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Reduced Sulfur Compound Oxidation by *Thiobacillus caldus*

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The oxidation of reduced inorganic sulfur compounds was studied by using resting cells of the moderate thermophile *Thiobacillus caldus* strain KU. The oxygen consumption rate and total oxygen consumed were determined for the reduced sulfur compounds thiosulfate, tetrathionate, sulfur, sulfide, and sulfite in the absence and in the presence of inhibitors and uncouplers. The uncouplers 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenyl-hydrazone had no affect on the oxidation of thiosulfate, suggesting that thiosulfate is metabolized periplasmically. In contrast, the uncouplers completely inhibited the oxidation of tetrathionate, sulfide, sulfur, and sulfite, indicating that these compounds are metabolized in the cytoplasm of *T. caldus* KU. *N*-Ethylmaleimide inhibited the oxidation of tetrathionate and thiosulfate at the stage of elemental sulfur, while 2-heptyl-4-hydroxyquinoline-*N*-oxide stopped the oxidation of thiosulfate, tetrathionate, and elemental sulfur at the stage of sulfite. The following intermediates in the oxidation of the sulfur compounds were found by using uncouplers and inhibitors: thiosulfate was oxidized to tetrathionate, elemental sulfur was formed during the oxidation of tetrathionate and sulfide, and sulfite was found as an intermediate of tetrathionate and sulfur metabolism. On the basis of these data we propose a model for the metabolism of the reduced inorganic sulfur compounds by *T. caldus* KU.

Thiobacillus caldus KU (5) is a moderately thermophilic acidophile found in environments such as coal spoil heaps (17), where the oxidative dissolution of sulfide minerals occurs. This bacterium obtains its carbon by reductive fixation of atmospheric CO_2. *T. caldus* is capable of oxidizing a wide range of reduced sulfur compounds, but it is incapable of oxidizing ferrous iron or pyrite. One of the products of the oxidation of reduced sulfur compounds is H_2SO_4, and this bacterium is able to live in acidic environments, down to pH 1.

One biotechnological application of acidophilic bacteria is the biooxidation of refractory sulfidic ores for the enhanced recovery of gold (13, 14). The gold is often associated with the iron sulfides pyrite (FeS_2) and arsenopyrite (FeAsS) as fine particles trapped within the mineral matrix. During the biooxidation process, the iron sulfides are oxidized to soluble ferric iron and sulfate, liberating the gold particles. It has been found that *T. caldus* is the primary sulfur oxidizer enriched from pilot scale bioleaching reactors operating at temperatures above 40°C (4). In a different pilot scale study of reactors operating between 45 and 50°C, *T. caldus* makes up approximately 10% of the total bacterial population (1).

Recent studies regarding the sulfur metabolism by bacteria of the genus *Thiobacillus* have focused on two acidophilic mesophilic species, *T. ferrooxidans* and *T. acidophilus*, and a moderately thermophilic neutrophile, *T. tepidarius*. It has been proposed that *T. acidophilus* is a suitable organism for use as a model of sulfur oxidation for thiobacilli (24). The enzyme which catalyzes the oxidation of thiosulfate ($S_2O_3^{2-}$) to tetrathionate ($S_4O_6^{2-}$) has been shown to be located in the periplasm of these bacteria (7, 16, 19).

Tetrathionate hydrolase, which catalyzes the metabolism of tetrathionate to sulfate, is located in the periplasm of *T. acidophilus* and *T. ferrooxidans* (7, 21), while in *T. tepidarius* it is located in the cytoplasm (16).

During our studies on the arsenical resistance mechanism of *T. caldus*, it was found that arsenite caused a transient production of sulfur in the culture medium of growing cells (6). In addition, we found that we were unable to effectively study the energetics of arsenic efflux because of a lack of understanding of the metabolism of *T. caldus*. We have therefore studied reduced inorganic sulfur compound metabolism in the moderately thermophilic acidophile *T. caldus* KU. Oxygen consumption and use of cellular poisons have led to a model of reduced sulfur compound oxidation in strain KU.

