Mechanism of Growth Inhibition by Tungsten in *Acidithiobacillus ferrooxidans*

Tsuyoshi Sugio, Hiroyuki Kuwano, Atsunori Negishi, Terunobu Maeda, Fumiaki Takeuchi, and Kazuo Kamimura

JSBA

Mechanism of Growth Inhibition by Tungsten in *Acidithiobacillus ferrooxidans*

Tsuyoshi Sugio,[1,†] Hiroyuki Kuwano,[1] Atsunori Negishi,[2] Terunobu Maeda,[2] Fumiaki Takeuchi,[3] and Kazuo Kamimura[4]

[1]*Science and Technology for Energy Converwion, Okayama University, Tsushima Naka, Okayama 700-8530, Japan*
[2]*Technical Research Institute, Hazama Corporation, 515-1 Nishimukai, Karima, Tsukuba 305-0822, Japan*
[3]*Administration Center for Environmental Science and Technology, Japan*
[4]*Faculty of Agriculture, Okayama University, Tsushima Naka, Okayama 700-8530, Japan*

Cell growth of three hundred iron-oxidizing bacteria isolated from natural environments was inhibited strongly by 0.05 mM, and completely by 0.2 mM of sodium tungstate (Na_2WO_4), respectively. Since no great difference in the level of tungsten inhibition was observed among the 300 strains tested, the mechanism of inhibition by Na_2WO_4 was studied with *Acidithiobacillus ferrooxidans* strain AP19-3. When resting cells of AP19-3 were incubated in 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) with 0.1 mM Na_2WO_4 for 1 h, the amount of tungsten bound to the cells was 12 μg/mg protein. The optimum pH for tungsten binding to the resting cells was 2~3. Approximately 2 times more tungsten bound to the cells at pH 3.0 than at pH 6.0. The tungsten binding was specifically inhibited by sodium molybdenum. However, copper, nickel, cadmium, zinc, manganese, cobalt, and vanadate did not disturb tungsten binding to the resting cells. The iron-oxidizing activity of AP19-3 was inhibited 24, 62, and 77% by 1, 5, and 10 mM of Na_2WO_4, respectively. Among the components of iron oxidation enzyme system, iron:cytochrome *c* oxidoreductase activity was not inhibited by 10 mM of Na_2WO_4. In contrast, the activity of cytochrome *c* oxidase purified highly from the strain was inhibited 50 and 72%, respectively, by 0.05 and 0.1 mM of Na_2WO_4. The amounts of tungsten bound to plasma membrane, cytosol fraction, and a purified cytochrome *c* oxidase were 8, 0.5, and 191 μg/mg protein, respectively. From the results, the growth inhibition by Na_2WO_4 observed in *A. ferrooxidans* is explained as follows: tungsten binds to cytochrome *c* oxidase in plasma membranes and inhibits cytochrome *c* oxidase activity, and as a results, the generation of energy needed for cell growth from the oxidation of Fe^{2+} is stopped.

Key words: tungsten; *Acidithiobacillus ferrooxidans*; inhibition site; cytochrome *c* oxidase

An iron-oxidizing bacterium, *Acidithiobacillus ferrooxidans*[1] (*Thiobacillus ferrooxidans*), is an acidophilic chemolithotrophic bacterium that can use both ferrous iron and reduced sulfur compounds as energy soruce and atmospheric carbon dioxide as carbon source. The bacterium has been known to play a crucial role in the bacterial leaching of sulfide ores.[2-6] Since the bacterium usually inhabits acidic drainage from mines which contains large amounts and many kinds of heavy metals, a bacterial strain that has an iron oxidation enzyme system resistant to heavy metals seems to be more useful for the increased bioleaching of sulfide ore. It has been known that *A. ferrooxidans* cells are in general resistant to many kinds of heavy metals including iron, copper, zinc and nickel, but sensitive to mercury, silver, and molybdenum.[7-12] *A. ferrooxidans* strains resistant to mercury, silver, and molybdenum ions were isolated and the mechanisms of bacterial resistance to these toxic metals have been investigated. The properties of mercury reductase, a flavoenzyme that reduces Hg^{2+} to less toxic metal mercury with NADPH as the electron donor, has been studied in a wide range of Gram-negative and positive bacteria.[13-16] Mercury reductase activity has been found in *A. ferrooxidans* cells.[9,11,17,18] The genes involved in the volatilization of mercury have been cloned and characterized in detail.[19-21] Recently, we showed that the *A. ferrooxidans* strain Funis 2-1, resistant to mercury ion, has a mercury resistant cytochrome *c* oxidase which is one of the most important components of the iron oxidation enzyme system of this bacterium, and proposed that both a cytochrome *c* oxidase resistant to Hg^{2+} as well as a mercury reductase, is responsible for the more rapid growth of resistant strain in Fe^{2+} medium containing mercury.[11,12] Both cell growth and iron-oxidizing activity of *A. ferrooxidans* were strongly inhibited by a low concentration of silver ion.[10] Since

the level of inhibition by silver ion was merkedly decreased by the addition of reduced form of glutathione (GSH), it was proposed that GSH plays a crucial role in the detoxification of the ion.[10] Recently, we proposed the mechanism of growth inhibiton by molybdenum ion in A. ferrooxidans. The Mo^{5+}, but not Mo^{6+}, tightly binds to plasma membranes of A. ferrooxidans cells and inhibits the activity of cytochrome c oxidase and as a result, stops energy generation needed for cell growth and maintenance. These results suggest the importance of the iron oxidation enzyme system in A. ferrooxidans as the site of inhibition by toxic heavy metals.

