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Existence of a New Type of Sulfite Oxidase Which Utilizes Ferric Ions as an Electron Acceptor in *Thiobacillus ferrooxidans*

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A new type of sulfite oxidase which utilizes ferric ion (Fe^{3+}) as an electron acceptor was found in iron-grown *Thiobacillus ferrooxidans*. It was localized in the plasma membrane of the bacterium and had a pH optimum at 6.0. Under aerobic conditions, 1 mol of sulfite was oxidized by the enzyme to produce 1 mol of sulfate. Under anaerobic conditions in the presence of Fe^{3+}, sulfite was oxidized by the enzyme as rapidly as it was under aerobic conditions. In the presence of *o*-phenanthroline or a chelator for Fe^{2+}, the production of Fe^{2+} was observed during sulfite oxidation by this enzyme under not only anaerobic conditions but also aerobic conditions. No Fe^{2+} production was observed in the absence of *o*-phenanthroline, suggesting that the Fe^{2+} produced was rapidly reoxidized by molecular oxygen. Neither cytochrome *c* nor ferricyanide, both of which are electron acceptors for other sulfite oxidases, served as an electron acceptor for the sulfite oxidase of *T. ferrooxidans*. The enzyme was strongly inhibited by chelating agents for Fe^{3+}. The physiological role of sulfite oxidase in sulfur oxidation of *T. ferrooxidans* is discussed.

From a geochemical viewpoint, ferric ion (Fe^{3+}) seems to be a particularly important metal ion because it has the ability to oxidize metal moieties of sulfide ores chemically to solubilize them into the acidic drainage of mines. The role of Fe^{3+} in the bacterial leaching of sulfide ores has been established (16). Iron-oxidizing *Thiobacillus ferrooxidans* is considered to supply almost all of the Fe^{3+} to the acidic drainage of mines because the chemical oxidation of Fe^{2+} by molecular oxygen is slow under the conditions. In this way, *T. ferrooxidans* plays a crucial role in converting inorganic metal compounds in mines.

Since a high concentration of soluble Fe^{3+} is always available for *T. ferrooxidans* in its environment, this bacterium is expected to possess a unique enzyme system in which Fe^{3+} is absolutely required to operate the metabolic systems of this bacterium. An absolute requirement of Fe^{3+} for sulfur oxidation in *T. ferrooxidans* was recently proposed. Sulfur:ferric ion oxidoreductase, which utilizes Fe^{3+} as an electron acceptor for the oxidation of elemental sulfur, was purified in an electrophoretically homogeneous state from iron-grown *T. ferrooxidans* AP19-3 (18, 20). The enzyme catalyzed a reaction in which 1 mol of elemental sulfur was oxidized with 4 mol of Fe^{3+} to produce 4 mol of Fe^{2+} and 1 mol of sulfite. The Fe^{2+} thus produced was thought to be oxidized by the iron oxidase of the strain to regenerate Fe^{3+}. On the other hand, sulfite, which, if accumulated in a bacterium damages the cells (22), was considered to be oxidized chemically by 2 mol of Fe^{3+} to produce sulfate and an additional 2 mol of Fe^{2+}.

We show that, in addition to this sulfite-decomposing system, iron-grown *T. ferrooxidans* AP19-3 possesses an alternative sulfite-decomposing system or a new type of sulfite oxidase. A bacterial sulfite oxidase reported previously utilizes both cytochrome *c* and ferricyanide as electron acceptors (2, 13, 26, 29). There have been no reports of a sulfite oxidase that utilizes Fe^{3+} as an electron acceptor. Detoxification of sulfite was shown to be important in *T. ferrooxidans* AP19-3 because the strain absolutely produces a large amount of sulfite during sulfur oxidation under not only aerobic conditions but also anaerobic conditions (22). The physiological role of sulfite oxidase in sulfur oxidation of this bacterium is also discussed.

MATERIALS AND METHODS

Microorganism. The iron-oxidizing bacterium *T. ferrooxidans* AP19-3 was used throughout this study (18–22, 25).

