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Studies on Auxin Protectors. V. On the Mechanism of IAA Protection by Protector-I of the Japanese Morning Glory

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Abstract. **The mechanism of auxin protection by auxin protector-I (Pr-I) of the Japanese morning glory was studied *in vitro*. Four lines of evidence indicate that Pr-I acts as a strong reductant which prevents the peroxidase-catalyzed oxidation of IAA: 1) The kinetics of the reaction are best explained on this basis. 2) The Pr-I-induced lag preceding auxin destruction by peroxidase is completely eliminated by a strong oxidant such as H_2O_2 at a concentration which does not appreciably affect the reaction rate. 3) Strong organic reductants mimic the Pr-I-induced lag. And 4) when the reaction rate is altered by varying the concentrations of the reactants, or the temperature, the length of the Pr-I-induced lag varies inversely with the reaction rate.**

In the Japanese morning glory there exist substances with relatively high molecular weights which inhibit the destruction of indoleacetic acid (IAA) both by commercial horseradish peroxidase and by enzymes present in Japanese morning glory stems (15). Experiments on young plants have indicated that the inhibition of enzymatic destruction of IAA varies inversely with the age of the tissue, *i.e.*, inhibition decreases as one descends along the stem, and the interference with IAA destruction correlates with the rate of internode and leaf elongation. Thus auxin protection is greatest in leaves and internodes elongating most rapidly, and diminishes in maturing and senescing tissue (21, 22). At least 3 substances (or complexes or polymers of the same substance) appear to be involved: protector-A (Pr-A), protector-I (Pr-I), and protector-II (Pr-II). Based on gel filtration studies Pr-A has a molecular weight exceeding 200 000 g/mole and has been found to be highly active in seeds. Activity in the shoot tips of mature plants is also high (22). Pr-I has a molecular weight of approximately 8000 g/mole, and Pr-II has a molecular weight of approximately 2000 g/mole (15). Only small quantities of Pr-I, and no Pr-II, could be detected at the shoot apexes, whereas these 2 protectors are found in large quantities in the juvenile tissue of the shoot, and appear to play an important role in stem and leaf elongation. In old stem or leaf tissue, only small quantities of Pr-I and II are found and Pr-A could not be detected (22).

Similar substances have been observed in other plant systems such as tobacco (11, 12), sunflower, cocklebur, coconut water (coconut milk), *Nicotiana rustica*, *N. glauca*, and *N. affinis* (16, and unpublished observations). Clearly the morphogenetic role of these substances should be explored further.

One of the questions that needs to be answered is how do the protectors exert their action? What is the molecular mechanism of auxin protection? This paper addresses itself to this question by investigating the action of Pr-I.

As working hypotheses, one might consider 3 possibilities: 1) The protector acts as an enzyme inhibitor. 2) The protector complexes with IAA, thus preventing enzymatic attack. 3) The protector acts in some other fashion.

The first 2 possibilities can be considered improbable on kinetic grounds: Examination of the curves depicting the destruction of IAA by commercial horseradish peroxidase (17) shows that the presence of the protector brings about a lag in destruction not a change in rate. If Pr-I were to complex with either IAA or peroxidase alone, thereby inhibiting the destruction of IAA, it would have to do so with something approaching 100% efficiency for a limited time (*i.e.*, during the lag when no destruction occurs) and then would have to dissociate (the complex) suddenly and act with zero efficiency (*i.e.*, as destruction proceeds at a rate comparable to controls).

The evidence presented below indicates that Pr-I exerts its protective action by virtue of the fact that it can act as an antioxidant. This interpretation is consistent with our previous observations (17), that Pr-I is rapidly inactivated by Mn^{3+} even in an atmosphere of nitrogen, while Mn^{2+} will do so only in the presence of oxygen.

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Materials and Methods

Source of Protector-I. The technique for obtaining a partially purified preparation of auxin protector-I of the Japanese morning glory (*Pharbitis nil* Choisy) has been described previously (17). In essence, the material consisted of an aqueous extract of young leaves which was subjected to gel filtration (Sephadex G-50, Pharmacia) and assayed for auxin protection as indicated below.

