The Mechanism of Indole-3-acetic Acid Oxidation by Horseradish Peroxidases*

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The oxygen-consuming oxidation of indole-3-acetic acid (IAA) occurred much faster in the presence of horseradish peroxidase C (neutral isoenzyme) than in the presence of horseradish peroxidase A (acidic isoenzyme). An intermediate oxidation product of IAA was found to be a hydroperoxide species that reacted with the ferric enzymes to form Compound I at second order rate constants of $6.8 \times 10^8$ M$^{-1}$ s$^{-1}$ for peroxidase A and $2.0 \times 10^8$ M$^{-1}$ s$^{-1}$ for peroxidase C at pH 4.4. The hydroperoxide concentration reached about one-half of the initial IAA concentration at the end of the oxygen-consuming reaction and then decreased slowly. The main intermediate of the enzymes observed during the oxygen-consuming reaction was Compound II, which oxidized IAA to its free radical at rate constants of $1.5 \times 10^9$ M$^{-1}$ s$^{-1}$ for peroxidase A and $1.2 \times 10^9$ M$^{-1}$ s$^{-1}$ for peroxidase C at pH 4.4. The results supported the mechanism that the oxygen consumption occurs mainly through the reaction of oxygen with the IAA free radical formed from the peroxidatic oxidation of IAA. The ferric enzymes were not reduced by IAA under strict anaerobic conditions in the presence of carbon monoxide but were reduced upon addition of a small amount of oxygen or hydrogen peroxide to the system. The results suggested that the ferric enzyme is reduced by the IAA free radical but not by IAA itself. From a comparison of reactivities of oxy peroxidase and Compound II we concluded that the catalytic cycle of ferrous and oxyperoxidases is not involved in the IAA oxidase reaction.

Since Kenten (1) reported in 1955 that horseradish peroxidase catalyzes aerobic oxidation of the plant hormone indole-3-acetic acid (IAA) in the absence of added hydrogen peroxide, considerable progress has been made toward understanding the mechanism of the reaction. The earlier studies have been reviewed by Ray (2) and Galston and Purves (3). Various investigators (4-10) have suggested that the free radical derived from IAA is an intermediate in the IAA degradation. Since the reaction is not inhibited by a catalytic amount of catalase (1, 7, 11), hydrogen peroxide may not be involved in the catalytic function of peroxidase. Therefore, it has been suggested (10-14) that the IAA oxidase activity of peroxidase is attributed to its oxygenase-like function where the oxygen binding to the enzyme is an essential process. On the other hand, we have suggested that a major function of peroxidase in the peroxidase-oxidase reaction is to produce free radicals of substrates through the peroxidase cycle consisting of the ferric enzyme and Compounds I and II (5, 9, 15).

In this paper, we present evidence to indicate that the peroxidase cycle functions during the IAA oxidase reaction and that direct oxygen activation by peroxidase is not involved in the reaction.

EXPERIMENTAL PROCEDURES

Peroxidases A and C were purified from wild horseradish roots by the method of Shannon et al. (16) with slight modification. Peroxidase A is a mixture of isoenzymes A1 and A2 according to the nomenclature of Shannon et al. (16). The ratio of $A_{403}$ to $A_{280}$ at 20°C was 4.0 for peroxidase A and 3.3 for peroxidase C. The mol cancellation was calculated on the basis of $E_{280}$ values of 107 for peroxidase A at 403 nm and 100 for peroxidase C at 403 nm (17). Ascorbic oxidase was prepared from Japanese cucumber by the method of Nakamura et al. (18). Catalase was prepared from beef liver. IAA and bovine serum albumin were purchased from Sigma Chemical Co. IAA was added to reaction solutions from a freshly dissolved 20 mM solution containing 12% ethanol or acetone.

