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Kinetics of Respiration Reaction of *Thiobacillus ferrooxidans* with Several Iron(II) Complexes as Electron Donors

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Respiratory reaction of *Thiobacillus ferrooxidans* which uses Fe^{2+} as the substrate in acidic solution was analyzed by a Michaelis-Menten type equation, and the catalytic constant, $k_{\text{B,cat}}$, of the bacterial cell was determined as $3.0 \times 10^6 \text{ s}^{-1}$ for the first time. The Michaelis constant, $K'_{\text{m,Fe}}$, for Fe^{2+} was determined to be $37 \mu\text{M}$, which is much smaller than the values so far reported. In addition to FeSO_4 , a number of iron complexes ($\text{Fe}(\text{CN})_6^{4-}$, Fe-citrate, and ferrocene derivatives) functioned as the substrates in the respiration of *T. ferrooxidans*. The $k_{\text{B,cat}}$ values with the iron complexes are in the same order of magnitude as that with Fe^{2+} , $0.71 - 3.0 \times 10^6 \text{ s}^{-1}$, while the $K'_{\text{m,Fe}}$ values are widespread over the range from $1 \mu\text{M}$ for $\text{Fe}(\text{CN})_6^{4-}$ to $120 \mu\text{M}$ for ferrocenecarboxaldehyde.

Key Words : *Thiobacillus ferrooxidans*, Iron Complexes, Respiration, Kinetic Analysis

1 Introduction

Thiobacillus ferrooxidans is a unique chemolithotrophic bacterium, which utilizes ferrous ion in sulfuric acid solution to obtain energy for supporting carbon dioxide fixation and growth.¹⁾ It is involved in the acid mine drainage, and has attracted considerable attention in applying to a process known as bacterial leaching. Since the cell yield is very low because of the culture on the energy-poor inorganic substrate, attempts have been made to enhance the bacterial growth rate. There have been a considerable number of papers dealing with kinetics of the ferrous ion oxidation by *T. ferrooxidans* to seek an optimum condition for the bacterial growth.²⁻⁹⁾ Electrochemical regeneration of ferrous ion has been shown to provide a method for enhanced bacterial growth.¹⁰⁻¹⁴⁾ We have recently shown that *T. ferrooxidans* catalyzes the electrochemical reduction of O_2 with hexacyanoferrate(II) as an electron transfer mediator between the bacterial cells and an electrode.¹⁵⁾ This indicates that hexacyanoferrate(II) can serve as a substrate for the bacterial respiration as ferrous ion does. Present paper deals with the determination of the catalytic constant and the Michaelis constant of the bacterial respiration reactions with several iron(II) complexes including ferrous ion and hexacyanoferrate(II). The constants provide a means of evaluating how effective the complexes are as the substrates of the bacterial cells.

2 Experimental

2.1 Bacterium and materials

Cultures of *T. ferrooxidans* were incubated in 500 mL Erlenmeyer flasks each containing 100 mL of 9 K medium¹⁶⁾ and 3 mL inoculum at 28°C on a rotary shaker at 200 rpm. Cells were harvested toward the end of their exponential phase of growth. In order to remove the in-

soluble ferric ion compounds, the cultures were filtered through a Toyo No. 2 filter paper. The filtrate was then centrifuged at $10000 \times g$ for 15 min. The cells were washed twice with 10 mM H_2SO_4 and suspended in a sulfuric acid solution of pH 3.0. The activity of the bacterial cells for the oxidation of ferrous ion remained unchanged at least for a month when the suspension was stored at 5°C . The bacterial concentration in the suspension was determined by measuring absorbance at 610 nm. The cell number contained in the suspension of unit absorbance was calculated to be 9.6×10^8 cells per cm^3 , or $1.6 \times 10^{-12} \text{ mol dm}^{-3}$ (M) by using a hemacytometer.

Ferrous citrate (Fe(II)-citrate) was prepared by the addition of FeSO_4 into a solution containing excess amount of citrate. Ferrocenecarboxylic acid (Fc-COOH) and ferrocenecarboxaldehyde (Fc-CO₂CHO) were purchased from Aldrich Co., and ferrocenylethanol (Fc-EtOH) was from TCI Co. All other chemicals used were of reagent grade and were used as received.

