Distribution of the Enzymes of Nitrogen Assimilation within the Pea Leaf Cell

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ABSTRACT

Protoplasts obtained from expanded leaves of Pisum sativum have been used for the isolation of cell organelles and the subsequent study of the intracellular distribution of the enzymes of nitrate assimilation. The protoplasts were ruptured in a suitable medium and the total lysate subjected to sucrose density gradient centrifugation. Of the total chlorophyll more than 80% was recovered in intact chloroplasts. Nitrate reductase and glutamate synthase were found to be located wholly in the chloroplast. Glutamine synthetase was distributed between the chloroplast and the cytoplasm, with a maximum of 60% of the former. A possible role of the cytoplasmic enzyme is discussed in relation to photosynthesis. There was no evidence for the association of nitrate reductase with any cell organelle or membrane fraction.

It is now generally considered that the assimilation of nitrate to amino acids in leaves is carried out by the enzymes nitrate reductase (EC 1.6.6.1), nitrate reductase (EC 1.7.1.1), glutamine synthetase (EC 6.3.1.2), and glutamate synthase (EC 1.4.1.7.1) (16, 17). The over-all transformation of $^{15}$NO$_3$ to $^{15}$N-amino N has been shown to be strongly light-dependent (6) and isolated intact chloroplasts can carry out all of the reactions of the pathway from nitrite to amino-N when they are incubated in an appropriate medium in the light (1–3, 12, 14, 18, 25). Enzyme distribution studies using density gradient centrifugation of mechanically isolated chloroplasts have shown that nitrate reductase (7, 15) and glutamine synthetase (15) are present in the intact chloroplast. Because only a small proportion (about 20%) of the total Chl is recovered in intact chloroplasts using these techniques it is not possible to decide whether all of the activity of these enzymes within the cell is located in the chloroplast. The previous results suggested that nitrate reductase might be wholly in the chloroplast but the same might not be true of glutamine synthetase (15).

An alternative approach has been used by Rathnam and Edwards (20, 21) using ruptured isolated protoplasts, which allows recovery of about 80 to 90% of the chloroplasts intact. Their results, based on differential centrifugation techniques, indicate that glutamine synthetase and glutamate synthase were wholly in the chloroplast. Because these studies were with C$_4$ plants where the enzymes are differentially distributed in the dimorphic chloroplasts (9, 20) they may not be comparable to previous results using C$_3$ plants. In addition, estimation of a whole range of enzyme activities in a single leaf homogenate or organelle preparation may lead to gross underestimation of the activity of some enzymes, particularly those that are relatively unstable in the homogenization medium chosen. Nitrate reductase and glutamate synthase are both in this category; to obtain reliable estimates of activity, complex, protective extraction media are required (22, 24).

This paper presents results on the distribution of the enzymes of N assimilation in pea leaf cells using density gradient centrifugation of ruptured isolated protoplasts, in a variety of media designed to retain maximum activity of the individual enzymes under study.

MATERIALS AND METHODS

PLANT MATERIAL

Peas (Pisum sativum cv. Feltham First) were obtained from commercial suppliers. After overnight soaking they were sown either in soil-free compost in pots, or in shallow trays of Vermiculite, and grown in a Saxcil constant environment cabinet (day/night 20/16 C, 70/90% RH, and 12/12 hr light regime, intensity 124 W m$^{-2}$ in the range 400–700 nm).

ISOLATION OF PROTOPLASTS

Fully expanded leaves were harvested after 15 to 20 days. The lower epidermis was removed with forceps, and the leaves floated on buffered sorbitol (0.4 M sorbitol containing 1 mM KH$_2$PO$_4$ and 5 mM MgCl$_2$ [pH 5.5]). After 5 min the leaves were transferred to shallow plastic trays containing buffered sorbitol plus 1.5% (w/v) Cellulysin (Calbiochem). The trays were placed in a water bath at 30°C and incubated in the dark for 2.5 to 3 hr without shaking. At the end of this time the medium was gently swirled and decanted through a coarse nylon net. The leaves were washed with buffered sorbitol and the filtered washings added to the previous filtrate. Protoplasts were collected by centrifugation at 200g for 2 min, and subsequently purified by one of two methods.