The oxygen concentration was measured with a Beckman polarographic oxygen sensor. Spectrophotometric measurements were performed with a Hitachi EPS-3 spectrophotometer and a Shimazu MPS 5000 spectrophotometer, both equipped with a cuvette compartment thermostatically controlled. Rapid reactions were measured with Union Giken rapid reaction analyzer, RA 1300. This instrument is equipped with a flow apparatus and can be used either as a rapid wavelength scan spectrophotometer or as a sensitive photometer at a fixed wavelength. The absorption spectra are measured by means of an image dissector with a maximum speed of 300 nm/ms and are memorized in a digital computer system. The analogue replica is afterwards obtained in an X-Y recorder. The dead time of the flow apparatus at an $N_2$ gas pressure used in this experiment was about 1 ms.

Strict anaerobic conditions were obtained in a Thunberg tube with the system of ascorbate and ascorbate oxidase or of glucose (20 $\mu$M) and glucose oxidase plus catalase. The tube had been degassed and filled with $N_2$ (99.999%) or CO gas. In the ascorbate oxidase system the addition of excess ascorbate was avoided by monitoring absorbance at 265 nm. 0.5 $\mu$M ascorbate was detectable. The reactions were carried out in 50 mM sodium acetate buffer (pH 4.4, at 20°C unless otherwise noted.

RESULTS

Fig. 1 shows time courses of the oxygen consumption which occurred when IAA was added to aerobic solutions of horseradish peroxidases A and C. No addition of cofactor or hydrogen peroxide was necessary for the reaction. Peroxidase $C$ was more active than peroxidase A. In the reaction of peroxidase A the rate of oxygen consumption reached a constant value after a distinct lag, which disappeared upon addition of a small amount of hydrogen peroxide. The addition of hydrogen peroxide, however, gave no significant effect on the rate of steady state reaction of either isoenzyme. A similar effect of hydrogen peroxide has been reported in the reactions of Omphalia (7) and tobacco (19) peroxidases. The reactions obeyed apparently zero order kinetics once the steady state was attained and the mole ratio of consumed oxygen to IAA added was about 0.75 in the two oxidase reactions. The lag
The lag period in the reaction of peroxidase A was shortened to about one-tenth when the enzyme concentration was increased from 1 μM to 10 μM. It has been reported that the lag period is elongated by addition of a small amount of ascorbate (9), ferulic acid (14), or a metabolite of the insecticide carbofuran (20, 21).

The second addition of IAA to the reaction solution containing peroxidase A caused rapid consumption of oxygen and the lag disappeared (Fig. 2A). If the addition of IAA was preceded by that of ascorbate there appeared a lag again, the duration of which was very sensitive to the amount of ascorbate. It should be noted that the rate at steady state and the total amount of consumed oxygen were independent of the amount of ascorbate. The lag time measured when 48 μM ascorbate was added is shown in A.

The critical concentration became clear when a similar experiment was conducted with peroxidase C. Fig. 3 shows that a lag began to appear sharply by addition of ascorbate above 44 μM. The inset of Fig. 3 shows that such increase in the lag period was observed at a much lower concentration of ascorbate when the reaction was started in a fresh solution. The results suggested that a hydroperoxide species was formed during the IAA oxidase reaction and served as a substrate in the peroxidase reaction. Many workers (6-10) have suggested without direct evidence that a hydroperoxide derived from IAA is an intermediate product in the IAA degradation. It can be seen from Fig. 3 that the hydroperoxide was not decomposed by a catalytic amount of catalase.

