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Reduction of Mo^{6+} with Elemental Sulfur by *Thiobacillus ferrooxidans*

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In the presence of phosphate ions, molybdic ions (Mo^{6+}) were reduced enzymatically with elemental sulfur by washed intact cells of *Thiobacillus ferrooxidans* to give molybdenum blue. The whole-cell activity that reduced Mo^{6+} was totally due to cellular sulfur:ferric ion oxidoreductase (SFORase) (T. Sugio, W. Mizunashi, K. Inagaki, and T. Tano, J. Bacteriol. 169:4916–4922, 1987). The activity of Mo^{6+} reduction with elemental sulfur was competitively inhibited by Fe^{3+}, Cu^{2+}, and Co^{2+}. The Michaelis constant of SFORase for Mo^{6+} was 7.6 mM, and the inhibition constants for Fe^{3+}, Cu^{2+}, and Co^{2+} were 0.084, 0.015, and 0.17 mM, respectively, suggesting that SFORase can reduce not only Fe^{3+} and Mo^{6+} but also Cu^{2+} and Co^{2+} with elemental sulfur.

The iron-oxidizing bacterium *Thiobacillus ferrooxidans* can oxidize metal ions directly and indirectly under acidic conditions in the presence of Fe^{3+} (3–5, 8). In an indirect mechanism, uranous oxide (UO_2), cuprous ions (Cu^+), stannous ions (Sn^{2+}), and antimony ions (Sb^{3+}) are oxidized by a potent oxidant for metal ions or Fe^{3+} chemically and then the Fe^{2+} thus produced is oxidized by iron oxidase of *T. ferrooxidans* to regenerate Fe^{3+} (5). The ability to oxidize metal ions both directly and indirectly makes the bacterium one of the most important microorganisms for bacterial leaching.

Recently, it was found that *T. ferrooxidans* has the ability not only to oxidize metal ions but also to reduce them (1, 6, 9, 13, 16). Sulfur:ferric ion oxidoreductase (SFORase), which utilizes Fe^{3+} as an electron acceptor for the oxidation of elemental sulfur, was purified from iron-grown *T. ferrooxidans* AP19-3 to an electrophoretically homogeneous state (9, 13). Evidence that this enzyme is involved in an aerobic sulfur oxidation in this strain has been accumulated (11, 14–18). In addition to a SFORase, this strain possesses another unique enzyme, sulfite:ferric ion oxidoreductase, that absolutely requires Fe^{3+} as an electron acceptor for the oxidation of sulfite (12). Since soluble Fe^{3+} is available when *T. ferrooxidans* grows in environments containing a large amount of metal sulfides, it is not unreasonable to think that *T. ferrooxidans* has evolved a unique enzyme system in which Fe^{3+} is required to operate the metabolic system. Tetravalent manganese (Mn^{4+}) was reduced with elemental sulfur by washed intact cells of *T. ferrooxidans* AP19-3 (16). A mechanism of Mn^{4+} reduction has been proposed (16) in which Fe^{2+} and sulfite, produced during the oxidation of elemental sulfur by SFORase (13, 15), chemically reduce Mn^{4+}. This redox reaction is thermodynamically feasible (7).

In this way, when *T. ferrooxidans* AP19-3 reduces Fe^{3+} or Mn^{4+} with elemental sulfur, the SFORase of the cells directly or indirectly plays a crucial role in these reactions. These results prompted us to search for another enzyme, in *T. ferrooxidans* AP19-3, which utilizes electron acceptors other than Fe^{3+}. Molybdic ion (Mo^{6+}) was selected as a metal of interest because the E^0 value for the MoO^{3+}/MoO_4^{2-} reaction (0.48 V) is similar to that of the Fe^{3+}/Fe^{2+} reaction (0.771 V) (7) and hence a biological reduction of Mo^{6+} by elemental sulfur may be expected in *T. ferrooxidans*. In this work, we show that washed intact cells of *T. ferrooxidans* AP19-3 could reduce Mo^{6+} with elemental sulfur and that the whole-cell activity to reduce Mo^{6+} was totally due to cellular SFORase (13).

When molybdic ions (Mo^{6+}) are reduced with an appropriate reducing agent in the presence of phosphate ions under acidic conditions, a blue color due to the production of molybdenum blue develops (2). A good linearity was obtained between the concentration of Mo^{6+} (0.1 to 1.5 μmol of Mo^{6+}), which was reduced chemically with sulfide in the presence of GSH, and the developed blue color. The reduction of Mo^{6+} with elemental sulfur by *T. ferrooxidans* AP19-3 was determined spectrophotometrically by measuring the amount of molybdenum blue produced during the oxidation of elemental sulfur by SFORase in the presence of Mo^{6+} ($Na_2MoO_4 \cdot 2\ H_2O$) and phosphate ions.