Although tungsten also inhibits growth of A. ferrooxidans strongly, there have been no reports on the mechanism of tungsten inhibition in the bacterium. It is interesting to study inhibitions of cell growth by tungsten and molybdenum because these metals are oxyanions and thus, different from Hg^{2+} and Ag^+. In this report, we first show that among the components of the iron oxidation enzyme system of A. ferrooxidans, tungsten binds more tightly to cytochrome c oxidase and inhibits iron oxidation of this bacterium, stopping energy generation needed for cell growth and maintenance.

Materials and Methods

Microorganisms, medium, and growth conditions. Three hundred strains of iron-oxidizing bacteria isolated from streams and soils in Japan and A. ferrooxidans ATCC 13661, ATCC 14119, ATCC 19859, ATCC 21834, ATCC 23270, and ATCC 33020 from the American Type Culture Collection were used in this study. To isolate iron-oxidizing bacteria, stream water or soil samples were incubated at 30°C under aerobic conditions in Fe^{2+} medium (pH 2.5) containing (per liter) 30 g of $FeSO_4 \cdot 7H_2O$, 3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl and 0.01 g of $Ca(NO_3)_2$.[21] When the Fe^{2+} in the culture medium was oxidized, samples were plated on 1% gellan gum plates containing (per liter) 30 g of $FeSO_4 \cdot 7H_2O$, 3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl, 0.01 g of $Ca(NO_3)_2$ and 0.3% of yeast extract. Rusty colonies appearing on the plate were picked. This process was repeated more than three times, and the final isolates are preserved on Fe^{2+} medium (pH 2.5) and used throughout this study. The methods used for large-scale production of cells has been described previously.[21]

Growth rate. After cultivation in Fe^{2+} medium (pH 2.5) with or without sodium tungstate at 30°C, cells were separated from the particles of ferric hydroxide by filtering with a no. 5B Toyo paper filter. The number of cells in the filtrates were counted with a microscope and hemacytometer (Kayagaki Irika

Kogyo Co., Ltd., Tolyo, Japan) after dilution with 0.1 N sulfuric acid when necessary.

Iron-oxidizing activity. The activity was measured at 30°C from the oxygen uptake rate in a biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH, USA). The reaction mixture was composed of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0), resting cells of A. ferrooxidans AP19-3 (0.25 mg of protein), and $FeSO_4 \cdot 7H_2O$ (33.3 mM). The activity was calculated by assuming that the initial solubility of oxygen in the reaction mixture is 0.223 μmol/ml at 30°C.

Fe^{2+}:cytochrome c oxidoreductase activity. The activity was measured at 30°C by the increase of absorbance at 552 nm due to the reduction of membrane-bound cytochrome c by a Shimadzu multipurpose spectrophotometer, model MPS-5000, with a 1-cm light path cuvette. To decrease the amount of reduced compounds in the plasma membranes, $KMnO_4$ was added to the membranes until reduced cytochrome c in the membranes was nearly completely oxidized. The reaction mixture contained 0.5 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0), 40 mg of plasma membranes from A. ferrooxidans AP19-3, 10 mM sodium tungstate, and 50 mM $FeSO_4 \cdot 7H_2O$. Total volume was 1.0 ml. The reaction was started by adding ferrous iron to the reaction mixture.

Cytochrome c oxidase activity. The activity was measured at 30°C by the decrease in absorbance at 550 nm due to oxidation of reduced mammalian cytochrome c (Sigma type VI from horse heart) in a Shimadzu UV-1200 spectrophotometer. The reaction mixture contained a purified cytochrome c oxidase from A. ferrooxidans AP19-3 (10 μg), reduced cytochrome c (0.4 mg), sodium phosphate buffer (pH 5.5, 2.25 ml). Total volume was 2.5 ml. The reduced cytochrome c was prepared by the reduction of cytochrome c with sodium ascorbate. After the redcution of cytochrome c, the reaction mixture was passed throuh a Sephadex G-25 column to remove excess sodium ascorbate. One unit of cytochrome c oxidase was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of reduced cytochrome c in one min.

Purification of cytochrome c oxidase. Resting cells of A. ferrooxidans AP19-3 were disrupted by passage twice through a French pressure cell at 1,500 kg/cm² 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 and cytosol fraction. The plasma membranes of A. ferrooxidans AP19-3 was incubated for 1.5 h with 0.1 M sodium acetate buffer (pH 4.0) containing 1.5% 1-o-octyl-D-glucopyranoside (OGL) and 50 mM

Na$_2$SO$_4$ and then centrifuged at 105,000 × g for 1 h. The supernatant thus obtained was dialyzed against 5 liters of 10 mM sodium acetate buffer (pH 4.0) containing 0.1% Nonidet P-40 for 6 h. After dialysis, the greenish precipitate in a dialysis bag was collected by centrifugation at 105,000 × g for 1 h. The activity of cytochrome c oxidase was found in this precipitate, which was solubilized with 0.1 M sodium acetate buffer (pH 4.0) containing 2% OGL. The enzyme fraction obtained was put on a CM-Toyopearl 650M column (3 by 10 cm) equilibrated with 0.1 M sodium acetate buffer (pH 4.0) containing 1.5% OGL. The enzyme solution from a CM-Toyopearl 650M column had a specific activity of 14.8 mU/mg protein and was used to study the effect of Na$_2$WO$_4$ on cytochrome c oxidase activity. One unit of cytochrome c oxidase was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of reduced cytochrome c in one min.

