

# Plant NAD(H)-Glutamate Dehydrogenase Consists of Two Subunit Polypeptides and Their Participation in the Seven Isoenzymes Occurs in an Ordered Ratio

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## ABSTRACT

The structure and function of NAD(H)-glutamate dehydrogenase in plants was studied by using grapevine (*Vitis vinifera* L. cv Sultanina) callus grown under different nitrogen sources. The enzyme consists of two subunit-polypeptides,  $\alpha$  and  $\beta$ , with similar antigenic properties but with different molecular mass and charge. The two polypeptides have molecular masses of 43.0 and 42.5 kilodaltons, respectively. The holoenzyme is hexameric and is resolved into seven isoenzymes by native gel electrophoresis. Two-dimensional native/SDS-PAGE revealed that the 1 and 7 isoenzymes are homohexamers and the isoenzymes 2 through 6 are hybrids of the two polypeptides following an ordered ratio. The total quantity of  $\alpha$ - and  $\beta$ -polypeptides and the isoenzymic pattern was altered by the exogenous nitrogen source. The sample derived from callus grown on nitrate or glutamic acid contained a slightly greater amount of  $\beta$ -polypeptide and of the more cathodal isoenzymes, whereas  $\alpha$ -polypeptide and the more anodal isoenzymes predominated in callus grown in the presence of either ammonium or glutamine. The anabolic reaction was correlated with the  $\alpha$ - and the catabolic reaction with the  $\beta$ -polypeptide; this could suggest that each isoenzyme exhibits anabolic and catabolic function of different magnitude. The isoenzymic patterns did not obey the expected binomial distribution proportions.

GDH<sup>1</sup> (EC 1.4.1.2.) catalyzes the amination of  $\alpha$ -ketoglutarate (synthetic reaction) and the deamination of glutamate (catabolic reaction). The magnitude of GDH contribution in ammonia assimilation in the plant cell is to date a subject of controversy, especially as Yamaya *et al.* (29) showed that the ammonia concentration in mitochondria is in the range of 5 to 10 mM, which is close to the  $K_m$  values of GDH. Furthermore, Munoz-Blanco and Cardenas (16), working with *Chlamydomonas reinhardtii*, have shown that under different trophic and stress conditions, the GDH aminating activity is adaptive and involved in the maintenance of intracellular levels of L-glutamate. Also, Loyola-Vargas *et al.* (13) have suggested that GDH is the key enzyme involved in ammonia assimilation in roots from nonstress plants.

GDH is a mitochondrial enzyme (15) and has been purified to homogeneity from a few plant species (6, 18, 21). Recently, we (10) purified to homogeneity the major of the seven

NAD(H)-GDH isoenzymes from a perennial plant species, grapevine (*Vitis vinifera* L.). The amination reaction was fully activated by about 100  $\mu$ M  $\text{Ca}^{2+}$ , whereas the deamination reaction was not affected by the addition of  $\text{Ca}^{2+}$ . We (11) also raised rabbit antiserum against leaf NAD(H)-GDH isoenzyme 1. The antiserum recognized the seven isoenzymes and precipitated all the enzyme activity from leaf, shoot, and root tissues. Protein blot following SDS-PAGE revealed the same protein band for the three tissues, with a molecular mass of 42.5 kD, which was considered to correspond to the NAD(H)-GDH subunit.

In this paper, we present evidence from more detailed electrophoretic studies suggesting that the seven grapevine GDH isoenzymes consist of two polypeptides with similar antigenic properties but with different molecular mass and charge; they occur in an ordered ratio and NADH-GDH/NAD-GDH activity is regulated by the differential quantitative participation of the two polypeptides in the seven isoenzymes.

## MATERIALS AND METHODS

### Plant Material

Grapevine (*Vitis vinifera* L. cv Sultanina) virus-free plants were grown *in vitro* and micropropagated by the one-node technique in Roubelakis medium (23). Leaf, shoot, and root tissues were used for enzymatic studies. In addition, green leaf, shoot, and root segments of 0.5 to 1.0 cm were positioned for callogenesis on a modified Murashige and Skoog medium (17) supplemented with plant growth regulators at 2/5  $\mu$ M 6-benzaminopurine/ $\alpha$ -naphthaleneacetic acid, respectively.

The effect of different nitrogen sources on the NAD(H)-GDH was investigated in an effort to reveal more information on the structure and the possible physiological role of the enzyme. Thus, calluses were grown for three generations of 4 weeks each on nitrate (20 mM)-containing medium as the sole nitrogen source and then transferred for 15 d to media containing different nitrogen sources, either inorganic  $\text{KNO}_3$  (20 mM),  $\text{NH}_4\text{Cl}$  (10 mM), or both (20 mM plus 10 mM, respectively) and organic, glutamic acid, or glutamine at 10 mM.

### Enzyme Extraction

Grapevine tissues were pulverized in liquid nitrogen with the aid of a mortar and pestle. The powder from leaf, shoot,

<sup>1</sup> Abbreviation: GDH, glutamate dehydrogenase.

and root tissues was suspended in 5 volumes (v/w) of ice-cold grinding medium consisting of 200 mM Tris-HCl, pH 8.0, 14 mM  $\beta$ -mercaptoethanol, 10 mM L-cysteine-HCl, 0.5 mM PMSF, 0.5 g PVPP/g fresh weight, and 0.5% (v/v) Triton X-100. Callus powder was suspended in 2 volumes (v/w) of grinding medium consisting of 100 mM Tris-HCl, pH 8.0, 14 mM  $\beta$ -mercaptoethanol, 5 mM DTT, 10  $\mu$ M leupeptin, 0.5 mM PMSF, 1 mM EDTA, 3 mM MgCl<sub>2</sub>, 0.5% (v/v) Triton X-100, and 0.2 g PVPP/g fresh weight. After homogenization in an Omnimixer homogenizer for four times, 12 s each, the samples were centrifuged at 25,000g for 20 min and the supernatants were used for enzyme assays, immunoprecipitation, and SDS-PAGE. For native and two-dimensional PAGE, the supernatants were precipitated with solid ammonium sulfate and the fraction of 35 to 70% saturation was dialyzed and used (10). All steps were carried out at 4°C.

