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Oxidation of Elemental Sulfur to Sulfite by *Thiobacillus thiooxidans* Cells

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Thiobacillus thiooxidans **cells oxidized elemental sulfur to sulfite, with 1 mol of O_2 consumption per mol of sulfur oxidized to sulfite, when the oxidation of sulfite was inhibited with 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.**

The acidophilic thiobacilli *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* both grow on elemental sulfur, but only the latter uses ferrous iron as an energy source. The oxidation of sulfur to sulfuric acid by *T. thiooxidans* with sulfite as the key intermediate was proposed (8, 9) on the basis of the sulfur-oxidizing enzyme and sulfite-oxidizing enzyme systems: $S^0 + O_2 + H_2O \rightarrow H_2SO_3$ and $H_2SO_3 + \frac{1}{2} O_2 \rightarrow H_2SO_4$. A similar system involving iron reduction and oxidation was proposed more recently for *T. ferrooxidans* (6), with the same overall stoichiometry. Despite the presence of the sulfur- and sulfite-oxidizing enzyme systems in these as well as in other thiobacilli (9), the mechanism of oxidation of inorganic sulfur compounds is still controversial (3). This controversy is probably due to the reactive nature of inorganic sulfur intermediates, which makes the direct demonstration of a partial oxidation reaction difficult. Nevertheless, *T. ferrooxidans* cells accumulate some sulfite during the oxidation of sulfur when the pH is raised (7).

This report presents direct evidence that sulfite is the oxidation product of sulfur in *T. thiooxidans* cells when the further oxidation of sulfite is inhibited. Under certain conditions, sulfur is nearly stoichiometrically oxidized to sulfite; i.e., the oxidation of sulfur to sulfite is totally dissociated from the oxidation of sulfite to sulfate.

T. thiooxidans (ATCC 8085) was grown for 5 days at 28°C without shaking on elemental sulfur powder spread on the surface of Starkey's medium 1 as described previously (8), and the cells were suspended in the same medium. O_2 consumption in a total reaction mixture volume of 1.2 ml containing 4 mg of wet cells in 50 mM potassium phosphate buffer of various pHs was measured polarographically by use of an Oxygraph (Gilson) with a Clark electrode. Sometimes growth medium adjusted to pH 2.3 with sulfuric acid or 0.1 M β-alanine–H_2SO_4 buffer of pH 3.0 was used, with results similar (data not shown) to those obtained with the phosphate buffer. Precipitated sulfur (BDH; low in iron) suspended in 0.05% Tween 80 at a concentration of 320 g/liter was used as a substrate (0.1 ml) to start the reaction. A solution of Na_2SO_3 (0.1 M) was prepared in 50 mM disodium EDTA, and 2.5 μmol of sulfite was used as a substrate. Sulfite was determined by the pararosaniline method (12) after the removal of cells and sulfur by centrifugation.

T. thiooxidans cells oxidize elemental sulfur equally well either at an acidic pH, which is required for growth, or at a neutral pH, at which no growth is possible (8). Various researchers reported 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) or antimycin A to be a strong inhibitor of sulfite oxidation in cell-free systems of thiobacilli (1, 4, 10, 11). The oxidation of elemental sulfur by *T. thiooxidans* cells was

TABLE 1. Effect of pH on the inhibition of sulfur and sulfite oxidation by *T. thiooxidans*[a]

Expt	Substrate	Inhibitor	nmol of O_2 consumed/min at pH:						
			2.3	3.0	4.0	5.5	7.0	8.0	8.5
1	Sulfur	None	45		38	46	44	45	46
		HQNO (20 μg)	6		22	28	32	34	34
2	Sulfur	None	34	37	34	39			
		HQNO (20 μg)	25 ↓ 0	28 ↓	28	32			
		Na_2SO_3 (0.2 μmol)	3			40			
3	Sulfur	None	49	46	44	51	52	50	50
		Na_2SO_3 (0.2 μmol)	2	4	9	37	51		
		Na_2SO_3 (1.0 μmol)				52	52	47	46
4	Sulfite	None	0	3		6	11		
		HQNO (20 μg)	0	0		0	0		

[a] The downward-pointing arrow indicates that the O_2 consumption rate decreased with time during the experiment, sometimes levelling off completely (↓ 0). The inhibitors were added before the substrate, except in experiment 1, in which HQNO was added during the oxidation of sulfur.