Analysis of tungsten bound to resting cells or cytochrome c oxidase. The amounts of tungsten bound to the cells, cytosol fraction, plasma membrane, and cytochrome c oxidase from *A. ferrooxidans* AP19-3 were measured by the thiocyanate method. The plasma membrane, cytosol fraction, and purified cytochrome c oxidase were obtained by the method described above. Sodium tungstate was added to 0.1 M β-alanine-SO$_4^{2-}$ buffer (pH 3.0) containing resting cells, cytosol, plasma membrane, and cytochrome c oxidase at a final concentration of 0.1 mM for 1 h. A white precipitate was produced when the cytosol fraction was treated with sodium tungstate at pH 3.0. The cells, cytosol fraction, plasama membrane, and purified cytochrome c oxidase thus treated with sodium tungstate were centrifuged at 105,000 × g for 1 h to wash out the tungsten not bounding to these fractions using 0.1 M β-alanine-SO$_4^{2-}$ buffer (pH 3.0). The fractions treated with tun-

gsten and washed with 0.1 M β-alanine-SO$_4^{2-}$ buffer (pH 3.0) three times were suspended in 1 ml of deionized water and then incubated for 10 min at 60°C in 20 ml of 20% stannous chloride in 12 N HCl, to which sodium thiocyanate and 10 ml of diisopropylether were added. The tungsten in the ether layer was measured by a Shimadzu UV-1200 spectrophotometer using the absorbance at 400 nm.

Protein content. Protein was measured by the method of Lowry *et al.* with crystalline bovine serum albumin as the reference protein.[22]

Results

Growth of iron-oxidizing bacteria in Fe^{2+} medium with sodium tungstate

Screening 300 iron-oxidizing bacterial strains, including six *A. ferrooxidans* strains from the American Type Culture Collection, for resistance to sodium tungstate (Na$_2$WO$_4$) was done. Na$_2$WO$_4$ inhibited both cell growth and iron oxidation of 300 iron-oxidizing bacterial strains in Fe^{2+} medium (pH 2.5) strongly at 0.05 mM, and completely at 0.2 mM, respectively. Interestingly, no marked difference in the level of tungsten inhibition was observed among these 300 strains. Therefore, we selected *A. ferrooxidans* AP19-3[24] as a representative strain for the study of growth inhibiton by Na$_2$WO$_4$. The effects of Na$_2$WO$_4$ on the growth of strain AP19-3 and on the oxidation of Fe^{2+} in Fe^{2+} medium (pH 2.5) are shown in Fig. 1.

Effects of Na$_2$WO$_4$ on iron oxidase activity

Iron oxidase is one of the most important enzymes in iron-oxidizing bacterium *A. ferrooxidans*. Therefore, the effects of Na$_2$WO$_4$ on iron-oxidizing activity was studied with resting cells of *A. ferrooxidans* AP19-3. The activity was inhibited by 24% and 62%
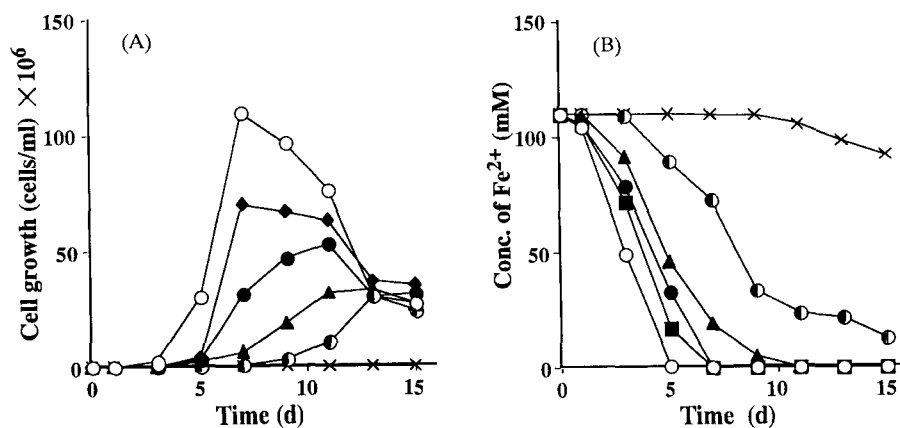

Fig. 1. Effects of Sodium Tungstate on the Growth of *A. ferrooxidans* AP19-3.
   Both cell growth (A) and Fe^{2+} concentration (B) in Fe^{2+} medium (pH 2.5) with or without sodium tungstate were measurred. Symbols: ○, Fe^{2+} medium without Na$_2$WO$_4$; Fe^{2+} medium with 0.01 mM (■), 0.02 mM (●), 0.05 mM (▲), 0.1 mM (◐), and 0.2 mM (×) of Na$_2$WO$_4$.

at 1 and 5 mM of Na_2WO_4, respectively (Fig. 2), indicating that the iron oxidation enzyme system absolutely needed for energy production was inhibited by Na_2WO_4 in this bacterium.