Media and cultivation conditions. The composition of a 10-times-concentrated basal salts solution was as follows: $(NH_4)_2SO_4$, 30 g; KCl, 1 g; K_2HPO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; $Ca(NO_3)_2$, 0.1 g; deionized water, 1,000 ml; and concentrated H_2SO_4, 25 ml. An Iron-salts medium used for the large-scale production of cells was prepared by adding 1 liter of the 10-times-concentrated basal salts solution, 8 liters of deionized water, and 300 g of $FeSO_4 \cdot 7H_2O$ to a 10-liter carboy. An active culture of iron-salts-grown *T. ferrooxidans* AP19-3 (1 liter) was inoculated into the 9 liters of iron-salts medium described above and cultured under aeration at 28°C for 144 h. Cultures from six carboys (ca. 60 liters) were filtered with Toyo no. 2 filter paper to remove the bulk of ferric precipitates and centrifuged with a Hitachi 18PR-52 continuous-flow rotor at 15,000 × *g* and a flow rate of 200 ml/min to yield ca. 0.35 g of cell protein. Harvested cells were washed three times with 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) and three times with 0.1 M sodium phosphate buffer (pH 7.5).

Analysis of sulfite-oxidizing activity. Iron-grown cells washed three times with 0.1 M sodium phosphate buffer (pH 7.5) were disrupted by passage two times through a French pressure cell at 1,500 kg/cm^2 and centrifuged at 12,000 × *g* for 20 min. The supernatant solution (crude cell extract) was further centrifuged at 105,000 × *g* for 60 min to obtain an orange supernatant solution (105,000 × *g* supernatant) and a red precipitate (105,000 × *g* precipitate). The latter was suspended in 0.1 M sodium phosphate buffer (pH 7.5). Since all of the sulfite-oxidizing activity of the cell extract was present in this 105,000 × *g* precipitate (plasma membrane

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fraction), we used this fraction as source of sulfite oxidase throughout this study.

The composition of the reaction mixture used for determining sulfite oxidase activity was as follows: 4 ml of 0.1 M sodium citrate buffer (pH 6.5), 1 to 10 mg of the 105,000 × *g* precipitate as protein, and 1 μmol of sodium sulfite. The total volume was 5 ml. Ferric sulfate (1 μmol) was added to the reaction mixture if necessary. The reaction was carried out at 30°C on a reciprocal shaker. A sample of the reaction mixture (0.5 ml) was withdrawn and centrifuged at 10,000 × *g* for 2 min, and the concentration of sulfite in the supernatant solution thus obtained (0.2 ml) was determined spectrophotometrically by the pararosaniline method (28). The amount of chemically oxidized sulfite was always checked with a reaction mixture containing the 105,000 × *g* precipitate boiled for 10 min instead of the native enzyme.

Sulfite oxidase activity was also measured by determining the amount of sulfate ion in the reaction mixture. The amount of sulfate ion was determined spectrophotometrically by a previously described method (18).

Sulfite oxidase activity was also measured spectrophotometrically by monitoring the reduction of $Fe(CN)_6^{3-}$ and cytochrome *c* by a modification of the method of Charles and Suzuki (2). The reaction mixture contained 2.5 ml of 0.5 M β-alanine, 105,000 × *g* precipitate, 1 mg of protein, 1.5 μmol of $K_3Fe(CN)_6$ or horse heart cytochrome *c* (type II-A; Sigma Chemical Co.), and 5 μmol of sodium sulfite. The total volume and the pH of the reaction mixture were 3 ml and 6.0, respectively. The reduction of $Fe(CN)_6^{3-}$ and cytochrome *c* was monitored at 400 and 550 nm, respectively, in a 1-cm cell with a Shimadzu UV-140 spectrophotometer.

