Role of Phenolic Inhibitors in Peroxidase-mediated Degradation of Indole-3-acetic Acid1, 2

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TSUNG T. LEE
Research Institute, Agriculture Canada, University Sub Post Office, London, Ontario, Canada, N6A 5B7

ABSTRACT

7-Hydroxy-2,3-dihydrobenzofuran derivatives, metabolites of a carbamate insecticide carbofuran, and five other phenolic inhibitors of indoleacetic acid (IAA) oxidase interfered with IAA-induced spectral change in the Soret band of horseradish peroxidase (HRP). The onset of IAA degradation required transformed HRP intermediates. The inhibitors, when added before IAA, protected HRP from reacting with IAA, thus preventing formation of highly reactive enzyme intermediates, and consequently, IAA degradation. When added after IAA, the inhibitors quickly reversed the IAA-induced spectral change of HRP and inhibited further IAA degradation.

The phenolic inhibitors differed in stability and reactivity. 7-Hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran, 3,7-dihydroxy-2,2-dimethyl-2,3-dihydrobenzofuran, catechol, protocatechuic acid, caffeic acid, ferulic acid, and scopoletin belonged to one group which produced only a temporary inhibition to IAA-induced spectral change of HRP and IAA degradation since the inhibitors were metabolized in the reaction. The length of the lag was dependent on the IAA, inhibitor, and enzyme concentrations. 3-Keto-7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran and 3-keto-carboutran belonged to the other group which produced a persistent inhibition.

Degradation of IAA required both the heme group and apoprotein of HRP. Reconstituted enzyme from bovine hemin and apoprotein or HRP after unfolding by urea or guanidine treatment were inhibited by the inhibitors in a way similar to the native HRP. The inhibition was reversible by higher concentrations of IAA, but the plot of 1/1 versus 1/s and 1/v versus i were curvilinear, reflecting the complex nature of a competitive inhibition.

In the study of the growth-promoting activity in plants of a carbamate insecticide carbofuran (2,2-dimethyl-2,3-dihydrobenzofuranyl-7-N-methylcarbamate), we found that certain metabolites of this compound inhibited the enzymic degradation of IAA and promoted growth in pea stem segment assays in the presence of IAA (5, 6). 7-Hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran and its derivatives were most interesting because they showed different kinetic behavior in the inhibition of IAA degradation, which has not been noted before. Therefore, this group of compounds, together with other known phenolic inhibitors, were used in a further study on the mechanism by which they inhibited the enzymic degradation of IAA.

MATERIALS AND METHODS

Horseradish peroxidase (EC 1.11.1.7) with a RZ value ($A_{280}$/$A_{275}$) of 2.9 was used. The reaction solution usually contained 1 to 2 μM HRP, 2 to 30 μM IAA, 25 mm K-phosphate (pH 5.9), and various concentrations of phenolic inhibitors. 2,4-Dichlorophenol and MnCl₂ were not used for the reaction unless with a low enzyme concentration ($10^{-10}$ M). The volume of the reaction solution was 2.5 or 3 ml. The temperature was maintained at 25 C by a thermostatically controlled cell compartment. The ΔA of HRP was monitored in the range from 350 to 600 nm or at selected wavelengths with a Unicam 8000 automatic recording spectrophotometer. ΔA at 418 nm was used as an indicator for the transient formation of HRP-II (8, 11). The wavelength 427 nm is the isosbestic point of ferroperoxidase, HRP-II and HRP-III, and therefore it was used to indicate the over-all change of HRP (8). The rate of IAA degradation was measured by the increase in $A_{340}$ nm or by decarboxylation of $[1^{14}C]IAA$. The radioactivity was determined by liquid scintillation counting. Removal of the prosthetic group from HRP was done by the method of Yonetani (13). Enzyme was reconstituted by mixing the apoprotein with bovine hemin at pH 7.5. The stability of the inhibitors was determined by spectrophotometry and TLC (6).

The chemicals used were from the following sources: HDB and 3-keto-carboutran (Niagara Chemicals, F.M.C.). DDB and KHDB (Dr. R. A. Chapman of this Institute). $[1^{14}C]IAA$ (Amersham/Searle). IAA (Calbiochem). HRP, bovine hemin, protocatechuic acid, caffeic acid, ferulic acid, and scopoletin (Sigma), and catechol (Fisher).

