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Oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus*

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Abstract. Oxidation of reduced sulphur compounds by *Thiobacillus acidophilus* was studied with cell suspensions from heterotrophic and mixotrophic chemostat cultures. Maximum substrate-dependent oxygen uptake rates and affinities observed with cell suspensions from mixotrophic cultures were higher than with heterotrophically grown cells. pH Optima for oxidation of sulphur compounds fell within the pH range for growth (pH 2–5), except for sulphite oxidation (optimum at pH 5.5). During oxidation of sulphide by cell suspensions, intermediary sulphur was formed. Tetrathionate was formed as an intermediate during aerobic incubation with thiosulphate and trithionate. Whether or not sulphite is an intermediate during sulphur compound oxidation by *T. acidophilus* remains unclear. Experiments with anaerobic cell suspensions of *T. acidophilus* revealed that trithionate metabolism was initiated by a hydrolytic cleavage yielding thiosulphate and sulphate. A hydrolytic cleavage was also implicated in the metabolism of tetrathionate. After anaerobic incubation of *T. acidophilus* with tetrathionate, the substrate was completely converted to equimolar amounts of thiosulphate, sulphur and sulphate. Sulphide- and sulphite oxidation were partly inhibited by the protonophore uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and by the sulfhydryl-binding agent *N*-ethylmaleimide (NEM). Oxidation of elemental sulphur was completely inhibited by these compounds. Oxidation of thiosulphate, tetrathionate and trithionate was only slightly affected. The possible localization of the different enzyme systems involved in sulphur compound oxidation by *T. acidophilus* is discussed.

Key words: *Thiobacillus acidophilus* – Acidophiles – Sulphur metabolism – Sulphide – Elemental sulphur – Thiosulphate – Tetrathionate – Trithionate – Sulphite – Hydrolytic polythionate cleavage

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The recent literature on sulphur oxidation by various neutrophilic and acidophilic thiobacilli clearly demonstrates that there is no uniformity in the pathways employed by different *Thiobacillus* species and that, in fact, vast differences may be found in the pathways involved (Kelly 1985 and 1989). This explains why many previous attempts to formulate a unifying route for the observed biological reactions have failed.

The acidophilic thiobacilli can thrive in environments with pH values as low as pH 1.5. From bioenergetic considerations, it can be expected that at least some of the reactions involved in the oxidation of reduced sulphur compounds will take place extracytoplasmically (Hooper and DiSpirito 1985), implying that the enzymes involved should be able to function while exposed to low pH values. Studies of sulphur compound oxidation by these bacteria are useful to obtain a better insight in their physiology. Such fundamental knowledge may contribute to an increased understanding of the role of these organisms in their natural environments and to the further development of biological leaching operations (Norris and Kelly 1988).

Thiobacillus ferrooxidans, an obligately chemolithoautotrophic organism, has been used frequently as an organism to study the physiology of growth in acidic environments (for a review see Ingledew 1982). However, during biochemical studies large amounts of biomass are often required. Due to the low energy availability per mol of substrate and limitation of substrate concentration, due to solubility and/or osmotic stress, biomass concentrations in *T. ferrooxidans* cultures grown on ferrous iron or reduced sulphur compounds are low.

To overcome the practical problems associated with the use of *T. ferrooxidans*, the facultative autotroph *Thiobacillus acidophilus* was introduced in our studies on sulphur oxidation by acidophiles. This organism was initially isolated as a contaminant of a ferrous iron-grown culture of *T. ferrooxidans* (Guay and Silver 1975). Like *T. ferrooxidans*, *T. acidophilus* is an acidophilic bacterium with a pH optimum of approximately 3. Growth substrates for autotrophic growth include elemental sulphur (Guay and Silver 1975), tetrathionate (Norris et al. 1986), thiosulphate and trithionate (Mason et al. 1987). Carbon

sources supporting heterotrophic growth include a number of monosaccharides, TCA-cycle intermediates and some amino acids (Guay and Silver 1975; Pronk et al. 1990a).

Aim of the present study was to investigate the kinetics and mechanism of sulphur compound oxidation by *T. acidophilus*.

Materials and methods

Organism and growth conditions

Thiobacillus acidophilus DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen as a liquid culture grown on glucose and maintained as described previously (Pronk et al. 1990a).