Production of Fe^{2+} during sulfite oxidation. The production of Fe^{2+} in the reaction mixture during sulfite oxidation was determined in the presence of *o*-phenanthroline (0.1 mM) under both aerobic and anaerobic conditions. The reaction mixture contained 4 ml of 0.5 M β-alanine, 5 mg of the 105,000 × *g* precipitate as protein, 0.5 μmol of ferric sulfate, 0.1 mM *o*-phenanthroline, and 1 μmol of sodium sulfite. The total volume and the pH of the reaction mixture were 5 ml and 6.0, respectively. All the components except *o*-phenanthroline and sulfite were put into a 30-ml flask. Sulfite and *o*-phenanthroline were kept in a small test tube separated from the other components of the reaction mixture. The reaction mixture was bubbled with nitrogen through a glass capillary tube for 15 min prior to the addition of sulfite and *o*-phenanthroline. The flask was shaken vigorously for 10 s to mix the sulfite and *o*-phenanthroline, and the reaction was carried out at 30°C with shaking. Fe^{2+} was determined colorimetrically by a modification of the *o*-phenanthroline method as previously described (15). The amount of chemically reduced Fe^{3+} was always checked with a reaction mixture containing the 105,000 × *g* precipitate (5 mg of protein) boiled for 10 min instead of the native enzyme.

Protein content. The protein content was determined by the biuret method (9) with crystalline bovine serum albumin as the reference protein.

RESULTS

Properties of *T. ferrooxidans* AP19-3 sulfite oxidase. The cell extract of *T. ferrooxidans* AP19-3 prepared by the method described in Materials and Methods was centrifuged at 105,000 × *g* for 60 min. All of the sulfite-oxidizing activity of the cell extract was observed in the 105,000 × *g* precipitate fraction (plasma membrane fraction). Sonic oscillation treatment of the 105,000 × *g* precipitate fraction (20 kc for 20 min) did not further solubilize the enzyme from the fraction, indicating that the enzyme is localized in the plasma membrane of the bacterium. The properties of sulfite oxidase were investigated with the plasma membrane fraction.

The optimum pH for *T. ferrooxidans* AP19-3 sulfite oxidase was 6.0 (Fig. 1). In contrast, the optimum pH for *Thiobacillus novellus* sulfite oxidase is 8.0 (2). The activity was completely destroyed by treating the membrane with protease for 2 h or heating it for 10 min at 58°C (data not shown). When sulfite was oxidized by the enzyme under aerobic conditions, equimolar amounts of sulfate were produced in the reaction mixture (data not shown).

Sulfite oxidation was observed under not only aerobic conditions but also anaerobic conditions (Fig. 2), suggesting that an electron acceptor other than molecular oxygen can serve as an electron acceptor in the case of the sulfite oxidase of this bacterium. The rate of sulfite oxidation under anaerobic conditions markedly increased when 0.2 μmol of Fe^{3+} per ml was added to the reaction mixture (Fig. 2). The results strongly suggest that the sulfite oxidase of this bacterium utilizes Fe^{3+} as an electron acceptor. To test whether Fe^{3+} is utilized as an electron acceptor, we studied the production of Fe^{2+} during the oxidation of sulfite under both aerobic and anaerobic conditions. In the presence of *o*-phenanthroline to trap Fe^{2+}, Fe^{2+} production was observed under both aerobic and anaerobic conditions (Fig. 3). The amount of Fe^{2+} produced under anaerobic conditions was greater than that produced under aerobic conditions. Under aerobic conditions, no Fe^{2+} production was observed in the absence of *o*-phenanthroline, suggesting that the Fe^{2+} produced by the enzyme is rapidly oxidized to Fe^{3+} by molecular oxygen.

T. ferrooxidans AP19-3 sulfite oxidase did not utilize either cytochrome *c* or ferricyanide, both of which are routinely utilized as electron acceptors for sulfite oxidase (2)

