Purification of Mitochondrial Glutamate Dehydrogenase from Dark-Grown Soybean Seedlings

Frank J. Turano*, Ralph Dashner†, Abha Upadhyaya, and Charles R. Caldwell

United States Department of Agriculture, Agricultural Research Service, Climate Stress Laboratory, Beltsville, Maryland 20705

Proteins in extracts from cotyledons, hypocotyls, and roots of 5-d-old, dark-grown soybean (Glycine max L. Merr. cv Williams) seedlings were separated by polyacrylamide gel electrophoresis. Three isoforms of glutamate dehydrogenase (GDH) were resolved and visualized in gels stained for CDH activity. Two isoforms with high electrophoretic mobility, GDH1 and GDH2, were in protein extracts from cotyledons and a third isoform with the lowest electrophoretic mobility, GDH3, was identified in protein extracts from root and hypocotyls. Subcellular fractionation of dark-grown soybean tissues demonstrated that GDH3 was associated with intact mitochondria. GDH3 was purified to homogeneity, as determined by native and sodium dodecyl sulfate-polyacrylamide gels. The isoenzyme was composed of a single 42-kD subunit. The pH optima for the reductive amination and the oxidative deamination reactions were 8.0 and 9.3, respectively. At any given pH, GDH activity was 12- to 50-fold higher in the direction of reductive amination than in the direction of the oxidative deamination reaction. GDH3 had a cofactor preference for NAD(H) over NADP(H). The apparent Michaelis constant values for a-ketoglutarate, ammonium, and NADH at pH 8.0 were 3.6, 35.5, and 0.07 mM, respectively. The apparent Michaelis constant values for glutamate and NAD were 15.8 and 0.10 mM at pH 9.3, respectively. To our knowledge, this is the first biochemical and physical characterization of a purified mitochondrial NAD(H)-dependent GDH isoenzyme from soybean.

GDH (EC 1.4.1.2-4.) has been the subject of numerous investigations (for review, see Stewart et al., 1980; Srivastava and Singh, 1987, and refs. therein), and it has been found in abundance in practically all plants investigated to date. GDH catalyzes the reversible reductive amination of a-ketoglutarate to form glutamate in the presence of the cofactor NAD(P)H. The enzyme exists as numerous isoenzymic forms (isoforms) that may differ in their cofactor preference and intracellular locations. In general, the NAD(H)-specific enzyme has been identified in the mitochondria, and the NADP(H)-specific enzyme has been identified in the chloroplast. The number and relative proportions of the different GDH isoforms can vary in plant tissues, depending on developmental and environmental conditions.

Based on their electrophoretic mobilities, three GDH isoforms, GDH1, GDH2, and GDH3, were identified in extracts of developing soybean (Glycine max) seeds obtained 35 d after flowering (McKenzie et al., 1981; McKenzie and Lees, 1981). The isoforms had different intracellular locations and cofactor affinities, but the isoforms had similar pH optima, relative molecular masses, and specificities for a-ketoglutarate and glutamate. McKenzie and Lees (1981) demonstrated that GDH activity from root extracts was associated with intact mitochondria and that the mitochondrial GDH co-migrated in a native polyacrylamide gel with GDH3 isolated from developing seeds, thus suggesting that the seed GDH3 was associated with mitochondria. Other researchers have also demonstrated mitochondrial GDH activities (King and Yung-Fan Wu, 1971; Duke and Ham, 1976). McKenzie and Lees (1981) demonstrated that GDH2 was associated with the chloroplast. Shailendra and Shargool (1991) have also reported a plastid GDH in soybean.

The results from the experiments conducted with soybean suggest that there are distinct mitochondrial and plastid GDH isoforms with different affinities for the same cofactors and substrates, and it is likely that these isoforms are encoded by distinct genes. Small, multigene families are common among enzymes associated with nitrogen metabolism. Several gene families have been identified and characterized for Gln synthetase (Cullimore et al., 1984; Coruzzi et al., 1988), glutamate synthase (Sakakibara et al., 1991; Gregerson et al., 1993), aspartate aminotransferase (Udvardi and Kahn, 1991; Gantt et al., 1992; Turano et al., 1992), and Asn synthetase (Tsai and Coruzzi, 1990). Furthermore, the presence of more than one GDH gene in plants is supported by results from genetic and molecular studies in maize (Pryor, 1979; Goodman et al., 1980; Sakakibara et al., 1995) and Arabidopsis (Cammaerts and Jacobs, 1983), which suggest that the GDH isoforms were composed of two distinct polypeptides or were encoded by two distinct loci. Recently, two distinct Arabidopsis expressed-sequence tags with high homology to bacterial and animal GDH genes were identified, thus providing conclusive evidence for the existence of multiple GDH genes in plants (Newman et al., 1994).