2.2 Measurement of the respiration activity of *T. ferrooxidans*

The concentrations of iron complexes in the bacterial suspension were measured by an electrochemical method using a film-coated electrode as described previously.¹⁷⁾ Polycarbonate membrane (10 μm thick, pore size 0.4 μm , Advantec Co.) and a glassy carbon electrode (3.0 mm i.d., No 11-2012, BAS Inc.) were used in preparing the film-coated electrode. A current-time curve was recorded at a fixed potential in an air-saturated stirred sulfuric acid solution (pH 3.0), into which aliquots of an iron complex stock solution and the cell suspension were injected successively. In case of Fe(II)-citrate complex, a pH 3.0 citric acid solution was used instead of the sulfuric acid solution. Sedimentation of the bacterial cells in the test solution was not observed during the course of

the electrochemical measurements. The measurements were conducted with a BAS CV-50W voltammetric analyzer in a one-compartment electrolysis cell with a sample volume of 1 mL. A platinum disk, and an Ag | AgCl | sat. KCl electrode were used as the auxiliary, and reference electrodes, respectively. All measurements were carried out at 25°C.

The concentration of dissolved O₂ in a *T. ferrooxidans* cell suspension was measured by means of a Clark-type electrode assembly (Oxygraph type 8, Central Science Co.).

3 Results and Discussion

3.1 Stoichiometry for the ferrous ion and hexacyanoferrate(II) oxidation through the respiration of *T. ferrooxidans*

It is known that *T. ferrooxidans* possesses a terminal oxidase,¹⁾ which reduces O₂ to produce 2 molecules of H₂O during the respiratory reaction. Thus over-all respiratory reaction scheme of *T. ferrooxidans* with ferrous ion as substrate is:

Figure 1 A shows that the current of an oxygen electrode immersed in a solution containing 0.50 mM Fe²⁺ starts to decrease by the addition of *T. ferrooxidans* cells and approaches about half the magnitude of the initial current. Considering that an air-saturated solution contains 0.25 mM O₂ at 25°C,¹⁸⁾ we can say that 0.12 to 0.13 mM O₂ was consumed by the bacterial respiration with 0.50 mM Fe²⁺. This confirms eq. (1) as the respiration reaction of *T. ferrooxidans*. Similar current decrease was also observed when the bacterial suspension contained 0.50 mM Fe(CN)₆⁴⁻ instead of Fe²⁺ (Fig. 1 B), revealing that not only Fe²⁺ but also Fe(CN)₆⁴⁻ serves as a substrate for the respiration of *T. ferrooxidans*.

Difference in the slope between curves A and B (Fig. 1) reflects the difference in the bacterial respiration rate with Fe²⁺ and Fe(CN)₆⁴⁻ as the substrate. The respiration rate should depend on the concentrations of the bac-

Fig. 1 O₂ consumption by *T. ferrooxidans* with (A) 0.50 mM FeSO₄ and (B) 0.50 mM K₄[Fe(CN)₆] as substrates. *T. ferrooxidans* cells: (A) 1.6×10^{-13} M, (B) 2.2×10^{-13} M.

terial cells and the substrate as well as the catalytic constant and Michaelis constant for the substrate as described below. The linear current decrease up to the inflection point (curve B) indicates that the value of the Michaelis constant for Fe(CN)₆⁴⁻ is very small (see below and Table 1).

3.2 Kinetics of the oxidation of iron(II) complexes by the respiration of *T. ferrooxidans*

The oxidation of Fe²⁺ by *T. ferrooxidans* cells is easily measured by the electrochemical method as depicted in Fig. 2. The current measured at 1.0 V in a pH 3.0 sulfuric acid solution started to appear by the addition of 1.5 mM FeSO₄ (arrow A) and attained a steady state, whose magnitude is proportional to the concentration of Fe²⁺. When 9.6×10^{-13} M of *T. ferrooxidans* cells were added (arrow B), the current begins to decrease because of the bacterial respiration. The initial rate, v_0 , of the Fe²⁺ consumption can be calculated from the initial slope of the current decrease. As shown in Fig. 3, v_0 is proportional to the concentration of the bacterial cell, [B]. Dependence of v_0 on the concentration of Fe²⁺, [Fe], is illustrated in Fig. 4, which shows a saturation tendency inherent in the enzyme kinetics. Thus, the dependence of

Fig. 2 A current-time (i - t) curve measured with a membrane-coated electrode at 1.0 V. A: FeSO₄ and B: *T. ferrooxidans* cells were successively added to make the final concentrations 1.5 mM and 9.6×10^{-13} M.