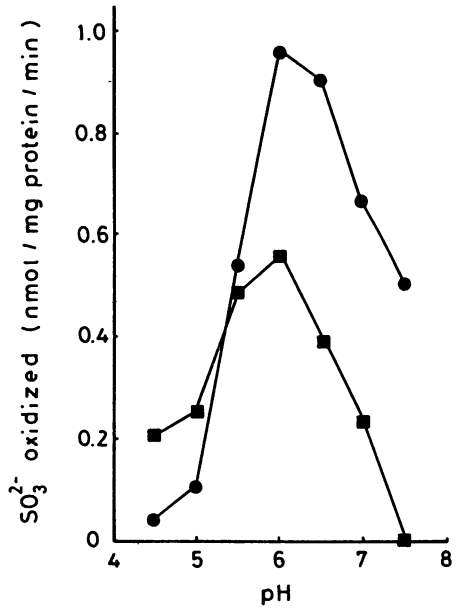

FIG. 1. Effect of pH on the activity of sulfite oxidase. Sulfite oxidase was measured with a plasma membrane fraction of iron-grown *T. ferrooxidans* AP19-3 (0.4 mg of protein per ml of reaction mixture) under aerobic conditions. The composition of the reaction mixture and the method used for analysis are described in the text. Symbols: ■, 0.1 M sodium phosphate buffer; ●, 0.1 M sodium citrate buffer.

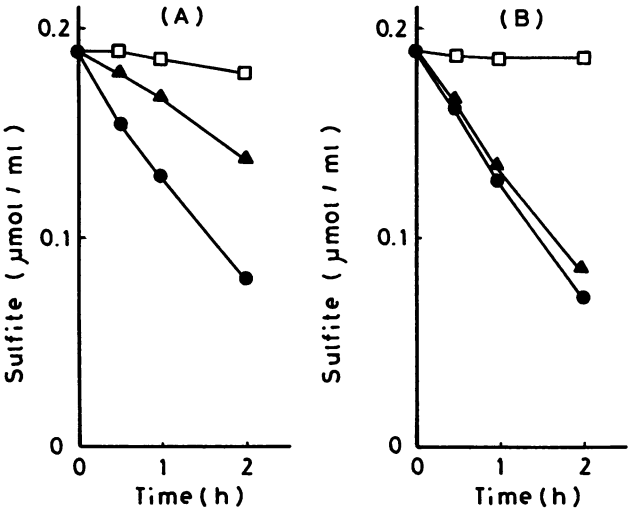

FIG. 2. Effect of ferric ions on the activity of sulfite oxidase. Sulfite consumed in the reaction mixture was measured in the absence (A) or in the presence (B) of ferric ions (0.2 μmol/ml) under aerobic and anaerobic conditions. The composition of the reaction mixture and the method used for analysis are described in the text. Symbols: ●, comsumption of sulfite measured under aerobic conditions; ▲, comsumption of sulfite measured under anaerobic conditions; □, comsumption of sulfite due to chemical oxidation determined with the enzyme boiled for 10 min under aerobic conditions.

(Fig. 4). In contrast, enzymatic reduction of Fe^{3+} by sulfite was detected in the same reaction mixture, except that cytochrome *c* or ferricyanide was exchanged for Fe^{3+}. The results strongly indicate that the sulfite oxidase observed in *T. ferrooxidans* AP19-3 is distinct from those observed previously in other bacteria. Sulfite oxidase was completely inhibited by chelating agents for Fe^{3+}, such as 4,5-dihydroxy-*m*-benzenedisulfonic acid disodium salt (Tiron) and

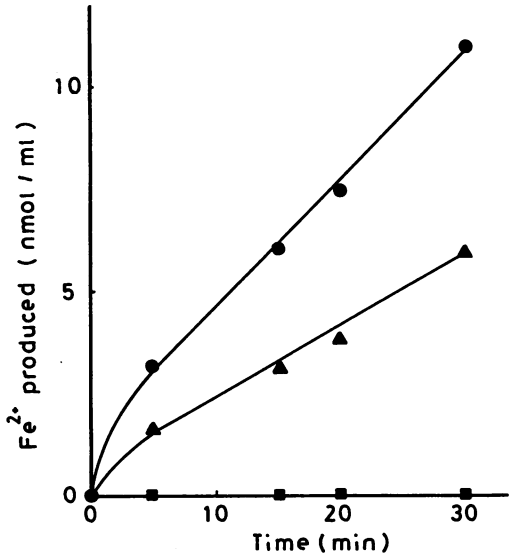

FIG. 3. Production of ferrous ions during the oxidation of sulfite. The production of Fe^{2+} was measured in the presence of *o*-phenanthroline (0.1 mM) and Fe^{3+} (0.5 μmol). Symbols: ▲, production of Fe^{2+} under aerobic conditions; ●, production of Fe^{2+} under anaerobic conditions; ■, production of Fe^{2+} under aerobic conditions without *o*-phenanthroline (0.1 mM).