The stoichiometric relationship in the reaction between the hydroperoxide and ascorbate is shown in Fig. 4. Inset A of Fig. 4 shows the formation of Compound I from the reaction of peroxidase C with the hydroperoxide. A kinetic trace measured with a stopped flow apparatus in the presence of a limited amount of the hydroperoxide is shown in inset R of Fig. 4. The presence of 200 μM IAA or ascorbate in the
hydroperoxide solution gave no significant effect on the kinetic trace of absorbance at 410 nm in 0.2 s at pH 7.4. In Fig. 4, the amount of Compound I is plotted against the hydroperoxide concentration calculated on the basis that the reaction mixture described in Fig. 3 contained 44 μM hydroperoxide. A control experiment with hydrogen peroxide is also shown in Fig. 4. The stoichiometric relationship implies that the critical concentration of ascorbate causing a sudden increase in the lag period was equal to the hydroperoxide concentration. Since the hydroperoxide removed an equimolar amount of ascorbate in a short time, it was possible to measure the hydroperoxide concentration at any time during the oxidase reaction, except for the very early stage of the reaction.

As reported already by others (6, 22-24), spectral changes occurred at least in two steps (Fig. 5). The first change corresponded to the oxygen consumption, the rate of which was different between the two isoenzymes (Figs. 5, A and B). The reactions yielded similar spectra having small bands at 281 and 289 nm. When the reaction of peroxidase A was started with an addition of a small amount of hydrogen peroxide, the 281 and 289 nm bands disappeared while a broad band at 301 nm increased (Fig. 5C). Morita et al. (24) reported that products from IAA varied with the concentration of Japanese radish peroxidases used for the reaction. Fig. 5D shows that the products varied with the concentration of peroxidase C but not with the rate of the reaction (Compare Fig. 5, A, B, and D). At any rate it was evident that a similar intermediate appeared having a spectrum with a flat absorption band between 270 and 280 nm and a sharp shoulder at 288 nm. In order to compare this spectral species with the hydroperoxide, time courses of the hydroperoxide concentration and absorbance at 274 nm are shown in Fig. 6, A and B.

Fig. 5. Repeated scan UV spectra in the IAA oxidase reaction. The reactions were started by the addition of peroxidase to an IAA solution, the spectrum of which is shown in each figure. Spectrum was scanned every 47 s from 235 nm at a rate of 800 nm/min. The time course follows the direction of the arrow indicated at 250 nm. 100 μM IAA. A, 1 μM peroxidase C; B, 1 μM peroxidase A; C, 1 μM peroxidase A and 50 μM hydrogen peroxide; D, 0.2 μM peroxidase C.

Fig. 6. Time courses of the hydroperoxide concentration (A) and the absorbance at 274 nm (B) in the IAA oxidase reaction. 1 μM peroxidase A (○) and 1 μM peroxidase C (●). A, The concentration of the hydroperoxide (R"OOH) was measured by adding different amounts of ascorbate to the reaction solution at times indicated in the abscissa. The hydroperoxide concentration was determined from the lag time versus ascorbate concentration plots as described in Fig. 3. To measure the hydroperoxide concentration in the reaction by peroxidase A, 1 μM peroxidase C was added with ascorbate so as to obtain a sharp inflection point. B, data were replotted from Fig. 5A (○) and 5B (●).
respectively. The result led to a conclusion that the intermediate spectrum is ascribable to the hydroperoxide that reacts with the ferric enzyme to form Compound I.

Ricard and Job (10) argued that the direct reduction of peroxidase by IAA is necessary for the oxidase reaction to proceed and also that most of Compound II is formed from a two-equivalent reduction of oxyperoxidase. Formation of Compound II and oxyperoxidase in the reaction of peroxidase C and IAA was investigated with a Union Giken rapid reaction analyzer. Experiments shown in Fig. 7 were performed in the presence of bovine serum albumin which inhibited degradation of peroxidase heme but had little effect on the rate of oxygen consumption. By a rapid scanning method, the formation of Compound II and oxyperoxidase can be measured from absorbance changes in the Soret band if the enzyme exists in a mixture of ferric, Compound II, and oxy-enzymes. Wavelengths of 461.7 and 452.7 were isosbestic between ferric and oxyenzymes and between Compound II and ferric enzymes and were used for assays of Compound II and oxyperoxidase, respectively. At these wavelengths, absorbance was not changed by the formation of a complex of peroxidase C and IAA. The formation of such a complex was observed spectrophotometrically by Ricard and Job (10). Spectrophotometric analyses of absorbance changes at those wavelengths indicated that Compound II was formed rapidly after a short lag and oxyperoxidase accumulated slowly and later than Compound II (Fig. 7B). The oxyperoxidase formation became evident as the pH decreased and the IAA concentration increased.

