Rapid Communication

Adenylosuccinate Synthetase: Site of Action of Hydantocidin, a Microbial Phytotoxin

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The site of action of hydantocidin was probed using Arabidopsis thaliana plants growing on agar plates. Herbicidal effects were reversed when the agar medium was supplemented with AMP, but not IMP or GMP, suggesting that hydantocidin blocked the two-step conversion of IMP to AMP in the de novo purine biosynthesis pathway. Hydantocidin itself did not inhibit adenylosuccinate synthetase or adenylosuccinate lyase isolated from Zea mays. However, a phosphorylated derivative of hydantocidin, N-acetyl-5'-phosphohydantocidin, was a potent inhibitor of the synthetase but not of the lyase. These results identify the site of action of hydantocidin and establish adenylosuccinate synthetase as an herbicide target of commercial potential.

Hydantocidin is a phytotoxin isolated from cultures of Streptomyces hydroscopicus (Nakajima et al., 1991). This compound is a potent, nonselective herbicide when applied to emerged plants and is similar in efficacy to glyphosate. Significantly, it is minimally toxic to mice, with an oral 50%-lethal dose of >1000 mg/kg (Nakajima et al., 1991). Thus, hydantocidin would be a useful herbicide were it economically synthesized. It nevertheless constitutes an important lead in the discovery of novel mechanisms for weed control, a practice currently exploiting only about 15 known enzymatic targets (Duke, 1990). It seemed that defining the mode of action of hydantocidin should reveal an effective new target enzyme.

Adenylosuccinate synthetase catalyzes the first step in the two-step conversion of IMP to AMP in the de novo purine biosynthesis pathway (Stayton et al., 1983). The crystal structure of the bacterial enzyme has been elucidated (Poland et al., 1993), and the kinetic mechanism has been well described in ongoing investigations (most recently, Kang and Fromm, 1995). It is known to be the site of action of several antibiotics, including hadacidin and alanosine (Stayton et al., 1983). Inhibition of adenylosuccinate synthetase by alanosine was shown to reveal an effective new target enzyme.

Materials and Methods

Materials

Hydantocidin (for structures, see Fig. 1) was synthesized as described (Mio et al., 1991). NAH1 and NAPH2 were synthesized by variations of that procedure to be published elsewhere. Hadacidin was synthesized as described (Jahngen and Rossomando, 1982). All other reagents for biochemical analysis and yeast adenylosuccinate lyase were purchased from Sigma. Seeds of Arabidopsis thaliana Columbia were obtained from Lehle Seeds (Tucson, AZ).

Growth Experiments

Arabidopsis seeds were grown in Murashige and Skoog mineral medium, pH 6.0, supplemented with 10 g/L Suc and solidified with 8 g/L phytagar. Growth experiments were conducted in triplicate, in 24-well sterile plates, with 1 mL of medium per well. Probe compounds illustrated in Figure 1 were prepared as 2 to 5 mM stock solutions, filter sterilized, and added to the sterile medium before it was dispensed into the wells. Supplements to the medium such as those listed in Table I were prepared as 5 (adenine) or 20 (all others) mg/mL stock solutions, filter sterilized, and added directly to the wells prior to dispensing the medium. Seeds (20–50 mg) were sterilized in 50-mL sterile polypropylene tube with 5-fold diluted commercial bleach.

1 Spectral data supporting the structure of NAH: 1H NMR (DMSO-d6) δ 2.44 (s, 3); 13C NMR (DMSO-d6) δ 171.3, 168.9, 153.1, 97.2, 89.2, 70.8, 70.3, 61.7, and 26.1.

2 Spectral data supporting the structure of NAPH: 1H NMR (CD3OD) δ 2.60 (s, 3); 13C NMR (CD3OD) δ 173.3, 171.1, 154.8, 99.4, 88.9, 73.0, 72.1, 67.0, and 26.7.

Abbreviations: EPPS, N-(Z-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; NAH, N-acetylhydantocidin; NAPH, N-acetyl-5'-phosphohydantocidin.

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followed by repeated washing in sterile water. About 12 seeds were added to each well using a 1000-μL pipetter. The 24-well plates were sealed and kept at 27°C for 7 to 10 d prior to scoring the growth by means of a visual estimate of the size of the plants compared to those grown in the absence of added compounds.