Chemostat cultivation

Mixotrophic chemostat cultures of *T. acidophilus* were grown in the mineral medium described by Pronk et al. (1990b) at 30 °C, at pH 3.0 and at a dilution rate of 0.05 h^{-1}. The medium contained 10 mM $Na_2S_2O_3 \cdot 5\ H_2O$ and 2.5 mM glucose as the growth-limiting substrates. Heterotrophic cultures were grown in the same mineral medium (without sodium thiosulphate), adjusted to pH 3.0 with H_2SO_4, with glucose as the growth-limiting substrate. High cell density (5 g dry wt $\cdot l^{-1}$) mixotrophic chemostat cultures of *T. acidophilus* were used for anaerobic incubations of cell suspensions with tetrathionate. These cultures were fed from two separate medium vessels. One contained 100 mM sodium thiosulphate and the other a mineral medium containing per liter of demineralized water: $(NH_4)_2SO_4$, 20 g; KH_2PO_4, 6.0 g; Na_2SO_4, 2.8 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; $CaCl_2 \cdot 2\ H_2O$, 0.55 g; EDTA, 0.15 g; $ZnSO_4 \cdot 7\ H_2O$, 45 mg; $CoCl_2 \cdot 2\ H_2O$, 3 mg; $CuSO_4 \cdot 5\ H_2O$, 3 mg; $NaMoO_4 \cdot 2H_2O$, 4 mg; $FeSO_4 \cdot 7\ H_2O$, 55 mg; H_3BO_3, 8 mg; KI, 1 mg; nitrilotriacetic acid, 25 mg; silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 0.2 ml. The mineral medium was adjusted to pH 3.0 with concentrated H_2SO_4 and autoclaved at 120 °C. Glucose was autoclaved separately at 110 °C and added to the mineral medium at a concentration of 200 mM. These mixotrophic cultures were grown at 30 °C, at pH 3.0 and at a dilution rate of 0.04 h^{-1}. The dissolved oxygen concentration was over 80% air saturation in all chemostat cultures used. The pH of the chemostat cultures was maintained at pH 3.0 by automatic titration with 4 M KOH.

The purity of the chemostat cultures was checked by phase contrast microscopy and immunofluorescence as described by Pronk et al. (1990b).

The dry weight of cell suspensions was determined with dried nitrocellulose filters as described previously (Pronk et al. 1990b).

Measurement of substrate-dependent oxygen consumption

Respiration rates of cell suspensions were assayed polarographically in a Biological Oxygen Monitor with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio, USA) at 30 °C. Assays were performed with cell suspensions (0.25 g dry wt $\cdot l^{-1}$ taken directly from substrate-limited chemostat cultures. Calculations were made on the basis of an oxygen concentration of 236 µM in air-saturated water at this temperature. The values presented have been corrected for the (low) endogenous respiration rates.

Oxidation of elemental sulphur by *T. acidophilus* was studied as oxygen uptake after the addition of solutions of elemental sulphur (S_8) in acetone. The acetone concentration in the reaction mixture did not exceed 1% (v/v). Control experiments showed that this acetone concentration influenced neither endogenous nor glucose-dependent oxygen uptake by *T. acidophilus*.

The oxygen uptake rates at different substrate concentrations were used for calculation of the K_s (apparent substrate saturation constant) and V_{max} (maximum substrate-dependent oxygen uptake rate) from a reciprocal plot according to Hanes (1932).

For adjusting the pH of cell suspensions, 2 N solutions of H_2SO_4 and KOH were used.

Oxygen uptake rates in the presence of inhibitors were assayed after 5 min incubation of the inhibitor with the cell suspension.

Anaerobic incubations of cell suspensions with trithionate

Cell suspensions (1.25 g dry wt $\cdot l^{-1}$) of mixotrophically grown *T. acidophilus* were flushed with argon for 15 min at 30 °C in a 10-ml thermostated reaction chamber. After addition of 1 mM trithionate, 0.5-ml samples were taken at desired time intervals and immediately mixed with 5 µl 1 M KOH to stop the reaction. Samples were then rapidly centrifuged in an Eppendorf bench-top centrifuge (5 min at 13,000 × *g*) before determinations of substrate concentrations were performed.

Anaerobic incubations of cell suspensions with tetrathionate

To remove sulphate, dense cell suspensions of mixotrophically grown *T. acidophilus* were centrifuged (10 min at 12,000 × *g*), washed twice with 25 mM potassium phosphate, pH 4.0, and resuspended in 1 l of the same buffer. Biomass concentration in the resulting suspension was 30 g dry wt $\cdot l^{-1}$. This cell suspension was transferred to a fermenter equipped with norprene tubing, stirred at 1250 rpm and flushed with nitrogen gas (1 l $\cdot$ min^{-1}) throughout the experiment. The dissolved oxygen concentration was monitored with a polarographic electrode and the pH was maintained at pH 4.0 by automatic titration of 1 M KOH. After addition of 10 mmol tetrathionate, through a septum, samples were taken at desired time intervals. Concentrations of thiosulphate, tetrathionate and sulphate were determined in the sample supernatant after centrifugation (5 min at 48,000 × *g*). For sulphur analysis, 0.25-ml samples were centrifuged at 48,000 × *g* for 5 min. The pellet was washed with 25 mM potassium phosphate, pH 4.0, recentrifuged and extracted overnight with acetone. KOH consumption was monitored with a buret.

