Mechanism of Tetravalent Manganese Reduction with Elemental Sulfur by *Thiobacillus ferrooxidans*

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Received August 10, 1987

Washed intact cells of iron-grown *T. ferrooxidans* AP19-3 could reduce manganese dioxide (*MnO₂*) enzymatically with elemental sulfur (*S°*) and the mechanism of *Mn⁴⁺* reduction was studied. The optimum pH for *Mn⁴⁺* reduction was 2.5. The amount of *Mn⁴⁺* reduced by the strain was proportional to the amount of *S°* added to the reaction mixture. An enzyme that directly catalyzes the reduction of *Mn⁴⁺* to *Mn²⁺* with *S°* was not found in the cell-free extract of this strain. *T. ferrooxidans* AP19-3 was found to possess sulfur : ferric ion oxidoreductase (*SFORase*), which catalyzes the reduction of *Fe³⁺* with *S°* to give *Fe²⁺* and sulfite. The reduction of *Mn⁴⁺* was inhibited by a specific inhibitor of *SFORase* or diamide, strongly suggesting that *SFORase* is involved in the *Mn⁴⁺* reduction in this strain. The involvement of *SFORase* in *Mn⁴⁺* reduction was further supported by the following results. Both the amounts of *Fe²⁺* and sulfite produced by *SFORase* were markedly reduced by *MnO₂*. The amount of *Mn⁴⁺* reduced increased 5.4-fold with 1 mM ferric ion and 10-fold with 2.5 mM cyanide or an inhibitor of iron oxidase, respectively. The sulfite and *Fe²⁺* were chemically oxidized by *MnO₂* instantly. From the results, it is concluded that the *Mn⁴⁺* reduction with *S°* in this strain occurs through two steps. In the first step *S°* is enzymatically oxidized by *SFORase* to give *Fe²⁺* and sulfite, and in the second step these two reduced compounds are chemically reduced by *MnO₂* to give *Mn²⁺*.

*Thiobacillus ferrooxidans* inhabits the drainage in acid mines and is used for bacterial leaching. Since a high concentration of soluble *Fe³⁺* is always available for *T. ferrooxidans* in its natural environment, there is a possibility that this bacterium possesses unique enzymes which utilize *Fe³⁺* to oxidize reduced inorganic compounds. It was found that *T. ferrooxidans* AP19-3 possesses sulfur : ferric ion oxidoreductase (*SFORase*), which catalyzes the reduction of ferric ion (*Fe³⁺*) with elemental sulfur to give *Fe²⁺* and sulfite. The enzyme actually operates during the growth of this strain on sulfur-salts medium have accumulated.1-3 Results supporting that this enzyme actually operates during the growth of this strain on sulfur–salts medium have accumulated. These results prompted us to search, in *T. ferrooxidans* AP19-3, for other enzyme systems which utilize electron acceptors other than *Fe³⁺* or molecular oxygen to oxidize elemental sulfur.

It is interesting to determine whether or not *T. ferrooxidans* possesses an enzyme system for reducing *Mn⁴⁺* to *Mn²⁺* because the content of manganese in the earth’s crust is relatively high (0.1%). Leaching of manganese dioxide by *T. thiooxidans*6-7 and *T. ferrooxidans*8-9 was studied, with cultures of these thiobacilli growing on sulfur-salts medium. A leaching mechanism for manganese was postulated for *T. thiooxidans*, in which *Mn⁴⁺* is reduced by inorganic sulfur intermediates formed during sulfur oxidation, such as sulfide and sulfite. However, there have been no reports on the precise mechanism of *Mn⁴⁺* reduction with washed intact cells of those thiobacilli.

In this work, we elucidate the mechanism of *Mn⁴⁺* reduction by washed intact cells of *T. ferrooxidans* AP19-3, in which the reduction of *Mn⁴⁺* to *Mn²⁺* with elemental sulfur occurs via two steps. In the first step, elemental sulfur is enzymatically oxidized with *Fe³⁺* by
MATERIALS AND METHODS

Microorganism. The obligate chemolithoautotroph, T. ferrooxidans AP19-3,[10,11] was used throughout this study.

