Oxidation of Indole-3-acetic Acid by Peroxidase: Involvement of Reduced Peroxidase and Compound III with Superoxide as a Product†

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ABSTRACT: Kinetic and spectral data establish that peroxidase may oxidize indole-3-acetic acid by either of two pathways depending on the enzyme/substrate ratio. When relatively low enzyme/substrate ratios are employed, the oxidation proceeds through a reduced peroxidase ⇄ compound III shuttle. Conversely, peroxidase operates through the conventionally accepted pathway involving native enzyme and compounds I and II only when high enzyme/substrate ratios are used. Compound III, a specific oxidase, constitutes the dominant steady-state form of peroxidase when the reduced peroxidase ⇄ compound III shuttle is operational. Activation of this shuttle also produces a flux of superoxide anion radical at the expense of molecular oxygen. Thus, important biological consequences may follow activation of this shuttle under physiological conditions.

Diverse mechanisms have been proposed for the aerobic oxidation of indole-3-acetic acid (IAA)[1] by peroxidase (EC 1.11.1.7). It is generally accepted, however, that the free radical of IAA is a reaction intermediate and that the enzyme undergoes various changes in redox state during reaction, with compound II of the enzyme being the predominant steady-state form (Dunford & Stillman, 1976; Nakajima & Yamazaki, 1979; Ricard & Job, 1975; Yamazaki & Yokota, 1973; Yamazaki, 1977). Utilization of oxygen is thought to result from a direct reaction between the IAA free radical and molecular oxygen with the initial formation of an IAA peroxy radical and, finally, an organic hydroperoxide (Nakajima & Yamazaki, 1979, 1980). It is claimed that the organic hydroperoxide may replace hydrogen peroxide as an essential reactant and that this explains the insensitivity of the aerobic oxidation of IAA to inhibition by catalytic amounts of catalase (EC 1.11.1.6) (Nakajima & Yamazaki, 1979).

In peroxidase–oxidase reactions with other substrates such as NADH and dihydroxyfumarate, utilization of oxygen results in the formation of $O_2^{-}\cdot$ rather than an organic hydroperoxide (Yamazaki & Yokota, 1973; Yamazaki, 1977). Formation of $O_2^{-}\cdot$ during oxidation of IAA has been considered unlikely because the scavenging enzyme superoxide dismutase (EC 1.15.1.1) had no inhibitory effects on the reaction whereas it is a potent inhibitor of the other peroxidase–oxidase reactions (Miller & Parups, 1971; Yamazaki & Yamazaki, 1973).

We studied the oxidation of IAA by peroxidase over an extremely wide range of concentrations of enzyme and substrate. Our results establish that peroxidase oxidizes IAA through the conventionally accepted pathway only when relatively high enzyme/substrate ratios (e.g., [HRP] > 1 μM; [IAA] $\sim 1 \times 10^{-4}$ M) are used. With lower enzyme/substrate ratios, oxidation proceeds through a previously undescribed reaction sequence in which reduced peroxidase and compound III participate as the major enzyme intermediates and $O_2^{-}\cdot$ is a major product. This novel reaction sequence may provide an insight into the mechanism of action of IAA as a plant growth regulator.

Materials and Methods

Ultrapure reagent grade water (Nanopure, Sybron/Barnstead) of specific resistance in excess of 17.5 mΩ cm^{-1} was used to make all solutions and to thoroughly wash all glassware. Solutions, and any transfers of these solutions, were made in a laminar flow, clean air cabinet. The experiments described cannot be repeated unless extreme care is taken to eliminate, as far as possible, trace organic and metal contaminants from all glassware and reagents.

Reagents. Horseradish peroxidase grade 1 (specific activity ca. 250 IU mg^{-1}) obtained from Boehringer Mannheim, was used in all experiments. Key experiments were repeated by using HRP type 8, acidic isoenzyme, and HRP type 9, basic isoenzyme supplied by Sigma Chemical Co. Two sources of IAA were used in all experiments (Sigma Chemical Co. and Calbiochem-Behring Corp.).

Superoxide dismutase, Cu–Zn type 1 from bovine blood (specific activity 3000 units mg^{-1}), was obtained from Sigma and analytical grade beef liver catalase (specific activity ca. 65 000 IU mg^{-1}) from Boehringer Mannheim. Care must be exercised with catalase as some samples oxidized IAA at an appreciable rate. The rate varied from sample to sample but increased as the pH of the reaction solution was varied from pH 5 to 7.

Hydrogen peroxide, 100 volumes of Aristar, was supplied by BDH Chemicals, and all buffers used were of analytical reagent grade.

Kinetics of IAA Oxidation. Oxidation of IAA by HRP was followed at 261 nm by using a Varian Techtron Model 635 UV–vis spectrophotometer fitted with a thermostated cell holder. Increase in absorbance at 261 nm is almost exactly proportional to substrate disappearance over a wide range of conditions (Ray & Thimann, 1955).