### Activity Assays and Protein Determination

GDH activity was determined in the aminating and the deaminating direction by following the absorption change at 340 nm (10). The standard amination reaction mixture contained 100 mM Tris-HCl, pH 8.0, 20 mM  $\alpha$ -ketoglutarate, 200 mM NH<sub>4</sub>Cl, 1.0 mM CaCl<sub>2</sub>, 0.2 mM NADH, enzyme solution, and deionized water to a final volume of 1.0 mL. The standard deamination reaction mixture contained 100 mM Tris-HCl, pH 9.3, 100 mM L-glutamate, 1.0 mM NAD<sup>+</sup>, 0.5 mM CaCl<sub>2</sub>, enzyme solution, and deionized water to a final volume of 1.0 mL. All assays were performed at 30°C. One unit of GDH activity was defined as the reduction or oxidation of 1.0  $\mu$ mol of coenzyme (NAD<sup>+</sup> or NADH, respectively) min<sup>-1</sup> at 30°C. Protein concentration was determined following 10% (w/v) TCA (final concentration) precipitation by the method of Lowry (12).

### Purification of NADH-GDH Isoenzyme 1 and Preparation of Antibodies

GDH isoenzyme 1 was purified to homogeneity from leaves of grapevine, and antibodies against this preparation were prepared as described previously (10, 11).

### Immunoprecipitation

GDH was immunoprecipitated from the different samples as previously described (11). The resulting immunoprecipitates were collected by centrifugation, washed, and resuspended in either Laemmli sample buffer (9) for SDS-PAGE or in O'Farrell lysis buffer (19) for two-dimensional PAGE.

### PAGE

Native PAGE of the partially purified GDH extracts was performed by the method of Davis (3) on 5% (w/v) resolving slab with 4% (w/v) stacking gel using the Bio-Rad mini Protean II model. The buffer system was 25 mM Tris, 192 mM glycine, adjusted to pH 8.1 with HCl. Running was at 4°C, 120 V for about 2 h and bands containing GDH activity were visualized with the tetrazolium system. The staining solution contained 100 mM Tris-HCl, pH 9.3, 55 mM L-glutamate, 0.45 mM NAD<sup>+</sup>, 0.48 mM MTT, 0.13 mM phen-

zine methosulfate, and 0.5 mM CaCl<sub>2</sub>. SDS-PAGE was performed according to Laemmli (9) with different concentrations of acrylamide on 1.5 mm thick gels. Samples were treated at 100°C for 5 min with 3% (w/v) SDS and 5% (v/v)  $\beta$ -mercaptoethanol and subjected to electrophoresis under constant current of 25 mA/slab gel. Silver staining of the proteins was performed as described by Wray *et al.* (28). Two-dimensional gel electrophoresis was run according to O'Farrell (19) except that isoelectric focusing gels contained 0.4% pH range 3.5 to 10, 0.8% pH range 5 to 7, and 0.8% pH range 7 to 9 Pharmalytes (Pharmacia).

To examine the polypeptide composition of each one of the isoenzymes, two-dimensional gel electrophoresis was performed using native 1.25 mm gels for the first dimension. Then, the gels were fixed in 40% (v/v) methanol and 10% (v/v) acetic acid and slightly stained with Coomassie brilliant blue R-250 to visualize the proteins. The gels were rinsed two times, 1 h each with nanopure d-water. The stained lanes were cut out with a razor, equilibrated for 20 min in Laemmli buffer, and subjected to the second dimension in SDS slab gels containing 7.5% (w/v) acrylamide.

### Protein Gel Blotting

After electrophoresis, the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.02% SDS) and the proteins were transferred to a nitrocellulose filter at 40 V overnight as described by Towbin *et al.* (27). Nitrocellulose blots were blocked with 2% (w/v) BSA in PBS, washed, incubated with first and second antibodies, and developed by standard methods (7). Antibodies used were monospecific against GDH (11). Densitometric analysis was performed by using a densitometer Quantimet (Cambridge Instruments).

## RESULTS

### Effect of Nitrogen Source on GDH Specific Activities

Transfer of callus from nitrate containing medium to culture media containing ammonium, as a nitrogen source, resulted in a significant increase in NADH-GDH specific activity and a decrease in NAD-GDH specific activity (Table I). This shift in GDH activities was found in all calluses and was independent of the initial donor tissue, used for callus initiation, namely leaf, shoot, and root.

Moreover, an increase in NADH-GDH and a decrease in NAD-GDH specific activity was observed in callus that was developed in the presence of both nitrate plus ammonium or glutamine. On the other hand, glutamic acid, which is the substrate for the deaminating reaction, exhibited the same effect as the nitrate (Table I). These differences in the specific activities of GDH were reflected on the ratio of aminating to deaminating enzyme activities (NADH-GDH/NAD-GDH) and were used to compare the effect of the nitrogen sources on GDH. As Table I shows, callus grown on nitrate or glutamate containing culture media had low NADH-GDH/NAD-GDH activity ratios. In contrast, callus grown on ammonium containing medium displayed high ratio value, whereas callus grown on nitrate plus ammonium or glutamine showed ratio values in between.

