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Kinetics and energetics of reduced sulfur oxidation by chemostat cultures of *Thiobacillus ferrooxidans*

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Abstract. Thiobacillus ferrooxidans was grown in chemostat cultures with thiosulfate and tetrathionate as the limiting substrates. The yields at steady state on both substrates at different dilution rates were calculated. In a few experiments the air supply was supplemented with 2% CO_2 (v/v). This resulted in a slightly increased yield.

Cells from the chemostat cultures were used to study the kinetics of thiosulfate, tetrathionate, sulfite and sulfide oxidation. With all substrates mentioned the Ks values were in the micromolar range. The values for thiosulfate and tetrathionate were 2 orders of magnitude lower that those published previously.

INTRODUCTION

The metabolism of reduced sulfur compounds by thiobacilli has been extensively studied. These investigations have been reviewed by London & Rittenberg (1964), Trudinger (1969), Suzuki (1974), Aleem (1975) and most recently by Kelly (1982; 1985).

Enzyme preparations catalyzing the oxidation of specific reduced sulfur compounds have been investigated by Lu & Kelly (1983), Suzuki (1965), Silver & Lundgren (1968), Tabita et al. (1969), Vestal & Lundgren (1971) and Oh & Suzuki (1977).

Data on the kinetics and energetics of reduced sulfur oxidation by these bacteria are rather scarce. Such data are of particular interest in the application of acidophiles such as *Thiobacillus ferrooxidans* in the leaching processes used for the recovery of metals from low grade ores and for coal desulfurization. Thiosulfate and tetrathionate oxidation by *T. ferrooxidans* have been studied by Eccleston & Kelly (1978) and thiosulfate oxidation by Bounds & Colmer (1972). The results of these studies showed that K_s values for both sulfur compounds were in the millimolar range. This is at least three orders of magnitude higher than those reported for *T. neapolitanus* and *T. versutus* (Beudeker et al. 1982).

Data on yield and maintenance energy obtained from thiosulfate- and tetrath-

ionate-limited cultures of *T. ferrooxidans* have been published by Kelly et al. (1977) and Eccleston & Kelly (1978).

It has recently been discovered that many strains of *T. ferrooxidans* are contaminated by *Thiobacillus acidophilus*, a facultative, autotrophic, acidophilic species, or by *Acidiphilium cryptum*, an acidophilic heterotroph (Arkesteyn 1980; Harrison 1981). Well identified pure cultures are now available, but it is not clear if, and to what extent, the results of older studies of *T. ferrooxidans* may have been influenced by the presence of contaminants.

In the course of a study on the removal of pyrite from coal, it was decided to reinvestigate the sulfur metabolism of a pure culture of *T. ferrooxidans*, as this is the main organism active in pyrite oxidation at moderate temperatures. In this paper, the kinetics and energetics of the oxidation of soluble, reduced sulfur compounds as model substrates for the sulfur part of pyrite, will be reported. It will be shown that our strain of *T. ferrooxidans* had a K_s for thiosulfate and tetrathionate two orders of magnitude lower than that reported in the literature.

MATERIALS AND METHODS

All experiments have been performed with *Thiobacillus ferrooxidans* ATCC 19859 (LMD 81.68). Before use the organism was thoroughly checked for purity by single cell isolations according to the method of Mackintosh (1978). In addition, purity was checked during subculturing on ferrous iron containing medium and during cultivation in the chemostat on different sulfur compounds, by making of serial dilutions in basal medium with ferrous sulfate at pH 1.8 and with tetrathionate or glucose at pH 3.0. The purity of the culture was occasionally checked by staining of the cells with fluorescent antibodies (Muyser et al. 1985).

A modified 9K medium (Silverman & Lundgren 1959) with the following composition was used as basal medium in g/l: $(NH_4)2SO_4$ 1.0; KCl 0.1; $Ca(NO_3)2$ 0.01; $MgSO_4.7H_2O$ 0.2; K_2HPO_4 0.5. To 1 litre of this medium 1 ml of a modified trace element solution according to Vishniac & Santer (1957) was added.

