other two forms.

Registry No. MAT, 9012-52-6; Met, 63-68-3; Adomet, 22365-11-3; Me_2SO, 67-68-5.

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Kinetic Study of the Interaction between Ribulosebisphosphate Carboxylase/Oxygenase and Inorganic Fluoride†

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ABSTRACT: The effect of inorganic fluoride on the reactions catalyzed by ribulosebisphosphate carboxylase/oxygenase has been characterized with the fully activated enzyme. Fluoride inhibits both reactions, and the concentration required to inhibit the activity of the magnesium-activated enzyme 50% is 2 mM when reactions are carried out at pH 8.3. Inhibition is strongly pH dependent with an apparent pK_a of 8.8. The inhibition kinetics were studied. It was found that inhibition is mixed but close to noncompetitive with respect to CO_2 and uncompetitive with respect to ribulose 1,5-bisphosphate. The mechanism of interaction between fluoride and the enzyme is discussed, and a model is proposed in which fluoride interferes with the reactions by displacing a catalytically important ligand, probably a water molecule, from the activator metal.

Ribulose-1,5-biphosphate carboxylase/oxygenase catalyzes the two reactions (Calvin & Massini, 1952; Andrews et al., 1973)

$$\text{ribulose bisphosphate} \xrightarrow{CO_2} \text{2,3-phosphoglycerate} \quad (1)$$

$$\text{ribulose bisphosphate} \xrightarrow{O_2} \text{3-phosphoglycerate} + \text{2-phosphoglycolate} \quad (2)$$

Reaction 1 is the primary step of CO_2 fixation in C_3 photosynthesis, and reaction 2 is believed to be the initial reaction of photorespiration (Lorimer, 1981). The substrates CO_2 and O_2 are competitive with each other with the consequence that O_2 inhibits CO_2 fixation in photosynthetic organisms (Badger & Andrews, 1974).

Both reactions have an absolute requirement for a divalent metal ion. Mg^{2+} is the best activator, but Mn^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} also support activity (Weissbach et al., 1956; Christeller, 1981).

Metal activation has been shown to occur by the slow reversible addition of CO_2 and a metal ion to the enzyme to form an enzyme–CO_2–M^{2+} complex, which is the catalytically active species (Lorimer et al., 1976). The CO_2 molecule in this activation reaction is bound as a carbamate (Lorimer & Miziorko, 1980) and does not react with ribulose 1,5-bis-

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phosphate (RuBP)[1] in reaction 1 (Lorimer, 1979). The role of metal in the reaction is not clear, but there are several indications of metal catalysis. These have been reviewed by Lorimer (1981).

Many metal-dependent enzymes are inhibited by inorganic fluoride (Wiseman, 1970), and in some cases, it has been shown that inhibition is caused by a metal–fluoride interaction (Maurer & Nowak, 1981; Dixon et al., 1980; Brändén et al., 1971). Inhibition of RuBP carboxylase by fluoride has been observed with a crude protein extract (Larsson, 1974) and with a partially purified enzyme (Springer, 1974). These findings could therefore be of interest in assigning a role for metal in the activated enzyme. However, the inhibition studies with RuBP carboxylase were made before the activation reaction was known, and their interpretation is complicated by the occurrence of both activation and catalysis during the assay. To evaluate the effect of fluoride on catalysis, it was therefore necessary to study the interaction between fluoride and the fully activated enzyme.

In this paper, we characterize the fluoride inhibition of purified and fully activated RuBP carboxylase. The effect of pH and different metal activators on inhibition, together with the inhibitory patterns with respect to CO_2 and RuBP, suggests that a metal-coordinated species, probably water, participates in the reactions.

Materials and Methods

Materials. RuBP tetrasodium salt, Bicine, and DTT were obtained from Sigma Chemical Co. $NaH^{14}CO_3$ was purchased from Amersham and the scintillator solution (Biofluor) from New England Nuclear. Sephacryl S-300, DEAE-Sepharose CL-6B, and Sephadex G-25 were from Pharmacia. All other chemicals were of analytical grade. Spinach was bought locally, and the leaves were stored at −20 °C. Buffers used in enzyme assays were prepared CO_2 free by refluxing at pH 4 for 1 h. After the buffer solution was cooled, the pH was adjusted to the desired value with CO_2-free NaOH (Long, 1961).

