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Mode of Action of the Toxin from *Pseudomonas phaseolicola*

I. TOXIN SPECIFICITY, CHLOROSIS, AND ORNITHINE ACCUMULATION[1, 2]

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ABSTRACT

The specificity of the *Pseudomonas phaseolicola* toxin for enzyme inhibition and its relationship to toxin-induced chlorosis in bean leaves (*Phaseolus vulgaris* L.) was examined. The toxin showed no significant inhibitory activity against glutamine synthetase, glutamine transferase, carbamyl phosphate synthetase, aspartate carbamoyltransferase, or arginase at concentrations 100-fold higher than that needed to inhibit ornithine carbamoyltransferase by 50%.

Protection from and reversal of toxin-induced chlorosis in bean leaves was attempted with several amino acids. Aside from protection with L-citrulline which was previously reported, only L-arginine-HCl and to a minor extent L-leucine and L-glutamine showed protection from chlorosis. L-Citrulline and L-arginine-HCl (but not L-glutamine and L-leucine) also reversed toxin-induced chlorosis.

Ultrastructurally, cells from toxin-treated chlorotic tissues showed no observable changes as compared to nontreated tissues. This, together with the ability of the two amino acids to reverse chlorosis, indicated that the toxin causes a reversible biochemical lesion in treated tissue.

While tissues from bean plants inoculated with *P. phaseolicola* showed a large accumulation of ornithine, toxin-treated tissues showed no accumulation of ornithine. The latter finding indicated that in addition to the ornithine carbamoyltransferase inhibitor, the pathogen may produce inhibitors of other ornithine metabolizing enzymes in inoculated tissues.

When bean plants (*Phaseolus vulgaris* L.) are infected by their pathogen *Pseudomonas phaseolicola* or injected with its toxic culture filtrates, they exhibit two symptoms: chlorosis and accumulation of ornithine (20, 28). Chlorotic pathogen infected tissues show a 440-fold increase in ornithine content, while chlorotic toxin-treated tissues show a 106-fold increase (28). Since the ornithine pool in plants is usually small (1), this striking increase indicates a derangement in the normal ornithine metabolism of affected tissues.

Ornithine is metabolized primarily through two pathways, although a third may exist (see Fig. 1). When radioactive ornithine is fed to plants, label appears in citrulline, arginine, glutamate, and proline (4). Appearance of label in citrulline and ultimately arginine is catalyzed by the enzymes of the ornithine cycle (13). The first enzyme of this pathway is ornithine carbamoyltransferase (E.C.2.1.3.3). Appearance of label in glutamate (and ultimately proline) involves reversal of ornithine biosynthesis; *i.e.*, formation of glutamic-γ-semialdehyde by γ-ornithine glutamate transaminase (2). Finally, appearance of label in proline occurs through the enzymes converting glutamate to proline (18). A third enzyme of ornithine catabolism, ornithine decarboxylase, may also occur in bean plants.

Accumulation of ornithine in infected tissues or those treated with toxic culture filtrates of the pathogen may be due to the inhibition of any one or all of these enzymes. In addition, inhibition of carbamoylphosphate synthetase or less directly that of glutamine synthetase could also lead to the same effect. Malfunction of either enzyme would retard the synthesis of carbamylphosphate (9, 15, 19), which in turn would affect carbamylation of ornithine to citrulline.

Patil *et al.* (22) previously reported that bean ornithine carbamoyltransferase is inhibited by a partially purified extracellular toxin from *P. phaseolicola* which also induces chlorosis in bean leaves. Further, toxin-induced chlorosis is prevented by citrulline, an end product of the OCT reaction, indicating a cause-effect relationship between OCT inhibition and chlorosis. Whereas OCT inhibition alone may be sufficient to explain chlorosis, it is possible that ACT, an enzyme which leads to the synthesis of pyrimidines, is also inhibited by the toxin. In the following paper (33), we report that the toxin specifically affects the carbamoylphosphate binding region of OCT, a region common to both ACT and OCT.

