Asparagine Synthetase in Corn Roots

INKE STULEN and ANN OAKS
Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

ABSTRACT

The level of asparagine synthetase is low in 10-mm root tips from corn seedlings (Zea mays W64 × W182F) but relatively high in mature root sections taken 20 to 35 mm from the tip. When root tips are excised there is a marked increase in asparagine synthetase over a 5-hour period. In mature root sections, on the other hand, the asparagine synthetase activity declines over the same 5-hour period. The increase in the root tip is sensitive to cordycepin, 6-methylpurine, and cycloheximide, which indicates that both RNA and protein synthesis are involved in the formation of asparagine synthetase in the root tip sections. The glutamine analogue asazernine also inhibits formation of the enzyme in root tips, as does glucose. The increase in the root tip is not sensitive to asparagine. Additions of glucose or asparagine have no effect on enzyme activity in extracts. When cycloheximide, asazernine, or glucose is added to the mature root sections there is no effect on recovered enzyme activity.

Previous work from our laboratory has shown that the synthesis of asparagine from [2-14C]acetate is inhibited by sugars in corn root tips but not in mature root sections (15, 17). Studies with various analogues of asparagine showed that the synthesis of asparagine was strongly inhibited by cycloheximide, but not markedly by asazernine (18, 25). Studies with various analogues of asparagine showed that the synthesis of asparagine was strongly inhibited by cycloheximide, but not markedly by asazernine (18, 25). These results suggest that some component involved in asparagine synthesis, i.e., the enzyme asparagine synthetase, required protein synthesis in root tip sections.

A glutamine-dependent asparagine synthetase which transfers the amide nitrogen of glutamine to aspartate in the presence of ATP and Mg2+ has been found in higher plants (8, 21, 23). The enzyme from lupin cotyledons had a very high affinity for glutamine although ammonium also could be used as a substrate (8).

The present experiments were designed to study the formation of asparagine synthetase in root tip and mature root sections. The results show that the initial level of asparagine synthetase in root tip sections is low compared to the level in mature root sections. The level of asparagine synthetase in root tip sections increases, but in mature root sections decreases after excision of the sections. As with the in vivo synthesis of asparagine the formation of asparagine synthetase in the root tip sections is inhibited by asazernine, cycloheximide, and glucose as well as by inhibitors of RNA synthesis. The previous experiments dealing with the in vivo formation of asparagine were interpreted to suggest that the asparagine synthetase from root tips was more responsive to glutamine than the mature root enzyme (18). The explanation for these results now appears to be the fact that new enzyme is being made in the root tip sections.

MATERIALS AND METHODS

Corn Roots. Seeds of Zea mays (W64 × W182F) were germinated in the dark for 68 hr at 27 C on Petri plates containing 0.9% agar made up in 0.1 strength Hoagland salts solution which included 10 mM (NH4)2SO4 and 10 mM KNO3. After that period the 10-mm tips and 20- to 35-mm mature sections were removed.

One-g samples were placed in 100 ml of 0.1 strength Hoagland salts solution containing 10 mM (NH4)2SO4 and 10 mM KNO3 at 27 C under gentle aeration for either 2 or 5 hr. The inhibitor compounds tested were made up in solution just before use and added to the Hoagland solution at zero time.

Enzyme Preparation. All steps were carried out at 0 to 4 C. Root sections were homogenized at a 1:2 (w/v) ratio with 0.1 M phosphate buffer (pH 7.5) containing 15% glycerol, 25 mM 2-mercaptoethanol, 0.8 mM aspartate, 1 mM ATP, and 10 mM MgCl2, with mortar and pestle. The homogenate was strained through one layer of Miracloth and centrifuged for 20 min at 12,000 g. Enzyme activity was assayed in the supernatant fraction.

Enzyme Assay. Enzyme activity was assayed according to the method described by Lea and Fowler (8) using 0.2 ml of enzyme preparation in a final volume of 0.62 ml containing 0.34 mM [UL-14C]aspartate. Glutamine was used as the amide donor at 10 mM unless indicated otherwise. A transaminase inhibitor, 1 mM α-amino oxyacetate was included in the assay mixtures.