α,α′-dipyridyl at 10 and 50 mM, respectively, suggesting that Fe^{3+} is utilized as an electron acceptor (data not shown).

Physiological role of sulfite oxidase in *T. ferrooxidans* AP19-3. We previously showed that when washed intact cells of *T. ferrooxidans* AP19-3 were incubated with elemental sulfur at pH 6.0 under aerobic conditions, a large amount of sulfite accumulated in the reaction mixture at the early stage of incubation, and that incubation of the cells with sulfite for 2 h markedly reduced the activities of both the iron oxidase- and ferric ion-reducing systems (22). After 2 h of incubation of the cells with elemental sulfur, the amount of sulfite accumulated in the reaction mixture rapidly decreased (Fig. 5). However, in the presence of 10 mM Tiron, which completely inhibits sulfite oxidase, the amount of sulfite did not eventually decrease but instead continuously increased, indicating that the rapid decrease in the amount of sulfite was due to the operation of sulfite oxidase in the plasma membrane.

DISCUSSION

In comparison with other bacteria, the iron-oxidizing thiobacillus *T. ferrooxidans* seems to possess a particularly high concentration of soluble iron in the periplasmic space because a large amount of Fe^{2+} in the environment was oxidized in the periplasmic space of this bacterium by the iron oxidase of the cells (3–7, 23, 24). This result suggests the possibility that *T. ferrooxidans* possesses unique Fe^{3+} dependent enzymes in the periplasmic space. Sulfur:ferric ion oxidoreductase (ferric ion-reducing system), which catalyzes the oxidation of elemental sulfur with Fe^{3+} as an electron acceptor, was purified to an electrophoretically homogeneous state from iron-grown *T. ferrooxidans* AP19-3 (18, 20), and evidence that the enzyme is involved in aerobic sulfur oxidation of this strain has accumulated (21, 22, 25). The observation that sulfur:ferric ion oxidoreductase is present in the periplasmic space of this strain (20) encouraged us to search for other oxidoreductases that require Fe^{3+} as an electron acceptor in *T. ferrooxidans*.

In this report, we showed the existence of a new type of sulfite oxidase which utilizes Fe^{3+} as an electron acceptor in the plasma membrane fraction of iron-grown *T. ferrooxidans*

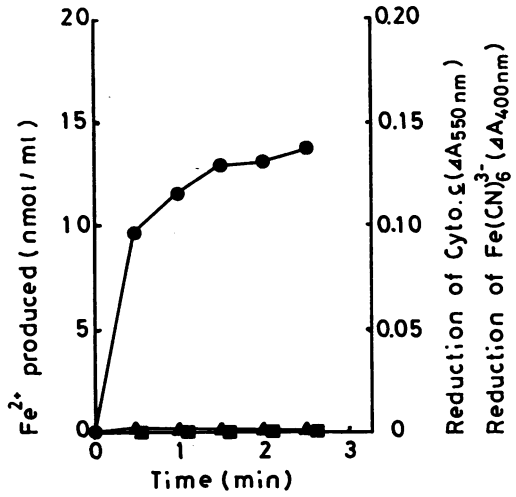

FIG. 4. Electron acceptor for sulfite oxidase. The reduction of ferric ions (●), horse heart cytochrome *c* (Cyto. c (▲), or ferricyanide (■) by sulfite was determined by the methods described in the text.