**Table 1.** Nitrogen Source-Dependent Changes in Specific Activities of NADH-GDH and NAD-GDH and in their Ratio in Grapevine Callus

Calluses were grown on culture medium containing 20 mM KNO<sub>3</sub>, as a nitrogen source, for three generations and then transferred for 15 d to the specific nitrogen source containing culture medium. Each value is the average of four determinations.

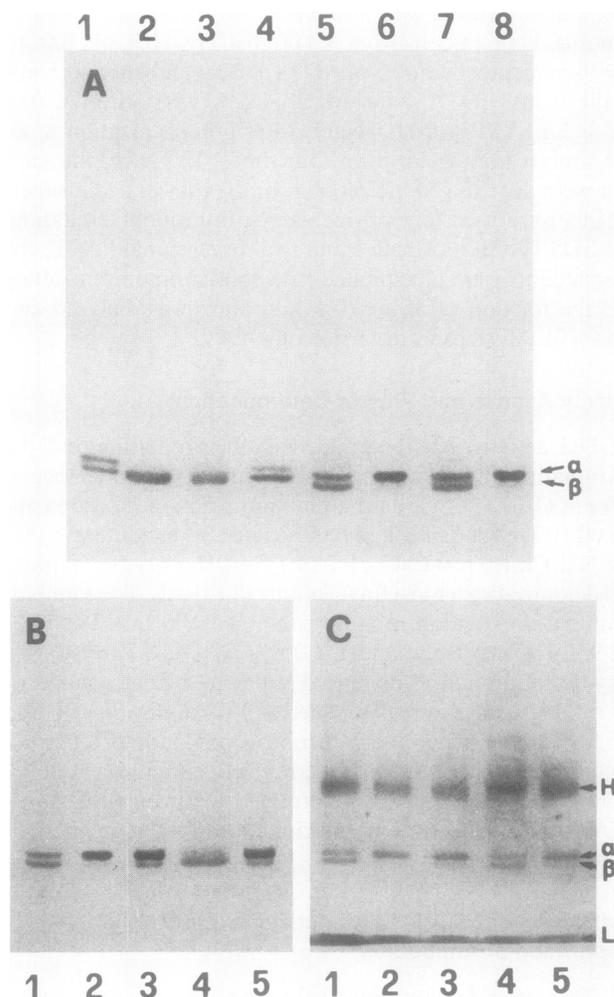
Treatment	Enzyme Specific Activities		NADH-GDH/NAD-GDH Activity Ratio
	NADH-GDH	NAD-GDH	
<i>munit · mg protein<sup>-1</sup></i>			
<b>Leaf callus</b>			
20 mM KNO <sub>3</sub>	152 ± 25	176 ± 18	0.9
10 mM NH <sub>4</sub> Cl	240 ± 20	62 ± 22	3.9
<b>Root callus</b>			
20 mM KNO <sub>3</sub>	136 ± 17	153 ± 20	0.9
10 mM NH <sub>4</sub> Cl	270 ± 15	63 ± 18	4.3
<b>Shoot callus</b>			
20 mM KNO <sub>3</sub>	136 ± 22	161 ± 17	0.8
10 mM NH <sub>4</sub> Cl	251 ± 14	59 ± 15	4.3
20 mM KNO <sub>3</sub> + 10 mM NH <sub>4</sub> Cl	224 ± 13	83 ± 10	2.7
10 mM Glutamic acid	130 ± 21	164 ± 19	0.8
10 mM Glutamine	208 ± 24	72 ± 16	2.9

### Polypeptide Composition of GDH

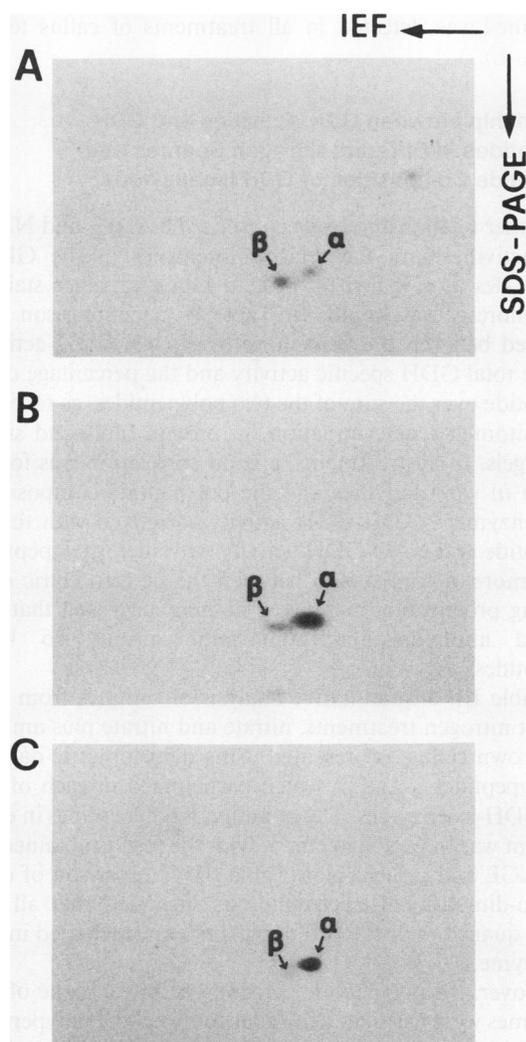
Callus samples grown on nitrate or ammonium containing medium with low and high NADH-GDH/NAD-GDH activity ratios, respectively, were analyzed by SDS-PAGE using different concentrations of polyacrylamide to examine whether any heterogeneity in the size of the enzyme subunits could be detected. Proteins were transferred on nitrocellulose sheets and GDH was immunodetected using antiserum against GDH isoenzyme 1 from grapevine leaves. In 14 and 10% acrylamide gels and in linear gradients of 10 to 18% acrylamide, only one GDH antigen was observed in the two samples. By using 7.5% acrylamide gels, GDH could be resolved into two bands with a small difference in mol wt. Based on their mobilities in the SDS gel, the 43.0 and 42.5 kD antigens were designated as being  $\alpha$ - and  $\beta$ -polypeptides, respectively. The sample derived from callus grown on nitrate medium contained a slightly greater amount of  $\beta$ -polypeptide, whereas the  $\alpha$ -polypeptide predominated in callus grown on ammonium medium. Based on these results, our remaining work was performed by using 7.5% acrylamide gels.

