In Vivo Conversion of 5-Oxoproline to Glutamate by Higher Plants

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ABSTRACT

L-(U-14C)-5-oxoprolin (pyrrolidine carboxylic acid or pyroglutamic acid) was infiltrated into detached leaves of a number of species and incubated for 1 to 6 hours. In every case, conversion to labeled glutamate and glutamine was observed. The amount converted varied from 1 to 64\% of the total label fed depending on the species. The ratio of glutamate-14C to glutamine-14C ranged from 5 in Viocia faba to 1 in sugar beet. This ratio could be affected by preinfiltrating various compounds before allowing the uptake of the 5-oxoprolin. When L-methionine-DL-sulfonimine was prefl to sugar beet leaves, the glutamate-glutamine ratio increased from 1 to 10. Prior treatment of V. faba leaves with asazeren resulted in essentially only labeled glutamine being recovered. Preinfiltration with NaF or ATP gave similar results in that the glutamate-glutamine ratio was greatly decreased. The results are consistent with glutamate being produced from the 5-oxoprolin and then being converted to glutamate.

A frequent constituent of extracts from higher plants is 5-OPH (pyrrolidine carboxylic acid or pyroglutamic acid) (13). It has been assumed that it is formed during the extraction procedure because of the ease with which glutamine or \( \gamma \)-glutamylpeptides are converted to 5-OP chemically (7). However, 5-OP can be formed enzymically from glutamate by glutamine synthetase (EC 6.3.1.2) in the absence of ammonia (9), \( \gamma \)-glutamyl-cysteine synthetase (EC 6.3.2.2) in the absence of an amino acid acceptor (13), and L-glutamic acid cyclotransferase (1). It can be produced from \( \gamma \)-glutamylamin amino acids by \( \gamma \)-glutamylcyclo-

transferase (EC 2.3.2.4) (14) and from L-glutamine or N-\( \gamma \)glutamyl peptides by L-glutamine cyclotransferase (EC 2.3.2.5) (11).

The catabolism of 5-OP has only recently been examined. An enzyme from rat kidney, 5-oxoprolinase, has been characterized which converts 5-OP to L-glutamate in the presence of ATP and Mg\( ^{2+} \) (17). To our knowledge, there are no studies of the catabolism of this compound in higher plants. This report describes the in vivo conversion of 5-OP-14C to glutamic acid and glutamine after infiltration into plant tissues. The reaction is widespread occurring in every species tested.

MATERIALS AND METHODS

Plant material was obtained from greenhouse specimens or germinating seedlings. Detached leaves were obtained by cutting the petiole under water. The cut end was placed in a small vial which contained the solution of the compound to be infiltrated. The fresh weight of leaf used was between 0.25 to 0.4 g. All feedings were normally carried out in the light at room temperatures (21 C). Seedlings were germinated in the dark and used when 5 to 8 days old. The root was removed at the junction of the hypocotyl, and the cut end was placed in a small vial containing the solution of labeled 5-OP or other addendum. Usually 0.3 to 0.5 \( \mu \)Ci of L-(U-14C)5-OP was the substrate in a volume of 0.1 ml. This gave an amount equal to 1.2 to 2 nmoles with 300,000 to 500,000 total cpm. If all the material was taken up before the end of the desired incubation time, distilled H\( _2 \)O was added.

At the end of the incubation, the plant material was homogenized with mortar and pestle in distilled H\( _2 \)O, and glacial acetic acid was added until approximately pH 3. The insoluble material in the homogenate was removed by centrifugation, and the supernatant solution was assayed for conversion of 5-OP to glutamic acid by a cation exchanger procedure of Van der Wef et al. (17). Aliquots of the eluate were counted in a Beckman LS-250 scintillation counter using the Triton X-114 counting mixture of Anderson and McClure (2) but using 0.8% butyl-PBD as the scintillant. Internal standards were used to correct for quenching losses.