The reaction was terminated after 20 min by the addition of 1 ml ethanol; the precipitated protein was removed by centrifugation. After evaporating the ethanol, the reaction mixture was passed through Dowex 1-acetate columns as described by Stee- ter (23). The effluent was mixed with 15 ml toluene-Triton X-110 (2:1) scintillation cocktail and counted. Figures obtained were corrected for the value of control tubes with boiled enzyme.

Protein was determined by the Folin method after Lowry et al. (10). BSA (fraction V) was used as the standard. Enzyme activity was expressed as nmol/asparagine formed/mg protein·20 min.

Identification of Product Formed. The radioactive product formed was identified by chromatography using the solvent systems butanol-acetic acid-water (3:1:1) and phenol-water (3:1) in the presence of ammonia vapor. Radioactive areas in the chromatograms were detected with a strip counter (Nuclear-Chicago Actigraph II). When α-amino oxyacetate was included

1 This research was supported by Grant A-2818 from the National Research Council of Canada and by a travel grant from the Dutch organization for Basic Research (ZW) for 1 S.
2 Present address: State University of Groningen, Department of Plant Physiology, Postbox 14, Haren (Gr), The Netherlands.
3 To whom correspondence should be addressed.
in the assay mixtures only one radioactive peak was obtained. It co-chromatographed with asparagine, and after mild hydrolysis with 1 N HCl or treatment with asparagase, it co-chromatographed with aspartic acid. Thus, the product appears to be asparagine.

Chemicals. Albizzine was a gift from P. G. Lea, Rothamsted Experimental Station, England. Cycloheximide, 6-methylpurine, and cordycepin were purchased from Sigma Chemical Co. Asazereine was purchased from Calbiochem. L-[U-14C]Aspartic acid was obtained from New England Nuclear. It was further purified over Dowex 1-X acate and Dowex 50-H⁺ columns before use. Asparagine was purchased from Sigma Chemical Co.

RESULTS

The results in Figures 1 and 2 show the kinetics of the level of asparagine synthetase in root tip and mature root sections after excision. In mature root sections asparagine synthetase level decreased rapidly during the first 2 hr of incubation. Between 2 and 5 hr there was a slight further decline in enzyme level. In root tip sections, on the other hand, there was a slight increase in asparagine synthetase level after 2 hr and a rapid increase in enzyme level between 2 and 5 hr.

Cycloheximide (3.6 μM) inhibited the increase in asparagine synthetase in the root tip almost completely, but had no effect on the decrease in enzyme level in mature root sections (Fig. 1). Similarly, 0.1 mM azaserine inhibited the development of the root tip enzyme and had no effect on the level of the mature root enzyme (Fig. 2).

In order to test whether RNA and/or protein synthesis was involved in the increase in asparagine synthetase in the root tip sections, the effects of some potential inhibitors of RNA and protein synthesis were tested. The effects of both asparagine and glucose were also tested in order to compare their effects on asparagine formation (13, 15) and asparagine synthetase formation (Table I).

The results in Table I show that the potential RNA inhibitors cordycepin and 6-methylpurine inhibited the potential formation of asparagine synthetase by 63 and 72%, respectively. Cycloheximide (a protein synthesis inhibitor) and azaserine (a glutamine analogue) inhibited the increase in enzyme level by 90%. Another glutamine analogue, albizzine, had virtually no effect on enzyme formation. Recent studies have shown that albizzine had no effect on the formation of asparagine from [2-14C]acetate in root tip sections (Oaks, unpublished results). Glucose (1%) appeared to inhibit enzyme formation by 58%. Asparagine (1 mM) had no influence on the formation of asparagine synthetase in the root tips. When glucose (1%) was added to the mature root sections there was no effect on the level of extractable enzyme. After 5 hr the level of asparagine synthetase was the same in both control and glucose-treated sections (14.7 nmol asparagine/mg protein·20 min).