Media and conditions for cultivation. The composition of the ten times concentrated basal salts solution used was as follows: (NH₄)₂SO₄, 30 g; KCl, 1 g; K₂HPO₄, 5 g; MgSO₄·7H₂O, 5 g; Ca(NO₃)₂, 0.1 g; deionized water, 1000 ml; and concentrated H₂SO₄, 25 ml. The iron-salts medium used for the large scale production of cells was prepared by adding 1 l of the ten times concentrated basal salts solution, 8 l of deionized water and 300 g of FeSO₄·7H₂O to a 10 l carboy. An active culture of iron-salts grown T. ferrooxidans AP19-3 (1 l) was inoculated into the 9 l of iron-salts medium described above, followed by culturing under aeration at 28°C for 144 hr. The cultures from six carboys (ca. 60 l) were filtered with Toyo filter paper (No. 2) in order to remove the bulk of ferric precipitates, followed by centrifugation in a Hitachi 18PR-52 continuous flow rotor (15,000 x g, flow rate, 200 ml/min), which yielded ca. 0.35 g of cell protein. The harvested cells were washed three times with 0.1 M β-alanine-SO₄⁻ buffer, pH 3.0.

Mn⁴⁺ reduction activity. Washed intact cells of iron-grown T. ferrooxidans AP19-3 were incubated with elemental sulfur and manganese dioxide (MnO₂) in the presence of sodium cyanide (2.5 mM). The Mn⁴⁺ reduction activity of the cells was determined by measuring the Mn²⁺ solubilized in the reaction mixture. The reaction mixture comprised 8 ml of 0.1 M β-alanine-SO₄⁻ buffer, pH 2.5; MnO₂, 20 mg; elemental sulfur, 500 mg; washed intact cells of iron-grown T. ferrooxidans AP19-3, 5 mg of protein; and sodium cyanide, 25 μmol. The volume was 10 ml. The amount of Mn²⁺ produced chemically was always checked throughout this study by using 10 min-boiled washed intact cells (5 mg of protein) in the reaction mixture instead of native washed intact cells (5 mg of protein). The reaction was carried out in a shaking bath at 30°C. One part of the reaction mixture was withdrawn and centrifuged at 12,000 x g for 2 min, and then the amount of Mn²⁺ in the supernatant thus obtained was determined by atomic absorption spectrometry with a Shimadzu AA-625-01 spectrophotometer with an air-acetylene flame.

RESULTS AND DISCUSSION

Enzymatic reduction of Mn⁴⁺ by washed intact cells of T. ferrooxidans AP19-3

When washed intact cells of T. ferrooxidans AP19-3 were incubated with elemental sulfur and manganese dioxide (MnO₂) in the presence of sodium cyanide (2.5 mM), divalent manganese ion (Mn²⁺) was solubilized into
Mechanism of Mn⁴⁺ Reduction by *T. ferrooxidans* AP19-3

Intact cells of *T. ferrooxidans* AP19-3 exhibit the activity of a ferric-ion reducing system (FIR system).¹ We purified a protein [sulfur : ferric ion oxidoreductase (SFORase)] which catalyzes the reduction of Fe³⁺ with elemental sulfur to give Fe²⁺ and sulfite from iron-grown *T. ferrooxidans* AP19-3, and clarified that SFORase corresponds to the FIR system.² Since no attempt to isolate cell-free enzyme system that directly reduces Mn⁴⁺ with elemental sulfur to give Mn²⁺ from this strain has been successful, whether or not SFORase is involved in the reduction of Mn⁴⁺ was studied.

