Inhibition of the Glycine Decarboxylase Multienzyme Complex by the Host-Selective Toxin Victorin

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Victoria blight of oats is caused by the fungus Cochliobolus victoriae. This fungus is pathogenic due to its ability to produce the host-selective toxin victorin. We previously identified a 100-kD protein that binds victorin in vivo only in susceptible genotypes and a 15-kD protein that binds victorin in vivo in both susceptible and resistant genotypes. Recently, we determined that the oat 100-kD victorin binding protein is the P protein of the glycine decarboxylase complex (GDC). In this study, we examined the effect of victorin on glycine decarboxylase activity (GDA). Victorin was a potent inhibitor of GDA. Leaf slices pretreated for 2 hr with victorin displayed an effective concentration for 50% inhibition (EC50) of 81 μM for GDA. Victorin inhibited the glycine-bicarbonate exchange reaction in vitro with an EC50 of 23 μM. We also identified a 15-kD mitochondrial protein that bound victorin in a ligand-specific manner. Based on amino acid sequence analysis, we concluded that the 15-kD mitochondrial protein is the H protein component of the GDC. Thus, victorin specifically binds to two components of the GDC, GDA in resistant tissue treated with 100 μg/mL victorin for 5 hr was inhibited 26%, presumably as a consequence of the interaction of victorin with the H protein. Victorin had no detectable effect on GDA in isolated mitochondria, apparently due to the inability of isolated mitochondria to import victorin. These results suggest that the interaction of victorin with the GDC is central to victorin’s mode of action.

INTRODUCTION

We have been investigating the plant disease victoria blight of oats because this host–pathogen interaction has several exploitable characteristics, including the fact that the determinants of specificity and host range are known in this disease interaction. Victoria blight is caused by the fungus Cochliobolus victoriae (Meehan and Murphy, 1946), which is pathogenic due to the production of the host-selective toxin victorin. Isolates of C. victoriae that produce victorin are pathogenic, whereas isolates that do not are nonpathogenic. Host response to purified victorin parallels host response to the pathogen. Furthermore, host response to the toxin, and therefore to the pathogen, is determined by a single gene (Vb). Only oat genotypes carrying the dominant Vb allele are sensitive to victorin. Consequently, the susceptible and resistant host response can be investigated in the absence of the pathogen, and the analysis of disease specificity can be simplified to an analysis of the interaction between a single fungal metabolite (victorin) with a single dominant plant gene (Vb) product.

An additional intriguing characteristic of victoria blight is that identifying the disease susceptibility gene (Vb) may lead simultaneously to identifying a disease resistance gene (Pc-2). Victoria blight became a major disease of oat during the 1940s, after the release of cultivars carrying the Pc-2 gene, which gives gene-for-gene–type disease resistance to the crown rust pathogen Puccinia coronata. Genotypes carrying the Pc-2 gene were susceptible to victoria blight, a previously undescribed disease. Various lines of evidence indicate that the Vb gene is either closely linked or identical to the Pc-2 gene (Rines and Luke, 1985). Thus, the Vb gene may condition susceptibility to one disease and resistance to another.

We have been studying the mode of action of victorin and attempting to identify the Vb gene product. The structure of victorin and procedures for producing biologically active 125I-labeled victorin have been established (Wolpert et al., 1988). Incubation of oat leaf tissue with 125I-labeled victorin resulted in identification of a 100-kD victorin binding protein (VBP) that covalently binds radiolabeled victorin in vivo in susceptible but not resistant genotypes (Wolpert and Macko, 1989). A molecular genetic analysis identified the 100-kD VBP as the P protein subunit of the multienzyme glycine decarboxylase complex (GDC) (Wolpert et al., 1994). Four proteins constitute the GDC: P protein, a 100-kD pyridoxal phosphate–containing enzyme; H protein, a 15-kD lipoamide-containing enzyme; T protein, a 45-kD tetrahydrofolate-containing enzyme; and L protein, a 61-kD lipoamide dehydrogenase (Oliver et al., 1990a). The GDC is located in the mitochondrial matrix and, along with serine hydroxymethyltransferase, catalyzes the conversion of two glycine molecules into serine.

Because victorin binds to the P protein, we decided to investigate whether victorin has any affect on the activity of the
multienzyme GDC. This study characterizes the effect of victorin on glycine decarboxylase activity (GDA).

