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Ethylene Enhances Reactivity of Superoxide with Peroxidase To Form the Oxy-Ferrous Complex†

Alan M. Smith,* Wendy L. Morrison, and Paul J. Milham

ABSTRACT: Ethylene and its analogues acetylene, carbon monoxide, and propylene inhibited the rate of oxidation of indole-3-acetic acid by peroxidase. Annulment of this effect by addition of superoxide dismutase showed that inhibition occurred only in the presence of the superoxide anion radical ($O_2^{-}\cdot$). Kinetic and spectral data established that ethylene and its analogues enhanced markedly the rate of reaction of $O_2^{-}\cdot$ with peroxidase. This reaction resulted in the formation of compound III, an oxy-ferrous complex of peroxidase. In the presence of indole-3-acetic acid, the interaction between ethylene, peroxidase, and $O_2^{-}\cdot$ activated the reduced peroxidase $\rightleftarrows$ compound III shuttle. $O_2^{-}\cdot$ is a major product of this shuttle, and compound III constitutes the dominant steady-state form of peroxidase. These interactions may help to explain the mechanism of action of ethylene as a plant growth regulator.

Peroxidase (EC 1.11.1.7) may oxidize indole-3-acetic acid (IAA)[1] by either of two pathways depending on the enzyme/substrate ratio (Smith et al., 1982). When relatively low enzyme/substrate ratios (e.g., [peroxidase] $< 4 \times 10^{-8}$ M; [IAA] $> 50\ \mu$M) are employed, the oxidation proceeds through a reduced peroxidase $\rightleftarrows$ 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 (e.g., [peroxidase] $> 2 \times 10^{-7}$ M; [IAA] < 0.5 mM) are used.

The oxy-ferrous[2] complex called compound III, which acts as a specific oxidase, constitutes the dominant steady-state form of peroxidase when the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle is operational. Activation of this shuttle also produces a flux of $O_2^{-}\cdot$ at the expense of molecular oxygen. We proposed that this novel reaction sequence may provide a molecular basis to explain the mechanism of action of IAA as a plant growth regulator (Smith et al., 1982).

The present study examines the effect of ethylene, another plant hormone, and some of its analogues on the oxidation of IAA by peroxidase. Ethylene was used because an intimate relationship is claimed to exist between it and IAA in their effects on plant growth (Abeles, 1973; Lieberman, 1979; Osborne, 1978). In fact, this relationship has been referred to as an IAA–ethylene feedback mechanism (Lieberman, 1979, 1980). Furthermore, ethylene and acetylene have been shown to interact with, and cause the suicidal destruction of, another hemoprotein, cytochrome P-450 (Ortiz de Montellano & Mico, 1980; Ortiz de Montellano et al., 1981).

Ethylene and its analogues[3] acetylene, propylene, and carbon monoxide were used with an extremely wide range of concentrations of IAA and peroxidase to ensure that oxidation would proceed both through the conventionally accepted pathway and through the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. Ethylene and its analogues affected the kinetics of oxidation of IAA and formation of enzyme intermediates only when the oxidation proceeded predominately through the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. These results and their potential biological significance are discussed.

Materials and Methods

The general methods of preparation and execution of these experiments have been described (Smith et al., 1982). Attention is again drawn to the stringent requirements for prevention of trace metal and organic contamination of glassware, reagents, etc.

Reagents. Suppliers and types of enzymes and reagents used have been specified previously (Smith et al., 1982). Boehringer Mannheim samples of horseradish peroxidase and the two specified sources of IAA were used in all experiments. The basic and acidic isoenzymes of peroxidase from Sigma Chemical Co. were used also in all key experiments.

† From the Biological and Chemical Research Institute, New South Wales Department of Agriculture, Rydalmere, 2116, New South Wales, Australia. *Received October 7, 1982.*

[1] Abbreviations: IAA, indole-3-acetic acid; $O_2^{-}\cdot$, superoxide anion radical; HRP, horseradish peroxidase; Fe_p^{2+}, reduced peroxidase; Fe_p^{3+}–$O_2^{-}\cdot$, CMPIII, compound III, an oxy-ferrous complex of peroxidase; Fe_p^{3+}–C_2H_4, complex between native peroxidase and ethylene; SOD, bovine Cu–Zn superoxide dismutase; H_2O_2, hydrogen peroxide; C_2H_4, ethylene.

[2] Terminology of Dunford & Pryor (1981).

[3] Terminology of Abeles (1973).