MATERIALS AND METHODS

Stock solutions. All stock solutions were prepared from commercially available chemicals. The sulfite solution was prepared in 50 mM EDTA to prevent autooxidation. The stock solutions of other reduced sulfur compounds were dissolved in water, except elemental sulfur, which was dissolved in acetone. The inhibitors and uncouplers utilized were dissolved in ethanol (96%, vol/vol). Control experiments showed that neither the final acetone concentration nor the final ethanol concentration adversely affected the oxidation of the substrates by *T. caldus.*

Bacteria, growth conditions, and preparation of resting cells. *T. caldus* KU (5) (DSM 8584 and ATCC 51756) was grown in batch culture in a medium consisting of the basal salts (grams per liter) $(NH_4)_2SO_4$ (3.0), $Na_2SO_4 \cdot 10H_2O$ (3.2), KCl (0.1), K_2HPO_4 (0.05), $MgSO_4 \cdot 7H_2O$ (0.5), and $Ca(NO_3)_2$ (0.01) and the following trace elements (milligrams per liter): $FeCl_3 \cdot 6H_2O$ (11.0), $CuSO_4 \cdot 5H_2O$ (0.5), HBO_3 (2.0), $MnSO_4 \cdot H_2O$ (2.0), $Na_2MoO_4 \cdot 2H_2O$ (0.8), $CoCl_2 \cdot 6H_2O$ (0.6), and $ZnSO_4 \cdot 7H_2O$ (0.9). The basal salts were adjusted to pH 2.5 with H_2SO_4 and autoclaved before the filter-sterilized trace elements were added. Tetrathionate (5 mM) served as the energy source except in one experiment in which 0.5% (wt/vol) sulfur (S^0) was used. The growth temperature was 45 ± 1°C, and the growth medium was sparged with CO_2-enriched (2%, vol/vol) air. Bacteria were harvested in late exponential phase (at an optical density at 440 nm of between 0.27 and 0.28), centrifuged, and washed with and resuspended in sulfate buffer (50 mM K_2SO_4 50 mM Na_2SO_4 adjusted to pH 3.0 with H_2SO_4). These resting cells were maintained in the sulfate buffer at 45°C for no longer than 2 h before use.

Assay of substrate-dependent oxygen consumption. The oxidation of reduced sulfur compounds was assayed with a Hansatech oxygen electrode at 45°C in the sulfate buffer with 50 μg (dry weight) of cells · ml^{-1} in a 1-ml reaction volume. The oxygen uptake rates were calculated from the initial linear oxygen consumption rate and were corrected for any basal level of oxygen consumption. Calculations were based on the assumption that air-saturated buffer contains 192 nmol

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TABLE 1. Oxidation of reduced sulfur compounds by resting *T. caldus* KU cells in the absence or in the presence of uncouplers or inhibitors

Oxidative substrate	Theoretical O_2 consumption (nmol)	Inhibitor or uncoupler	Oxidation rate (nmol O_2/min · mg)[a]	Total O_2 consumed (nmol)[a]
$S_4O_6^{2-}$ (25 nmol)	87.5	None	403 ± 11	77 ± 3
		CCCP (20 μM)	0	0
		NEM (6 mM)	83 ± 1	13 ± 1
		HQNO (10 μM)	140 ± 27	60 ± 2
$S_2O_3^{2-}$ (100 nmol)	200	None	866 ± 29	159 ± 7
		CCCP (20 μM)	602 ± 18	25 ± 1
		NEM (6 mM)	340 ± 28	26 ± 2
		HQNO (10 μM)	337 ± 17	139 ± 7
S^0 (60 nmol)	90	None	451 ± 7	85 ± 2
		CCCP (20 μM)	0	0
		NEM (6 mM)	0	0
		HQNO (10 μM)	192 ± 13	61 ± 3
SO_3^{2-} (50 nmol)	25	None	300 ± 29	19 ± 1
		CCCP (20 μM)	0	0
		HQNO (10 μM)	0	0
S^{2-} (50 nmol)	100	None	458 ± 23	94 ± 5
		CCCP (20 μM)	226 ± 1	26 ± 1
		NEM (6 mM)	421 ± 1	20 ± 1

[a] Mean value ± standard deviation of at least three experiments.

of $O_2 \cdot ml^{-1}$ at 45°C. Substrate-dependent oxygen uptake rates in the presence of inhibitors and uncouplers were assayed after a 3-min incubation of the cell suspension with the inhibitors or uncouplers. The experiment was run until the oxygen consumption rate returned to the base level, and the difference in the O_2 concentration was considered to be total O_2 consumed.