Determination of sulphur compound concentration

Thiosulphate, tetrathionate and trithionate were determined according to the method of Sorbö (1957) as modified by Kelly et al. (1969).

Sulphide and sulphite were determined according to Trüper and Schlegel (1964).

Production of long-chain intermediary sulphur compounds from different substrates was investigated by monitoring the OD_{430} in a Hitachi double-beam spectrophotometer (model 100–60, Hazeu et al. 1988). The same suspension of cells without substrate was placed in the reference cuvette.

Solutions of elemental sulphur in acetone were assayed by cyanolysis (Sorbö 1957).

Sulphate and thiosulphate were assayed with a Waters HPLC

using a Machery-Nagel anion exchange column (Nucleosil, 250 × 4 mm), eluted with 0.04 M sodium salicylate, pH 4.0 (flowrate 1 ml · min^{-1} at 20 °C). Detection was performed with an RI detector (Waters 410, detection limits approximately 0.05 mM for sulphate and 0.5 mM for thiosulphate).

Chemicals

Trithionate was prepared as described by Wood and Kelly (1986). Carbonyl cyanide *m*-chlorophenylhydrazone and *N*-ethylmaleimide were obtained from Aldrich (Aldrich Chemie n.v./s.a., Brussels, Belgium). 2,4-Dinitrophenol was obtained from Baker (J. T. Baker Chemicals, Deventer, Holland). All other chemicals were reagent grade and obtained from commercial sources.

Results

Kinetics of sulphur compound oxidation

From previous work on the oxidation of reduced sulphur compounds, it is well-known that, at higher substrate concentrations, many chemical interactions between the sulphur compounds may occur (Roy and Trudinger 1970). Control experiments indicated that at the substrate concentrations used in this study (generally lower than 500 µM), chemical oxidation of the sulphur compounds did not contribute significantly to the observed substrate conversion rates (data not shown).

Substrate inhibition was not observed with any of the sulphur compounds tested, except for sulphite. Sulphite oxidation was strongly inhibited at concentrations above 50 µM. Thiosulphate-, tetrathionate-, trithionate- and sulphite-dependent oxygen uptake by *Thiobacillus acidophilus* exhibited the stoichiometries expected for complete oxidation to sulphate. The rates of sulphur and sulphide oxidation decreased to values only slightly above the endogenous respiration rates before the oxygen uptake (i.e. substrate oxidation and electron transfer to oxygen), corresponding to complete conversion to sulphate, had been reached.

Sulphide-dependent oxygen uptake by *T. acidophilus* followed a biphasic pattern. Oxygen uptake rates during the first phase were higher than in the second phase. Initial sulphide-dependent oxygen uptake rates, observed with cells from mixotrophic chemostat cultures, were approximately two-fold higher than the V_{max} of heterotrophically grown cells (Table 1). The apparent K_s values of cell suspensions from heterotrophic and mixotrophic cultures were similar.

The maximum rate of elemental sulphur-dependent oxygen uptake observed with heterotrophically grown cells was lower than with cells from mixotrophic cultures. In addition, the latter cells exhibited a lower K_s for elemental sulphur (Table 1).

Cell suspensions of *T. acidophilus* grown in mixotrophic chemostat cultures exhibited a biphasic pattern of thiosulphate- and trithionate-dependent oxygen uptake. As with sulphide oxidation, oxygen uptake rates during the first phase were higher than in the second phase. Because of the small amount of oxygen consumed during the first phase of thiosulphate and trithionate oxidation, initial rates of oxygen uptake could only be determined accurately at substrate concentrations above 50 µM. This resulted in higher K_s values than the K_s values observed with heterotrophically grown cells (Table 1), which exhibited a monophasic oxidation pattern with thiosulphate and trithionate. The V_{max} values of heterotrophically grown cells were, for both substrates, significantly lower than those of mixotrophically grown cells (Table 1).

The maximum tetrathionate-dependent oxygen uptake rates observed with cells from mixotrophic and heterotrophic chemostat cultures were similar. However, the K_s of mixotrophically grown cells appeared to be slightly lower than that of heterotrophically grown cells (Table 1).

Mixotrophically grown cells of *T. acidophilus* exhibited very low rates of sulphite oxidation under physiological conditions (30 °C, pH 3) (Table 1, Fig. 1). In theory, this observed maximum oxygen uptake rate for extracellularly added sulphite could account for only 35% of the flux of sulphur atoms to sulphate in the mixotrophic cultures. Of course, these data are not necessarily representative for the oxidation rate of intracellularly formed sulphite. Sulphite oxidation rates of heterotrophically grown cells were too low to allow accurate calculation of kinetic parameters (data not shown).