Experiments were made in 3-mL capacity, 1-cm path-length cuvettes. HRP was used routinely at 2.3×10^{-8} and 2.3×10^{-7} M. IAA concentrations used varied between 20 μM and 0.5 mM. Oxidation rates of higher concentrations of substrate could not be measured accurately because of their high optical density at 261 nm. Effects of SOD, catalase, SOD plus catalase, and H_2O_2 on kinetics of IAA oxidation were measured. Most experiments were done at 25 °C in 50 mM citrate buffer, pH 5.0. Key experiments were repeated at pH 4.0 and

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[1] Abbreviations: IAA, indole-3-acetic acid; $O_2^{-}\cdot$, superoxide anion radical; HRP, horseradish peroxidase; Fe_p^{3+}, native peroxidase; Fe_p^{2+}, reduced peroxidase; Fe_p^{2+}–CO, carbon monoxide adduct of reduced peroxidase; Fe_p^{3+}–$O_2^{-}\cdot$, CMPIII, compound III of peroxidase; SOD, bovine superoxide dismutase; H_2O_2, hydrogen peroxide.

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5.8 in citrate and at pH 5.8 in 50 mM phosphate buffer. In some experiments, temperature was varied between 10 and 35 °C. Rates of oxidation were measured over the linear range of absorbance changes at 261 nm assuming that complete oxidation of 0.1 mM IAA causes a ΔA of 0.22. This value was obtained by reacting 0.1 mM IAA with 2.3×10^{-8} M HRP under our standard conditions for 2 h. These concentrations of reactants gave linear rates of oxidation for up to 20 min.

A major limitation to the use of this method to measure oxidation of IAA is that if any treatment used caused an alteration in product distribution then this would be reflected in an apparent change in the kinetics of oxidation. To overcome this problem, similar experiments were done in an oxygen electrode, and oxygen uptake was used to measure the kinetics of oxidation of IAA (Yamazaki & Yamazaki, 1973).

Oxygen Uptake Studies. An oxygen electrode, fitted with a glass reaction chamber (Rank Bros, Cambridge, United Kingdom), was used in this study. In most experiments, HRP was used at 4.2×10^{-8} M in 50 mM citrate buffer, pH 5.0, at 25 °C and with concentrations of IAA ranging from 20 μM to 5 mM. Effects of SOD, catalase, SOD plus catalase, and H_2O_2 on oxygen uptake were also measured. Oxygen uptake in these experiments was linear for at least 5 min. With the high concentrations of IAA, the rate of oxidation increased dramatically when the oxygen concentration fell below about 50 μM.

Spectral Studies of Enzyme Intermediates. Changes in the redox state of HRP, as indicated by the formation of different enzyme compounds (Yamazaki & Yokota, 1973) during oxidation of IAA, were followed by using a Hewlett-Packard Model 8450 UV–vis spectrophotometer fitted with a Model 7245A printer plotter and a Model 9875A cartridge tape unit. The unique design features of this spectrophotometer enabled instantaneous recording of the complete spectrum between 200 and 800 nm. The spectrum of HRP could be monitored even in the visible range with enzyme concentrations as low as 2.3×10^{-8} M and concentrations of IAA ranging from 1 μM to 5 mM when experiments were done in 10-cm path-length cuvettes. When concentrations of both HRP and IAA were varied, spectral changes in the enzyme could be followed over a wide range of enzyme/substrate ratios.

Most experiments were done in 50 mM citrate, pH 5.0, at 25 °C, but some studies were done at pH 4.0 in citrate and at pH 5.8 in 50 mM phosphate buffer. The effect of SOD and H_2O_2 on the spectrum of HRP during oxidation of IAA was examined. Catalase effects could not be monitored properly because its spectrum overlies the spectrum of HRP.

A series of experiments was done under atmospheres of nitrogen or carbon monoxide to determine whether Fe_p^{2+} was involved as an enzyme intermediate under the various reaction conditions. Buffer, degassed by boiling for 10 min and cooled under N_2, was used, and measurements were made in 1-cm path-length cuvettes. Care was taken to restrict reentry of oxygen or loss of carbon monoxide. HRP was used at 2.3×10^{-8}, 2.3×10^{-7}, 2.3×10^{-6}, 4.2×10^{-8}, 4.2×10^{-7}, and 4.2×10^{-6} M; IAA concentrations ranged from 0.1 to 5 mM.

In all experiments, spectral data were recorded at 10 and 30 s and 1, 2, 5, 7.5, and 10 min after the oxidation of IAA began. Where substrate was not limiting, additional spectra were recorded every 2 min from 10 to 20 min after the oxidation started.

All the experiments reported in this paper, whether done with the spectrophotometers or the oxygen electrode, were repeated on numerous occasions. The specific result presented is typical for the particular experiment.