Analysis of intermediates and end products. The substrate utilization and product formation analyses of the reduced sulfur compounds in the presence and in the absence of uncouplers and inhibitors were carried out with the sulfate buffer. S^0 production from sulfide (S^{2-}) was measured as the increase of A_{430} (7). For the other reduced sulfur compounds, samples were taken at various time points and filtered (0.22-μm-pore-size acetate filter) or added to Eppendorf tubes placed in an ethanol ice bath to stop bacterial oxidation. Tetrathionate was analyzed by cyanolysis (26) as modified (11). Elemental sulfur concentration was determined by cyanolysis as described previously (7). High-pressure ion chromatography with a Milton Roy pump and conductivity meter, a Dionex ion PAC ASA A precolumn, and an ASA 4A anion column with a suppressor column was used for thiosulfate analysis. The eluent used for high-pressure ion chromatography was 0.8 mM Na_2CO_3–3 mM $NaHCO_3$. Sulfite was analyzed spectrophotometrically with *para*-rosaniline as the indicator (27).

RESULTS

Oxidation of reduced sulfur compounds by resting cells. Experiments with tetrathionate-grown resting cells in the O_2 electrode were carried out to gain an insight into the oxidation of the reduced sulfur compounds by strain KU.

The rate of oxygen consumption with tetrathionate as the substrate was linear over the first half of the time course, decreasing towards the end of the reaction. Increasing the concentration of tetrathionate from 12.5 nmol increased the oxygen consumption rate up to 600 nmol of tetrathionate, at which concentration the O_2 consumption rate decreased because of apparent substrate inhibition. Thiosulfate-dependent oxygen utilization exhibited a biphasic pattern, indicating that tetrathionate was produced from thiosulfate oxidation as seen with other thiobacilli (16, 19). The initial linear O_2 consumption rate was considered to be the thiosulfate oxidation rate, which was higher than with tetrathionate as the substrate (Table 1). The oxygen consumption rate with S^0 was higher than the O_2 consumption rate with tetrathionate as the substrate but lower than the rate obtained with thiosulfate as the substrate (Table 1). At pH 3.0, the oxygen consumption rates for sulfite and sulfide were in the same range as for tetrathionate and S^0 (Table 1). This was the pH optimum for sulfite oxidation (over a pH range of 2.0 to 6.0), but the oxygen consumption rate for sulfide increased to 909 ± 98 nmol of O_2 $min^{-1} \cdot mg$ (dry weight) of $cells^{-1}$ at pH 4.5 (Fig. 1).

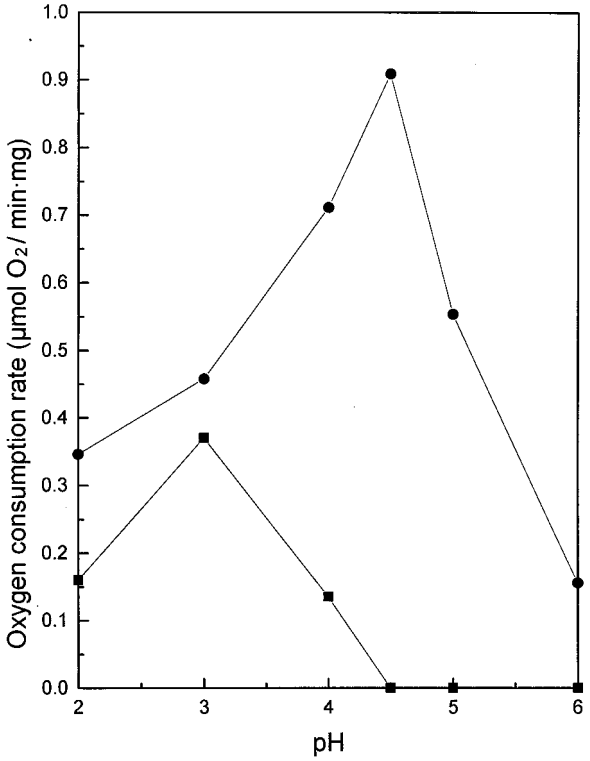

FIG. 1. Sulfide (●) and sulfite (■) oxidation by *T. caldus* KU in sulfate buffer at various pH values.

The oxidation of each of these reduced sulfur compounds by tetrathionate-grown *T. caldus* KU exhibited near-stoichiometric values of total O_2 consumption, as expected for the complete oxidation to sulfate (Table 1).

