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Competitive Inhibition of Ferrous Iron Oxidation by *Thiobacillus ferrooxidans* by Increasing Concentrations of Cells

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The oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) with dioxygen (O_2) by various strains of *Thiobacillus ferrooxidans* was studied by measuring the rate of O_2 consumption at various Fe^{2+} concentrations and cell concentrations. The apparent K_m values for Fe^{2+} remained constant at different cell concentrations of laboratory strains ATCC 13661 and ATCC 19859 but increased with increasing cell concentrations of mine isolates SM-4 and SM-5. The latter results are explained by the competitive inhibition of the Fe^{2+}-binding site of a cell by other cells in the reaction mixture. Possible mechanisms involving cell surface properties are discussed.

Thiobacillus ferrooxidans oxidizes ferrous iron to ferric iron to obtain energy for growth ($2Fe^{2+} + \frac{1}{2}O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$), an important reaction in the bacterial leaching of metals from sulfide ores. Its unique chemolithotrophic life and its role in metal recovery have been reviewed (1, 3–6, 12, 14, 15). The mechanism of iron oxidation proposed (6–8) involves the oxidation of ferrous iron outside the cell membrane at pH 2 and the reduction of O_2 inside at pH 6.5. Cytochrome oxidase in the membrane mediates the transfer of electrons from outside to inside, and the remaining components of the iron oxidase system are believed to be located outside the cytoplasm (8). These include polynuclear Fe^{3+} iron coat on the cell wall and rusticyanin (a copper protein), *c*-type cytochromes, and possibly a recently purified Fe^{2+}-cytochrome *c*-552 reductase (2) in the periplasm. The details of the mechanism are not yet established.

In our study to characterize the properties of various *T. ferrooxidans* strains isolated from a mine site (11) we encountered an unusual effect of cell concentration on the apparent Michaelis constants (K_m values) for Fe^{2+} in the oxidation of ferrous iron. The effect can be best explained by the assumption that a bacterial cell functioning as an Fe^{2+}-oxidizing enzyme system can also interact with another cell in such a way that it competes with Fe^{2+} for the Fe^{2+}-binding site of the latter cell.

MATERIALS AND METHODS

Microorganisms. Four strains of *T. ferrooxidans* were used in this study. Strains SM-4 and SM-5 were isolated from water samples at the Flin Flon mine of the Hudson Bay Mining and Smelting Co. Ltd. (11). The two laboratory strains were ATCC 13661 and ATCC 19859.

Medium and culture conditions. The medium used for the growth of *T. ferrooxidans* (13) contained 0.4 g of $(NH_4)_2SO_4$, 0.1 g of K_2HPO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, and 33.3 g of $FeSO_4 \cdot 7H_2O$ per liter and was adjusted to pH 2.3 with H_2SO_4. The ferrous sulfate solution (33.3 g/100 ml) was filter sterilized separately and mixed with the autoclaved remaining medium components.

A culture was grown in six 1-liter Erlenmeyer flasks each containing 400 ml of medium (10% inoculum) at 25°C on a rotary shaker at 130 rpm. Cells were harvested towards the end of the log phase by centrifugation at 12,000 × *g* for 10 min after the removal of insoluble ferric iron by decantation followed by low-speed centrifugation (100 × *g* for 10 min). Cells were washed twice with 10 ml of pH 3.0 water-H_2SO_4 (ATCC 13661 and SM-5) or pH 2.3 medium without $FeSO_4 \cdot 7H_2O$ (ATCC 19859 and SM-4). Finally, a cell suspension was made to a concentration of 50 mg of wet cells per ml in pH 3.0 water or pH 2.3 medium (no $FeSO_4 \cdot 7H_2O$). For the preparation of cells with no Fe^{2+}-oxidizing activity 0.5 ml of SM-4 cell suspension was heated for 10 min at 80°C, and the inactivated dead cells were washed with 10 ml of pH 2.3 medium (no $FeSO_4 \cdot 7H_2O$) before resuspension in the original volume.