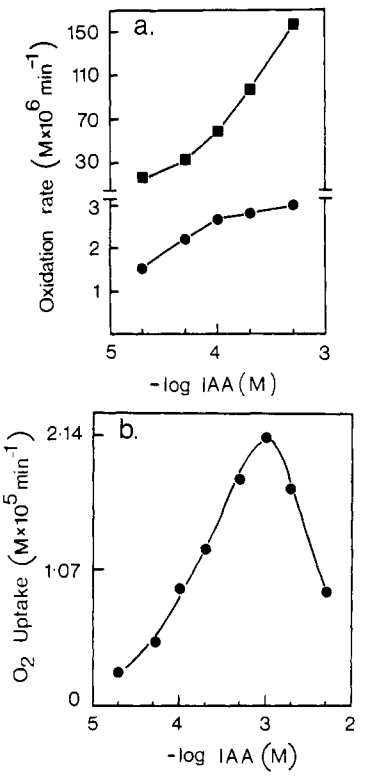

FIGURE 1: Effect of enzyme/substrate ratios on rate of oxidation of IAA by HRP in 50 mM citrate, pH 5, at 25 °C. (a) (●) 2.3×10^{-8} M HRP; (■) 2.3×10^{-7} M HRP; oxidation rate measured at 261 nm. (b) (●) 4.2×10^{-8} M HRP; oxidation rate determined from oxygen uptake.

Results

Kinetics of IAA Oxidation. The rate of oxidation of IAA catalyzed by 2.3×10^{-8} M HRP, as measured at 261 nm in the spectrophotometer, increased only marginally when concentrations of substrate were increased above 0.1 mM. Conversely, with the 2.3×10^{-7} M HRP, the oxidation rate of IAA increased sharply over this same range of substrate concentration (Figure 1a). The oxidation rate of IAA increased by much more than an order of magnitude when the concentration of HRP was increased from 2.3×10^{-8} to 2.3×10^{-7} M (Figure 1a). Similar results were obtained by using different buffers, pH values, temperatures, and sources of IAA and HRP. Double-reciprocal plots of the spectrophotometric data in Figure 1a gave values of K_m = 19 μM IAA and V_m = 3.2 μM min^{-1} for 2.3×10^{-8} M HRP and K_m = 0.37 mM IAA and V_m = 0.27 mM min^{-1} for 2.3×10^{-7} M HRP. Valid K_m values may be defined for oxidations catalyzed by peroxidase, although such reactions contain no hint of reversibility or of complex formation (Dunford & Stillman, 1976). Spectral evidence indicated that the products of oxidation of IAA were identical with both these concentrations of HRP. Therefore, the rate-limiting step in the oxidation of IAA was different at each of these enzyme concentrations.

Variations in substrate concentration also markedly affected the kinetics of oxidation when 4.2×10^{-8} M HRP oxidized IAA over a concentration range of 20 μM to 5 mM in the oxygen electrode. Substrate inhibition was evident at concentrations of IAA in excess of 1 mM (Figure 1b).

HRP Spectrum. The classical spectral changes recorded in the literature (Yamazaki & Yokoto, 1973) occurred when 2.3×10^{-7} M or higher concentrations of HRP were used to oxidize IAA at concentrations up to 0.5 mM in citrate buffer,

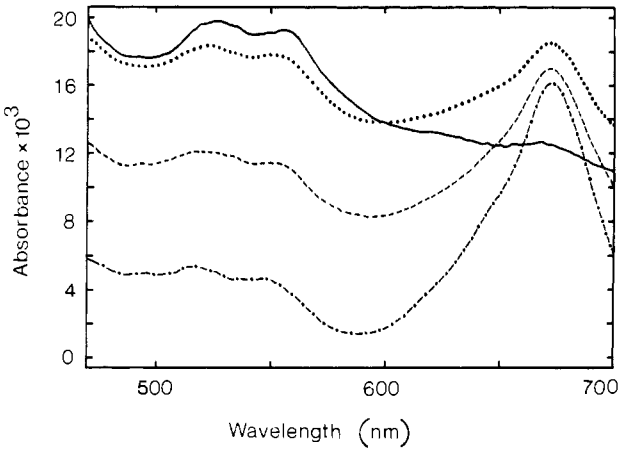

FIGURE 2: Spectrum of 1×10^{-6} M HRP during oxidation of 0.1 mM IAA in 50 mM citrate, pH 5, at 25 °C after (—) 10 s, (···) 30 s, (- - -) 1 min, and (-·-) 5 min (1-cm light path). λ_{max}(CMPII) 527 and 554 nm; λ_{max}(CMPIV) 670 nm.