RESULTS AND DISCUSSION

It has been known that IAA reacts with HRP under aerobic conditions causing absorbance changes in the Soret band of HRP (2, 9, 11). The ferroporphyrin group of HRP has an absorption maximum at 403 nm. The IAA-induced transient spectral change has been attributed to formation of intermediates HRP-III and HRP-II depending on IAA concentration and pH (8). 7-Hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran and related compounds tested were found to interfere with the IAA-induced spectral change of HRP. Figure 1 shows the reversible shift in absorption peaks of HRP following successive additions of IAA and HDB. Under the specified condition, when HRP was mixed with IAA the absorption peak changed from 403 to 418 nm within 45 sec. The peak remained at 418 nm for 8 min although the intensity decreased with time. In contrast, addition of HDB 2 min after IAA shifted the peak from 418 nm back to 403 nm. Therefore, this type of inhibition is reversible by higher concentrations of IAA or the presence of HDB.

ABBREVIATIONS: HRP: horseradish peroxidase; HDB: 7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran; DDB: 3,7-dihydroxy-2,2-dimethyl-2,3-dihydrobenzofuran; KHDB: 3-keto-7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran.

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The pH was at 5.9 and the temperature 25 C.

403 nm in 1 min. The time course of the change represented by ΔA at 427 nm is shown in Figure 2. It increased rapidly after addition of IAA and reached a peak in 1 min followed by a gradual decline until IAA was completely degraded. With the addition of HDBB, however, the effect of IAA on the heme protein was rapidly reversed before the depletion of IAA.

Mixing the inhibitor HDBB with HRP in the absence of IAA did not change the A of HRP, but mixing HDBB with HRP followed by addition of IAA delayed IAA-induced spectral change of HRP and IAA degradation. It is crucial to know whether the inhibitor-induced delay in ΔA of HRP was related to IAA degradation. With low concentrations of HDBB as the inhibitor, parallel tests showed a close relationship between the two (Fig. 3). A high rate of IAA degradation occurred only after a noticeable ΔA in HRP as indicated by the increase at 418 nm. The ΔA represented a mixture of enzyme forms with absorption maxima at 403 nm, 418 nm, and perhaps other wavelengths. The transformation of a small number of HRP molecules with absorption maximum from 403 to 418 nm by reacting with IAA in the lag period would not be detectable by the method used. Therefore, the spectral change of HRP certainly occurred before IAA degradation. The results obtained by two different methods for IAA degradation, the release of 14C2O from [1-14C]IAA and the increase in A at 261 nm, were similar. The rate of IAA degradation reached a steady level when the A of HRP at 418 nm reached a peak. When a slightly higher concentration of HDBB was used, the lag periods for the spectral change of HRP and IAA degradation were both extended. The slow increase in A at 261 nm in the long lag period was due to a low rate of IAA degradation which was readily detectable in the later part of the long lag period. On the other hand, the formation of a small amount of HRP intermediates during the lag period was not detectable by the spectral change at 418 nm.

The delay in IAA degradation by HDBB was associated with the delay in absorbance change of HRP (Fig. 3). The results support the view that the enzyme intermediates rather than the native HRP degraded IAA (7, 9, 12). The inhibitor HDBB protected the heme group of the enzyme from reacting with IAA thus preventing the formation of highly reactive enzyme intermediates and consequently preventing IAA degradation.

Note also that the length of the inhibitor-induced lag was not proportional to the inhibitor concentration (Fig. 3). It appears to agree with the finding of Gelinas (3) that with ferulic acid as the inhibitor, a linear increase in inhibitor concentration resulted in a logarithmic increase in the lag period. The results suggest that the inhibition occurred in more than one step. This is further supported by the observations that the IAA-induced spectral change of HRP was rapidly reversed by subsequent addition of DDDB (Figs. 1 and 2).

To confirm the observation (Fig. 3) that the delay by HDBB in the IAA-induced spectral change of HRP was related to the delay in IAA degradation, other phenolic inhibitors were tested. The kinetic behavior of six phenolic compounds, catechol, protocatechuic acid, caffeic acid, ferulic acid, scopoletin, and DDDB in the IAA-HP system was similar to that of HDBB. These compounds all produced a lag period for the IAA-induced ΔA of HRP and IAA degradation. After a lag period which varied in length with the inhibitors and concentrations used, a noticeable increase in A at 418 nm occurred before or concurrent with a rapid increase in the rate of IAA degradation. Interaction of scopoletin and IAA on the spectral change of HRP has been reported by Sirois and Miller (10).