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Ethylene, acetylene, oxygen-free nitrogen, and carbon dioxide were obtained from Commonwealth Industrial Gases and carbon monoxide and propylene from Matheson.

Effect of Ethylene on Kinetics of IAA Oxidation. Buffer (4 mL), 50 mM, was dispensed into 14-mL capacity glass vials which were sealed with Teflon-lined, silicon disks (Pierce Chemical Co., Rockford, IL). Ethylene was injected through the disks with a glass, gas-tight syringe, in injections of up to 0.5 mL, to give desired final concentrations of ethylene in the headspace. Before injection of ethylene, an equal volume of air was syringed from the headspace to ensure pressure equilibration. Control vials had oxygen-free nitrogen injected into them in place of ethylene. All vials were shaken vigorously to equilibrate the gas concentration in the headspace with the buffer, and they were incubated in a water bath at the desired temperature.

Experiments were made in 3-mL capacity, 1-cm path-length cuvettes. Gas-equilibrated buffer (2.8 mL) was dispensed into cuvettes and substrate added, and cuvettes were capped and their contents mixed. IAA concentrations ranged from 20 μM to 0.5 mM. HRP at 2.3×10^{-8} or 2.3×10^{-7} M was the final reagent added.

Oxidation of IAA by HRP in the presence and absence of different concentrations of ethylene was followed at 261 nm in the spectrophotometer as already described (Smith et al., 1982). Most experiments were done at 25 °C in 50 mM citrate buffer, pH 5.0. Key experiments were repeated at pH 4.0 and 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. HRP concentrations were varied also in some experiments. Effects of additions of superoxide dismutase (SOD) (EC 1.15.1.1), catalase (EC 1.11.1.6), and SOD plus catalase on kinetics of IAA oxidation in the presence and absence of ethylene were also measured with the spectrophotometer.

Effect of Ethylene on Oxygen Uptake. An oxygen electrode, fitted with a glass reaction chamber (Rank Bros., Cambridge, United Kingdom), was used to study the effect of ethylene on the kinetics of oxidation of IAA by HRP.

The ethylene and control treatments were prepared as described in the spectrophotometer 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. Additions of SOD, catalase, and SOD plus catalase on the effects of ethylene on oxygen uptake were also measured.

Spectral Studies on Effects of Ethylene on Enzyme Intermediates. The Hewlett-Packard Model 8450 UV–vis spectrophotometer and accessories were used as already described (Smith et al., 1982) to study the spectrum of HRP during oxidation of IAA in the presence and absence of ethylene. Effects of addition of SOD in these experiments were also recorded. Catalase additions could not be monitored because its spectrum overlies the spectrum of HRP.

Effects of Ethylene Analogues on Kinetics of Oxidation of IAA. Effects of acetylene, carbon monoxide, and propylene on the kinetics of oxidation of IAA by HRP were measured both in the spectrophotometer and in the oxygen electrode. The Hewlett-Packard Model 8450 spectrophotometer was used also to monitor any effects of these analogues on the formation of peroxidase intermediates during oxidation of IAA.

These ethylene analogues were equilibrated with buffer in vials as described for the ethylene experiments. HRP was used at 2.3×10^{-8}, 4.2×10^{-8}, and 2.3×10^{-7} M with a range of concentrations of IAA. Experiments were made in 50 mM citrate buffer, pH 5.0, at 25 °C. Effects of additions of SOD, catalase, and SOD plus catalase on kinetics of oxidation of IAA in the presence of the ethylene analogues were also measured.

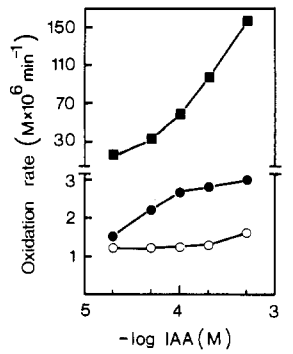

FIGURE 1: Effect of ethylene (50 mL L^{-1}) on rate of oxidation of IAA by HRP at a range of enzyme/substrate ratios in 50 mM citrate, pH 5, at 25 °C; oxidation rates measured at 261 nm. (●) 2.3×10^{-8} M HRP, control; (○) 2.3×10^{-8} M HRP plus ethylene; (■) 2.3×10^{-7} M HRP, control; 2.3×10^{-7} M HRP plus ethylene gave identical result to control.