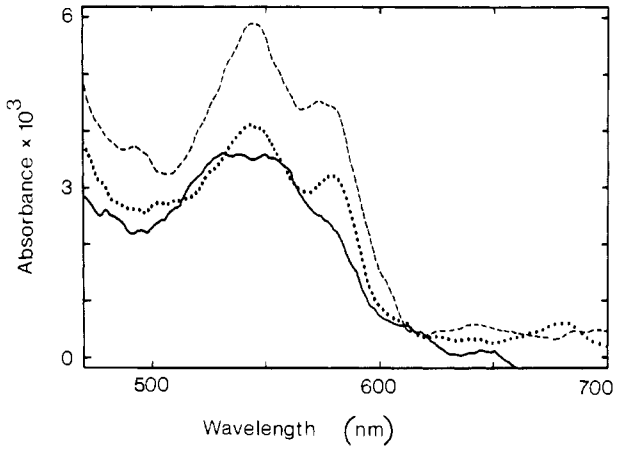

FIGURE 3: Increase in compound III formation by 4.2×10^{-8} M HRP after 10-s oxidation of IAA at (—) 50 μM, (···) 0.5 mM, and (- - -) 5 mM in 50 mM citrate, pH 5, at 25 °C (10-cm light path). λ_{max}(CMPII) 527 and 554 nm; λ_{max}(CMPIII) 546 and 583 nm.

pH 5, at 25 °C. Only the spectrum of the Fe_p^{3+}, or native enzyme, was seen when HRP was added to buffer. After IAA was added, compound II initially dominated the spectrum. Compound IV, or P-670, appeared within 30 s and increased rapidly in concentration (Figure 2). As the concentration of P-670 increased , there was a concomitant decrease in the rate of oxidation of IAA.

Vastly different spectral changes occurred when HRP was used at concentrations up to 4.2×10^{-8} M and IAA in excess of 50 μM. When concentrations up to 4.2×10^{-8} M HRP were added alone to either citrate or phosphate buffers, virtually all the enzyme was present as compound II and not as Fe_p^{3+}. Only the Fe_p^{3+} spectrum was visible, however, if these low concentrations of HRP were added to reagent grade water. Similarly, only the Fe_p^{3+} spectrum was seen if 3.3×10^{-9} M catalase was added to the citrate and phosphate buffers before the HRP was introduced. Thus, these buffers, freshly made in a clean system, contained sufficient H_2O_2 to oxidize virtually all the native form of peroxidase when HRP was added at concentrations up to 4.2×10^{-8} M.

When concentrations of IAA in excess of 50 μM were added to either citrate buffer or phosphate buffer containing up to 4.2×10^{-8} M HRP, most of the enzyme was rapidly transformed to compound III which predominated over compound II and P-670 throughout the reaction (Figure 3). More compound III formed, and it formed more rapidly when the concentration of IAA was increased above 50 μM (Figure 3). Compound II predominated over compound III for most of the reaction only when IAA was used at concentrations below 10 μM. Even with these low concentrations of IAA (1–10 μM), some compound III still formed.

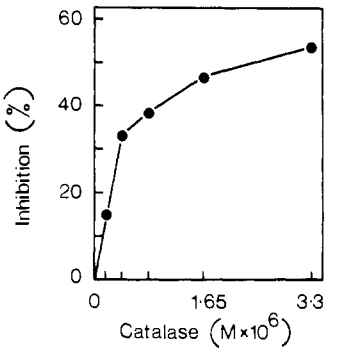

FIGURE 4: Catalase inhibition of oxidation of 0.1 mM IAA by 2.3×10^{-8} M HRP in 50 mM citrate, pH 5, at 25 °C; oxidation rate measured at 261 nm.

These experiments were repeated under an atmosphere of carbon monoxide and the Fe_p^{2+}–CO spectrum was clearly present in both the Soret (λ_{max} 423 nm) and visible (λ_{max} 542 and 572 nm) regions when IAA in excess of 50 μM was reacted with HRP at concentrations of up to 4.2×10^{-8} M. More of the Fe_p^{2+}–CO complex formed, and it formed faster the higher the IAA concentration used. A similar trend occurred with the 10^{-7} M range of HRP except that the Fe_p^{2+}–CO complex formed rapidly only when the IAA was used at concentrations in excess of 0.5 mM. With the 10^{-6} M range of HRP, only a trace of the Fe_p^{2+}–CO complex formed and then only when 5 mM IAA was used as substrate.

When these experiments were repeated under N_2 but in the absence of carbon monoxide, the classical Fe_p^{2+} spectrum appeared in the Soret (λ_{max} 440 nm) and visible (λ_{max} 557 nm, shoulders 510 and 580 nm) regions when either 2.3×10^{-7} or 4.2×10^{-7} M HRP was reacted with 1 mM or higher concentrations of IAA. Fe_p^{2+} was not detected with either the 10^{-8} M or 10^{-6} M range of HRP even when IAA was used at 5 mM. Oxidation of IAA proceeded slowly for at least 15 min with the 10^{-8} M HRP range, indicating that sufficient oxygen was present to sustain the reaction. Under such conditions, Fe_p^{2+} would be unlikely to accumulate even if it was an intermediate in the reaction (Phelps et al., 1974). Conversely, with the 10^{-6} M HRP range oxidation of IAA stopped within 10 s, indicating that any residual oxygen was utilized. Fe_p^{2+} still did not accumulate and thus appears unlikely to be an important intermediate when these high concentrations of HRP were used to catalyze oxidation of IAA.

