Proposed Model for the Peroxidase-Catalyzed Oxidation of Indole-3-acetic Acid in the Presence of the Inhibitor Ferulic Acid

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ABSTRACT

Linear increments in ferulic acid concentration produce logarithmic increases in the ferulic acid-induced lag periods prior to the peroxidase-catalyzed oxidation of indole-3-acetic acid in a system containing 2,4-dichlorophenol and MnCl₂ in acetate buffer at pH 5.6. Maintaining the ratio of indole-3-acetic acid to ferulic acid constant at 100 while linearly raising the ferulic acid concentration results in linear increases in the lag period. Both indole-3-acetic acid and ferulic acid are substrates of horseradish peroxidase in the presence of H₂O₂, and indole-3-acetic acid competitively inhibits the oxidation of ferulic acid. A model for the enzymatic oxidation of indole-3-acetic acid catalyzed by peroxidase is proposed.

It is well established that plants contain enzymes capable of oxidizing IAA in vitro (7). Although these enzymes are generally known as IAA oxidase, all appear to be peroxidases, with the possible exception of an enzyme from tobacco roots (16). Commercial HRP oxidizes IAA and has been used in numerous studies, including those reported here.

Even though the in vivo role of IAA oxidase remains hypothetical, considerable work has been done on the nature and distribution within the plant of IAA oxidase inhibitors. These are phenolic compounds which interfere with the peroxidase-catalyzed oxidation of IAA in vitro by introducing a lag prior to the onset of IAA oxidation (5, 11, 15, 18–20, 21). Most inhibitors are of low molecular weight, although Stonier et al. (18, 20) reported phenolic inhibitors from Pharbitis with molecular weights of 5,000 to 10,000, based upon behavior on sephadex gels. The association of high inhibitor concentrations with actively growing tissues suggests that inhibitors promote growth by preventing IAA destruction by endogenous IAA oxidase—the “auxin protector” concept of Stonier and Yoneda (20).

Several workers have sought to clarify the mechanism(s) by which phenolic inhibitors delay the peroxidase-catalyzed oxidation of IAA (15, 18–20, 21). The generally accepted theory is that phenolic inhibitors trap free radical intermediates which would otherwise contribute to the oxidation of IAA. The results of the present study suggest an alternative mechanism is possible.

MATERIALS AND METHODS

All reactions were conducted at 30°C in 50 mM sodium acetate buffer, pH 5.6. IAA (prepared fresh) was dissolved in 95% (v/v) ethanol, then made to water with distilled water. FA (Aldrich Chemicals) was diluted from a stock solution containing 5% (v/v) ethanol. In experiments where IAA and/or FA concentration was varied, the final ethanol concentration was maintained constant throughout. HRP (Sigma, type II) was diluted from a 1.0 mg/ml stock solution. Two different enzyme preparations having slightly different activities (but both with measured R₂ values of 1.2) were used during the course of these studies. Therefore, results from different experiments are not necessarily quantitatively comparable. DCP (Eastman Organic Chemicals) and MnCl₂ were added routinely to promote IAA oxidation, except in experiments with H₂O₂.

Spectrophotometric Experiments. Kinetic experiments employed a Coleman 46 spectrophotometer equipped with automatic cell positioner, thermostatted cell compartment, and Perkin-Elmer 56 recorder.

Colorimetric Experiments—Residual IAA was measured with Salkowskii reagent according to the method of Gordon and Weber (6). Two ml of test solution were mixed with 4 ml of reagent, and absorbance was measured at 525 nm on a Coleman 46 spectrophotometer equipped with a Gilson transferor. Color development was allowed to occur in darkness for about 1 hr before measuring absorbance.

DMACA reagent prepared according to the method of Meudt and Gaines (9) was used to measure the accumulation of IAA oxidation products. Two ml of test solution were added to 2 ml of reagent, and the samples were allowed to stand in darkness about 1 hr before absorbance was measured at 562 nm was determined as described above.