Basic Assay for Protector-I. Unless otherwise indicated, the reaction mixture used to assay for auxin destruction, or the inhibition of destruction, consisted of a mixture of dichlorophenol (DCP) (Eastman Organic Chemicals), manganese chloride (Baker Analyzed), and indole-3-acetic acid (Calbiochem), each at a concentration of 0.1 mM, and horseradish peroxidase (Calbiochem, RZ value 0.84) at a final concentration of 0.2 μg/ml. All reactions were carried out at 30° in a Dubnoff shaker, and buffered at pH 6.1 with 0.02 M potassium phosphate (Baker Analyzed). The reaction mixture in each 50 ml Erlenmeyer flask was adjusted to a total volume of 10 ml. Destruction of IAA was followed by removing samples at various time intervals and assaying by means of Salkowski reagent; optical density at 540 mμ was used to detect the amount of IAA in the reaction mixture.

Other Chemicals. Glutathione (Calbiochem), Dithiothreitol (Calbiochem), $FeSO_4 \cdot 7H_2O$ (Baker Analyzed), and $CoCl_2 \cdot 6H_2O$ (Baker Analyzed) were utilized in the concentration and manner indicated below. Stock solutions of these compounds were made up on the day they were used in water boiled to exclude air, and were kept under nitrogen at 4° until added to the reaction mixture. Riboflavin (Eastman Organic Chemicals), and hydrogen peroxide (30 %, Baker Analyzed) were also made up fresh each day and refrigerated until use.

Preincubation Experiments. In some of the experiments one, or a combination of reagents, were preincubated for varying periods of time in buffer. At the end of such a preincubation period, the rest of the reagents were added to complete the basic assay as described above (see below).

Results

The data presented below involve the following 4 concepts: 1) The efficiency of IAA protection is related inversely to the efficiency with which IAA is oxidized by peroxidase. That is, anything that normally decreases the rate of IAA oxidation by peroxidase, *e.g.*, decreased temperatures, decreased enzyme concentration, superoptimal or suboptimal concentrations of other reagents, also results in an increase in the Pr-I-induced lag period. 2) The Pr-I-induced lag period can be eliminated by the addition of strong oxidants such as hydrogen peroxide, or riboflavine in the presence of light. 3) Two organic reductants, glutathione and dithiothreitol (3), are able to mimic Pr-I, *i.e.*, they also produce a lag phase preceding IAA oxidation. So can riboflavine if light is excluded from the system. Inorganic reductants such as Fe^{2+} and Co^{2+} show no effect. 4) In accordance with the evidence that Pr-I acts as an antioxidant, some preincubation experiments were performed to ascertain which of the components in the reaction system interact with Pr-I. Experiments reported previously (17) have shown that Pr-I interacts with manganese.

Effect of Temperature on Pr-I-Induced Lag. Table I shows the effect of decreasing the temperature from 30° to 20°. In the absence of protector, the oxidation of IAA by horseradish peroxidase was slowed approximately by a factor of 2, *i.e.*, it took approximately twice as long to destroy half of the Salkowski-measurable IAA. In the presence of Pr-I the lag was increased but not necessarily by a corresponding factor of 2.

Table I. *Effect of Temperature on Pr-I-Induced Lag*

Conc of Pr-I	Temp	Lag time
	deg	*min*
0 (control)	30	0
0 "	20	0[1]
2.5 %	30	15
2.5 "	20	30
5.0 "	30	90
5.0 "	20	>240

[1] Although lag time was not affected by temp, reaction rate was roughly halved (see text).

Effect of Varying the Concentration of Enzyme. Figure 1 shows the effect of varying the concentration of peroxidase with a concomitant doubling or halving of the reaction rate in the absence of Pr-I. In the presence of Pr-I there is a corresponding decrease or increase of the lag period. Note the

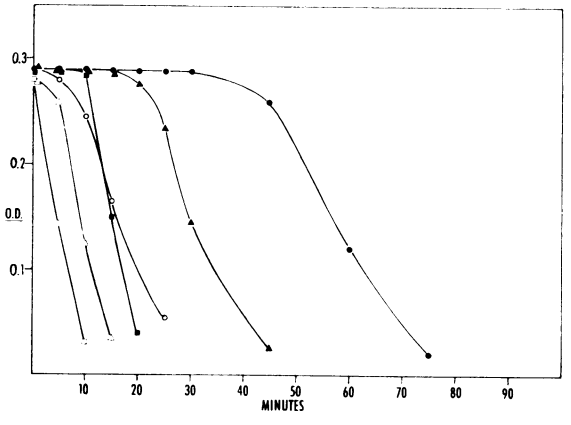

FIG. 1. Effect of enzyme concentration on lag: The peroxidase concentration in 10 ml of reaction mixture is 0.4 μg (□), 0.2 μg (△), and 0.1 μg (○). Solid symbols represent the corresponding enzyme concentration but in the presence of 0.4 ml Pr-I. Note that with decreasing enzyme concentrations, there is an increasing lag.