Enzyme Purification. RuBP carboxylase was purified from spinach leaves as follows: Leaves (0.8 kg) and 1.5 L of 0.1 M Tris-HCl at pH 8.3 were homogenized in a Waring Blender for 1 min. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 10000*g* for 20 min. Solid ammonium sulfate was added to the supernatant, and the fraction obtained between 20% and 40% saturation was collected and dissolved in 50 mM Tris-HCl at pH 7.7 containing 0.1 M NaCl, 1 mM EDTA, and 1 mM DTT (referred to as "buffer" in this section). For the removal of phenolic compounds, the protein solution was passed through a Sephadex G-25 column (20 × 12 cm) in buffer, and the eluted protein was fractionated with ammonium sulfate as described above. The precipitated protein was dissolved in buffer and subjected to gel filtration on a Sephacryl S-300 column (70 × 5 cm) equilibrated with buffer. Fractions containing RuBP carboxylase were pooled and applied to a DEAE-Sepharose CL-6B column (30 × 5 cm) equilibrated with buffer. Proteins were eluted with a linear gradient of 0.1–0.6 M NaCl in 2 L. RuBP carboxylase containing fractions, eluting at 0.35–0.4 M NaCl, were pooled, solid ammonium sulfate to 70% saturation was added, and the protein was collected by centrifugation. The pellet was suspended in buffer containing 70% ammonium sulfate and 10 mM DTT, and the enzyme was stored as a suspension at 4 °C. RuBP carboxylase isolated by this procedure was homogeneous as judged by sodium dodecyl sulfate gel electrophoresis and had a specific activity of 1.5–2 μmol of CO_2 fixed mg^{-1} min^{-1} at 25 °C and pH 8.3.

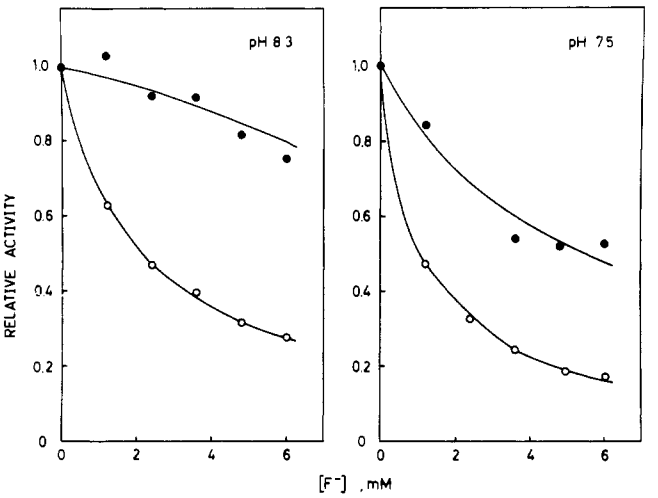

FIGURE 1: Effect of inorganic fluoride on the magnesium-dependent (○) and manganese-dependent (●) carboxylase activities at pH 8.3 and 7.5, respectively. The reaction mixture contained 0.5 mM RuBP, 10 mM $NaHCO_3$, 10 mM $MgCl_2$ or 5 mM $MnCl_2$, and NaF as indicated in 50 mM Bicine or Tris buffer. Reactions were initiated by the addition of 40 μg of magnesium-activated or 250 μg of manganese-activated enzyme. Activities are expressed as relative to the activity with no fluoride present.