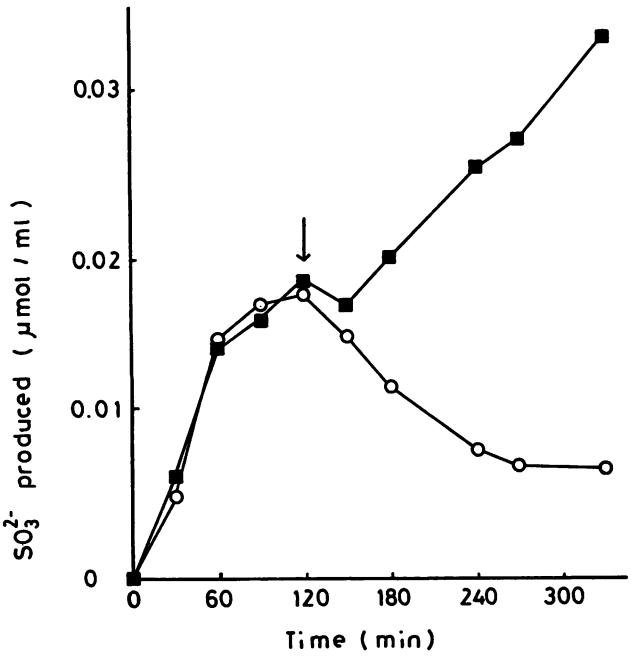

FIG. 5. Effect of 4,5-dihydroxy-*m*-benzene-disulfonic acid disodium salts (Tiron) on the production and decomposition of sulfite during aerobic oxidation of elemental sulfur. The composition of the reaction mixture and the methods used for analysis are described in the text. After 2 h of incubation of washed intact cells of *T. ferrooxidans* AP19-3 with elemental sulfur under aerobic conditions, 10 mM Tiron was added at the point indicated by the arrow (■); cells with no Tiron added were also tested (○).

AP19-3. We first assumed that all of the sulfite formed as an intermediate during sulfur oxidation in *T. ferrooxidans* AP19-3 was oxidized chemically by Fe^{3+} in the periplasmic space (18, 20). So, the results obtained indicate that in addition to chemical sulfite decomposition, an enzymatic sulfite oxidation system is also present in this strain. *T. ferrooxidans* AP19-3 sulfite oxidase was markedly distinguished from those obtained from other bacteria, such as *T. novellus* (2, 17, 26, 29), *Thiobacillus thiooxidans* (1, 8, 10), *Thiobacillus thioparus* (13), *T. ferrooxidans* (27), *Thiobacillus versutus* (11, 12), and *Thiobacillus concretivorus* (14). Namely, *T. ferrooxidans* AP19-3 sulfite oxidase could not utilize cytochrome *c* and ferricyanide as electron acceptors. In contrast, the sulfite oxidases previously described cannot utilize Fe^{3+} as an electron acceptor. It is interesting that when Fe^{3+} was added to the reaction mixture, *T. ferrooxidans* sulfite oxidase could oxidize sulfite under anaerobic conditions as rapidly as under aerobic conditions. Sulfite oxidation in *T. novellus* is carried out under aerobic conditions via two cellular components, namely, sulfite:cytochrome *c* oxidoreductase and cytochrome oxidase, and this produces energy for the cells.

Under aerobic conditions, intact cells of *T. ferrooxidans* AP19-3 produced a large amount of sulfite during sulfur oxidation at a pH at which the iron oxidase of the cells scarcely operated (above pH 5.0) (22). If the sulfite produced was not rapidly taken away from the cells, the amounts of both iron oxidase and sulfur:ferric ion oxidoreductase were markedly reduced (22). The results obtained in Fig. 5 strongly suggest that both the production and the decomposition of sulfite simultaneously occur during sulfur oxidation in this strain. Namely, the sulfite oxidase of this strain can decompose a harmful sulfite absolutely produced during sulfur oxidation to a harmless sulfate. The fact that the sulfite oxidase of this strain is localized in the plasma membrane is important for the detoxification of sulfite because the enzyme must be in the plasma membrane to block sulfite from entering the cytoplasm of the cells.

Thus, it can be said that the Fe^{3+}-dependent sulfite oxidase of *T. ferrooxidans* AP19-3 has two physiological roles: (i) detoxification of sulfite produced during sulfur oxidation and (ii) production of Fe^{2+} by the reduction of Fe^{3+} by sulfite and oxidation of the Fe^{2+} produced by iron oxidase to produce energy for the cells. The problem of what percentage of sulfite is oxidized by this enzymatic process is now unclear.

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