Participation of Metals in Peroxidase-catalyzed Oxidations*

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The requirement for manganese in peroxidase-catalyzed oxidations has hitherto been considered to be very specific. Various investigators (1-6) have tested a number of other ions (Zn, Mg, Cu, Co, Fe, Ni, Cd, Ca, Al, Sn, and molybdate) in different peroxidase-catalyzed oxidations, but with the exception of a slight stimulation of reduced diphosphopyridine nucleotide oxidation by cobaltous ions, these ions have proved inactive or inhibitory.

In view of the suggestions that the peroxidase-catalyzed oxidations of dicarboxylic acids (7) and the peroxidase-catalyzed oxidation of indoleacetic acid (8) depend on the formation of manganic ions, it is interesting that the peroxidase system of Kotten and Mann (9) oxidized manganese only. Ions of copper, cobalt, zinc, nickel, and iron were not affected. However, Yamada and Ota (10) have reported the oxidation of ferrous ions by a preparation from rice roots; they considered the responsible enzyme to be a peroxidase.

The possibility that cerium might be oxidized by a peroxidase system and might replace manganese in peroxidase-catalyzed oxidations was considered because of the closeness of the oxidation-reduction potential of cerium to that of manganese, and because of the known initiation of oxidations by ceric ions (11). It has been found that cerous ions will replace manganous ions in the three peroxidase-catalyzed oxidations examined.

EXPERIMENTAL

Purified horseradish peroxidase was obtained from Nutritional Biochemicals Corporation (activity stated by supplier was 60 units per mg by the assay of Polis and Shmukler (12)), and enzyme solutions were prepared by dissolving 10 mg of the powder in 100 ml of distilled water. When stored under toluene at 5°C, the enzyme solutions were stable for periods as long as 2 months.

Gas exchange was measured manometrically at 30°C (13). The volume of the reaction mixture was 3.0 ml and the reactions were started by addition of the enzyme from the side arm.

The oxidation of cerium was measured by following the increase in absorbancy of the cerium-citrate complex at 272 μm. The reaction mixture, in a 1-cm cuvette, consisted of 0.5 ml of 0.1 M citrate buffer pH 7.0, 0.2 ml of 0.01 M CeCl₃, 0.5 ml of 0.001 M resorcinol, 0.2 ml of 0.03 per cent H₂O₂, enzyme solution, and water to make a total volume of 3.5 ml. The reaction was started by the addition of the enzyme, and readings were taken in a Beckman DU spectrophotometer at timed intervals.

Absorption spectra were determined with a Cary model 11 recording spectrophotometer or with a Beckman DU spectrophotometer.

RESULTS

A water solution of cerous chloride shows a characteristic absorption spectrum in the ultraviolet region with an absorption maximum at 232 μm (14). In the presence of citrate at pH 7.0, the formation of a complex was indicated by a shift of the absorption spectrum to longer wave lengths with a new absorption maximum at 272 μm (Fig. 1). The spectral change was complete when the cerium citrate ratio was 1:2. (A spectral shift upon the addition of ethylenediaminetetraacetic acid to a solution of cerous chloride, with a new peak appearing at 280 μm, was also observed, but this complex was not used in the study of the oxidation of cerium.)

It is difficult to establish the absolute absorption of the ceruria of cerium and citrate, because ceric salts tend to decompose near neutrality, and because they may oxidize certain constituents of the medium, such as citrate. Ceric sulfate dissolved in citric acid solution (final pH 1.9), and the same solution adjusted with KOH to pH 7.0, yielded the spectra shown in Fig. 1. It should be noted that the ceric-citrate complex absorbs more strongly at 272 μm than does the cerous-citrate complex (comparative measurements reported in this paper were made at 272 μm). Because of the difficulties indicated, the data should not be interpreted in terms of absolute reaction rates.

Under the same conditions as those used for the oxidation of manganese, the oxidation of cerium occurred. These conditions include the presence of horseradish peroxidase, hydrogen peroxide, and a phenolic component. The oxidation of the cerium was indicated by an intensification of the optical density at 272 μm. For example, in the presence of 0.5 μmole of cerous chloride (other conditions as described in the "Experimental" section), the optical density increased from 0.45 to 0.68 during the 90 seconds after addition of the enzyme. The effects of enzyme concentration (Fig. 2) and cerium concentration (Fig. 3) were measured.

Since manganic ions have been postulated to initiate peroxidase-catalyzed oxidations (8), it was thought that ceric ions, produced enzymically by the peroxidase system, might be equally effective. Maclachlan and Waygood (8) have shown the effect of manganous concentration on the oxidation of indoleacetic acid. Table I shows the effect of cerium concentration on oxidations of indoleacetic acid by horseradish peroxidase. In experiments in which both metal ions were tested at concentrations of 0.001
The rate of indoleacetic acid oxidation by horseradish peroxidase was greater in the presence of cerium than in the presence of manganese: 17% at pH 4.5 (acetate buffer) and 24% at pH 6.0 (succinate buffer).

The oxidation of 2-nitropropane was followed by measuring oxygen uptake manometrically. In agreement with the report of Little (6), the requirement for metal ions and a phenolic compound was not absolute, but both of these enhanced oxidation, apparently by acting as cofactors. Fig. 4 shows that manganous and cerous ions are equally effective in enhancing the oxidation of 2-nitropropane.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>µmoles Ce³⁺⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish</td>
<td>25 47 57 57 73</td>
</tr>
</tbody>
</table>

* Values are µl of O₂ uptake per 10 minutes.

The rate of DPNH oxidation at pH 5.0 in succinate buffer depends upon the concentrations of manganous or cerous ions; the effect of varying the concentration of cerous ions is shown in Fig. 6. At pH 7.5 there is inhibition of the oxidation by cerous and cobaltous ions and by excessive concentrations of manganese. These effects are presented in Table II.