T. caldus KU grown on sulfur exhibited a reduced oxygen consumption rate for thiosulfate (108 ± 9 nmol of O_2 min^{-1} · mg [dry weight] of $cells^{-1}$) and insignificant oxygen consumption rates for tetrathionate and sulfide (8 ± 2 nmol of O_2 min^{-1} · mg [dry weight] of $cells^{-1}$ and basal level, respectively) compared with the rates obtained with tetrathionate-grown strain KU. This suggests that the enzymes for the oxidation of thiosulfate, tetrathionate, and sulfide are inducible. The oxygen consumption rate for sulfite was comparable to that obtained with tetrathionate-grown *T. caldus* KU (data not shown). The elemental sulfur oxidation rate was higher in sulfur-grown cells than in tetrathionate-grown cells (600 ± 47 compared with 451 ± 7 nmol of O_2 min^{-1} · mg [dry weight] of $cells^{-1}$).

On the basis of the oxygen consumption data, we have further investigated the pathway and cellular locations of the reduced sulfur compound metabolism through the use of cellular poisons.

Oxidation of reduced sulfur compounds in the presence of uncouplers. The uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) exhibited the same effects on reduced sulfur compound oxidation, but data are presented only for CCCP. CCCP (20 μM) completely inhibited tetrathionate-dependent oxygen consumption (Table 1), and 10 μM CCCP reduced the rate by 65% (data not shown). Total oxygen consumed during the first phase of oxygen consumption with thiosulfate as the substrate was unaffected by the addition of 20 μM CCCP, but the rate was reduced to 70%, and 80 μM CCCP also reduced the rate to approximately 70%. The subsequent oxidation of tetrathionate produced from thiosulfate was completely inhibited. CCCP (20 μM) completely inhibited S^0- and sulfite (SO_3^{2-})-dependent oxygen consumption (Table 1), but 80 μM CCCP was required to completely inhibit the oxidation of S^{2-}. CCCP (20 μM) reduced the S^{2-} oxygen consumption rate to 49% of the rate for the control, and the total oxygen consumed indicated that the oxidation stopped at the redox level of sulfur.

Oxidation of reduced sulfur compounds in the presence of inhibitors. *N*-Ethylmaleimide (NEM) (6 mM) reduced the overall O_2 consumption of strain KU with 25 nmol of tetrathionate as the substrate to 13 ± 1 nmol of O_2 and with 100 nmol of thiosulfate to 26 ± 2 nmol of O_2. The oxygen consumption rates with these two substrates were also substantially reduced (Table 1). Upon the addition of 4 mM NEM, sulfur oxidation was greatly reduced, and 6 mM NEM completely inhibited sulfur oxidation. Six millimolar NEM was required to completely inhibit the oxidation of S^0 that was formed during the oxidation of S^{2-} with only 20 ± 1 nmol of O_2 consumed from 50 nmol of S^{2-} (Table 1).

2-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) (10 μM) completely inhibited oxygen consumption with sulfite as the substrate (Table 1). The oxidation rates of tetrathionate, thiosulfate, and elemental sulfur were all reduced by 10 μM HQNO (Table 1). In addition, the total oxygen consumed with each of these substrates was reduced, indicating that the complete oxidation of these substrates did not occur but was stopped at SO_3^{2-}.

Analysis of the intermediates and end products. To further analyze the pathway of reduced sulfur compound oxidation by *T. caldus* KU, we assayed for the intermediates formed.

As indicated above from the oxygen consumption data and the effect of CCCP, tetrathionate is the first intermediate formed during the oxidation of thiosulfate. Within approximately 5 min, 1 mM thiosulfate was fully oxidized and a slightly less than stoichiometric equivalent of tetrathionate was produced (Fig. 2A). The tetrathionate produced was completely oxidized after a total of 22 min. When 200 μM DNP was added, the tetrathionate produced was not oxidized by *T. caldus* KU (Fig. 2B).