The amount of glutamic acid and glutamine-14C produced was determined by ascending chromatography on glass plates (20 \( \times \) 20 cm) coated with MN 300 cellulose powder from Macherey-Nagel Co. Solvent systems used were isopropanol-formic acid-H\( _2 \)O (20:1:5) most commonly, or butanol-acetic acid-H\( _2 \)O (90:10:29). At times electrophoresis in 0.6 m formic acid at 1000 v was also employed. The radioactive areas were located by using a Radiochromatogram Spark Chamber from Birchover Instruments Ltd. fitted with a Polaroid camera. The Polaroid print was then superimposed on the chromatogram by means of a print projector, and the radioactive areas were outlined. The cellulose powder within the area was removed by suction and transferred to scintillation vials and suspended in a scintillation mixture of 0.8% butyl-PBD in toluene for counting.

L-(U-14C)5-OP was purchased from New England Nuclear. Alternatively, it was synthesized from L-(U-14C)glutamine or L-(U-14C)glutamic by the procedures of Greenstein and Winitz (4). All other chemicals were obtained from commercial sources.

RESULTS

5-OP Conversion and Distribution among Plant Families. Table I shows the distribution of the ability to convert 5-OP to glutamate among representatives of seven plant families (monocot and dicot); all were able to convert infiltrated 5-OP to glutamate and glutamine. Leaves were customarily used but seedlings and cotyledon slices were also able to perform these reactions. The amounts converted during infiltration time varied greatly. Chenopodium leaf was consistently the most effective and sometimes (Table I) 64\% of the total label fed was converted to glutamate plus glutamine in 2 hr.
Products of 5-OP Conversion. There were usually only two labeled compounds present in the eluate from the cation exchange column. These were identified as glutamic acid and glutamine by co-chromatography in butanol-acetic acid-water, isopropanol-formic acid-H₂O, and phenol-NH₄-H₂O, by ascending chromatography. Further identification was made by use of co-electrophoresis in 0.6 M formic acid at 1000 v for 25 min with known standards of glutamate and glutamine. Sometimes a third radioactive compound was formed which by co-chromatography and co-electrophoresis was identified as γ-aminobutyrate. That this conversion was enzymatic and not an artifact of isolation was proven by a control preparation in which plant material was homogenized and adjusted to pH 3 with acetic acid and then ¹⁴C-5-OP was added, and the isolation of products was carried on as usual. No conversion to glutamic acid or glutamine was ever discerned.

The relative amounts of glutamate and glutamine formed depended on the species used. Table I shows the ratio of labeled glutamate to glutamine found with leaves of different plants. The results show that in V. faba leaves glutamate predominates, whereas in sugar beet the amount of glutamine formed was equal to glutamate. There was some variation in this ratio within the same plant from experiment to experiment, but the general pattern in Table I was consistently followed.

The sequence in which the products appeared was examined. Although it appeared most likely that glutamate gave rise to glutamine, it was also possible that 5-OP could be converted directly to glutamine. Because sugar beet leaves produced such large amounts of glutamine, they were selected to investigate this aspect. Sugar beet leaves were preinfiltrated with either water only, or L-glutamate or methionine sulfoximine solutions. The latter compound resembles glutamine in structure and is a sensitive, specific inhibitor of glutamine synthetase (15). If glutamate-¹⁴C is the first product, then it should be diluted by the infiltrated L-glutamate in one case, and the conversion of labeled glutamate to glutamine should be inhibited in the second case. The results of Table II show that these predictions are accurately followed.

Prefeeding with methionine sulfoximine gives the same amount of conversion as with L-glutamate, but the ratio of glutamate to glutamine is much larger.

Effect of ATP and Other Nucleotides on 5-OP Conversion. Since the 5-OPase of animal tissues required ATP and Mg²⁺ to cleave 5-OP to glutamate, the effect of ATP and Mg²⁺ on the in vivo conversion in V. faba leaves was examined by perfusing leaves with each compound separately and together and then transferring to vials containing ¹⁴C-5-OP. In the presence of ATP a large decrease in the glutamate-glutamine ratio was observed.

Table II. Effect of Methionine Sulfoximine and Glutamic Acid on Ratio of Glutamic Acid and Glutamine Formed from ¹⁴C-5-OP

Individual sugar beet leaves were preinfiltrated for 1 hr at 21 C in the light with 0.1 ml of H₂O containing the compound to be tested. At the end of this period, the leaves were transferred to vials containing the usual amount of L-¹⁴C-5-OP. After incubation for 2 hr the leaves were extracted and analyzed for the labeled amino acids formed.