An important problem in the mechanism of IAA oxidase reaction might be to identify a chemical species that reduces the ferric enzyme and to clarify the role of its reduction in the overall oxidase reaction. Ricard and Nari (25, 26) reported that peroxidase is reduced simply by an addition of IAA. It was also found that hydroquinone, NADH, 5-hydroxyindole-3-acetate, and chlorogenic acid inhibited both the oxygen-induced formation of peroxidase A caused by an addition of hydrogen peroxide. It should be noted that ascorbate inhibited the oxygen-induced reduction but not the hydrogen peroxide-induced reduction. The strict anaerobiosis in these experiments was obtained with an enzyme system of ascorbate oxidase or glucose oxidase plus catalase. In an anaerobic solution obtained with commercial carbon monoxide without using these enzyme systems, an appreciable amount of ferriperoxidase was reduced simply by an addition of IAA. It was also found that hydroquinone, NADH, 5-hydroxyindole-3-acetate, and chlorogenic acid inhibited both the oxygen-induced formation of carbon monoxide complex and the IAA oxidase reaction, just as ascorbate did. The results do not conform with the mechanism of Ricard and Job (10), who concluded that the inhibition by ascorbate is ascribable to its specific binding to peroxidase.

Marklund et al. (27) reported that there is marked difference between peroxidases A and C in the rate of reactions of the enzymes with organic hydroperoxides or peracids (Table I). The rate constants for the reactions of hydrogen peroxide with peroxidase A and C were both independent of pH between 4 and 8, being measured at 1.0 × 10⁶ M⁻¹ s⁻¹ for peroxidase A and 2.0 × 10⁶ M⁻¹ s⁻¹ for peroxidase C. These values are in agreement with those reported by others (27-29).

Fig. 8B shows pH dependence of the rate constants for the reactions of IAA with Compound II of peroxidases A and C. The rate constant (kₐ) was measured according to the following equation:

\[ \frac{d[R^{-}]}{dt} = 2 k_{a} [\text{Compound II}] [RH] \]
**Indole-3-acetic Acid Oxidation by Peroxidases**

**TABLE I**

| Peroxidase               | Peroxidase A | Peroxidase C | Ratio | pH | Reference |
|--------------------------|--------------|--------------|-------|----|-----------|
| Hydrogen peroxide        | 1.0          | 20           | 0.05  | 7  | This paper |
|                          | 2.0          | 14.6         | 0.15  | 7  | (27)      |
| Methylhydroperoxide      | 0.17         | 1.3          | 0.13  | 7  | (27)      |
| Ethylhydroperoxide       | 0.08         | 4.0          | 0.02  | 7  | (27)      |
| n-Propylhydroperoxide    | 0.037        | 4.8          | 0.008 | 7  | (27)      |
| Hydroxyethylhydroperoxide| 0.011        | 0.45         | 0.024 | 4.6| (27)      |
| p-Nitroperoxybenzoic acid| 0.47         | 37           | 0.013 | 4.5| (27)      |
| m-Nitroperoxybenzoic acid| 0.89         | 17           | 0.05  | 5.8| This paper |
| Hydroperoxide from IAA   | 0.0019       | 2.0          | 0.0007| 7  | This paper |
|                          | 0.0068       | 2.0          | 0.0034| 4.4| This paper |

*Schonbaum and Lo (37) reported a value of 2.1 x 10^7 M^-1 s^-1 at pH 6.1 and 5°C.

