Intracellular Localization of Three L-Glutamate Dehydrogenase Isozymes from *Chlamydomonas reinhardtii*1

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**ABSTRACT**

The intracellular localization of the activity and synthesis of three isozymes of NAD(P)+-glutamate dehydrogenase from the unicellular green alga *Chlamydomonas reinhardtii* cw-92 has been established. Isozyme activities have been located within mitochondria by using differential centrifugation techniques and discontinuous Percoll gradient separations. Experiments with protein synthesis inhibitors cycloheximide, rifampicin, chloramphenicol, and actinomycin D, under dark and carbon starvation conditions, revealed that synthesis of the three isozymes was likely to occur in cytosol as precursor proteins that are then transported and processed inside the mitochondria.

In green algae and higher plants the GS3 (EC 6.3.1.2)/GOGAT (EC 1.4.7.1) cycle is considered the main route for assimilation of ammonium, both exogenously supplied and derived from nitrate reduction or from photorespiration. In contrast, the possible role of GDH in the process has not been resolved (9).

In *Chlamydomonas reinhardtii*, ammonia assimilation takes place mainly through the GS/GOGAT cycle in both illuminated and darkened cells, whereas GDH has been proposed to play a catabolic rather than an anabolic role (2). Recently, it was suggested that the possible anabolic function of GDH is connected with carbon rather than with nitrogen metabolism and that the role of GDH is to maintain the internal concentration of L-glutamate whenever the GS/GOGAT cycle activity is insufficient to meet the demand (15). In addition, we have demonstrated the existence of at least three NAD(P)+-GDH isozymes that respond differently to different environmental and stress conditions (16), and more recently, we purified them to electrophoretic homogeneity (14).

The subcellular localization of GDH is of the utmost inter-

1 This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (PB90-0780-CO2-01) and Consejería de Educación y Ciencias (grupo No. 3249), Junta de Andalucía, Spain, to J.C. Z.R. is a fellow of Ministerio de Educación y Ciencia, Spain.

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3 Abbreviations: GS, glutamine synthetase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; PEP, phosphoenolpyruvate.

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**MATERIALS AND METHODS**

**Organisms and Growth Conditions**

*Chlamydomonas reinhardtii* wild-type cell strain 6145c (Ruth Sager, Sidney Farber Center, Boston, MA) and a cell-wallless mutant strain cw-92 (D.D. Kaska, University of California, Santa Barbara) were grown at 25°C phototrophically in continuous white light (40 W/m²), at pH 7.2, in liquid medium with air enriched in CO₂ (5%, v/v) and 10 mM NH₄Cl as the N₂ source (14). Growth was followed by measuring turbidity of the cultures at 660 nm.

**Cell Extracts Preparation**

Cells were harvested by centrifugation at 15,000g for 10 min at 4°C, and after washing with distilled water, they were disrupted by freezing at −40°C and thawing with gentle stirring in 0.1 M Tris-HCl buffer, pH 8.0, containing 2.5 mM MgCl₂, 0.5 mM EDTA, 3 mM DTE, 0.2% Triton X-100, and 1 mM PMSF. The suspension was centrifuged at 27,000g for 15 min at 4°C, and the resulting supernatant was used as material to determine total and GDH isozyme activity.

To measure GDH and marker enzyme activity in purified
chloroplasts and mitochondria, organelles were broken by freezing and thawing as described above in 0.1 M potassium-phosphate buffer, pH 7.7, containing 2.5 mM MgCl₂, 0.5 mM EDTA, 3 mM DTE, and 1 mM PMSF.

Chloroplast Isolation and Purification

One liter of exponentially growing cells (cw-92) were harvested by centrifugation at 4000g for 5 min at room temperature. Cells were washed with 25 mM Hepes-KOH, pH 7.5, supplemented with 2 mM NaCl, and, after centrifugation, the cell pellet was resuspended in 50 mL of ice-cold disruption buffer (50 mM Hepes-KOH [pH 7.2], 300 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, and 1% BSA). Two milliliters of the algal suspension were withdrawn for GDH activity and marker enzyme assays. Cells were then transferred to a precooled Yeda press bomb and equilibrated at 200 psi (N₂) for 4 min. The lysate was let out at an even flow of about 2 mL s⁻¹.