Despite detailed information on the physical and biochemical properties of the enzyme, relatively little is

1 Present address: National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Building 6, Room 324, MSC 2745, 9000 Rockville Pike, Bethesda, MD 20705.

* Corresponding author; e-mail fturano@asrr.ars.usda.gov; fax 1–301–504–7521.

Abbreviations: Cat, catalase; GDH, glutamate dehydrogenase; TPI, triose phosphate isomerase.
known about the molecular regulation and gene structure of GDH in plants. Furthermore, the physiological role of GDH in plants is not fully understood. The lack of understanding of the physiological role of GDH in plants can be partially attributed to more interest in the other enzymes associated with nitrogen assimilation, contradictory results from different investigations, and, until recently, the lack of molecular tools (antibodies, mutants, and cDNA probes) for analyses. In this report, a GDH isoform identified in the roots and hypocotyls of dark-grown soybean seedlings was purified to homogeneity and the pure enzyme was physically and biochemically characterized. This study is part of a broader effort in the laboratory to understand the cellular, developmental, and environmental factors that control GDH isoenzyme activation and/or gene expression in soybean. Fundamental biochemical studies are required to gain a basic understanding of the cellular factors, i.e. pH, substrate, and cofactor availability, that control enzyme activity. Furthermore, such studies can provide a means of developing the molecular tools for detailed analyses of the molecular regulation and gene structure of GDH in the future.

MATERIALS AND METHODS

Plant Material and Crude Protein Extractions

Soybean (Glycine max L. Merr. cv Williams) seeds were germinated on paper towels moistened with deionized water in the dark for 5 d at 25°C under aseptic conditions. After 5 d, cotyledons, hypocotyls, and roots were separated and protein samples were extracted from each of the organs. The samples (200 mg) were ground in 400 µL of buffer containing 40 mM Tris-HCl, pH 7.2, 1 mM EDTA, 5% (v/v) glycerol, and 0.01 mg/mL bromphenol blue. Triton X-100 was added to each sample to a final concentration of 0.05% (v/v) to disrupt organelles. The samples were incubated on ice for 30 min. Debris was removed from the sample by centrifugation at 13,000g for 10 min.

Protein Determinations and Enzymatic Activity Assays

Protein concentrations were determined using a Bio-Rad2 or Pierce protein assay kit. The amination and deamination reactions were used to determine GDH activity and cofactor preference as described by Loulakakis and Roubelakis-Angelakis (1990b). However, due to the high background in crude protein preparations and lower activity associated with the deamination reaction, the amination reaction was used routinely to determine activity and the amount of GDH loaded onto gels. In the amination reaction, GDH activity was determined in the presence of 40 mM NH₄Cl or 50 mM (NH₄)₂SO₄, 13 mM α-ketoglutarate, 0.25 mM NADH, and 1 mM CaCl₂ in 100 mM Tris-HCl or 100 mM KPO₄, pH 8.0, and the decrease in A₃₄₀ was recorded for 1 min. In the deamination reaction, GDH activity was determined in the presence of 35 mM glutamate, 0.25 mM NAD, and 1 mM CaCl₂ in 100 mM Tris-HCl or 100 mM KPO₄, pH 9.3, and the increase in A₃₄₀ was recorded for 1 min. One unit of GDH activity is defined as the reduction or oxidation of 1 micromole of coenzyme (NAD/NADP or NADH/NADPH, respectively) per min at 30°C. To determine cofactor specificity, NADP(H) replaced NAD(H) at the same concentrations as described above.