In this communication we examine the effect of *P. phaseolicola* toxin on several enzymes related to the ornithine cycle, describe protection from and reversal of toxin-induced chlorosis by citrulline and arginine *in vivo*, and discuss the relevance of the findings in relation to toxin inhibition of OCT and chlorosis on the one hand and the reported accumulation of ornithine on the other.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from commercial sources and were reagent grade. Carbamoylphosphate (Na^+),

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[3] Abbreviations: CAP: carbamoylphosphate; CPS: carbamoylphosphate synthetase; ACT: aspartate carbamoyltransferase; GS: glutamine synthetase; GT: glutamine transferase; OCT: ornithine carbamoyltransferase; ARG: arginase; OD: ornithine decarboxylase; OTA: ornithine transaminase.

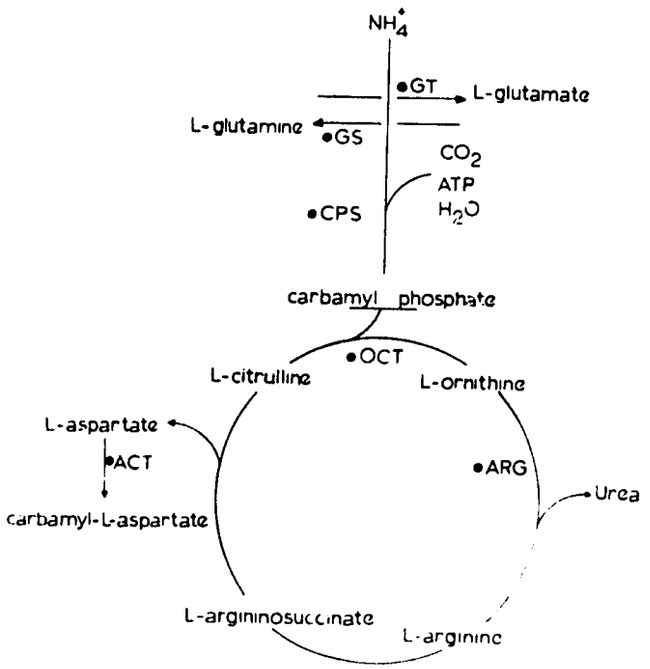

FIG. 1. The ornithine cycle and related enzymes. GT: glutamine transferase; GS: glutamine synthetase; CPS: carbamyl phosphate synthetase; OCT: ornithine carbamoyltransferase; ACT: aspartate carbamoyltransferase; ARG: arginase.

ornithine, citrulline, arginine, acetyl glutamate, 2,2′ iminodiethanol, ATP, and DPNH were obtained from Sigma as were lactic dehydrogenase (rabbit muscle), glutamine synthetase (sheep), and pyruvate kinase (rabbit skeletal muscle). Arginase (beef liver) was obtained from Mann.

Enzyme and Toxin Preparation. Preparation of the acetone powder (21) and crude extracts (33) was as previously described. The bean crude extracts were used as a source of CPS and ACT. Since activity of GS and GT in the above crude extracts was very low, similarly prepared crude extracts of pea (*Pisum sativum*) seedlings were used as a source of these enzymes from a plant source.

Toxin Preparation. The preparation of the toxin is described elsewhere (23). It was from the same batch of standard preparation as used in the kinetic studies of OCT inhibition reported in the next paper (33). After complete hydrolysis in 6 N HCl under vacuum, it contained 42 nmoles of alanine equivalents per μl. For purposes of comparison toxin concentrations are referred to in terms of units. One unit of toxin is that amount of toxin which gives 50% inhibition of OCT under standard assay conditions (33). The above toxin preparation contained 10 units per μl.