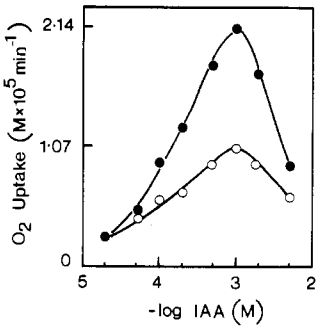

FIGURE 2: Effect of ethylene (50 mL L^{-1}) on uptake of oxygen during oxidation of a range of concentrations of IAA by 4.2×10^{-8} M HRP in 50 mM citrate, pH 5, at 25 °C. (●) Control; (○) plus ethylene.

All experiments reported, whether done in the presence of ethylene or its analogues, were repeated on numerous occasions. The result presented is typical for the particular experiment.

Results

Effects of Ethylene on Kinetics of Oxidation of IAA. Addition of ethylene had complex effects on the rate of oxidation of IAA catalyzed by HRP in both the spectrophotometer and oxygen electrode studies (Figures 1 and 2). In the spectrophotometer studies, ethylene inhibited the rate of oxidation of IAA at concentrations ranging from 20 μM to 0.5 mM when HRP was used at 2.3×10^{-8} M but had no effect when the concentration of HRP was increased to 2.3×10^{-7} M (Figure 1). In the oxygen electrode where 4.2×10^{-8} M HRP was used to catalyze oxidation of an extremely wide range of concentrations of IAA (20 μM to 5 mM), ethylene caused an inhibition in the oxidation rate at concentrations of IAA ranging from 50 μM to 5 mM (Figure 2).

Similar results occurred with ethylene irrespective of the type of HRP, source of IAA, buffer, pH, or temperature used. Spectral data confirmed that the products of oxidation were the same in the presence or absence of ethylene.

Results in Figures 1–3 show clearly that the degree of inhibition of oxidation rate of IAA caused by ethylene is a complex function of the concentration of IAA used. Maximum inhibition occurred with intermediate concentrations of IAA and declined with lower and higher concentrations of IAA. Ethylene caused no more than 50–60% inhibition in the rate

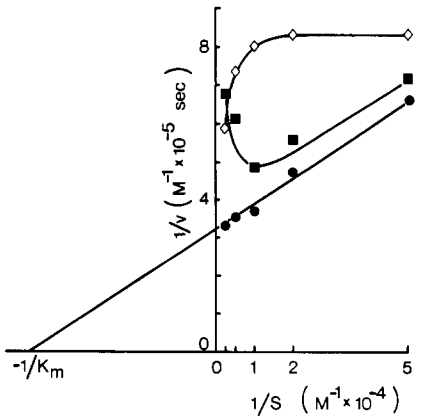

FIGURE 3: Double-reciprocal plots of rates of oxidation of a range of IAA concentrations by 2.3 $\times 10^{-8}$ M HRP in the (◇) presence of ethylene, 50 mL L^{-1} (data obtained from Figure 1), (■) presence of SOD, 1 $\times$ 10^{-7} M, and (●) control rate (data obtained from Figure 1) in the absence of both. Rates measured at 261 nm in 50 mM citrate, pH 5, at 25 °C. *S* is the concentration of IAA.

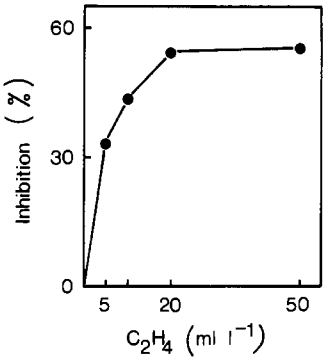

FIGURE 4: Relationship between ethylene concentration and inhibition in oxidation rate of 0.1 mM IAA by 2.3 $\times$ 10^{-8} M HRP as measured at 261 nm in 50 mM citrate, pH 5, at 25 °C.

of oxidation of IAA in any experiment.

Maximum inhibition of oxidation rate occurred at a concentration of ethylene of about 20 mL L^{-1} in the gas phase (Figure 4). A double-reciprocal plot of data in Figure 4 indicated a $K_i \sim 5$ mL L^{-1} for ethylene.

Interpretation of these complex ethylene effects became clearer when these results were compared with our previous studies on the oxidation of IAA by HRP (Smith et al., 1982). It appears probable that ethylene caused an inhibition in the oxidation rate of IAA only under conditions where the Fe_p^{2+} $\rightleftarrows$ CMPIII shuttle was activated but not when the conventionally accepted pathway (Figure 7) was predominant.