The ending of the HDBB-induced lag was due to breakdown of the inhibitor in the IAA-HP system (6). In the early stage of

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**Fig. 1.** Reversible changes in absorption spectrum of the heme portion of horseradish peroxidase as affected by IAA and 7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran. 1: HRP (1.2 μM) in 2.3 ml K-phosphate (25 μM); 2: 45 sec after 20 μl of IAA (2 mM) was added to 1; 3: 4 min after the addition of IAA; 4: in a separate cuvette, 1 min after the addition of 10 μl of HDBB (2 mM) which was added 2 min after IAA, the concentrations of HRP and IAA were the same as 1 and 2. The pH was 5.9 and the temperature 25 C.

**Fig. 2.** Changes in A of horseradish peroxidase following successive additions of IAA and 7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran. The 2.5-ml reaction solution initially contained 1.2 μM HRP and 25 mM K-phosphate. At zero time, 10 μl of IAA (2 mM) were added (line 1), and at the end of 2 min 5 μl of HDBB (2 mM) were added (line 2). The pH was 5.9 and the temperature 25 C.

**Fig. 3.** Correlation of IAA-induced spectral change of HRP and IAA degradation as affected by two concentrations of 7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran. The 2.5-ml reaction solution contained 1.2 μM HRP, 0.16 mM IAA, 25 mM K-phosphate, and 5.7 μM (——) or 7.6 μM (——) of HDBB. The pH was 5.9 and the temperature 25 C.
the lag period when IAA degradation was negligible, HDDB largely remained, but it disappeared rapidly near the end of the lag period when several IAA degradation products were detectable. A similar result was found with DDBB. Both HDDB and DDBB were not metabolized in vitro without HRP or IAA. Breakdown of catechol, ferulic acid, and scopoletin in peroxidase-catalyzed IAA oxidation has been reported (3, 9, 10). From the similarity in inhibition kinetics, it is assumed that protocatechuic acid and caffeic acid were also degraded in the active IAA-HRP system.

A common feature of the seven phenolic inhibitors tested was that the induced inhibition to peroxidase-mediated IAA degradation was limited by depletion of the inhibitors in the enzyme system. However, among the benzofuran derivatives tested, two keto compounds, KHDBB and 3-keto-carbofuran, were found to be stable in the IAA-HRP system; the induced inhibition of IAA degradation was persistent and without a distinct lag period (6). We therefore compared the two types of phenolic inhibitors on the spectral change of HRP induced by IAA. For the control without inhibitor, the A at 418 nm increased rapidly after mixing with IAA and reached a peak in 50 sec followed by a decline in two distinct stages (Fig. 4A). First, the A decreased sharply for 3 min then remained at a low level for 3 min but the maximum stayed at 418 nm. In the second stage when IAA was depleted, the A peak shifted back to 403 nm; thus the A at 418 nm decreased sharply. The effects of HDDB (Fig. 4A, line 1) and DDBB (line 2) were similar except for the difference in the length of induced lag period which agreed with that for IAA degradation (6). Both compounds showed only a limited inhibition, although HDDB was more inhibitory than DDBB. After the lag, the A at 418 nm reached a peak as high as that of the control. Corresponding changes in A at 403 nm are shown in Figure 4B.

In contrast, the effect of KHDBB was different. The slow increase and the low level of A at 418 nm (Fig. 4A, line 3) reflected a partial persistent inhibition of IAA-HRP interaction. This was confirmed by repeated scan of the absorption spectrum in the Soret region which showed that during the 10-min period when the A at 418 nm finally reached the highest point, the absorption maximum shifted only slightly from 403 nm to the right and never changed to 418 nm as did with HDDB or DDBB. The curve represented a mixture of native HRP protected by the keto inhibitor and enzyme intermediates as a result of reacting with IAA. When IAA was depleted, the enzyme returned to the native state as reflected by the decrease in A at 418 nm in the final stage. Little change in A at 403 nm with this inhibitor is demonstrated in Figure 4B (line 3). A similar effect was observed with 3-keto-carbofuran at higher concentrations.

Clearly, the two types of phenolic inhibitors differed in kinetic behavior as expressed in the spectral change of HRP as well as the rate of IAA degradation.