Catalase Effects. Catalase at concentrations of 1.65×10^{-7} M or higher caused a marked inhibition in the rate of oxidation of 0.1 mM IAA by 2.3×10^{-8} M HRP (Figure 4). Conversely, concentrations of catalase at up to 3.3×10^{-6} M had no inhibitory effects on the rate of oxidation of 0.1 mM IAA when the concentration of HRP was increased to 2.3×10^{-7} M.

Oxygen uptake during oxidation of 1 mM IAA by 4.2×10^{-8} M HRP was also inhibited markedly by additions of 1.65×10^{-7} M or higher concentrations of catalase. When the concentration of IAA was increased to 5 mM, catalase additions of even 3.3×10^{-6} M had no inhibitory effects on oxygen uptake with 4.2×10^{-8} M HRP.

Additions of catalase, therefore, produced variable effects on reaction kinetics of the oxidation of IAA depending on the enzyme/substrate ratio used in any particular experiment.

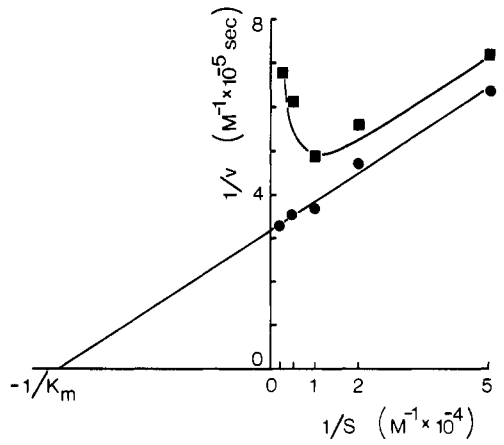

FIGURE 5: Double-reciprocal plots of rates of oxidation of a range of IAA concentrations by 2.3×10^{-8} M HRP in the (■) presence and (●) absence of 1×10^{-7} M SOD. Rates measured at 261 nm in 50 mM citrate, pH 5, at 25 °C.

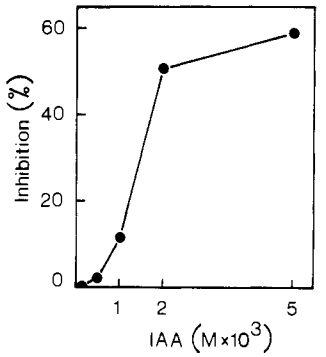

FIGURE 6: SOD, 1×10^{-7} M, inhibition in oxygen uptake when 4.2×10^{-8} M HRP oxidized a range of IAA concentrations in 50 mM citrate, pH 5, at 25 °C.

SOD Effects. SOD added at 1×10^{-7} M (active protein) inhibited the oxidation of IAA within the 10^{-8} M HRP range as measured by either oxygen uptake or spectral changes at 261 nm. Inhibition caused by SOD increased markedly as the concentration of IAA increased (Figures 5 and 6). Higher concentrations of SOD did not increase the degree of inhibition, but lower concentrations caused less inhibition.

Conversely, SOD caused no inhibition of the oxidation rate when 2.3×10^{-7} M HRP was used to oxidize IAA at concentrations ranging from 20 μM to 0.5 mM. Thus, the inhibitory effect of SOD on reaction kinetics appears to be correlated with the type of mechanism that dominated the oxidation of IAA.

Additions of SOD did not cause any measurable changes in the Soret or visible spectrum of HRP in any of these experiments. Compound III continued to dominate the spectrum in the presence of SOD within the 10^{-8} M HRP range whereas compound II and P-670 dominated the spectrum within the 10^{-7} M HRP range.

SOD plus Catalase Effects. Virtually all the inhibitory effects caused by addition of 1×10^{-7} M SOD could be prevented by the simultaneous addition of low concentrations of catalase which, when added alone, had neither inhibitory nor stimulatory effects on the rates of oxidation of IAA catalyzed by HRP. Thus, the addition of 6.6×10^{-8} M catalase at the start of the reaction prevented the inhibitory effects caused by 1×10^{-7} M SOD when 2.3×10^{-8} M HRP oxidized 0.1 mM IAA under our standard conditions in the spectrographic studies. Catalase at concentrations lower than 6.6×10^{-8} M had either no effect or only partially reversed the inhibitory effect of the SOD treatment. Similar results were obtained in the oxygen uptake studies where vastly different concentrations of reactants were used (Table I).