With the exception of sulphite, the pH optima for the

Table 1. Apparent substrate saturation constants (K_s) and maximum oxygen uptake rates (V_{max}) for oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus*. Cells had been grown mixotrophically on 2.5 mM glucose and 10 mM thiosulphate (0.25 g dry wt · l^{-1}) or heterotrophically on 10 mM glucose (0.6 g dry wt · l^{-1}), in substrate-limited chemostat cultures at a dilution rate of 0.05 h^{-1}. Substrate-dependent oxygen consumption rates were determined in a Biological Oxygen Monitor

Substrate	Mixotrophically grown cells		Heterotrophically grown cells	
	K_s (µM)	V_{max} (nmol O_2 · min^{-1} · (mg dry wt)$^{-1}$)	K_s (µM)	V_{max} (nmol O_2 · min^{-1} · (mg dry wt)$^{-1}$)
Sulphide	5	268	7	150
Elemental sulphur	2	94	18	46
Thiosulphate	133[a]	310	8	76
Tetrathionate	1	56	5	52
Trithionate	72[a]	175	9	66
Sulphite	13	17	nd	nd

nd; not determined; [a] determined at relatively high substrate concentrations (see text)

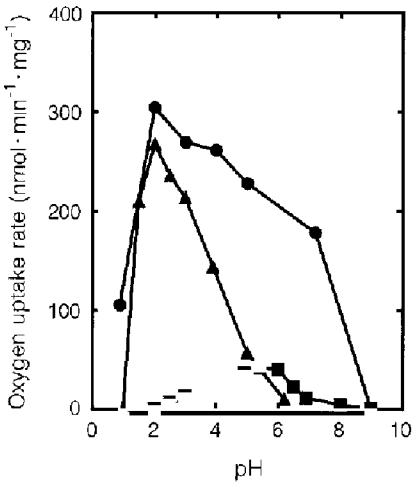

Fig. 1. pH Optima for the oxidation of sulphide, thiosulphate and sulphite by intact cells. *Thiobacillus acidophilus* was grown mixotrophically as described in the legend to Table 1. Substrate concentrations were 50 µM sulphide, 250 µM thiosulphate and 40 µM sulphite. ●: sulphide; ▲: thiosulphate; ■: sulphite

oxidation of sulphur compounds by *T. acidophilus* all fell within the pH range for growth (pH 2–5). Oxidation of sulphide had a very broad pH optimum, whereas the pH optimum for oxidation of thiosulphate was much sharper (Fig. 1). Surprisingly, the pH optimum for sulphite oxidation was higher than the pH optima for the oxidation of the other sulphur compounds (Fig. 1).

Formation and metabolism of tetrathionate

Oxidation of tetrathionate by cell suspensions of *T. acidophilus* was sulphate-dependent. Tetrathionate-dependent oxygen uptake rates decreased strongly upon washing of cell suspensions in a sulphate-free solution. However, 25 mM potassiumphosphate could replace this need for sulphate totally.

The stoichiometry of oxygen uptake during the distinct phases of thiosulphate and trithionate oxidation may give an indication of the identity of intermediary products. During the first, rapid phase of thiosulphate and trithionate oxidation, 0.23 ± 0.02 mol O_2 and 0.28 ± 0.05 mol O_2 were consumed per mol of substrate, respectively. The lower oxygen uptake rates during the second phase of both compounds corresponded with the oxygen uptake rates observed with tetrathionate as a substrate. These observations were consistent with the formation of tetrathionate as an intermediate during the oxidation of both thiosulphate and trithionate. Indeed, product analysis revealed an almost quantitative conversion of thiosulphate and trithionate to tetrathionate during the first phase of oxidation.

In the neutrophiles *Thiobacillus tepidarius* (Lu and Kelly 1988) and *Thiobacillus neapolitanus* (Trudinger 1964a) trithionate is initially hydrolysed to give thiosulphate and sulphate. In these organisms thiosulphate is subsequently oxidized to tetrathionate (Wood and Kelly 1986; Trudinger 1961). During anaerobic incubation of *T. acidophilus* with trithionate, thiosulphate was identified as the main product (Fig. 2). A small amount of tetrathionate was also produced (Fig. 2), probably as a result of a chemical reaction between thiosulphate and trithionate, yielding tetrathionate and sulphite (Naito et al. 1975). Indeed, when mixtures of low concentrations of thiosulphate and trithionate were incubated in the absence of cells, formation of small amounts of tetrathionate was also observed (data not shown).

