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Purification and Some Properties of Sulfite:Ferric Ion Oxidoreductase from *Thiobacillus ferrooxidans*

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Sulfite:ferric ion oxidoreductase in the plasma membrane of *Thiobacillus ferrooxidans* AP19-3 was purified to an electrophoretically homogeneous state. The enzyme had an apparent molecular weight of 650,000 and was composed of two subunits (M_rs, 61,000 and 59,000) as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Michaelis constants of sulfite:ferric ion oxidoreductase for Fe^{3+} and sulfite ions were 1.0 and 0.071 mM, respectively. Sulfite:ferric ion oxidoreductase suffered from end product inhibition by 1 mM Fe^{2+}.

The iron-oxidizing bacterium *Thiobacillus ferrooxidans* inhabits acid mine drainage containing a large amount of ferrous and ferric ions. A unique enzyme, hydrogen sulfide: ferric ion oxidoreductase (SFORase), which uses ferric ion as an electron acceptor for oxidation of sulfide, was recently purified to an electrophoretically homogeneous state (15, 18, 20) from iron-grown *T. ferrooxidans* AP19-3. Originally, SFORase was thought to oxidize elemental sulfur directly, but now it is thought to actually use sulfide (18). SFORase was found to be present not only in *T. ferrooxidans* but also in *Leptospirillum ferrooxidans* and the moderately thermophilic iron-oxidizing bacterial strains BC1, TH3, and Alv (22). One of the reaction products of SFORase is sulfite ion, and the harmful effect of this ion on the growth of *T. ferrooxidans* AP19-3 has been noted (4, 17, 21). Thus, it is reasonable to expect that *T. ferrooxidans* AP19-3 possesses an enzyme system that can oxidize the toxic sulfite ion to the harmless sulfate ion. We reported the existence of a novel sulfite oxidase in the plasma membrane of *T. ferrooxidans* AP19-3 which uses Fe^{3+} as an electron acceptor (19). Sulfite oxidase from *T. ferrooxidans* AP19-3 was markedly distinguished from those obtained from other bacteria, such as *T. novellus* (2, 14, 23, 27), *T. thiooxidans* (1, 5, 6), *T. thioparus* (11), *T. ferrooxidans* (24), *T. versutus* (8–10), and *T. concretivorus* (12); namely, *T. ferrooxidans* AP19-3 sulfite oxidase could not utilize cytochrome *c* or ferricyanide as an electron acceptor (19). In contrast, the sulfite oxidases previously described cannot use Fe^{3+} as an electron acceptor. The Fe^{3+}-dependent sulfite oxidase found in *T. ferrooxidans* AP19-3 was considered to have two physiological roles: (i) detoxification of the harmful sulfite ion produced during sulfur oxidation by SFORase and (ii) production of Fe^{2+} by reducing Fe^{3+} with sulfite ion, and the Fe^{2+} thus formed is oxidized by iron oxidase to supply energy for cell growth (19). Thus, to clarify the mechanisms of sulfur oxidation by the industrially important bacterium *T. ferrooxidans* it is important to characterize the sulfite:ferric ion oxidoreductase of *T. ferrooxidans* AP19-3. This work shows the purification, to an electrophoretically homogeneous state, of sulfite:ferric ion oxidoreductase, which uses Fe^{3+} as an electron acceptor, from iron-grown *T. ferrooxidans* AP19-3. The results show that sulfite:ferric ion oxidoreductase activity was specifically inhibited by Fe^{2+} and strongly suggest that this enzyme plays an important role in the regulation of sulfur use by Fe^{2+} in *T. ferrooxidans*.

T. ferrooxidans AP19-3 was used throughout this study (16). The composition of the iron-basal salts medium used for large-scale production of cells and the method for cultivation were described previously (19). The activity of sulfite: ferric ion oxidoreductase, which catalyzes the oxidation of sulfite ion with Fe^{3+} as an electron acceptor to produce Fe^{2+} and sulfate ion, was determined by measuring either sulfite ion oxidized or Fe^{2+} produced in the reaction mixture. Sulfite was determined spectrophotometrically by the pararosaniline method (26). The composition of the reaction mixture used for sulfite oxidation was as follows: enzyme, 0.25 μmol of $NaHSO_3$, 0.5 μmol of Fe^{3+}, and 2.7 ml of 0.1 M sodium citrate-NaOH buffer (pH 6.0). The total volume was 3.0 ml. The reaction was carried out under aerobic conditions at 30°C on a reciprocal shaker. A sample of the reaction mixture was withdrawn and centrifuged at 12,000 × *g* for 2 min. The supernatant solution thus obtained was used to determine the sulfite concentration. The amount of sulfite chemically oxidized was always checked with a reaction mixture containing enzyme that had been boiled for 10 min instead of the native enzyme.