RESULTS

Effect of Victorin on in Vivo Glycine Decarboxylation

The activity of the GDC was assayed in vivo by incubating leaf slices with toxin for 2 hr before a 1-hr pulse with carbonyl-labeled 14C-glycine. The resulting dose response showed that toxin concentrations of ~60 ng/mL inhibit glycine decarboxylation >90% in susceptible genotypes, as shown in Figure 1A. Victorin had an effective concentration for 50% inhibition (EC50) of GDA of 0.07 ng/mL (81 pM) in leaf slices exposed to toxin for 2 hr. Increasing the amount of time leaf slices were incubated with toxin increased the inhibition of GDA. For example, GDA in leaf slices incubated for 2 hr in buffer followed by 5 hr with toxin and a 1-hr pulse with 14C-glycine was inhibited 14% by 0.005 ng/mL victorin. GDA was inhibited 61% in leaf slices treated with 0.01 ng/mL of toxin under the same conditions. Thus, following a 5-hr exposure to toxin, the EC50 of victorin for GDA was below 0.01 ng/mL (12 pM).

A comparison with previously described inhibitors of glycine decarboxylation indicated that victorin is as inhibitory to GDA at nanomolar concentrations as the known glycine decarboxylation inhibitors cysteine, serine, or glycine hydroxamate are at millimolar concentrations. As shown in Figure 1B, 1.2 nM victorin (1 ng/mL) inhibited GDA to approximately the same extent as 100,000-fold higher concentrations of the other GDA inhibitors.

Because victorin is a potent phytotoxin, treatment of sensitive genotypes with victorin ultimately leads to cell death. Presumably, as a consequence of victorin's interaction with its site of action, many cellular activities are indirectly perturbed before cell death occurs. Effects of victorin on sensitive tissues include electrolyte leakage, membrane depolarization, extracellular polysaccharide production, increased respiration, and inhibition of dark CO2 fixation (Walton and Earle, 1985; Wolpert et al., 1988; Ullrich and Novacky, 1991). To investigate the possibility that the effect of victorin on GDA in vivo is the result of general cellular disruption, as opposed to a specific effect of victorin directly on GDA, we compared the in vivo effect of victorin on GDA with its effect on light-dependent CO2 fixation, another organellar process. We found that the in vivo effect of victorin on light-dependent CO2 fixation was ~350 times less inhibitory than that on GDA (Figure 2). The EC50 for the inhibition of light-dependent CO2 fixation was 66 ng/mL (81 nM) as compared with 0.19 ng/mL (0.23 nM) for GDA after exposure to toxin for identical times, as shown in Figure 2.

In Vitro Effects of Victorin on GDA

A mitochondrial matrix extract was used for glycine-dependent bicarbonate exchange reactions. Glycine–bicarbonate exchange activity was examined because the reaction is dependent only upon the P and H proteins of the GDC, and the T and L proteins are not required (Sarojini and Oliver, 1983). Thus, the reaction can be performed aerobically, unlike glycine decarboxylation reactions, which require an anaerobic environment to avoid oxidation of tetrahydrofolate, a required cofactor. Extracts from susceptible plants were incubated for 75 min with or without toxin, after which the glycine exchange reaction was initiated; the reaction was allowed to continue for 15 min, as illustrated in Figure 3A. The EC50 of victorin for inhibition of glycine exchange activity was 19 µg/mL (23 µM). Victorin inhibited the glycine–bicarbonate exchange reaction in samples from both susceptible and resistant genotypes. This is...
consistent with in vitro binding studies that demonstrated victorin binding by P protein in extracts from both genotypes (Wolpert and Macko, 1989).

During binding studies with victorin, we observed that in vitro binding of \(^{125}\)I-victorin by the P protein was stimulated \(~23\%\) by higher salt concentrations, as illustrated in Figure 3B. Because the P protein bound victorin more effectively in high-salt conditions, we examined the effect of adding salt to the exchange reactions. The presence of 25 mM KCl resulted in a 96% increase in exchange activity. Victorin was a more effective inhibitor of exchange activity in the presence of KCl (Figure 3A). Thus, conditions that increased binding of toxin to the P protein resulted in increased inhibition of the exchange reaction by victorin.