Desalting and Activation of the Enzyme. An aliquot of the suspension was centrifuged, and the pellet was dissolved in 50 mM CO_2-free Bicine or Tris buffer at pH 8.3. Bicine buffer was used in experiments with Mg^{2+} as activator, and Tris buffer was used for the other metals. The solution was passed through a small Sephadex G-25 column (15 × 1 cm) equilibrated with the appropriate buffer, and the protein fraction was collected. The enzyme was diluted to a suitable concentration, and metal and $NaHCO_3$ were added. The final concentration of metal was 10 mM in experiments with Mg^{2+} and 5 mM for Mn^{2+}, Co^{2+}, or Ni^{2+} experiments. The final concentration of $NaHCO_3$ was 10 mM. The enzyme was then activated (Lorimer et al., 1976) for at least 20 min before it was used.

Assays. Carboxylase and oxygenase assays were performed as described by Lorimer et al. (1977). All reactions were run at 25 °C, and the carboxylase reactions were carried out under a nitrogen atmosphere. The assay solution used in oxygenase assays was equilibrated with air, $[O_2]$ = 0.25 mM. The detailed composition of each assay is given with each experiment.

Protein Determination. The concentration of RuBP carboxylase was calculated from the absorption at 280 nm and $A_{280}^{1\%}$ = 16.4 (Paulsen & Lane, 1966).

Data Analysis. Equations were fit to the data with nonlinear least-squares methods.

Results

Effect of Different Metal Activators and pH on Fluoride Inhibition. To obtain an indication of whether fluoride inhibition is caused by interaction between fluoride and the metal, we performed experiments with different metal activators. The inhibition of magnesium- and manganese-dependent carboxylase activities is shown in Figure 1. Here, it can be seen that with Mn^{2+} as activator, the enzyme is not as sensitive to fluoride as with Mg^{2+}. With Co^{2+} or Ni^{2+} as activator, only slight inhibition (activity >85% of the control

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; RuBP, ribulose 1,5-bisphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

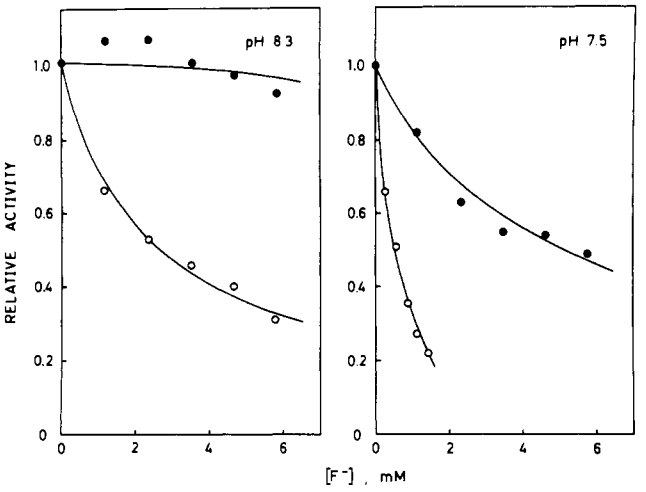

FIGURE 2: Effect of inorganic fluoride on the magnesium-dependent (○) and manganese-dependent (●) oxygenase activities at pH 8.3 and 7.5, respectively. Reaction mixtures contained 0.5 mM RuBP, 10 mM $MgCl_2$ or 5 mM $MnCl_2$, and NaF as indicated in 50 mM Bicine or Tris buffer. Reactions were initiated by the addition of 150 μg of magnesium- or manganese-activated enzyme in 10 μL. Activities are expressed as relative to the activity with no fluoride present.

at 6 mM NaF) was observed. Similar experiments were performed for the oxygenase reaction with Mg^{2+} or Mn^{2+} as activator. The effect of fluoride is shown in Figure 2.

These results show that the inhibitory effect of fluoride on both the carboxylase and oxygenase reactions is dependent on the kind of metal activator present. For both reactions, stronger inhibition is observed at pH 7.5 than at pH 8.3. With the manganese-activated enzyme, both reactions are equally affected at pH 8.3 and 7.5. This is also the result obtained with the magnesium-activated enzyme at pH 8.3, while at pH 7.5, the magnesium-dependent oxygenase activity is more sensitive than the carboxylase activity. It can also be seen that the pH at which the reaction is carried out affects the sensitivity to fluoride. We have, however, limited further study of the pH dependency to the carboxylase reaction, since investigation of the oxygenase reaction is complicated by the carry-over of bicarbonate to the assay with the activated enzyme.