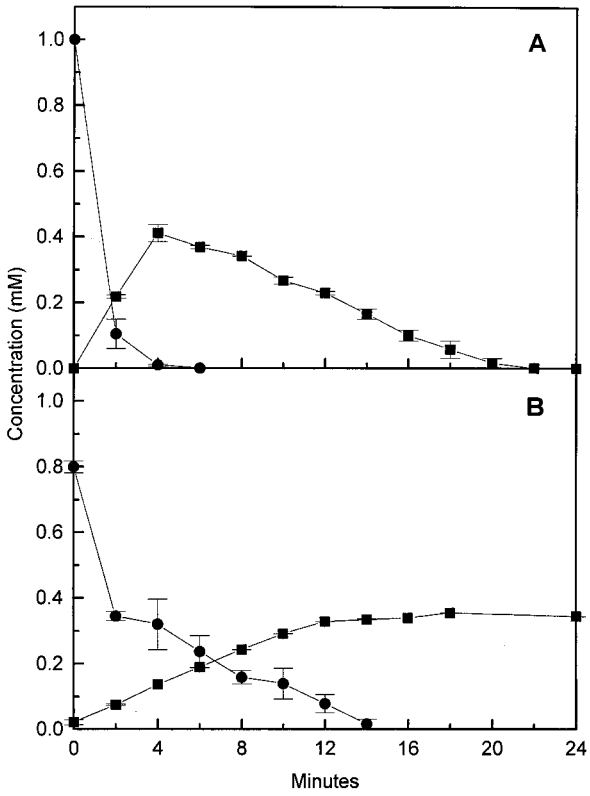

FIG. 2. Oxidation of thiosulfate (●) to tetrathionate (■) by *T. caldus* KU in the absence (A) and in the presence (B) of 200 μM DNP. Each datum point is a mean value of two experiments, with the error bars indicating the standard error.

In the presence of 6 mM NEM, 19 nmol of tetrathionate was consumed in 15 min and a stoichiometric amount of S^0 was produced (Fig. 3). In the presence of 10 μM HQNO, *T. caldus* KU produced 78 nmol of sulfite from 81 nmol of S^0 (Fig. 4). When 10 μM HQNO was added to *T. caldus* KU in the presence of 71 nmol of tetrathionate, a stoichiometric amount of sulfite (137 nmol) was produced (Fig. 5).

A transient production of sulfur was observed during the oxidation of 50 nmol of S^{2-}, as indicated by an increase in A_{430} of a suspension of *T. caldus* KU (data not shown). In the presence of 4 mM NEM and 50 nmol of S^{2-}, a larger accumulation of sulfur occurred before it was subsequently oxidized, and 6 mM NEM completely inhibited further oxidation of the accumulated sulfur. The presence of accumulated S^0 was confirmed by analysis of the culture medium (data not shown).

DISCUSSION

The metabolism of reduced sulfur compounds by mesophilic acidophilic thiobacilli (24) and the neutrophilic thiobacilli (10) has been well studied. In contrast, little information concerning the oxidation of reduced sulfur compounds in moderately thermophilic acidophiles has been published. The growth of the moderately thermophilic acidophile *T. caldus* BC13 on

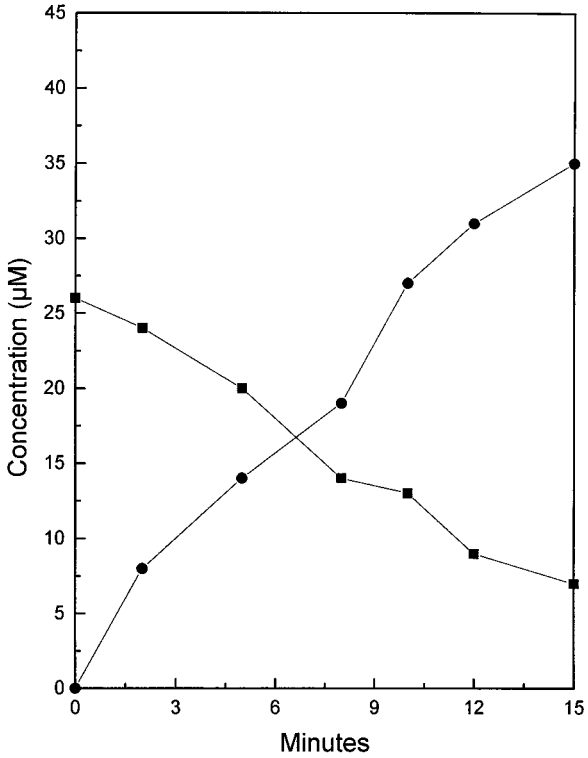

FIG. 3. Accumulation of sulfur (●) during the metabolism of tetrathionate (■) by *T. caldus* KU in the presence of 6 mM NEM. The data were obtained from two separate experiments.