Gel Electrophoresis, Gel Staining Procedures, and Western Blot Analysis

During the purification procedure, proteins were routinely separated by native PAGE and SDS-PAGE on the Phast (Pharmacia) system to determine the relative purity of GDH. Gradient gels (8–25 or 4–15% polyacrylamide) or homogeneous gels (7.5% polyacrylamide) were used for non-denaturing (native) gels, and gradient gels (4–15% polyacrylamide) or homogeneous gels (12.5 or 7.5% polyacrylamide) were used for denaturing (SDS) gels. Proteins were visualized by silver stain on the Phast system as described by the manufacturer. Protein bands containing GDH activity were visualized in native polyacrylamide gels by incubation in 100 mM Tris-HCl, pH 9.3, with 50 mM glutamate, 0.5 mM NAD, 0.25 mM nitroblue tetrazolium, and 0.1 mM phenazine methosulfate for 15 to 60 min at 37°C (Hartmann et al., 1973). Identical gels were incubated in the GDH stain solution minus glutamate as controls. In some instances, NADP replaced NAD in the GDH stain solution to identify distinct isoforms that may use a different cofactor or those isoforms that may utilize either cofactor. To identify different GDH isoforms in various dark-grown tissues or to purify GDH3 by preparative gel electrophoresis, proteins were separated in a native 6% polyacrylamide gel as described by Laemmli (1970), except that SDS was omitted and the acrylamide:BIS ratio was 125:1. In both cases, gels were stained for GDH activity as described above. Pure GDH3 was resolved in a SDS 7.5% polyacrylamide gel as described by Laemmli (1970).

Western blot analysis was conducted as described by Turano et al. (1990). Proteins were separated by SDS-PAGE in 7.5% gels as described by Laemmli (1970). Rabbit serum raised against grape leaf NADH-GDH was provided by Loulakakis and Roubelakis-Angelakis (1990a).

Enriched Mitochondrial Fraction

Mitochondria were isolated from 10 g of 5-d-old roots and hypocotyls. The tissues were homogenized with a Polytron (Brinkmann) in two volumes of mitochondrial extraction buffer containing 0.3 M mannitol, 3 mM β-mercaptoethanol, 1 mM EDTA, 0.1% BSA (w/v), and 0.6% (w/v) insoluble PVP in 30 mM Mops, pH 7.5, per gram fresh weight. BSA was omitted from the buffer when the preparation was used for purification of GDH3. The homogenate was filtered through one layer of Miracloth (Calbiochem). Debris was removed from the resulting suspension by centrifugation at 500g. Mitochondria were concentrated by centrifugation at 12,000g. The resulting
crude mitochondrial pellet was used for subcellular localization or for purification of GDH3.

Subcellular Localization

The mitochondrial pellet was washed twice in 5 mL of mitochondrial resuspension buffer containing 0.3 mM mannitol, 1 mM EDTA, 1 mM MgCl₂, 25 mM HEPES, pH 7.8, 0.1% (w/v) BSA, and 0.05% (v/v) β-mercaptoethanol. The mitochondrial suspension (5 mL) was layered onto a discontinuous Percoll gradient (21, 26, 32, 47, and 60%). After centrifugation at 45,000g for 45 min at 4°C, three distinct organelle bands were removed and assayed for GDH activity and specific organelle marker enzymes. The mitochondrial marker enzyme Cyt c oxidase (Smith, 1955), the plastid marker enzyme TPI (Miřín, 1974), and the peroxisomal/glyoxosomal marker enzyme Cat (Beers and Sizer, 1952) were assayed as previously described by Turano et al. (1991).

Protein Purification

The crude mitochondrial pellet from 80 to 100 g of tissue was washed as described above and resuspended in mitochondrial lysis buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% (v/v) Tween 20. The suspension was passed through a tuberculin syringe (22-gauge) two to three times and then subjected to a freeze-thaw cycle to ensure maximum solubilization of membrane proteins. The solubilized mitochondrial suspension was clarified by centrifugation. The pellet was washed twice in a minimum of 100 mL Tris-HCl, pH 7.5, and the supernatants were pooled.