Effect of Ethylene on Enzyme Intermediates. Spectral studies of enzyme intermediates of HRP confirmed that ethylene inhibited the oxidation of IAA only when the reaction proceeded predominately through the Fe_p^{2+} $\rightleftarrows$ CMPIII shuttle. Under these conditions, compound III (λ_{max} 546 and 583 nm) constituted the major steady-state form of the enzyme. Ethylene treatments caused faster formation of, and a higher final concentration of, compound III. Thus, ethylene caused approximately 14% more compound III to form in 1 min when 2.3 $\times$ 10^{-8} M HRP oxidized 0.1 mM IAA under our standard conditions (Figure 5).

Compound II (λ_{max} 527 and 554 nm) was the major steady-state form of the enzyme under conditions where ethylene treatments did not inhibit the rate of oxidation of IAA. Compound II dominates the spectrum when peroxidase operates through the conventionally accepted pathway (Figure 7, pathway 1). Ethylene treatments caused no discernible change in the concentration of compound II.

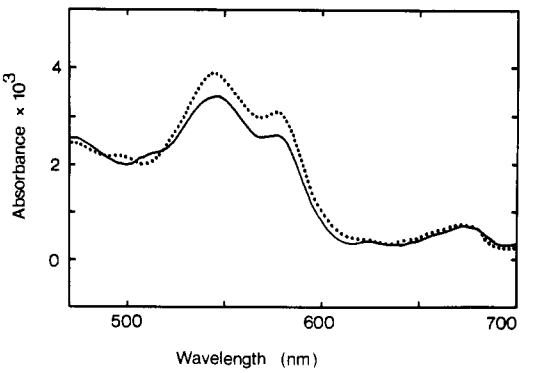

FIGURE 5: Increase in compound III formation caused by ethylene after 1-min oxidation of 0.1 mM IAA catalyzed by 2.3 $\times$ 10^{-8} M HRP in 50 mM citrate, pH 5, at 25 °C (10-cm light path). (···) Ethylene, 50 mL L^{-1}; (—) control. λ_{max}(CMPIII) 546 and 583 nm.

Table I: Effects of Additions of SOD,[a] Catalase,[b] and SOD plus Catalase on Ethylene Inhibition of Oxidation[c] of 0.1 mM IAA Catalyzed by 2.3 $\times$ 10^{-8} M HRP in 50 mM Citrate Buffer, pH 5.0, at 25 °C

treatment	IAA oxidation rate (M $\times$ 10^6 min^{-1})	inhibition (%)
control	2.32	
C_2H_4	1.18	49
C_2H_4 + SOD	1.64	29
C_2H_4 + SOD + catalase	2.34	0
C_2H_4 + catalase	1.18	49
SOD	1.86	20
SOD + catalase	2.34	0
catalase	2.32	0

[a] SOD concentration 1 $\times$ 10^{-7} M (active protein). [b] Catalase concentration 6.6 $\times$ 10^{-8} M. [c] Oxidation rate measured at 261 nm.

Table II: Effect of SOD Additions on Ethylene Inhibition of Oxygen Uptake during Oxidation of 1 mM IAA Catalyzed by 4.2 $\times$ 10^{-8} M HRP in 50 mM Citrate Buffer, pH 5.0, at 25 °C

treatment[a]	oxygen uptake (M $\times$ 10^5 min^{-1})	inhibition (%)
control	1.97	
C_2H_4	0.90	54
C_2H_4 + SOD, 1 $\times$ 10^{-8} M	1.00	49
C_2H_4 + SOD, 5 $\times$ 10^{-8} M	1.20	39
C_2H_4 + SOD, 7.5 $\times$ 10^{-8} M	1.46	26
C_2H_4 + SOD, 1 $\times$ 10^{-7} M	1.97	0
C_2H_4 + SOD, 1 $\times$ 10^{-7} M (minus catalase)	1.77	10

[a] Control and treatments contained 6.6 $\times$ 10^{-8} M catalase which had no effect on reaction kinetics when added alone at this concentration.

The presence of ethylene did not perturb the spectrum of HRP in the absence of IAA.