tetrathionate and sulfur (23), as well as the growth of strain BC13 in chemostats on tetrathionate and thiosulfate (22), has been described previously. We report here a more thorough study of the metabolism of reduced sulfur compounds by *T. caldus.*

The rates of oxidation of the reduced sulfur compounds by tetrathionate-grown *T. caldus* KU were higher than those reported for *T. ferrooxidans* (7) and *T. tepidarius* (16). The total amount of oxygen consumed (Table 1) for each of the reduced sulfur compounds was consistent with their complete oxidation to sulfate (SO_4^{2-}) by strain KU. In some cases, 100% of the theoretical oxygen consumption was not met, presumably because of channelling of some electrons to the production of reducing equivalents, such as NAD(P)H.

After growth of *T. caldus* KU on elemental sulfur, the bacteria oxidize S^0 faster than those grown on tetrathionate. In addition, the oxidation rates of tetrathionate and S^{2-} are dramatically reduced and that of thiosulfate is slightly reduced in S^0-grown *T. caldus*, indicating that these activities are inducible. Similar results were also found for *T. ferrooxidans* (9). The enzymes for the oxidation of the reduced sulfur compounds seem to be constitutively expressed in *T. acidophilus* (19). The low rate of thiosulfate oxidation can be explained by the observation that very small concentrations of thiosulfate are produced when the oxidation of S^0 to SO_3^{2-} is followed by the reaction between S^0 and SO_3^{2-} to produce $S_2O_3^{2-}$ (25).

The oxidation of thiosulfate by *T. caldus* KU exhibited a biphasic pattern of oxygen consumption, indicating the accumulation of an intermediate, with the degree of inflection in the oxygen consumption rate being less than that reported for other organisms. In *Sulfolobus* strain LM the inflection in the oxygen consumption rate is significantly larger than in *T. caldus* (22). It has been reported that the oxidation of other reduced sulfur compounds follows a biphasic pattern because of the accumulation of intermediary sulfur (19). Tetrathionate-grown strain KU has a higher oxidation rate for sulfur than for tetrathionate, reducing the likelihood of intermediary sulfur accumulation.

The optimum pH for the oxidation of SO_3^{2-} by *T. caldus* KU (Fig. 1) differs from the results reported for *T. acidophilus* and *T. thiooxidans*, for which the optimum is approximately pH 6 (12, 19). *T. ferrooxidans*, in contrast, also has a pH optimum of 3.0 for sulfite oxidation (24). Strain KU exhibited a pH optimum for S^{2-} oxidation of 4.5, which is similar to that of *T. acidophilus* (24) and *T. ferrooxidans* (8).

From the results of the effects of inhibitors and uncouplers and intermediate analyses, we propose a model of reduced sulfur compound oxidation by *T. caldus* KU (Fig. 6). In this model we have indicated intermediates of the oxidation of these compounds and inferred cellular locations of the enzymes involved.

T. caldus KU oxidized 1 mM thiosulfate to 0.4 mM tetrathionate (Fig. 2A), strongly suggesting that tetrathionate is an intermediate in thiosulfate oxidation. In the presence of 200 μM DNP, no further oxidation of the tetrathionate occurs (Fig. 2B). The tetrathionate produced is trapped by the addition of the DNP as with *T. tepidarius* (15) but not with the acidophiles *T. ferrooxidans* (7) and *T. acidophilus* (19), for which thiosulfate oxidation is inhibited at the level of sulfur by uncouplers. Since the uncouplers do not inhibit thiosulfate oxidation, it appears that the thiosulfate-oxidizing enzyme is located in the periplasm of *T. caldus* KU as in other thiobacilli (16, 20).