**Extraction and Isolation of Adenylosuccinate Synthetase and Lyase**

Adenylosuccinate synthetase was partially purified from 4- or 5-d-old etiolated seedlings of Zea mays. All procedures were performed at 4°C. Seedlings were homogenized in a Waring blender with an equal volume (gram of tissue = milliliter of buffer) of 50 mM EPPS, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 5 mM sodium bisulfite, 5 mM DTT, 0.1 mM PMSF, and 100 mL/L glycerol. The extract was expressed through Miracloth (Calbiochem) sandwiched between layers of cheesecloth. To each liter of extract was added 4 g of potassium phosphate, pH 7.0, containing 1 mM DTT (KP buffer). Final concentrations of substrates were 3 mM aspartate, 0.2 mM IMP, and 0.2 mM GTP, but the sequence of addition varied as described in the figure legends (see “Results”). Anion-exchange HPLC was used to separate substrates from products and quantitate product formation. Following a 15-min reaction, 100 μL of the reaction mixture was injected into a Hamilton PRP-X100 column equilibrated with 3% acetonitrile. Reactants were eluted with a convex gradient (curve = –2) to 6% 1 M potassium phosphate, pH 7, 12% 2 M KCl, and 3% acetonitrile delivered during a span of 30 min at a flow rate of 0.8 mL/min by a Perkin-Elmer 410 BIO pump. The column eluate was monitored at 270 nm using a Beckman DU7 spectrophotometer.

Adenylosuccinate lyase was assayed in a reaction mixture of 1 mL of KP buffer containing 0.15 mM adenylosuccinate. The rate of the reaction was measured by monitoring the disappearance of the substrate at 280 nm.

**RESULTS**

**Arabidopsis Growth Experiments**

The mode of action of hydantocidin and other related compounds (Fig. 1) was initially probed by observing the growth of Arabidopsis seedlings in the presence of herbicidal compounds and putative antidotes. In the first phase, minimum lethal concentrations (those causing 95 to 100% inhibition of growth) of hydantocidin, NAH, NAPH, and hadacidin were determined to be 2, 12, 8, and 50 μM, respectively. The ability of a range of classes of biosynthetic products to reverse growth inhibition was then tested. Despite the similarity between the symptoms caused by hydantocidin and glyphosate, aromatic amino acids in any combination did not reverse the growth inhibition of Arabidopsis caused by hydantocidin (not shown), consistent with the lack of structural similarity between hydantocidin

**Enzyme Assays**

Adenylosuccinate synthetase was assayed in the presence of 50 mM EPPS, pH 7.5, 10 mM potassium phosphate, 1 mM MgCl₂, 0.1 mM EDTA, and 100 mL/L ethylene glycol (assay buffer). Final concentrations of substrates were 3 mM aspartate, 0.2 mM IMP, and 0.2 mM GTP, but the sequence of addition varied as described in the figure legends (see “Results”). Anion-exchange HPLC was used to separate substrates from products and quantitate product formation. Following a 15-min reaction, 100 μL of the reaction mixture was injected into a Hamilton PRP-X100 column equilibrated with 3% acetonitrile. Reactants were eluted with a convex gradient (curve = –2) to 6% 1 M potassium phosphate, pH 7, 12% 2 M KCl, and 3% acetonitrile delivered during a span of 30 min at a flow rate of 0.8 mL/min by a Perkin-Elmer 410 BIO pump. The column eluate was monitored at 270 nm using a Beckman DU7 spectrophotometer.

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**Figure 1.** Structures of compounds used in this study.
and any of the intermediates of the shikimate pathway. On the other hand, consistent with hydantocidin bearing a structural similarity to a nucleoside, inhibition of Arabidopsis growth caused by hydantocidin was readily reversed by the purine mononucleotides (not shown).

More detailed studies showed that growth inhibition was effectively reversed by AMP but not by GMP, IMP, or GTP (Fig. 2). The most effective reversion occurred with AMP, at a minimum optimal concentration of 0.5 mM. The pattern of protection by putative antidotes from the growth inhibition caused by NAH, NAPH, and hadacidin was similar to that observed with hydantocidin (Table I). Clearly, AMP, adenosine, and adenine were effective in reversing the inhibition caused by all of the compounds, whereas IMP and GMP were ineffective. Pyrimidines were also ineffective (not shown). These results suggest that hydantocidin exerts its herbicidal effect by inhibiting the conversion of IMP to AMP (Fig. 3). This hypothesis is supported by the knowledge that hadacidin is an inhibitor of adenylosuccinate synthetase (Markham and Reed, 1977). Because adenine and adenosine readily reversed the inhibition caused by these compounds, we eliminated the salvage pathway for synthesis of AMP (Fig. 3) from further consideration.

**Enzyme Assays**

To determine which of the two enzymes that convert IMP to AMP were inhibited by hydantocidin and its derivatives, the enzymes were extracted and assayed for inhibition. The compounds illustrated in Figure 1 were tested as potential inhibitors of adenylosuccinate lyase from yeast as well as maize after preincubation with the enzyme from 0 to 60 min. At 1 mM, well in excess of the concentration required for 100% inhibition of Arabidopsis growth, none of these compounds inhibited adenylosuccinate lyase from either source (data not shown).