Media for continuous cultivation containing thiosulfate or tetrathionate were autoclaved at 110 °C and pH 7.0 in order to prevent degradation of the sulfur compounds.

The organism was pregrown in shake flasks in medium with 5 mM tetrathionate at pH 4.5. Chemostat cultures were run at pH 3.0 with 20 mM thiosulfate or 10mM tetrathionate in the feed. All cultures were made at 30 °C.

For continuous cultivation a Biolafitte (Poissy, France) fermentor with a working volume of 1 litre, or a magnetically driven fermentor with a working volume of 1.5 litres was used (Harder et al. 1974). The pH was controlled at pH 3.0 by the automatic addition of 4 N NaOH or 2 N H_2SO_4. The fermentors

were aerated with air or with 2% CO_2 in air. The dissolved oxygen concentration was always between 80 and 100% as monitored with an oxygen electrode. The CO_2 content of air or of other gas mixtures was measured with a Beckman model 864 Infrared analyser.

Samples from the fermentor were immediately centrifuged at 20,000 g for 3 min. The supernatants were used for analysis of residual substrates or intermediates or stored at −20 °C if necessary. Dry weight determinations of biomass were carried out by filtering a measured volume of suspension over dried preweighed membrane filters (Sartorius, pore size 0.2 μ). The filters were dried to constant weight at 70 °C. As an alternative the carbon content of media, supernatants and cultures was determined with a Beckman Total Organic Carbon Analyser model 915-B. The elemental composition of thiosulfate-grown cells, as analysed with a CHN-analyser was: 47.5% C, 6.6% H, 12.9% N with an ash content of 5.03%.

The optical density of suspensions was measured at 430 nm with medium as a blank.

The respiration rate of cell suspensions after the addition of substrates or inhibitors was measured with a biological oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio). Samples from substrate-limited chemostat cultures were used without treatment, unless otherwise stated. Occasionally cells were washed by centrifuging and resuspending in basal medium at the required pH. No significant difference between washed and unwashed cells was observed for thiosulfate oxidation. All measurements were carried out at least in duplicate at different substrate concentrations, and were repeated at least twice with different samples from the same fermentor at steady state conditions. The oxygen consumption rates at different substrate concentrations were used for calculation of the K_s and V_{max} from a double reciprocal plot. A correlation coefficient of at least 95% was considered as a condition for calculation of K_s and V_{max} in this way. As the double reciprocal plot at concentrations between 0 and 100 μM did not always result in a straight line, indicating that the kinetics might be complex, the K_s was estimated as the substrate concentration at which the oxygen consumption rate was half of the maximal value.

The following analytical procedures were applied: Thiosulfate and tetrathionate were determined according to the method of Sörbo (1957) as modified by Kelly et al. (1969), because the tetrathionate analysis was found unreliable in Sörbo's method. The analysis was carried out at room temperature, however, and separate calibration curves were made for both compounds to allow the calculation of the amount of thiocyanate produced. Sulfite was analysed by the method of Trüper & Schlegel (1964). Glycolic acid was demonstrated by the method of Calkins (1943).

Thiosulfate and tetrathionate were supplied by Fluka.

Table 1. Apparent yield (g dry weight/mol substrate consumed) for chemostat cultures of *Thiobacillus ferrooxidans* grown at pH 3.0 and various dilution rates.

limiting substrate	dilution rate (h^{-1})	Y (g/mol)
$Na_2S_2O_3$	0.016	4.9
	0.021	4.9
	0.031	6.3*
	0.037	5.5
$K_2S_4O_6$	0.006	6.3*
	0.008	9.9*
	0.012	9.7*
	0.020	8.6
	0.023	9.0
	0.026	8.9–9.9
	0.030	10.3
	0.030	12.3–13.6*

* culture sparged with air + 2% CO_2

RESULTS

Characteristics and yield data of chemostat cultures of Thiobacillus ferrooxidans

The yield data, expressed as g dry weight/mol substrate consumed for different substrates and conditions are summarized in Table 1. All cultures were substrate-limited, as could be concluded from the absence of residual substrate, and the linear response of biomass concentration to an increase of the growth limiting substrate. The number of data was considered to be too limited for accurate calculation of the maximal yield and maintenance energy. It should be noted that the yield values of cultures supplied with extra CO_2 tended to be somewhat higher than those aerated with air. This increase in yield was partly caused by a slightly lower carbon content of the supernatant and a higher total carbon content of the culture. In supernatants of cultures, aerated with air, a low concentration of glycolic acid (2–5 mg/l) could be demonstrated.