DNP and CCCP affect the membrane potential of *T. aci-*

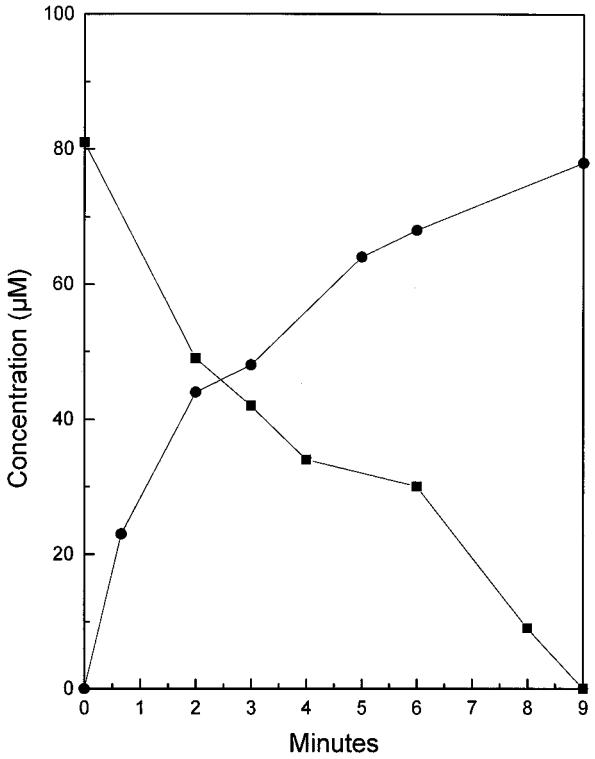

FIG. 4. Oxidation of sulfur (■) and accumulation of sulfite (●) by *T. caldus* KU in the presence of 10 μM HQNO. The data represent two different experiments.

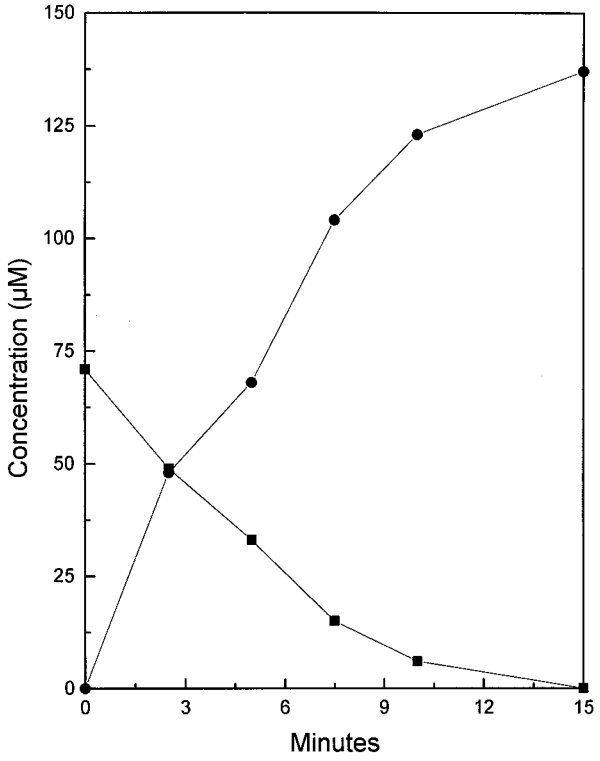

FIG. 5. Oxidation of tetrathionate (■) and accumulation of sulfite (●) by *T. caldus* KU in the presence of 10 μM HQNO. The data were obtained from two experiments.

dophilus when grown with glucose as the growth substrate (18). The inhibitory effect of CCCP on tetrathionate oxidation by *T. caldus* KU implies that this compound must be transported across the cellular membrane before oxidation. Therefore, the tetrathionate hydrolase enzyme may be located in the cytoplasm of the cell or at least on the inner surface of the cell membrane. In general, the mesophilic acidophilic thiobacilli do not transport the tetrathionate into the cytoplasm, whereas the two thermophilic thiobacilli do.

CCCP inhibits the oxidation of S^{2-}, with 20 μM CCCP stopping the oxidation at the redox level of S^0 but requiring a higher concentration (80 μM) to completely inhibit the oxidation of S^{2-}. CCCP (20 μM) inhibited all S^0- and SO_3^{2-}-dependent oxygen consumption. The effect of CCCP on sulfide, sulfur, and sulfite suggests that their oxidation also occurs within the cytoplasm. It is also possible that an energized membrane is required for the metabolism of these compounds by reductive reactions, as has been shown for the metabolism of elemental sulfur by *T. ferrooxidans* (2).

NEM stopped the oxidation of tetrathionate at the level of S^0, as could be seen from the analysis of the production of S^0 from tetrathionate (Fig. 3). NEM also inhibited the oxidation of S^{2-} at the level of S^0, as shown by spectrophotometric data and confirmed by analysis of the cell suspensions used in those reactions. NEM reduced the oxidation rates of sulfur compounds in *T. caldus* more than in other acidophiles (3, 19, 22). These results suggest that sulfhydryl groups are involved in the oxidation of the reduced sulfur compounds, specifically sulfur.