Figure 1A shows the polypeptides of NAD(H)-GDH from extracts of different tissues and from calluses, grown in the presence of either nitrate or ammonium. All samples contained the two GDH polypeptides, indicating that the enzyme has the same qualitative content in the tested tissues; however, the two polypeptides differed quantitatively in the various samples. In leaf, shoot, and root extracts, the  $\beta$ -polypeptide was the predominant GDH antigen, with slightly greater quantities of the  $\alpha$ -polypeptide in the root extract compared to leaf and shoot extracts. The presence of nitrate and ammonium in the culture media had the same effect on the GDH polypeptides of leaf, root, and shoot callus.

Next, we examined the composition of GDH polypeptides in response to different nitrogen sources. It should be men-



**Figure 1.** Polypeptide composition of GDH from different grapevine tissues. A and B, Protein blot analysis after 7.5% acrylamide SDS gel electrophoresis. NAD(H)-GDH polypeptides were probed with anti-GDH serum. About 16 munits of NADH-GDH were loaded per well. C, Silver staining of NAD(H)-GDH after 7.5% acrylamide gel electrophoresis. About 60 munits of NADH-GDH were immunoprecipitated as described in "Materials and Methods" and loaded per well. A, Enzyme extracts from nitrate grown shoot callus (lane 1), leaf (lane 2), shoot (lane 3), root (lane 4), nitrate grown leaf callus (lane 5), ammonium grown leaf callus (lane 6), nitrate grown root callus (lane 7), and ammonium grown root callus (lane 8). B and C, Enzyme extracts or immunoprecipitates from shoot callus grown on culture media containing 20 mM KNO<sub>3</sub> (lanes 1), 10 mM NH<sub>4</sub>Cl (lanes 2), 20 mM KNO<sub>3</sub> plus 10 mM NH<sub>4</sub>Cl (lanes 3), 10 mM glutamate (lanes 4), and 10 mM glutamine (lanes 5), as nitrogen sources. H, Heavy chain of immunoglobulin G (IgG); L, light chain of IgG;  $\alpha$ ,  $\alpha$ -GDH polypeptide;  $\beta$ ,  $\beta$ -GDH polypeptide.



**Figure 2.** Two-dimensional urea-isoelectric focusing/SDS-PAGE of ammonium sulfate fractionated extracts from grapevine shoot callus. The first dimension was performed in the presence of 9.5 M urea and the second dimension on 7.5% SDS-polyacrylamide slab gels. The proteins were transferred on nitrocellulose membranes and probed with anti-GDH serum. Enzyme extracts were from shoot callus grown on culture media containing as nitrogen source 20 mM  $\text{KNO}_3$  (A), 20 mM  $\text{KNO}_3$  plus 10 mM  $\text{NH}_4\text{Cl}$  (B), and 10 mM  $\text{NH}_4\text{Cl}$  (C).

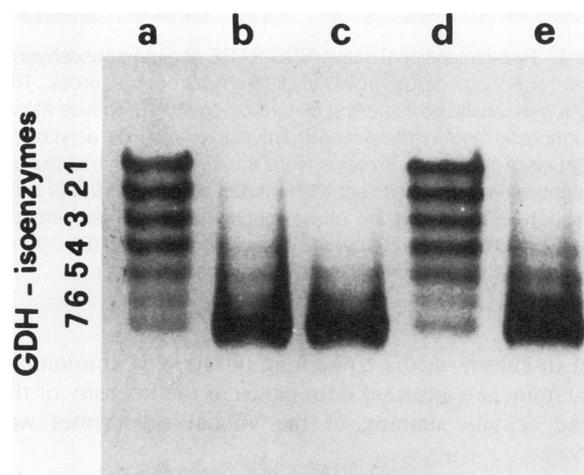
tioned that in all subsequent studies, callus derived from shoot tissue was used. Figure 1B shows a protein blot of extracts from calluses grown on different nitrogen sources. In the presence of ammonium, or ammonium combined with nitrate or glutamine, the  $\alpha$ -polypeptide predominated, while in the presence of nitrate or glutamic acid, the  $\beta$ -polypeptide was slightly more than the  $\alpha$ -polypeptide (Fig. 1B). Furthermore, in order to verify the above findings, GDH from the same samples was immunoprecipitated with anti-GDH serum, the proteins were separated with SDS-PAGE, and silver stained (Fig. 1C). The intensities and composition of the polypeptides were comparable to those obtained by the protein blot (Fig. 1B, C).

