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

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Oxidation of ferrous iron by *Thiobacillus ferrooxidans* SM-4 was inhibited competitively by increasing concentrations of ferric iron or cells. A kinetic analysis showed that binding of one inhibitor did not exclude binding of the other and led to synergistic inhibition by the two inhibitors. Binding of one inhibitor, however, was affected by the other inhibitor, and the apparent inhibition constant increased with increasing concentrations of the other inhibitor.

*Thiobacillus ferrooxidans* oxidizes ferrous iron to ferric iron with atmospheric oxygen: $4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$, an important reaction in the bacterial leaching of metals from sulfide ores (5, 10). The product of oxidation, Fe$^{3+}$, inhibits Fe$^{2+}$ oxidation competitively (1). Our recent work has demonstrated that Fe$^{2+}$ oxidation by some *T. ferrooxidans* strains is also competitively inhibited by increasing concentrations of cells (8).

We have now studied the effect of increasing cell concentrations on competitive inhibition of Fe$^{2+}$ oxidation by Fe$^{3+}$. The results agree with the concept that both Fe$^{3+}$ and cells compete with Fe$^{2+}$ for the Fe$^{2+}$-binding site of Fe$^{2+}$-oxidizing cells, but binding of one inhibitor does not exclude binding of the other, although the apparent inhibition constant for the latter increases with increasing concentrations of the former.

**MATERIALS AND METHODS**

**Microorganism.** The *T. ferrooxidans* strain used in this study, SM-4, was isolated from a sulfide ore mine site (4) and grown in a medium with FeSO$_4$·7H$_2$O (33.3 g/liter) as described previously (4, 6).

**Ferrous-iron-oxidizing activity.** The rate of Fe$^{2+}$ oxidation was determined by measuring the rate of O$_2$ consumption at 25°C in a Gilson oxygraph with a Clarke electrode. The reaction was performed in growth medium containing the following minus FeSO$_4$·7H$_2$O: 0.4 g of (NH$_4$)$_2$SO$_4$, 0.1 g of K$_2$HPO$_4$, and 0.1 g of MgSO$_4$ per liter (adjusted to pH 2.3 with H$_2$SO$_4$). A total volume of 1.2 ml contained microliter volumes of a cell suspension (50 mg of wet cells per ml), 50 mM Fe$_2$(SO$_4$)$_3$, and 0.1 M FeSO$_4$·7H$_2$O at pH 2.3. The reaction was started by addition of Fe$^{2+}$ as the substrate, and the initial linear rate of O$_2$ consumption (nanomoles of O$_2$ per minute) was taken as the reaction rate. The reaction rate was plotted against the concentration of Fe$^{2+}$ in double-reciprocal Lineweaver-Burk plots (3).

**RESULTS**

Oxidation of Fe$^{2+}$ by *T. ferrooxidans* SM-4 cells was competitively inhibited by Fe$^{3+}$ at three fixed concentrations of cells (0.25 [A]; 0.50 [B], and 1.00 [C] mg of cells) as shown in the double-reciprocal reaction rate -Fe$^{2+}$ concentration plots of Fig. 1. The replot of the slope against the Fe$^{3+}$ concentration (Fig. 2) indicated that increasing cell concentrations lowered both the slopes and y intercepts but increased the values of apparent inhibition constants for Fe$^{3+}$ (the positive value of x intercepts). The secondary replot of the slope change from Fig. 2 showed that the slope increased with the reciprocal of the cell concentrations (Fig. 2, inset).

When the data were plotted at fixed Fe$^{3+}$ concentrations (0 [A], 2 [B], 8 [C], and 16 [D] mM Fe$^{3+}$), the patterns in Fig. 3 were obtained in which increasing cell concentrations lowered both the y intercepts and slopes and characteristically increased the apparent $K_p$ values (the positive value of the reciprocal of the x intercepts), similarly to our previous results (8).