difference in the shapes of the curve when comparing the reaction involving 0.4 μg peroxidase in the presence of Pr-I, with 0.1 μg peroxidase in the absence of Pr-I: In both cases approximately half the Salkowski-detectable IAA has disappeared in 15 minutes. However, in the former case, the reaction starts later (*i.e.*, following a lag of about 10 min) and is completed sooner.

Effect of Varying the Concentration of Other Reagents in the System. In addition to peroxidase, the reaction mixture contained IAA, Mn^{2+}, and DCP, each at a concentration of 0.1 mM. The effect of varying the concentration of IAA on the Pr-I-induced lag period has been reported previously (16), as has the interaction between Mn and Pr-I (17). In the absence of Pr-I, increasing or decreasing the concentration of DCP causes a decrease in the rate of peroxidase-catalyzed IAA destruction. Maximum (optimum) rates of destruction were observed at a ratio of 1 mole of DCP per 2 moles of IAA. Table II shows the change in reaction rates brought about by changing the concentration of DCP (from 0.013–0.4 mM) in the presence of 0.1 mM IAA and Mn^{2+}, and the corresponding effect on the lag period produced by the presence of Pr-I.

Table II. *Effect of Varying Concentrations of Dichlorophenol on Both Reaction Rates of IAA Destruction and Pr-I induced Lag Periods*

IAA, Mn^{2+}, and enzyme present at usual concentrations (see Materials and Methods section). Pr-I added as 0.4 ml per 10 ml reaction mixture.

	Conc of DCP					
	0.013	0.025	0.05	0.1	0.2	0.4,
			mM			
50% IAA destruction (min) (no Pr-I)	18.5	11.5	9.5	10	13.5	25
Length of Pr-I induced lag (min)	20	14	8	8	18	>30

Effect of H_2O_2 on Pr-I-Induced Lag. The addition of 0.01 mM H_2O_2 to the reaction mixture caused a complete elimination of the Pr-I-induced lag. In the absence of Pr-I, destruction of IAA proceeded at a rate comparable to controls lacking H_2O_2 (fig 2). This was true even in the absence of Mn. Hydrogen peroxide, by itself, did not oxidize IAA over the time periods tested. At higher concentrations (0.1 mM), hydrogen peroxide could substitute partially for Mn, *i.e.*, in the presence of peroxidase and DCP, the presence of 0.1 mM H_2O_2 accelerated IAA destruction, but only during the first few minutes of the reaction.

Effect of Riboflavine on IAA Destruction and Protection. Like H_2O_2, the addition of 0.1 mM riboflavine in the presence of light, completely eliminated the Pr-I-induced lag. However, unlike H_2O_2, in the absence of Pr-I, riboflavine speeded up the destruction of IAA in the light and would, by itself, bring about the destruction of IAA.

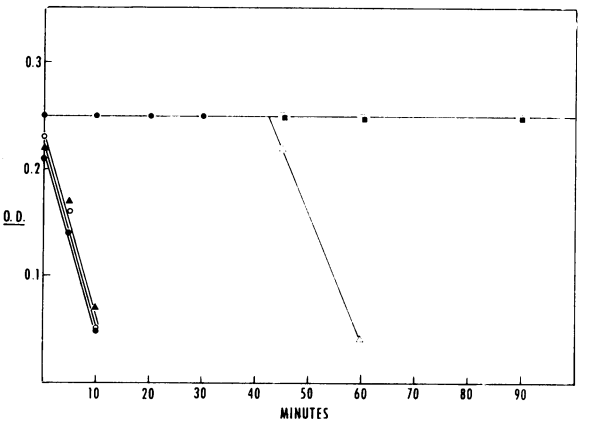

FIG. 2. Effect of 0.01 mM H_2O_2 on Pr-I induced lag: enzyme only (○), enzyme + H_2O_2 (●), enzyme + Pr-I (△), enzyme + Pr-I + H_2O_2 (▲), neither enzyme nor Pr-I (□), neither enzyme nor Pr-I, but H_2O_2 (■). Note H_2O_2 does not oxidize IAA, nor affect rate of IAA oxidation but does completely eliminate Pr-I induced lag.

