A Plastidial Localization and Origin of L-Glutamate Dehydrogenase in a Soybean Cell Culture

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ABSTRACT

The subcellular distribution of L-glutamate dehydrogenase (GDH, EC 1.4.1.3.) was studied in SB3 soybean (Glycine max) cells using subcellular fractionation techniques. Compounds that inhibit protein synthesis either on 80S or 70S ribosomes were also used to give a preliminary idea of which subcellular fraction is involved in GDH synthesis. It was found that whereas cycloheximide and puromycin considerably reduced the total amount of protein synthesized by the cells, they did not appear to inhibit the synthesis of GDH. In the presence of chloramphenicol, both GDH activity and protein level in the cells were considerably reduced, suggesting that this enzyme was synthesized in organelles and not in the cytosol. Streptomycin, which inhibits plastid protein synthesis, also inhibited synthesis of GDH, indicating that a fraction of GDH activity was plastidial in origin. This is supported by the data on subcellular distribution of the enzyme, which showed that a major fraction of GDH is found in the plastidial fraction, although some activity is found associated with the mitochondrial fraction also. Since a major fraction of GDH activity was found in the plastidial fraction, we studied protein synthesis using isolated plastids and 35S-methionine. Using antibodies raised against purified GDH, we identified a 35S-labeled 41-kilodalton polypeptide synthesized by plastids as GDH.

GDH is frequently found distributed in most plant parts. Although the glutamine synthetase (EC 6.3.1.2)/glutamate synthase (EC 1.4.1.14) system is now believed to be the major route of ammonia assimilation, the presence of high levels of GDH in plants has been considered to play either an aminating or a deaminating role during certain environmental or nutritional conditions (26, 27). GDH has also been shown to function when ammonia is in excess (16). The enzyme has been found to be localized in different plant organs and has been shown to exist in several isoforms in various plants (26). The subcellular localization of GDH has also been studied in various plants and the enzyme has been found to be distributed in mitochondria, plastids, and cytosol (26, 27, and references therein). In most cases, the NAD/NADP-specific enzyme was found to be mitochondrial, whereas the chloroplastic form is found to be NADP/NADPH-dependent. However, using mixed substrate methods, it has been shown that both for the mitochondrial and chloroplastic enzymes, NADH and NADPH bind to the same active site, suggesting a dual coenzyme specificity (27). In a recent study, GDH was found to be localized exclusively in the chloroplast of Chlamydomonas, and the enzyme was shown to be equally active with NAD and NADP (7). An ammonium inducible NADP-GDH was also reported to be located in the chloroplasts of Chlorella, however, its subunit-precursor was shown to be synthesized in the cytosol (19).

In previous reports from this laboratory, the characteristics of SB1 and SB3 cell cultures of soybean, and the properties of glutamate dehydrogenase from these cell lines, were described (10, 21, 22). It was shown that SB3 cells grown in MNHA medium show an induction of GDH activity (10). Also, chloramphenicol was found to inhibit GDH activity at 96 h of cell growth. The aim of the present work was to study the subcellular localization of GDH using inhibitors of protein synthesis and subcellular fractionation of soybean cell protoplasts. The presence of higher GDH activity in the plastid fraction and the inhibition of the enzyme by chloramphenicol and streptomycin indicated that GDH was plastidial in origin. Thus, we studied protein synthesis by isolated plastids. The results presented here show that in SB3 cells, a 41-kD GDH polypeptide that shows a positive reaction with GDH antibodies is synthesized by isolated plastids.

MATERIALS AND METHODS

Plant Material

The SB3 soybean (Glycine max L. cv Mandarin) cell culture was used in this study. The cells were grown as suspension cultures in 100 mL B5 or MNHA medium as described earlier (21).

Addition of Inhibitors

Chloramphenicol (5 mM), streptomycin (1 mM), cycloheximide (1 mg/mL), and puromycin (0.4 mg/mL) were used to find out the site of synthesis of GDH in soybean cells. These inhibitors were first dissolved in a small volume of growth medium, filter sterilized, and then added to the medium to give a desired concentration.