Immunodetection of GDH after being resolved by two-

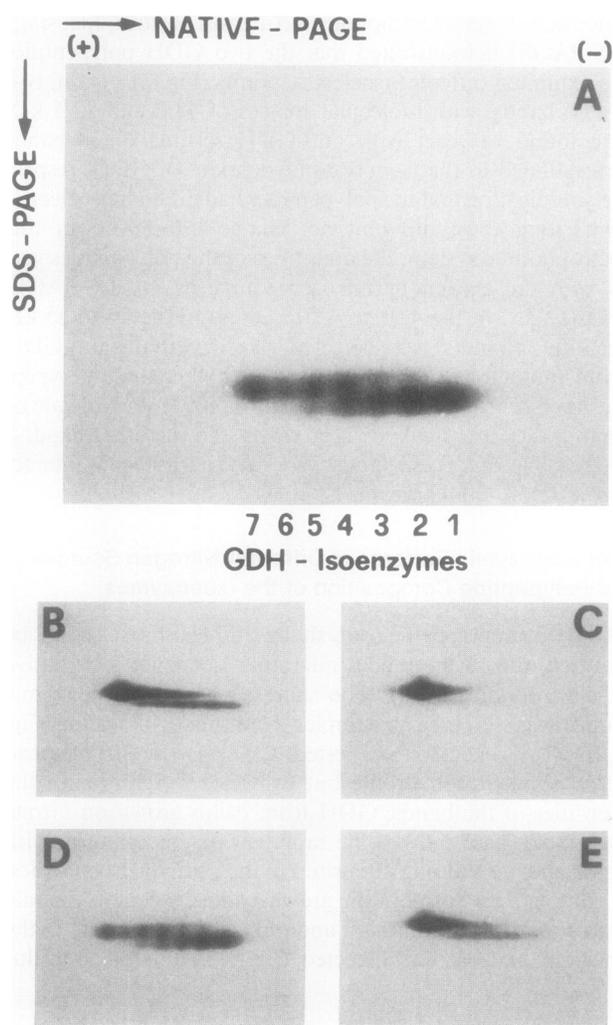
dimensional gel electrophoresis (urea-isoelectric focusing/SDS-PAGE) demonstrated that the two GDH polypeptides also exhibited different isoelectric points (Fig. 2). Again, two protein bands, with molecular masses of 43.0 and 42.5 kD, were found to react with anti-GDH serum. These bands corresponded to the  $\alpha$ - and  $\beta$ -polypeptides of GDH, respectively, indicating that the polypeptides had different isoelectric points and, thus, different net charge. Moreover, in this electrophoretic system, the quantities of the polypeptides varied with the exogenous nitrogen source as in SDS-PAGE. Occasionally, in the 42.5 to 43.0 kD and in the 30 to 35 kD area, minor band(s) were detected to react with the anti-GDH serum (not shown). It is not known whether these polypeptides represent unique molecules with a physiological role or whether they are breakdown products. On the other hand, it was possible that these bands were due to unspecific interaction with anti-GDH serum.

#### GDH Isoenzymic Patterns in Different Nitrogen Sources and Polypeptide Composition of the Isoenzymes

GDH from grapevine root, shoot, and leaf tissues could be analyzed into seven anodal migrating isoenzymes by native gel electrophoresis (10). The same seven band isoenzymic pattern was observed in samples from grapevine callus (Fig. 3). In all nitrogen sources tested, the seven isoenzymes had the same migration profile but exhibited differences in the intensities of the bands. GDH from callus grown on nitrate or glutamic acid showed the same isoenzymic pattern, with slightly greater staining intensities in the cathodal isoenzymes. In contrast, on ammonium grown callus, the more anodal isoenzyme was predominant and only a small quantity of the other isoenzymes was detected (Fig. 3). Finally, in callus



**Figure 3.** Effect of nitrogen source on the GDH isoenzymic pattern in grapevine shoot callus. Callus was grown on culture medium containing 20 mM  $\text{KNO}_3$ , as a nitrogen source, for three generations and then transferred for 15 d to culture media containing 20 mM  $\text{KNO}_3$  (a), 20 mM  $\text{KNO}_3$  plus 10 mM  $\text{NH}_4\text{Cl}$  (b), 10 mM  $\text{NH}_4\text{Cl}$  (c), 10 mM glutamate (d), or 10 mM glutamine (e). About 30 units of ammonium sulfate fractionated enzyme extracts were resolved in 5% acrylamide native gel electrophoresis. Gel was stained for GDH activity as described in "Materials and Methods."



**Figure 4.** Two-dimensional native/SDS-PAGE of glutamate dehydrogenase from shoot callus grown in different nitrogen sources. The samples were analyzed in the first dimension on 5% acrylamide native electrophoresis and in the second dimension on 7.5% acrylamide SDS gel electrophoresis. Proteins were transferred on nitrocellulose membranes and probed with anti-GDH serum. Enzyme extracts were from shoot callus grown in culture media containing as nitrogen source 20 mM  $\text{KNO}_3$  (A), 20 mM  $\text{KNO}_3$  plus 10 mM  $\text{NH}_4\text{Cl}$  (B), 10 mM  $\text{NH}_4\text{Cl}$  (C), 10 mM glutamate (D), and 10 mM glutamine (E).

grown in culture media containing nitrate plus ammonium or glutamine, a consistent dominance in the intensity of the enzyme activity staining of the anodal isoenzymes was observed.