Effects of SOD, Catalase, and SOD plus Catalase Additions on Ethylene Inhibition. Ethylene inhibition of the oxidation of IAA was annulled by the addition of SOD (1 $\times$ 10^{-7} M) plus catalase (6.6 $\times$ 10^{-8} M) at the start of the reaction in both the spectrophotometer and oxygen electrode studies (Tables I and II). SOD, added alone at 1 $\times$ 10^{-7} M, partially alleviated the ethylene inhibition (Tables I and II). Reversal

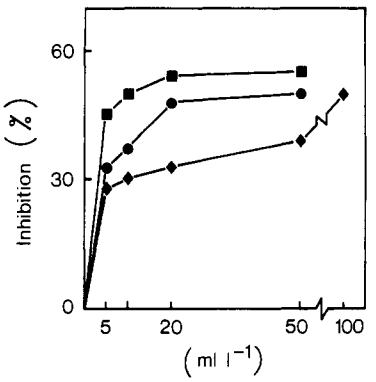

FIGURE 6: (■) Carbon monoxide, (●) acetylene, and (♦) propylene inhibition in rate of oxidation of 0.1 mM IAA by 2.3×10^{-8} M HRP in 50 mM citrate, pH 5, at 25 °C; oxidation rate measured at 261 nm.

of the ethylene effect could not be improved by addition of more SOD. Lower concentrations of SOD, however, removed less of the ethylene effect irrespective whether or not catalase was applied simultaneously (Table II). Catalase added at 6.6×10^{-8} M in the absence of SOD had no effect on inhibition caused by ethylene (Table I).

Under the same experimental conditions but in the absence of ethylene, SOD at 1×10^{-7} M caused a significant inhibition in the rate of oxidation of IAA (Table I and Figure 3). The degree of inhibition caused by SOD increased dramatically as the concentration of IAA increased (Figure 3).

Spectral data obtained in experiments where ethylene inhibited reaction kinetics indicated that compound III still dominated the steady-state spectrum of peroxidase when SOD (1×10^{-7} M) was present. Compound III, however, formed more slowly and reached a lower final concentration in these treatments than when compared with ethylene alone treatments. SOD addition in these experiments also caused a discernible increase in the formation of P-670 of peroxidase (λ_{max} 670 nm). Spectral information on peroxidase could not be obtained in the catalase and SOD plus catalase treatments because the catalase spectrum overlies the spectrum of HRP.

Effects of Ethylene Analogues on Kinetics of Oxidation of IAA. Acetylene, carbon monoxide, and propylene each caused inhibition of the oxidation rate of IAA under experimental conditions where ethylene was inhibitory (Figure 6). Each of these gases caused inhibition in the oxidation rate of IAA only under conditions where the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle was activated. They had no effect on reaction kinetics when the oxidation proceeded predominately through pathway 1 (Figure 7). Carbon monoxide was more effective as an inhibitor than acetylene, and both gases were considerably more effective than propylene (Figure 6).

The inhibitory effects of these ethylene analogues were alleviated by the addition of SOD (1×10^{-7} M) and completely abolished by the addition of SOD (1×10^{-7} M) plus catalase (6.6×10^{-8} M). Catalase added alone at 6.6×10^{-8} M had no effect on the inhibition in oxidation rate of IAA caused by these gases. Thus, these three gases behaved the same as ethylene in these experiments. Spectral data confirmed that these three gases also increased formation of compound III of peroxidase but only under conditions where the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle was operational. SOD additions prevented this increase in the formation of compound III.

Discussion

Kinetic data showed that ethylene and its analogues, acetylene, carbon monoxide, and propylene, inhibited the rate of oxidation of IAA by peroxidase under certain experimental

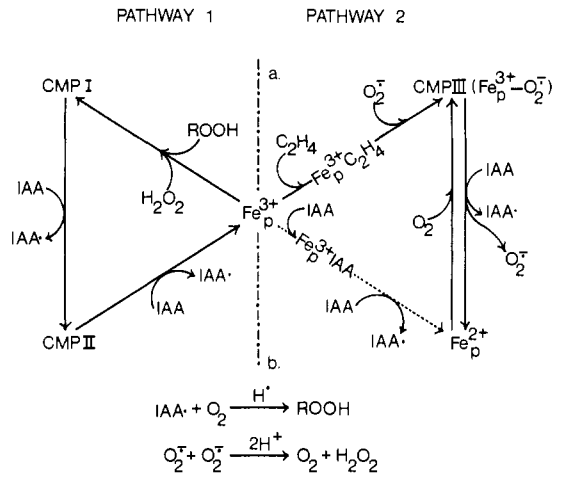

FIGURE 7: Pathways for oxidation of IAA catalyzed by peroxidase. Pathway 1, established reaction sequence; pathway 2a, our mechanism for activation of $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle by interaction between peroxidase, ethylene, and O_2^-·; pathway 2b, activation of this shuttle by elevated concentrations of IAA in the absence of ethylene. 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 from either pathway 1 or 2 from a reaction between the IAA free radical and oxygen. O_2^-· formed via pathway 2 spontaneously dismutates to form O_2 and H_2O_2. In pathway 2b, the step between Fe_p^{3+} and Fe_p^{2+} is shown as a broken line. Fe_p^{2+} is an integral intermediate in this pathway, but its formation from multiple binding of IAA molecules to Fe_p^{3+} is speculative (Smith et al., 1982).