Amounts of tungsten bound to A. ferrooxidans AP19-3

To clarify the mechanism of tungsten inhibition more precisely, the amounts of tungsten bound to *A. ferrooxidans* AP19-3 cells were studied. Resting cells of AP19-3 were incubated for 1 h in 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) containing 0.1 mM of Na_2WO_4. After incubation with Na_2WO_4, cells were washed three times with 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) and then the concentration of tungsten bound to the cells was measured. The amounts of tungsten bound to the resting cells increased with incubation time
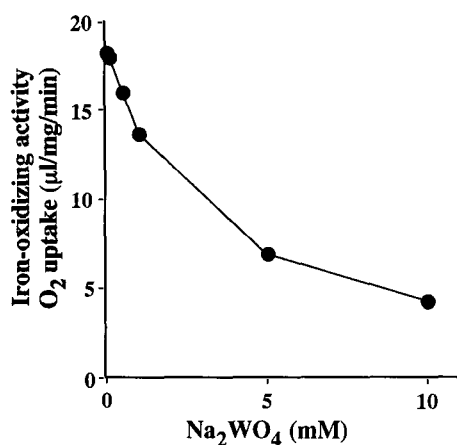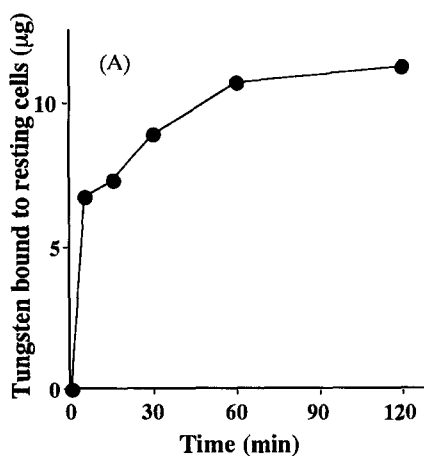

Fig. 2. Effects of Sodium Tungstate on Iron-oxidizing Activity of *A. ferrooxidans* AP19-3.
   Iron-oxidizing activity was measured with resting cells of *A. ferrooxidans* AP19-3.

(Fig. 3(A)) and with the amounts of cells used (Fig. 3 (B)). When incubated with 0.1 mM of Na_2WO_4 at pH 3.0 for 1 h, the maximum amount of tungsten bound to *A. ferrooxidans* AP19-3 cells was 12 μg/mg protein.

Effects of pH on tungsten binding to A. ferrooxidans AP19-3 cells

The effects of pH on tungsten binding to *A. ferrooxidans* cells were studied with resting cells of strain AP19-3. The amount of tungsten bound to cells at acidic pH was larger than at neutral and alkaline pHs (Fig. 4). Approximately 2 times more tungsten bound to the cells at pH 3 compared with pH 6. The same experiment was done with resting cells of *A. ferrooxidans* Funis 2-1.[25] The level of growth inhibition by Na_2WO_4 in Funis 2-1 cells was nearly the same as that in AP19-3. The same pH profile for the binding of tungsten to the cells was observed between the two iron-oxidizing bacterial strains.

Effects of Na₂WO₄ on iron:cytochrome c oxidoreductase activity

The iron oxidation enzyme system of *A. ferrooxidans* is divided into two parts: one, iron:cytochrome c oxidoreductase and the other, cytochrome c oxidase. To study the site of inhibition by Na_2WO_4 of the iron oxidation enzyme system more precisely, effects of Na_2WO_4 on the activities of iron: cytochrome c oxidoreductase and cytochrome c oxidase were studied. Just after the addition of 5 nmol of Fe^{2+} to oxidized plasma membranes (20 mg protein) of *A. ferrooxidans* AP19-3 in the presence of potassium cyanide (5 mM), absorption peaks at 595 nm due to the α-peak of cytochrome c oxidase and at 552, 525, and 418 nm due to α-, β-, and γ-peaks of membrane bound cytochrome c increased, indicating
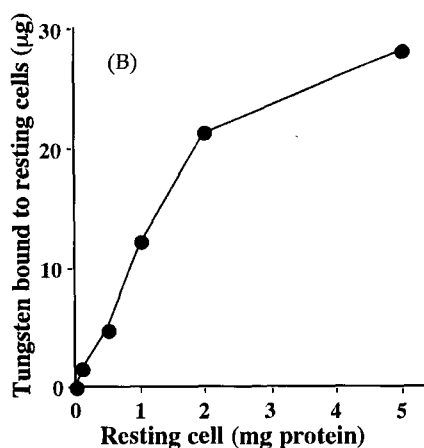