Victorin Binding to the H Protein

Previous work revealed that a 15-kD protein also bound \(^{125}\)I-victorin in vivo and in vitro (Wolpert and Macko, 1989). However, unlike the binding to the 100-kD VBP that occurs in vivo only in susceptible genotypes, binding to the 15-kD VBP occurs in both susceptible and resistant genotypes. During purification of the P protein from mitochondria, we observed that a 15-kD VBP was also enriched in the mitochondrial fraction. We purified this protein from isolated mitochondria and determined that it bound \(^{125}\)I-victorin in a ligand-specific manner, as shown in Figure 4A. The N-terminal amino acid sequence identified the first 30 amino acid residues of the 15-kD VBP purified from mitochondria (Figure 4B). The amino acid sequence showed 77% amino acid identity with the first 30 amino acid residues of the mature H protein of the GDC deduced from a cDNA isolated from pea and 73% with the deduced mature form of the Arabidopsis H protein (Figure 4B) (Kim and Oliver, 1990; Srinivasan and Oliver, 1992). These results indicated that victorin binds in a ligand-specific manner to the H protein component of the GDC from oats. Thus, victorin binds to two different components of the GDC in a ligand-specific manner.
Figure 4. Analysis of the 15-kD Oat Victorin Binding Protein.

(A) An autoradiograph of a polyacrylamide gel following electrophoresis of purified 15-kD VBP. Prior to electrophoresis, samples were incubated in the presence of 3 μCi/mL of ¹²⁵I-labeled victorin for 1 hr. Binding assays were conducted in the presence of 0, 0.1, 1, 10, and 100 ng/mL of unlabeled victorin. The marker at left indicates the H protein.

(B) N-terminal amino acid sequence of the mature 15-kD VBP isolated from susceptible oat leaf tissue (A.s.); N-terminal amino acid sequence of mature H protein deduced from cDNAs cloned from pea (P.s.) and Arabidopsis (A.t.). The numbers above the amino acid sequences indicate amino acid positions.

Our current hypothesis is that the 15-kD protein labeled in vivo is the same protein labeled in vitro, the H protein.

Glycine Decarboxylase in Roots and Etiolated Tissue

Victorin is toxic to roots (Hawes, 1983), a non-photorespiratory tissue. We have found that etiolated blades treated with victorin in the dark are sensitive to toxin. Because non-green tissues are sensitive to victorin, we sought to confirm that oats, like other plants, have the GDC present in non-green tissue. The GDC is known to be strongly light induced in plants, and green tissue has ~10-fold more GDC than etiolated tissue (Walker and Oliver, 1986). Mitochondria were isolated from roots, etiolated blades, and etiolated blades that had been exposed to light for 14 hr before harvesting. Mitochondrial protein was separated using SDS-PAGE and analyzed by protein blotting. P protein was detected with anti-100-kD VBP antibody in both roots and etiolated blades, as seen in Figure 5. Thus, as in other plants, the oat GDC is present in non-green tissues and is light inducible (lane 3).

Treatment of Isolated Mitochondria with Victorin

The effect of victorin on GDA in intact mitochondria isolated from susceptible and resistant oat blades was evaluated. Victorin appeared to have no effect on GDA in isolated mitochondria (data not shown). GDA was not inhibited in isolated mitochondria pretreated with 100 μg/mL of victorin for 1 hr before the addition of ¹⁴C-glycine. Failure of isolated mitochondria to import victorin might explain the lack of a detectable effect of toxin on isolated mitochondria. To evaluate toxin uptake, we examined whether ¹²⁵I-victorin would label P protein in intact, isolated mitochondria. Because P protein is a matrix protein, it becomes freely soluble if mitochondria lose their integrity. Also, it has been established that soluble P protein binds victorin (Wolpert and Macko, 1989). Therefore, it was necessary to determine whether any labeled P protein detected in toxin-treated mitochondrial preparations was within the matrix of intact mitochondria or in the soluble fraction as a consequence of mitochondrial lysis.

Mitochondria were incubated with ¹²⁵I-victorin for 40 min and then centrifuged. The mitochondrial supernatant and pellet were separated and analyzed. We compared the distribution of labeled and unlabeled P protein between the mitochondrial pellets and supernatants. Labeled P protein was quantitated by densitometric scans of autoradiographs of SDS-polyacrylamide gels. Total P protein was estimated by densitometric...
scans of the 100-kD protein band detected in Brilliant Blue G–stained SDS–polyacrylamide gels. To ensure that the 100-kD protein scanned was the P protein, we separated mitochondrial proteins by two-dimensional gel electrophoresis. Only one protein that migrated with an apparent mass of 100 kDa was detected in stained gels. This protein was immunoreactive with the P protein antibody (D.A. Navarre and T.J. Wolpert, unpublished results). Therefore, the 100-kDa band detectable on Brilliant Blue G–stained SDS–polyacrylamide gels is primarily the P protein. This finding is consistent with the observation that the GDC is the most abundant mitochondrial enzyme in green tissues (Walker and Oliver, 1986). Quantitation of P protein by direct staining was considered more accurate than protein blotting because transfer of P protein from SDS–polyacrylamide gels to membranes is not quantitative.