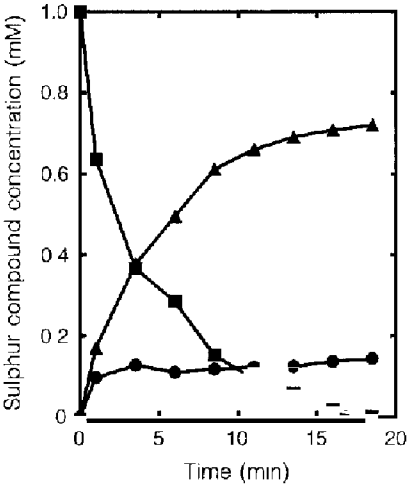

Fig. 2. Formation of thiosulphate and tetrathionate during anaerobic incubation of a *Thiobacillus acidophilus* cell suspension (1.25 g dry wt · l^{-1}) with 1 mM trithionate. *T. acidophilus* was grown mixotrophically as described in the legend to Table 1. Thiosulphate and tetrathionate were analysed by cyanolysis and trithionate concentration was calculated by subtracting thiosulphate and tetrathionate concentrations from total sulphane sulphur concentration (Sorbö 1957; Kelly et al. 1969). ▲: thiosulphate; ●: tetrathionate; ■: trithionate

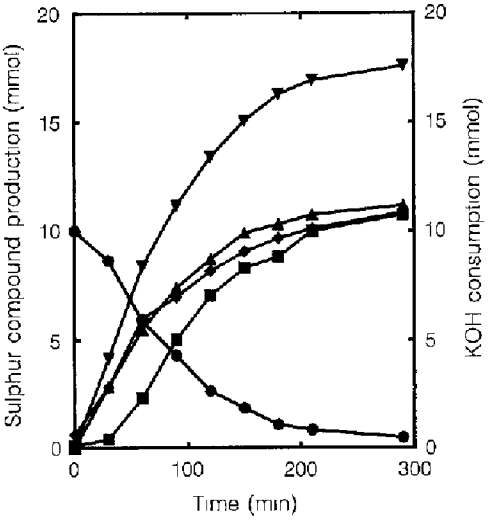

Fig. 3. Formation of products and KOH consumption during incubation of 10 mM tetrathionate in an anaerobic cell suspension (30 g dry wt · l^{-1}) of *Thiobacillus acidophilus*. The organism was grown mixotrophically on 100 mM glucose and 50 mM thiosulphate in a substrate-limited chemostat culture at a dilution rate of 0.04 h^{-1}. ●: tetrathionate; ▲: thiosulphate; ■: sulphur; ◆: sulphate; ▼: KOH

During anaerobic incubation of tetrathionate in cell suspensions of *T. acidophilus*, tetrathionate was stoichiometrically converted to thiosulphate, sulphate and some form of sulphur (Fig. 3). Formation of sulphide and sulphite could not be detected. KOH consumption revealed that two moles of H^+ were released per mol of tetrathionate metabolized (Fig. 3). These results suggest that tetrathionate metabolism is initiated by a cleavage reaction according to the equation:

$$S_4O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + S + SO_4^{2-} + 2\,H^+ \quad (1)$$

Formation of sulphur could also be monitored as an increase of OD_{430}, when tetrathionate was incubated with anaerobic cell suspensions and when thiosulphate, trithionate or tetrathionate was incubated in aerobic cell suspensions with 100 µM NEM.

Formation of intermediary sulphur from sulphide

As mentioned above, sulphide-dependent oxygen uptake exhibited a biphasic pattern. Biphasic oxygen uptake was particularly evident with cells from heterotrophic cultures. The oxygen consumption during the first phase of sulphide oxidation by cells from such cultures corresponded to 0.57 ± 0.08 mol per mol sulphide. With cell suspensions from mixotrophic cultures, it was more difficult to determine the transition from first to second phase and oxygen uptake during the first phase tended to be larger.

Oxidation of sulphide by *T. acidophilus* was accompanied by a transient increase of the OD_{430} of cell suspensions (Fig. 4). The time spans over which the increase and decrease of the OD_{430} occurred, corresponded with the first and second phase observed during oxygen uptake experiments, respectively. The decrease of the OD_{430} during the second phase of sulphide oxidation was much slower with cells from heterotrophic cultures than with mixotrophically grown cells (Fig. 4). The biphasic oxygen uptake pattern observed during sulphide oxidation, combined with the transient increase of the optical density of the cell suspensions, suggests that, as during anaerobic incubation of tetrathionate, some intermediary sulphur compound was formed. The lower rates of oxygen uptake during the second phase of sulphide oxidation would then reflect the complete oxidation of this intermediate to sulphate. The stoichiometry observed during the first phase of sulphide oxidation by cells from heterotrophic cultures indicates that sulphide is initially oxidized to the redox level of elemental sulphur. The larger amount of oxygen consumed during the first phase of sulphide oxidation by cells from mixotrophic cultures is probably due to simultaneous oxidation of sulphide to sulphur and intermediary sulphur to sulphate.