conditions (Figures 1–4 and 6). Spectral data confirmed that this inhibition occurred only under conditions where the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle (Smith et al., 1982) was activated (Figure 5). When the oxidation proceeded predominately through the conventionally accepted pathway (Figure 7, pathway 1), these gases had no effect on the reaction kinetics (Figure 1).

The results obtained when SOD, catalase, and SOD plus catalase were added in these experiments indicated that O_2^-· must be present before ethylene and its analogues are able to inhibit the rate of oxidation of IAA. Thus, SOD, at 1×10^{-7} M, prevented most of the inhibitory effects of these gases on the rates of oxidation of IAA (Tables I and II). Low concentrations of catalase were required also in most experiments to completely restore reaction rates. The concentration of catalase used had no direct effect on the reaction but was adequate to remove H_2O_2 that accumulates from SOD-catalyzed dismutation of O_2^-· (Fridovich, 1975, 1976). Accumulation of H_2O_2 can increase the formation of P-670, a catalytically inactive form of peroxidase (Smith et al., 1982). Spectral data confirmed that P-670 did accumulate in the presence of SOD. This essential requirement for O_2^-· explains why ethylene and its analogues inhibited the reaction kinetics only when a reasonable proportion of the IAA was oxidized via the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. This shuttle provided the essential source of O_2^-· (Smith et al., 1982).

The complex relationship that occurred in these experiments between degree of inhibition in oxidation rate caused by ethylene and the concentration of IAA used (Figures 1–3) is now explicable. Sufficient IAA must be used to activate the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle to supply a flux of O_2^-·; otherwise ethylene is ineffective. Thus, a threshold concentration of IAA was necessary before ethylene inhibited the oxidation rate. At the highest concentrations of IAA used, virtually all the peroxidase was transformed directly into the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle (Smith et al., 1982). Addition of ethylene no longer appreciably increased participation of this shuttle under these conditions, and its inhibitory effects diminished (Figures 2 and 3). Ethylene appeared most effective in inhibiting reaction

kinetics when oxidation of IAA was partitioned equally between pathway 1 and the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. When all the peroxidase is converted to the shuttle, ethylene treatments could halve the rate of oxidation of IAA. Compound III still constituted the major steady-state form of peroxidase when oxidation of IAA was evenly partitioned between the two pathways. IAA reacts much faster with the enzyme intermediates of pathway 1 than it does with compound III in the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle (Smith et al., 1982; Yamazaki & Yokota, 1973). This explains why under conditions where ethylene caused about 50% inhibition in the rate of oxidation it caused only about 14% increase in the steady-state concentration of compound III (cf. Figures 1 and 2 with 5).

A mechanism that is compatible with these data may now be proposed to explain how ethylene and its analogues interact with peroxidase to affect the kinetics of oxidation of IAA (Figure 7, pathway 2a). Spectral and kinetic data confirm that ethylene and its analogues switch oxidation of IAA from pathway 1 to pathway 2a and, thus, enhance activation of the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle. This conclusion is supported both by the increase in the speed of formation and final concentration of compound III and by the concomitant reduction in the rate of oxidation of IAA [$k(IAA_{CMPII:CMPIII}) \simeq 25$; Yamazaki & Yokota, 1973]. Furthermore, addition of SOD to the ethylene and analogue treatments stopped this increase in compound III formation and prevented these gases inhibiting the oxidation of IAA.

No spectral evidence was obtained for binding of ethylene and its analogues to native peroxidase as indicated in our model. This suggests that if binding occurs, these gases are associated with the protein chain rather than with the iron porphyrin portion of the molecule. Most substrates of peroxidase bind to distinct, though related, hydrophobic regions of the protein and cause little perturbation of the optical spectrum (Schejter et al., 1976; Williams et al., 1977). This substrate binding pocket has been called an "oily droplet" portion of the protein (Williams et al., 1977). Ethylene and its analogues, with their hydrophobic characteristics, could be expected to partition readily into such a pocket. NMR studies should verify binding to and locate binding sites of ethylene and its analogues on native peroxidase.