The majority of labeled P protein (100-kD VBP) was found in the supernatant, as shown by the autoradiograph in Figure 6B, whereas the pellet contained only a small amount of labeled P protein. However, as expected, the majority of the P protein was in the mitochondrial pellet and not in the mitochondrial supernatant, as shown in Figure 6A. Comparison of the densitometric analysis of the autoradiographs and Brilliant Blue G–stained polyacrylamide gels revealed that 76% of the P protein but only 7% of the total labeled P protein was detected in the mitochondrial pellet, whereas 93% of the labeled P protein was detected in the supernatant (Table 1). Thus, when isolated mitochondria are treated with victorin, 93% of the labeled P protein is found in the intact mitochondrial supernatant, whereas the majority of the P protein is in the intact mitochondria.

We sought to determine whether the small amount of labeled P protein in the pellet was due to victorin that had been imported into intact mitochondria or whether this binding was attributable to externally labeled P protein adsorbed to the mitochondrial surface and/or to a small proportion of membrane-damaged mitochondria in the pellet. Protease treatments were unsuccessful in determining whether the small amount of labeled P protein associated with the mitochondrial pellet was compartmentalized within mitochondria, because protease concentrations sufficient to digest labeled P protein known to be externally associated with the mitochondria resulted in loss of mitochondrial integrity (data not shown). Thus, as an alternative approach, mitochondria were frozen and thawed three times and then treated with 125I-victorin (Figure 6B), as described above. In this instance, 74% of the P protein was in the supernatant and 98% of the labeled P protein was also in the supernatant (Table 1). Even though the mitochondria had been subjected to three freeze–thaw cycles, 4% of the labeled P protein remained in the pellet (Figure 6B). The most plausible interpretation of these results is that victorin is not imported by intact isolated mitochondria and that the small amount of label found in the mitochondrial pellets is due to a combination of permeabilized mitochondria and/or compartmentalized P protein associated with mitochondrial membranes.

Vicorin import into isolated mitochondria was not stimulated in the presence of ADP or ATP or with various mitochondrial substrates, including glycine, succinate, and malate (D.A. Navarre and T.J. Wolpert, unpublished results).

**Effect of Victorin on Glycine Decarboxylation in Resistant Genotypes**

Because the 15-kD VBP labeled in vitro was identified as the GDC H protein, it is possible that the 15-kD VBP labeled in vivo in both susceptible and resistant genotypes is the H protein. If the H protein is the protein labeled in vivo in resistant genotypes, then victorin might also have an effect on GDA in resistant genotypes, despite the fact that the P protein is not labeled. We measured the effect of high victorin concentrations on GDA in leaf slices from resistant genotypes. Leaf slices from the resistant genotype X424 were incubated with 100 μg/mL of toxin for 5 hr before measuring GDA. An inhibitory effect was observed that was far less pronounced than that seen in the susceptible line X469 and required much higher toxin concentrations. In one representative experiment (10 replications per treatment), in which samples received 100 μg/mL of toxin, GDA was inhibited 26.5% relative to control samples without toxin (SE = 1.5%, P = 9 × 10^−11). Although crude preparations of C. victoriae extracts have been shown to have effects on resistant plants, no effect has been previously observed with purified victorin concentrations as high as 100 μg/mL (Scheffer and Livingston, 1984). This effect is most likely due to the interaction of victorin with the H protein subunit of the GDC. No effect on GDA was observed in resistant plants with the toxin concentrations used to generate the dose response for sensitive plants (Figure 1A). Leaves from resistant plants treated with 100 μg/mL of victorin displayed no visible symptoms.
was determined by scanning the SDS-polyacrylamide gels. The relative amount of radiolabeled P protein in the supernatant versus pellet fraction was determined by scanning an autoradiograph of the SDS-polyacrylamide gels. Data represent the average of two replicates.

### Table 1. 

<table>
<thead>
<tr>
<th>Mitochondrial Fraction</th>
<th>Total</th>
<th>Total</th>
<th>125I-Victorin Binding by P Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact pellet</td>
<td>76</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Supernatant from intact pellet</td>
<td>24</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Pellet from freeze-thawed sample</td>
<td>26</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Supernatant from freeze-thawed sample</td>
<td>74</td>
<td>96</td>
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Isolated mitochondria were either intact or freeze-thawed three times and then labeled with 125I-victorin for 40 min. Samples were separated into supernatant and pellet fractions and resolved by SDS-PAGE. The relative amount of 100-kD protein in the supernatant versus pellet was determined by scanning the SDS-polyacrylamide gels. The relative amount of radiolabeled P protein in the supernatant versus pellet fraction was determined by scanning an autoradiograph of the SDS-polyacrylamide gels. Data represent the average of two replicates.