A washout experiment was carried out to determine μ_{max}. During a gradual increase of the dilution rate in the tetrathionate-limited chemostat at pH 2.9, a white precipitate of sulfur on the wall of the fermentor appeared at $D = 0.065$ h^{-1}, but almost no sulfur could be observed in the culture liquid. Residual tetrathionate was still not detectable. Washout of the culture was observed at $D = 0.14$ h^{-1}.

Although the cultures were inoculated with a strain of *T. ferrooxidans* maintained in medium with ferrous sulfate as the sole energy source, the cells from thiosulfate- or tetrathionate-limited chemostat cultures could no longer oxidize

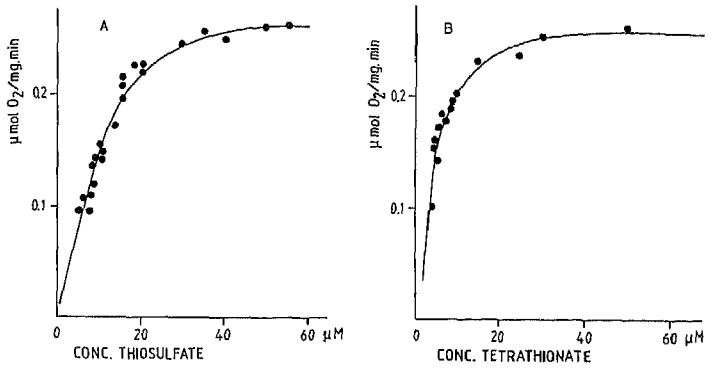

Fig. 1. Relationship between specific oxygen consumption rate (µmol/mg · min) and substrate concentration as observed in the biological oxygen monitor for cells grown in thiosulfate-limited chemostat cultures at $D = 0.02\ h^{-1}$.
Fig. 1a. thiosulfate oxidation and *Fig. 1b.* tetrathionate oxidation.

ferrous ions after a few volume changes. Similarly, iron-limited cells could poorly or not oxidize thiosulfate or tetrathionate (unpublished). Batch iron-grown cells were capable of oxidizing sulfide, sulfur and sulfite very well, but showed little thiosulfate, and no tetrathionate oxidizing capacity. Serial dilutions of the cells in media with ferrous sulfate or tetrathionate demonstrated, however, that the cells had not lost their capacity to grow on both substrates.

Kinetics of the oxidation of thiosulfate, tetrathionate, sulfide and sulfite by chemostat-grown cells of Thiobacillus ferrooxidans

The relationship between initial oxygen consumption rate and concentration of thiosulfate or tetrathionate for thiosulfate-limited cells grown at a dilution rate of $0.02\ h^{-1}$ is presented in Figs. 1a–1b. At tetrathionate concentrations between 0 and 5 µM, it sometimes took several minutes for the cells to respond,

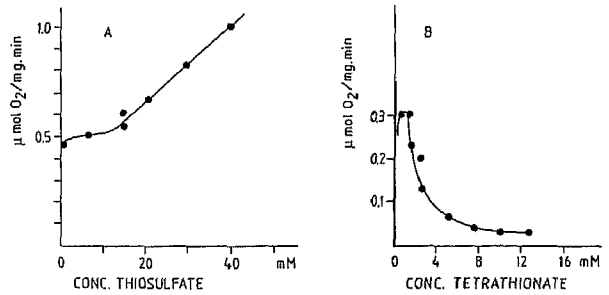

Fig. 2. Relationship between oxygen consumption rate and higher thiosulfate (*Fig. 2a*) and tetrathionate (*Fig. 2b*) concentrations for tetrathionate-limited cells, grown at $D = 0.02\ h^{-1}$.