Our results show that, in the presence of ethylene (and its analogues), native peroxidase reacts extremely rapidly with $O_2^{-}\cdot$ to form compound III. In the absence of ethylene, Fe_p^{3+} has been claimed to react with $O_2^{-}\cdot$ but with the relatively slow rate constant of $k \sim 3 \times 10^7$ M^{-1} s^{-1} (Yamazaki & Yokota, 1973). A careful pulse radiolysis study, however, indicated a rate constant for this reaction orders of magnitude smaller than 10^7 M^{-1} s^{-1} (Bielski et al., 1974). SOD, on the other hand, reacts very rapidly with $O_2^{-}\cdot$ with a $k \sim 2 \times 10^9$ M^{-1} s^{-1} which is among the fastest known rate constants for reaction between an enzyme and its substrate (Chance et al., 1979; Fridovich, 1975, 1976). In our experiments with ethylene present, 7.5×10^{-8} M SOD was required to compete equally with 4.2×10^{-8} M HRP for $O_2^{-}\cdot$ (Table II). Separate experiments established that ethylene has no detrimental effects on the scavenging ability of SOD for $O_2^{-}\cdot$ under these experimental conditions (unpublished data). Thus, a rate constant of $k \sim 3.5 \times 10^9$ M^{-1} s^{-1} may be estimated for the reaction between the Fe_p^{3+}–C_2H_4 complex and $O_2^{-}\cdot$. A similar rate constant for this complex was derived from the spectrophotometer experiments where about 3.3×10^{-8} M SOD was required to compete equally with 2.3×10^{-8} M HRP for $O_2^{-}\cdot$.

Although in these experiments, the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle provided the source of $O_2^{-}\cdot$ for the Fe_p^{3+}–C_2H_4 complex, many other sources of $O_2^{-}\cdot$ would be available under physiological conditions (Fridovich, 1976). Thus, compound III may form even in the absence of IAA provided peroxidase, ethylene, and $O_2^{-}\cdot$ are present. Once converted to compound III, peroxidase will cycle through the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle whenever IAA (or, perhaps, other specific reductants) and oxygen are present. An initial interaction between peroxidase, ethylene, and $O_2^{-}\cdot$ may result in an autocatalytic production of $O_2^{-}\cdot$. The flux of $O_2^{-}\cdot$ is produced at the expense of molecular oxygen whenever IAA is present. Thus, as discussed previously (Smith et al., 1982), important biological consequences may result from even a small amount of peroxidase transformed into the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle under physiological conditions.

The regulatory effects of ethylene on plant growth have been recognized for at least 80 years, but its mechanism of action remains obscure (Abeles, 1973; Lieberman, 1979, 1980; Osborne, 1978). The novel interactions that we have described between ethylene and peroxidase suggest that this enzyme may play a significant role in the action of ethylene as a plant hormone. Further support for this hypothesis comes from the interactions that occur between peroxidase and both ethylene and IAA (Figure 7). An intimate relationship is known to exist between ethylene and IAA in their effects on plant growth (Abeles, 1973; Lieberman, 1979; Osborne, 1978).

In our experiments relatively high concentrations of ethylene (and its analogues) were required to convert all the native peroxidase to the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle (Figures 4 and 6). We argue, however, that this is an artifact of our experimental system. So that appreciable differences in reaction kinetics could be measured, sufficient ethylene was required to ensure that all the Fe_p^{3+} cycling via pathway 1 was converted rapidly to compound III in the presence of a low flux of $O_2^{-}\cdot$. Much lower concentrations of ethylene caused measurable inhibition of reaction kinetics after about 15 min or more of reaction. By this time, however, substrate was depleted, and meaningful measurements of reaction kinetics could not be made. These restrictions would not apply under physiological conditions, and biologically active concentrations of ethylene could promote a slow conversion of Fe_p^{3+} to compound III provided $O_2^{-}\cdot$ is present. As already discussed, important biological consequences may result from even a small amount of peroxidase transformed into the $Fe_p^{2+} \rightleftarrows$ CMPIII shuttle.