### DISCUSSION

In this study, victorin was found to be a potent inhibitor of GDA in vivo, with an EC50 of 0.07 ng/mL (81 pM) (Figure 1A) in leaf slices pretreated for 2 hr with victorin. GDA inhibition by as little as 5 pg/mL victorin was observed after a 5-hr toxin pretreatment. We evaluated the possibility that inhibition of GDA might be an indirect effect of victorin. Because glycine decarboxylation occurs in an organelle, we compared GDA with another organellar process, light-dependent CO2 fixation, based on the assumption that an indirect perturbation of organellar functions would be similar for all organelles. Light-dependent CO2 fixation was ~350 times less sensitive to toxin (Figure 2) and was thus presumed to be an indirect effect and indicative of generalized cellular disruption. Additional evidence that victorin directly inhibited the GDC was shown by the in vitro inhibition of glycine-bicarbonate exchange (EC50 of 23 μM). Furthermore, victorin binding by the P protein was enhanced by high-salt concentrations, as was the inhibition of glycine-bicarbonate exchange activity by victorin. To our knowledge, no effect of victorin on cell-free preparations has been reported (Scheffer and Livingston, 1984).

Victorin inhibited GDA at lower concentrations in vivo than in vitro. This may be due in part to the fact that the glycine-bicarbonate exchange reaction was used for in vitro assays and the glycine decarboxylation reaction for in vivo assays. However, a more likely explanation may be that the inhibition of GDA in vivo might cause perturbations upstream in the photorespiratory pathway and disrupt the cooperative interactions of chloroplasts, peroxisomes, and mitochondria that occur during photosynthesis. If glycine concentrations rise due to the inhibition of GDA, the plant may shunt glycine and glycine precursors into different metabolic pathways. Inhibition of GDA may also have deleterious effects on mitochondria, such as reduced ability to import exogenous substrates, including glycine. Victorin may also be less inhibitory in vitro due to differences in the structure of the GDC in vivo versus in vitro. In vivo, the GDC is composed of 42 subunits and has a molecular mass >1.3 MD (Oliver et al., 1990b). As pointed out by Neuburger et al. (1991), the recognition processes of the intact complex in the mitochondrial matrix, where it exists in a concentrated state (130 mg/mL in pea), are different from those of the dissociated form of the complex in vitro. Thus, the interaction of victorin with the complex in vivo versus in vitro is possibly quite different. This is consistent with our previous finding that in vivo the P protein binds victorin only in sensitive genotypes, whereas in vitro its specificity is lost and P protein from both susceptible and resistant plants binds toxin. Another explanation for the observation that victorin inhibited GDA at lower concentrations in vivo than in vitro might be that victorin is modified to a more toxic form in vivo. Finally, the fact that picomolar amounts of victorin inhibited GDA in vivo may indicate that victorin is concentrated by the plant—an event that does not occur when GDA is assayed with enzyme extracts.

### Victorin Labels Mitochondria in Vivo but Not in vitro

Given the effect of victorin on GDA in leaf slices and on mitochondrial matrix extracts, we were surprised to find that victorin had no discernible effect on GDA in isolated mitochondria. This is consistent with earlier findings that indicated that victorin did not directly affect respiratory activity in isolated mitochondria, despite the respiratory burst observed with treated, intact tissue (Wheeler and Hanchey, 1966). We examined victorin binding by isolated mitochondria to determine whether toxin uptake could explain the discrepancy between the inhibition of GDA both in vivo and in vitro but the lack of effect on GDA in isolated mitochondria. Victorin did not appear to be imported by isolated mitochondria (Figure 6). A small amount of 125I-victorin binding (7% of total binding) was associated with intact mitochondria (Figure 1B); however, we believe this binding is due to damaged mitochondria and/or liberated P protein associated with the outside of mitochondrial membranes.