Table 2. Calculated and estimated* K_s and V_{max} values obtained from experiments with thiosulfate- or tetrathionate-limited cells of *Thiobacillus ferrooxidans* grown at different dilution rates, at pH 3.0.

growth substrate	dilution rate (h^{-1})	substrate investigated	K_s (μM)	V_{Max} (μmol O_2/mg dry weight.min.)
$Na_2S_2O_3$	0.02	$Na_2S_2O_3$	9*	0.27*
		$K_2S_4O_6$	4.2*	0.26*
	0.03	$Na_2S_2O_3$	20*	0.26*
		$K_2S_4O_6$	15*	0.22*
		Na_2SO_3	59	0.24
		Na_2S	4.7	0.22
$K_2S_4O_6$	0.02	$Na_2S_2O_3$	16	0.27
		$K_2S_4O_6$	3.7	0.29
		Na_2SO_3	35	0.18
	0.03	$Na_2S_2O_3$	14.5	0.23
		$K_2S_4O_6$	3.5*	0.19*
		Na_2SO_3	30*	0.25*
		Na_2S	3.4	0.30

or they did not react. With the other substrates such a minimum threshold concentration was not observed. Data obtained with higher substrate concentrations are given in Figs 2a–2b, for tetrathionate-limited cells. High concentrations of tetrathionate (above 1–1.5 mM) were found to be inhibitory. Thiosulfate oxidation, on the contrary, increased linearly with concentrations above 10 mM. Comparison of Figs 1 and 2 shows that the oxidation kinetics of these two substrates are rather complex. Kinetic data relevant for the micromolar range of substrate concentrations were calculated, if possible, from a Lineweaver-Burk plot of Fig. 1, and from similar data obtained at other dilution rates or with other substrates, or were estimated. No kinetic data were estimated from the experiments in the millimolar range. Calculated and estimated values from a number of experiments have been summarized in Table 2. It was found that during prolonged cultivation in the chemostat the affinity of the cells for tetrathionate gradually increased from about 200 to less than 10 μM after 100–200 volume changes. When the experiment was repeated, the same adaptation was observed. The affinity for thiosulfate, sulfite and sulfide remained almost constant from the start of the experiment.

The oxidation kinetics of thiosulfate by thiosulfate-grown cells was also studied at different pH values. The K_s values decreased from 1–3 mM at pH 4.3 via 10–20 μM at pH 3.0 (Table 2) to less than 10 μM at pH 1.9.

As pyrite is a natural substrate for *T. ferrooxidans*, it might be expected that during sulfur oxidation high iron concentrations would occur. The influence of ferrous and ferric sulfate on thiosulfate oxidation was therefore studied. It

was found that at concentrations of 75 mM, these salts did not change the K_s for thiosulfate significantly, but caused a decrease of V_{max} of 20–30%.

Stoichiometry of reduced sulfur oxidation

In the oxygen uptake experiments discussed in the previous paragraph it was noted that the total oxygen uptake was always 10 to 30% less, than would be expected from the complete oxidation to sulfate of the substrate supplied. Only sulfite was oxidized stoichiometrically to sulfate. If the oxidation is coupled to energy generation 10–15% of the available electrons might be used for CO_2 reduction. Therefore it must be assumed that some intermediate reduced sulfur compound such as elemental sulfur had been formed even at the micromolar concentration used. These observations together with the observed production of sulfur during the washout experiment, stimulated the investigation of the mechanism of reduced sulfur oxidation by this organism. Detailed studies on possible intermediates, and on the oxidation pathway, will be published separately.