The supernatant was applied to an Affi-Gel Blue (Bio-Rad) column (3 mL bed volume) preequilibrated in wash buffer containing 10 mM Tris-HCl, pH 7.5, 1% (v/v) glycerol, 100 μM CaCl₂, 100 μM EDTA, and 150 μM β-mercaptoethanol. The column was washed with 5 bed volumes of wash buffer before eluting the bound proteins with 5 bed volumes of 10 mM NAD and 500 mM glutamate in concentrated (10×) wash buffer. The affinity chromatographic procedures were performed at room temperature. The eluted sample was dialyzed overnight against 1 L of 5 mM Tris-HCl, pH 7.5, with 1% (v/v) glycerol at 4°C and was concentrated in a Speed-Vac (Savant, Farmingdale, NY). A 400-μL sample, representing approximately 100 units of GDH activity, was loaded onto a 10 cm × 8 cm Mini-Gel (Bio-Rad) with a native 6% polyacrylamide resolving gel. The gel was run for 3 h at 10 mA. The sides of the gel were cut off and stained for GDH activity. After realigning the gel, a section of the unstained gel that corresponded to the organelle band located between the 47 and 60% Percoll interface, which migrated with 73% of the Cyt c oxidase activity (Table I). A small amount (16%) of NADH-dependent GDH activity was associated with the band located between the 26 and 32% Percoll interface, which contained 81% of the TPI activity. Only 2% of the NADH-dependent GDH activity was associated with the band located between the 47 and 60% Percoll interface, which contained 81% of the Cat activity. These data indicate that GDH3 was primarily associated with Cyt c oxidase activity in intact mitochondria. Similar results were obtained with Suc gradients (data not shown), in which the mitochondrial fraction contained approximately 60% of the total NADH-dependent GDH activity applied to samples could be frozen until needed with a minimal loss of GDH activity.

RESULTS

Organ-Specific Accumulation

Proteins extracted from cotyledons, hypocotyls, and roots of 5-d-old dark-grown soybeans were separated by PAGE. Three isoforms were visualized in gels stained specifically for GDH activity (Fig. 1). Two isoforms with relatively high electrophoretic mobilities, GDH1 and GDH2, were identified in extracts from cotyledons. One isoform with the lowest electrophoretic mobility, GDH3, was identified in hypocotyl and root preparations.

Subcellular Localization of GDH3

Since only GDH3 activity was identified in hypocotyls and roots of dark-grown soybean seedlings, these tissues were used as a source of material for the subcellular localization of the isoenzyme. Mitochondria were separated from plastids and peroxisomes by centrifugation on a discontinuous Percoll gradient as described above. After centrifugation, the three distinct organelle bands were collected from the gradient. Each sample was assayed for NADH- and NADPH-dependent GDH, Cyt c oxidase, TPI, and Cat activity. Much of the NADH-dependent GDH activity (66%) was observed in a band located between the 32/47% Percoll interface, which migrated with 73% of the Cyt c oxidase activity (Table I). A small amount (16%) of NADH-dependent GDH activity was associated with the organelle band located between the 26 and 32% Percoll interface, which contained 81% of the TPI activity. Only 2% of the NADH-dependent GDH activity was associated with the organelle band located between the 47 and 60% Percoll interface, which contained 81% of the Cat activity. These data indicate that GDH3 was primarily associated with Cyt c oxidase activity in intact mitochondria. Similar results were obtained with Suc gradients (data not shown), in which the mitochondrial fraction contained approximately 60% of the total NADH-dependent GDH activity applied to...
GDH3 was purified to homogeneity. When the same polyacrylamide gel, a single polypeptide was visible after identified a single polypeptide (42 kD) that cross-reacted pure GDH3 (Fig. 2C, lane 4) separated in a 7.5% SDS-PAGE analysis of crude protein preparations (Fig. 2C, lane 3) and present (Fig. 2B, lane 3); these results also suggest that the single polypeptide that constituted pure GDH3 was not an artifact obtained during purification.

Similar results were obtained on native homogeneous gels staining the gel with silver (Fig. 2C, lane 2). Western blot of 7.5 or 12% polyacrylamide (data not shown).

Distribution of soybean GDH3 and the marker enzymes Cyt c oxidase (Cyt c ox), TPI, and Cat in bands obtained from a Percoll step-gradient

The results presented in this table are representative of three separate experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>NADH</th>
<th>NADPH</th>
<th>Cyt c ox</th>
<th>TPI</th>
<th>Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched mitochondria</td>
<td>100 (300)*</td>
<td>100 (45)</td>
<td>100 (45)</td>
<td>100 (225)</td>
<td>100 (40)</td>
</tr>
<tr>
<td>26/32% Percoll</td>
<td>16</td>
<td>13</td>
<td>81</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>32/47% Percoll</td>
<td>66</td>
<td>70</td>
<td>73</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>47/60% Percoll</td>
<td>2</td>
<td>n.d.</td>
<td>5</td>
<td>1</td>
<td>60</td>
</tr>
</tbody>
</table>

* The value in the parentheses represents the total activity for each enzyme in the enriched mitochondrial preparation that was loaded onto the gradient. A preparation enriched for mitochondria was obtained by differential centrifugation (see “Materials and Methods”). The enriched mitochondrial preparation (equivalent to 300 units of NADH-GDH activity) was loaded on a discontinuous Percoll gradient. One unit of GDH activity equals the oxidation of 1 micromole of NADH or NADPH per minute at 30°C. n.d., Not detectable.