Enzyme Extraction

The enzyme extracts were prepared according to the procedures described by Chiu and Shargool (2). The crude extracts were subjected to ammonium sulfate fractionation and

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2 Abbreviations: GDH, L-glutamate dehydrogenase (EC 1.4.1.3); MNHA, medium containing no nitrate and 10 mM ammonium as sole source of nitrogen.
the pellets suspended in 25 mM Tricine (pH 7.5) containing 1 mM EDTA and 1% β-mercaptoethanol were passed through Sephadex G-50 columns as described earlier (10). The enzyme preparations were kept on ice and used for GDH assays and protein estimations.

**Enzyme Assay**

GDH activity assay was according to the method modified from Dougall (6). The activity in the aminating direction was measured by monitoring NADPH oxidation at 340 nm in presence of α-ketoglutarate, ammonium chloride, and 1 mM calcium. The enzyme was highly active with both NADH and NADPH. The activity in the deaminating direction using NAD or NADP, however, was reported to be very low (10). The unit of enzyme activity used is the amount of enzyme that catalyzes the utilization of 1 μmol coenzyme/min at 30°C.

**Protein Estimation**

Protein was estimated using the dye binding method of Bradford (1).

**Protoplast Preparation**

Protoplasts were prepared from 48-h-old cells using the methods described earlier (11). However, the concentration of enzymes was increased since cell wall digestion was very slow. The final concentration of enzymes used for the production of protoplasts was as follows: cellulase, 2.5%; hemicellulase, 1.5%; and pectinase, 1.5%. Also, driselase (0.5%) was included in the enzyme mixture. The protoplasts were purified as described by Jain et al. (11).

**Subcellular Fractionation**

Purified protoplasts were resuspended in breakage buffer containing 0.3 M mannitol, 1 mM EDTA, 0.1% BSA, 0.5% cysteine, and 50 mM Hepes (pH 7.4). The protoplasts were lysed by passing through a 20-μm nylon net held on top of a 24-gauge needle, using a 10-mL syringe.

A portion of the protoplast lysate was retained and the rest centrifuged at 3000g for 20 min in a Sorvall RC-2B centrifuge using an SS-34 rotor. The 3000g pellet was resuspended in a small volume of suspend buffer (breakage buffer without cysteine), centrifuged at 200g for 10 min, and the pellet discarded. The supernatant was then centrifuged at 3000g for 20 min to get a plastid-enriched pellet. An additional purification of plastids was done on a linear 20 to 60% sucrose gradient (23). The intact plastidial band formed at a sucrose density of 1.21 g/mL was collected and resuspended in suspend buffer.

The first 3000g supernatant was centrifuged at 12,000g for 20 min in a Sorvall SS-34 rotor. The pellet was utilized as the crude mitochondrial preparation and the supernatant as the soluble fraction. The crude mitochondrial pellet was resuspended in a small volume of suspend buffer.

**Sonication**

The organelles were lysed by sonication for 15 s in a Sonifier® Cell Disrupter (Model W185; Heat Systems Ultrasonics Inc., New York).

**Assay of Marker Enzymes**

Triose phosphate isomerase was used as a marker enzyme for plastids (11, 15). The enzyme activity was measured by monitoring NADH oxidation at 340 nm as described earlier (8). Succinate dehydrogenase, a marker for mitochondria, was assayed by monitoring extinction of dichloroindophenol (3). The unit of enzyme activity in each case is defined as in the reference cited.

**Protein Synthesis by Isolated Plastids**

For protein synthesis, plastids were purified from a 3000g pellet on a 25 to 85% Percoll gradient essentially according to the method of Dietz and Bogorad (5). The intact plastid band was collected, washed with suspended buffer, and finally resuspended in a small volume of suspend buffer to give protein concentration of 2 mg/mL. Plastids thus purified were devoid of mitochondrial contamination since the activity of succinate dehydrogenase was barely detectable.

For in organello translation, 50 μL of purified plastid preparation (100 μg of protein) was labeled with 25 μCi of 35S-methionine (1134 Ci/mmol; New England Nuclear) for 1 h at 25°C with externally added ATP and 19 amino acids. The concentration of individual components of the incubation mixture was the same as described by Leaver et al. (14). To check for the possible contamination of plastids with bacteria, 20 mM sodium acetate was added to the incubation mixture and ATP was omitted (14). Also, inhibitors of protein synthesis (cycloheximide, chloramphenicol, and streptomycin, 0.4 mM each) were added to the reaction mix separately. The reaction was terminated after 1 h by adding cold unlabeled methionine (10 mM). Plastids were centrifuged and stored at −80°C.

