Effect of L-Canavanine on Nitrate Reductase in Corn Roots

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ABSTRACT

L-Canavanine inhibits the appearance of nitrate reductase (NADH-nitrate oxidoreductase, EC 1.6.6.1) in both root tips and mature root sections of corn (Zea mays L.). Ten-fold more canavanine was required to cause a 50% reduction in the level of nitrate reductase activity (NRA) in root tips than in mature root sections. For example with one particular batch of seeds 500 μM canavanine was effective in root tips whereas only 50 μM was required in mature root sections. In root tips arginine (1 mM) completely reversed the effect of 1 mM canavanine. In mature root sections higher concentrations of arginine (approximately 5 mM) were required for a complete reversal of the canavanine effect. Additions of canavanine to roots after a period of 3 hours with 5 mM KNO₃ resulted in a loss of NRA. NO₃⁻ protected nitrate reductase from this inactivation in both root tip and mature root sections.

L-Canavanine, an analog of arginine, occurs in certain species of the family Leguminosae (9). In plant species in which it is a foreign substance, it interferes with normal metabolism of the plant and inhibits growth (3, 5, 16, 18, 19). Since the inhibition of growth of organisms by canavanine is often reversed by arginine (9), it has been suggested that canavanine interferes with the biosynthesis and/or utilization of arginine (14, 16, 18, 19). Recently Weaks and Hunt (18) observed only partial reversal of canavanine inhibition of root elongation by arginine in Zea mays and suggested that canavanine inhibition is more complex than a simple competition with arginine in protein biosynthesis.

Our studies have shown that in Zea mays the appearance of NRA⁴ required protein synthesis (8) and hence represents a simple system for examining the effect of canavanine. In addition the induction of the enzyme is extremely sensitive to canavanine (7). Our results show that canavanine reduces the level of NRA in the root tissues and that its effect can be completely reversed by arginine additions.

MATERIALS AND METHODS

Seed of Zea mays L. (var. W64A × W182E), was supplied originally by the Warwick Seed Company of Blenheim, Ontario, and more recently by the Wisconsin Seed Foundation of Madison. After washing the seed twice with distilled H₂O to remove excess fungicide, they were planted on NO₃⁻-free 0.9% (w/v) agar as described previously (1). After 64 hr, at 26 C the enzyme was induced in the roots according to the procedure outlined earlier (1, 8). The induction medium contained 5 mM KNO₃, L-Canavanine sulfate (Sigma) and arginine were added to the induction medium when required. In studies concerned with the inactivation of nitrate reductase, the enzyme was first induced with 5 mM KNO₃ in 0.10-strength Hoagland solution. After a 3-hr induction period, the roots were transferred to the treatment solution. At appropriate time intervals, the root tips (0 to 10-mm sections) and mature root sections (25–35 mm from the root tip) were harvested, frozen immediately in liquid N₂, and stored at -20 C. Extracts from mature root sections were prepared and assayed immediately after the liquid N₂ treatment while root tip sections were stored overnight before extraction.

Preparation of Cell-free Extracts. The root sections were weighed and homogenized with mortar and pestle in 4 volumes of 0.1 m HEPES buffer (pH 7.4) containing 0.5 mM EDTA and 5 mM cysteine. The extracts were centrifuged at 30,000g for 15 min and the supernatant solution served as the enzyme source.

In some experiments, where NO₃⁻-induced NADH-Cyt c reductase (NADH-CR) was isolated, the root sections were homogenized in 4 volumes of 0.05 m HEPES (pH 7.5) containing 0.4 mM sucrose, 0.1% (w/v) BSA, 0.5 mM EDTA, 0.1 mM CaCl₂, and 5 mM cysteine (15). The extracts were centrifuged at 10,000g for 10 min. The supernatant was further centrifuged at 272,000g for 60 min. NR and associated component activities were recovered in the final supernatant fraction. The supernatant solution thus obtained was purified partially by precipitation with 40% (NH₄)₂SO₄ (10). The pellet was taken up in a known volume of 0.1 m HEPES buffer (pH 7.4) containing 0.5 mM EDTA.

Enzyme Assays. NADH-nitrate reductase (NADH-NR) was assayed as described previously (1). FMNH₂-nitrate reductase (FMNH₂-NR) and NO₃⁻-induced NADH-CR were assayed as described previously (2).

Protein Determination. Soluble protein was precipitated with 10% (w/v) trichloroacetic acid, the pellet was redissolved, and the protein content determined by the method of Lowry et al. (4) using BSA, fraction V, as the standard.

NO₃⁻ Determination. Nitrato was reduced to nitrite with a partially purified NR obtained from maize leaves (7) and the NO₃⁻ thus obtained was assayed by the usual procedure (1).