**Fig. 10.** Dependence of the ratio of the rate of oxygen consumption to that of IAA free radical formation upon the enzyme concentration. The ratio was calculated from the results in the inset. The rate of IAA free radical formation was assumed to equal 2 k_{c}[Compound II] [IAA]. The concentration of Compound II was measured as described in Fig. 7B.

**DISCUSSION**

Diverse features of the IAA oxidase reaction are probably ascribable to the fact that free radical mechanism is involved in the reaction. We have proposed a general mechanism for the peroxidase-oxidase reaction (15, 31) and a peculiar pattern of the NADH oxidase reaction catalyzed by peroxidase C has been simulated with a computer from nine rate equations (32). Contrary to the NADH oxidase reaction, the IAA oxidase reaction is insensitive to superoxide dismutase (9, 12). Hydrogen peroxide accumulates in the NADH oxidase reaction (32), while a hydroperoxide derived from IAA accumulates in the IAA oxidase reaction. The hydroperoxide corresponds with intermediate A in the Ray's mechanism (6). Since Ray and Thimann (22) have shown that the formation of intermediate A is accompanied by decarboxylation, the intermediate is probably a hydroperoxide form of skatole. Major reactions involved in the IAA oxidase reaction are, therefore, formulated as follows:

\[
Peroxidase + R'OOH \longrightarrow k_{1}(2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \rightarrow \text{Compound I} + R'OH \quad (1)
\]

\[
\text{Compound I} + RH \xrightarrow{\text{fast}} \text{Compound II} + R' \quad (2)
\]

\[
\text{Compound II} + RH \xrightarrow{k_{2}(1.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1})} \text{Peroxidase} + R' \quad (3)
\]

\[
R' + O_2 \rightarrow R'OOH + CO_2 \quad (4)
\]

\[
R'OOH + RH \rightarrow R'OOH + R' \quad (5)
\]

where RH and R' denote IAA and its free radical. The rate of free radical formation was assumed to correspond to that of the reaction of peroxidase A with the hydroperoxide derived from IAA. 0.1 µM enzyme, 7.8 µM hydrogen peroxide. The rate was measured from similar experiments shown in inset B of Fig. 4. B, the k_{1} value was measured from the overall kinetics by the method described elsewhere (5). The rate of IAA free radical formation was assumed to equal that of iron free radical formation. The rate constants in parentheses are for the reactions of peroxidase A.

It has been suggested (7-10) that the oxygen consumption in the IAA oxidase reaction is attributed to the incorporation of oxygen into a free radical of IAA, R' + O_2 \rightarrow R'OOC. In order to check this point, the ratio of the rate of oxygen consumption to the rate of free radical formation through the peroxidase cycle was calculated from data shown in the inset of Fig. 10. Fig. 10 shows that the ratio depended on the concentration of peroxidase C. The ratio might be closely correlated with propagation and termination of chain reactions. At low concentrations of the enzyme the ratio became higher than 2, indicating an involvement of a chain propagating reaction: R'OOH + RH \rightarrow R'OOH + R'.