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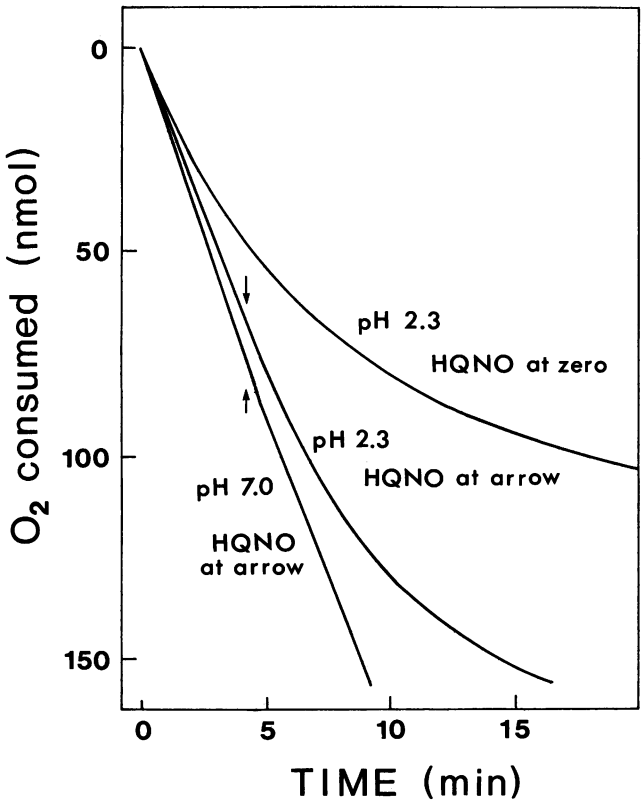

FIG. 1. Sulfur oxidation by *T. thiooxidans* cells in the presence of HQNO. HQNO (20 μg) was added to the 1.2-ml reaction mixture as indicated. The oxidation continued linearly in the absence of HQNO (data not shown).

inhibited by HQNO (Sigma; 10 mg dissolved in 1 ml of dimethyl sulfoxide [DMSO]) more strongly under acidic conditions (Table 1, experiments 1 and 2). Since sulfite was inhibitory to sulfur oxidation only at an acidic pH (Table 1, experiment 3) and the oxidation of sulfite was inhibited by HQNO independently of pH (Table 1, experiment 4), the inhibition of sulfur oxidation by HQNO was thought to be at the level of sulfite oxidation. The oxidation of sulfur in the presence of HQNO would be expected to slow down as the concentration of sulfite increased with time during sulfur oxidation under acidic conditions. The results shown in Fig. 1 and Table 1 agree with this interpretation.

Sulfite did accumulate in the presence of HQNO during sulfur oxidation at either pH 2.3 or pH 7.0, with an approximate stoichiometry of 1 mol of sulfite per mol of O_2 (Table 2, experiments 1 and 2). Very little sulfite accumulated in the absence of HQNO. Finally, when the amount of elemental sulfur as a substrate was limited (Table 2, experiments 3 and 4), the molar ratio of sulfur added to O_2 used to sulfite formed was 1:1:1, in agreement with the equation $S^0 + O_2 + H_2O \rightarrow H_2SO_3$ at pH 7.0 in the presence of HQNO. At pH 2.3, the oxidation was incomplete because of strong progressive inhibition by HQNO, and the amount of sulfite recovered was lower. In the absence of HQNO, very little sulfite accumulated and the amount of O_2 consumed was approximately 1.5 times the amount of sulfur oxidized in moles, in agreement with the complete oxidation of sulfur to sulfate: $S^0 + 1\frac{1}{2}\ O_2 + H_2O \rightarrow H_2SO_4$.

The direct demonstration of elemental sulfur oxidation to sulfite by *T. thiooxidans* cells confirms our original proposal (8, 9) and establishes the central role of sulfite in inorganic sulfur metabolism.

Corbett and Ingledew (2) reported the inhibition of sulfur oxidation but not ferrous iron oxidation by *T. ferrooxidans* cells after 4 h of preincubation with HQNO. Our preliminary results confirm their findings. The inhibition, however, seems to be more complex than that in *T. thiooxidans*, since the sulfite-oxidizing system is not inhibited by HQNO or antimycin A (5). Final results will be reported after more work.