The reduction of Mo^{6+} by washed intact cells was determined by the method described below. The reaction was performed under aerobic conditions in the presence of 5 mM sodium cyanide, which could completely inhibit iron oxidase at this concentration, and as a result, inhibited reoxidation of molybdenum blue to Mo^{6+} by iron oxidase. The reaction mixture contained 8 ml of 0.1 M β-alanine sulfate buffer (pH 3.0), washed intact cells of iron-grown *T. ferrooxidans* AP19-3, 5 to 20 mg of protein, 50 μmol of sodium cyanide, 10 μmol of NaH_2PO_4, 50 to 500 mg of elemental sulfur, and 50 to 400 μmol of $NaMoO_4 \cdot 2H_2O$. The total volume was 10.0 ml. The reduction of Mo^{6+} by SFORase purified from *T. ferrooxidans* AP19-3 was determined by the method described below. The reaction mixture contained 4.5 ml of 0.1 M sodium phosphate buffer (pH 6.5), SFORase purified at the stage of Mono Q column chromatography (13), 11 μg of protein, 0.2 mg of bovine serum albumin, 100 mg of elemental sulfur, 20 μmol of reduced glutathione, 2.5 μmol of 4,5-dihydroxy-*m*-benzenedisulfonic acid disodium salt (Tiron), and 10 μmol of $Na_2MoO_4 \cdot 2H_2O$. The total volume was 5.0 ml. The reaction was carried out under aerobic conditions by shaking the reaction mixture at 30°C. A sample of the reaction mixture (1.0 ml) was centrifuged at 12,000 × *g* for 1 min to discard solid elemental sulfur. A 0.1-ml volume of 2.0 N HCl was added to 0.8 ml of the supernatant solution obtained by centrifugation. A blue color developed and was

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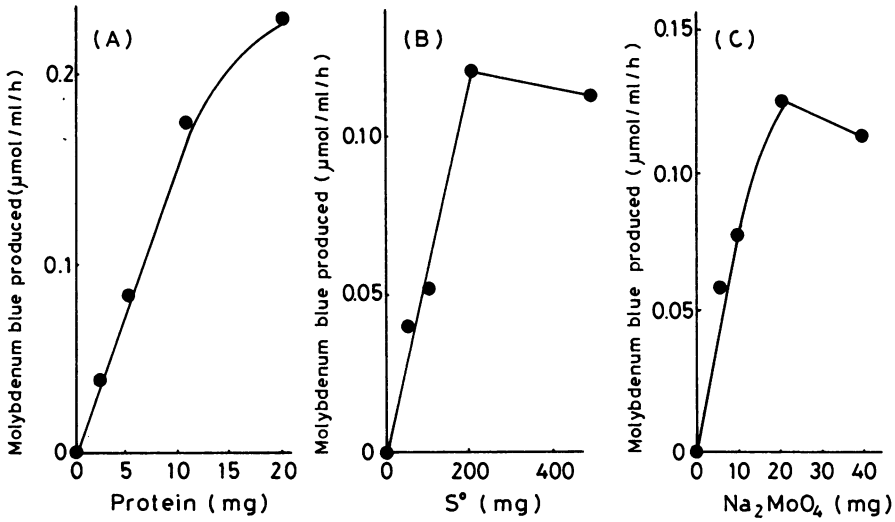

FIG. 1. Effects of the concentration of cells, elemental sulfur, and Mo^{6+} on the activity of Mo^{6+} reduction by washed intact cells of *T. ferrooxidans* AP19-3. (A) Effect of cell concentration on the activity of Mo^{6+} reduction. (B) Effect of elemental sulfur concentration on the activity of Mo^{6+} reduction. (C) Effect of Mo^{6+} concentration on the activity of Mo^{6+} reduction.

measured in a Shimadzu UV-140 spectrophotometer at 660 nm. The amount of molybdenum blue produced chemically was always checked by using 10-min-boiled purified enzyme instead of native purified enzyme.

Washed intact cells of *T. ferrooxidans* AP19-3 (10), which was grown on iron-salts medium without sodium molybdate, reduced Mo^{6+} with elemental sulfur under acidic conditions to give molybdenum blue. The pH optimum of Mo^{6+} reduction with elemental sulfur at pH 3.0 corresponds well to that of the SFORase (9, 13) (data not shown). The rate of Mo^{6+} reduction was proportional to the concentration of cells, elemental sulfur, and Mo^{6+} in the reaction mixture (Fig. 1).