As has been observed with the obligately autotrophic acidophile *T. ferrooxidans* (Hazeu et al. 1988), the oxidation of intermediary sulphur, formed during sulphide oxidation, was completely inhibited by the sulfhydryl-binding agent *N*-ethylmaleimide (NEM) (Fig. 4). This compound also completely inhibited the oxidation of elemental sulphur and, to a lesser extend, the oxidation of sulphide and tetrathionate by *T. acidophilus* (Fig. 4, Table 2). NEM had a profound effect on the stoichiometry of sulphide-dependent oxygen uptake. In the presence of the inhibitor, only approximately 0.5 mol of oxygen was consumed per mol of sulphide added, corresponding to the incomplete oxidation of sulphide to the redox level of elemental sulphur. Oxidation of other sulphur compounds was not substantially inhibited by NEM (Table 2).

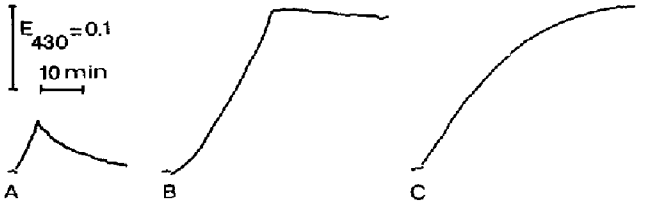

Fig. 4A–C. Recorder tracings of the increase of OD_{430}, due to formation of intermediary sulphur, during oxidation of sulphide by intact cells of *Thiobacillus acidophilus*. The reference cuvette contained the same cell suspension without substrate. The initial sulphide concentration was 200 µM. Cells had been cultivated as described in the legend to Table 1. **A.** Mixotrophically grown cells. **B.** Heterotrophically grown cells. **C.** Mixotrophically grown cells. Sulphide was added after preincubation with 100 µM NEM

Table 2. Effects of the uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and the sulfhydryl-binding agent *N*-ethylmaleimide (NEM) on the initial oxygen uptake rates during oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus*. Cells were taken from a mixotrophic culture, cultivated as described in the legend to Table 1. Substrate-dependent oxygen consumption rates were determined in a Biological Oxygen Monitor. Concentrations of inhibitors were 100 µM DNP, 125 µM CCCP and 100 µM NEM. Results are given as a percentage of the oxygen consumption rate without inhibitors

Substrate	DNP (%)	CCCP (%)	NEM (%)
Sulphide	18	21	61
Elemental sulphur	0	7	8
Thiosulphate	119	106	98
Tetrathionate	119	67	76
Trithionate	101	65	92
Sulphite	62	60	91

Effects of uncouplers on sulphur compound oxidation

To investigate whether energy-requiring reactions (e.g. active transport or activation of substrate molecules) are involved in the metabolism of any of the sulphur compounds tested, the effects of the protonophore uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were studied. The concentrations of the uncouplers used in the experiments completely inhibited glucose-dependent oxygen uptake.

The oxidation of the sulphur oxyanions thiosulphate, trithionate and tetrathionate was not inhibited by DNP

(Table 2). CCCP caused a significant decrease of the trithionate and tetrathionate oxidation rates (Tables 2).

Oxidation of sulphide was strongly inhibited by uncouplers (Table 2). The uncouplers also effected the stoichiometry of sulphide-dependent oxygen uptake. In the presence of DNP or CCCP, oxygen consumption corresponded to the amount required for the incomplete oxidation of sulphide to elemental sulphur. Also the oxidation of exogeneously added elemental sulphur was completely inhibited by uncouplers (Table 2).

Sulphite-dependent oxygen uptake rates were inhibited by about 40% after addition of uncouplers (Table 2).

Discussion

Kinetics of sulphur compound oxidation

Thiobacillus acidophilus grown in mixotrophic chemostat cultures exhibited very low apparent substrate saturation constants for the sulphur compounds studied (Table 1). These kinetic parameters for oxidation of reduced sulphur compounds by *T. acidophilus* were comparable with those determined with its obligately autotrophic counterpart, *Thiobacillus ferrooxidans* (Hazeu et al. 1986). This, together with the constitutive nature of oxidation of reduced sulphur compounds in *T. acidophilus* (Table 1), may be advantageous in the competition of *T. acidophilus* with obligate autotrophs in environments where both inorganic sulphur compounds and organic substrates are available.