The inhibition of IAA degradation by HDDB and KHDBB was not limited to the native HRP (6). When the inhibitors were added at different stages of the reaction, at which certain transient forms of HRP might be predominant, a similar inhibition was observed. It appears that the inhibitors had greater affinities than IAA not only for the native HRP but also for the enzyme intermediates. This agrees with the interpretation made earlier for Figure 3 in which a small increase in the inhibitor concentration induced more than a linear increase in the lag period. Presumably, HRP-III was the key intermediate which controlled the rate of formation of HRP-II and HRP-I, which, in turn, controlled the rate of breakdown of IAA and labile phenolic inhibitors.

Removal of the prosthetic group of HRP by acid butanone destroyed the enzyme activity for IAA degradation in the apoenzyme portion, but the enzyme activity recovered after reconstitution of a holoenzyme from bovine hemin and the apoprotein. The result agreed with that of Ku et al. (4). Both HDDB and KHDBB inhibited IAA degradation by the reconstituted enzyme in a similar way as that by the native HRP. Thus clearly, not only the heme group was involved in IAA degradation, but the apoenzyme was also required. Preliminary results showed a binding of IAA to the protein moiety of HRP (unpublished). Binding of donor molecules to the protein portion of HRP had been suggested by Chance a long time ago (1). It appears that there is more than one site for IAA. How the phenolic inhibitors affect its binding is not known.

Unfolding of HRP molecules by treatment with high concentrations of urea and guanidine did not change the response of the enzyme to inhibition by HDDB or KHDBB. Heating the enzyme solution at 60 C for 5 min with or without 10 mM urea also showed no effect. It appears that the inhibitor-IAA relationship was not affected by a conformational change.

Increasing IAA concentrations reversed the inhibition by both types of inhibitors, but a complete reversal was not observed.

Fig. 4. Comparison of the effects of 7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran and related compounds on IAA-induced spectral change of HRP. A: 418 nm; B: 403 nm. The 2.5-mL reaction solution contained 1.2 mM HRP, 8 mM IAA, 25 mM K-phosphate, and 1.6 mM HDBB (line 1), DDBB (line 2), or KHDBB (line 3). The pH was 5.9 and the temperature 25 C.

Fig. 5. Effect of IAA concentration on the rate of IAA degradation in the presence or absence of 3-keto-7-hydroxy-2,2-dimethyl-3,3-dihydrobenzofuran. v represents A418/min and s is the IAA concentration in mM. The 3-mL reaction solution contained 0.067 to 1.4 mM IAA, zero or 33 mM KHDBB, 0.2 mM 2,4-dichlorophenol, 0.2 mM MnCl2, 25 mM K-phosphate, and 2.5 g HRP. The pH was 5.9 and the temperature 25 C.
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Fig. 6. Effect of inhibitor concentration on the rate of IAA degradation. v represents ΔA381 min⁻¹ and i is the concentration of 3-keto-7-hydroxy-2,2-dimethyl-2,3-dihydrobenzofuran in 10 μM. The 3-mI reaction solution contained zero to 133 μM KHDDB, 33 μM IAA, 50 μM 2,4-dichlorophenol, 50 μM MnCl₂, 25 mm K-phosphate, and 0.5 μg HRP. The pH was 5.9 and the temperature 25°C.

With the second type of inhibitor, KHDDB, tested in a broad range of IAA concentrations, a Lineweaver-Burk plot of 1/v against 1/i is shown in Figure 5. Note a substrate inhibition in the high concentration range. With a low concentration of IAA, the plot was also not completely linear. It seems to reflect at least in part the participation of IAA in multiple steps of reaction. In the presence of the inhibitor, the plot (Fig. 5, line 2) was further curved. Similar results have been observed with the other type of inhibitors, such as HDDB (6) and scopoletin (10).

The effect of increasing inhibitor concentration on the rate of IAA degradation is shown in Figure 6. With KHDDB as the inhibitor, the 1/v versus i plot showed basically a competitive inhibition although the inhibitor at a high concentration produced more inhibition than would be expected. Thus it appears that the nonlinearity in both kinds of plot reflects a complex nature of the reaction, in which the phenolic inhibitors may compete with IAA at various steps and the different enzyme intermediates may differ in affinities for the inhibitor and IAA.

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LITERATURE CITED