The effects of varying the manganese and resorcinol concentrations on the oxidation of DPNH was examined. Fig. 7 shows...
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The effect of metallic cofactors on the oxidation of 2-nitropropane. The reaction mixture consisted of 50 pmols of succinate buffer, pH 5.0, 3 pmols of metal chloride, 2 pmols of resorcinol, 5 pmols of 2-nitropropane, 0.3 ml of horseradish peroxidase solution in a total volume of 3.0 ml. The nitropropane solution was prepared from a 10^{-3} \text{M} solution which was kept at pH 13 overnight and adjusted to pH 5.0 and to 5 \times 10^{-3} \text{M} just before the experiment. \( \nabla \), without resorcinol; \( \Delta \), with Mn\(^{2+}\); \( \bigcirc \), with Ce\(^{3+}\).

Fig. 4

The effect of pH on the oxidation of DPNH. 5 pmols of MnCl\(_2\) or CeCl\(_3\), 0.3 ml of horseradish peroxidase solution in each cuvette. \( a \), Ce\(^{3+}\); \( b \), Mn\(^{2+}\); \( \Delta \), succinate buffer; \( \bigcirc \), tris(hydroxymethyl)aminomethane buffer.

Fig. 5

that the optimal concentration of manganous ions depends on the concentration of resorcinol; as the concentration of resorcinol was increased, the optimal concentration of manganese increased. Akazawa and Conn (5) considered the mechanism of DPNH oxidation to be different from the peroxidase-catalyzed oxidation of indoleacetic acid because of the lower optimal concentration of manganese required for the oxidation of DPNH. The low optimal concentration they observed probably resulted from the low concentration of resorcinol used. The interaction of phenolic and metallic compounds in the oxidation of DPNH is the same as that observed by Hillman and Galston (15) and confirmed by Pilet (16) for the enzymic oxidation of indoleacetic acid and provides evidence that the mechanisms of the two reactions are in fact similar.

DISCUSSION

A hypothesis unifying the mechanisms of peroxidase-catalyzed oxidations would be attractive; as manganese is common to all of these reactions, the mechanism of its action is probably critical for a general scheme.

It has been suggested that manganic ions are essential in peroxidase-catalyzed oxidations (7, 8). Points in favor of this idea are the appearance of manganic ions in the oxidation of dicarboxylic acids (7), and the inhibition of indoleacetic acid oxidation by the chelators citrate and pyrophosphate (17). Analogous reactions of cobaltic ions with formic acid (18), manganic ions with

![Graph showing O_2 uptake over time](image)

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<tr>
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<th>Mn(^{2+})</th>
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<th>Ce(^{3+})</th>
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<tr>
<td>only 5 pmols Mn(^{2+})</td>
<td>0.110</td>
<td>0.110</td>
<td>0.110</td>
</tr>
<tr>
<td>+ 5 pmols metal</td>
<td>0.100</td>
<td>0.000</td>
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</tr>
<tr>
<td>+ 10 pmols metal</td>
<td>0.090</td>
<td>0.075</td>
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<tr>
<td>+ 10 pmols metal</td>
<td>0.080</td>
<td>0.065</td>
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* Values are change in optical density at 340 nm per minute.

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The nonenzymic oxidation of indoleacetic acid by manganic ions has been observed (20). Oxalic acid (19) and ceric ions with 2,3-butanediol (11) have been reported. Finally, the nonenzymic oxidation of indoleacetic acid at different manganese concentrations (2) remains that the mechanism of oxidation is not the same at high and at low concentrations of the metal ions. The present evidence allows the interpretation that the oxidations proceed with the participation of manganic ions when the manganese concentration is high. The difference in optimal pH we have observed for DPNH oxidation by catalase (21,22), that the optimal concentration of manganese, is not inhibited by citrate and pyrophosphate, and is inhibited by ethylenediaminetetra-acetic acid. However, in favor of the suggestion that the peroxidatic mechanism is the same for the oxidation of indoleacetic acid and DPNH, it should be noted that inhibition of indoleacetic acid oxidation by catalase has been reported (21, 22), that the optimal concentrations of manganese for DPNH and indoleacetic acid oxidation are of the same order provided the concentration of the phenolic free radical is the same, and that our unpublished tests found for indoleacetic acid oxidizing enzymes. A further similarity of the peroxidase-catalyzed oxidations of indoleacetic acid and DPNH is the lag period frequently observed before oxidation starts. This lag period may represent, according to the scheme of Maclachlan and Waygood (8), a period during which the concentration of manganic ions is building up, or according to the mechanism of Akazawa and Conn (5), a period during which the concentration of the phenolic free radical RO· is rising.

**SUMMARY**

1. Horseradish peroxidase catalyzes the oxidation of cerous ions by hydrogen peroxide in the presence of low concentrations of resorcinol.

2. Manganous ions can be replaced by cerous ions in the peroxidase-catalyzed oxidations of indoleacetic acid, 2-nitropropane, and reduced diphosphopyridine nucleotide.

3. In the oxidation of reduced diphosphopyridine nucleotide, cerous ions were most effective at pH 5.0 and manganous ions were most effective at pH 7.5. Cobaltous ions were ineffective at pH 5.0, and only slightly effective at pH 7.5. At pH 7.5, the oxidation of reduced diphosphopyridine nucleotide in the presence of manganous ions was inhibited by cerous, cobaltous, and excess manganous ions.

4. The optimal concentration of manganese for the peroxidase-catalyzed oxidation of reduced diphosphopyridine nucleotide increased as the concentration of the phenolic compound was increased.

**REFERENCES**

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