HQNO completely inhibits the oxidation of sulfite to sulfate, suggesting that the ubiquinone-cytochrome *b* complex is involved in the transfer of electrons from SO_3^{2-} to oxygen. HQNO slows both the oxygen uptake rate and reduces the total oxygen consumed for each of the other reduced sulfur

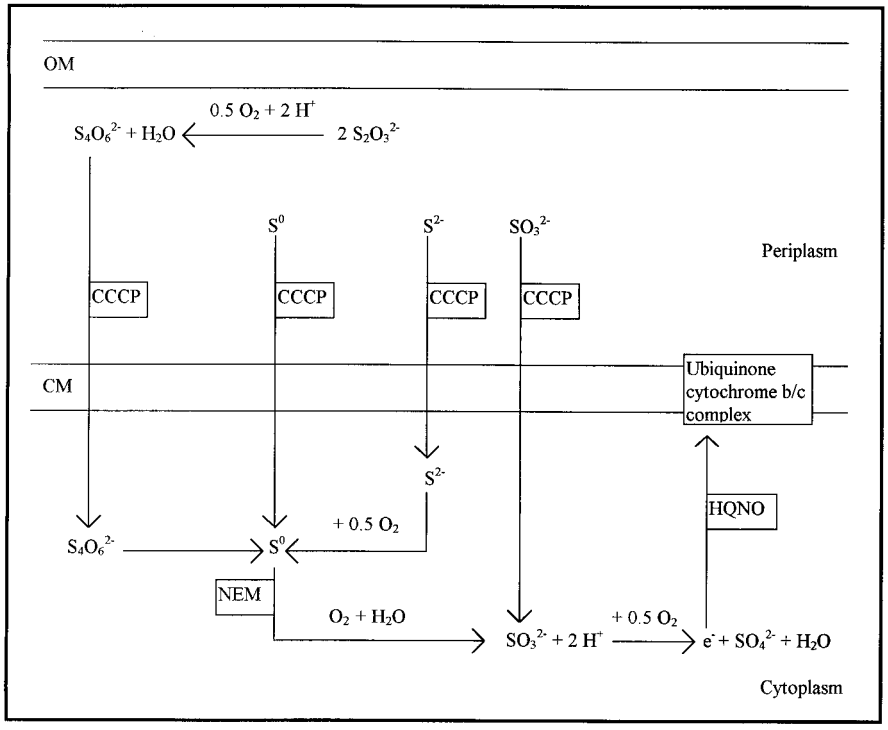

FIG. 6. Proposed model for the metabolism of reduced inorganic sulfur compounds by *T. caldus* KU. Boxes on arrows indicate at which step the compound shown exerts its inhibitory effect. OM, outer membrane; CM, cytoplasmic membrane.

compounds. A similar effect of HQNO has been observed with both *T. tepidarius* (15) and *T. thiooxidans* (3). The production of SO_3^{2-} from S^0 occurred in the presence of HQNO, indicating that SO_3^{2-} was an intermediate of S^0 oxidation. In addition, SO_3^{2-} was formed in stoichiometric amounts from tetrathionate in the presence of HQNO. These data, coupled with the effect of NEM on tetrathionate oxidation, indicate that tetrathionate is oxidized to sulfate via S^0 and SO_3^{2-} by *T. caldus* KU.

Exactly how tetrathionate is metabolized by *T. caldus* KU to sulfate via S^0 and SO_3^{2-} remains unclear. Attempts to carry out the hydrolysis of tetrathionate under anaerobic conditions (19) with strain KU were not successful, as no metabolism of the tetrathionate was observed. A possible reason for this is that the uptake of the tetrathionate into the cytoplasm requires a charged membrane, which is not maintained under anaerobic conditions. Initial results with cell extracts of strain KU, at pH 7, have suggested that the metabolism of tetrathionate occurs via a hydrolysis reaction, converting the tetrathionate into thiosulfate and sulfur with the production of 2 H^+. Enzyme purification studies will clarify the mechanism of tetrathionate metabolism as well as verify the cellular locations of the other reactions proposed in the model (Fig. 6).

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