In contrast, in the dark, riboflavine inhibited IAA destruction. This was true both in the presence and absence of Pr-I. Figure 3 shows that riboflavine acts as an inhibitor of IAA destruction until it is exposed to light. At that point, the presence of Pr-I, is ineffective in preventing IAA destruction.

IAA-Destruction as Affected by Organic Reducing Agents. The addition of reduced glutathione (0.05–0.2 mM) produced a lag similar to that produced by Pr-I. However, following the lag period, the rate of destruction did not coincide with that of the controls, rather it usually proceeded more slowly. Reduced dithiothreitol (0.01–0.3 mM), on the other hand, mimicked Pr-I in that the lag period was followed by a rate of IAA destruction comparable to controls. At higher concentrations, dithiothreitol interfered with the Salkowski reaction so that at a concentration of 1.0 mM, the IAA seemed to "disappear" almost immediately, and then "reappear"

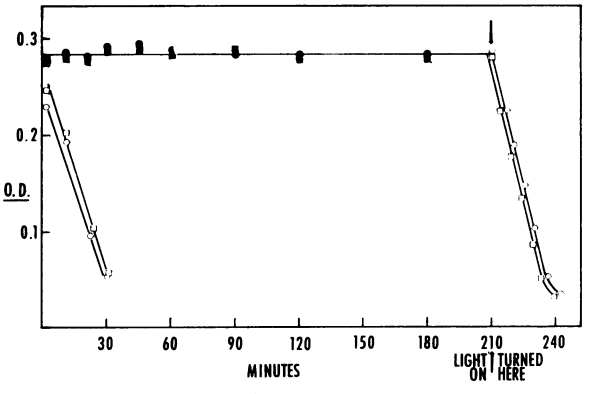

FIG. 3. Effect of riboflavin on Pr-I-induced lag in the light and in the dark: Riboflavin added in the presence of light (○), and in the dark (●), and the combination of riboflavin and Pr-I in the light (□), and dark (■). Note that Pr-I is rendered completely ineffective in the light.

over a period of many hours, and then disappear again after 24 hours as further oxidation inactivated the dithiothreitol.

Effects of Inorganic Reducing Cations. In the presence of manganese neither Fe^{2+} ions, added as 0.1 mM $FeSO_4$, nor Co^{2+} ions, added as 0.1 mM $CoCl_2$, produced any appreciable lag. In the absence of manganese, addition of Co^{2+} neither enhanced nor diminished the rate of IAA destruction under the conditions of the experiments.

Preincubation Experiments. In our earlier report (17) we described experiments in which Pr-I was preincubated with $MnCl_2$ prior to the addition of the other 3 reagents (IAA, peroxidase, DCP). Under these conditions, in the presence of oxygen, Pr-I was inactivated by the Mn. In other words, Pr-I interacted directly with the manganese ions (probably Mn^{3+}). Similar preincubation experiments were carried out with IAA, peroxidase, and DCP.

Preincubating Pr-I with either IAA or DCP caused no appreciable difference in lag period when compared to controls (Pr-I shaking by itself in buffer, which causes some inactivation of the protector). Preincubating Pr-I with the enzyme lengthened the lag period. However, a similar increase could be produced by shaking the enzyme in buffer by itself, prior to adding Pr-I and the other reagents. Furthermore, preincubating the enzyme by itself also caused a lag of about 4 minutes to appear even in the absence of any added Pr-I. Thus these experiments yielded no data which demonstrated a direct interaction between Pr-I and IAA, peroxidase, or DCP.

Discussion

The most important single clue to the molecular mechanism of Pr-I action is the shape of the curve that reflects the destruction of IAA by peroxidase in the presence of the protector: The rate of the reaction is not appreciably changed. Instead, the presence of Pr-I brings about a delay in the disappearance of Salkowski-detectable IAA. Such a phenomenon tends to rule out, as we have indicated in the introduction, the likelihood that Pr-I is acting simply either as an enzyme inhibitor, or that it complexes with IAA thereby preventing enzymatic attack.