Vicorin import by mitochondria in vivo is indicated by the observations that (1) the 100-kD VBP is labeled in vivo and is the P protein component of a mitochondrial enzyme complex (Wolpert et al., 1994); (2) the 15-kD VBP labeled in vivo may be the H protein component of the same enzyme complex; and (3) in vivo labeling with biotinylated victorin shows differential labeling of intact mitochondria from susceptible and resistant genotypes (H. Israel, T.J. Wolpert, and V. Macko, unpublished data). Possible mechanisms for victorin import into mitochondria might include the requirement of a carrier protein, another cellular component, a particular mitochondrial substrate or cofactor, or victorin modification before mitochondrial import. In considering the latter possibility, it is interesting that the glyoxylic acid residue covalently bound to the dichloroleucine
residue of victorin C is important for its activity (Wolpert et al., 1988). Glyoxylic acid is the immediate precursor to photorespiratory glycine. Furthermore, Kono et al. (1986) identified a form of victorin (victorin M) in which the glyoxylic acid residue is replaced with a glycine residue; this suggests that victorin M may be deaminated to form victorin C.

If the mitochondrion is the site of action for victorin, then genotypic specificity could occur at the point of victorin import into mitochondria or the cell. However, if the 15-kD protein labeled in vivo in susceptible and resistant genotypes is the same as the 15-kD protein (H protein) labeled in vitro, then victorin is apparently imported into the mitochondria of both susceptible and resistant genotypes. Supporting this possibility is the fact that high concentrations of victorin inhibited GDA in resistant plants. These data suggest that differential mitochondrial uptake of victorin is not the basis for the genotype-specific effects of victorin or the explanation for genotype-specific binding of victorin to the 100-kD P protein from susceptible genotypes.

**Interactions of Victorin and the GDC**

The interaction of victorin with GDC may be sufficient to cause cell death. However, phytotoxicity could result as a consequence of three possibilities. First, victorin is selectively metabolized by resistant genotypes and rendered nontoxic. Conceivably, the GDC could be responsible for such detoxification. It has been suggested that victorin is degraded by resistant plants (Wheeler, 1969). In a second possibility, victorin is metabolized by this enzyme complex in susceptible genotypes, and a resulting victorin metabolite causes cell death. Supporting this possibility is the existence of victorin forms with glyoxylic acid or glycine residues—both photorespiratory substrates. Furthermore, that victorin binds two different components of GDC may be indicative of processing by the complex. The H protein, with its flexible lipoamide arm, interacts with the active sites of the P, T, and L proteins. Thus, toxin might also interact with T and L proteins in addition to P protein. We have found that victorin binds to two components of GDC. The other two proteins of the GDC have molecular masses of 45 kD (T protein) and 61 kD (L protein) in pea (Bourguignon et al., 1988)—intriguingly close in size to the 45- and 65-kD VBPs detected by Akimitsu et al. (1992) using anti-victorin antibody. An explanation for the detection of different VBPs by the different approaches may be due to the nature of victorin detection used and metabolism of victorin by the plant. Our approach can detect proteins that bind victorin only if the 125I-labeled group remains on the part of victorin bound, whereas the anti-victorin antibody could detect a different part of the victorin molecule.

A third possibility is that cell death is a direct consequence of the inhibition of the GDC. GDC photorespiratory mutants have been shown to be lethal (Somerville and Ogren, 1982). Thus, nonfunctional GDC can be lethal in plants. Victorin is toxic to roots (Hawes, 1983), a non-photorespiratory tissue, and etiolated leaf blades. Because non-photorespiratory tissues are sensitive to victorin, we must consider the possibility that the GDC has non-photorespiratory biochemical functions that lead to cell death if disrupted. The GDC is found in roots and etiolated tissues of oat and other plants and in C4 plants, which suggests non-photorespiratory roles in plants. Furthermore, the GDC is found in a wide range of organisms from bacteria to humans and, thus, obviously has functions unrelated to photorespiration. Defective GDC is the cause of hyperglycinemia, a lethal, incurable disease in humans (Kume et al., 1988). In *Escherichia coli*, 15% of all carbon atoms assimilated from glucose are thought to enter the glycine-serine pathway; this pathway is also the major source of one-carbon units in the cell (Wilson et al., 1993). Although photorespiration may not be necessary for victorin-mediated cell death, we might expect that under photorespiratory conditions, green tissue would be less tolerant to the inhibition of GDA and consequently more sensitive to victorin. Green tissue may be more sensitive to toxin because it has ~10-fold more GDA than does etiolated tissue (Walker and Oliver, 1986). Interestingly, leaf protoplasts have been reported to be 10 times more sensitive to victorin than root tissue (Hawes, 1983).