Purification and Characterization of GDH3

A protocol that included the isolation of mitochondria, affinity chromatography, and preparative PAGE was devised for the purification of GDH3. This procedure resulted in a 1134-fold purification and 11% recovery of GDH3 (Table II). GDH3 eluted from a preparative PAGE appeared as a single band on silver- and activity-stained native polyacrylamide gels (Fig. 2A, lanes 4 and 7, respectively). The relative molecular weight on a native gradient of 7.2 and 8.9 at pH 8.0 and 9.3, respectively. Likewise, there was a preference for NADP over NADP that favored the amination reaction compared with activity in the deamination reaction.

Purified protein preparations were used to determine pH optima, cofactor specificities, and K_m values. The optimum pH for the amination reaction was determined to be 8.0, but the optimum pH for the deamination reaction was 9.3 (Fig. 3). At any pH tested, the estimate of enzyme activity were 12 to 55 times higher in assays that favored the amination reaction compared with activity in the deamination reaction.

To determine cofactor preference, GDH assays were conducted in both directions using NAD(H) and NADP(H) at the two pH optima (Table III). GDH3 had a preference for NADH over NADPH, which could be expressed as a ratio of 7.2 and 8.9 at pH 8.0 and 9.3, respectively. Likewise, there was a preference for an NAD over NADP (ratio 17 at pH 9.3) by the enzyme. Furthermore, estimates of GDH3 activity measured by the amination reaction were about 35 times higher with NADH at pH 8.0 and 17 times higher at pH 9.3 than estimates of GDH activity using NAD in the corresponding deamination reactions. A similar trend was observed with estimates of GDH activity using NADPH, in which the estimates of activity were at least 33 times higher compared with estimates of activity using NADP.

An estimation of K_m values was conducted (Table IV). The apparent K_m values for each of the substrates were affected by pH. The apparent K_m values for α-ketoglutarate, ammonium, and NADH at pH 8.0 were 36, 35.5, and 0.07 mM, respectively; but at pH 9.3 the apparent K_m values for those substrates were 12.3, 20.1, and 0.06 mM, respectively. The apparent K_m values for glutamate and NAD were 15.8 and 0.1 mm at pH 9.3 and 7.2, and 0.12 mM at pH 8.0, respectively.

Table I. Distribution of soybean GDH3 and the marker enzymes Cyt c oxidase (Cyt c ox), TPI, and Cat in bands obtained from a Percoll step-gradient

<table>
<thead>
<tr>
<th>Sample</th>
<th>NADH-GDH Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>381</td>
<td>1002</td>
<td>0.38</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Crude mitochondria</td>
<td>316</td>
<td>53</td>
<td>5.96</td>
<td>83</td>
<td>15.7</td>
</tr>
<tr>
<td>Affi-Gel Blue chromatox</td>
<td>194</td>
<td>2.5</td>
<td>77.6</td>
<td>51</td>
<td>204</td>
</tr>
<tr>
<td>Electroleozone</td>
<td>41</td>
<td>0.095</td>
<td>431</td>
<td>11</td>
<td>1134</td>
</tr>
</tbody>
</table>

* One unit of GDH activity equals the oxidation of 1 micromole of NADH per minute at 30°C. GDH activity was determined in the presence of 50 mM (NH_4)_2SO_4, 13 mM α-ketoglutarate, 0.25 mM NADH, and 1 mM CaCl_2 in 100 mM Tris-HCl, pH 8.0. b, c, Not applicable.
Purification of Mitochondrial Soybean Glutamate Dehydrogenase