Comparison Experiment. An experiment was designed to permit simultaneous measurements of changes in IAA, FA, and IAA oxidation products during the course of the reaction. A beaker containing the following concentrations of reactants in a final volume of 180 ml was allowed to equilibrate for about 1 hr in a water bath at 30°C: 0.1 mM IAA, 0.1 mM DCP, 0.1 mM MnCl₂, 7.0 mM FA, and 50 mM acetate buffer, pH 5.6. To start the reaction, the beaker was placed on a magnetic stirrer and 30 ml of ice cold HRP was poured in, producing a final HRP concentration of 0.5 µg/ml. An accurate elapsed-time clock and the spectrophotometer recorder were started the moment the HRP was added and allowed to run throughout the experiment (25 min). Immediately after adding the HRP, the beaker was returned to the water bath for the duration of...
the experiment. Starting at 30 sec and each 30 sec thereafter throughout the experiment, 2-ml samples were removed from the beaker and mixed alternately with Salkowski reagent (to follow loss of IAA) or DMACA (to follow increase in IAA oxidation products). Tubes were stored in darkness for 1 hr to allow color development, then examined at 525 nm (Salkowski) or 562 nm (DMACA).

In addition to the colorimetric estimation with DMACA, IAA oxidation products were also studied spectrophotometrically at 262.5 nm. Disappearance of FA was followed at 320 nm. The beaker in the water bath was connected to the spectrophotometer cell by a Gilson transferor. Samples (4 ml) were pumped to the spectrophotometer where change in absorbance was recorded first at 262.5 nm (IAA oxidation products) for about 20 sec, then at 320 nm (FA) for about 20 sec. The sample was then returned to the beaker and replaced by a fresh aliquot, whereupon absorbance changes were again recorded at 262.5 nm and 320 nm. This process, which required about 1 min, was repeated continuously throughout the experiment.

Pumping samples to and from the beaker eliminated several sources of error which would attend retaining the same sample in the spectrophotometer throughout (e.g., temperature differences between the spectrophotometer and water bath, different surface to volume ratios for samples in a cuvette compared to the beaker). Final absorbance data were calculated from the recording by subtracting the previously measured absorbance of water blanks at 262.5 nm and 320 nm.

RESULTS

In the presence of IAA, DCP, and MnCl₂ (but without H₂O₂), FA delays the onset of the HRP-catalyzed oxidation of IAA. The relationship between FA concentration and lag period (time to approximate 50% loss of IAA as measured by Salkowski reagent) is shown in Figure 1. As reported previously by Mudd and Burris (11), the length of the lag period is not directly proportional to FA concentration (Fig. 2, inset). However, the log of the lag period is directly proportional to FA concentration (Fig. 2).

IAA oxidation was also studied spectrophotometrically at 262.5 nm (accumulation of IAA oxidation products) and 320 nm (disappearance of FA). Oxidation products begin forming within 1 min after adding HRP in the absence of FA (Fig. 3, inset), but their appearance is preceded by a lag in the presence of FA (Fig. 3, inset). Plotting the log of the lag in minutes (time to increase of 0.05 A) at 262.5 nm as a function of FA concentration yields a straight line (Fig. 3).

In the absence of FA, oxidase increases at 320 nm (near the absorption maximum of FA but where interference by IAA absorption is slight) as IAA oxidation products are produced. In the presence of FA, absorption at 320 nm decreases (reflecting loss of FA) to a minimum, then increases as oxidation products accumulate (Fig. 4). Plotting the log of the lag in minutes (time to minimum absorbance at 320 nm) as a function of FA concentration yields a straight line (Fig. 4, inset).

Enzyme inactivation during FA oxidation might explain the
Loss of FA. The absorbance of FA during the reaction mixture in absorbance of FA (curve 2). Second experiment (bars) measured HRP activity (purpurogallin formation) remaining in IAA oxidation mixtures at various times during the lag. Both IAA oxidation mixtures contained 0.1 mM IAA, 0.1 mM DCP, 0.1 mM MnCl₂, 7.0 μM FA (except for curve 1) and 0.5 μg HRP/ml in a final volume of 3.0 ml (curves) or 18 ml (bars). Once the length of the lag had been determined spectrophotometrically, 15 ml of an identical reaction mixture were placed in a beaker in a water bath at 30 C. The reaction was started by the addition of HRP. At the times indicated by the bars, 0.2-ml samples of reaction mixture were added to 2.8 ml of the following mixture in spectrophotometer cells: 10 ml of 10 mM pyrogallol, 2.0 ml of 15% (v/v) H₂O₂, and 44.0 ml of H₂O. The increase in absorbance at 430 nm (purpurogallin formation) was linear for at least 5 min. The height of the bars indicates the total increase in absorbance at 430 nm in 5 min (averages of three determinations). The sample at 1 min is arbitrarily shown as 100% activity.