Discussion

Recently, 5-OP has been implicated as a part of a cyclic mechanism for transporting amino acids into kidney cells (10). This cycle involves transpeptidation reactions forming γ-glutamyl dipeptides. The enzyme γ-glutamyltranspeptidase has been de-
Table III. Comparison of Effect of NaF and Various Nucleotides on Ratio of Glutamate to Glutamine from 5-OP

All the compounds tested were perfused into detached V. faba leaves in a volume of 0.1 ml which was adjusted to that of the distilled H2O control. pH 5. All solutions contained 10 μmoles of the indicated compound and feeding continued for 2 hr, then the leaves were transferred to vials containing 14C-5-OP and incubated for 2 hr in the light at 21 C followed by assay for conversion to amino acids as usual.

<table>
<thead>
<tr>
<th>Prefeeding Treatment</th>
<th>Glutamate/Glutamine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>2</td>
</tr>
<tr>
<td>H2O + NaF</td>
<td>0.2</td>
</tr>
<tr>
<td>H2O + ATP</td>
<td>0.3</td>
</tr>
<tr>
<td>H2O + UTP</td>
<td>0.7</td>
</tr>
<tr>
<td>H2O + CTP</td>
<td>2</td>
</tr>
<tr>
<td>H2O + GTP</td>
<td>1.4</td>
</tr>
</tbody>
</table>

1 Any γ-aminobutyrate present was counted and added to the glutamate total.

Table IV. Effect of Various Inhibitors and NH4+ on Ratio of Glutamate to Glutamine Formed from 5-OP

Excised leaves of V. faba were placed in vials containing 0.1 ml of water plus the indicated compound. Prefeeding time was 2 hr in the light at 21 C, then transfer to vials containing L-[14-C]-5-OP for 2 hr and assay as previously described.

<table>
<thead>
<tr>
<th>Prefeeding Treatment</th>
<th>Glutamate/Glutamine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>1</td>
</tr>
<tr>
<td>H2O + 5 μmoles of azaserine</td>
<td>0.3</td>
</tr>
<tr>
<td>H2O + 5 μmoles of aminooxyacetate</td>
<td>0.7</td>
</tr>
<tr>
<td>H2O + 10 μmoles of NH4Cl</td>
<td>1.2</td>
</tr>
</tbody>
</table>

1 Includes any 14C-γ-aminobutyrate which might be formed.

scribed in higher plants (16), and γ-glutamylidipeptides are common constituents of higher plants (3). In many animal tissues γ-glutamylcyclotransferase cleaves these idipeptides to form 5-OP (13). This enzyme has not yet been reported in plants; however, the cleavage of N-L-glutaminyl peptides to form 5-OP is catalyzed by the enzyme L-glutamine cyclotransferase which has been purified from papaya latex (11). L-glutamine is also a substrate for this enzyme. It appears reasonable that 5-OP is a normal constituent of higher plants. The results presented in the study clearly show that higher plants are capable of converting 5-OP to glutamate and glutamine.

Some of the properties of this conversion can be deduced from the results of the prefeeding experiments. The results from using methionine sulfoximine suggest that 5-OP is cleaved to form glutamate and then is converted to glutamine. Preinfiltrating either ATP or NaF gave a most surprising result. In both instances, the ratio of glutamate to glutamine produced from 5-OP was markedly lowered and essentially to the same degree. Plasmalemma membrane-associated ATPases are well known (6). The infiltrated NaF would probably inhibit this enzyme effectively increasing the ATP concentration at this site. The same response would probably result from the addition of exogenous ATP. In some manner this increased concentration of ATP is reflected in a greater fraction of the 5-OP which is cleared being converted to glutamine.

Other assumptions which are suggested by the results are that some of the glutamate must be formed by glutamate synthase (5, 12) from the labeled glutamine since azaserine, which inhibits amide nitrogen transfer (8), decreases the glutamate-glutamine ratio dramatically. The lack of effect of aminooxyacetate indicates that pyridoxal phosphate is not involved in the reactions studied.

Efforts to obtain cell-free preparations capable of cleaving 5-OP or glutamate have given ambiguous results. Some attempts have given positive indications, but these were nonreproducible and variable in amount. We do not know whether the conversion of 5-OP to glutamate is ATP-dependent as in the animal system, and further work will be required to clarify the characteristics of the plant reaction.

LITERATURE CITED