The pH dependency of the fluoride inhibition of the magnesium-dependent carboxylase activity was investigated as follows: A control reaction and a reaction in the presence of 4.8 mM NaF were run at several pH values. The ratio of activity in the presence of fluoride to activity in the control reaction (V/V_0) was calculated and is shown as a function of pH in Figure 3.

The pH profile of inhibition can be described by an acid–base equilibrium between two enzyme forms with different sensitivities to fluoride. The solid curve in Figure 4 shows the simulated pH profile obtained by assuming $V/V_0 = 0.13$ for the acidic form, $V/V_0 = 1.0$ for the basic form, and $pK_a = 8.8$. The parameters were obtained by fitting a theoretical titration curve to the data.

Inhibition Patterns. The inhibition by fluoride with respect to CO_2 or RuBP concentration was investigated, and the result was evaluated by fitting the equation $v = V/[K_s/s(1 + i/K_i) + 1 + i/K_i']$ to the data. Here, s = the concentration of limiting substrate, i = the concentration of NaF, and V, K_s, K_i, and K_i' are adjustable parameters. The result obtained with CO_2 as the limiting substrate is shown in Figure 4 as a double-reciprocal plot with solid lines drawn according to the set of parameters that gave the best fit. These parameters are given in the figure legend. With RuBP as the limiting substrate, the result shown in Figure 5 was obtained.

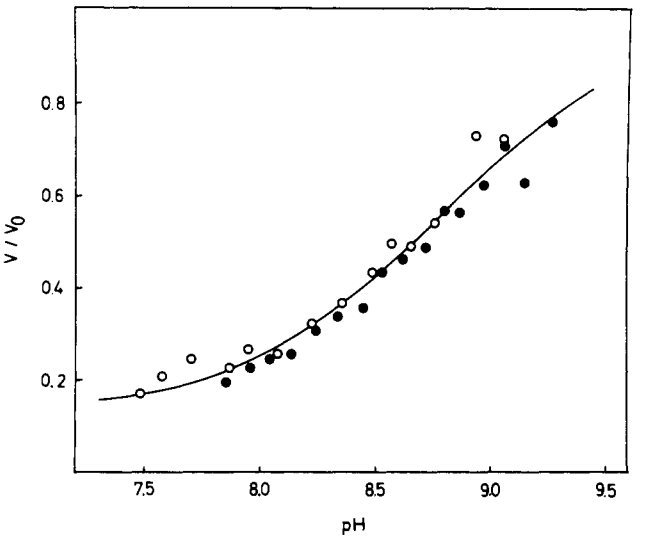

FIGURE 3: Effect of pH on the fluoride inhibition of the magnesium-dependent carboxylase activity. Inhibition is expressed as the ratio between activity in an assay containing 4.8 mM NaF (V) and a control reaction (V_0) run at the same pH. The other constituents of the assay were 0.5 mM RuBP, 10 mM $NaHCO_3$, and 10 mM $MgCl_2$. Reactions were initiated by the addition of 40 μg of activated enzyme. (○) and (●) represent the results obtained in two separate series of experiments, and the solid curve is a theoretical titration curve with $V/V_0 = 0.13$ for the acidic form, $V/V_0 = 1.0$ for the basic form, and $pK_a = 8.8$.

