The Inhibition of Ferroxidase by Trivalent and Other Metal Ions*

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SUMMARY

The inhibitory effects of trivalent and other metal ions on ferroxidase (ceruloplasmin EC 1.12.3.1.) activity were investigated. All trivalent cations tested inhibited ferroxidase activity but the strongest inhibitors had an ionic radius of 0.81 Å or less. The inhibition by Al(III) was found to be mixed competitive and uncompetitive with respect to the substrate, Fe(II). The uncompetitive feature of the inhibition was not due to competition by Al(III) with the other substrate, oxygen. A mechanism for the mixed inhibition by Al(III) is proposed consistent with these results. A comparison of the strong cationic inhibitors provides the following metal ion series in order of decreasing effectiveness of inhibition: In(III) > ZrO(II) > Al(III) > Sc(III) > Ga(III)

Al(III) and In(III) were reported by Curzon (1) partially to inhibit the activity of ferroxidase (ceruloplasmin, EC 1.12.3.1.) with N,N-dimethyl-p-phenylenediamine as the substrate. The role of Fe(II) as a substrate and an activator for ferroxidase (2) has raised the question concerning the effects of other metal ions on ferroxidase activity. In the preceding paper (3), divalent metal ions were reported to activate ferroxidase at low concentrations and inhibit at higher concentrations. Preliminary results indicated that inhibition of ferroxidase by Al(III), In(III), and other polyvalent cations was stronger than expected from earlier reports (1, 2). In view of the availability of a highly sensitive spectrophotometric assay for ferroxidase activity, and the proposed biological role for this protein in iron metabolism (4, 5), the effects of trivalent and other metal ions on ferroxidase activity were investigated.

* This investigation was supported in part by HE 08344 from the National Heart Institute. Partial support of C. T. Huber by Biochemistry Training Grant GM-1087 is also gratefully acknowledged. The work is taken in part from the dissertation submitted by C. T. Huber to Florida State University in January 1970 toward the degree of Doctor of Philosophy. It is Paper 35 in a series from this laboratory on copper biosystems.
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EXPERIMENTAL PROCEDURE

Ferroxidase—Ferroxidase was prepared by the procedure of Deutsch, Kasper, and Walsh (6), modified as previously described (3). Stock solutions of ferroxidase were prepared in 0.20 M Chelex-treated sodium acetate buffer, pH 6.0. The As 3979-A 610 ratio of the crystalline human ferroxidase was 22.0 ± 0.3 and the concentration was estimated from the absorption at 610 nm by εm = 9000 (3).

Reagents—Reagents used were of reagent grade purity except for ascorbate (USP) and ZrO(NO₃)₂-2H₂O (chemically pure).

Kinetic Measurements—The spectrophotometric assay for ferroxidase activity was described in the preceding paper (3). The metal ions were incubated for 100 sec with ferroxidase before addition of ascorbate and Fe(II) unless otherwise noted. Oxygen measurements and rates of uptake were obtained with a Beckman oxygen sensor mounted in a thermostated closed chamber. The solution was rapidly stirred by a magnetic stirrer.

Metal Ion Solutions—The stock solutions were made up in dilute H₂SO₄ (pH 1.4) to prevent hydrolysis to complex polynuclear species (7, 8). No pH change was observed upon addition of a small amount of these solutions to the reaction mixture. The hydrolysis of metal ions to polynuclear species in the reaction mixture is assumed negligible in the short times and low concentrations used. The independence of the degree of inhibition from the incubation time (100 to 600 sec) supports this assumption. The one exception, Sc(III), shows increasing inhibition up to about 300 sec and then remains unchanged up to 600 sec.

Ultracentrifuge Measurements—Sedimentation velocity measurements were performed in a Spinco model E analytical ultracentrifuge.

RESULTS

The effects of various cations on ferroxidase activity are listed in Table I, together with the corresponding ionic radii. The oxidation-reduction potential of the Fe(II)-Fe(III) couple permits the transfer of electrons from ascorbate to ferroxidase (2), but the potentials of ionic couples for the other metal ions in Table I are not in the proper range (with the exception of the potential for the VO(II), 2H⁺/V(III), H₂O half-cell reaction).