Figure 2. Electrophoretic analyses of GDH3 by native and SDS-PAGE. A, Enzyme preparations containing GDH3 (0.5 units/lane) were separated by PAGE in a native 4 to 15% gradient gel. The proteins in lanes 1 through 4 were stained with silver. Proteins in lanes 5 through 7 were stained for NADH-GDH activity. Molecular mass markers (lane 1) are as follows: ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), and BSA (66 kD). Proteins from crude mitochondrial preparations are in lanes 2 and 5, Affi-Gel Blue-purified preparations are in lanes 3 and 6, and gel/electroeluted purified GDH are in lanes 4 and 7. B, Enzyme preparations containing GDH3 (0.5 units/lane) were separated by SDS-PAGE in a 4 to 15% gradient gel. Protein samples from crude mitochondria (lane 1), Affi-Gel Blue chromatography (lane 2), and gel/electroelution (lane 3) preparations were visualized by silver stain. Molecular mass markers (lane 4) are as follows: phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and B-lactalbumin (14.4 kD). C, Enzyme preparations containing GDH3 (10 units/lane) were separated by SDS-PAGE in a 7.5% homogeneous gel. The molecular mass markers (lane 1) are as described above. Pure (gel/electroeluted) GDH3 (lane 2) was visualized by silver stain. One polypeptide (42 kD) cross-reacted with rabbit serum raised against grape leaf NADH-GDH (Loulakakis and Roubelakis-Angelakis, 1990a) in the crude (lane 3) and pure (lane 4) preparations of GDH3.

DISCUSSION

In this investigation protein extracts from germinating soybeans contained three GDH isoforms that were resolved by native PAGE and were designated GDH1, GDH2, and GDH3, according to their electrophoretic mobilities toward the anode. The isoforms exhibited organ-specific activity: GDH1 and GDH2 were localized in protein extracts from cotyledons, whereas GDH3 was identified in both hypocotyls and roots. In earlier studies by McKenzie et al. (1981) and McKenzie and Lees (1981), three GDH isoforms with distinct electrophoretic mobilities were identified in protein extracts from developing soybean seeds. In their studies GDH1 was detected in developing seeds and not in roots or leaves, GDH2 was identified in leaves and developing seeds, and GDH3 was identified in roots and developing seeds. Their results, as well as those presented here, demonstrate the organ-specific activity of different GDH isoforms. A summary of the results from the combined studies suggest that (a) GDH1, the fastest migrating isoform, is specific to the cotyledonary tissue in both developing seeds and germinating seedlings, (b) GDH2 is associated with both cotyledons and leaves, and (c) the slowest migrating isoform, GDH3, is in roots and hypocotyls.

In this study the intracellular location of GDH3 from hypocotyl and root extracts was determined using discontinuous Percoll gradients. The isoform was associated with intact mitochondria. Other investigators (Duke and Ham, 1976) have identified a mitochondrial GDH isoform from soybean roots, but the specific isoform was not determined by electrophoretic mobility. McKenzie and Lees (1981) demonstrated that GDH3 was localized in root mitochondria. A mitochondrial isoform was identified in the cotyledons of 11-d-old soybeans (King and Yung-Fan Wu, 1971), but again, the specific isoform was not determined by electrophoretic mobility. In this study we were unable to definitively determine the cellular locations of GDH1 and GDH2 due to difficulties associated with the clear separation of organelles from cotyledons on Percoll or Suc gradients. Similar complications were reported previously.

Figure 3. Determination of pH optima for soybean GDH3. The optimum pH for the amination reaction (NADH-dependent) was determined to be 8.0 and the optimum pH for the deamination reaction (NAD-dependent) was determined to be 9.3. All data are expressed as relative amount of activity versus NADH-GDH activity at pH 8.0. (See “Materials and Methods” for specific details of the reaction conditions.)
by McKenzie and Lees (1981). Furthermore, McKenzie and Lees (1981) were not able to localize GDH2 and GDH3 directly from extracts of developing seeds. The localization of GDH2 and GDH3 were determined by analogy from other plant parts, namely leaves and roots, respectively.

Initial estimates of the relative molecular mass were determined by native PAGE on a series of homogeneous and gradient gels (F.J. Turano and R. Dashner, unpublished results). The relative molecular mass of the native protein was $280 \pm 20$ kD. These data are similar to physical data reported for a GDH isoform isolated from the plastids of soybean cell culture (Shargor and Jain, 1989); the relative molecular weight was $263 \pm 6$ kD and it was composed of 41-kD subunits. Moreover, these data are consistent with those from other plant GDHs. In general, plant GDH isoforms have relative molecular weights ranging from 208 to 300 kD and are composed of four subunits of about 60 kD or six subunits of about 45 kD (Stewart et al., 1980; Srivas-
tava and Singh, 1987). GDH enzymes are usually composed of homogeneous monomers. Recently, however, NADH-GDH from grape was shown to be composed of two different polypeptides (Loulakakis and Roubelakis-Angelakis, 1991). The two polypeptides (about 42 kD each, designated $\alpha$ and $\beta$) were clearly resolved by 7.5% SDS-PAGE. However, all of our results suggest that soybean GDH3 is composed of a 42-kD subunit and is most likely a hexameric protein.