**SDS-PAGE, Fluorography, and Western Blotting**

For SDS-PAGE, The labeled plastids were suspended in a small volume of SDS buffer (13), sonicated for 15 s, and boiled for 3 min. The labeled polypeptides were then separated on 10% gels by SDS-PAGE (13). Equivalent amounts of protein (75 μg) of each sample were loaded onto the gels. For fluorography, the gels were enhanced with En'hance (New England Nuclear), dried, and exposed to Kodak X-Omat film for 12 to 15 d at −80°C.

For Western blotting, the polypeptides separated by SDS-PAGE were transferred to nitrocellulose membranes using a Bio-Rad Transblot cell. The nitrocellulose membranes were then subjected to immune-blot assay using antibodies raised against purified GDH (22) and goat anti-rabbit IgG horse-radish peroxidase conjugate following manufacturer's instructions (Bio-Rad Canada Ltd.).
RESULTS

Effect of Inhibitors of Protein Synthesis on GDH

The effect of cycloheximide and chloramphenicol on GDH production by SB3 cells at 0 to 120 h of growth in B5 and MNHA medium is shown in Figure 1, A and B. It is clear that GDH activity increases from 0 to 120 h and that chloramphenicol significantly inhibits GDH activity. It is also evident that when cells are grown in MNHA medium, GDH production is stimulated (compare activity in Fig. 1, A and B) and that this ammonia-induced GDH production is also inhibited by chloramphenicol. Cycloheximide did not inhibit the enzyme and in fact, it seemed to stimulate GDH production slightly.

The cells were also grown in the presence of puromycin and streptomycin for 96 h and the results are shown in Table I. Also, the data on the effects of cycloheximide and chloramphenicol are included in the table to compare the relative effects of different inhibitors on cell growth, protein content, and GDH production. It is evident that the growth of cells (based on weight of cells per flask) was inhibited by all the inhibitors in B5 medium. Cycloheximide (1 mg/mL medium) and puromycin (0.4 mg/mL medium) inhibited cell growth by 45 to 50%. The inhibitory effect of chloramphenicol was more pronounced than that of any other inhibitor (approximately 75% inhibition of growth). Streptomycin inhibited the growth of cells by 60%. The soluble protein content, in general, was also reduced by these inhibitors (Table I). In cycloheximide- and puromycin-treated cells, the specific activity of GDH was comparable with that in control experiments, however, a considerable inhibition was observed in chloramphenicol- and streptomycin-treated cells.

Subcellular Distribution of GDH

The relative distribution of GDH, triose phosphate isomerase, and succinate-dehydrogenase in different subcellular fractions of SB3 protoplasts is shown in Table II. A major fraction of GDH activity as well as the highest specific activity was found associated with the 3,000g pellet, which contains mainly plastids. The major fraction of triose phosphate iso-

Table I. Effect of Inhibitors of Protein Synthesis on Cell Growth, GDH, and Protein in SB3 Cells Grown in B5 Medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight of Cells</th>
<th>GDH (crude extract)</th>
<th>Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 mL Flask</td>
<td>units/g cells</td>
<td>mg/g cells</td>
<td>units/mg protein in Sephadex G-50 fraction</td>
</tr>
<tr>
<td>Control</td>
<td>25.0 ± 3.5</td>
<td>0.277 ± 0.03</td>
<td>1.44 ± 0.18</td>
<td>0.290 ± 0.03</td>
</tr>
<tr>
<td>+CH*</td>
<td>14.0 ± 2.6</td>
<td>0.230 ± 0.04</td>
<td>1.09 ± 0.13</td>
<td>0.310 ± 0.03</td>
</tr>
<tr>
<td>+PU</td>
<td>12.5 ± 1.8</td>
<td>0.202 ± 0.03</td>
<td>0.905 ± 0.08</td>
<td>0.305 ± 0.03</td>
</tr>
<tr>
<td>+CM</td>
<td>6.0 ± 1.5</td>
<td>0.056 ± 0.02</td>
<td>0.800 ± 0.08</td>
<td>0.105 ± 0.01</td>
</tr>
<tr>
<td>+SM</td>
<td>10.5 ± 3.0</td>
<td>0.120 ± 0.02</td>
<td>0.901 ± 0.10</td>
<td>0.180 ± 0.02</td>
</tr>
</tbody>
</table>

* CH, cycloheximide; PU, puromycin; CM, chloramphenicol; SM, streptomycin.
merase was also found in this fraction. Some GDH activity was detected in the 12,000g pellet, the crude mitochondrial fraction (20–26% of total activity), however, the activity units as well as specific activity were very low in the soluble fraction. The specific activity of GDH in plastids purified on a sucrose gradient was also very high.