Enzyme Assays. CPS was assayed by measuring the rate of DPNH oxidation in a system coupled with pyruvate kinase and lactate dehydrogenase according to Fahien and Cohen (7). In a final volume of 1 ml the reaction mixture contained the following: 10 mM $MgCl_2$, 2.5 mM phosphoenol pyruvate, 50 mM glycylglycine, 0.16 mM DPNH, 0.1 M $KHCO_3$, 0.1 M KCl, 10 mM acetylglutamate, 5 mM ATP, 12 mM NH_4Cl, 0.01 mg of pyruvate kinase, 0.06 mg of lactate dehydrogenase, and enough dialyzed bean crude extract to oxidize 1.6 μmoles of DPNH per min. Under these conditions, neither pyruvate kinase nor lactate dehydrogenase were limiting. The reaction (23 C) was started by adding the bean enzyme to a 1-ml cuvette positioned in a Beckman DB recording spectrophotometer. The blank contained all reactants except DPNH. Under the above conditions the rate of the reaction was linear with time for 10 min. Appropriate controls were used to examine the possibility that the toxin inhibits lactate dehydrogenase or pyruvate kinase. Neither enzyme is inhibited by the toxin. The bean crude extract contained a trace of ATPase activity, but this did not interfere with the assay. That the oxidation of DPNH observed under the conditions of the assay was due to CPS was confirmed by testing feedback inhibitors (arginine and UTP) at a concentration of 10 μmoles per assay. Both reduced the oxidation of DPNH, confirming that bean crude extracts contained CPS.

ACT was assayed by measuring ureidosuccinic acid-diacetyl monoximine complex as described by Sheperdson and Pardee (29), except that the reaction mixtures were incubated at 37 C. The amount of bean crude extract added to reaction mixtures was such as to yield 1 to 2 μmoles of Pi per 30 min. The blank consisted of all reactants except aspartate. Since mercaptoethanol interferes with the ATC assay all assays were conducted in its absence. Arginase was assayed by measuring urea formation according to Brown and Cohen (3). Two milliliters of arginase (1 mg/ml) were dialyzed overnight in 10 mM tris-HCl, pH 9.0. Under the conditions of the assay, which was allowed to run for 10 min at 25 C, 0.133 μmole of urea was formed. GT and GS were assayed by the methods of Stumpf *et al.* (32) and Elliot (5) respectively, except that assays were conducted in tris-HCl at pH 9.0. Both enzymes were preincubated with appropriate concentrations of toxin for 20 min before starting the assays. Incubation with all components of the system together was for 20 min at 37 C. Under these conditions the end-product formed (expressed as glutamylhydroxamate) was 0.12 μmole per min for the GS reaction and 0.177 μmole per min for the GT reaction.

For determining the effect of toxin on ornithine metabolism of bean leaves, a total of 1,213 units of toxin were injected in several individual injections with a hypodermic needle intercellularly from the underside of primary leaves of 12-day-old bean plants. Toxin-treated areas which showed faint chlorosis in 10 days were cut out and freeze-dried under vacuum. Comparable areas from opposite side of each primary leaf which did not receive the toxin were also cut out and freeze-dried similarly. Each sample (0.25 g) of the freeze-dried tissue (treated or nontreated) was blended in 20 ml of hot 95% ethanol, and the homogenate was filtered. The filtrate was evaporated until a green precipitate was observed to which 10 ml of distilled water were added. The precipitate was centrifuged, washed, and discarded, and the combined supernatants were evaporated to 1 ml under vacuum. To this an equal volume of 25% sucrose in 0.1 N HCl was added, and the sample was applied to the column of a Technicon amino acid analyzer. Amino acid concentrations were expressed as μmoles per mg freeze-dried tissue.

For electron microscopic study leaf tissue was diced into 1 mm pieces and aspirated in formaldehyde-glutaraldehyde fixative (11) for 30 min. Material was transferred to fresh fixative and fixed for an additional 4 hr at about 4 C. Samples were washed in 50 mM phosphate buffer, pH 7.2, and postfixed for 2 hr in 1% osmium tetroxide at about 4 C. After washing in phosphate buffer, samples were dehydrated with a graded series of ethanol, treated with propylene oxide, and embedded in epoxy resin. Thin sections were obtained with a diamond knife in a Porter-Blum MT-2B ultramicrotome and were placed on copper grids. Sections were routinely stained for 5 min in aqueous 2% uranyl acetate and for 3 min with lead citrate (26). The sections were examined and photographed in a Hitachi HS-8-1 electron microscope operated at 50 kv.