The purification of Mo^{6+}-reducing enzyme from iron-grown *T. ferrooxidans* was studied. The method of purification was the same as that used for SFORase (13). In all the steps of purification, such as the 105,000 × *g* supernatant, $(NH_4)_2SO_4$ fractionation, Sephadex G-100 column chromatography, and fast protein liquid chromatography on a Mono Q column, the activity of Mo^{6+} reduction always appeared at the same fraction as that of SFORase, and the increase of purity of Mo^{6+} reduction was proportional to that of SFORase (data not shown), indicating that SFORase could utilize both Fe^{3+} and Mo^{6+} as an electron acceptor for the oxidation of elemental sulfur. A purified SFORase at the stage of Mono Q column chromatography (13) reduced Mo^{6+} with elemental sulfur under optimal conditions for a SFORase reaction (in the presence of GSH at pH 6.5), giving sulfite and molybdenum blue (Fig. 2). No activity was observed in the absence of GSH. Although the role of GSH in the oxidation of elemental sulfur by SFORase is still unsolved, an absolute requirement of GSH for Mo^{6+} reduction supports the involvement of SFORase in the reduction of Mo^{6+}. When 1 μmol of sulfite was produced, 4.6 to 5.4 μmol of molybdenum blue was produced. We could precisely determine the amount of molybdenum blue produced in the reaction mixture, but it is difficult to give a precise stoichiometry for Mo^{6+} reduction, because the composition of molybdenum blue is very complex. The compounds obtained by mild reduction of Mo^{6+}, in which the mean oxidation state of Mo is between 5 and 6, are thought to be the blue ones [e.g., $MoO_{2.0}(OH)$ and $MoO_{2.5}(OH)_{0.5}$] (2).

The activity of Mo^{6+} reduction was competitively inhibited by Fe^{3+}, Cu^{2+}, and Co^{2+} (Fig. 3). The Michaelis constant of SFORase for Mo^{6+} was 7.6 mM, and the inhibition constants for Fe^{3+}, Cu^{2+}, and Co^{2+} were 0.084, 0.015, and 0.17 mM, respectively, suggesting that SFORase is able to reduce not only Fe^{3+} and Mo^{6+} but also Cu^{2+} and Co^{2+}. The activity of the SFORase was completely inhibited by a specific chelating agent for Fe^{3+} or 4,5-dihydroxy-*m*-benzenedisulfonic acid disodium salt (Tiron) at 5 mM. However, if 2 mM Mo^{6+} was added to the reaction mixture, 30% of the SFORase activity was restored (data not shown). Furthermore, SFORase, dialyzed with 5 mM of Tiron and then with 0.1 M sodium phosphate buffer (pH 6.5) three times to discard the Tiron, showed no SFORase activity. However, if 1 mM Mo^{6+} or Fe^{3+} was added to the Tiron-dialyzed SFORase, 13 or 14% of the SFORase activity was restored, respectively (data not shown), supporting our

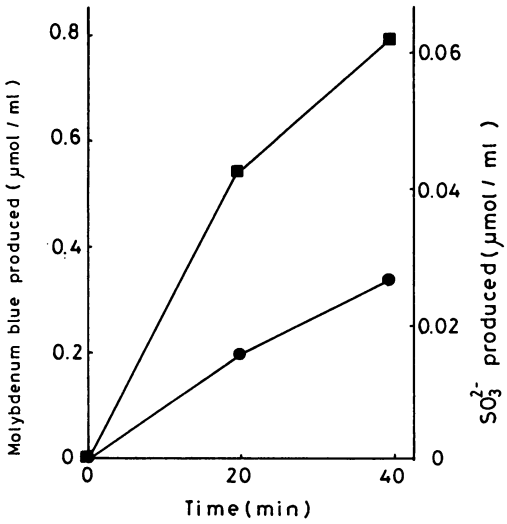

FIG. 2. Sulfite and molybdenum blue formation during the oxidation of elemental sulfur with Mo^{6+} by SFORase purified from *T. ferrooxidans* AP19-3. Symbols: ●, production of molybdenum blue; ■, production of sulfite.