Ferrous iron-oxidizing activity. The rate of Fe^{2+} oxidation was determined by measuring the rate of oxygen (O_2) consumption in a Gilson oxygraph with a Clarke electrode at 25°C. The standard reaction mixture in a volume of 1.2 ml

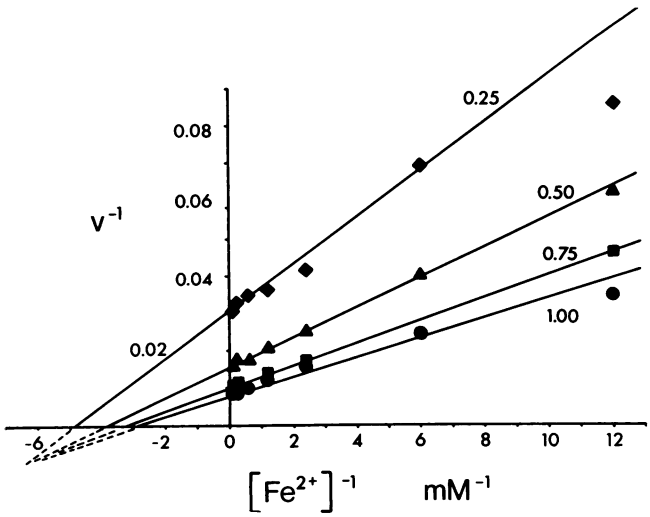

FIG. 1. Effect of Fe^{2+} concentration on the Fe^{2+}-oxidizing activity of various concentrations of SM-4 cells. The O_2 consumption rate (v) was determined as nanomoles of O_2 per min at 25°C as described in Materials and Methods at various Fe^{2+} concentrations. The amounts of cells used were 0.25 (◆), 0.50 (▲), 0.75 (■), and 1.00 (●) mg of wet cells in a total volume of 1.2 ml.

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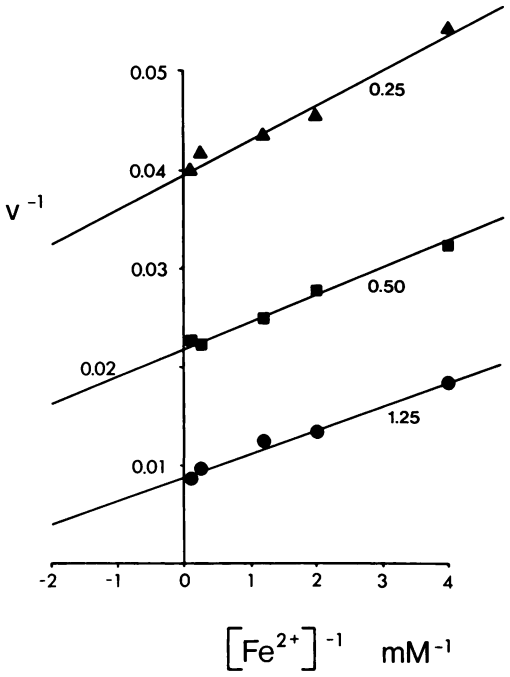

FIG. 2. Effect of Fe^{2+} concentration on the Fe^{2+}-oxidizing activity of various concentrations of SM-5 cells. Experimental conditions were the same as those described in the legend to Fig. 1, except for the amounts of cells (0.25 [▲], 0.50 [■], and 1.25 [●] mg).