RESULTS

Effect of Canavanine on Induction of Nitrate Reductase. Addition of 1 mM canavanine to the induction medium resulted in a marked reduction of NR levels in both the root tip (60%) and mature root (80%) sections (Table I). Arginine or lysine alone usually caused a slight increase of NR activity in root tip sections and a decrease of the activity in mature root sections. Additions of arginine reversed completely the canavanine effect in root tip sections (Fig. 1). In mature root sections, there was a clear reversal by arginine but higher concentrations of arginine were required. Lysine, which is known to reverse canavanine inhibition in other systems (14, 19), was less effective than arginine. Homoaarginine, the higher homolog of arginine, did not inhibit the induction of NR (Table I). Higher levels of NO₃⁻ were seen in roots treated with canavanine, indicating that canavanine interfered more with NR activity than with NO₃⁻ uptake. Concentrations of canavanine giving 50% inhibition in the medium...
Table 1. Effect of Canavanine on the Induction of Nitrate Reductase Activity in Corn Roots.

Seedlings were grown on minus-nitrate agar for 64 hr at 26°C and then were transferred to an induction medium containing 5 mM KNO₃ for 3 hr. The control values for NRA in the root tips were 185 nmoles NO₃⁻/hr/mg protein⁻¹ and in the mature root sections 97 nmoles NO₃⁻/hr/mg protein⁻¹. The control NO₃⁻ concentrations were 2.9 nmoles for the root tips and 1.3 nmoles ml⁻¹ for the mature root sections.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root Tips NRA</th>
<th>Mature Root Sections NRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Canavanine 1 mM</td>
<td>93</td>
<td>97</td>
</tr>
<tr>
<td>Canavanine 5 mM</td>
<td>99</td>
<td>108</td>
</tr>
<tr>
<td>Canavanine 10 mM</td>
<td>122</td>
<td>134</td>
</tr>
<tr>
<td>Canavanine 50 mM</td>
<td>112</td>
<td>123</td>
</tr>
<tr>
<td>Control 10 mM KNO₃</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Canavanine 0.5 mM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Canavanine 1 mM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Canavanine 0.5 mM</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Lysine 10 mM</td>
<td>122</td>
<td>127</td>
</tr>
</tbody>
</table>

Effect of Canavanine on Inactivation of NR. The effect of canavanine on NR inactivation was studied in the presence and absence of NO₃⁻. Previous results have shown that NRA in mature root sections varied between seed lots, and apparently between varieties (12) but the same trend was always observed, i.e., the inactivation in mature root sections was much more sensitive to canavanine than was the induction in the root tip. A similar variability in the effectiveness of canavanine has been reported for the maize embryo system (19).

Figure 2 shows the time course of the induction of the NADH-NR activity and its component activities, namely FMNH₂-NR and a NO₃⁻-induced NADH-Cyt c reductase. There is appreciable activity after 1 hr and an apparent steady-state is achieved by 3 hr for each of the three activities. With NR-NR and FMNH₂-NR there is essentially no increase in activity when 1 mM canavanine is added to the system. The increase in NO₃⁻-induced NADH-CR, on the other hand, was not inhibited by canavanine over the initial 2 hr of the experiment, but the activity of the final control activity had been achieved. There was no further increase over a subsequent 4-hr interval. This suggests that there may be a pool of Cyt c reductase which is activated by NO₃⁻ and that after 2 hr we see the effects of canavanine on the actual synthesis of that portion of the enzyme.

DISCUSSION

The action of canavanine as an arginine analog has been summarized in three recent review articles (3,5,9). In our system,
arginine is more effective than lysine in reversing the canavanine inhibition of the induction of NR. This is consistent with earlier observations on the effect of canavanine on the growth of maize embryos (19). Canavanine could be active in virtually any reaction where arginine is a substrate and hence at any of a number of steps it could lead to an abnormal metabolism. Each of these reactions would presumably have a unique specificity or affinity for canavanine and arginine.

Weak and Hunt observed that canavanine inhibited DNA, RNA, and protein synthesis in Glycine max (17) and that arginine gave only partial reversal of canavanine-induced inhibition of growth (18). They suggested that canavanine inhibition was more complex than a simple competition with arginine in protein synthesis. In the present study, 1 mm canavanine inhibited the induction of NR by about 60% (Table I). In root tips, the inhibition was reversed completely by an equimolar concentration of arginine; with mature root sections higher concentrations of arginine were required. This suggests that the canavanine effect in a defined system such as the induction of NR is direct and is related to protein synthesis. Rosenthal (personal communication) has direct evidence that canavanine is incorporated into protein in plant systems.

The effect of canavanine in our system was very similar to the effect of tungstate (2). For example, tungstate, an analog of molybdate, is incorporated into the NR protein instead of molybdate to yield an inactive protein (6). Enzyme made previously is degraded in a fashion characteristic of the tissue and the degradation is usually retarded by NO₃⁻ additions (2, 11, 13). The current results with canavanine show that NO₃⁻ also protects preexisting NR from degradation and hence suggest that canavanine is incorporated into the NR-protein to yield an inactive enzyme.

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