Amino Acid Studies. Protection studies were done by injecting 5 μmoles (in 10 μl H_2O) of the following amino acids in

trifoliate leaves of 2- to 3-week-old bean plants (23) in the same spot where toxin injection was made. In all cases 10 units (in 10 μl H_2O) of toxin were used. The amino acids were: L-alanine, L-arginine-HCl, L-citrulline, L-cysteine-HCl, L-leucine, L-isoleucine, L-methionine, L-proline, L-threonine, L-serine, and L-valine. In addition, L-glutamine was also used at the same concentrations. At the concentration used, L-tyrosine, L-tryptophan, L-histidine-HCl, L-lysine-HCl, and glycine were phytotoxic to bean leaves. For reversal studies 5 μmoles of each of L-citrulline, L-arginine, L-leucine, and L-phenylalanine were separately injected in the center of the fully developed chlorotic halos (48 hr after toxin injection).

RESULTS

Enzyme Studies. As our studies on OCT inhibition in the succeeding paper (33) indicate, the toxin specifically affects the CAP region of the enzyme. Therefore, it was of interest to investigate whether it has affinity for other CAP enzymes as well. To test the activity of the toxin the same preparation as used in the kinetic studies (33) was employed. Toxin in amounts of 10 and 100 units was preincubated with the appropriate enzyme for appropriate periods of time prior to starting the reaction. No significant inhibition of CPS or ACT (10% for CPS; none for ACT) was observed even with 100 units of toxin.

Although among the CAP enzymes tested the toxin appeared to be specific for OCT alone, it is possible that it inhibits other enzymes of the ornithine cycle. However, OCT alone has been extracted from bean tissues. Among the commercially available enzymes of the urea cycle is arginase (beef liver), which was used in the present studies. Under the conditions of the assay toxin even in amounts 100-fold higher than needed to inhibit OCT by 50% inhibits arginase by less than 10%.

In the conversion of glutamate to glutamine as well as in the formation of carbamoylphosphate, the size of intracellular ammonia pools is important (8). *Pseudomonas tabaci*, a pathogen of tobacco plants, produces an inhibitor (30) of glutamine synthetase. The structure of the compound, which is a low molecular weight peptide, was recently reported (31). Since *P. phaseolicola* toxin is also a low molecular weight peptide (Patil, unpublished data), we tested it on glutamine synthetase and transferase. As in the case of CPS and ATC neither GS nor GT was inhibited significantly by the toxin.

The results so far indicate that OCT inhibition by the toxin is intimately related to chlorosis and that the reported (20, 28) accumulation of ornithine is a result of the OCT inhibition. However, it is unlikely that OCT inhibition alone could be the cause of the abnormally high ornithine concentrations in affected tissues. Thus, besides OCT the toxin must inhibit other ornithine utilizing enzymes. But in the following paper (33) we show that the toxin is specific for a region of the OCT molecule which affects the CAP site and not the ornithine site. Therefore, logically, the toxin may not inhibit other ornithine enzymes and chlorosis would not be accompanied by ornithine accumulation. In order to see whether in fact the toxin used in these studies causes ornithine accumulation, we injected it in bean leaves as described in "Materials and Methods." The amino acid profiles of samples showed that the control tissue (no toxin) contained 5.5×10^{-5} μmoles of ornithine per mg dry weight of tissue, in agreement with reported values (20), and 4.8×10^{-6} μmoles per mg dry weight in toxin-treated tissues. Because of this surprising finding, which is contradictory to published reports, it was important to see if ornithine accumulates in chlorotic tissues produced by infection of the pathogenic isolate used in the present studies.

Plants were inoculated (20) with a washed cell suspension of a 14-hr culture of the pathogen. The chlorotic halos which appeared in 2 to 3 days after inoculation were cut out, extracted, and the extract was analyzed as described. On a per mg dry weight basis, the halo tissue showed 4.0×10^{-4} μmoles of ornithine, almost 10-fold more than the control tissue.