Carbon dioxide usually antagonizes the effects of ethylene under physiological conditions (Abeles, 1973; Lieberman, 1979, 1980). About 10% (v/v) carbon dioxide is required to annul the physiological effects caused by 1 μL L^{-1} of ethylene. Not surprisingly, this concentration of carbon dioxide was without effect on our experiments where ethylene was used routinely at a concentration of 50 mL L^{-1}.

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Registry No. Peroxidase, 9003-99-0; indole-3-acetic acid, 87-51-4; superoxide, 11062-77-4; ethylene, 74-85-1; acetylene, 74-86-2; carbon monoxide, 630-08-0; propylene, 115-07-1.

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Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*: Spatial Relationship of the Mutase and Dehydrogenase Sites†

Richard I. Christopherson, Elizabeth Heyde, and John F. Morrison*

ABSTRACT: The inhibition of the bifunctional enzyme chorismate mutase–prephenate dehydrogenase (4-hydroxyphenylpyruvate synthase) by substrate analogues has been investigated at pH 6.0 with the aim of elucidating the spatial relationship that exists between the sites at which each reaction occurs. Several chorismate and adamantane derivatives, as well as 2-hydroxyphenyl acetate and diethyl malonate, act as linear competitive inhibitors with respect to chorismate in the mutase reaction and with respect to prephenate in the dehydrogenase reaction. The similarity of the dissociation constants for the interaction of these compounds with the free enzyme, as determined from the mutase and dehydrogenase reactions, indicates that the reaction of these inhibitors at a single site prevents the binding of both chorismate and prephenate. However, not all the groups on the enzyme, which are responsible for the binding of these two substrates, can be identical. At lower concentrations, citrate or malonate prevents reaction of the enzyme with prephenate, but not with chorismate. Nevertheless, the combining sites for chorismate and prephenate are in such close proximity that the diethyl derivative of malonate prevents the binding of both substrates. The results lead to the proposal that the sites at which chorismate and prephenate react on hydroxyphenylpyruvate synthase share common features and can be considered to overlap.

Chorismate mutase–prephenate dehydrogenase (EC 5.4.99.5 and EC 1.3.1.12; 4-hydroxyphenylpyruvate synthase) is a bifunctional enzyme that catalyzes the two sequential reactions along the tyrosine biosynthetic pathway which are shown in eq 1. The enzyme from *Escherichia coli* has a molecular

$$\text{chorismate} \rightarrow \text{prephenate} \xrightarrow{\text{NAD}} \text{4-hydroxyphenylpyruvate} + \text{NADH} + \text{H}^+ + \text{CO}_2 \quad (1)$$

weight of 88 000 and is composed of two apparently identical subunits (SampathKumar & Morrison, 1982a). The results of early studies by Koch et al. (1972) suggested that the sites for the mutase and dehydrogenase activities of the enzymes from *Aerobacter aerogenes* and *E. coli* were either contiguous or close to each other. The kinetic data obtained by Heyde & Morrison (1978) with the enzyme from *A. aerogenes* were consistent with the two reactions occurring at a single site or at two separate sites with similar kinetic properties. The idea of the reactions occurring at the same site or at two sites in close proximity was supported by the results of computer simulation of progress curves for the conversion of chorismate to hydroxyphenylpyruvate and by the finding that a small, but significant, proportion of prephenate formed from chorismate is converted directly to hydroxyphenylpyruvate (Heyde, 1979).

Further evidence that the mutase and dehydrogenase activities occur at the same or similar sites comes from the findings that the two activities are lost coordinately under a variety of inactivating conditions. The loss of each activity as a result of the reaction of iodoacetamide with a single sulfhydryl group on each subunit can be prevented by the addition of prephenate or of NAD plus tyrosine (Heyde, 1979; Hudson & Davidson, 1981). Attempts to obtain a mutant enzyme that possesses only prephenate dehydrogenase activity were unsuccessful although it was possible to obtain an enzyme that lacks dehydrogenase activity because of alteration at the NAD binding site. A protein lacking both activities could also be obtained (Rood et al., 1982).

The aforementioned results tend to indicate that the two catalytic activities of chorismate mutase–prephenate dehydrogenase do not take place at distinct sites. Thus, the enzyme differs from chorismate mutase–prephenate dehydratase which has been shown by a variety of procedures to have separate, noninteracting sites (Duggleby et al., 1978). The purpose of the present investigation was to gain additional information about the spatial relationship of the sites at which the mutase and dehydrogenase reactions occur by using as inhibitors analogues of both chorismate and prephenate. The

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra ACT 2601, Australia. *Received October 4, 1982.*

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