The fact that it is possible to mimic this phenomenon with strong organic reducing agents, and that strong oxidants such as H_2O_2, and riboflavine in the presence of light, completely neutralize Pr-I, implies that the protector acts as an electron donor. This is consistent with our previous report that Pr-I is rapidly inactivated by Mn^{3+} even under nitrogen, and by Mn^{2+} in the presence of oxygen (17). This finding is also consistent with the reports of Pilet (13) who found that glutathione decreases IAA-oxidase activity of carrot cultures and extracts, and Betz (2) who found that not only glutathione, but also ascorbic acid, NADH, cysteine, and thioglycolic acid are effective inhibitors of peroxidase-catalyzed oxidation of IAA. Betz also showed that the oxidized forms of glutathione, NAD, and cystine fail to act as inhibitors, and concluded that it is the reducing capability of these substances which caused the inhibition. Betz did not publish the kinetics of the reaction and suggested that the inhibition is a case of allosteric inhibition.

The preincubation experiments indicate that although Pr-I interacts directly with manganese, Pr-I does not seem to interact directly with either IAA, DCP, or the enzyme, when these reagents are preincubated singly with the protector. Yet it cannot be concluded that the effect of Pr-I is solely upon the manganese: This is shown again by the shape of the curve representing IAA destruction. Clemetson and Andersen (4) for example, have demonstrated that certain plant phenols such as rutin and quercetin prevent the oxidation of ascorbic acid (*i.e.*, protect it) by chelating with copper. In their case, however, the removal of the metalic catalyst results in a decreased rate of ascorbic acid oxidation, not a lag (see their fig 5, p 364). Similarly, in our experiments, as well as those first reported by Wagenknecht and Burris (19) and numerous other workers since (*e.g.*, as reviewed by 6, 10), removing the manganese from the system merely slows the rate of the reaction. In contrast, the presence of Pr-I introduce a lag and therefore Pr-I must do more than merely interact with the manganese.

It is to the first set of experiments that we can look for a clue: The slower the reaction, the longer the lag. Apparently Pr-I either donates electrons more readily than IAA thereby preventing the oxidation of IAA (by keeping the other reactants in a reduced state), or Pr-I replenishes electrons lost by IAA in a reversible step ultimately leading to its oxidized form (*e.g.*, an intermediate along the lines suggested by Meudt (8, 9). This means that as long as a significant number of Pr-I molecules are present, no IAA is irreversibly oxidized. Pr-I, like glutathione and dithiothreitol, constitutes a reservoir of reducing power with a finite capability dependent on concentration. Any factor which speeds up the rate of the reaction thus speeding the transfer of electrons during the process of oxidation, causes a decrease in the time during which Pr-I protects IAA: Once Pr-I has been depleted of electrons, it becomes inactive, and the oxidation of IAA proceeds at rates comparable to that of the controls containing no Pr-I. Conversely, anything which interferes with the reaction, slowing the flow of electrons, decreases the rate of electron donation by Pr-I and thereby enhances the lag period. Increases in the lag period can be brought about by decreasing the temperature, decreasing the concentration of the enzyme, or adding superoptimal, or suboptimal concentrations of cofactors.

The apparent instability of the IAA-protector substances reported by us previously (15), is now explicable in terms of the inactivation of Pr-I as a

result of oxidation. This is demonstrated by the extremely rapid inactivation of Pr-I by low (0.01 mM) concentrations of H_2O_2 which have virtually no effect on the rate of the enzymatic oxidation of IAA in the absence of Pr-I. Similar results were obtained by preincubating Pr-I with riboflavine in the light, but not in the dark.

The evidence presented above, although strongly suggestive, remains indirect. Aside from the interaction with manganese, reported by us earlier (17), no obvious mechanism of the reaction is suggested by the data. The shape of the curve reflecting IAA destruction implies an intervention on the part of Pr-I at more than 1 site in the electron transport from IAA to the electron acceptor. It may be that intervention at any 1 site by itself merely brings about a reduction in the rate of destruction (as with manganese), but that the combination involves synergistic interactions such that the reaction rate is reduced virtually to zero, hence the lag in IAA destruction. This could also explain why doubling the concentration of Pr-I brings about a much greater than 2-fold lag. Thus one may ask, does the inhibition of the IAA oxidation involve in addition, an allosteric inhibition of the peroxidase as suggested by Betz (2)? Or is one dealing with the formation of free IAA* radicals as suggested by several workers (*e.g.* 1, 5, 7, 20) and the protector donates electrons to this radical in a charge transfer reaction as shown to occur with other indole compounds (18)? The complex question of possible molecular mechanisms involving antioxidants, and the relation of antioxidants to growth, has been reviewed by Siegel and Porto (14). We hope to be able to clarify some of these questions in the future.

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