The interaction of victorin with the GDC could conceivably have many consequences. Inhibiting the complex in photorespiratory conditions could result in a rapid inhibition of photosynthesis, which in turn will have its own cascade of effects. Furthermore, inhibiting the GDC may prevent the T protein from replenishing the mitochondrial pool of tetrahydrofolate. In addition, if toxin interacts with or inhibits the L protein, then pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase may also be affected because the L protein is also a constituent of these two complexes (Bourguignon et al., 1992). Interestingly, of the four GDC proteins, the L protein is expressed the most in non-green tissue (Bourguignon et al., 1992).

Thus, several lines of evidence suggest that victorin's interaction with the multienzyme GDC is pivotal: (1) victorin is a potent inhibitor of GDA in vivo; (2) victorin inhibits GDC glycine-bicarbonate exchange in vitro; (3) in addition to binding to the P protein, 125I-victorin binds to a 15-kD protein, which we have identified as the H protein of the GDC, and therefore, victorin binds in a ligand-specific manner to two components of the GDC in vitro; (4) victorin is bound in vivo by the P protein in susceptible but not resistant cultivars; (5) victorin inhibits GDA in resistant tissue; (6) all known forms of victorin have structural components (glyoxylic acid or glycine) that are key substrates of the photorespiratory pathway.

Our current hypothesis is that the mitochondrion is the primary site of action of victorin, specifically at the GDC, and that a GDC component may be the product of the Vb gene. However, the GDC or a component of the GDC could also be the site of action without being the product of the Vb gene. In this scenario, the Vb gene product would presumably directly or indirectly mediate the interaction of victorin with a GDC protein. Alternatively, a GDC protein could be the product of the Vb gene without being the site of action. In this instance, the GDC component might modify toxin in susceptible genotypes only, and the resulting metabolite could act elsewhere.
METHODS

Plant Material

Oat seedlings were grown in a growth chamber for 5 to 7 days under a 16-hr photoperiod at 24°C. The resistant oat line X424 and susceptible lines X469 and Park were used. Etiolated plants were grown for 1 week at 24°C in a growth chamber and fertilized with nutrient solution (Moore, 1981). Etiolated plants receiving light treatment were exposed to light for 14 hr on day 7 before being harvested. Roots were excised from seedlings grown for 6 to 8 days on cheese cloth over an aerated nutrient solution (Moore, 1981).

Isolation of Mitochondria

Leaves from 5- to 7-day-old seedlings were homogenized with a cold mortar and pestle in cold isolation buffer (400 mM mannitol, 0.3% PVP, 1 mM EDTA, 0.1% defatted BSA, 30 mM 3-[N-morpholino]-propanesulfonic acid [Mops], pH 7.5). The homogenate was filtered through two layers of cheesecloth and centrifuged at 800g for 5 min. The supernatant was collected and centrifuged at 12,000g for 10 min. The pellet was gently resuspended in isolation buffer and fractionated on Percoll gradients essentially as described by Douce et al. (1987). The purified mitochondria were collected and rinsed twice with suspension buffer (300 mM mannitol, 10 mM Mops, 1 mM EDTA, 2 mM MgCl2, pH 7.2).

Glycine Decarboxylation Assays

Leaf slices prepared as described by Wolpert et al. (1988) were used for the in vivo assay of glycine decarboxylation. Ten leaf slices were aliquoted into serum vials containing 500 µL of 10 mM Mops, pH 7.2, and allowed to equilibrate for 1 hr with gentle shaking inside a 30°C water bath incubator. Victorin was added, and the vials were incubated for 2 hr, which was followed by the addition of 500 nCi of 1-14C-glycine (51 mCi/mmol; ICN, Costa Mesa, CA). Glycine decarboxylation was terminated after 1 hr with 100 µL of 50% (v/v) acetic acid. Liberated CO2 was trapped on filter paper impregnated with 150 µL of 2 N NaOH. Filters containing trapped CO2 were placed in 4 mL of CytoScint ES (ICN) and counted in a liquid scintillation spectrometer.