Fig. 3. Effects of Incubation Time (A) and Cell Concentration (B) on the Amount of Tungsten Bound to *A. ferrooxidans* AP19-3 Cells.
   (A) Resting cells (1 mg of protein) of *A. ferrooxidans* AP19-3 were incubated in 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) with 0.1 mM of Na_2WO_4 for the time indicated in the figure. (B) Resting cells from 0.1 to 5 mg of protein of *A. ferrooxidans* AP19-3 were incubated in 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) with 0.1 mM of Na_2WO_4 for 1 h. The amounts of tungsten bound to the cells were measured as described in Materials and Methods.

that both cytochrome c oxidase and cytochrome c were reduced rapidly by Fe^{2+} (Fig. 5(A)). After addition of Fe^{2+}, cytochrome c in the plasma membranes remained in a redcued form for 60 min under aerobic conditions, suggesting that cytochrome c oxidase activity was inhibited by potassium cyanide. The same experiment was done in the presence of 10 mM Na_2WO_4 (Fig. 5(B)). The cytochrome c in the membranes was reduced rapidly by Fe^{2+} in the presence of Na_2WO_4, indicating that iron:cytochrome c oxidoreductase activity was not inhibited by Na_2WO_4.
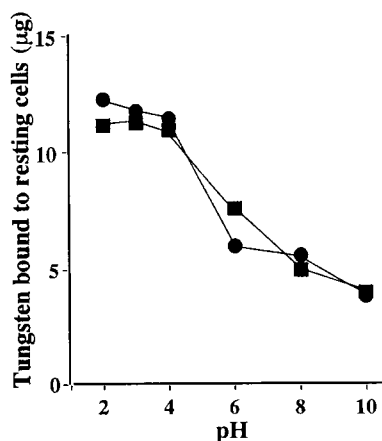

Fig. 4. Effects of pH on the Binding of Tungsten to Resting Cells of *A. ferrooxidans*.

Resting cells (1 mg of protein) of *A. ferrooxidans* AP19-3 (●) and Funis 2-1 (■) were incubated in 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) with 0.1 mM Na_2WO_4 for 1 h. The amounts of tungsten bound to the cells were measured as described in Materials and Methods.

Effects of Na_2WO_4 on cytochrome c oxidase activity

Cytochrome c oxidase was purified from *A. ferrooxidans* AP19-3 after solubilizaton of the enzyme from plasma membranes with 1.5% 1-o-octyl-D-glucopyranoside (OGL). Compared with plasma membranes, the enzyme was purified 13-fold with a specific activity of 14.8 mU/mg protein and a yield of 2%. When reduced with sodium hydrosulfite, a purified cytochrome c oxidase showed two absorption peaks characteristic to cytochrome c oxidase of *A. ferrooxidans* at 595 nm (α-band) and 439 nm (γ-band). We have previously purified cytochrome c oxidase to an electrophoretically homogeneous state from plasma membranes of *A. ferrooxidans* AP19-3 after solubilizaton of the membranes with 1% Nonidet P-40.[26] The purified enzyme had a specific activity of 24 mU/mg protein. The activity of cytochrome c oxidase purified highly from strain AP19-3 was inhibited approximately 50 and 72% by 0.05 and 0.1 mM Na_2WO_4, respectively (Fig. 6).

The amounts of tungsten bound to cytosol fractions, plasma membranes, and a highly purified cytocrome c oxidase from *A. ferrooxidans* AP19-3 were measured (Table). A small amount of tungsten bound to the proteins in the cytosol fraction. In contrast, almost all the tungsten bound to resting cells was present in the plasma membranes. The plasma membrane was solubilized with 1.5% OGL to isolate cytochrome c oxidase. The amounts of tungsten bound to plasma membranes, the fraction solubilized with 1.5% OGL and a highly purified cytochrome c oxidase were 8, 33 and 191 μg/mg protein, respectively. Interestingly, the increase of the amount of
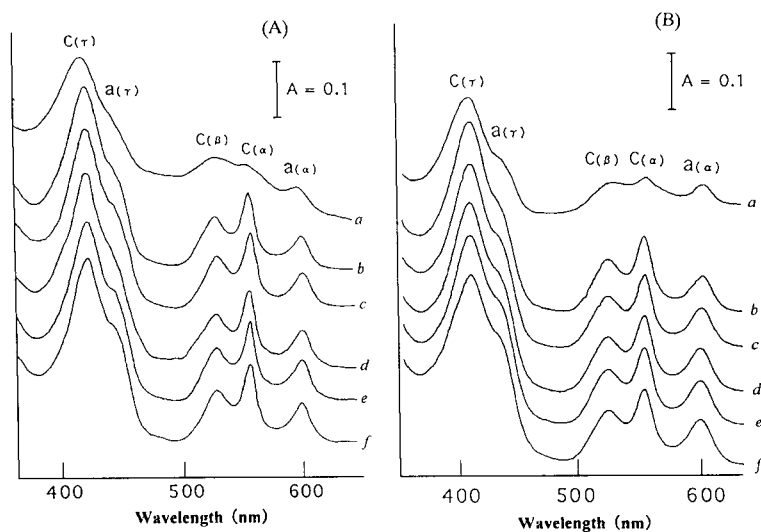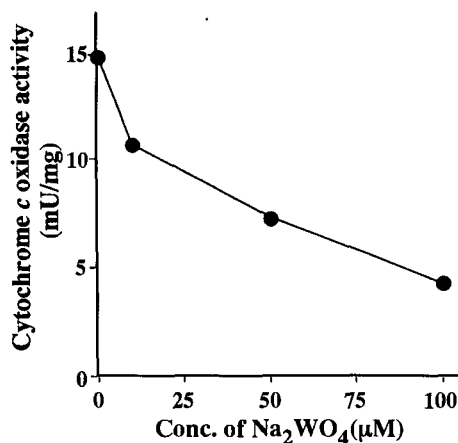

Fig. 5. Effect of Sodium Tungstate on Fe^{2+}:cytochrome c Oxidoreductase Activity of *A. ferrooxidans* AP19-3.