When the reaction rate, $v$ (nanomoles of O$_2$ per minute), was converted to the specific activity or rate $v_s$ (divided by the concentration of cells), and plotted in a double-reciprocal manner against the Fe$^{2+}$ concentration (8), the resulting plots (Fig. 4) showed competitive inhibition by increasing concentrations of cells at four fixed Fe$^{3+}$ concentrations of 0 to 16 mM (A to D). The replot of the slope against the cell concentrations (Fig. 5) indicated an increase in the apparent inhibition constants for cells in Fe$^{3+}$ and stronger inhibition by cells at higher Fe$^{3+}$ concentrations (Fig. 5, insets).

These results can be explained if Fe$^{3+}$ and cells both compete with Fe$^{2+}$ for the Fe$^{2+}$ oxidation sites of cells and binding of one inhibitor (Fe$^{3+}$ or cells) does not exclude binding of the other (cells or Fe$^{3+}$) but only inhibits it. Segel (7) discussed the inhibition of an enzyme (E) by two competitive inhibitors ($I$ and $X$) with respect to the substrate ($S$) in the formation of a product ($P$):

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_3} E + P, E + I \xrightarrow{k_4} EI, E + X \xrightarrow{k_5} EX,$$

$$\frac{k_6}{k_2} \frac{k_7}{k_8} \frac{k_9}{k_{10}} \frac{k_{11}}{k_4}$$

$EI + X \xrightarrow{k_{12}} EIX$, and $EX + I \xrightarrow{k_{13}} EIX$

The formation of $EIX$ distinguishes this synergistic system from inhibition by mutually exclusive inhibitors.

The rate equation derived by Segel (7) is rearranged as:
The O₂ consumption rate (v) was determined as nanomoles of O₂ per minute at 25°C, as described in Materials and Methods, at various Fe³⁺ concentrations. The concentrations of Fe³⁺ were 0, 2, 8, and 16 mM, as shown. The amounts of cells used were 0.25, 0.50, and 1.00 mg of wet cells in a total volume of 1.2 ml in A, B, and C, respectively.

\[
v = \frac{k_3[E][S]}{[S] + K_s(1 + \frac{[I]}{K_I} + \frac{[X]}{K_x} + \frac{([I][X])}{(\alpha K_s K_x)})}
\]

where \(v\) is the reaction rate; \([E]\), \([S]\), \([I]\), and \([X]\) are concentrations of \(E\), \(S\), \(I\) and \(X\); and \(K_s\) and \(K_I\) are inhibition constants for \(I\) and \(X\), respectively. \(K_s\) is the dissociation constant for \(S\). \(k_s/k_{k_4}\) and \(k_s/k_{k_6}\) are the Michaelis constant when \(k_{3s}k_3\). When binding of one inhibitor does not affect binding of the other, \(\alpha = 1\). \(K_I = k_3/k_4 \approx k_1/k_{10}\), and \(K_s = k_s/k_{k_6} \approx k_s/k_{k_6}\).

We have previously derived a rate equation for competitive inhibition of \(T. ferrooxidans\) Fe³⁺ oxidation by increasing \(T. ferrooxidans\) cell concentrations (8):

\[
v = \frac{k_3'[C][Fe^{2+}]}{K_m[1 + ([C]/[K_s'] + ([C][Fe^{2+}])/([C][Fe^{3+}])(\alpha k_4/K_p)]}
\]

where \([C]\) is the concentration of cells, \(k_3'\) is \(k_3\) times the number of enzyme per cell, \([Fe^{3+}]\) the concentration of \(Fe^{3+}\), and \(K_s'\) is \(K_s\) divided by the number of inhibitor per cell.

With \(Fe^{3+}\) as the second competitive inhibitor, the rate equation becomes:

\[
v = \frac{k_3'[C][Fe^{2+}]}{[Fe^{2+}] + K_m(1 + ([C]/[K_s'] + ([C][Fe^{2+}])/([C][Fe^{3+}])(\alpha k_4/K_p))}
\]

where \(K_m\) is the inhibition constant for \(Fe^{3+}\) and \([Fe^{3+}]\) is the concentration of \(Fe^{3+}\).