Oxidation of sulphide, thiosulphate and trithionate by cell suspensions of mixotrophically grown *T. acidophilus* proceeded in two phases, reflecting the transient accumulation of intermediary sulphur compounds. It should be realized that the occurrence of biphasic oxygen uptake patterns depends on the substrate concentration added and on the grown conditions. For example, biphasic oxygen uptake patterns were not observed with very low concentrations of thiosulphate or during thiosulphate oxidation by cells from heterotrophic cultures. Apparently the rate of tetrathionate degradation by these cell suspensions was equal to the rate of tetrathionate formation from thiosulphate and trithionate. Significant accumulation of intermediary sulphur compounds did not occur under substrate-limited growth conditions in chemostat cultures (Pronk et al. 1990b).

Localization of sulphur compound-oxidizing enzyme systems

The pH optima for the oxidation of sulphur compounds by *T. acidophilus* cells fell within the pH range for growth (pH 2–5) of this acidophile (Fig. 1). A notable exception was sulphite, which was hardly oxidized at pH 3, the pH at which the cells had been grown (Fig. 1). Maximum sulphite oxidation rates were observed at pH 5.5. A high pH optimum for sulphite oxidation has also been reported for the acidophilic chemolithoautotroph *Thiobacillus thiooxidans* (Kodama and Mori 1968). The uncoupler sensitivity of sulphite oxidation (Table 2) may indicate that an energized membrane is required, possibly for energy-dependent uptake of sulphite. If it is assumed that the carrier protein here involved in the uptake of sulphite is, in fact, a sulphate carrier, the sulphite has to be in the dissociated form (SO_3^{2-}) to be recognized by the carrier protein. Considering the dissociation constants of H_2SO_3 ($pK_1 = 1.77$; $pK_2 = 7.21$) a pH optimum for sulphite uptake is then expected around pH 7. However, in view of the decreased metabolic activity at this neutral pH, the lower pH optimum for sulphite-dependent oxygen uptake (i.e. sulphite uptake and sulphite oxidation) is not surprising.

The oxidation of thiosulphate, trithionate and tetrathionate was not inhibited by DNP (Table 2). The inhibition of tetrathionate and trithionate oxidation by CCCP is therefore probably a pleiotropic effect. The stimulation of thiosulphate oxidation by DNP and CCCP can be explained by uncoupling of respiratory control. The insensitivity of the oxidation of the sulphur oxyanions towards uncouplers suggests that the oxidation of these compounds does not require membrane energization. Our present hypothesis is therefore, that, with the exception of sulphite, initial oxidation of the sulphur oxyanions occurs extracytoplasmically.

The oxidation of sulphide and elemental sulphur by cell suspensions was strongly inhibited by uncouplers (Table 2), suggesting that sulphide is actively transported across the membrane and oxidized in the cytoplasm.

Pathways of sulphur compound oxidation in Thiobacillus acidophilus

T. acidophilus employs the metabolic sequence from trithionate via thiosulphate to tetrathionate (Fig. 5). We recently purified trithionate hydrolase and thiosulphate oxidoreductase (Meulenberg, unpublished). These enzymes catalyse the hydrolysis of trithionate to thiosulphate and the oxidation of thiosulphate to tetrathionate, respectively.

Oxidation of trithionate via thiosulphate to tetrathionate has previously been demonstrated in a variety of obligately autotrophic thiobacilli (*T. thiooxidans:* Okuzumi 1966, Okuzumi and Kita 1965; *T. tepidarius:* Lu and Kelly 1988; *T. neapolitanus:* Trudinger 1961 and 1964a; *T. ferrooxidans:* Sinha and Walden 1966; for a review see Pronk et al. 1990c). Thiosulphate metabolism in *T. acidophilus* differs significantly from that in the neutrophilic facultative autotroph *Thiobacillus versutus*. Oxidation of thiosulphate by the latter organism does not involve tetrathionate as an intermediate (Lu and Kelly 1984).

The formation of equimolar amounts of thiosulphate, sulphur and sulphate from tetrathionate under anaerobic conditions (Fig. 3 and 5) has also been demonstrated with the obligately autotrophic neutrophile *T. neapolitanus* (Trudinger 1964b). A hydrolytic cleavage of tetrathionate, yielding disulphane monosulphonic acid ($S_3O_3^{2-}$) and sulphate has recently been suggested for