Reduction of membrane bound cytochrome c by 5 nmol of Fe^{2+} was measured under aerobic conditions in a 1 ml of reaction mixture containing oxidized plasma membranes of *A. ferrooxidans* AP19-3 (20 mg of protein), KCN (0.25 μmol) and with 10 mM of Na_2WO_4 (B) or without Na_2WO_4 (A). Line a shows a spectrum of oxidized plasma membranes of *A. ferrooxidans* AP19-3 (20 mg of protein) without Fe^{2+} addition. Lines $b \sim f$ show spectra of plasma membranes of *A. ferrooxidans* AP19-3 (20 mg of protein) after the addition of Fe^{2+}: b, 1 min; c, 5 min; d, 15 min; e, 30 min and f, 60 min.

Table. The Amounts of Tungsten Bound to Resting Cells, Cytosol Fractions, Plasma Membranes, and a Purified Cytocrome c Oxidase from A. ferrooxidans AP19-3

Cell fractions	Tungsten bound (μg/mg protein)	Cytochrome c oxidase activity (mU/mg)
Resting cells	12	
Cytosol fraction	0.5	
Plasma membranes	8	1.1
Fraction solubilized with 1.5% OGL	33	3.0
Purified cytochrome c oxidase	191	14.8

Fig. 6. Effect of Sodium Tungstate on Cytochrome c Oxidase Activity of A. ferrooxidans AP19-3.

The most highly purified cytochrome c oxidase from a CM Toyopearl 650 column (specific activity, 14.8 mU/mg protein) was used.

Tungsten bound to resting cells (μg/mg protein)

Fig. 7. Effects of Heavy Metals on Tungsten Binding of A. ferrooxidans AP19-3.

Resting cells of A. ferrooxidans AP19-3 (1 mg of protein) was treated with 1 mM sodium tungstate or with 1 mM sodium tungstate plus 1 mM heavy metal at pH 3.0 for 1 h. After the treatment, cells were washed three times with 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) and the tungsten bound to the cells was measured by the method described in Materials and Methods.
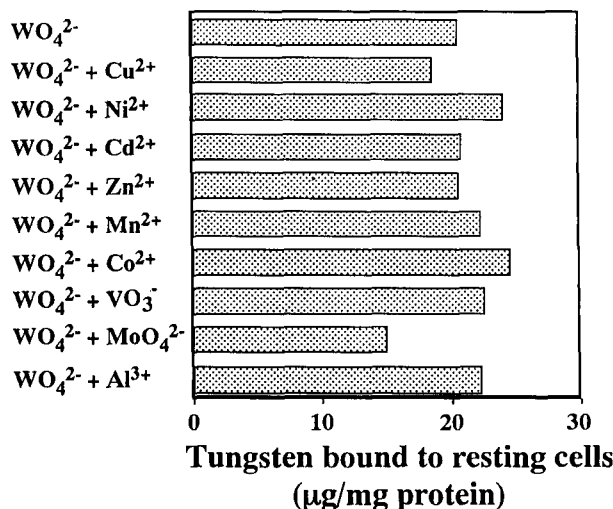
tungsten bound to these fractions corresponded well with the increment of cytochrome c oxidase activity, suggesting that tungsten bound to cytochrome c oxidase and caused enzyme inhibition.

Effects of metal ions on the binding of tungsten to resting cells of A. ferrooxidans

To study the specificity of tungsten binding to resting cells of A. ferrooxidans, the amount of tungsten bound to the cells was measured in the presence of different metal ions. The same concentration of cupric, cadmium, zinc, and manganase ions as that of sodium tungstate (1 mM) did not disturb tungsten binding to the cells (Fig. 7). Nickel and cobaltous ions slightly increased the amounts of tungsten bound to the cells. Molybdenum ion, which is an oxyanion like sodium tungstate, inhibited tungsten binding 27%. However, vanadate, which is also an oxyanion, did not affect the tungsten binding to the cells of A. ferrooxidans AP19-3. These results suggest the existence of protein(s) to which tungsten binds specifically in the cells.