The possibility that the inhibition of the level of extractable enzyme produced by the various compounds (Table I) is caused by an effect on enzyme activity rather than on enzyme formation was ruled out by determining the effects of these compounds on the activity of asparagine synthetase in vitro using the same concentrations (Table II). None of the compounds had any substantial effect at these concentrations. Similarly, preincubation of the inhibitors with the root tip enzyme had no effect on the activity of the enzyme (Table II). Azaserine, which is considered to act as an irreversible inhibitor (20) and might therefore have decreased the level of extracted enzyme by irreversible binding, had no effect on enzyme activity at a concentration of 0.1 mM. Both glutamine analogues, azaserine and albizzine, had an inhibitory effect on asparagine synthetase activity in vitro at concentrations of 25 mM and higher (Table III). Since cycloheximide has also been considered to be a glutamine analogue in this system (18) it was also tested for its ability to inhibit asparagine synthetase. At concentrations much higher than those required to inhibit asparagine synthesis in vivo, it inhibited asparagine synthetase in vitro. Asparagine had

---

Table I. Influence of various compounds on the formation of asparagine synthetase in root tips.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Level tested</th>
<th>Increase in Aem synthetase level between 0-5 hr</th>
<th>Increase in Aem synthetase level between 0-5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>3.6 μM</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>6-Methylpurine</td>
<td>0.1 mM</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>0.1 mM</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Azaserine</td>
<td>0.1 mM</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Albizzine</td>
<td>0.1 mM</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Glucose</td>
<td>50 μM</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.0 mM</td>
<td>102</td>
<td>102</td>
</tr>
</tbody>
</table>

---

Fig. 1. Influence of cycloheximide (3.6 μM) on kinetics of the level of asparagine synthetase in excised root sections. Experimental setup as described under "Materials and Methods." A: root tips; B: mature sections.

Fig. 2. Influence of azaserine (0.1 mM) on kinetics of the level of asparagine synthetase in excised root sections. Experimental setup as described in Figure 1. A: root tips; B: mature sections.
The low which after and synthetase no effect data recovery was from inhibitors. Azaserine 0.1 mM Glucose 0.1 mM 3.6 105 99 101 1.0 96 100 102 0.1 102 101 0.1 98 99 99 50 98 99 98 1.0 95 102 98

Table III. The inhibitory action of the glutamine analogues azaserine and albizline on the activity of asparagine synthesis in vitro.

The glutamine analogues were added at zero time to the reaction mixture. Glutamine concentration used was 2.5 mM. Root tip enzyme was extracted from tip sections incubated for 3 hr; mature enzyme was prepared from freshly harvested sections. For the experiment where the effect of cycloheximide was tested, eluates from Sephadex G-75 columns were used (Stulen and Oaks, in preparation). The other assays were performed with crude extracts.

<table>
<thead>
<tr>
<th>Glutamine</th>
<th>Level tested (mM)</th>
<th>Asparagine synthetase activity as % of control (control = 100)</th>
<th>tip enzyme</th>
<th>mature enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azaserine</td>
<td>25 77 80</td>
<td></td>
<td>50 58 62</td>
<td>100 35 38</td>
</tr>
<tr>
<td>Albizline</td>
<td>25 58 66</td>
<td></td>
<td>50 44 38</td>
<td></td>
</tr>
<tr>
<td>Cycloheximde</td>
<td>10 61 58</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

no effect on the activity of the enzyme at a concentration of 1 mM.