Fig. 4. Absorbance changes at 320 nm in the absence (1, control) and presence of 5.0 μM FA (2). In the absence of inhibitor (1), absorbance increases immediately at 320 nm due to accumulation of IAA oxidation products which absorb slightly at 320 nm. In the presence of FA (2) accumulation of IAA oxidation products is delayed. During the lag the decrease in absorbance at 320 nm reflects loss of FA. The subsequent increase results from accumulation of oxidation products. The inset shows the log of the lag at 320 nm (time to minimum absorbance) plotted as a function of FA. The reaction mixture contained 0.1 mM IAA, 0.1 mM MnCl₂, 0.5 μg HRP/ml, and FA as indicated in a final volume of 3.0 ml.

Fig. 5. Inactivation of HRP during the lag. An initial experiment (curves) was performed to determine the lag prior to accumulation of IAA oxidation products (λ 262.5 nm) caused by 7.0 μM FA (curve 2). Each point represents a single measurement from a recording. A second experiment (bars) measured HRP activity (purpurogallin formation) remaining in IAA oxidation mixtures at various times during the lag. Both IAA oxidation mixtures contained 0.1 mM IAA, 0.1 mM DCP, 0.1 mM MnCl₂, 7.0 μM FA (except for curve 1) and 0.5 μg HRP/ml in a final volume of 3.0 ml (curves) or 18 ml (bars). Once the length of the lag had been determined spectrophotometrically, 15 ml of an identical reaction mixture were placed in a beaker in a water bath at 30 C. The reaction was started by the addition of HRP. At the times indicated by the bars, 0.2-ml samples of reaction mixture were added to 2.8 ml of the following mixture in spectrophotometer cells: 10 ml of 10 mM pyrogallol, 2.0 ml of 15% (v/v) H₂O₂, and 44.0 ml of H₂O. The increase in absorbance at 430 nm (purpurogallin formation) was linear for at least 5 min. The height of the bars indicates the total increase in absorbance at 430 nm in 5 min (averages of three determinations). The sample at 1 min is arbitrarily shown as 100% activity.

Fig. 6. Comparison of the timing of IAA disappearance (Salkowski) with accumulation of IAA oxidation products (DMACA at 562 nm and spectrophotometrically at 262.5 nm) and loss of FA (λ 320 nm). The reaction mixture contained 0.1 mM IAA, 0.1 mM DCP, 0.1 mM MnCl₂, 7.0 μM FA, and 0.5 μg HRP/ml in a final volume of 180 ml. Absorbance at 320 nm was recorded on expanded scale (0.0–0.4 A) and is shown multiplied 10 times to aid comparison. The increase in absorbance at 320 nm at the end of the lag results from oxidation products. Increasingly long lag periods as FA concentration is increased. This interpretation is not supported by the data of Figure 1, which show that the rate of IAA disappearance is nearly the same after a long lag as for the control. However, since the HRP-catalyzed oxidation of IAA involves nonenzymatic-free radical reactions, other evidence of enzyme activity during the lag was obtained (Fig. 5). HRP loses about 20% of its activity towards pyrogallol during a lag of about 25 min. Loss of activity could result either from enzyme denaturation or enzyme inhibition by FA oxidation products, which are transferred with the enzyme. Inactivation is more rapid as IAA oxidation products accumulate, resulting in a loss of about 50% activity at the end of the experiment. Regardless of its cause, decrease in enzyme activity during the lag is insufficient to account for the logarithmic increases accompanying linear increments in FA, but enzyme inactivation could become an important factor in very long lags.

All three methods (loss of IAA, loss of FA, appearance of oxidation products) of measuring the effect of FA on IAA oxidation yield qualitatively similar results, but do not measure the same aspect of the reaction. Comparing increase in oxidation products (λ 262.5 nm) with loss of IAA from the same reaction mixture shows that IAA disappearance is approximately 50% complete before oxidation products begin accumulating (Fig. 6). Using DMACA to detect IAA oxidation products indicates that increase in absorbance at 262.5 nm corresponds with the appearance of DMACA-positive products (Fig. 6).