As Table I indicates, several metal ions are potent inhibitors of ferroxidase activity. All of the trivalent cations tested displayed some inhibition on short exposure to ferroxidase except Cr(III)
### Table I

**Inhibitory effect of metal ions on ferroxidase activity**

The final concentration of the reactants was: 0.083 M Chelex-treated sodium acetate buffer (pH 6.0), 2.0 μM Fe(II), 100 μM Chelex-treated ascorbate, 0.120 μM Chelex-treated ferroxidase, and 100 μM of each metal ion (none in the control except for 2.0 μM Fe(II)). The time of incubation of metal ion with ferroxidase before addition of ascorbate and Fe(II) was 100 sec except where noted. The various anions had no effect at the concentrations used. The values in the table have been corrected for the non-enzymic rates.

<table>
<thead>
<tr>
<th>Cation (100 μM)</th>
<th>Activity % of control</th>
<th>Ionic radius*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc(III)</td>
<td>10</td>
<td>0.81</td>
</tr>
<tr>
<td>Y(III)</td>
<td>45</td>
<td>0.92</td>
</tr>
<tr>
<td>La(III)</td>
<td>61</td>
<td>1.14</td>
</tr>
<tr>
<td>Ce(III)</td>
<td>65</td>
<td>1.07</td>
</tr>
<tr>
<td>ZrO(II)</td>
<td>2</td>
<td>1.50</td>
</tr>
<tr>
<td>VO(II)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cr(III)</td>
<td>102</td>
<td>0.63</td>
</tr>
<tr>
<td>Rh(III)</td>
<td>98</td>
<td>0.68</td>
</tr>
<tr>
<td>Al(III)</td>
<td>5</td>
<td>0.51</td>
</tr>
<tr>
<td>Ga(III)</td>
<td>22</td>
<td>0.62</td>
</tr>
<tr>
<td>In(III)</td>
<td>5</td>
<td>0.81</td>
</tr>
</tbody>
</table>

* Values from Reference 9.

### Table II

**Reversibility of metal ion inhibitors of ferroxidase**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Plot of V against [E]</th>
<th>Reversibility upon dilution</th>
<th>Reversibility with Sephadex G-25</th>
<th>Reversibility upon treatment with Chelex-100 after Sephadex G-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al(III)</td>
<td>Linear through the origin</td>
<td>+</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>Ga(III)</td>
<td>Nonlinear</td>
<td>+</td>
<td>73</td>
<td>100</td>
</tr>
<tr>
<td>In(III)</td>
<td>Linear through the origin</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Se(III)</td>
<td>Linear through the origin</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZrO(II)</td>
<td>Nonlinear</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Plot of velocity against enzyme concentration with AlCl₃ as the inhibitor of ferroxidase activity. The final concentration in the reaction mixture was: 0.083 M Chelex-treated acetate buffer (pH 6.0) and 100 μM Chelex-treated ascorbate. The ferroxidase was Chelex-treated and the incubation time of the enzyme at 30° with AlCl₃ was 100 sec.
The final concentration of reductants was 0.088 mM Chelex-treated sodium acetate buffer (pH 6.0), 100 μM Chelex-treated ascorbate, and 2.10 μM Chelex-treated ferroxidase. The data in Table III indicate that Al(III) can compete with Fe(II) for the site of reduction. The competitive part of the mixed inhibition is probably due to competition with Fe(II) in both its role as a substrate and as an activator.

The competitive component of the mixed inhibition of Al(III) with Fe(II) may involve the site of reduction, the site of activation by Fe(II) (3), or both sites on the enzyme. If Al(III) can compete with Fe(II) for the site of reduction, the steady state concentration of cycling ferroxidase in the oxidized form should increase. The data in Table III indicate that Al(III) can compete with Fe(II) for the site of reduction. The competitive part of the mixed inhibition is probably due to competition with Fe(II) in both its role as a substrate and as an activator.

The over-all scheme of mixed inhibition by Al(III) is shown in Fig. 4. The competition by Al(III) with Fe(II) can occur at the enzyme reduction site (or sites) and this is represented in the top line (E_{el} through E_{el}). If there is only one reduction site, inhibited forms E_{el} through E_{el} are the same species. Competitive inhibition may also occur at the site of activation by Fe(II), giving rise to enzyme form E_{el}. Since there is no competition by Al(III) with respect to O₂, the simplest scheme for the competitive part of the inhibition is illustrated in Fig. 4 with the inhibitor binding to both forms E_{el} and E_{el} with the same association constant K. The inhibited complexes E_{el} and E_{el} can then react with a second Al(III) with the identical association constant K. For the competitive part of the inhibition the scheme in Fig. 4 generates the following expression:

$$\frac{1}{v_{max}} = \frac{1}{240} k_{XIII}$$

in which k_{XIII} is the rate constant for the rate-limiting step for the activated reaction in the absence of inhibitors. Kᵣ and Kᵦ are equilibrium constants for the association of the two Al(III) cations with the enzyme. K_{XV} is the equilibrium constant for the interconversion of E_{XIV} and the enzyme form in the activated pathway with which Al(III) binds, to E_{XIV} and k_{XV} is the corresponding rate constant for that reaction. This expression produces the curve drawn through the extrapolated points in Fig. 3b and fits these data closely.

Table III

<table>
<thead>
<tr>
<th>Al(III)</th>
<th>Fe(II)</th>
<th>ΔXIV loss upon addition of Fe(II)</th>
<th>Inhibition of rate of oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 μM</td>
<td>0 μM</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>2.0 μM</td>
<td>200 μM</td>
<td>38%</td>
<td>92.5%</td>
</tr>
<tr>
<td>100 μM</td>
<td>0 μM</td>
<td>67%</td>
<td>92%</td>
</tr>
<tr>
<td>100 μM</td>
<td>200 μM</td>
<td>67%</td>
<td>92%</td>
</tr>
</tbody>
</table>

* This value should be termed per cent of apparent inhibition since in the uninhibited system the ascorbate concentration is limiting (9).

88 mM Chelex-treated sodium acetate buffer at pH 6.0, 100 μM Chelex treated ascorbate, 100 μM Fe(II), and 0.120 μM Chelex-treated ferroxidase at 30°. The degree of inhibition by 2.0 μM Al(III) did not vary with the partial pressure of O₂ in the reaction mixture. The competitive component of the mixed inhibition of Al(III) with Fe(II) may involve the site of reduction, the site of activation by Fe(II) (3), or both sites on the enzyme. If Al(III) can compete with Fe(II) for the site of reduction, the steady state concentration of cycling ferroxidase in the oxidized form should increase. The data in Table III indicate that Al(III) can compete with Fe(II) for the site of reduction. The competitive part of the mixed inhibition is probably due to competition with Fe(II) in both its role as a substrate and as an activator.

The over-all scheme of mixed inhibition by Al(III) is shown in Fig. 4. The competition by Al(III) with Fe(II) can occur at the enzyme reduction site (or sites) and this is represented in the top line (E_{el} through E_{el}). If there is only one reduction site, inhibited forms E_{el} through E_{el} are the same species. Competitive inhibition may also occur at the site of activation by Fe(II), giving rise to enzyme form E_{el}. Since there is no competition by Al(III) with respect to O₂, the simplest scheme for the competitive part of the inhibition is illustrated in Fig. 4 with the inhibitor binding to both forms E_{el} and E_{el} with the same association constant K. The inhibited complexes E_{el} and E_{el} can then react with a second Al(III) with the identical association constant K. For the competitive part of the inhibition the scheme in Fig. 4 generates the following expression:

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The Mechanism of Inhibition by Al(III)

FIG. 4. The mechanism of mixed inhibition by Al(III) showing competition with Fe(II) for the site (or sites) of ferroxidase in the top line (E1 through E4). Possible competition of Al(III) with Fe(II) for the activation site is shown with E12I as the inhibited form. The two Al(III) cations which are responsible for the uncompetitive component of the inhibition are shown binding the forms E4 and E14 on the unactivated and activated pathways, respectively. The conversion of enzyme form E12 to E13 is the essentially irreversible rate-limiting step in the unactivated pathway while conversion of E13 to E14 in the corresponding step in the activated pathway with k_{13} > k_{12} (3).