After centrifugation at 1000g for 30 s, the pellet was resuspended in the disruption buffer and layered onto a 70% (5 mL) and 45% (4 mL) discontinuous Percoll gradient (Percoll in 50 mM Hepes-KOH [pH 8.0], 300 mM sorbitol, 1 mM Na₂P₂O₇, 5 mM EDTA, 1 mM MgCl₂, and 1% BSA). Ascorbate and DTT were added to the gradient solution to a final concentration of 5 mM each. Centrifugation was carried out using 15-mL Corex tubes in a swing-out rotor (JS-13.1, Beckman) at 6000g and 4°C for 20 min. After centrifugation, the chloroplast fraction was collected from the 45 to 70% interface and diluted 4-fold with the cell disruption buffer. Chloroplasts were pelleted by allowing the rotor to accelerate up to 500g and then stopping the run. The pellet was washed once with disruption buffer, and after a second centrifugation under the same conditions, chloroplasts were resuspended in 5 mL of 50 mM Hepes-KOH (pH 8.0), 300 mM sorbitol, and stored on ice.

Mitochondria Isolation and Purification

To isolate mitochondria, cw-92 cells were grown, harvested, and washed under the same conditions as described above. The washed cells were resuspended in 30 mL of ice-cold disruption buffer (25 mM Hepes-KOH [pH 7.4], 250 mM sucrose, and 2 mM EDTA). The algal suspension was transferred to a precooled Yeda press, equilibrated at 80 psi (N₂) for 4 min, and then the lysate was let out at a flow of about 1 mL s⁻¹.

After centrifugation at 10,000g for 10 min, the pellet was resuspended in the disruption buffer and layered onto a 60, 30, and 20% discontinuous Percoll gradient (Percoll in 25 mM Hepes-KOH, pH 7.4). Sucrose was added to the gradient solution to a final concentration of 250 mM. Centrifugation was carried out at 4°C during 20 min using 15-mL Corex tubes in the same swing-out rotor as described above at 9800g. After centrifugation, the mitochondrial fraction was collected from the 30 to 60% interface and diluted with 25 to 30 mL of disruption buffer. Mitochondria were pelleted at 12,000g for 10 min and then resuspended in 1 mL of 50 mM Hepes-KOH (pH 8.0), 300 mM sorbitol, and stored on ice.

Intactness Assays

Chloroplast intactness was assayed using the Hill reaction with ferricyanide as the electron acceptor (10). Oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech Ltd, Norfolk, UK). Intact chloroplasts and osmotically shocked chloroplasts, equivalent to 15 µg of Chl, were transferred to an assay medium consisting of the disruption buffer, to which 1000 units mL⁻¹ of bovine liver catalase, 2.5 mM NH₄Cl, and 2.5 mM K₃Fe(CN)₆ were added. Measurements were done under a light intensity of 50 W/m².

Enzyme Assays

NADH-GDH (EC 1.4.1.2) was determined spectrophotometrically at 37°C by following NADH oxidation at 340 nm according to the method of Moyano et al. (14). GDH isozymes were separated electrophoretically at pH 8.9 in 6% acrylamide gel rods under conditions previously described (14). Activity was located on the gels as colored bands produced by the dehydrogenase reaction of GDH as described by Muñoz-Blanco et al. (16). Blue band quantitation, as a percentage of total GDH, was performed by integrating them in a Beckman DU-70 spectrophotometer equipped with a gel-scanning program (16).

Fumarase (EC 4.2.1.2) (19) and PEP carboxylase (EC 4.1.1.31) (1) were measured according to published procedures. Triton X-100 (0.1%, w/v) was included in the mixture reaction to solubilize membrane-bound proteins.

Analytical Determinations

Protein was determined colorimetrically by the method of Lowry et al. (12), using BSA as a standard. Chl was extracted in absolute ethanol, and its concentration was calculated using the absorption coefficients of Wintermans and de Mots (20).