Reduction of Fe^{3+} with sulfite ion by sulfite:ferric ion oxidoreductase was determined by measuring the Fe^{2+} produced in the reaction mixture by the *o*-phenanthroline method (13). The composition of the reaction mixture, which was in a cuvette, was as follows: 0.75 ml of 0.5 M β-alanine, enzyme, 0.1 ml of 50% glycerol, 16.6 μl of 0.1% *o*-phenanthroline, 84 nmol of $NaHSO_3$, and 0.33 μmol of Fe^{3+}. The total volume and pH of the reaction mixture were 1.0 ml and 6.0, respectively. After incubation of the reaction mixture at 30°C for 10 min, the reaction was started by adding the Fe^{3+} solution. Fe^{2+} production was measured with a Shimadzu UV-1200 spectrophotometer by monitoring the change in A_{470} for 1 min. The amount of chemically reduced Fe^{2+} was always checked with the reaction mixture containing enzyme that had been boiled for 10 min instead of the native enzyme. One unit of sulfite:ferric ion oxidoreductase was defined as the amount of enzyme that produces 1 μmol of Fe^{2+} in 1 min.

Sulfite:ferric ion oxidoreductase was purified as described below. All purification steps were done at 4°C unless other-

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wise indicated. Iron-grown cells of *T. ferrooxidans* AP19-3 washed three times with 0.1 M sodium phosphate buffer (pH 7.5) were disrupted by passage twice through a French pressure cell at 1,500 kg/cm^2 and centrifuged at 12,000 × *g* for 20 min. The supernatant solution (cell extract) was further centrifuged at 105,000 × *g* for 60 min to obtain the plasma membrane. Sulfite:ferric ion oxidoreductase in the plasma membrane was solubilized with 10 mM sodium phosphate buffer (pH 7.5) containing Nonidet P-40, glycerol, and KCl at 1%, 20%, and 1 M, respectively. Nonidet P-40 was added to the plasma membrane at a concentration of 0.7 mg/mg of protein. After incubation of the plasma membrane preparation with the Nonidet P-40 solution at 18°C for 1 h, it was centrifuged at 105,000 × *g* for 60 min. The supernatant solution thus obtained was dialyzed three times against 3 liters of 10 mM sodium phosphate buffer (pH 7.5) containing 0.1% Nonidet P-40 and centrifuged to pellet the precipitate formed in the dialysis bag. The clear supernatant thus obtained was concentrated with polyethylene glycol 6000 by use of a dialysis bag. The 0.1% Nonidet P-40-solubilized fraction was then applied to a DEAE-Toyopearl 650S column (1.5 by 15 cm) equilibrated with 10 mM sodium phosphate buffer containing 0.1% Nonidet P-40 (pH 7.5). After the column was washed with the same buffer (40 ml), enzyme was eluted by a linear gradient of 0 to 0.5 M NaCl (150 ml). The enzyme activity eluted at 0.2 to 0.3 M NaCl containing 0.1% Nonidet P-40. The active fractions from the column were combined and concentrated with polyethylene glycol 6000. The concentrated active fractions were applied to a Sephacryl S-500 gel filtration column (1.5 by 74 cm) equilibrated with 10 mM sodium phosphate buffer containing 0.1% Nonidet P-40 (pH 7.5). The active fractions from the Sephacryl S-500 column were collected and concentrated with polyethylene glycol 6000 by use of a dialysis bag. The concentrated enzyme solution from the previous step was electrophoresed in a 4% polyacrylamide gel. One of the gels was stained with Coomassie brilliant blue to determine the site of sulfite:ferric ion oxidoreductase in the gel. The part of the gel (5 mm long) containing sulfite:ferric ion oxidoreductase was cut out and disrupted in a small amount of 10 mM sodium phosphate buffer, pH 7.5. The sulfite:ferric ion oxidoreductase was extracted and then concentrated with polyethylene glycol 6000. The protein content was determined by the method of Lowry et al. with crystalline bovine serum albumin as the reference protein (7).