Fig. 3 Plots of v_0 vs. the concentration of *T. ferrooxidans* cells. FeSO₄: 1.5 mM.

Table 1 Kinetic parameters for Fe(II) oxidation by *Thiobacillus ferrooxidans* cells.

Substrate	FeSO ₄	K ₄ [Fe(CN) ₆]	Fe(II)-citrate	Fc-COOH	Fc-CO ₂ CHO	Fc-EtOH
$k_{B,cat}/s^{-1}$	3.0×10^6	1.5×10^6	7.1×10^5	2.6×10^6	1.6×10^6	1.1×10^6
$K'_{m,Fe}/\mu M$	37	1	7.7	51	120	20

Fig. 4 Dependence of v_0 on the concentration of FeSO₄. *T. ferrooxidans*: 1.6×10^{-13} M.

v_0 on the concentrations of the bacterial cell and Fe²⁺ can be expressed by a Michaelis-Menten type equation written by

$$v_0 = k_{B,cat} [B] [Fe] / (K'_{m,Fe} + [Fe]) \quad (2)$$

where $k_{B,cat}$ is the catalytic constant for a *T. ferrooxidans* cell and $K'_{m,Fe}$ the apparent Michaelis constant for the iron compound. The values of $k_{B,cat}$ and $K'_{m,Fe}$ obtained by nonlinear regression of the experimental data by eq. (2) are given in Table 1. When Fe(CN)₆⁴⁻, Fe-citrate, Fc-COOH, Fc-CH₂CHO, and Fc-EtOH are used as the substrate, similar behavior to that for FeSO₄ was observed and the results were analyzed by eq. (2). Table 1 summarizes the $k_{B,cat}$ and $K'_{m,Fe}$ values for these iron complexes. Interestingly, the $k_{B,cat}$ values are in the same order of magnitude for all the iron complexes examined, $0.71 - 3.0 \times 10^6$ s⁻¹. On the other hand, the $K'_{m,Fe}$ values are widespread over the range from 1 to 120 μM. The $K'_{m,Fe}$ value for FeSO₄, 37 μM, is much smaller than those reported by many workers.^{2-7,13} The reason for the large discrepancy between our $K'_{m,Fe}$ value and those by others is not clear. However, we may say that the spectrophotometric method^{2,4,7} or oxygen electrode method^{3,5,6,13} employed previously are not appropriate for the measurements of Fe²⁺ concentrations as low as 30 μM. Our method as exemplified in Fig. 2 can measure the concentrations of iron complexes down to less than 1 μM.

This is the first report for the measurement of the $k_{B,cat}$ values, which express the respiration rates of *T. ferrooxidans* cell with the iron complexes as the substrates. We have shown that a number of bacterial species, *Gluconobacter industrius*, *Pseudomonas fluorescens*, and *Acetobacter aceti* give $k_{B,cat}$ values comparable to those in Table 1 with organic compounds, glucose and ethanol, as the substrates.¹⁹ Thus, we may say that the respiration reac-

tion of *T. ferrooxidans* using the iron complexes as the substrate is as fast as the ordinary bacterial respirations using organic substrates.

The oxygen consumption rates in the respiration reaction were measured under the same experimental conditions as above (Fig. 2) with an oxygen electrode. The current at the oxygen electrode decreased linearly by the addition of the bacterial cells to the solution until the current reached one tenth of the initial current magnitude, which corresponds to the O₂ concentration, [O₂], of about 25 μM. This fact indicates that the bacterial respiration rate is independent of the O₂ concentration at least at [O₂] > 25 μM and accordingly that the apparent Michaelis constant for O₂, $K'_{m,O}$, has a value smaller than 25 μM. This assures that the respiration rate of *T. ferrooxidans* is not limited by the concentration of O₂ under air-saturated conditions (0.25 mM).

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