RESULTS AND DISCUSSION

Three different NAD(P)⁺-GDH isozymes have been found in C. reinhardtii that respond differently to different nutritional and stress conditions (16). These isozymes have been isolated and purified until electrophoretic homogeneity and their physicochemical and kinetic properties have been reported (14, 16). To locate these isozymes within the cell, we have used a cell wall-deficient mutant (cw-92) that contains the three NAD(P)⁺-GDH activities and has the advantage that large amounts of intact chloroplasts (75% on a Chl basis) and mitochondria (85–90% based on the fumarase assay) can be obtained in a Yeda press without treatment with autolysine.

Isolation and Purification of Chloroplasts from C. reinhardtii

cw-92 chloroplasts were purified by centrifugation through a discontinuous Percoll gradient, and a fraction enriched in intact chloroplasts was isolated in the 45 to 70% interface of the gradient, as described in "Materials and Methods." After the material was thoroughly washed, the Chl content of the
chloroplast preparation was only 6 to 10% of that of the starting material.

On the basis of the ferricyanide-dependent oxygen evolution reaction, an intactness of chloroplasts of 85 to 90% was calculated (data not shown), a proportion similar to that found by other methods (8). Purity of chloroplasts was determined by following marker enzyme activities of cytosol (PEP carboxylase) and mitochondria (fumarase) (Table I). By comparing the specific activities in the homogenate and in chloroplasts, we calculated the contamination of the chloroplasts by cytosol and mitochondria to be 6 to 13%, which is slightly lower than that found by Klein et al. (8). When NADH-GDH activity was determined in purified chloroplasts, it was only 10% of that of the homogenate on a protein basis, and a similar value of 12% of activity was found on the basis of Chl content. In contrast, Fisher and Klein (4) found in chloroplasts a value of 97% of NAD(P)⁺-GDH activity on the basis of Chl content. These authors isolated chloroplasts from Chlamydomonas cells kept under dark conditions during 9 h, after which time a higher yield of chloroplasts than in the light was found. In chloroplasts, GDH may be involved in protein degradation or amino acid turnover (2), but its physiological role is still not completely understood (4). In C. reinhardtii 6145c, we found that total GDH activity was strongly induced under dark conditions (16). However, we did not see any increase in GDH activity in chloroplasts isolated from cells kept in the dark during 12 h.

On the other hand, when isozymes were separated by electrophoresis of different fractions of cw-92 cells kept either in darkness or in light, we found only faint bands of GDH in the chloroplast fraction (Fig. 1, A and C), possibly due to a weak contamination of chloroplasts with mitochondria and/or cytosol. Our method has the advantage over that of Fischer and Klein (4) in that no protoplasts obtained with autolysine or lysis of the protoplasts with digitonin was required before separation of organelles by differential centrifugation. Therefore, we did not need any correction for enzyme data, nor did we see mitochondria as major contaminants of our chloroplast preparation.

**Isolation and Purification of Mitochondria from C. reinhardtii**

Mitochondria from cw-92 were isolated as described in "Materials and Methods." A mitochondrial fraction was collected from the 30 to 60% interface of a Percoll discontinuous gradient, with a yield of 66% on the basis of total fumarase activity in the homogenate. Mitochondria consumed oxygen in the presence of 3 mm L-malate in a reaction dependent on ADP (29 nmol/min with 35 μM ADP) with a respiratory quotient of 1.70. Oxygen consumption was inhibited by 50 μM KCN, an additional indication that the isolated fraction is enriched in mitochondria. Chl was practically absent (<0.9% of total in cells), and PEP carboxylase activity was not detected. In addition, the specific activity of the marker enzyme of mitochondrial activity, fumarase, increased about 32-fold in comparison with that of homogenate (Table I); all of these results indicated a high-purity mitochondria fraction.