As expected, maize adenylosuccinate synthetase was strongly inhibited by 10 μM hadacidin (Fig. 4). Neither hydantocidin (Fig. 4) nor NAH (not shown) inhibited the synthetase at a concentration of 0.5 mM. This was surprising because, as noted above, hydantocidin did not inhibit adenylosuccinate lyase. Based on the hypothesis that hydantocidin could exert its effect by mimicking a nucleotide, the phosphorylated derivative NAPH (Fig. 1) was synthesized. Consistent with the results presented in Table I, NAPH almost completely inhibited adenylosuccinate production at a concentration of 2 μM (Fig. 4). As with the maize enzyme, adenylosuccinate synthetase isolated from E. coli and rat liver was inhibited by NAPH and hadacidin but not by NAH or hydantocidin (data not shown).

The degree of inhibition of maize adenylosuccinate synthetase by NAPH was observed to increase with time, the duration of which was a function of the concentration of NAPH (Fig. 5). In contrast, the degree of inhibition by hadacidin was not measurably time dependent (Fig. 5). To determine whether the time dependence was due to mod-

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**Figure 2.** Inhibition of Arabidopsis growth by hydantocidin and its reversal by AMP. I, IMP; H, hydantocidin; C, control (no supplements). Antidotes were present at 1 (columns 1 and 5) or 3 (columns 2 and 6) mg/mL. Hydantocidin was present at 2 μM. The left two columns probe the effect of antidotes alone; the center two, the effect of hydantocidin alone; and the right two, hydantocidin and antidotes in combination.

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Table 1. Effect of various antidotes on the inhibition of growth of Arabidopsis caused by hydantocidin, NAH, NAPH, or hadacidin

Hydantocidin, NAH, NAPH, and hadacidin were present at 2.12, 8, and 50 μM, respectively. None of the antidotes had any effect on the growth of Arabidopsis without the inhibitors. Growth was evaluated by means of a visual estimate of the size of the plants compared to those grown in the absence of added compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antidote</th>
<th>Percent Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Hydantocidin, NAH, NAPH, or hadacidin</td>
<td>None</td>
<td>0–5</td>
</tr>
<tr>
<td>AMP</td>
<td>80–100</td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>0–5</td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

*For structures, see Figure 1. Antidotes were present at 2 mg/mL, except adenine, which was at 0.5 mg/mL.*

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were started promptly with the addition of phosphorylation rather than acetylation accounts for its inhibitory activity. Because hydantocidin was more potent in the Arabidopsis bioassay than NAH, it is possible that 5'-phosphohydantocidin would be a better inhibitor than NAPH.

The interpretation that these results elucidate the mode of action of hydantocidin rests on the assumption that plants are able to phosphorylate hydantocidin, which has not been investigated. The situation is similar to that of the antibiotics 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (Balzarini et al., 1993) and ribavirin (Streeter et al., 1978), which are antiviral compounds that must be phosphorylated in vivo to become inhibitors of IMP dehydrogenase. Prior to the discovery of the mechanism of their phosphorylation in vivo, those compounds were shown to be inactive on the target enzyme unless phosphorylated. The low level of toxicity of hydantocidin to life forms other than plants may arise from an inability to phosphorylate hydantocidin or an ability to rapidly dephosphorylate 5'-phosphohydantocidin. Selective toxicity is apparently not due to differential inhibition of adenylosuccinate synthetase by NAPH, because the enzyme isolated from E. coli or rat liver was inhibited by NAPH (not shown). Inhibition of wheat germ adenylosuccinate synthetase by hadacidin and growth inhibition of wheat seedlings and its reversal by adenine derivatives have been reported (Hatch, 1967).

While this manuscript was in review, a report appeared showing a similar pattern of reversion of growth inhibition due to hydantocidin in Arabidopsis growth tests (Heim et al., 1995). In addition, treatment of Arabidopsis plants with hydantocidin resulted in reduced incorporation of [14C]Gly into adenine but not guanine nucleotides and depleted pools of ATP. As in our study, hydantocidin did not inhibit adenylosuccinate synthetase or lyase, but an inhibitor of the synthetase was extracted from plants treated with hydantocidin. Our finding that a phosphorylated derivative of hydantocidin is a potent inhibitor of the synthetase is complemented by an experiment in the earlier report in which an undefined inhibitor of the synthetase was formed in a time-dependent manner in a mixture of hydantocidin, ATP, MgCl₂, and an extract of Setaria faberi.

LITERATURE CITED


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