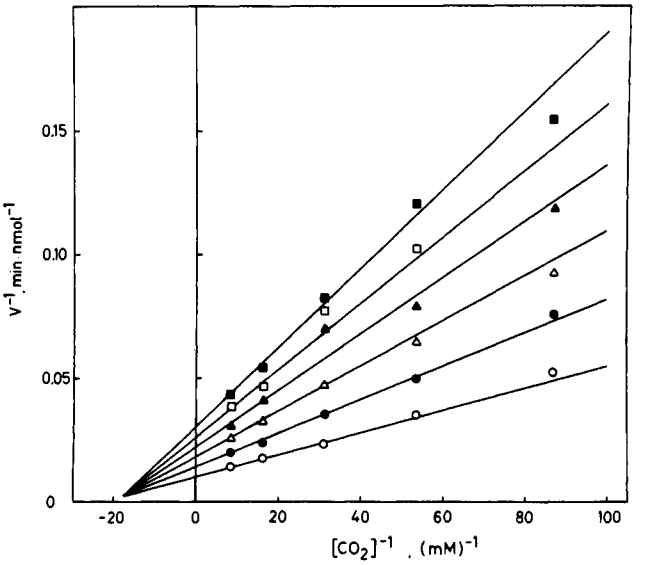

FIGURE 4: Double-reciprocal plot of inhibition with respect to $[CO_2]$. The reaction mixture contained 0.5 mM RuBP, $NaHCO_3$ as indicated, 10 mM $MgCl_2$, and 0 (○), 1.2 (●), 2.4 (△), 3.6 (▲), 4.8 (□), or 6 (■) mM NaF in 50 mM Bicine buffer. The pH was 8.3, and reactions were initiated by the addition of 40 μg of activated enzyme. The solid lines are drawn according to $V = 2.2$ μmol mg^{-1} min^{-1}, $K_s = 45$ μM, $K_i = 2.4$ mM, and $K_i' = 3.0$ mM.

From these results, it can be seen that inhibition is mixed but approaching noncompetitive with respect to CO_2 and is uncompetitive with respect to RuBP.

Discussion

Sensitivity to fluoride is a common property among metal-dependent enzymes and is often ascribed to interaction between fluoride and the metal. There are, however, examples of inhibition by F^- of enzymes not dependent on the metal, and these show that an observed inhibition is not necessarily caused by the metal–fluoride interaction (Wiseman, 1970).

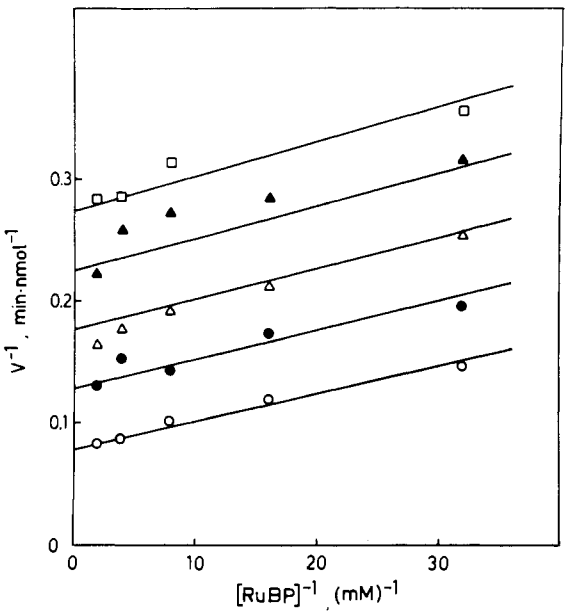

FIGURE 5: Double-reciprocal plot of inhibition with respect to [RuBP]. The reaction mixture contained 10 mM $NaHCO_3$, 10 mM $MgCl_2$, RuBP as indicated, and 0 (○), 1.2 (●), 2.4 (△), 3.6 (▲), or 4.8 (□) mM NaF in 50 mM Bicine buffer. The pH was 8.3, and reactions were initiated by the addition of 6 μg of activated enzyme. The solid lines are drawn according to $V = 2.0$ μmol mg^{-1} min^{-1}, $K_s = 20$ μM, $K_i = 19$ mM, and $K_i' = 2.0$ mM.

In this investigation, we have studied the effect of fluoride on RuBP carboxylase activated with different metals. With Mg^{2+} as activator, the enzyme is more sensitive than with Mn^{2+} as activator, and with Co^{2+} or Ni^{2+} as activator, the enzyme is only slightly inhibited. The enzyme's sensitivity to fluoride thus parallels the stability of the fluoride complex of the activator metal (Smith & Martell, 1976), indicating that fluoride inhibits the enzyme by binding to the metal.