In vitro glycine decarboxylase activity was measured by the glycine-bicarbonate exchange reaction (Sarojini and Oliver, 1983). Percoll-purified mitochondria from 50 to 100 g of tissue were rinsed with wash buffer (400 mM mannitol, 5 mM Tris, 5 mM Mops, 1 mM EDTA, 2 mM DT; pH 7.5) and suspended in 1.5 mL of extraction buffer (5 mM Tris, 5 mM Mops, 1 mM EDTA, 2 mM DT; pH 7.5); the matrix protein was released with three or four freeze-thaw cycles. After each cycle, the samples were centrifuged at 3 min at 12,000 rpm in a Sorvall (Norwalk, CT) MC 12V microcentrifuge. The supernatant was saved, and the pellet was reextracted. Supernatants were pooled and centrifuged at 100,000g for 1 hr to pellet mitochondrial membranes. The resulting mitochondrial matrix extract was used in the glycine-bicarbonate exchange reactions. Assays were conducted in a 100-µL reaction volume containing 40 µg of protein, 50 µM pyridoxal phosphate, 2 mM DT, 20 mM glycine, and 3 µCi of NaH14CO3 (3 mCi/mmol) in extraction buffer. Samples were incubated with victorin for 75 min at 30°C, after which glycine, pyridoxal phosphate, and NaH14CO3 were added. The reaction was stopped with 50% (v/v) acetic acid after 15 min. Protein concentrations were determined with the Bio-Rad protein assay with BSA as the standard (Bradford, 1976).

Light-Dependent CO2 Fixation Assay

For comparing the effect of victorin on light-dependent CO2 fixation versus glycine decarboxylation, 10 leaf slices were incubated inside a covered, shaking water bath for 1 hr in 500 µL of 10 mM Mops, pH 7.2, followed by a 90-min toxin treatment. Samples were then incubated for 20 min with either 500 nCi of 14C-glycine (51 mCi/mmol) or 1 µCi of 14C-bicarbonate (1 mCi/mmol). In the CO2 fixation assays, vials were transferred to high light (11,000 lux) for the duration of the reaction and 5 min before the addition of bicarbonate. Reactions were stopped with 100 µL of 50% (v/v) acetic acid; for the CO2 fixation assay, the remaining acid-stable radioactivity was counted. Light-dependent CO2 fixation reactions were conducted in scintillation vials under high light intensity, and a water bath was used to minimize transfer of radiant heat.

Labeling of Mitochondria with 125I-Victorin

Isolated mitochondria were used immediately for 125I-victorin binding studies. Radiolabeled victorin was prepared as described previously by Wolpert and Macko (1989). Mitochondria (50 µg of protein) that were either intact or ruptured with three freeze-thaw cycles were incubated for 40 min in suspension buffer at 25°C in a 30-µL reaction containing 6 mM DTT and 6 µCi/mL 125I-victorin. Samples were then centrifuged at 4°C for 5 min at 8000 rpm in a microcentrifuge. The supernatant was removed, and the pellet was washed twice with 1.5 mL of cold suspension buffer and pelleted as described above. Samples were mixed with 10 µL of gel loading buffer (92.5 mM Tris-HCl, pH 9.8, 2.3% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 10% [w/v] sucrose) and then separated by electrophoresis in 14% polyacrylamide gels containing SDS. Radiolabeled proteins were analyzed by autoradiography, and proteins were stained with colloidal Brilliant Blue G reagent (Sigma). Scans of gels and autoradiographs were generated with a Bio-Photronics Gelprint 2000i imaging system, and bands were quantified with GPTools v30 software (Bio-Photronics, Ann Arbor, MI).

Purification and Identification of the 15-kD Victorin Binding Protein

Mitochondrial matrix protein was prepared as described above. Matrix protein was adjusted to 100 mM KCl by the addition of solid KCl and applied to a hollow-fiber, centrifugal concentrator (RCF-ConFilt; Bio-Molecular Dynamics, Beaverton, OR). The majority of matrix proteins were retained by the concentrator, but the 15-kD victorin binding protein (VBP) was not. The material that had passed through the concentrator was mixed with three volumes of 20 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, pH 8.0 (buffer A), and applied directly to a Mono-Q anion exchange column (Pharmacia), which had been equilibrated in buffer A. The protein was then eluted with a 120-min linear gradient of 0 to 1.0 M NaCl in buffer A. Fractions were analyzed by SDS-PAGE, and fractions containing the 15-kD protein were pooled. In vitro binding was assessed by the addition of 3 µCi/L of 125I-victorin to protein fractions and incubating the samples for 1 hr at 25°C. The reaction was terminated by mixing samples with gel loading buffer. Samples
were then subjected to electrophoresis on 14% polyacrylamide gels containing SDS. Radiolabeled proteins were analyzed by autoradiography. The N-terminal amino acid sequence was determined on an Applied Biosystems (Foster City, CA) model 475A gas-phase protein sequencer operated by the Central Service Lab of the Center for Gene Research and Biotechnology (Oregon State University).

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