In this study pure preparations of GDH3 were separated by SDS-PAGE in a 7.5% polyacrylamide gel, conditions that resolved two 42-kD polypeptides in grape (Loulakakis and Roubelakis-Angelakis, 1991), but only one polypeptide was apparent. We observed similar results using different gel systems (Phast [Pharmacia], Protein I and Mini-Protein II [Bio-Rad]) and a wide range of polyacrylamide concentrations using homogeneous (5, 5.5, 6.7, 7.0, up to 12%) and gradient gels (4–15%, 10–15%, and 8–25%). Western blot analysis of crude protein preparations from soybean roots and hypocotyls and pure GDH3 separated by 7.5% SDS-PAGE identified a single 42-kD polypeptide that cross-reacted with rabbit serum raised against grape leaf NADH-GDH (Loulakakis and Roubelakis-Angelakis, 1990a). These data suggest that GDH3 was composed of a single polypeptide that was an artifact obtained during purification of GDH3 or due to preferential purification of one polypeptide. Likewise, our preliminary results from two-dimensional electrophoresis suggest that GDH3 is composed of a single polypeptide (F.J. Turano, unpublished results). In grape, the ratio of the $\alpha$ and $\beta$ polypeptides was shown to change when calli were maintained on different nitrogen sources (Loulakakis and Roubelakis-Angelakis, 1991). The $\alpha$ subunit was almost exclusively present in calli grown on medium containing 10 mM NH$_4$Cl, but the $\beta$ subunit was predominant in protein extracts from calli maintained on 20 mM KNO$_3$. If soybean GDH3 were similar to the grape isoenzymes, i.e. composed of two distinct polypeptides, the presence of a single polypeptide in this experiment could be due to the absence of either nitrogen source during germination of the seeds. Therefore, the existence of a second GDH3 polypeptide cannot be ruled out at this time.

An alternative explanation for the appearance of one band in pure preparations of GDH3 by SDS-PAGE could be the inability to resolve two polypeptides of very similar or identical molecular weight. This hypothesis provides a plausible explanation for why soybean GDH does not resolve into numerous distinct bands on native polyacrylamide gels, as do grape (Loulakakis and Roubelakis-Angelakis, 1990b, 1991) or Arabidopsis (Cammaerts and Jacobs, 1983, 1985; F.J. Turano, unpublished results) NADH-GDH isoenzymes. Grape NADH-GDH isoenzymes are composed of two subunits of different molecular weight that can be resolved by SDS-PAGE. The two subunits combined in different ratios to form hexameric complexes that can be resolved into seven isoenzymes by native PAGE (Loulakakis and Roubelakis-Angelakis, 1991). Similarly, two nonallelic genes are responsible for the formation of seven isoenzymes in Arabidopsis (Cammaerts and Jacobs, 1983).

GDH3 had two pH optima, one at 8.0 for the amination reaction and the other for the deamination reaction at 9.3. The existence of two pH optima is not uncommon for GDH isoenzymes (Stewart et al., 1980); similar findings have been observed with GDH from grape leaves (Loulakakis and Roubelakis-Angelakis, 1990) and soybean cotyledons (King and Yung-Fan Wu, 1971). Estimates of enzyme activity were 12 to 55 times higher in assays that favored the amination reaction compared with activity in the deamination reaction at the same pH.

GDH3 had a preference for NAD(H) over NADP(H); the NADH-to-NADPH ratio was slightly greater than 7. Our results are consistent with results from studies of other plant mitochondrial GDH isoforms, which usually show a preference for NAD(H) (Stewart et al., 1980; Srivastava and

### Table III. Cofactor specificity of soybean GDH3

<table>
<thead>
<tr>
<th>Sample</th>
<th>GDH Activity</th>
<th>GDH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADH</td>
<td>NADPH</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>345</td>
<td>48</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>294</td>
<td>33</td>
</tr>
</tbody>
</table>

*One unit of GDH activity equals the reduction or oxidation of 1 micromole of coenzyme per minute at 30°C. *n.d., Not detectable. –, Not applicable.