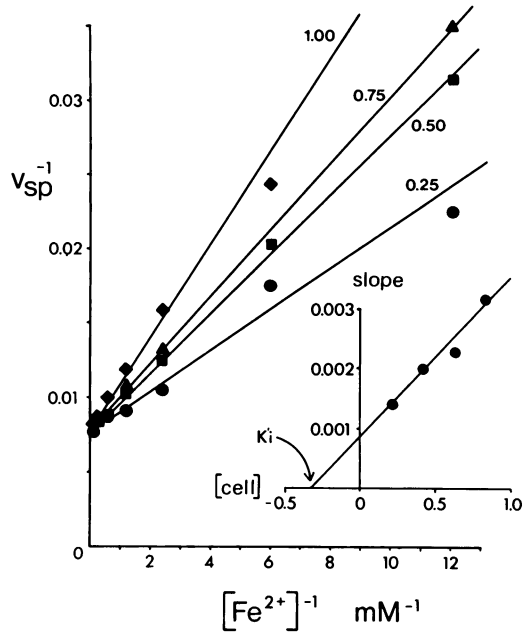

FIG. 3. Competitive inhibition of Fe^{2+}-oxidizing activity of SM-4 cells by increasing concentrations of cells. The specific activity or rate (v_{sp}) was calculated from the data in Fig. 1 as nanomoles of O_2 per minute per milligram of wet cells per milliliter. The insert is a replot of the slope to obtain the inhibition constant (K_i') value in milligrams of wet cells per milliliter.

contained pH 2.3 medium (no $FeSO_4 \cdot 7H_2O$), microliter volumes of cell suspension (50 mg of wet cells per ml), and 0.1 M $FeSO_4 \cdot 7H_2O$ at pH 2.3. The reaction was started with the addition of Fe^{2+} as the substrate, and the initial linear rate of O_2 consumption (nanomoles of O_2 per min) was taken as the reaction rate (v). The reaction rate was plotted against the concentration of Fe^{2+} in double-reciprocal Lineweaver-Burk plots (10).

RESULTS

The rate of Fe^{2+} oxidation (nanomoles of O_2 consumed per min) at a fixed concentration of cells increased with increasing concentrations of Fe^{2+} in all strains, following typical saturation kinetics, and the double-reciprocal rate-Fe^{2+} concentration plots were linear, as expected with standard Michaelis-Menten kinetics (10). When the Fe^{2+} oxidation rate was determined at various concentrations of cells, however, the results with mine isolates SM-4 and SM-5 were anomalous and different from those with laboratory strains ATCC 13661 and ATCC 19859.

The double-reciprocal rate-Fe^{2+} concentration plots at various SM-4 cell concentrations (Fig. 1) and SM-5 cell concentrations (Fig. 2) showed a family of lines which intersected on the x axis at different points, i.e., the K_m values for Fe^{2+} increased with increasing cell concentrations. If the reaction rate was converted to the specific activity or rate of Fe^{2+} oxidation (nanomoles of O_2 per minute per milligram of wet cells; v_{sp}) and plotted in a double-reciprocal manner against the Fe^{2+} concentration (Fig. 3 and 4), a family of lines intersected on the y axis at the same point characteristic of typical competitive inhibition. Thus, the Fe^{2+}-oxidizing activity of a unit cell (or enzyme system) was competitively inhibited by other cells with respect to Fe^{2+} concentrations.

Laboratory strains ATCC 13661 and ATCC 19859, on the other hand, showed no anomalous results in their double-

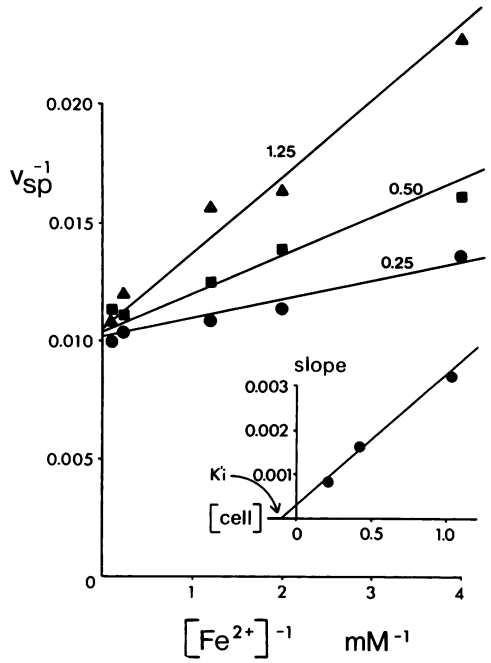

FIG. 4. Competitive inhibition of Fe^{2+}-oxidizing activity of SM-5 cells by increasing concentrations of cells. The data in Fig. 2 were used as described for Fig. 1 data in the legend to Fig. 3.