To determine the participation of the  $\alpha$ - and  $\beta$ -polypeptides in the composition of each of the seven GDH isoenzymes, the isoenzymes were resolved by two-dimensional native/SDS-PAGE and immunodetected on protein blots (Fig. 4). The more anodal isoenzyme contained only  $\alpha$ -polypeptide, whereas the more cathodal one contained only  $\beta$ -type of GDH polypeptides. The remaining five isoenzymes, from the more cathodal to the more anodal one, consisted of both polypeptides; in fact, as  $\alpha$ -polypeptide increased, the  $\beta$ -polypeptide of GDH decreased. The same polypeptide analysis of the

isoenzymes was detected in all treatments of callus tested (Fig. 4A-E).

#### Relationship between GDH Activities and GDH Polypeptides in Different Nitrogen Sources and Polypeptide Composition of GDH Isoenzymes

A further relationship between *in vitro* NADH- and NAD-GDH activities and the relative intensities of the GDH-polypeptides as revealed by protein blot and silver stained electrophoresis was found. In Table II, a comparison was attempted between the percentage of NADH-GDH activity over the total GDH specific activity and the percentage of  $\alpha$ -polypeptide over the sum of the two polypeptides, as revealed by densitometric determination on protein blots and silver stained gels. In all treatments, a good correlation was found between *in vitro* activities and the polypeptide composition of the enzyme; NADH-GDH activity correlated with the  $\alpha$ -polypeptide and NAD-GDH activity with the  $\beta$ -polypeptide. Furthermore, a comparison between the densitometric data following protein blot and silver staining suggested that the prepared antibodies had the same affinity to both polypeptides.

In Table III, a quantitative analysis of samples from two different nitrogen treatments, nitrate and nitrate plus ammonium grown callus, is presented using densitometric data of the polypeptides  $\alpha$  and  $\beta$ , which participated in each of the seven GDH-isoenzymes. The quantity of polypeptides in each treatment was in good agreement with the results obtained by SDS-PAGE and protein blot (Table II). Comparison of one- and two-dimensional electrophoresis suggested that all the existing quantities of the two polypeptides participated in the holoenzyme.

Moreover, the polypeptide percentages for each one of the isoenzymes were calculated in order to reveal the polypeptide composition of the GDH isoenzymes (Table III). From these values, the proposed model of  $\alpha$ - and  $\beta$ -polypeptide participation in the seven isoenzymes was further proved; the isoenzymes 1 and 7 are homohexamers and the isoenzymes 2 through 6 are hybrids consisting of  $\alpha$ - and  $\beta$ -polypeptides at ratios of 1:5, 2:4, 3:3, 4:2, and 5:1, respectively. Similar results

**Table II.** Relationship between GDH Activities and GDH Subunit-Polypeptide Composition in Extracts from Grapevine Shoot Callus Grown on Different Nitrogen Sources

The percentage of NADH-GDH was calculated from the data in Table I and the percentage of  $\alpha$ -polypeptide was calculated from densitometric measurements of Western blots or silver stained 7.5% acrylamide SDS-gels. Each value is the average of three independent measurements.

Treatment	Percentage of NADH-GDH	Percentage of $\alpha$ -Subunit	
		Western blot	Silver stained gel
20 mM $\text{KNO}_3$	46	42	44
10 mM $\text{NH}_4\text{Cl}$	81	91	89
20 mM $\text{KNO}_3$ + 10 mM $\text{NH}_4\text{Cl}$	73	81	80
10 mM Glutamic acid	44	38	39
10 mM Glutamine	74	84	82

were obtained from all the remaining treatments tested (data not shown). Based on these ratios of  $\alpha$ - and  $\beta$ -polypeptides in the seven isoenzymes and on the frequency of each polypeptide in the two nitrogen sources ( $P_{\alpha}$ ,  $P_{\beta}$ , respectively, see Tables II, III), the expected proportions of the seven isoenzymes from the binomial distribution could be calculated (Table IV). The observed total GDH protein of each isoenzyme, as a sum of the polypeptides  $\alpha$  and  $\beta$ , was not in accordance with the expected proportions (Table IV); thus, it could be suggested that the two polypeptides were not randomly associated in the structure of the seven isoenzymes.

## DISCUSSION

The structure of GDH from animal and fungal systems has been extensively studied. In vertebrate liver and *Neurospora crassa*, the NAD-GDH consists of six and four identical subunits with molecular mass of 56 and 116 kD, respectively. With plant GDH, there is a general agreement that it is a metalloprotein with a molecular weight ranging from 208,000 to 270,000 (25); however, there is a discrepancy in the literature regarding the number and the characteristics of the subunits. For example, as six electrophoretic bands appeared after cross-linking, the enzyme was suggested to be a hexamer (8); in contrast, Scheid *et al.* (24), using SDS-PAGE and EM studies, have suggested that GDH from *Lemna* and *Pisum* seeds is a tetramer of four identical subunits with a molecular weight of 58,000. Furthermore, the isoenzymic profile of the enzyme varies with plant species as well as other nutritional and environmental conditions (for review see ref. 26). In legumes, it has been suggested that GDH isoenzymic pattern is the result of an adaptation of the cell to nitrogen metabolism, suggesting a physiological role of GDH isoenzymes in the regulation of nitrogen metabolism. Furthermore, a hypothesis has been proposed for the anabolic and catabolic role of GDH isoenzymes in plants (4, 5, 14), whereas studies of gene analysis have suggested that two nonallelic genes are responsible for the synthesis of two types of subunits that are

**Table IV.** Comparison between the Observed and the Expected from the Binomial Distribution Proportions of NAD(H)-GDH Isoenzymes Protein Content

Extracts from grapevine callus grown on culture media containing 20 mM KNO<sub>3</sub> or 20 mM KNO<sub>3</sub> plus 10 mM NH<sub>4</sub>Cl were used. The observed sum of polypeptide content ( $\alpha + \beta$ ) for each isoenzyme was calculated from data of Table III. The expected  $\alpha + \beta$  frequencies were calculated from the binomial distribution using  $P_{\alpha} = 0.42$ ,  $P_{\beta} = 0.58$  for nitrate-grown callus and  $P_{\alpha} = 0.81$ ,  $P_{\beta} = 0.19$  for nitrate plus ammonium grown callus.