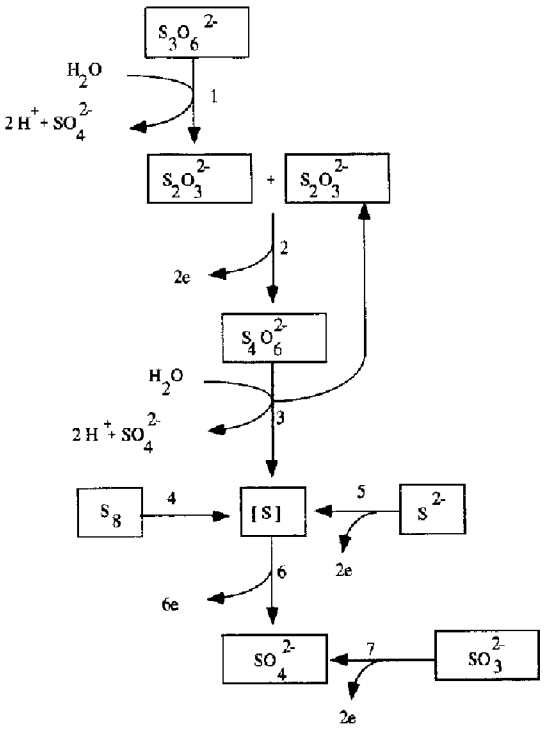

Fig. 5. Working hypothesis for the metabolic pathways of sulphur compound oxidation in *Thiobacillus acidophilus*, involving two hydrolytic cleavages (reaction *1* and *3*) and four oxidative steps (reactions *2,5–7*). Reaction *4* represents the activation of elemental sulphur into a form susceptible to further oxidation

T. ferrooxidans (Steudel et al. 1987; Hazeu et al. 1988). Alternatively, an initial reduction of tetrathionate, yielding thiosulphate has been proposed (Trudinger 1964b, Steudel et al. 1987). Spontaneous chain-elongation of sulphane monosulphonic acids ($S_3O_3^{2-}$ and $S_2O_3^{2-}$) would then result in production of hydrophilic sulphur and sulphite (Steudel et al. 1987; Hazeu et al. 1988; Steudel and Prenzel 1989). In our experiments formation of sulphur seems to be somewhat delayed compared to formation of thiosulphate and sulphate (Fig. 3). This might reflect chain-elongation of sulphane monosulphonic acids. However, we have not been able to demonstrate the production of sulphite predicted by the model of Steudel et al. (1987). This, together with the observed quantitative relationships between the product concentrations after anaerobic incubation of tetrathionate, which were unchanged even after 9 h of incubation makes it unlikely that sulphur was formed via spontaneous chain-elongation of thiosulphate (Steudel and Prenzel 1989) or disulphane monosulphonic acid (Steudel et al. 1987; Steudel and Prenzel 1989). Our observations suggest that thiosulphate, sulphur and sulphate are produced in one single step during tetrathionate metabolism (Fig. 5). The observed delay of sulphur production may well occur because initially the density of the growing sulphur nuclei is too small to allow precipitation by centrifugation.

Hazeu et al. (1988) reported that intermediary sulphur ('biological sulphur') was formed during the oxidation of tetrathionate by cell suspensions of *T. ferrooxidans*. During aerobic incubation of cell suspensions of *T. acidophilus* with tetrathionate, no formation of intermediary sulphur could be detected. This suggests that, during oxidation of tetrathionate, the rate of sulphur oxidation equals the rate of sulphur formation. Increase of OD_{430}, due to formation of intermediary sulphur, could be detected when further oxidation of sulphur was inhibited by NEM or exclusion of oxygen.

Transient accumulation of intermediary sulphur was observed during oxidation of sulphide by *T. acidophilus* (Fig. 4). Similar results have been reported for *T. ferrooxidans* (Hazeu et al. 1988). The observed stoichiometries of sulphide-dependent oxygen consumption indicated that the intermediary sulphur is of the same redox level as elemental sulphur. The subsequent oxidation of intermediary sulphur exhibited a number of similarities with the oxidation of exogeneously added elemental sulphur: both processes were uncoupler-sensitive and could be inhibited by NEM (Table 2). These similarities suggest that the oxidation pathways of intermediary sulphur and elemental sulphur share a common intermediate (Fig. 5). The almost complete inhibition of elemental sulphur oxidation by uncouplers has previously been described for other sulphur-oxidizing bacteria. From their work on *T. ferrooxidans*, Bacon and Ingledew (1989) recently suggested that an energized membrane is required for activation of the chemically inert S_8 ring structure. Alternatively, the uncoupler sensitivity of elemental sulphur oxidation may reflect the involvement of active transport mechanisms.

At present, it is unclear whether or not sulphite is an intermediate during the oxidation of sulphur compounds by *T. acidophilus*. Accumulation of sulphite during the oxidation of any of the sulphur compounds tested was not observed (results and shown). Moreover, the sulphite oxidation rates observed at physiological pH values were not observed (results not shown). Moreover, the sulphite sulphur atoms during sulphur compound oxidation. However, it is possible that the observed oxygen uptake rates give an underestimate of the oxidation capacity for intracellularly generated sulphite.

Further research will be focused on the enzymes involved in the metabolism of the inorganic sulphur compounds by *T. acidophilus* and on the intermediates formed during the oxidation of sulphide and elemental sulphur.

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