Purification I. The protoplasts were resuspended in Tricine-sorbitol (0.4 M sorbitol, 50 mM Tricine [pH 7.5], and 1 mM MgCl$_2$), and 5 to 10 ml layered onto 5 ml of 25% sucrose (in 50 mM Tricine [pH 7.5], 1 mM MgCl$_2$) in glass centrifuge tubes. After centrifugation at 300g for 2 min the protoplasts at the interface were removed with a Pasteur pipette. They were then diluted with Tricine-sorbitol, collected by centrifugation at 200g, and resuspended in a small volume of Tricine-sorbitol.

Purification II. Protoplasts were resuspended in 0.5 M sucrose (in 50 mM Tricine [pH 7.5], 5 mM MgCl$_2$) and placed in glass centrifuge tubes (120 × 13 mm); 2 ml of 0.4 M sucrose plus 0.1 M sorbitol were layered on top, followed by 2 ml of 0.5 M sorbitol (both solutions containing 50 mM Tricine [pH 7.5], 5 mM MgCl$_2$). After centrifugation at 200g for 4 min, intact protoplasts at the upper interface were removed with a Pasteur pipette. This method yielded protoplasts essentially free of chloroplasts and other debris in a good yield (about 50% of extracted Chl), whereas the first method produced less pure protoplasts in smaller yield. Purification II was used in all of the later separations with essentially the same distribution as with purification I protoplasts.

DENSITY GRADIENT CENTRIFUGATION

Protoplasts prepared by either method were ruptured by four passes through a 30-μm nylon net on the tip of a syringe (20, 21).
BSA (in Tricine-sorbitol) was added after breakage to a concentration of 0.1% and an aliquot of the ruptured protoplasts (2–5 ml) layered onto a sucrose density gradient in a 38 ml cellulose nitrate tube. Gradients were prepared as described (15), and centrifuged in an SW 27 rotor for 4 min at 4,000g (maximum) and 10 min at 16,000g (maximum). Tubes were fractionated by upward displacement using an ISCO fractionator, and 1.5- or 2.2-ml fractions collected.

**VARIATIONS FOR SPECIFIC ENZYMES**

This basic method was varied according to the enzyme under study. For glutamine synthetase and nitrate reductase the method was as described above. For glutamate synthase, the purified protoplasts were washed, broken, and density gradients run in sucrose and sorbitol solutions made up in 50 mm Tricine (pH 7.2), 1 mm MgCl₂, 1 mm Na₂EDTA, 0.5 mm PMSF and 10 mm β-mercaptoethanol. For nitrate reductase, plants were watered with 10 mM KNO₃ for 24 hr before harvesting and 2 mM KNO₃ was added to the digestion medium. After purification, all sucrose and sorbitol solutions were made up in 50 mm Tricine (pH 7.8), 5 mm cysteine, 0.5 mm Na₂EDTA, 0.5 mm PMSF, and 1 mm MgCl₂. After breaking, casein was used in place of BSA to a final concentration of 0.5%.

**ENZYME ASSAYS**

Nitrate reductase was assayed by the method of Dalling et al. (7), nitrite reductase by the method of Bourne and Miflin (4). Glutamine synthetase was assayed in a Mg²⁺-dependent synthetase assay. The assay mixture contained, in a final volume of 0.86 ml, 5 μmol aspartate, 45 μmol MgCl₂, 6 μmol hydroxylamine, 87 μmol glutamate, 45 μmol imidazole (pH 7.2), and up to 200 μl enzyme. Tubes were incubated at 30°C for 40 min and the reaction stopped by the addition of 0.86 ml FeCl₃ reagent (0.37 M FeCl₃, 0.67 M HCl, 0.2 M trichloroacetic acid). After centrifugation for 5 min at 1,000g, the A of the supernatant was measured at 500 nm, and compared to that of an authentic glutamyl-hydroxamate standard under the same conditions. Glutamate synthase was assayed as previously described (25) with 10 mM amino-oxacycetate added to the assay medium to inhibit transaminase activity. NAD-glutamate dehydrogenase was assayed as described by Miflin (15) for NADP-dependent activity, Cyt oxidase by the method of Hackett (8), and catalase by the method of Luck (13). Chl was estimated in 95% ethanol using the extinction coefficients of Wintermans and DeMots (26), protein by the dye-binding assay of Bradford (5), and sucrose concentration determined by refractometry.