DISCUSSION

Chemostat cultures

In the course of a study of the sulfur metabolism of *T. ferrooxidans* it was decided to check some of the basic characteristics of chemostat cultures of a strain of this organism grown on sulfur compounds. This was done because many reputedly pure cultures of *T. ferrooxidans* had been reported to be contaminated with acidophilic heterotrophs. The results of the investigations presented here essentially confirm the data published in earlier work, as regards μ_{max}, yields and the effect of CO_2 on the yields, but the affinity constants for the growth-limiting substrates were dramatically different. The yields on tetrathionate were similar to those published by Eccleston & Kelly (1978) and Kelly et al. (1977), who reported a yield on tetrathionate of 8 g/mol at $D = 0.02\ h^{-1}$, which increased to about 10 g/mol during aeration with air containing 9% CO_2. From data of Tuovinen (1977) a yield of 5.9 g/mol for thiosulfate and of 9.8 g/mol for tetrathionate could be calculated. Yields of other thiobacilli grown on thiosulfate, such as *T. neapolitanus* and *T. versutus*, fall in the same range (Hempfling & Vishniac 1967; Kuenen 1979). The increase in yield of *T. ferrooxidans* during aeration with CO_2-enriched air as observed by us (Table 1) and others (Eccleston & Kelly 1978) raised the question of whether CO_2 rather than the energy source had been growth limiting. The fact that an increase in the concentration of the energy source gave a linear response in the observed yield seemed to rule out this possi-

bility. As an additional check for possible CO_2 limitation the theoretically necessary CO_2 supply was calculated. The CO_2 supply of our fermentor, when sparged with 700 ml air/min was 22.3 mg/h (6.1 mg C/h). At a biomass concentration of 80 mg/l (47.5% C) and at $D = 0.02\ h^{-1}$ the carbon requirement of the culture would be 0.76 mg C/l.h, corresponding to 2.79 mg CO_2. The mass transfer of CO_2 in this type of fermentor was estimated to be 5.83 mg C/l.h, at a known Kla of at least 60 h^{-1}, and a solubility at 30°C and atmospheric pressure of 8.1 µM for 0.03% CO_2 in air. (Kla is the product of the mass transfer coefficient Kl (m/s) for gasses from air to liquid, and the specific surface a (m^2/m^3).) From these calculations it can be concluded that both the availability and the mass transfer of CO_2 were sufficient.

An alternative explanation of the CO_2-effect might be inefficient CO_2 fixation at relatively low CO_2 concentrations. Indeed the presence of a low concentration of glycollate in cultures aerated with excess air indicated that under these conditions of sub-optimal CO_2/O_2 ratio the ribulose bisphosphate carboxylase in the cells was also acting as an oxygenase. This has previously been reported for *Thiobacillus neapolitanus* (Beudeker et al. 1981). The latter organism excreted 4.3 mg glycollate at comparable cell densities during growth in excess air. Electron micrographs of our cells, when grown under normal growth conditions in the chemostat did not contain appreciable numbers of carboxysomes (unpublished). However, when the dissolved oxygen concentration was allowed to fall from the normal 80–100% to 60% air saturation, the yield decreased and numerous carboxysomes were observed. From previous work with *T. neapolitanus* (Beudeker et al. 1981), it is known that an increase in the number of carboxysomes is a response to a decrease in the available CO_2 concentration in the steady state culture. Thus it can be inferred that *T. ferrooxidans* grown at air saturation did not behave as though carbon limited. The dependence of the yield on the CO_2 content of the air might therefore be explained by either or both of two factors:

i. a very low affinity of the cells for CO_2, which would lead to an inefficient coupling between energy generation and carbon fixation at low CO_2 concentrations,
ii. oxygenase activity of ribulose bisphosphate carboxylase, which leads to glycollate production and consequently energy losses.

Kinetics of thiosulfate, tetrathionate, sulfite and sulfide oxidation. Work by Arkesteyn (1980) and by Huber et al. (1984) provided strong evidence that *T. ferrooxidans*, when growing on pyrite, oxidizes not only the ferrous iron, but also the sulfur moiety of the pyrite. Therefore, insight into the kinetics of sulfur compound oxidation is of importance, from both a theoretical and an applied point of view, in order to understand and model the growth of *T. ferrooxidans* on its natural substrate, pyrite. Only limited published data on the kinetics of re-

Table 3. Kinetic data on the oxidation of reduced sulfur compounds by whole cells or by isolated enzymes from *Thiobacillus ferrooxidans*.