Comparing the timing of FA disappearance (absorbance decrease at 320 nm) with the disappearance of IAA (Salkowski, λ 525 nm) shows a steady decrease in FA during the lag before IAA disappearance (Fig. 6). The slow decrease in FA is followed by a more rapid loss which corresponds with the onset of IAA oxidation as shown by comparison of the FA curve (320 nm) with that of IAA (Salkowski, λ 525 nm). The point of minimum absorbance at 320 nm corresponds to the first increases at 262.5 nm and the appearance of DMACA-positive...
increments in FA at a constant IAA/FA ratio (Fig. 8). The lag produced by a given FA concentration is seen to be much shorter when the IAA/FA ratio remains at 100 than under conditions of constant IAA (Fig. 4, inset). Significantly, the length of the lag increases linearly with linear increments of FA when the IAA/FA ratio remains constant. The deviation at the highest concentrations of IAA and FA is reproducible, but unexplained.

The importance of the IAA/FA ratio in determining the lag suggests a competition between IAA and FA for some reaction component. Both IAA and FA are substrates of HRP in the presence of H₂O₂. If IAA and FA compete for a site on the enzyme they would be expected to act as competitive inhibitors of each other in the presence of H₂O₂. The initial rate of FA oxidation over a range of concentrations was measured with and without the addition of IAA (Fig. 9). Lineweaver-Burk plots show that IAA does behave as a competitive inhibitor of FA oxidation in that IAA inhibition is eliminated when FA concentration is increased to 0.1 mM.

**DISCUSSION**

A proposed model for the HRP-catalyzed oxidation of IAA in the presence of FA is presented in Figure 10. The model is intended to explain the enzymatic phase of IAA oxidation under a variety of experimental conditions. Although FA was the only inhibitor studied, the model is applicable to all phenolic inhibitors, with the possible exception of high molecular weight inhibitors. The component reactions of the model are represented by reactions 1 through 3 and are discussed individually.

HRP is assumed to have two substrate sites: a peroxide site where H₂O₂ attaches, leading to the formation of HRP-I from free HRP; and a donor site where AH₂ (often a phenolic compound) complexes, leading to the production of free radicals (AH·), water, and free HRP (1, 2). Reaction 1 represents the typical peroxidase reaction cycle. The subsequent nonen-
enzymatically with thereby accounting for the overall cycle under which the initial consumption of HRP-I and HRP-II must approach zero. It is probable that the initial oxidation of IAA occurs via some mechanism other than the typical peroxidase reaction cycle under these conditions. Yokota and Yamazaki (22) and Ricard and Nari (14) demonstrated that HRP-III, a generally inactive complex of O2 and ferroperoxidase, has a high activity towards IAA, resulting in the formation of IAA-free radicals, free HRP, and H2O2 (reaction 2a). I propose that the HRP and H2O2 produced upon decomposition of HRP-III can then form HRP-I and initiate the typical peroxidase reaction cycle (reaction 2b) with the potential oxidation of two donor molecules and regeneration of free HRP. The donor molecules in reaction 2b could be IAA, yielding a potential of three oxidized IAA molecules for each molecule of HRP-III decomposed. There is evidence for the formation of HRP-I and HRP-II in the work of Fox et al. (4), who showed that addition of IAA to free HRP caused spectral changes indicative of rapid decomposition of HRP-I, its conversion to HRP-II, and the ultimate regeneration of free HRP. Although Fox et al. specifically report finding no evidence of HRP-III, it is probable that its rapid decomposition upon addition of IAA would have escaped detection. The participation of HRP-III in the system of Fox et al. is supported by the observed requirement for O2 in the formation of HRP-I and HRP-II. The fact that catalase had no effect on HRP-I and HRP-II formation suggests that the H2O2 resulting from decomposition of HRP-III remains bound to the enzyme, represented by brackets in reactions 2a and 2b.

Following changes in FA concentration (320 nm) during the lag period in a system containing IAA, DCP, MnCl2, and HRP (but lacking exogenous H2O2) shows that FA concentration decreases steadily during the lag period before rapid IAA oxidation (Fig. 6). The proposed mechanism of FA oxidation under these conditions is represented by reactions 2a and 2b. IAA is required to initiate the decomposition of HRP-III to HRP-I and HRP-II, which in turn react with FA as electron donor to effect FA oxidation. It is clear from the proposed mechanism why no FA oxidation occurs without IAA (Gelinas, unpublished). Similarly, Siros and Miller (17) found no oxidation of scopoletin unless a 10- to 50-fold excess of IAA was present. Natural inhibitors from Zea mays behave in a similar way, remaining unaffected by HRP unless IAA is present (5). It is also clear from the model that addition of small amounts of H2O2 would eliminate the lag since HRP-I would be formed directly in large amounts and FA oxidation would be rapid (no longer limited by the rate of formation of HRP-III).