In double-reciprocal form, the equation becomes:

\[
v^{-1} = \frac{v^{-1}}{[Fe^{2+}]^{-1}}
\]

FIG. 3. Effect of Fe²⁺ concentration on the Fe²⁺-oxidizing activity of various concentrations of cells at fixed concentrations of Fe³⁺. The data were the same as in Fig. 1. Three lines in each graph represent the data at 0.25, 0.50, and 1.00 mg of wet cells in a total volume of 1.2 ml. Four graphs (A through D, respectively) represent the data obtained with 0, 2, 8, and 16 mM Fe³⁺.

FIG. 4. Competitive inhibition of Fe²⁺-oxidizing activity of cells by increasing concentrations of cells. The specific activity or rate (v⁻¹) was calculated on the basis of the data in Fig. 3 as nanomoles of O₂ per minute per milligram of wet cells per milliliter. The other conditions were as described in the legend to Fig. 3.
Thus, plots of \( 1/v \) versus \( 1/[\text{Fe}^{3+}] \) at a fixed \([C]\) and different values of \([\text{Fe}^{3+}]\) should intersect on the y axis at \( 1/(k_3'[C]) \) with slopes increasing with \([\text{Fe}^{3+}]\), which is typical of competitive inhibition patterns (Fig. 1). The slope replots against \([\text{Fe}^{3+}]\) (Fig. 2) should intersect the y axis at \( (K_m'[k_3'[C]]) + K_m'(k_3'[C]) \) and the x axis at \( -K_g'/(1 + [C]/K_i') \) and have slopes equal to \( (K_m'[k_3'[C]]/K_i' + K_m'(k_3'[C]/K_i')) \). The secondary replots of the slopes against \([C]\) (Fig. 2, inset) should have a slope of \( K_m'(k_3'(\alpha K_i'/K_g')) \) and an intercept on the y axis at \( K_m'(k_3'(\alpha K_i'/K_g')) \) and on the x axis at \( -1/(\alpha K_i') \).

The plots at a fixed \([\text{Fe}^{3+}]\) and various values of \([C]\) (Fig. 3) produced patterns different from Fig. 1, and the y intercept and slope both decreased with increasing \([C]\) because of the \([1/C]\) term in equation 4. In our previous paper (8), we used the specific activity or rate, \( v_{sp} \), by dividing \( v \) by the cell concentration, \([C]\), to demonstrate competitive inhibition. Equation 4 becomes:

\[
1/v = 1/k_3'[C] + K_m'/k_3'[C] \{1 + [C]/K_i' + [\text{Fe}^{3+}]K_{if}' \}
\]

Thus, plots of \( 1/v \) versus \( 1/[\text{Fe}^{3+}] \) should intersect on the y axis at \( 1/(k_3'[C]) \) with slopes increasing with \([\text{Fe}^{3+}]\), which is typical of competitive inhibition patterns (Fig. 1).

The slope replots against \([\text{Fe}^{3+}]\) (Fig. 2) should intersect the y axis at \( (K_m'[k_3'[C]]) + K_m'(k_3'[C]) \) and the x axis at \( -K_g'/(1 + [C]/K_i') \) and have slopes equal to \( (K_m'[k_3'[C]]/K_i' + K_m'(k_3'[C]/K_i')) \). The secondary replots of the slopes against \([C]\) (Fig. 2, inset) should have a slope of \( K_m'(k_3'(\alpha K_i'/K_g')) \) and an intercept on the y axis at \( K_m'(k_3'(\alpha K_i'/K_g')) \) and on the x axis at \( -1/(\alpha K_i') \).

The rate and kinetic constants obtained from the results are shown in Table 1. An \( \alpha \) value larger than 1 indicates that binding of one inhibitor decreases the binding constant of the other inhibitor (7), i.e., increases the apparent inhibition constant. This is evident in Fig. 2 and 5, in which the apparent \( K_i' \) and \( K_g' \) (positive values of \( x \) intercepts) increased with increasing concentrations of the other inhibitor.