Discussion

Sodium tungstate completely inhibited the growth

of A. ferrooxidans cells in Fe^{2+} medium (pH 2.5) at 0.2 mM. No great difference was observed in the level of growth inhibition by tungsten among 300 strains of iron-oxidizing bacteria tested and thus, we could not obtain an A. ferrooxidans strain resistant to tungsten from our stock cultures of iron-oxidizing bacteria that grew in the Fe^{2+} medium supplemented with 0.2 mM of sodium tungstate. Using A. ferrooxidans AP19-3 as a representative strain for the study of growth inhibiton by Na_2WO_4, the following findings were obtained in this report: (1) Tungsten bound more tightly to A. ferrooxidans cells at acidic pH than neutral or alkaline pHs. (2) Most of the tungsten bound to resting cells was present in the plasma membranes of this bacterium. (3) Approximately 16~24 times more tungsten bound to cytochrome c oxidase of A. ferrooxidans AP19-3 compared with resting cells and plasma membranes of this bacterium. (4) The activities of iron oxidase and cytochrome c oxidase, but not iron:cytochrome c oxidoreductase, were inhibited by sodium tungstate. Since cytochrome c oxidase is one of the most important

components in the iron oxidation enzyme system of *A. ferrooxidans*,[26-30] the mechanism of inhibition of cell growth by Na_2WO_4 of *A. ferrooxidans* is explained as follows: tungsten binds to cytochrome *c* oxidase in plasma membranes and inhibits cytochrome *c* oxidase activity, and as a results, the generation of energy needed for cell growth and cell maintenance from the oxidation of Fe^{2+} is stopped.

We have stressed the importance of cytochrome *c* oxidase in bacterial resistance to bisulfite ion, molybdenum ion, and mercury ion in *A. ferrooxidans*.[11,31-33] These ions toxic to the growth of *A. ferrooxidans* cells can inhibit cytochrome *c* oxidase activity strongly. However, interestingly, *A. ferrooxidans* strains resistant to bisulfite ion, molybdenum ion, and mercury ion have a cytochrome *c* oxidase resistant to each of these ions.[11,31-33] Unfortunately, we could not obtain this time an *A. ferrooxidans* strain resistant to tungsten from our stock cultures. Since it was found that a large amount of tungsten (191 μg/mg protein) bound to cytochrome *c* oxidase of *A. ferrooxidans* AP19-3 at pH 3.0, the cause that we could not find a tungsten resistant strain is probably the chemical properties of tungsten. Namely, tungsten bound easily and tightly to cytochrome *c* oxidase of this bacterium at acidic pH, and thus, inhibited the enzyme activity strongly. We have shown that cytochrome *c* oxidase from *A. ferrooxidans* AP19-3 is composed of three subunits of apparent molecular weights of 53,000 Da (α), 24,000 Da (β), and 19,000 Da (γ).[26] It has been reported that α- and β-subunits of cytochrome *c* oxiase have a heme binding catalitic site.[34] Therefore, to clarify the mechanism of tungsten inhibition more precisely, it is important to specify the subunit of cytochrome *c* oxidase to which tungsten binds, and the work on this is now under way.

References

1) Kelly, D. P. and Wood, D. P., Reclassification of some species of *Thiobacillus* to the newly desingated genera *Acidithiobacillus* gen. nov. *Int. J. Syst. Evol. Microbiol.*, **50**, 511-516 (2000).

2) Torma, A. E., The role of *Thiobacillus ferrooxidans* in hydrometallurgical processes. *Adv. Biochem. Eng.*, **6**, 1-38 (1977).

3) Lundgren, D. G. and Silver, M., Ore leaching by bacteria. *Ann. Rev. Microbiol.*, **34**, 263-283 (1980).

4) Hutchins, S. R., Davidson, M. S., Brierley, J. A., and Brierley, C. L., Microorganisms in reclamation of metals. *Ann. Rev. Microbiol.*, **40**, 311-336 (1986).

5) Brierley, C. L., Microbiological mining. *Sci. Am.*, **247**, 42-49 (1982).

6) Nicolaidis, A. A., Microbial mineral processing: The opportunities for genetic manipulation. *J. Chem. Tech. Biotechnol.*, **38**, 167-185 (1987).

7) Tuovinen, O. H., Niemela, S. I., and Gyllenberg, H. G., Tolerance of *Thiobacillus ferrooxidans* to some metals. *Antonie van Leeuwenhoek*, **37**, 489-496

8) Imai, K., Sugio, T., Tsuchida, T., and Tano, T., Effect of heavy metal ions on the growth and iron-oxidizing activity of *Thiobacillus ferrooxidans*. *Agric. Biol. Chem.*, **39**, 1349-1354 (1975).

9) Olson, G. J., Iverson, W. P., and Brinckman, F. E., Volatilization of mercury by *Thiobacillus ferrooxidans*. *Current Microbiol.*, **5**, 115-118 (1981).

10) Sugio, T., Tano, T., and Imai, K., Isolation and some properties of silver ion-resistant iron-oxidizing bacterium *Thiobacillus ferrooxidnas*. *Agric. Biol. Chem.*, **45**, 2037-2051 (1981).

11) Takeuchi, F., Iwahori, K., Kamimura, K., and Sugio, T., Isolation and some properties of *Thiobacillus ferrooxidans* strains with differing levels of mercury resistance from natural environments. *J. Biosci. Bioeng.*, **88**, 387-392 (1999).

12) Iwahori, K., Takeuchi, F., Kamimura, K., and Sugio, T., Ferrous iron-dependent volatilization of mercury by the plasma membrane of *Thiobacillus ferrrooxidans*. *Appl. Environ. Microbiol.*, **66**, 3823-3827 (2000).