Isoenzymes	Nitrate		Nitrate Plus Ammonia	
	Observed $\alpha + \beta$	Expected $\alpha + \beta$	Observed $\alpha + \beta$	Expected $\alpha + \beta$
1	19.0	3.8	3.2	0.005
2	19.7	16.5	4.5	0.1
3	13.7	30.0	5.2	1.3
4	16.3	29.0	6.6	7.3
5	12.6	15.7	8.8	23.3
6	6.9	4.5	15.6	39.7
7	11.8	0.5	56.1	28.2

arbitrarily associated in a hexameric complex giving rise to seven isoenzymes (1, 2, 20).

Previous reports from our laboratory have shown that GDH from leaf, root, and shoot tissues of *V. vinifera* L. consist of seven isoenzymes. The molecular mass of the native enzyme was 252 kD and consisted of 42.5 kD subunits (10). Moreover, following purification of the major GDH isoenzyme (which showed the lowest electrophoretic mobility) and antibody preparation, immunological studies revealed that GDHs from various plant organs were closely related proteins and confirmed that the enzyme was a hexamer consisting of 42.5 kD subunits, as revealed by SDS-PAGE with 10% or 10 to 18% gradient gels (11). However, when electrophoresis was performed in 7.5% polyacrylamide gels (this work), it was clear that GDH was composed of two polypeptides with small but distinct differences in both the mol wt and the charge (Figs.

**Table III.** Subunit-Polypeptide Composition of GDH-Isoenzymes in Grapevine Shoot Callus

Callus was grown on culture media containing 20 mM KNO<sub>3</sub> or 20 mM KNO<sub>3</sub> plus 10 mM NH<sub>4</sub>Cl, as nitrogen source. The polypeptide content of the isoenzymes was calculated by densitometry of the two-dimensional native/SDS polyacrylamide gels (Fig. 4). Subunit-polypeptide percentage designates the ratio of each polypeptide over the sum  $\alpha + \beta$  polypeptides in every isoenzyme. Values represent the means from three independent measurements of different samples.

Isoenzymes	Nitrate				Nitrate Plus Ammonia				Subunit Participation in Hexamer $\alpha:\beta$
	Subunit content		Subunit %		Subunit content		Subunit %		
	$\alpha$	$\beta$	$\alpha$	$\beta$	$\alpha$	$\beta$	$\alpha$	$\beta$	
1	0.0	19.0	0.0	100.0	0.0	3.2	0.0	100.0	0 6
2	3.2	16.5	16.2	83.8	0.8	3.7	17.7	82.3	1 5
3	4.5	9.2	32.8	67.2	1.8	3.4	34.5	65.5	2 4
4	8.3	8.0	50.9	49.1	3.4	3.2	51.5	48.5	3 3
5	8.5	4.1	67.5	32.5	5.8	3.0	66.0	34.0	4 2
6	5.7	1.2	82.6	17.4	13.1	2.5	84.0	16.0	5 1
7	11.8	0.0	100.0	0.0	56.1	0.0	100.0	0.0	6 0
Total	42.0	58.0			81.0	19.0			

1, 2). Thus, the hypothesis of Cammaerts and Jacobs (1, 2) and Ratajczak *et al.* (22) that GDH is composed of two subunits is supported. Furthermore, by using two-dimensional native/SDS-PAGE it was confirmed that the two polypeptides are associated in an ordered ratio so that the isoenzymes 1 and 7 are composed of polypeptides  $\beta$  and  $\alpha$ , respectively, and the isoenzymes 2 through 6 (from the less to the more electrophoretically mobile) are hybrids consisting of  $\alpha$ - and  $\beta$ -polypeptides at ratios of 1:5, 2:4, 3:3, 4:2, and 5:1, respectively (Fig. 4, Table III).

Studies with *in vitro* plants grown on culture media containing only nitrates or only ammonium or a combination of both as nitrogen sources showed that ammonium resulted in an increase in NADH-GDH activity in leaf, shoot, and root enzyme (11). A comparable effect of ammonium on NADH-GDH activity was observed in the callus system used in this study. On the other hand, the activity of the catabolic reaction decreased in the presence of ammonium (Table I). Studies of the isoenzymic patterns of GDH revealed that, in the presence of ammonium or glutamine in the culture medium, isoenzyme 7 predominated, whereas isoenzyme 1 was almost absent; in the presence of only nitrates or glutamic acid, all the isoenzymes were present, with a slight predominance of the cathodal isoenzymes (Fig. 3). This effect of ammonium on isoenzymic pattern reflected an increase in polypeptide  $\alpha$  as revealed by SDS-PAGE and two-dimensional electrophoresis (Figs. 1 and 4, respectively). Moreover, comparison of GDH activities and the polypeptide content showed a correlation of the catabolic reaction with the  $\beta$ -polypeptide and of the synthetic reaction with the  $\alpha$ -polypeptide (Table II), indicating that isoenzyme 1 has more catabolic function, whereas isoenzyme 7 has more anabolic function toward amination of  $\alpha$ -ketoglutaric acid; the isoenzymes 2 through 6 exhibited activities that correlated to their  $\alpha$ - and  $\beta$ -polypeptide composition. Further work using *in vivo* labeling of proteins has shown that the increase in the intensity of the more anodal isoenzymes in the presence of ammonium was due to *de novo* synthesis of the  $\alpha$ -polypeptide (our manuscript in preparation). Therefore, we suggest that ammonium does not cause activation of GDH, as has been proposed (26), but that it induces expression of polypeptide  $\alpha$ , and therefore the regulation of GDH is exerted by differential expression of the respective isoenzymes.