Fig. 6 and 7 are Dixon plots as described by Segel (7) drawn to confirm our interpretation of the results. The reciprocal of \( v_{sp} \), at an \([\text{Fe}^{3+}]\) of 0.42 mM was plotted against \([\text{Fe}^{3+}]\) at three different \([C]\) values (Fig. 6). The changing slope indicates that the two inhibitors are not exclusive of each other in the inhibition. The lines intersect at the negative \( x \) coordinate corresponding to \( K_g' \), i.e., a 3.2 mM \([\text{Fe}^{3+}]\). Similar plots against \([C]\) at four different \([\text{Fe}^{3+}]\) concentrations of 0, 2, 8, or 16 mM, as shown.

**TABLE 1.** Rate and kinetic constants

<table>
<thead>
<tr>
<th>Constant</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>( k_3' )</td>
<td>200 nmol of O(_2) per min per mg of wet cells</td>
</tr>
<tr>
<td>( K_m' )</td>
<td>70 ( \mu )M ([\text{Fe}^{3+}])</td>
</tr>
<tr>
<td>( K_i' )</td>
<td>135 ( \mu )g of wet cells per ml</td>
</tr>
<tr>
<td>( K_{if}' )</td>
<td>640 ( \mu )M ([\text{Fe}^{3+}])</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>5</td>
</tr>
</tbody>
</table>

**FIG. 5.** Replot of slopes from Fig. 4 against the concentration of cells. The concentrations of \([\text{Fe}^{3+}]\) used for the lines were 0, 2, 8, and 16 mM, as shown. The inset is the secondary replot of the slope and intercept against the concentration of \([\text{Fe}^{3+}]\).

**FIG. 6.** Plot of the reciprocal of \( v_{sp} \) against the concentration of \([\text{Fe}^{3+}]\). The data were obtained from Fig. 4 at a fixed \([\text{Fe}^{2+}]\) concentration of 0.42 mM. Each line represents values at a cell concentration of 0.21, 0.43, or 0.83 mg of wet cells per milliliter, as shown.

**FIG. 7.** The plot of the reciprocal of \( v_{sp} \) against the concentration of cells. The data were obtained from Fig. 4 at a fixed \([\text{Fe}^{2+}]\) concentration of 0.42 mM. Each line represents values at an \([\text{Fe}^{3+}]\) concentration of 0, 2, 8, or 16 mM, as shown.
values produced Fig. 7. Four lines of different slopes intersected at the negative x coordinate \( \alpha K' \), i.e., \( 0.66 \text{ mg of wet cells per ml} \). These values are very close to the values expected on the basis of those in Table 1.

When the specific activity or rate \( (v_s) \) was used, inhibition by either \( \text{Fe}^{2+} \) or cells was greater when the other inhibitor concentration, \([C]\) or \([\text{Fe}^{3+}]\), was higher; i.e., there was a synergistic inhibition effect (Fig. 6 and 7).

**DISCUSSION**

There are complex interactions during \( \text{Fe}^{2+} \) oxidation by *T. ferrooxidans* among the cells as the \( \text{Fe}^{2+} \)-oxidizing catalyst, \( \text{Fe}^{3+} \) as the substrate, \( \text{Fe}^{2+} \) as the product of oxidation and a competitive inhibitor of \( \text{Fe}^{3+} \) oxidation, and also the cells as a competitive inhibitor of \( \text{Fe}^{3+} \) oxidation. These interactions should affect the rate of bacterial leaching of sulfide minerals in addition to other factors, such as the air supply, nutrients, temperature, pH, and toxic metals.

The results of this study indicate that, with *T. ferrooxidans* SM-4, both cells and \( \text{Fe}^{3+} \) act as competitive inhibitors of \( \text{Fe}^{3+} \) in its oxidation by cells and that inhibition is not mutually exclusive; i.e., both inhibitors can bind the \( \text{Fe}^{3+} \)-oxidizing system simultaneously, resulting in stronger inhibition than by each separately because of the formation of an additional inactive form \( (\text{Fe}^{3+}) \). Binding of the second inhibitor, however, is inhibited by the first inhibitor, since \( \alpha \) is larger than 1. Therefore, the apparent inhibition constants increase with the increasing concentration of the other inhibitor.