Plastid Protein Synthesis

Figure 2 shows the pattern of polypeptides synthesized by SB3 cell plastids. It is clear that purified plastids can actively synthesize several polypeptides in the presence of externally added ATP and other required components. Approximately 21 polypeptides were detected by fluorography (Fig. 2, lane 1). Cycloheximide, an inhibitor of cytosolic protein synthesis, did not affect plastid protein synthesis (Fig. 1, lane 2). No radioactive incorporation was detected when ATP was not included in the incubation mixture and sodium acetate was used as substrate (Fig. 1, lane 3). Streptomycin (Fig. 2, lane 4) and chloramphenicol (Fig. 2, lane 5) both inhibited the incorporation of 35S-methionine into proteins by isolated plastids as evidenced by very low intensity of polypeptides in the fluorographs.

Western Blot Analysis and Identification of GDH Polypeptide

Labeled plastid proteins separated by SDS-PAGE were transblotted onto a nitrocellulose membrane and treated with GDH antibodies using goat anti-rabbit IgG horseradish peroxidase immune blot assay. The results are shown in Figure 3. A polypeptide that showed positive reaction with GDH antibodies was detected and is marked as GDH in Figure 3. This polypeptide corresponded to a 41-kD band in the fluorograph (Fig. 2, arrows). It is also important to note that the intensity of GDH polypeptide in the Western blot appeared to be lower in the presence of inhibitors of organelle protein synthesis—streptomycin and chloramphenicol (Fig. 3, lanes 4 and 5, respectively) and also, much lower when ATP was not included in the incubation mixture (Fig. 3, lane 3). On the other hand, the apparent intensity of this band was higher when plastids were incubated in complete reaction mixture without inhibitor or when cycloheximide was used (Fig. 3, lanes 1 and 2, respectively). A similar pattern of intensity of this band was observed in the fluorographs (Fig. 2).

DISCUSSION

In rice roots, Kanamori et al. (12) reported that chloramphenicol inhibited the production of ammonium-induced GDH by 50%, whereas cycloheximide inhibited the synthesis of the enzyme by 80%. In other studies, puromycin and cycloheximide (24, 25), but not chloramphenicol, were shown to inhibit ammonium-induced GDH activity suggesting a cytosolic origin of the ammonium-induced enzyme. This is interesting since our studies on the effects of these inhibitors on GDH production by SB3 cells show that chloramphenicol, but not cycloheximide or puromycin, inhibits GDH production by cells grown in B5 or MNHA medium (Fig. 1, A and B; Table 1). These results suggest that a significant portion of the enzyme is not cytosolic in origin, but instead originates in organelles, either plastids or mitochondria.

Since GDH activity was apparently inhibited by chloramphenicol, suggesting either a mitochondrial or plastidial origin of GDH, we used streptomycin, an inhibitor of plastidial protein synthesis (18), in an effort to further delineate the site of GDH synthesis. The results are shown in Table 1. It is evident that streptomycin (1 mm) also inhibited GDH production significantly, suggesting a plastidial origin of the enzyme. However, inhibitor studies alone do not confirm the subcellular localization of a particular protein, since they sometimes show side effects on cell metabolism. To support our observation that a major fraction of GDH activity was plastidial in origin, the subcellular fractionation of protoplasts was carried out. Table II clearly indicates that a major fraction of the GDH activity was found in the 3000g pellet, which contains mostly plastids as evidenced by the marker enzymes. In the plastids obtained from sucrose density gradient fractionation, the GDH specific activity was significantly higher than the crude plastid pellet. However, some GDH activity