When NADH-GDH activity was assayed in this mitochondrial fraction, a specific activity increase of about 15-fold in relation to homogenate was found (Table I), which strongly suggests that GDH activity in C. reinhardtii is located in mitochondria. When proteins from the mitochondrial fraction were separated by electrophoresis on acrylamide gels and stained for GDH activity, the three NAD(P)⁺-GDH isozymes were present (Fig. 1). This indicates that in C. reinhardtii the three NAD(P)⁺-GDH isozymes are located in mitochondria, in contrast to their localization in chloroplasts described by Fischer and Klein (4). This localization of NAD(P)⁺-GDH in mitochondria is consistent with the elevation of total GDH.

### Table I. Specific Activity of NAD(P)⁺-GDH and Marker Enzymes in Chloroplasts and Mitochondria of C. reinhardtii

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity</th>
<th>Homogenate</th>
<th>Chloroplasts</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarase</td>
<td>1375 ± 244</td>
<td>181 ± 37</td>
<td>44340 ± 1595</td>
<td></td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>193 ± 20</td>
<td>12 ± 3</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>GDH</td>
<td>165 ± 17</td>
<td>11 ± 3</td>
<td>2470 ± 310</td>
<td></td>
</tr>
</tbody>
</table>

* N.D. Not detected.
under dark conditions (16) and with a possible biosynthetic role of GDH when the GS/GOGAT cycle in the chloroplast (in the dark) is not operative (4).

**Effect of Inhibitors of Protein Synthesis on the Induction of NAD(P)+-GDH Isozymes of C. reinhardtii**

When cells of *C. reinhardtii* 6145c phototrophically grown with 10 mM NH₄Cl were kept in the dark in the presence of cycloheximide, an inhibitor of protein synthesis in cytosolic 80S ribosomes (5, 13), GDH₁ and GDH₂ synthesis was prevented (Fig. 2A). However, rifampicin, a specific inhibitor of chloroplastic RNA polymerase (5), was without effect (Fig. 2C), and in the presence of chloramphenicol, an inhibitor of protein synthesis in chloroplastic 70S ribosomes (5, 6, 13), both activities were slightly enhanced (Fig. 2B). After 7 h under dark conditions, actinomycin D, an inhibitor of DNA-dependent RNA synthesis (5), had the same effect as cycloheximide on the induction of isozyme activities (results not shown).

Under carbon starvation conditions, GDH₂ and GDH₃ synthesis was inhibited in the presence of cycloheximide (Fig. 3A), rifampicin was without effect, and chloramphenicol slightly increased both activities (Fig. 3, B and C).

These results suggest that those three isozymes of NAD(P)+-GDH are products of nuclear genes and that their synthesis takes place in the 80S cytosolic ribosomes, because cycloheximide inhibits protein synthesis in cytosol (6, 13).

*Figure 2.* Effect of protein synthesis inhibitors on the induction of NAD(P)+-GDH isozymes of *C. reinhardtii* under dark conditions. Wild 6145c cells phototrophically grown with 10 mM ammonium (Aₕc = 1.0–1.2) were harvested and resuspended in fresh medium with CO₂ and ammonium and kept in the dark in the presence of A, 5 μg/mL of cycloheximide; B, 1 mg/mL of chloramphenicol; C, 250 μg/mL of rifampicin. O, treated; ●, control without inhibitor. Concentrations of inhibitors were maintained by periodic additions every 7 to 8 h. Activity was determined as described in “Materials and Methods.” Similar results were found in three different experiments.

*Figure 3.* Effect of protein synthesis inhibitors on the induction of NAD(P)+-GDH isozymes of *C. reinhardtii* under carbon starvation conditions. Experimental conditions were as in the legend of Figure 2, except that cells grown in ammonium were resuspended in the light in a fresh medium containing ammonium without any carbon supply.

These results together with those found in the studies of localization indicate that the three NAD(P)+-GDH activities are located in mitochondria, but their synthesis is likely to occur in cytosol. NAD(P)+-GDH proteins synthesized in cytosol, as precursors or as subunits, and then transported into and processed within the chloroplasts have been described in *Chlorella sorokiniana* (17).

**ACKNOWLEDGMENT**

We thank Ms. C. Santos and I. Molina for secretarial assistance.

**LITERATURE CITED**

fractionation of *Chlamydomonas reinhardtii* with emphasis on the isolation of the chloroplast. Plant Physiol 72: 481–487