TABLE IV
Properties of some strong metal ion inhibitors of ferroxidase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Apparent K_{RST}</th>
<th>[I] giving 50% inhibition</th>
<th>Effect on visible and ultraviolet spectrum of ferroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>μM</td>
</tr>
<tr>
<td>Al(III)</td>
<td>6.2 × 10^{-12} M²</td>
<td>~50 (dependent on [E])</td>
<td>2.1</td>
</tr>
<tr>
<td>Ga(III)</td>
<td>4.7 × 10^{-7} M</td>
<td>0.45</td>
<td>7.1</td>
</tr>
<tr>
<td>In(III)</td>
<td></td>
<td></td>
<td>133</td>
</tr>
<tr>
<td>Sc(III)</td>
<td></td>
<td></td>
<td>393</td>
</tr>
<tr>
<td>ZrO(II)</td>
<td>0.5-2.0 (dependent on [E])</td>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

a The values refer to the spectrophotometric assay with the reaction mixture containing the following final concentrations: 0.120 μM Chelex-treated ferroxidase, 0.083 M Chelex-treated sodium acetate buffer (pH 6.0), 100 μM Chelex-treated ascorbate, and 100 μM Fe(II) at 30°C.

b Chelex-treated ferroxidase, 7.8 μM, in 0.083 M Chelex-treated sodium acetate buffer, pH 6.0, at 30°C.

Reversibility of the inhibition by In(III) and Sc(III) is indicated by the plots of linear velocity against enzyme concentration shown in Fig. 5. Plots of 1/v against inhibitor concentration at 100 μM Fe(II) for the two inhibitors are shown in Fig. 3a. The plot is linear within experimental error for In(III), but that for Sc(III) shows some nonlinearity, which may indicate a complicated inhibition scheme similar to that of Al(III). Plots of velocity against enzyme concentration in the presence of the two strong inhibitors Ga(III) and ZrO(II) are shown in Fig. 6 and indicate nonlinearity even though the inhibition is reversible upon dilution. No significant differences due to order of addition were obtained in the inhibition exhibited by Al(III), In(III), and Sc(III). However, Ga(III) and ZrO(II) displayed 16% and 22% less inhibition, respectively, in the systems in which they
FIG. 5. Velocity of the ferroxidase reaction at 30° plotted against enzyme concentration with Sc(III) and In(III) as inhibitors. The final concentration in the reaction mixture was: 0.083 M Chelex-treated acetate buffer (pH 6.0), 100 µM Chelex-treated ascorbate, and 100 µM Fe(II).

FIG. 6. Velocity of the ferroxidase reaction at 30° plotted against enzyme concentration with Ga(III) and ZrO(II) as inhibitors. The final concentration in the reaction mixture was: 0.083 M Chelex-treated acetate buffer (pH 6.0), 100 µM Chelex-treated ascorbate, and 100 µM Fe(II).

were not previously incubated. The inhibition by Sc(III) increased slowly as the reaction proceeded but this increase did not diminish with longer incubation times before the addition of substrate. The inhibition by Al(III) showed a slow, gradual tendency to diminish as the reaction proceeded. A summary of some of the properties of the inhibitors is given in Table IV. It should be noted that these cationic inhibitors had no effect on the visible or ultraviolet spectrum of ferroxidase.

The apparent potent inhibition by VO(II) indicated in Table I is actually a lag in the enzymic reaction which is readily observable at VO(II) concentrations of 25 µM or less. Reduction of Fe(III) by ascorbate and strong binding of Fe(III) by apotransferrin made the usual spectrophotometric assays impossible for testing inhibition by Fe(III). The most convenient method available was to monitor the oxygen uptake in the oxygen electrode in the presence and absence of 55 µM apotransferrin at pH 6.0 in 0.20 M acetate buffer. With 30 µM Fe(II) as substrate, 0.490 µM ferroxidase generated its own inhibitor Fe(III). The apparent inhibition was 60%, showing that Fe(III) is also a strong cationic inhibitor.

DISCUSSION

The results show that all trivalent ions tested can inhibit ferroxidase activity. The strongest trivalent inhibitors have an ionic radius of 0.81 Å or less. A suggestion of a relationship between the ionic radius and extent of inhibition is found in the series Sc(III), Y(III), and La(III) in Table I. These metals are consecutive members of Group IIIa in the Periodic Table. The ionic radii show a gradual increase with atomic weight while the ability to inhibit ferroxidase decreases. Hydrolysis may complicate such a comparison but the smallest cation, Sc(III), has a much greater tendency to hydrolyze than do the larger lanthanide ions (14). If hydrolysis is significant, it would be expected to interfere to the greatest extent with Sc(III) and so would not invalidate the comparison. It is of interest that Løvstad and Frieden (15) reported a similar inverse correlation between the degree of inhibition of rat ceruloplasmin by certain anions, e.g. Cl-, Br-, I-, and NO₃-, with the hydrated ionic radius of these anions.

