Site of Synthesis of the Enzymes of the Pyrimidine Biosynthetic Pathway in Oat (Avena sativa L.) Leaves

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ABSTRACT

Heat-bleached oat (Avena sativa L. cv Porter) leaves lacking 70S chloroplast ribosomes have been used to demonstrate that four chloroplast-localized enzymes of pyrimidine nucleotide biosynthesis: aspartate carbamoyltransferase, dihydroorotase, orotidine phosphoribosyltransferase, and orotidine-5'-phosphate decarboxylase, are synthesized on cytoplasmic ribosomes. Two other chloroplast enzymes, carbamoyl phosphate synthetase, involved in both pyrimidine and arginine biosynthesis, and ornithine carbamoyltransferase, an enzyme of arginine biosynthesis, were also shown to be made on 80S ribosomes.

Most of the steps of the pathway of de novo pyrimidine nucleotide biosynthesis in pea leaves occur in the chloroplasts. The first two enzymes of the pathway, ACTase and DHOase, are found only in chloroplasts (2) as are the last two, OPRTase and ODCase (2). Only DHOdeHase, the third enzyme of the pathway, is not chloroplastic; it is found in the mitochondria of pea leaves (2). CPSase, which provides carbamoyl phosphate for both pyrimidine and arginine biosynthesis, and OCTase, the committed step of the arginine pathway, are also strictly chloroplastic in pea leaves (17). Although conclusive data on the subcellular localization of these enzymes in other plants is unavailable, CPSase and ACTase have been found in the chloroplasts of a variety of species (16), and CPSase and OCTase were detected in plastids of cultured soybean cells (15). It seems very likely that these enzymes are plastidic in all plants.

A variety of grass seedlings have been shown to become bleached when grown at elevated temperatures. Rye (7), barley, wheat, and oats (6) grown at temperatures of 28 to 30°C, depending on the species, develop this chlorosis. The cause is apparently a very specific loss of 70S chloroplastic ribosomes; the rest of the cell is virtually unaffected (5). The bleached plastids are structurally intact and contain many proteins imported from the cytosol (7). They therefore provide a powerful tool for determining whether or not specific chloroplast proteins are synthesized within the chloroplast. We have used these plants to demonstrate that the chloroplastic enzymes of pyrimidine biosynthesis, as well as CPSase and OCTase, are all made on 80S cytosolic ribosomes.

MATERIALS AND METHODS

Plant Material. Oat (Avena sativa L. cv Porter) seeds were a gift of Dr. Mark Sorrels. Following surface sterilization with 2% Clorox, they were germinated in vermiculite in a growth chamber with a 12 h light/12 h dark cycle at 21 to 23°C to produce normal green plants. To produce bleached plants, after surface sterilization the seeds were germinated on cheesecloth in a container covered with clear plastic at room temperature overnight, and then the container was placed in an incubator with the temperature at 30 to 35°C with a 12 h light/12 h dark cycle.

Preparation of Crude Extract. Plants, either green or bleached, were harvested and cut into small pieces with scissors. The green tips of the bleached plants were removed and discarded. The plants were then ground in a mortar and pestle in a small volume of a medium containing 30 mM Hepes-KOH (pH 7.2), 5 mM MgCl2, 1 mM EGTA, 0.05% (w/v) BSA, and 20% (v/v) insoluble PVPP hydrated with grinding medium (1 g/10 ml). Glass beads were added to the mortar and pestle to aid in grinding. The homogenate was filtered through cheesecloth and centrifuged at 12,000g for 10 min. The supernatant was desalted, using centrifuge columns of Sephadex G-50 Medium (10), into 30 mM Hepes-KOH (pH 7.2), 25 mM KCl, 5 mM MgCl2 prior to use in enzyme assays.

Preparation of Intact Chloroplasts. The procedure used was essentially identical to that described earlier (2) except that a discontinuous gradient of 45 to 85% (v/v) Percoll was used.

Enzyme Assays. ACTase, DHOase, and OPRTase/ODCase (coupled assay), were assayed as described earlier (2).

Table 1. Enzyme Activities in Crude Extracts of Green and Bleached Oat Leaves

Extracts were prepared as detailed in "Materials and Methods." Each number represents the mean of two different extractions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (nmol/mg protein h)</th>
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<tbody>
<tr>
<td></td>
<td>Green</td>
</tr>
<tr>
<td>RubPCase</td>
<td>50,161</td>
</tr>
<tr>
<td>Sucrose phosphate synthase</td>
<td>103</td>
</tr>
<tr>
<td>CPSase</td>
<td>26</td>
</tr>
<tr>
<td>ACTase</td>
<td>16</td>
</tr>
<tr>
<td>DHOase</td>
<td>58</td>
</tr>
<tr>
<td>OPRTase/ODCase</td>
<td>9</td>
</tr>
<tr>
<td>OCTase</td>
<td>805</td>
</tr>
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</table>