Ultrastructurally, there are no apparent differences between cells of chlorotic toxin-treated tissue and cells of normal tissue of bean leaves. Particular attention was given to the structure of chloroplasts since chlorosis presumably indicates a lack of functional chloroplasts. However, the appearance of the stroma, the peripheral vesicles, and the granal and stromal lamellae was normal in the chlorotic toxin-treated tissue. Frequency and distribution of starch grains was also similar with more starch occurring in spongy mesophyll cells than in palisade parenchyma cells of both toxin-treated and nontreated tissues. Two tissue samples of each of three separate toxin treatments produced similar results.

Protection and Reversal Experiments. We previously reported that citrulline protects toxin-treated bean leaves from chlorosis (22). This indicated that the inhibition of OCT in toxin-treated leaves is closely related to chlorosis. In order to examine the specificity of the protection phenomenon, we used several naturally occurring amino acids as well as glutamine in these experiments. Aside from citrulline, only arginine and to a minor extent leucine and glutamine, offered protection.

Although the amino acids were provided to tissues 3 to 4 hr after toxin treatment, a possibility remained that they interfered with uptake of the toxin. To investigate this possibility reversal experiments were performed. Leaves were injected with 10 units of toxin. At this concentration halos developed in 48 hr. All four compounds which showed protection were then individually injected (5 μmoles) in the center of the halos. Figure 2a shows the typical chlorotic halo produced by the toxin. Areas b, c, d, and e show the regreening of the tissue with arginine, citrulline control (no toxin), regreening of toxin produced halo by citrulline, and arginine control (no toxin) respectively. Neither glutamine nor leucine, both of which showed slight protection, reverse chlorosis.

DISCUSSION

The present studies show that the *P. phaseolicola* toxin is a highly specific inhibitor. Of the various enzymes related to the ornithine cycle it inhibits only OCT. Since the reaction catalyzed by OCT is a bimolecular reaction, the enzyme molecule has two distinct regions at its active site, one for CAP and another one for ornithine. The toxin could be specific for either one or both of these regions. If it is specific for the CAP region, it might be expected to inhibit other enzymes which have similar CAP regions at their active sites. On the other hand, specificity for the ornithine region of OCT might indicate possible inhibition of other ornithine-metabolizing enzymes.

Our studies on the kinetics of OCT inhibition reported in the following paper (33), indicate that the toxin is specific to a sensitive site on the OCT molecule which affects the CAP region of the active site. Therefore, one would expect that other CAP enzymes might be inhibited by the toxin. But the data show that ACT is not inhibited by the toxin.

While the toxin may inhibit OCT directly, it could also do it indirectly if it inhibits GS or CPS. Pea seedlings utilize ammonia-N only about 10% as efficiently as the amide-N of glutamine (12). Further, in *Chlorella pyrenoidosa* (9), *Agaricus bisporus* (15), and pea seedlings (19) the carbamyl-N-atom of citrulline is largely derived from glutamine. Inhibition of GS would thus cause deficiency of CAP, as would inhibition of CPS. Since neither of these enzymes is inhibited, it appears

FIG. 2. Reversal of toxin induced chlorosis in bean leaves by citrulline and arginine. a: Toxin-induced halo; b: reversal by arginine; c: citrulline control (no toxin), d: regreening of toxin-produced halo by citrulline; and e: arginine control (no toxin).

that OCT inhibition alone is responsible for the chain of events that leads to chlorosis.

The action of the *Pseudomonas tabaci* toxin in tobacco leaves is interesting in this respect. The toxin, one of the few naturally occurring β-lactams (31), induces chlorosis in leaves and inhibits GS of tobacco *in vitro* (30). Toxin-treated leaves are protected from chlorosis by glutamine. Inhibition of glutamine synthetase would thus have a dual effect on the metabolism of toxin-treated tissues. One, a reduction in protein synthesis, since glutamine is a protein amino acid; and two, reduction in CAP concentration and in turn in citrulline and arginine synthesis. To the extent the latter effect predominates in treated tobacco tissues, the mode of action of the *P. tabaci* toxin would be the same as that of the *P. phaseolicola* toxin.