Table I: Effects of Addition of SOD, Catalase, and SOD Catalase on Oxygen Uptake during Oxidation of 5 mM IAA by 4.2×10^{-8} M HRP and 50 mM Citrate Buffer, pH 5.0, at 25 °C

treatment	$d[O_2]$ ($\times 10^5$) (M min^{-1})/dt	inhibition (%)
control	0.82	
1×10^{-7} M SOD	0.33	60
1.65×10^{-6} M catalase	0.83	0
1×10^{-7} M SOD + 1.65×10^{-6} M catalase	0.85	0
1×10^{-7} M SOD + 6.6×10^{-7} M catalase	0.51	38

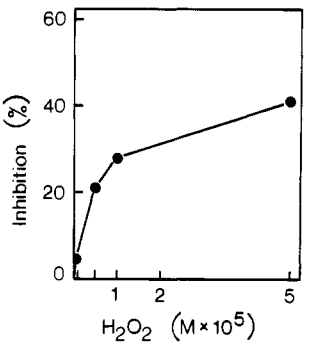

FIGURE 7: H_2O_2 inhibition of the oxidation rate of 0.1 mM IAA by 2.3×10^{-8} M HRP in 50 mM citrate, pH 5, at 25 °C; oxidation rate measured at 261 nm.

H_2O_2 Effects. When added at the start of the reaction at concentrations from 2 to 50 μM, H_2O_2 inhibited the rate of oxidation of 0.1 mM IAA by 2.3×10^{-8} M HRP as measured at 261 nm in the spectrophotometer (Figure 7). The inhibitory effects of the H_2O_2 additions were prevented by adding 6.6×10^{-8} M catalase after the H_2O_2 but before the oxidation of IAA began. Addition of catalase after oxidation began only partially removed the inhibitory effects of the H_2O_2. H_2O_2 at concentrations from 0.01 to 1 μM had neither inhibitory nor stimulatory effects on the rate of oxidation of IAA under these experimental conditions.

Spectral studies on HRP under the conditions of these experiments showed that additions of H_2O_2 from 2 to 50 μM caused an increase in the rate of formation and final concentration of P-670. Additions of lower concentrations of H_2O_2 caused no detectable spectral changes in HRP. When H_2O_2 was added in excess of 0.1 mM, an increase in formation of compound III occurred even in the absence of IAA.

H_2O_2 added at 10 μM and 0.1 mM also inhibited oxygen uptake by 16% and 44%, respectively, when 4.2×10^{-8} M HRP catalyzed the oxidation of 1 mM IAA in the oxygen electrode.

Discussion

Kinetic and spectral data obtained in the study established that peroxidase may oxidize IAA by either of two pathways. The accepted pathway (pathway 1, Figure 8) dominates the reaction when relatively high enzyme/substrate ratios (e.g., [HRP] > 2×10^{-7} M; [IAA] < 0.5 mM) are used. A second pathway which we call the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle (pathway 2, Figure 8) operates when low enzyme/substrate ratios (e.g., [HRP] < 4×10^{-8} M; [IAA] > 50 μM) are used. Other researchers have argued that peroxidase oxidizes IAA by different pathways when enzyme/substrate ratios are varied (Miller & Parups, 1971). No spectral information was

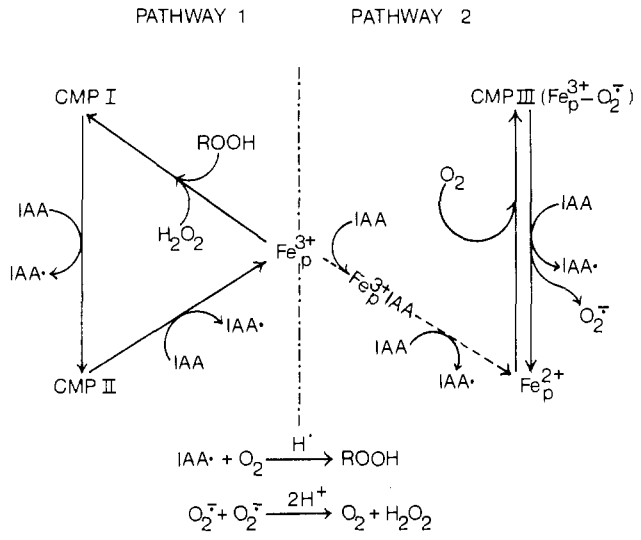

FIGURE 8: Pathways for oxidation of IAA by peroxidase: pathway 1, accepted reaction sequence; pathway 2, our model of the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. In pathway 1, either H_2O_2 or the organic hydroperoxide may react with Fe_p^{3+} to form CMPI. The organic hydroperoxide may form in either pathway from a reaction between the IAA free radical and oxygen. $O_2^{-}\cdot$ produced via pathway 2 spontaneously dismutates to form O_2 and H_2O_2. In pathway 2, the step between Fe_p^{3+} and Fe_p^{2+} is shown as a broken line. Fe_p^{2+} is an integral intermediate in pathway 2, but its formation as a consequence of multiple binding of IAA molecules to Fe_p^{3+} is speculative.

presented, however, to enable identification of the peroxidase intermediates involved in the oxidation under their low enzyme/substrate ratios.