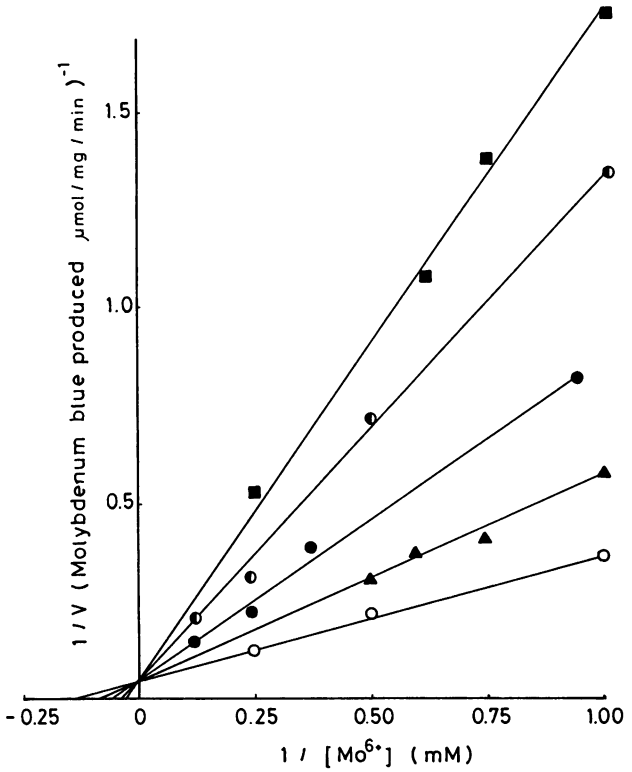

FIG. 3. Effect of Fe^{3+}, Cu^{2+}, and Co^{2+} on the velocity of molybdenum blue formation by SFORase purified from *T. ferrooxidans* AP19-3. Fe^{3+} at 0.1 mM (●) or 0.2 mM (◐), 0.05 mM Cu^{2+} (■), 0.1 mM Co^{2+} (▲), or no metal (○) was added to the reaction mixture.

conclusion that Mo^{6+} is an alternative electron acceptor for SFORase. The activity of Mo^{6+} reduction by SFORase was measured in the presence of 0.5 mM Tiron (at this concentration, ca. 19% inhibition of SFORase activity was observed) because Mo^{6+} reduction was increased by Tiron. Possibly, Tiron, a specific chelating agent for Fe^{3+}, chelated with Fe^{3+} in the SFORase and, as a result, accelerated a transfer of electrons from elemental sulfur to Mo^{6+} instead of transferring electrons to Fe^{3+}.

In this work, the mechanism of sulfur oxidation by *T. ferrooxidans* was studied with a highly purified SFORase, and it was found that in the presence of GSH, the enzyme utilizes not only Fe^{3+} but also Mo^{6+} as an electron acceptor for the oxidation of elemental sulfur. Furthermore, the ability of SFORase to reduce Cu^{2+} and Co^{2+} with elemental sulfur was also implied by the result that the reduction of Mo^{6+} by SFORase was competitively inhibited not only by Fe^{3+} but also by Cu^{2+} and Co^{2+}. We previously showed that the reduction of Fe^{3+} with elemental sulfur by washed intact cells of *T. ferrooxidans* AP19-3 was competitively inhibited by Cu^{2+} and that the growth inhibition by Cu^{2+} was completely restored by adding Fe^{3+} to sulfer-salts medium (17).

For the following reasons, Fe^{3+} but not Mo^{6+} seems to be an intrinsic electron acceptor for SFORase: (i) the specific activity of Fe^{3+}, Mo^{6+}, and Mn^{4+} reduction with washed intact cells of *T. ferrooxidans* AP19-3 was 3.3, 0.13, and 0.16 μmol of Fe^{2+}, molybdenum blue, or Mn^{2+} per mg of protein per h, respectively; therefore, Fe^{3+} is 20-fold superior to Mo^{6+} or Mn^{4+} as an electron acceptor for SFORase; (ii) the reduction of Mo^{6+} with elemental sulfur by SFORase was competitively inhibited by Fe^{3+}, and an extremely low inhibition constant for Fe^{3+} was obtained, indicating that an enzyme-Fe^{3+} complex is more difficult to separate into its components than an enzyme-Mo^{6+} complex; and (iii) a high concentration of Fe^{3+} is present in the environment of *T. ferrooxidans* containing a large amount of metal sulfides.

The mechanism of oxidation of elemental sulfur by *T. ferrooxidans* was partially clarified by this study. The properties of SFORase are distinct from those of sulfur:oxygen oxidoreductase purified from *Thiobacillus thiooxidans* and *Thiobacillus thioparus* by Suzuki and Silver (19–21), except that GSH is absolutely required for enzymatic activity. When elemental sulfur is oxidized, the SFORase utilizes Fe^{3+}, but sulfur:oxygen oxidoreductase utilizes molecular oxygen as an electron acceptor. Since GSH is commonly required for a elemental sulfur oxidation, a study of the role of GSH in sulfur oxidation by SFORase may provide valuable information in clarification of the oxidation mechanism of elemental sulfur.

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