 1971; Nakamura *et al.*, 1995; de Jong *et al.*, 2000). As already mentioned, by applying a dioxygenase for sulfur oxidation, only two of the six electrons that were transferred in the redox reactions from sulfur to sulfate can be fed into the respiratory chain. Consequently, about 50 % of the ΔG of approximately -500 to -550 kJ per mol sulfur atoms accompanying the complete sulfur oxidation (Kelly, 1999) cannot be used for energy conservation. At first sight, this seems to be an unnecessary loss for the cell and alternative mechanisms for sulfur oxidation in acidophilic bacteria, e.g. a reverse sulfite reductase, have been proposed (Kelly, 1999). However, the dioxygenase is the only sulfur-converting enzyme so far identified in acidophilic sulfur bacteria. Although research on this field has been going on for more than 40 years, no alternative pathway has been demonstrated. Obviously, the ΔG of about -250 kJ per mol sulfite oxidized to sulfate provides sufficient energy for lithotrophic growth on sulfur.

In our model, free sulfide is oxidized to elemental sulfur by a separate sulfide : quinone oxidoreductase, which is located at the periplasmic site of the cytoplasmic membrane. Although this enzyme has only been isolated from neutrophilic sulfur bacteria, strong evidence exists that it is equally present in acidophilic bacteria. (1) Sulfide-oxidizing membrane preparations of *Atb. thiooxidans* absolutely require ubiquinone (Moriarty & Nicholas, 1969). (2) By comparing the gene sequences of various sulfide : quinone oxidoreductases, an ORF in the genome of *Atb. ferrooxidans* was identified which has high sequence similarity to known sulfide dehydrogenase genes (Nübel *et al.*, 2000; Griesbeck *et al.*, 2002). Recently, a reaction mechanism for sulfide : quinone oxidoreductase with free polysulfides instead of

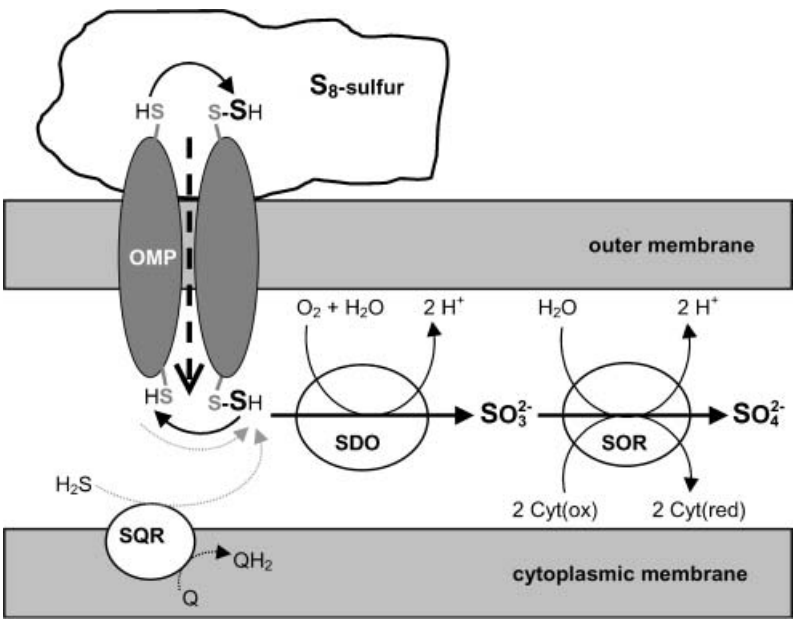

Fig. 6. Proposal of a biochemical model for sulfur oxidation in *Acidithiobacillus* and *Acidiphilium* spp. In this scheme extracellular elemental sulfur (S_8) is mobilized as persulfide sulfane sulfur by special outer-membrane proteins (OMP) and oxidized by periplasmic sulfur dioxygenase (SDO). The resulting sulfite is oxidized to sulfate by sulfite : acceptor oxidoreductase (SOR), which probably uses cytochromes as electron acceptors (Cyt). Free sulfide is oxidized by a separate dehydrogenase (SQR), which uses quinones (Q) as electron acceptors.

elemental sulfur as the initial oxidation product has been proposed (Griesbeck *et al.*, 2002). It is argued that these water-soluble sulfur compounds would be sufficiently mobile to cross the outer membrane, probably by diffusion, whereas elemental sulfur would precipitate in the periplasm. Thus, the polysulfide mechanism could explain why elemental sulfur is only formed extracellularly in species which do not oxidize sulfide further to sulfite or sulfate, such as *Rhodobacter capsulatus* (Griesbeck *et al.*, 2002). However, polysulfides are not stable in acidic and neutral solutions and immediately decompose to elemental sulfur and sulfide (Steudel, 1996, 2000). Consequently, elemental sulfur will accumulate in the periplasmic space even if polysulfides are the initial product of sulfide oxidation. In our model, the zero valence sulfur formed from sulfide, whether as the initial product or after decomposition of polysulfides, does not precipitate because it reacts with the thiol groups of the outer-membrane proteins and forms persulfide sulfur. In fact, the membrane binding of sulfur in the zero valence state has been demonstrated in sulfide oxidation experiments with cell-free extracts from strains of *Atb. thiooxidans* (Moriarty & Nicholas, 1969, 1970). If no further oxidation occurred, e.g. in the presence of inhibitors, it is likely that the persulfide sulfur can be transported outside the cell via the same route as extracellular sulfur enters the cell (see above). The existence of a reversible transport mechanism is supported by studies where complete sulfide oxidation in *Atb. thiooxidans* was inhibited and elemental sulfur accumulated extracellularly (Chan & Suzuki, 1993).

To clarify the exact biochemical and genetic basis of elemental sulfur oxidation in acidophilic bacteria, we plan to isolate the enzyme system responsible from different leaching bacteria. The most promising species for such an isolation is *Acp. acidophilum* due to its extraordinarily high activity. In addition, *Acp. cryptum* and possibly other obligately heterotrophic species of this genus are an interesting object for further studies, because the enzyme system seems to be present in these organisms, too.

ACKNOWLEDGEMENTS

This work was supported by grants to W. S. from BMBF via UBA (1490954), DBU (05333) and the Max-Buchner-Forschungsstiftung (DECHEMA eV).

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