A prediction of the model is that oxidation of FA must be accompanied by oxidation of at least as many molecules of IAA. However, the amount of IAA oxidized during the lag period induced by the highest FA concentration (9 µM) is too small to be detected by the Salkowski reagent under the conditions employed. The FA-induced lag period prior to the rapid oxidation of IAA is interpreted as the time required for the IAA/FA ratio to increase (as a result of FA oxidation) to a point where IAA can compete effectively with FA for the donor site of HRP-I and HRP-II. Inherent in this interpretation is the assumption that the donor site of HRP-I and HRP-II has a much greater affinity for FA than for IAA, resulting in preferential oxidation of FA in IAA-FA mixtures.

The model predicts that IAA and FA should compete for the donor site of HRP-I and HRP-II. It is difficult to perform standard kinetic studies on the effect of FA on IAA oxidation in the absence of exogenous H2O2, since the initial rate of IAA oxidation approaches zero during the lag period. However,
IAA should competitively inhibit FA oxidation when most of the enzyme is present as HRP-I and HRP-II, a condition achieved by addition of exogenous H$_2$O$_2$. The results (Fig. 9) show that IAA does inhibit FA oxidation and that increasing FA concentration to 0.1 mm overcomes the inhibition, as expected for competitive inhibition. However, Lineweaver-Burk plots in the presence of IAA are curvilinear. A similar result was obtained by Sirois and Miller (17) who showed that scopoletin acts as a competitive inhibitor of IAA oxidation, but that Lineweaver-Burk plots in the presence of scopoletin were curvilinear. The cause(s) of the deviation from linearity in the presence of inhibitor remains unclear.

If competition for the donor site of HRP-I and HRP-II were the only mechanism through which FA inhibits IAA oxidation, linear increases in FA concentration would be expected to cause linear increases in lag period, rather than the logarithmic increases observed (Fig. 2). FA must therefore interfere with more than one step in IAA oxidation, a point made previously by Stonier and Yoneda (20). I propose that FA and IAA compete for the donor site of HRP-III, but that FA forms an inactive complex (reaction 3), which may be the "modified-HRP" of Sirois and Miller (17). As the IAA/FA ratio decreases, FA would compete not only for the donor site of HRP-I and HRP-II, but it would also displace IAA from the donor site of HRP-III, thereby reducing the concentration of HRP-I and HRP-II and further slowing FA oxidation. The effect of IAA concentration on the lag produced by a given FA concentration (Fig. 7) supports this interpretation. Increasing IAA from 0.05 mm to 0.15 mm causes an approximate 50% reduction in lag. Further increases in IAA from 0.15 mm to 0.5 mm reduce the lag by only about 15%. The initial rapid decrease is interpreted as IAA displacement of FA from the donor site of HRP-III, thereby increasing the concentration of effective enzyme. The subsequent gradual decrease in lag as IAA increases is interpreted as competition between IAA and FA for the donor site of HRP-I and HRP-II, the donor site of HRP-III now being essentially saturated with IAA.

Another prediction of the model is that the rate of FA oxidation should increase as the lag progresses, since IAA would compete more effectively for the donor site of HRP-III, thereby increasing the amount of HRP-I and HRP-II available to oxidize FA. Although this prediction may be borne out (Fig. 6), the most rapid increase is coincident with the onset of rapid IAA oxidation and could result from some other mechanism. The model is in agreement with the finding that the rate of IAA oxidation varies sigmoidally as a function of IAA concentration in the presence of scopoletin (17).

Examination of the model suggests that linear increases in FA would produce linear increases in lag period if competition between IAA and FA for the donor site of HRP-III remained constant. This condition is achieved by increasing FA while maintaining the same IAA/FA ratio by simultaneously increasing IAA. Under these conditions the lag period is seen to vary linearly with linear increases in FA (Fig. 8).

The promoting effects of MnCl$_2$ and DCP (and other phenols) probably are exerted through the regeneration of HRP$^{+}$ and nonenzymatic-free radical chain reactions (21). The proposed model provides a framework for interpretation of seemingly contradictory observations on the enzymatic oxidation of IAA and suggests several avenues for further experimentation.

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LITERATURE CITED