Identification of the different redox states of peroxidase relies on their characteristic visible spectra which have low extinction coefficients (Dunford & Stillman, 1976). Therefore, most previous work on the HRP intermediates involved in the oxidation of IAA has been restricted to high enzyme/substrate ratios (e.g., [HRP] > 1 μM; [IAA] $\sim 10^{-4}$ M). This probably explains why Nakajima & Yamazaki (1979) concluded that Fe_p^{2+} and compound III were not involved in the oxidation of IAA by HRP. Their experiments were done with enzyme/substrate ratios that in our experience would ensure major participation of pathway 1.

The kinetic evidence and spectral evidence strongly support our proposed reaction sequence for pathway 2. Compound III constituted the steady-state form of peroxidase in experiments where pathway 2 predominated (Figure 3). The marked reduction in the rate of oxidation of IAA under such conditions is consistent with the differences recorded in the reaction rate of IAA with compound III ($k = 7.1 \times 10^2$ M^{-1} s^{-1}) as compared with compound II ($k = 1.8 \times 10^4$ M^{-1} s^{-1}), the rate-limiting step in pathway 1 (Yamazaki & Yokota, 1973). Spectral evidence from experiments done under N_2 or carbon monoxide indicated that Fe_p^{2+} is an integral intermediate in pathway 2. The significance of Fe_p^{2+} formation during the oxidation of IAA by HRP requires cautious interpretation because peroxidase may be reduced directly by IAA free radicals in a minor side reaction (Yamazaki & Yokota, 1973). This reaction does not provide a satisfactory explanation for the formation of Fe_p^{2+} in our experiments. Reaction rates and, thus, IAA free radical flux were greatest with 10^{-6} M HRP, but only a trace of Fe_p^{2+} was detected in experiments where these high enzyme concentrations were used. Conversely, high concentrations of Fe_p^{2+} formed rapidly with lower concentrations of enzyme provided that the enzyme/substrate ratios favored participation of pathway 2. Fe_p^{2+} reacts rapidly with oxygen to form compound III (Phelps et al., 1974) and would be undetectable as an enzyme intermediate in experiments done in the presence of oxygen. Under reduced oxygen tension, Fe_p^{2+} may react directly with compound III to form Fe_p^{3+} (Phelps et al., 1974). This reaction may explain why the oxidation rate increased sharply when the concentration of dissolved oxygen fell below about 50 μM in experiments where 4.2×10^{-8} M HRP catalyzed the oxidation of IAA at concentrations in excess of 0.5 mM in the oxygen electrode. Peroxidase would be diverted from pathway 2 to pathway 1 by such a reaction, and the oxidation rate of IAA could be expected to increase.

Addition of SOD in these experiments provided compelling evidence that $O_2^{-}\cdot$ is an important product of pathway 2 but not of pathway 1. As enzyme/substrate ratios were lowered and more of the oxidation proceeded via pathway 2, SOD addition caused an increased inhibition of reaction rates (Figures 5 and 6). This inhibition in reaction rates was prevented by the simultaneous addition of low concentrations of catalase which had no direct effect when added alone. These results indicate that SOD indirectly inhibited the reaction rates by increasing the steady-state concentration of H_2O_2 through catalytic dismutation of $O_2^{-}\cdot$. P-670, a catalytically inactive form of peroxidase (Nakajima & Yamazaki, 1980), increased in concentration when H_2O_2 was present in excess of 2 μM. Ray (1962) found also that concentrations of H_2O_2 in excess of ~ 5 μM inhibited the rate of oxidation of IAA by peroxidase. Even in the presence of SOD, compound III remained the dominant steady-state form of peroxidase in experiments where oxidation of IAA proceeded mainly through pathway 2. Compound III may form from a relatively slow reaction between Fe_p^{3+} and $O_2^{-}\cdot$ (Bielski et al., 1974; Yamazaki & Yokota, 1973), but obviously, this reaction is not responsible for compound III formation in our experiments.

Experiments done under identical conditions in both the spectrophotometer and the oxygen electrode showed that 1.5 mol of O_2 was consumed per mol of IAA oxidized when the oxidation proceeded predominantly through pathway 2. Conversely, a ratio of 1/1 was recorded when the oxidation was resticted to pathway 1. These ratios agree exactly with predictions that are implicit in our proposed reaction sequences in Figure 8. It should be noted that in pathway 2 spontaneous dismutation of $O_2^{-}\cdot$ will return 1 mol of O_2 for every 2 mol of $O_2^{-}\cdot$ produced.