13) Babich, K., Engle, M., Skinner, J. S., and Laddaga, R. A., Deletion mutant analysis of the *Staphylococcus aureus* plasmid p1258 mercury resistance determinant. *Can. J. Microbiol.*, **37**, 624-631 (1991).

14) Schottel, J., Mandal, A., Clark, D., Silver, S., and Hedges, R. W., Volatilization of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. *Nature* (London) **251**, 335-337 (1974).

15) Schottel, J. L., The mercury and organomercurial detoxifying enzymes from a plasmid-bearing strain of *Escherichia coli*. *J. Biol. Chem.*, **253**, 4341-4349 (1978).

16) Silver, S. and Pheng, L. T., Bacterial heavy metal resistance: new surprises. *Annu. Rev. Microbiol.*, **50**, 753-789 (1996).

17) Booth, J. E. and Williams, J. W., The isolation of a mercuric ion-deducing flavoprotein from *Thiobacillus ferrooxidans*. *J. Gen Microbiol.*, **130**, 725-730 (1984).

18) Olson, G. J., Porter, F. D., Rubinstein, J., and Silver, S., Mercuric reductase enzyme from a mercury-volatilizing strain of *Thiobacillus ferrooxidans*. *J. Bacteriol.*, **151**, 1230-1236 (1982).

19) Douglas, E. R. and Kusano, K., Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol. Rev.*, **58**, 39-55 (1994).

20) Inoue, C., Kusano, T., and S. Silver, Mercuric ion uptake by *Escherichia coli* cells producing *Thiobacillus ferrooxidans* MerC. *Biosci. Biotechnol. Biochem.*, **60**, 1289-1292 (1996).

21) Kusano, T., Inoue, C., and Silver, S., Constitutive synthesis of a transport function encoded by the *Thiobacillus ferrooxidans* merC gene cloned in *Escherichia coli*. *J. Bacteriol.*, **172**, 2688-2692 (1990).

22) Sugio, T., Katagiri, T., Moriyama, M., Zhen, Y. L., Inagaki, K., and Tano, T., Existence of a new type of sulfite oxidase which utilizes ferric ion as an electron acceptor in *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **54**, 153-157 (1988).

23) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and

Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).

24) Sugio, T., Domatsu, C., Munakata, O., Tano, T., and Imai, K., Role of a ferric ion-reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.*, **49**, 1401-1406 (1985).

25) Sugio, T., Kanao, T., Furukawa, H., Nagasawa, T., and Blake II, R. C., Isolation and identification of an iron-oxidizing bacterium which can grow on tetrathionate medium and the properties of a tetrathionate-decomposing enzyme isolated from the bacterium. *J. Ferment. Bioeng.*, **82**, 233-238 (1996).

26) Iwahori, K., Kamimura, K., and Sugio, T., Isolation and some properties of cytochrome *c* oxidase purified from a bisulfite ion resistant *Thiobacillus ferrooxidans* strain OK1-50. *Biosci. Biotechnol. Biochem.*, **62**, 1081-1086 (1998).

27) Ingledew, W. J., Cox, J. C., and Halling, P. J., A proposed mechanism for energy conservation during Fe^{2+} oxiation by *Thiobacillus ferro-oxidans*: Chemiosmotic coupling to net H^+ influx. *FEMS Microbiol. Lett.*, **2**, 193-197 (1977).

28) Ingledew, W. J., *Thiobacillus ferrooxidans*. The bioenergetics of an aciophilic chemolithotroph. *Biochem. Biophys. Acta*, **683**, 89-117 (1982).

29) Hooper, A. B. and Dispirito, A. A., In bacteria which grow on simple redutants, generation of a pro-

ton gradient involves extracytoplasmic oxidation of substrate. *Microbiol. Rev.*, **49**, 140-151 (1985).

30) Kai, M., Yano, T., Tamegai, H., Fukumori, Y., and Yamanaka, T., *Thiobacillus ferrooxidans* cytochrome *c* oxidase: Purification, and molecular and enzymatic features. *J. Biochem.*, **112**, 816-821 (1992).

31) Sugio, T., Iwahori, K., Uemura, S., Makino, I., and Tano, T., Inhibition of iron-oxidizing activity by bisulfite ion in *Thiobacillus ferrooxidans*. *Biosci. Biotechnol. Biochem.*, **58**, 2109-2110 (1994).

32) Sugio, T., Makino, I., Iwahori, K., Uemura, S., Takai, M., and Tano, T., Isolation and some properties of an iron-oxidizing bacterium *Thiobacillus ferrooxidans* resisttant to bisulfite ion. *Biosci. Biotechnol. Biochem.*, **59**, 435-438 (1995).

33) Ng Kim Yong, M. Oshima, R. C. Blake, II, and T. Sugio, Isolation and some properties of an iron-oxidizing bacterium, *Thiobacillus ferrooxidans*, resistant to molybdenum ion. *Biosci. Biotechnol. Biochem.*, **61**, 1523-1526 (1997).

34) Appia-Ayme, C., N. Guiliani, J. Ratouchniak, and V. Bonnefoy, Characterization an operon encoding two *c*-type cytochromes, an aa_3-type cytochrome oxidase, and rusticyanin in *Thiobacillus ferrooxidans* ATCC 33020. *Appl. Environ. Microbiol.*, **65**, 4781-4787 (1999).