In the sedimentation studies involving relatively high concentrations of Al(III) and ferroxidase, an increase in the sedimentation coefficient of the single peak should be apparent if a significant amount of dimer or higher aggregate were formed and in rapid equilibrium. From an association constant for the ferroxidase-Al(III) complex which was estimated from the extrapolated value for inhibition with 1.0 µM Al(III) (see Fig. 2), it was calculated that all of the ferroxidase should be in the dimeric form if dimerization of ferroxidase were occurring. The fact that the sedimentation coefficient seems to decrease slightly suggests that Al(III) binding to ferroxidase changes the shape or solvation of the enzyme. A conformational change is attractive as a mechanism of uncompetitive inhibition with respect to Fe(II) since no competition by Al(III) with the other substrate, O₂, was observed. Involvement of 2 molecules of Al(III) in the...
uncompetitive part of the mixed inhibition obtained with Al(III) is the simplest explanation consistent with the experimental data. However, binding of a single Al(III) to activated forms of the enzyme on alternative activated pathways, with different binding constants, is also a possible explanation if the activated forms are separated by essentially irreversible steps from enzyme forms to which Fe(II) and O₂ bind as substrates. An over-all scheme of inhibition which is consistent with all of the data is shown in Fig. 4.

The inhibition by In(III) and Sc(III) is reversible while the plots of velocity against enzyme concentration (Fig. 6) with Ga(III) and ZrO(II) as inhibitors are nonlinear. Since inhibition by Ga(III) and ZrO(II) is reversible upon dilution, the nonlinearity in the plots of velocity against enzyme concentration is interpreted as the result of pseudoirreversible inhibition (16). The inhibition by Al(III) shows a similar pseudoirreversibility at relatively high concentrations of Al(III) and ferroxidase (Fig. 1).

The concentration of inhibitors giving 50% inhibition listed in Table IV represents an inexact means of comparison but furnishes the following series for the inhibition of 120 nM ferroxidase at 100 μM Fe(II):

\[ \text{In(III)} > \text{ZrO(II)} > \text{Al(III)} > \text{Sc(III)} > \text{Ga(III)} \]

The work of Plumb and Harris (11) indicates that the mechanisms of exchange of the two cations are different. This might account for the apparent discrepancy in the inhibitory behavior.

Acknowledgment—The authors wish to thank Dr. N. S. Incardona for performing the ultracentrifuge experiments.

REFERENCES

15. Løvstad, R., and Frieden, E., in press.
CORRECTIONS

In the paper by M. K. Sahib and C. R. Krishna Murti (Vol. 244, No. 17, Issue of September 10, 1969, page 4730), on page 4733, line 22 in the right-hand column, the sentence beginning “Histidine pyruvate aminotransferase . . .” should be replaced with the following:

“In contrast to histidine ammonia lyase, histidine pyruvate aminotransferase did not alter either with the age of the rats or the protein content of the diets ingested by them, as reported earlier by Rao, Deodhar, and Hariharan (22a).”


In the paper by Philip T. Cohen and Nathan O. Kaplan (Vol. 245, No. 11, Issue of June 10, 1970, page 2825), the first author’s given name is spelled incorrectly. It should be “Philip” not “Phillip.” On page 2835, right-hand column, the word “not” should be inserted in line 8 of the second paragraph of the “Discussion” so that the sentence reads:

“Two lines of evidence led the earlier workers to believe the enzyme was not a flavoprotein.”

In the paper by Zenon Schneider, Earl G. Larsen, Gail Jacobson, B. Connor Johnson, and J. Pawelkiewicz (Vol. 245, No. 13, Issue of July 10, 1970, page 3388), the definition of DBCC given in Footnote 1 on page 3388 is incorrect. The correct definition is:

“DBCC, 5,6-dimethylbenzimidazolylcobamide 5’-deoxyadenosyl coenzyme.”

In the paper by C. Thomas Huber and Earl Frieden (Vol. 245, No. 15, Issue of August 10, 1970, page 3979), Equation 1 in the right-hand column on page 3981 is incorrect and should be replaced by the following:

\[
\frac{[E]}{V_{\text{max}}} = \frac{1}{240} \frac{k_{\text{III}}}{k_{\text{III}}} + \frac{(K_n[I] + K_nK_0[I])(K_{\text{XY}} + k_{\text{XY}})}{K_{\text{XY}} + k_{\text{XY}}} (1)
\]
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