Organism	Substrate	K_s(mM)	Reference
Thiobacillus ferrooxidans	$Na_2S_2O_3$	20–50	Bounds & Colmer (1972)
Ferrobacillus ferrooxidans	$Na_2S_2O_3$	20–50	Bounds & Colmer (1972)
Ferrobacillus ferrooxidans	$Na_2S_2O_3$	5–7	Bounds & Colmer (1972)
Thiobacillus ferrooxidans	$Na_2S_2O_3$	1.2–25	Eccleston & Kelly (1978)
	$K_2S_4O_6$	0.13–8.33	Eccleston & Kelly (1978)
Thiobacillus ferrooxidans	$Na_2S_2O_3$	0.005–0.025	this study
	$K_2S_4O_6$	0.004–0.025	this study
	Na_2SO_3	0.03–0.06	this study
	Na_2S	0.005	this study
enzyme			
thiosulfate-oxidizing enzyme from *T. ferrooxidans*		0.9	Silver & Lundgren (1968)
rhodanese from *T. ferrooxidans*		0.58	Tabita et al. (1969)
sulfite oxidase		0.58	Vestal & Lundgren (1971)

duced sulfur oxidation by whole cells of *T. ferrooxidans* are available (Bounds & Colmer 1972; Eccleston & Kelly 1978) and these were obtained from tests at relatively high substrate concentrations. The studies reported here provide a set of values obtained from experiments at low concentrations, which are more relevant for the growth of *T. ferrooxidans* under 'natural' conditions, or during the oxidation of poorly soluble compounds such as pyrite.

Table 3 shows that the observed K_s values for thiosulfate and tetrathionate were one or two orders of magnitude lower than those previously published. It should be noted that the K_s values given are the result of a chain of enzyme conversions leading to oxygen consumption via the respiratory chain. That these values are representative of the actual situation during growth in the chemostat is indicated by the fact that thiosulfate and tetrathionate were not detectable in the cultures. Also at a dilution rate of 0.02 h^{-1}, the actual rate of oxygen consumption by the thiosulfate-limited culture was about 0.12–0.14 μmol O_2/mg dry weight.min, half of the maximal value observed in Fig. 1. Although it is clear that the K_s values are dependent on the growth conditions, such as pH, and the strain (or mixed culture) used, it appears that, over a broad range of pH values, the K_s value for reduced sulfur compounds, including sulfide, of *T. ferrooxidans* is at least two orders of magnitude lower than the K_s values for ferrous iron. This raises the question of the relative importance of sulfur versus iron oxidation of *T. ferrooxidans* during growth on pyrite.

A particularly interesting observation was the adaptation of *T. ferrooxidans* to increasingly lower tetrathionate concentrations during prolonged cultiva-

tions. This may reflect the selection of more efficient strain(s). Comparable phenomena have been observed during continuous cultivation of other organisms (Dijkhuizen & Hartl 1983).

The V_{max} values for different substrates (Table 1) were lower than those found for thiosulfate and tetrathionate by Eccleston & Kelly (1978), but this is a consequence of the concentrations tested. When the initial rate for thiosulfate is taken into consideration, then the oxidation step of thiosulfate to tetrathionate is very rapid. This may have consequences for poorly buffered media containing high thiosulfate concentrations (10 mM), as the pH can rise from 3 to 5.8 weithin 10 minutes. Bounds & Colmer (1972) reported the same phenomenon.

Our strain of *T. ferrooxidans* was oxidizing thiosulfate at high concentrations, but comparable concentrations of tetrathionate were strongly inhibitory (Fig. 2). Eccleston & Kelly (1978) reported a constant oxidation rate for tetrathionate at pH 3.0 in the same concentration range.

The stoichiometry of thiosulfate and tetrathionate oxidation resulting in insufficient oxygen uptake has also been observed for '*Thiobacillus* X' (*T. neapolitanus*) by Trudinger (1964). Although the exact reason for this is unknown, it can be expected that part of the electrons might be directed via the 'reversed flow' for the production of reduced NAD(P), and thus are not detected in respiration. From the elemental composition of the cells, it can be calculated that during growth on thiosulfate with a yield of 6 g/mol, about 12.5% of the electrons will be required for reduction of CO_2 to the oxidation level of biomass.

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