1 Supported by grant PCM 8304418 from the National Science Foundation.
2 Current address: Agronomy Department, 310 Curtis Hall, University of Missouri-Columbia, Columbia, MO 65211.
3 Abbreviations: ACTase, aspartate carbamoyltransferase (EC 2.1.3.2); DHOase, dihydroorotase (EC 3.5.2.3); OPRTase, orotidine phosphoribosyltransferase (EC 2.4.2.10); ODCase, orotidine-5'-phosphate decarboxylase (EC 4.1.1.23); DHOdeHase, dihydroorotate dehydrogenase (EC 1.3.3.1); CPSase, carbamoyl phosphate synthetase (EC 6.3.5.5); OCTase, ornithine carbamoyltransferase (EC 2.1.3.3); PVPP, polyvinylpyrrolidone; RubBPCase, ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39); RubBP, ribulose-1,5-bisphosphate; Taps, tri(hydroxymethyl)-methylaminopropane sulfonic acid.
Table II. Detection of Activities of Pyrimidine Biosynthetic Enzymes, CPSase and OCTase, in Intact Chloroplasts Isolated from Green Oat Leaves

Chloroplasts were isolated as explained in "Materials and Methods," broken, and desalted prior to use in the assays.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (nmol/mg Chl·h)</th>
</tr>
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<tbody>
<tr>
<td>Aspartate carbamoyltransferase</td>
<td>17</td>
</tr>
<tr>
<td>Dihydroorotase</td>
<td>37</td>
</tr>
<tr>
<td>Orotate phosphoribosyltransferase/orotidine-5'-phosphate decarboxylase</td>
<td>2.6</td>
</tr>
<tr>
<td>Carbamoyl phosphate synthetase</td>
<td>11</td>
</tr>
<tr>
<td>Ornithine carbamoyltransferase</td>
<td>1480</td>
</tr>
</tbody>
</table>

The reaction mixture for CPSase consisted of 30 mM glutamine, 6 mM ATP, 9 mM MgCl₂, 10 mM KHCO₃, 3 mM ornithine, 35 mM Tricine (pH 8.1), and extract in a total volume of 200 µl. Following incubation at 37°C, the reaction was stopped by addition of 10 µl of 2 M NH₄OH (pH 6.5), and the solution incubated at 95°C for 10 min to convert carbamoyl phosphate to hydroxyurea. This latter compound was then detected by the colorimetric procedure of Pierson (12).

The assay for OCTase was modified from Martin et al. (9). The reaction mixture consisted of 35 mM Tricine-KOH (pH 8.1), 3 mM ornithine, 4 mM Li₂-carbamoyl phosphate and extract in a total volume of 1 ml. After incubation at 37°C, the citrulline formed was determined colorimetrically by the method of Prescott and Jones (13). RuBPCase was assayed using a modification of the method of Pierce et al. (11). The reaction mixture components were 100 mM Taps-KOH (pH 8.2), 20 mM MgCl₂, 10 mM KHCO₃ (containing 0.4 µCi Na₂¹⁴CO₃) and extract in a total volume of 480 µl. These components were mixed together in a scintillation vial, which was then capped with a serum stopper and allowed to sit for 3 min to activate the enzyme. The reaction was started by addition of 20 µl of 12.5 mM RuBP through the stopper, and stopped after 1 min at room temperature by injection of 200 µl 2 N HCl. Blanks were run by addition of HCl prior to RuBP.

Sucrose-phosphate synthase was assayed by the method of Rufty and Huber (14) and protein was determined using the Pierce BCA reagent.

RESULTS AND DISCUSSION

The activity of RuBPCase in the heat-bleached leaves was less than 0.8% of that in green leaves on a protein basis (Table I). The lack of RuBPCase in the bleached leaves was confirmed by SDS gel electrophoresis, which showed that both the large and small subunits were missing in the bleached leaves (data not shown). Sucrose-phosphate synthase, a cytoplasmic marker, was present at higher levels on a protein basis in the bleached leaves (Table I). This is expected, since RuBPCase and other chloroplast-synthesized proteins make up a substantial portion of the protein in the green plants. CPSase, ACTase, DHCase, OPRTase/ODCase, and OCTase were all also found at higher levels in the bleached than in the green plants (Table I), indicating that they are still synthesized on 80S cytoplasmic ribosomes. It is reasonable to further conclude that they are all encoded by nuclear DNA.

To confirm the chloroplastic location of these enzymes in oats, they were assayed in extracts of intact oat chloroplasts (Table II). All were detectable in these extracts, although some had lower levels of activity than in the crude extract. This may be accounted for in part by the difficulty in obtaining substantial quantities of intact chloroplasts, which meant that these assays were done with less protein and run for longer periods of time. The most extreme difference between the crude extract and the chloroplast preparation is in OPRTase/ODCase activity; this is probably due to interference with this assay by RuBPCase in the chloroplast extract as explained earlier (2). The data presented here do not absolutely rule out the possibility that cytosolic isozymes of these enzymes exist in oat leaves. However, all of these enzymes have been shown to be strictly chloroplastic in pea leaves (2, 17) and it seems very likely that the same is true in oats.

This finding is not unexpected. To date, the only products of chloroplast transcription and translation identified are either components of the chloroplast protein-synthesizing machinery or proteins involved in photosynthesis (8). The vast majority of chloroplast proteins are encoded in the nucleus, including many enzymes of carbon metabolism (7), shikimate dehydrogenase, an enzyme of aromatic amino acid biosynthesis (7), the enzymes of proline biosynthesis (from L-α-aminoadipic acid (4), the NADP-dependent proline biosynthesis, and the enzymes of fatty acid biosynthesis (3). The chloroplast-localized enzymes of pyrimidine biosynthesis and CPSase and OCTase can now be added to this list.

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LITERATURE CITED