**DISCUSSION**

The initial level of asparagine synthetase in mature root sections is high compared to the level in root tip sections. Likewise, synthesis of asparagine from [2-14C]acetate was very low in root tips, but high in mature regions of the root (14). The present study shows a striking difference in kinetics of asparagine synthetase level between root tip and mature root sections after excision. Root tip sections, which represent a meristematic region of the root where growth and hence RNA and protein synthesis are important factors, show a marked increase in asparagine synthetase with time. Asparagine synthetase from [2-14C]acetate increased for at least 6 hr when root tip sections were excised from the rest of the seedlings (17). The data on the incorporation of acetate carbon into asparagine, which can be considered to be a measure for the functioning of asparagine synthetase in vivo, agree with the data on the recovery of extractable asparagine synthetase presented here. Proof that the formation of asparagine synthetase in the tip represents new synthesis was obtained from the experiments with various inhibitors. Cordycepin, an inhibitor of RNA synthesis, inhibited formation of the enzyme in the tips. The results with 6-methylpurine also show that RNA synthesis is an essential prerequisite for the formation of the enzyme. However, protein synthesis may also be affected by this compound (3, 13). The results with cycloheximide, which is widely used to inhibit protein synthesis (19), also indicate that protein synthesis is involved in the formation of asparagine synthetase in the root tips. Cycloheximide interferes with other processes as well (12, 18, 22), and in particular it has been considered to act as a glutamine analogue (7, 18, 22). In our experiments it has been shown to inhibit asparagine synthetase in vitro but at concentrations much higher than those required to block the in vivo formation of asparagine (Table III).

Azaserine caused an accumulation of glutamine in both root tip and mature root sections (18) which indicated that in both sections the functioning of some glutamine-requiring enzymes may be inhibited. Cycloheximide additions also result in increased levels of glutamine (18) a result recently confirmed by Jones (7). Azaserine has been shown to inhibit in micromolar quantities the activities of glutamine-requiring enzymes involved in nucleic acid biosynthesis in mammalian and bacterial cells (5, 9). In the intact animal azaserine inhibits purine synthesis as well (2). Tomisek et al. concluded from their experiments with _Escherichia coli_ that nucleic acid synthesis is probably the primary site of action of azaserine (24). Because it is an amino acid analogue it could also inhibit protein synthesis; e.g. the nitrate-dependent appearance of nitrate reductase in corn roots is also sensitive to azaserine (16).

From our results it is apparent that the asparagine synthetase reaction is some 250- to 1,000-fold less sensitive to azaserine than is the actual biosynthesis of asparagine in the root tip sections. This observation suggests that the in vivo inhibitions that we are seeing in the root tip sections are related to an inhibition of enzyme formation and not to an inhibition of the enzyme, per se. This is corroborated by the fact that mature root sections which have high levels of asparagine synthetase are insensitive to compounds which appear to be having an effect on enzyme formation.

Glucose inhibited the formation of asparagine synthetase in the tips too. Tentatively, the glucose effect might be explained as an example of "catabolite repression," e.g. repression of enzyme synthesis by catabolites formed from glucose (11). Since asparagine accumulates in situations which favor protein synthesis, asparagine synthetase might be considered a catabolic enzyme. The induction of nitrate reductase, which might be considered an anabolic enzyme, was enhanced by glucose in intact corn roots. (1).

In summary, we may say that all compounds inhibiting the incorporation of acetate carbon into asparagine in root tip sections inhibited the formation of asparagine synthetase as well. Asparagine, which had no effect on its own synthesis in _in vivo_ (15), had no effect on either the formation of asparagine synthetase or the activity of the enzyme. The differences in inhibition of asparagine formation between root tip and mature root sections can be explained by the fact that new enzyme is being made in the root tip sections.

**LITERATURE CITED**

22. ROSS C 1968 Influence of cycloheximide upon pyridine nucleotide metabolism and RNA synthesis in cocklebur leaf discs. Biochim Biophys Acta 166: 40-47
CORRECTIONS

Vol. 60: 157–164, 1977
Caldwell, Martyn M., C. Barry Osmond, and Dixie L. Nott. C₄ Pathway Photosynthesis at Low Temperature in Cold-tolerant Atriplex Species.
Page 162, column 2, paragraph 2, lines 13–16 should be corrected to read: In A. confertifolia, however, malate pools are about twice the size of the aspartate pools and at low temperatures the pool of either C₄ acid is about twice as large as that at high temperature.
Page 163, column 1, Figure 10 legend, line 3 should be corrected to read: about 27 C (○) and about 9 C (●).

Vol. 60: 680–683, 1977
Page 680, column 2, paragraph 5, lines 4–6 should be corrected to read: Glutamine (10 mM) and ATP (1.0 mM) were included in the assay mixture and the final concentration of aspartate was 0.8 mM. The hybrid used was W64A x W182E.