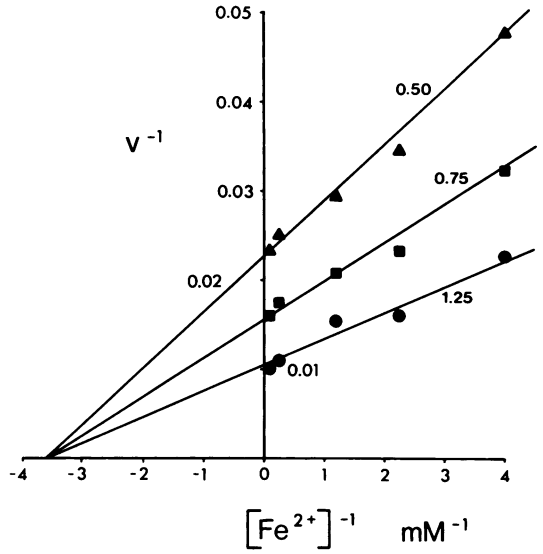

FIG. 5. Effect of Fe^{2+} concentration on the Fe^{2+}-oxidizing activity of various concentrations of ATCC 13661. Experimental conditions were the same as those described in the legend to Fig. 1, except for the amounts of cells.

reciprocal rate-Fe^{2+} concentration plots at various cell concentrations (Fig. 5 and 6). A family of lines converged on the x axis, and the K_m values were unchanged by various cell concentrations.

A more direct demonstration of a competitive inhibition by cells was attempted by carrying out the experiments in the presence of heat-inactivated (80°C for 10 min) SM-4 cells. These inactivated dead cells inhibited the Fe^{2+} oxidation of active SM-4 cells competitively with respect to Fe^{2+} concentrations (Fig. 7). The extent of inhibition was lower than that expected of the results from Fig. 3, probably because of the effect of heat treatment. SM-4 cells inactivated at 100°C for 5 min showed a high degree of aggregation, settling readily, and had little inhibitory action on the Fe^{2+} oxidation of active cells.

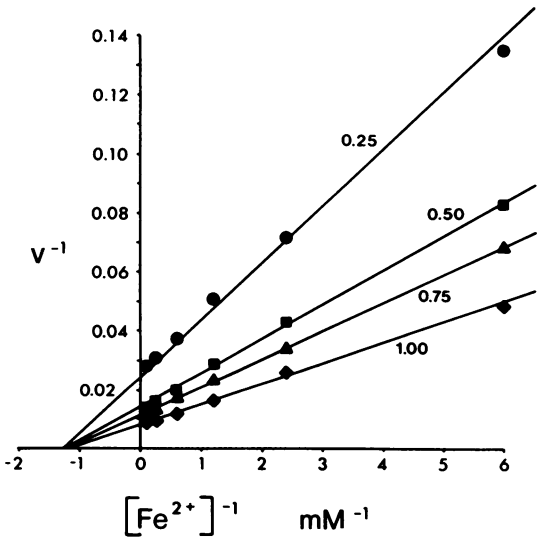

FIG. 6. Effect of Fe^{2+} concentration on the Fe^{2+}-oxidizing activity of various concentrations of ATCC 19859. Experimental conditions were the same as those described in the legend to Fig. 1.