When *T. ferrooxidans* cells grow in a \( \text{Fe}^{2+} \)-containing medium, the rate of \( \text{Fe}^{2+} \) oxidation is initially governed by the Michaelis-Menten equation \( v = k_s' [C]/(\text{Fe}^{2+}) + K_m \), but when the concentrations of cells and \( \text{Fe}^{3+} \) increase, the apparent \( K_m \) values increase because of competitive inhibition by cells and \( \text{Fe}^{3+} \). The high concentration of \( \text{Fe}^{3+} \) used in normal growth medium is essential to overcome this effect. Precipitation of insoluble \( \text{Fe}^{5+} \) (5, 10) reduces the concentration of \( \text{Fe}^{3+} \) and therefore its inhibitory effect.

The rate of \( \text{Fe}^{2+} \) oxidation in a culture growing in \( \text{Fe}^{2+} \) increases when the cell concentration increases, but the specific activity \( (\text{Fe}^{2+} \) oxidation rate per milligram of cells) decreases because of competitive inhibition by cells when the \( \text{Fe}^{2+} \) concentration is not saturating. In our culture of SM-4 in \( \text{Fe}^{2+} \)-containing medium, the cell concentration reached 20 to 50 \( \mu \text{g of wet cells per ml} \), which is below the \( K_i \) value of 135 \( \mu \text{g of wet cells per ml} \); therefore, the inhibition was not very significant. The inhibition becomes more significant in experiments in which concentrated cell suspensions are used at low \( \text{Fe}^{3+} \) concentrations, as in this work. Since only *T. ferrooxidans* strains recently isolated demonstrated this anomalous property (8), it is possibly related to their mode of growth on the surface of sulfide ores.

We have studied the effect of cell concentration on pyrite oxidation, and the results will be reported later.

The \( K_m \), \( K_{ip} \), and \( K_{ip}' \) values shown in Table 1 are minimum values and are considerably lower than those previously reported for apparent \( K_m \) values of 0.33 to 9.4 \( \mu \text{M} \) or higher \( \text{Fe}^{2+} \) (1, 9), apparent \( K_{ip} \) values of 1.2 to 28 \( \mu \text{M} \) \( \text{Fe}^{3+} \) (1, 9), and a \( K_{ip}' \) value for the SM-4 strain of 0.33 \( \mu \text{M of wet cells per ml} \) (8). The last value was perhaps higher because of the lower activity of the cells \( (k'_{ip}, 125 \text{ instead of 200 nmol of O}_2 \text{ per min per mg of wet cells}) \) or simply because different batches of cells were used. The relatively low \( K_{ip}' \) value raises the possibility that the observed inhibition by cells was caused by \( \text{Fe}^{3+} \) introduced with the cell suspension. The possibility of inhibition by free \( \text{Fe}^{3+} \) was eliminated by calculating the effect of low concentrations of \( \text{Fe}^{3+} \) found in the cell suspension. This does not exclude the possible cause of inhibition suggested in our previous paper (8), i.e., that the special \( \text{Fe}^{3+} \) coat on the surface of the cells makes contact with \( \text{Fe}^{2+} \)-oxidizing cells. Inhibition by \( \text{Fe}^{3+} \) formed from \( \text{Fe}^{2+} \) was also considered negligible, since the oxidation rate was determined as the initial linear reaction rate before accumulation of any significant concentration of \( \text{Fe}^{3+} \).

It is interesting that \( \text{Fe}^{3+} \) inhibition of \( \text{Fe}^{2+} \) oxidation by *T. ferrooxidans* was found to be less pronounced at lower temperatures (2). The apparent \( K_{ip}' \) value dramatically increased (15-fold) when the temperature was lowered from 27 to 5°C. Perhaps secondary binding of inhibitors to form \( \text{EIX} \) is more difficult at lower temperatures (higher \( \alpha \) values), although it is possible that \( K_{ip}' \) itself is affected by temperature.

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**LITERATURE CITED**