Our experiments do not explain why pathway 2 is activated only when there is a large excess of substrate to enzyme (i.e., at low enzyme/substrate ratios). Spectral studies confirmed that under such conditions peroxidase is reduced rapidly to Fe_p^{2+} which, in our model, is a prerequisite for operation of pathway 2. Burns et al. (1976) were unable to show direct reduction of Fe_p^{3+} by IAA, but their experiments were done with high enzyme/substrate ratios that would have precluded activation of pathway 2. Our model (Figure 8) proposes that the reduction of Fe_p^{3+} by IAA at low enzyme/substrate ratios depends upon multiple binding of substrate molecules to the native enzyme. Peroxidase possesses a large substrate binding pocket (Williams et al., 1977) which could feasibly accommodate more than one molecule of IAA. Binding of a substrate molecule causes a conformational change to the protein structure of peroxidase (Williams et al., 1977) which may conceivably favor direct electron transfer to the iron porphyrin center from another bound substrate molecule. NMR studies using low enzyme/substrate ratios compatible with activation of pathway 2 should resolve this question of multiple binding of IAA to Fe_p^{3+}.

Confusion abounds in the literature with regard to the essential requirement of H_2O_2 for oxidation of IAA by peroxidase (Yamazaki, 1974). In this respect it is significant that our results show that buffers, even when freshly prepared in a clean system, may contain in excess of 10^{-8} M H_2O_2. In experiments under our standard conditions (2.3×10^{-8} and 4.2×10^{-8} M HRP; 0.1 and 1 mM IAA, respectively), where oxidation proceeds simultaneously through both pathways 1 and 2, catalytic amounts of catalase inhibited the rate of oxidation of IAA, indicating that H_2O_2 is required for the maximum rate of reaction (Figure 4). Conversely, in experiments where virtually only pathway 1 (2.3×10^{-7} M HRP; 0.1 mM IAA) or pathway 2 (4.2×10^{-8} M HRP; 5 mM IAA) operated, catalase had no inhibitory effects on reaction kinetics. The reaction sequences described in Figure 8 offer an explanation for these apparent contradictory results. When oxidation is restricted to pathway 1, there is no continual source of H_2O_2, and presumably, the reaction is carried by the organic hydroperoxide. Similarly, catalase is without effect when oxidation is restricted to pathway 2 because IAA and oxygen are then the essential reactants rather than either H_2O_2 or the organic hydroperoxide. Under conditions where oxidation proceeds simultaneously through both pathways, H_2O_2 will be produced slowly from dismutation of $O_2^{-}\cdot$ formed in the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. Provided H_2O_2 reacts faster than the organic hydroperoxide with native peroxidase, catalase may be expected to slow reaction kinetics as recorded under our standard conditions.

An important feature of pathway 2 is that once peroxidase enters the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle it remains there whenever IAA (or, perhaps, some other reductant) and oxygen are present. Peroxidase then behaves as a terminal oxidase because it both binds oxygen and accepts electrons, although $O_2^{-}\cdot$ is the product rather than either H_2O or H_2O_2. Other research has shown $O_2^{-}\cdot$ production from compound III in the presence of adrenaline (Rotilio et al., 1975).

Peroxidase is a ubiquitous enzyme in plants and fungi (Saunders et al., 1964), and activation of the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle under physiological conditions could have considerable biological significance. This shuttle produces a flux of $O_2^{-}\cdot$ at the expense of molecular oxygen. Irrefutable evidence now exists that production of $O_2^{-}\cdot$ has far-reaching biological consequences that can be either deleterious or beneficial (Fridovich, 1976; Halliwell, 1979; Michelson et al., 1977). Whether these effects are directly or indirectly caused by $O_2^{-}\cdot$ is open to argument (Bors et al., 1980; Fee, 1980). Furthermore, compound III which acts as a specific oxidase (Yamazaki & Yokota, 1973) constitutes the dominant steady-state form of peroxidase whenever the shuttle is activated. Compound III may be considered as an enzyme-bound form of $O_2^{-}\cdot$, and we have shown that free $O_2^{-}\cdot$ may be regenerated in the presence of IAA. When bound in compound III, $O_2^{-}\cdot$ is protected from dismutation by SOD (Rotilio et al., 1975) and, thus, may be transported within cells. Reactivity of this enzyme-bound $O_2^{-}\cdot$ is vastly different from that of free $O_2^{-}\cdot$ which is a very poor oxidant but a good reductant (Sawyer & Gibian, 1979). These differences in reactivity are consistent with the failure of $O_2^{-}\cdot$ to directly oxidize IAA (unpublished data) whereas facile oxidation occurs with compound III. If this shuttle operates under physiological conditions, the formation of $O_2^{-}\cdot$ and compound III may provide a molecular basis to explain the mechanism of action of IAA as a plant growth regulator.

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