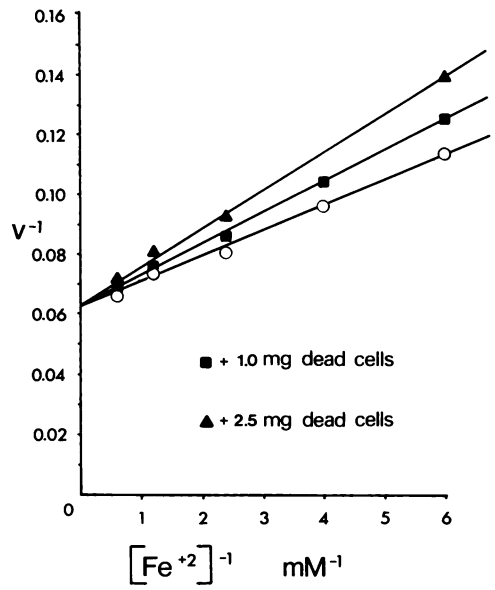

FIG. 7. Competitive inhibition of Fe^{2+}-oxidizing activity of SM-4 cells by heat-inactivated cells. Experimental conditions were the same as those described in the legend to Fig. 1, except that the amount of active SM-4 cells used was 0.25 mg. Where indicated, heat-inactivated (80°C for 10 min) dead cells were present at 1 or 2.5 mg in the reaction mixture. ○, No dead cells were present.

The experimental results may be explained as follows.

If each *T. ferrooxidans* cell has m number of an enzyme system (E) capable of oxidizing Fe^{2+} (S) to Fe^{3+} (P) with dioxygen ($E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P + O_2$ consumption), the rate of O_2 consumption (v) is expressed in a steady-state rate equation as follows:

$$v = \frac{k_3[E][S]}{[S] + K_m} = \frac{mk_3[C][S]}{[S] + K_m} \quad (1)$$

where K_m is the Michaelis constant, $(k_2 + k_3)/k_1$, and [E], [S], and [C] are concentrations of the enzyme system, Fe^{2+}, and cells, respectively.

If each cell also acts as a competitive inhibitor of another cell with respect to Fe^{2+} ($E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI$ instead of $E + S \rightleftarrows ES$) and $[I] = n[C]$, the rate equation becomes:

$$v = \frac{k_3[E][S]}{[S] + K_m\{1 + (I/K_i)\}} = \frac{mk_3[C][S]}{[S] + K_m\{1 + (n[C]/K_i)\}} \quad (2)$$

where K_i is the inhibition constant, k_5/k_4.

If $mk_3 = k_3'$ and $K_i/n = K_i'$, then the equation becomes:

$$v = \frac{k_3'[C][S]}{[S] + K_m\{1 + ([C]/K_i')\}} \quad (3)$$

In the double-reciprocal form the equation becomes:

$$\frac{1}{v} = \frac{1}{k_3'[C]} + \frac{K_m}{k_3'[C]}\{1 + ([C]/K_i')\}\left(\frac{1}{[S]}\right) \quad (4)$$

Thus, plots of $1/v$ versus $1/[S]$ at different cell concentrations should show a family of lines with intercepts on the y axis at $1/(k_3'[C])$ ($k_3'[C]$ = the maximal reaction rate, V_{max}, at the cell concentration [C]) and on the x axis at $-1/\left(K_m\{1 + ([C]/K_i')\}\right)$ (Fig. 1 and 2).

TABLE 1. Rate and kinetic constants for Fe^{2+} oxidation

Strain	k_3' (nmol of O_2/min per mg of wet cells)	K_m for Fe^{2+} (mM)	K_i' (mg of wet cells/ml)
ATCC 13661	88	0.28	
ATCC 19859	143	0.80	
SM-4	125	0.11	0.33
SM-5	100	0.30	0.10

If the observed reaction rate, v, is converted to the specific activity or rate, v_{sp}, by dividing v by the cell concentration, [C], the following equation is obtained:

$$v_{sp} = \frac{k_3'[S]}{[S] + K_m\{1 + ([C]/K_i')\}} \quad (5)$$

In the double-reciprocal form the equation becomes:

$$\frac{1}{v_{sp}} = \frac{1}{k_3'} + \frac{K_m}{k_3'}\{1 + ([C]/k_i')\}\left(\frac{1}{[S]}\right) \quad (6)$$

Now the plots of $1/v_{sp}$ versus 1/[S] at different cell concentrations should show a family of lines converging on the y axis at $1/k_3'$ with changing slopes corresponding to $(K_m/k_3')\{1 + ([C]/K_i')\}$ (Fig. 3 and 4). The slope replots against [C] should intersect on the y axis at K_m/k_3' and on the x axis at $-K_i'$ (Fig. 3 and 4).