Sulfite:ferric ion oxidoreductase is localized in the plasma membranes of *T. ferrooxidans* AP19-3 (19). It was purified from iron-grown cells; the results of a typical purification are summarized in Table 1. Total activity in the DEAE-Toyopearl fraction doubled, probably because this step removed an unknown material which precipitated the Fe^{3+} used as electron acceptor for sulfite:ferric ion oxidoreductase. The procedure gave 34-fold purification over the plasma membrane. The purified enzyme was homogeneous as determined by polyacrylamide gel electrophoresis (PAGE) (Fig. 1A). The apparent molecular weight of sulfite:ferric ion oxidoreductase was 650,000 on the basis of data from HiLoad 16/60 Superdex 200 prep grade (FPLC; Pharmacia) column chromatography. Two bands close to each other, with molecular weights of 61,000 and 59,000, were observed after analysis by sodium dodecyl sulfate-PAGE (Fig. 1B), indicating that the enzyme was composed of two subunits of different molecular weights. The enzyme had an optimum pH of 6.5, and the activities of sulfite oxidation and Fe^{2+} production by the purified enzyme were proportional to the amount of sulfite and Fe^{3+} added to the reaction mixture.

TABLE 1. Summary of purification procedure for sulfite:ferric ion oxidoreductase from iron-grown *T. ferrooxidans* AP19-3

Step	Total protein (mg)	Total activity (U)	Sp act[a] (U/mg)	Yield (%)
Plasma membrane	2,422	4.84	0.002	100
Solubilized fraction with 1% Nonidet P-40	579	5.79	0.010	120
DEAE-Toyopearl	205	10.66	0.052	220
Sephacryl S-500	20	1.64	0.082	34
PAGE gel cut[b]	5.1	0.35	0.068	7.2

[a] Sulfite:ferric ion oxidoreductase activity was determined by measuring Fe^{2+} produced in the reaction mixture.

[b] After PAGE, the enzyme fraction on the gel was removed and extracted with 0.1 M sodium phosphate buffer (pH 7.5) supplemented with 1% Nonidet P-40 and glycerol.

The Michaelis constants of sulfite:ferric ion oxidoreductase for sulfite and Fe^{3+} were 0.071 and 1.0 mM, respectively (data not shown). The purified enzyme showed an absorption maximum at 410 nm in an oxidized form (Fig. 2). The absorption maximum at 410 nm disappeared, and an absorption maximum at 418 nm appeared, with greatly reduced

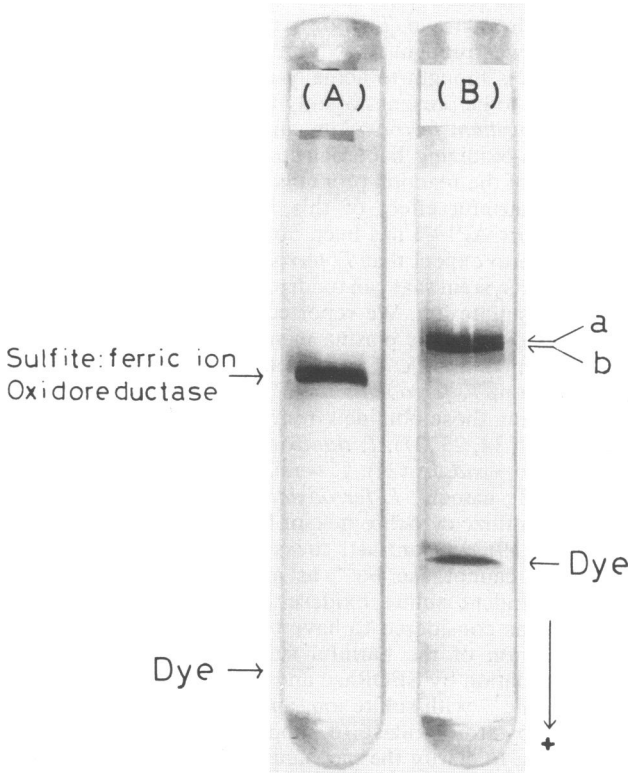

FIG. 1. PAGE of purified sulfite:ferric ion oxidoreductase. (A) Disc gel electrophoresis was done in a 7.5% polyacrylamide gel at pH 9.4 and at 2 mA per tube for 1.5 h. Purified sulfite:ferric ion oxidoreductase from a PAGE gel cut fraction (65 μg of protein) was placed on the gel. (B) Sodium dodecyl sulfate-PAGE was done in a 10% polyacrylamide gel at 2 mA per tube. Purified sulfite:ferric ion oxidoreductase from a PAGE gel cut fraction (110 μg of protein) was placed on the gel. Subunit M_rs: a, 61,000; b, 59,000. Subunit molecular weights were determined by the method of Weber and Osborn (25).