**RESULTS**

The protoplasts isolated from expanded pea leaves were capable of CO₂ fixation (0.07 μmol CO₂ fixed mg Chl⁻¹, hr⁻¹) and
ammonia assimilation (9 μmol mg Chl⁻¹ hr⁻¹). Using gentle homogenization techniques virtually all of the protoplasts could be ruptured with only minimal breakage of chloroplasts. The intact chloroplasts were located in the gradient at their known density of 1.21 g/cm² as found previously with these gradients (15) (Fig. 1). The distribution of the enzymes of nitrate assimilation in the density gradients is shown in Figures 1 to 3. Figure 1 shows that the intact chloroplast band was largely pure with minimal contamination by mitochondria (indicated by Cyt oxidase and glutamate dehydrogenase activity markers) or microbodies (catalase). Nitrite reductase (Fig. 2), glutamate synthase and glutamine synthetase (Fig. 3) all show a band of activity coinciding with the band of intact chloroplasts whereas nitrate reductase (Fig. 2) does not appear to be associated with any organelle.

To calculate the total distribution of the various enzymes between the chloroplast, the cytoplasm and the other particulate fractions the gradients were divided into three regions (as described in the legend of Table 1) and the total activity in each region calculated. The distributions from a number of gradients is shown in Table 1. The results show that the proportion of the total activity of the enzymes nitrite reductase, glutamate synthase, and NADPH-dependent triose-P dehydrogenase that was recovered in region III (intact chloroplasts) was the same as for Chl. The distribution of nitrite reductase was not affected by increasing the total amount of enzyme in the leaf by the addition of nitrate to the growth medium (as described under “Materials and Methods” for the nitrate reductase measurements). In contrast to the above three enzymes considerably less of the total glutamine synthetase was in region III and more than 40% of the activity was recovered in the supernatant fraction. The behavior of the mitochondrial markers was as expected but only half of the microbody marker catalase was recovered within the gradient. This may be due either to incomplete sedimentation of smaller microbodies during the short centrifugation or to microbody breakage; however, even if all of the activity in region I was due to microbody breakage the recovery of catalase in a particulate form is still considerably higher than some previous reports (23).

On the evidence presented in Table 1 and Figure 2 the vast majority of nitrate reductase does not appear to be associated with the chloroplasts. In a confirmatory experiment chloroplasts were isolated by differential centrifugation at 4,000g for 60 sec and assayed in the presence and absence of 0.03% Triton X-100 and in the light and dark. None of the various treatments significantly enhanced the nitrate reductase activity of the chloroplasts which contained 5% of the total enzyme of the original homogenate but 86% of the Chl. In similar experiments the supernatant of a 10,000g (maximum) 20-min centrifugation was recentrifuged at 100,000g (maximum) for 2 hr; less than 1% of the glutamine synthetase or nitrate reductase was recovered in the pellet produced. We considered it unlikely that the reduced activity in organelles is associated with membrane fractions.

The effect of the different isolation media was pronounced: for example, nitrate reductase activity could not be measured in either the simple Tricine-sorbitol medium or in that used for glutamate synthase whereas the activity of the latter enzyme was markedly reduced in Tricine-soribitol. Conversely the activity of all other enzymes was very low in the nitrate reductase medium. Besides affecting the total recovery of enzyme activity the distribution of activity within the different regions was also altered, presumably because the enzyme within the chloroplast was protected from the deleterious compounds present in the supernatant.

In all of the experiments for which the results are presented the enzyme activity recovered from the gradient was calculated and found to be 85% or more of that applied.