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TABLE 2. Stoichiometry of sulfite formation from sulfur by *T. thiooxidans*[a]

Expt	Substrate	pH	HQNO (20 μg)	Oxidation rate (nmol of O_2 consumed/min)	Time (min)	Total O_2 (nmol)	Total sulfite (nmol)
1	Sulfur (32 mg), suspended	2.3	−	38	8	300	12
		2.3	+	27 ↓ 3	18	170	150
		7.0	−	38	8	300	0
		7.0	+	30	11	305	324
2	Sulfur (32 mg), suspended	7.0	−	34	9	300	19
		7.0	+	25	12	300	339
3	Sulfur in DMSO[b] (4.5 μg = 140 natom[c])	2.3	−	72	4	210	ND[d]
		2.3	+	13 ↓ 2	20	100	51
		7.0	−	82	3	210	ND
		7.0	+	47	3	140	120
4	Sulfur in DMSO[b] (5.1 μg = 160 natom)	2.3	−	72	7	230	0
		2.3	+	13 ↓ 4	24	140	61
		7.0	−	88	6	230	14
		7.0	+	50	8	160	167

[a] See Table 1, footnote *a*, for an explanation of the downward-pointing arrow.
[b] Sulfur was dissolved in DMSO, which had no effect on sulfur oxidation.
[c] The atomic weight of sulfur is 32, so 32 ng of S is 1 natom and 4.5 μg of sulfur is 40 natom; we cannot use the term moles, since sulfur can exist as S_8, etc.
[d] ND, not determined.

REFERENCES

1. **Adams, C. A., G. M. Warnes, and D. J. D. Nicholas.** 1971. A sulfite-dependent nitrate reductase from *Thiobacillus denitrificans*. Biochim. Biophys. Acta **235:**398–406.
2. **Corbett, C. M., and W. J. Ingledew.** 1987. Is $Fe^{3+/2+}$ cycling an intermediate in sulfur oxidation by Fe^{2+}-grown *Thiobacillus ferrooxidans*? FEMS Microbiol. Lett. **41:**1–6.
3. **Kelly, D. P.** 1989. Physiology and biochemistry of unicellular sulfur bacteria, p. 193–217. *In* H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Science Tech Publishers, Madison, Wis.
4. **Kodama, A., T. Kodama, and T. Mori.** 1970. Studies on the metabolism of a sulfur-oxidizing bacterium. VII. Oxidation of sulfite by a cell-free extract of *Thiobacillus thiooxidans*. Plant Cell Physiol. **11:**701–711.
5. **Sugio, T., T. Hirose, Y. L. Zhen, and T. Tano.** 1992. Purification and some properties of sulfite:ferric ion oxidoreductase from *Thiobacillus ferrooxidans*. J. Bacteriol. **174:**4189–4192.
6. **Sugio, T., W. Mizunashi, K. Inagaki, and T. Tano.** 1987. Purification and some properties of sulfur:ferric ion oxidoreductase from *Thiobacillus ferrooxidans*. J. Bacteriol. **169:**4916–4922.
7. **Sugio, T., M. Noguchi, and T. Tano.** 1987. Detoxification of sulfite produced during the oxidation of elemental sulfur by *Thiobacillus ferrooxidans*. Agric. Biol. Chem. **51:**1431–1433.
8. **Suzuki, I.** 1965. Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. Biochim. Biophys. Acta **104:**359–371.
9. **Suzuki, I.** 1974. Mechanisms of inorganic oxidation and energy coupling. Annu. Rev. Microbiol. **28:**85–101.
10. **Takakuwa, S.** 1976. Studies on the metabolism of a sulfur-oxidizing bacterium. IX. Electron transfer and the terminal oxidase system in sulfite oxidation by *Thiobacillus thiooxidans*. Plant Cell Physiol. **17:**103–110.
11. **Tano, T., T. Ito, H. Takesue, T. Sugio, and K. Imai.** 1982. B-type cytochrome, an electron carrier in the sulfite-oxidation system of *Thiobacillus thiooxidans*. J. Ferment. Technol. **60:** 181–187.
12. **West, P. W., and G. C. Gaeke.** 1956. Fixation of sulfur dioxide as disulfitomercurate (II) and subsequent colorimetric estimation. Anal. Chem. **28:**1816–1819.