Patil *et al.* (22) previously reported that citrulline protects toxin injected bean leaves from chlorosis. This observation can be interpreted in two ways. First, externally provided citrulline simply alleviates deficiency of the amino acid caused by OCT inhibition, thereby allowing the tissue to resume arginine and in turn protein synthesis. Alternatively, injected citrulline is broken down to CAP and ornithine via the reverse OCT reaction and then the attendant increased level of CAP protects OCT from the toxin. In the next paper (33) we show that the toxin is a competitive inhibitor of CAP. The equilibrium of the OCT reaction in mammalian tissues, however, greatly favors the formation of citrulline (25). Also, the V_{max} of the forward reaction of bean OCT is approximately six times larger than that of the reverse reaction (23). Therefore, the latter explanation does not seem plausible.

Furthermore, in the current study we have shown that arginine also protects leaves from chlorosis. The possibility that arginine is converted to citrulline in this case cannot be seri-

ously considered, since arginine desimidase has been reported only from microorganisms (17). That the former explanation is the more plausible of the two is also supported by results of the reversal experiments. When either arginine or citrulline was supplied to fully developed chlorotic halos, both compounds at least partially induced regreening of the chlorotic tissues. Whichever explanation is accepted, it seems clear that even 48 hr after injection the toxin does not cause irreversible damage in affected tissues. The ultrastructure results reinforce this view, since there are no observable differences between treated and control tissues. Lesemann and Rudolph (14) recently reported that a polysaccharide toxin isolated from *P. phaseolicola* shows no differences other than ribosomal aggregation as compared to controls. Thus, it can be postulated that the toxin inhibits OCT in treated tissues leading to citrulline and in turn arginine deficiency. A reduction in arginine concentration lowers the synthesis of proteins, including enzymes of chlorophyll synthesis. This lack of synthesis combined with the natural turnover results in chlorosis. Since there is no structural damage to chloroplasts, supplying citrulline and arginine allows them to resume chlorophyll synthesis. Thus, the data indicate that the toxin produces a specific but reversible biochemical lesion in the pathway of chlorophyll synthesis.

Although the cause-effect relationship between presumed citrulline and arginine deficiency and chlorosis in toxin-treated tissues is thus strongly implicated, the possibility exists that the toxin inhibits chloroplastic protein synthesis (16), including that of the enzyme ribulosediphosphate carboxylase. The latter is synthesized on chloroplastic ribosomes in the chloroplast (6).

The lack of ornithine accumulation in the present studies is surprising but not inconsistent with the finding that our toxin preparation does not compete for the ornithine region of the OCT active site. Therefore, the other ornithine enzymes may not be inhibited by it and should be able to metabolize the increased ornithine pool resulting due to OCT inhibition. The fact that the toxin used in these studies does not inhibit ornithine decarboxylase of *E. coli* (Seymour Cohen, personal communication) supports this contention.

Another explanation for the discrepancy between the reported results of ornithine accumulation and those presented here may be that it is due to the difference in toxin preparations. Rudolph and Stahmann (28) used crude culture filtrates in their early work. It is, therefore, possible that the *P. phaseolicola* filtrates contain more than one inhibitor and that our isolation procedure separates the OCT inhibitor from other inhibitors. It is also possible that *in vivo* the pathogen produces a toxin which has two separate regions: one specific for the CAP region, and another for the ornithine region of the active site of various enzymes; and that during the isolation procedure and/or storage the function specific for the ornithine region is modified or lost. The finding that ornithine accumulates in inoculated tissues is consistent with this reasoning. Whatever the reason for the reported (20, 28) high accumulation of ornithine, it does not seem to be caused by OCT inhibition alone and indicates that it is not related to chlorosis.

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