Table IV. Apparent $K_m$ values of soybean GDH3 with different substrates at pH 8.0 or 9.3

<table>
<thead>
<tr>
<th>pH</th>
<th>Amination Reaction</th>
<th>Deamination Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$-KG</td>
<td>NH$_4$</td>
</tr>
<tr>
<td>8.0</td>
<td>3.6</td>
<td>35.5</td>
</tr>
<tr>
<td>9.3</td>
<td>12.3</td>
<td>20.1</td>
</tr>
</tbody>
</table>
Sirigh, 1987). In addition, these findings are in agreement with results from western blot analysis (Fig. 2C), in which GDH3 cross-reacted with rabbit serum raised against grape leaf NADH-GDH (Loulakakis and Roubelakis-Angelakis, 1990a). The rabbit serum raised against grape leaf NADH-GDH does not cross-react with NADPH-GDH from soybean or Arabidopsis (F.J. Turano, unpublished results).

McKenzie and Lees (1981) demonstrated that NADPH-dependent activity of GDH3 was less than 5% of the NADH-dependent activity, which relates to a NADH-to-NADPH ratio of greater than 20. The difference between the results from their study and this study may reflect isoenzymic variants among different soybean varieties or variations in the experimental procedures.

The $K_m$ values for $\alpha$-ketoglutarate, ammonium, and NADH at the optimal pH 8.0 for the amination reaction were 3.6, 35.5, and 0.07 mM, respectively. The $K_m$ for glutamate and NAD at the optimal pH 9.3 for the deamination reaction were 15.8 and 0.10 mM, respectively. These values are similar to those observed for other plant NADH-GDH isoforms (Stewart et al., 1980; Srivastava and Singh, 1987). When estimates of the $K_m$ for $\alpha$-ketoglutarate, ammonium, and NADH were conducted at pH 9.3, the optimal pH for the deamination reaction, the values were 12.3, 20.1, and 0.06 mM, respectively. Estimates of the $K_m$ for glutamate and NADH were 7.2 and 0.12 mM at pH 8.0, the optimal pH for the deamination reaction. In both cases the most dramatic changes in apparent $K_m$ values were for the substrates $\alpha$-ketoglutarate, ammonium, and glutamate, and not the cofactors NADH and NAD. These results suggest that the interaction between pH and available substrate could control catabolic or metabolic NAD(H)-GDH activity in the mitochondria.

Results from recent studies contain data to support both a catabolic and a metabolic role of NAD(H)-GDH in plant nitrogen metabolism. The specific cellular factors that control NAD(H)-GDH activity remain elusive, but the general environmental factors are more defined. NAD-GDH activity may play a complementary role to Gln synthetase/glutamate synthase in the reassimilation of excess ammonia released during stress conditions or during specific developmental stages (Yamaya et al., 1986; Rhodes et al., 1989). However, Robinson et al. (1991, 1992) demonstrated that glutamate oxidation may provide carbon skeletons, in the form of $\alpha$-ketoglutarate, to the tricarboxylic acid cycle in cells exposed to carbon-limited conditions. A better understanding of how these pH and substrate availability and other cellular factors (i.e. Ca$^{2+}$, tricarboxylic acids, nucleotides, etc.) interact to affect GDH activity may give greater insight into the physiological role of GDH in plants. However, it is important to determine the location of GDH in the mitochondria and obtain accurate estimates of mitochondrial pH and substrate concentrations before considering how these factors control GDH activity in vivo.

**ACKNOWLEDGMENTS**

The authors would like to thank Drs. Melinda Martin, Benjamin F. Matthews, H. David Husic, and Michael J. Muhitch for critical review of the manuscript; and Drs. Perry Kregan (U.S. Department of Agriculture/Agricultural Research Service, Soybean and Alfalfa Research Laboratory, Beltsville, MD) and William Kenworthy (Agronomy Department, University of Maryland, College Park, MD) for providing a supply of Williams soybean seed.

Received June 11, 1996; accepted August 7, 1996.

**LITERATURE CITED**


King J, Yung-Fan Wu W (1971) Partial purification and kinetic properties of glutamic dehydrogenase from soybean cotyledons. Phytochemistry 10: 915-928


Loulakakis KA, Roubelakis-Angelakis KA (1990a) Immunohoracterization of NADH-glutamate dehydrogenase from *Vitis vinifera* L. Plant Physiol 94: 109-113


Miflin B (1974) The localization of nitrite reductase and other...