The oxygenase and carboxylase activities are similarly affected by fluoride at pH 8.3, but at pH 7.5, the magnesium-dependent oxygenase activity is apparently more sensitive than the carboxylase activity. However, this result must be interpreted with care as CO_2, a competitive inhibitor of O_2 in the oxygenase reaction, always is present in the assays due to carry-over with the activated enzyme. The same amount of bicarbonate is carried over the pH 8.3 and 7.5, but the resulting concentration of CO_2 is higher at pH 7.5. The interaction between F^- and CO_2 inhibition of the oxygenase activity must therefore be studied more closely before conclusions on the oxygenase mechanism can be drawn.

The pH dependence on inhibition of the carboxylase reaction is more informative. As shown in Figure 3, the effect of fluoride may be described as being controlled by a single dissociable group. A simple explanation of the observed pH dependency and the result that fluoride probably binds at the metal is that fluoride and a protonatable ligand compete for the metal ion:

$$ML \overset{H^+}{\rightleftharpoons} MLH \overset{F^-}{\rightleftharpoons} MF + LH$$

In this scheme, catalysis requires coordination of L or LH, but the bond between the metal and L in the MLH complex is sufficiently weak to allow displacement of LH from the metal by F^-. To discuss the nature of the ligand L, we must first consider the inhibitory patterns with respect to CO_2 and RuBP.

Inhibition is mixed with respect to CO_2, but as can be seen from Figure 4, the pattern is close to a noncompetitive one. This is also reflected in the relative values of K_i and K_i'. The similarity between these values shows that CO_2 and F^- bind almost independently to the enzyme. As the most probable binding site for fluoride is the metal, the inhibition with respect to CO_2 suggests that binding of this substrate does not involve direct coordination to the metal. This conclusion agrees with the ^{13}C NMR results of Miziorko & Mildvan (1974), from which a second-sphere coordination of rapidly exchanging CO_2 to enzyme-bound Mn^{2+} was proposed.

The uncompetitive inhibition with respect to RuBP indicates that fluoride combines with the enzyme after RuBP is bound. This increase in affinity for fluoride could be caused by a change in the metal environment induced directly or indirectly by the binding of RuBP.

From the kinetic results, it is clear that the protonatable ligand in the suggested scheme cannot be the substrate CO_2. RuBP is also a less likely candidate since its coordination would have given rise to competitive inhibition with respect to this substrate. Furthermore, the ligand is not a part of a carboxylated intermediate, as inhibition would then have been uncompetitive also with respect to CO_2. Possible protonatable ligands left to consider are then an amino acid side chain or a water molecule. From the observed pK_a of 8.8, which is within the range unsually observed with metal-coordinated water (Hanzlik, 1976), the latter alternative seems more likely. This view is also supported by the observation (Miziorko & Mildvan, 1974) of metal-coordinated water in the manganese-activated enzyme.

Current ideas about the RuBP carboxylase/oxygenase mechanism include a hydrolytic cleavage of an initially formed 2-carboxy- or 2-peroxy-3-oxopentitol 1,5-bisphosphate. This step is probably initiated by the attack of water or hydroxyl ion at carbon atom 3 of the intermediate (Lorimer, 1981). The results presented here suggest that a metal-coordinated water molecule or hydroxyl ion is important to catalysis, and it therefore seems reasonable to propose that the attacking species in hydrolysis is delivered from the metal. The mechanism of hydrolysis would resemble the reaction mechanism of carbonic anhydrase, where a metal-coordinated hydroxide ion most probably is a reactive species (Pocker & Sarkanen, 1978). One of the many potential functions for the metal in the activated enzyme might thus be to activate the water that participates in both reactions.

Registry No. RuBP, 24218-00-6; carbon dioxide, 124-38-9; fluoride, 16984-48-8; ribulosebisphosphate carboxylase, 9027-23-0; ribulosebisphosphate oxygenase, 39335-11-0.

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

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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.

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[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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