DISCUSSION

The use of ruptured protoplasts for organelle isolation, particularly for chloroplasts, is a valuable technique (9, 19, 21) and when used with suitable rupturing media designed to maximize enzyme activity it provides the best method for studying the distribution of enzymes within cells. The results obtained provide a picture of the spatial arrangement of the enzyme of nitrate assimilation in which nitrate reductase is outside the chloroplast and nitrite reductase and glutamate synthase wholly within. Glutamine synthetase appears to be present both in and outside the chloroplast; using the data presented in Table I, and allowing for chloroplast breakage, a maximum of about 60% of the total glutamine synthetase is present in the chloroplast. The remainder of the activity seems to be elsewhere in the cell but there is no evidence from experiments reported here or from experiments with mechanically isolated mitochondria (11) of any activity associated with the mitochondria. The distribution of nitrate reductase is predominantly in the supernatant; from our results a maximum of 10% of the activity, assayable with NADH, could be chloroplastic but whether this is a genuine association with the organelle is not certain. The failure of the nitrate reductase and glutamine synthetase present in a 10,000g supernatant to sediment at 100,000g for 2 hr suggests that these enzymes are not closely associated with any of the cell membranes.

The distribution of glutamine synthetase in both the chloroplast and cytoplasm confirms the suggestion made earlier based on separations of mechanically isolated chloroplasts (15). This distribution differs from that reported for Panicum miliaceum in which 70 to 80% of the enzyme could be accounted for in intact plastids (20). The difference could be due to the use of different rupturing media or to a true species difference. Reasons for the latter may lie in the function of the extrachloroplastic enzyme. As has been pointed out (17) the process of photorespiration, which involves the oxidative conversion of 2 molecules of glycine to 1 of serine, 1 of CO₂, and 1 of NH₃ leads to the release of large amounts of ammonia by the mitochondria. Evidence (11) indicates that this ammonia is not reassimilated within the mitochondria but is reincorporated into the organic form via glutamine synthetase;
Fig. 3. Distribution of glutamine synthetase (●) and glutamate synthase (○) in sucrose density gradients of whole homogenates of ruptured pea leaf protoplasts. The top and bottom parts of the figure represent different experiments using different isolation techniques. CA and CO represent the position of the major peaks of catalase and Cyt c oxidase activity, respectively.

Canvin and Atkins (6) have shown that $^{15}$NH$_3$ can be assimilated in the dark and they have suggested that this may occur outside the chloroplast via mitochondrial glutamate dehydrogenase. An alternative hypothesis is that the extrachloroplastic glutamine synthetase, again supported by mitochondrial ATP production, could be the assimilatory enzyme involved. In the leaf of

reconstitution experiments suggested that this could occur via the cytoplasmic form of glutamine synthetase using ATP produced by the mitochondria during the oxidative decarboxylation of glycine. The lower proportion of nonchloroplastic glutamine synthetase in C$_4$ plants (20) could be related to the presumed lower flux of carbon along the photorespiratory pathway in these species (10).

# Table I: Enzyme Distribution in Sucrose Density Gradients

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No. of Expts</th>
<th>Distribution in Gradient (%) Recovered Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Region I</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>9</td>
<td>2.6 (2.5)2</td>
</tr>
<tr>
<td>Nitrite reductase</td>
<td>4</td>
<td>10.5 (3.5)</td>
</tr>
<tr>
<td>Glutamate synthase</td>
<td>2</td>
<td>19.5 (6.4)</td>
</tr>
<tr>
<td>Triose-P-dehydrogenase (NADPH)</td>
<td>1</td>
<td>18.0 (0)</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>6</td>
<td>41.8 (11.4)</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>5</td>
<td>8.6 (7.8)</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (NADPH)</td>
<td>2</td>
<td>13.0 (4.2)</td>
</tr>
<tr>
<td>Catalase</td>
<td>4</td>
<td>16.0 (15.7)</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>2</td>
<td>93.5 (2.1)</td>
</tr>
</tbody>
</table>

1 Regions defined as: I - volume corresponding to the volume of protoplast lysate applied to the gradient; II - top of the gradient down to the beginning of the intact chloroplast band; III - intact chloroplast band down to the bottom of the gradient (see Figure 1).

2 Figures in brackets denote the standard deviations of the mean.
an intact plant in the light ammonia is likely to be produced within the chloroplast by nitrite reductase and assimilated in situ by the chloroplastic glutamine synthetase.

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LITERATURE CITED