The rate and kinetic constants obtained from these results are shown in Table 1. The rate constant, k_3', was relatively constant among the four strains of *T. ferrooxidans*. The K_m values for Fe^{2+} for the ATCC strains were not affected by cell concentrations, while the K_m values for SM-4 and SM-5 were the minimum values which increased with increasing cell concentrations. The K_i' values were the concentrations of cells which doubled the experimental K_m values; thus, SM-5 cells with a smaller K_i' value could compete better than could SM-4 cells with Fe^{2+} for the Fe^{2+}-binding sites.

DISCUSSION

The results obtained in this work agree with the idea of competitive inhibition of *T. ferrooxidans* Fe^{2+} oxidation by other *T. ferrooxidans* cells which compete with Fe^{2+} for the Fe^{2+}-binding sites of the Fe^{2+}-oxidizing cells. Mine isolates SM-4 and SM-5 showed this anomalous kinetic property when cell concentrations were varied; SM-5, with a lower K_i' value, showed a more dramatic effect than did SM-4 (Fig. 1 and 2). Laboratory strains ATCC 13661 and ATCC 19859 maintained the same K_m values for Fe^{2+} at different cell concentrations (Fig. 5 and 6); therefore, they did not inhibit the binding of Fe^{2+} to the Fe^{2+}-oxidizing sites, at least at the cell concentrations used in this study.

The apparent K_m values for Fe^{2+} reported by various workers for *T. ferrooxidans* vary from 0.43 to 9.4 mM (9). This variation may have resulted from the use of different cell concentrations, although differences in strains, culture conditions, and assay conditions may have been responsible for the variation.

The reason for the competitive inhibition observed is not clear but may lie in the unusual surface properties of *T. ferrooxidans* cells. The cells are believed to be covered with a coat of Fe^{3+} complex on the surface (7, 8). *T. ferrooxidans* cells are known to adsorb to solid surfaces, and the adsorption is found to be inhibitory to the Fe^{2+}-oxidizing activity of cells (16). It is possible that the Fe^{2+}-oxidizing activity of *T. ferrooxidans* cells is inhibited by their contact with other cells. The inhibitory component could be the Fe^{3+} coat on the surfaces of the cells. Soluble ferric iron competitively inhibits the ferrous iron oxidation of *T. ferrooxidans* (9). The observed competitive inhibition by heat-inactivated cells is in agreement with the postulate. Heat-killed cells of *T. ferrooxidans* have been reported to adsorb to solid surfaces (16). The exact mechanism of inhibition, however, requires further investigation.

A different response by laboratory strains versus that of mine isolates may indicate a difference in their cell surface properties. Laboratory strains have been maintained in a medium with high concentrations of soluble ferrous iron for many years, while mine isolates have been living in contact with mineral surfaces such as pyrite (FeS_2) and chalcopyrite ($CuFeS_2$) until recently (11). The former may have lost some adsorption properties, requiring higher concentrations of cells for inhibition than those used in our experiments. In fact, the harvesting of cells was easier with laboratory strains because of their ready dissociation from the ferric iron precipitate. The cells of mine isolates, on the other hand, required more extensive washing of the precipitate for dissociation and sometimes (not in this study) washing with ferrous sulfate at 0.1 M. It is interesting that the laboratory strains could not grow on the sulfide ore containing pyrite and chalcopyrite, while SM-4 and SM-5 could (11). Perhaps the anomalous result observed with these mine isolates may be related to their ability to attack sulfide minerals.

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