The effects of diamide [diazenedicarboxylic acid bis-(N,N-dimethylamide)] on the SFORase activity and on the amount of Mn⁴⁺ reduced were studied with washed intact cells of *T. ferrooxidans* AP19-3. Diamide is known to rapidly oxidize GSH stoichiometrically in such cells.¹⁵⁻¹⁸ If SFORase is involved in Mn⁴⁺ reduction, diamide should inhibit the Mn⁴⁺ reduction because GSH is absolutely

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The activities of sulfur: ferric ion oxidoreductase (SFORase) (A) and Mn⁴⁺ reduction (B) were determined with washed intact cells of iron-grown _T. ferrooxidans_ API9-3. Symbols: diamide was added to the reaction mixture at 1 mM (A) and 5 mM (B), respectively. No diamide was added to reaction mixture (○).

The involvement of SFORase in Mn⁴⁺ reduction was further supported by the following results. If SFORase is involved in Mn⁴⁺ reduction, the amounts of Fe²⁺ and sulfite produced by SFORase should be decline by required for the SFORase activity. As shown in Fig. 4, the SFORase activity decreased to 60.9% and 41.8% in the presence of 1 mM and 5 mM diamide, respectively, and the Mn⁴⁺ reduction activity also decreased with 1 mM and 5 mM diamide, to 83.5% and 48.2%, respectively, strongly suggesting that SFORase is involved in Mn⁴⁺ reduction in this strain.

The reduction of MnO₂ was carried out with washed intact cells of iron-grown _T. ferrooxidans_ API9-3.

The sulfur: ferric ion oxidoreductase (SFORase) activity was determined with washed intact cells (A) and partially purified SFORase, _i.e._, at the stage of ammonium sulfate fractionation, from iron-grown _T. ferrooxidans_ AP19-3 (B), respectively.

Symbols: MnO₂ (△, 0.1 mg; □, 1 mg; ×, 3.5 mg; ▲, 5 mg; ■, 10 mg; ●, 20 mg; ○, 50 mg) was added to a 10 ml (A) or 5 ml (B) reaction mixture. No MnO₂ was added to the reaction mixture (○).
MnO₂ because Fe²⁺ and sulfite are chemically oxidized by MnO₂ instantly (data not shown). The production of Fe²⁺ by SFORase of washed intact cells was completely abolished by 3.5 mg of MnO₂ added to the reaction mixture (Fig. 5A). The amount of sulfite produced by partially purified SFORase from iron-grown T. ferrooxidans AP19-3 was also greatly decline by MnO₂ (Fig. 5B).

If SFORase is involved in Mn⁴⁺ reduction, the amount of Mn⁴⁺ reduced should increase when Fe³⁺ is supplied to the reaction mixture because Fe³⁺ is absolutely required as an electron acceptor for elemental sulfur. As shown in Fig. 6, the amount of Mn⁴⁺ reduced increased with the addition of Fe³⁺ to the reaction mixture.

If SFORase is involved in Mn⁴⁺ reduction, the amount of Mn⁴⁺ reduced should increase when cyanide is added to the reaction mixture because cyanide strongly inhibits iron oxidase of T. ferrooxidans and as a results, Fe⁴⁺ produced by SFORase is not oxidized by molecular oxygen but by MnO₂ added to the reaction mixture chemically. Cyanide enhanced the activity of Mn⁴⁺ reduction ca. 10-fold at 2.5 mM (Fig. 7). The reduction of Mn⁴⁺ was also observed under aerobic conditions in the absence of cyanide, suggesting that ca. 14% and ca. 86% of Fe²⁺ produced by SFORase is oxidized by MnO₂ and molecular oxygen, respectively.

From the results obtained in this study, we propose the mechanism of Mn⁴⁺ reduction by elemental sulfur in T. ferrooxidans AP19-3 shown in Fig. 8. The reduction of Mn⁴⁺ occurs through two steps, an enzymatic one and non-enzymatic one, in this strain. In the first step, elemental sulfur is enzymatically oxidized by SFORase to give Fe²⁺ and sulfite, and in the second step, these two reduced compounds non-enzymatically reduce Mn⁴⁺ to Mn²⁺.

REFERENCES