<table>
<thead>
<tr>
<th>Medium</th>
<th>Enzyme</th>
<th>Total Units</th>
<th>3000g Pellet</th>
<th>12000g Pellet</th>
<th>12000g Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>S.A.*</td>
<td>%</td>
<td>S.A.</td>
</tr>
<tr>
<td>B5</td>
<td>GDH</td>
<td>1.36</td>
<td>72.0</td>
<td>0.45 ± 0.06 (0.58)</td>
<td>20.1</td>
</tr>
<tr>
<td>TPI</td>
<td></td>
<td>1.73</td>
<td>56.2</td>
<td>0.43 ± 0.07</td>
<td>7.6</td>
</tr>
<tr>
<td>SDH</td>
<td></td>
<td>82.50</td>
<td>9.5</td>
<td>8.30 ± 0.50</td>
<td>59.5</td>
</tr>
<tr>
<td>MNHA</td>
<td>GDH</td>
<td>2.42</td>
<td>65.0</td>
<td>0.63 ± 0.08 (0.78)</td>
<td>26.1</td>
</tr>
<tr>
<td>TPI</td>
<td></td>
<td>1.88</td>
<td>62.3</td>
<td>0.45 ± 0.06</td>
<td>10.5</td>
</tr>
<tr>
<td>SDH</td>
<td></td>
<td>87.50</td>
<td>12.0</td>
<td>4.03 ± 0.60</td>
<td>62.0</td>
</tr>
</tbody>
</table>

* S.A., specific activity; TPI, triose phosphate isomerase; SDH, succinate dehydrogenase.
was also detected in the mitochondrial fraction. The presence of GDH in higher plant mitochondria has been well documented by several workers (4, 9, 17, 26, 27, 29), and the fact that a fraction of GDH in SB3 cells could be mitochondrial cannot be ruled out at present. However, the results presented here suggest that a major fraction of GDH is of plastidial origin.

The localization of GDH in higher plant and algal chloroplasts has also been reported (7, 19, 27). In one study, the subunit-precursor of chloroplastic GDH was shown to be synthesized in the cytosol (19). However, studies dealing with nongreen plastids and their enzymatic characteristics have received less attention (28). Although the presence of GDH in nongreen plastids was shown earlier (20, 28), these authors did not determine the actual site of plastidial GDH synthesis.

The distribution of GDH in soybean plastids and its inhibition by streptomycin and chloramphenicol led us to ask whether the enzyme subunit was actually being synthesized in the plastids. Thus, we studied protein synthesis by isolated plastids. The results presented indicate that isolated soybean plastids are able to synthesize several polypeptides in the presence of externally added ATP and that cycloheximide does not inhibit the incorporation of the label into proteins (Fig. 2). It is also clear that none of the polypeptides could be identified as the products of bacterial protein synthesis since no radioactive incorporation was observed when ATP was omitted and sodium acetate was used as substrate (Fig. 2, lane 3). The inhibition pattern of streptomycin and chloramphenicol (Fig. 2, lanes 4 and 5, respectively) was also similar, suggesting that plastids were not contaminated with mitochondria, since one would expect a differential inhibition by these two inhibitors if mitochondrial contamination was sufficient enough to count for the synthesis of some polypeptides.

Western blot analysis of plastid translation products using GDH antibodies showed that a band corresponding to the 41-kD polypeptide gave a positive reaction with GDH antibodies (Fig. 3). The staining intensity of this band in the Western blots (Fig. 3) followed the same pattern as that of a 41-kD band in the fluorograph (Fig. 2), the intensity being low in presence of streptomycin and chloramphenicol or when ATP was omitted, thus suggesting that this polypeptide was being actively synthesized by the plastids during incubation with 35S-methionine. A positive reaction with GDH antibodies, and the fact that the molecular mass (41 kD) is identical to that recently published for the GDH subunit from SB3 cells (22), strongly suggest that this band is in fact GDH.

The results presented here strongly suggest that at least one GDH in soybean cells is plastidial in origin. Although the plant chloroplast genome is known to be of large size, and several proteins including those involved in photosynthetic functions are known to be synthesized by chloroplasts, not much information on protein synthesis by nongreen (nonphotosynthetic) plastids is available. Thus, additional studies on the isolation of mRNA from nongreen plastids and its in vitro translation in cell-free system should yield valuable information. This was beyond the scope of the present study and remains a subject of future investigation.

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LITERATURE CITED