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inhibited by a large amount of catalase (1, 11, 35). Schonbaum (36) has shown that peroxidase C forms spectroscopically distinct complexes with aromatic hydroxamic acids, hydrazides, amides, and α-hydroxyketones and also that peracids structurally related to those compounds react with peroxidase C at high rates to form Compound I (37). Peroxidase A has a smaller affinity for the hydroxamic acids (38) and reacts with the peracids or organic hydroperoxides at much slower rates (27) in comparison with peroxidase C. This tendency becomes more pronounced in the reaction with the hydroperoxide C. The rate of the IAA oxidase reaction is more pronounced in the reaction with the hydroperoxide free radical (R'OO·) than in peracids or organic hydroperoxides at much slower rates (27) in comparison with peroxidase C. This tendency becomes more pronounced in the reaction with the hydroperoxide derived from IAA (Table I). Marked differences in the IAA oxidase activity of the two isoenzymes can be accounted for in terms of the k1 and k3 values. The rate of the IAA oxidase reaction at steady state is mainly controlled by the k3 value. The reaction reaches the steady state after the hydroperoxide accumulates to a certain concentration. Since Reaction 2 is not rate-limiting, the critical concentration can be roughly calculated according to the equation, k1[hydroperoxide] = k2[IAA]. The calculated concentration of the hydroperoxide is 92 μM for peroxidase A and 0.6 μM for peroxidase C when [IAA] = 100 μM. These values do not contradict the results shown in Figs. 1 and 6. The effect of hydrogen peroxide on the IAA oxidase reaction shown in Fig. 1 can be explained by the fact that the k1 value for peroxidase A differs widely from thehydrogen peroxide to the hydroperoxide derived from IAA. Compound II appears exponentially after IAA is added to an aerobic solution of peroxidase C (Fig. 7B). This seems to reflect the fact that the hydroperoxide (H2O2) is formed according to an autocatalytic mechanism, as can be seen in Reactions 1 to 5. The detailed analysis of similar reactions has been performed in the NADH oxidase reaction catalyzed by peroxidase (32, 39). It can be said from the result in Fig. 10 that the chain propagation (Reaction 5) may work efficiently in the early stage of the reaction where the formation of hydroperoxide free radical (H2O2·) is extremely slow. We assume that a trace amount of the hydroperoxide is necessary to start the reaction (9).

It has been reported that oxyperoxidase is formed during the peroxidase-oxidase reactions (15, 31) and reacts with IAA at a relatively high rate in comparison with other electron donors (40, 41). Specific interaction between peroxidase and indole derivatives has been reported (10, 42). Therefore, the mechanism that peroxidase behaves like an oxygenase to form an active ternary complex of the enzyme, IAA, and oxygen becomes very attractive. Ricard and Job (10) reported that the formation of Compound II is preceded by that of oxyperoxidase in the IAA oxidase reaction. Our results shown in Fig. 7, A and B seem to contradict their conclusion. The discrepancy may arise from the difference in experimental conditions and also from complexity in spectral analysis of the enzyme intermediates (10). Since Compound II reacts with IAA about 10 times as fast as oxyperoxidase does (41), the concentration of oxyperoxidase would become 10 times that of Compound II in the steady state if Compound II is formed via oxyperoxidase. The slow accumulation of oxyperoxidase shown in Fig. 7A implies that its formation is a side reaction and has no significant role in the catalytic oxidation of IAA. It seems likely that the ferric enzyme is reduced by the IAA free radical (R·) formed in Reactions 2 and 3 (5) and the reduced enzyme then reacts with oxygen to form oxyperoxidase (43, 44).

The mechanism of ascorbate oxidation by the hydroperoxide derived from IAA is not clearly understood. The stoichiometric relationship shown in Fig. 4 may suggest that the peroxidases catalyze the oxidation of ascorbate by the hydroperoxide. This simple explanation, however, is not true because 1) the ascorbate inhibition is much stronger than expected from competition of IAA and ascorbate for Compound II and 2) the ascorbate inhibition occurs in the presence of oxygen (Fig. 8A) but not in the presence of hydrogen peroxide under anaerobic conditions (Fig. 8B). It seems that ascorbate reacts with the hydroperoxide free radical. The addition of ascorbate does not change the amount of oxygen consumption but decreases the amount of the hydroperoxide accumulation.

Products of the IAA oxidation are not thoroughly analyzed in this experiment. The fact that the mole ratio of oxygen consumed to IAA added is about 0.75 may suggest the formation of indole-3-methanol (R'OH) as a product of Reaction 1. The major end product 3-methylenoxindole is formed through Reaction 6, as suggested by Hinman and Lang (8). Minor products may be formed from disproportionation of the hydroperoxide free radical when the rate of its formation is high.

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878 Indole-3-acetic Acid Oxidation by Peroxidases

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