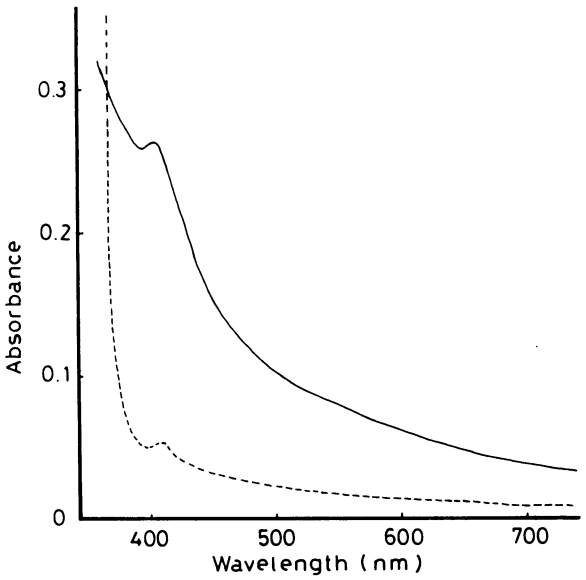

FIG. 2. Absorption spectra of purified sulfite:ferric ion oxidoreductase. Symbols: ——, purified sulfite:ferric ion oxidoreductase (1.3 mg/ml); – – –, purified sulfite:ferric ion oxidoreductase (1.3 mg/ml) reduced with sodium hydrosulfite.

absorbance at 375 to 600 nm, when the purified enzyme was reduced with sodium hydrosulfite.

The activity of sulfite:ferric ion oxidoreductase was not inhibited by 40 μM 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide or 1.0 μM antimycin A, specific inhibitors of electron transfer in the cytochrome bc_1 segment of respiratory chains (3). Instead, slight stimulation was observed (data not shown), suggesting that cytochrome bc_1 was not involved in sulfite oxidation by *T. ferrooxidans*. The characteristic absorption maxima due to reduced-type cytochrome *b* were not observed in Fig. 2. The effects of metal ions on the activity of sulfite:ferric ion oxidoreductase were studied. A significant inhibition effect by the following metal ions at 1 mM was not observed: uranous oxide, nickel, lead, cadmium, magnesium, and cobaltous ions. Zinc, molybdic, silver, and cupric ions caused 28 to 36% drops in activity (Table 2). In contrast, ferrous and mercuric ions at 1 mM completely inhibited the activity. Fe^{2+} at 0.5 mM inhibited the activity by ca. 50%. Complete inhibition of sulfite oxidase by 5 mM Fe^{2+} had previously been observed when the plasma membrane was used as an enzyme source instead of purified sulfite:ferric ion oxidoreductase (17).

It is interesting that sulfite:ferric ion oxidoreductase activity was specifically inhibited by Fe^{2+} because Fe^{2+} is one of the end products of sulfite oxidation by this enzyme. It is known that *T. ferrooxidans* is one of the most important bacteria for bacterial leaching of sulfide ore. This is mainly due to the bacterial ability to oxidize both Fe^{2+} and inorganic sulfur compounds. However, recently we showed that in sulfur-basal salts medium supplemented with 0.108 M Fe^{2+}, *T. ferrooxidans* AP19-3 used only Fe^{2+} but not sulfur as an energy source for growth (17). This inhibition of sulfur use by a high concentration of Fe^{2+} can be explained by inhibition of sulfite:ferric ion oxidoreductase by Fe^{2+}. The result that sulfite:ferric ion oxidoreductase was specifically inhibited by Fe^{2+} strongly suggests that this enzyme plays an important role in the regulation of sulfur use by *T. ferrooxidans* in the presence of Fe^{2+}.

TABLE 2. Effects of metal ions on sulfite:ferric ion oxidoreductase activity

Addition	Sulfite:ferric ion oxidoreductase activity (sulfite oxidized [nmol/mg/min])[a]
None	33.0
$NiSO_4$	31.3
$Pb(NO_3)_2$	30.9
$CdSO_4$	30.4
$MgSO_4 \cdot 7H_2O$	29.6
$UO_2SO_4 \cdot 3H_2O$	27.0
$CoSO_4 \cdot 7H_2O$	24.8
$ZnSO_4 \cdot 7H_2O$	23.7
$Na_2MoO_4 \cdot 2H_2O$	22.7
$CuSO_4$	22.2
$AgNO_3$	21.0
$FeSO_4 \cdot 7H_2O$	0.0
$HgCl_2$	0.0

[a] Sulfite:ferric ion oxidoreductase was determined by measuring sulfite ion in the reaction mixture as described in the text. The metal ion concentration used was 1 mM. Purified sulfite:ferric ion oxidoreductase from a Sephacryl S-500 fraction was used.

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