In conclusion, data presented in this report give some answer to hypotheses, which have appeared in the literature, on the possible structure of plant NAD(H)-GDH by showing its two subunit-polypeptides ordered participation to the hexameric structure of the enzyme with varying coenzyme specificity for each isoenzyme. The existence of two nonallelic genes that control the synthesis of GDH subunits in *Arabidopsis thaliana* has been suggested based on genetic analysis data (1). However, one could not rule out the possibility that the difference in mol wt and charge, which cause different reactivity of the polypeptides when they associate to hexamers, may arise from differential posttranscriptional or post-translational modification of a single gene's product; *e.g.* the difference could represent specific proteolytic cleavage (of about five amino acids) which could change the electrophoretic behavior and reactivity or a small prosthetic group (*e.g.* phosphate). Thus, further work is required before being able

to definitely characterize the two polypeptides as one- or two-gene products. As a first approach for answering this point, we have in progress work for the sequencing of the two polypeptides.

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#### LITERATURE CITED

1. Cammaerts D, Jacobs M (1983) A study of the polymorphism and the genetic control of the glutamate dehydrogenase isoenzymes in *Arabidopsis thaliana*. *Plant Sci Lett* **31**: 65-73
2. Cammaerts D, Jacobs M (1985) A study of the role of glutamate dehydrogenase in the nitrogen metabolism of *Arabidopsis thaliana*. *Planta* **163**: 517-526
3. Davis BJ (1964) Disc electrophoresis II. Method and application to human serum proteins. *Ann NY Acad Sci* **121**: 404-427
4. Hartman T (1973) Endogenously and exogenously caused alteration of the isoenzyme pattern of NADH-specific glutamic dehydrogenase in shoots of *Pisum sativum*. *Planta* **111**: 129-136
5. Hartman T, Nagel M, Ilert HJ (1973) Organ specific multiple forms of glutamic dehydrogenase in *Medicago sativa*. *Planta* **111**: 119-128
6. Itagaki T, Dry IB, Wiskich JT (1988) Purification and properties of NAD-glutamate dehydrogenase from turnip mitochondria. *Phytochemistry* **27**: 3373-3378
7. Kanellis AK, Solomos T, Mehta AM (1989) Decreased cellulose activity in avocado fruit subjected to 2.5% O<sub>2</sub> correlates with lower cellulase protein and gene transcript levels. *Plant Cell Physiol* **30**: 817-823
8. Kindt R, Pahllich E, Asched I (1980) Immunochemical investigations with highly purified glutamate dehydrogenase from pea seeds by means of the Ouchterlony test. *J Exp Bot* **31**: 419-423
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680-685
10. Loulakakis KA, Roubelakis-Angelakis KA (1990) Intracellular localization and properties of NADH-glutamate dehydrogenase from *Vitis vinifera* L. Purification and characterization of the major leaf isoenzyme. *J Exp Bot* **41**: 1223-1230
11. Loulakakis KA, Roubelakis-Angelakis KA (1990) Immunocharacterization of NADH-glutamate dehydrogenase from *Vitis vinifera* L. *Plant Physiol* **94**: 109-113
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275
13. Loyola-Vargas VM, Yanez A, Caldera J, Oropeza C, Robert ML, Quiroz J, Scorer KN (1988) Nitrogen metabolism in *Canavalia ensiformis* L. DC. II. Changing activities of nitrogen assimilating enzymes during growth. *J Plant Physiol* **132**: 289-293
14. Mazurkova H, Ratajczak W, Ratajczak L (1980) Glutamate dehydrogenase characteristics in the organs and root nodules of *Lupinus luteus* L. *Acta Physiol Plant* **2**: 167-177
15. Mifflin BJ, Lea PJ (1980) Ammonia assimilation. In PK Stumpf, E Conn, eds, *The Biochemistry of Plants*, Vol 5. New York, Academic Press, pp 169-203
16. Munoz-Blanco J, Cardenas J (1989) Changes in glutamate dehydrogenase activity of *Chlamydomonas reinhardtii* under different trophic and stress conditions. *Plant Cell Environ* **12**: 173-182
17. Murashige I, Skoog F (1962) Revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant* **14**: 473-497
18. Nagel M, Hartman T (1980) Glutamate dehydrogenase from

- Medicago sativa* L. Purification and comparative kinetic studies of organ-specific multiple forms. *Z Naturforsch* **35**: 406–415
19. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**: 4007–4021
  20. Pahlich E (1972) Evidence that the multiple molecular forms of glutamate dehydrogenase from pea seedlings are conformers. *Planta* **104**: 78–88
  21. Pahlich E, Joy KW (1971) Glutamate dehydrogenase from pea roots: purification and properties of the enzyme. *Can J Biochem* **49**: 127–138
  22. Ratajczak L, Koroniak D, Mazurowa H, Ratajczak W, Prus-Glowacki W (1986) Glutamate dehydrogenase isoforms in lupine roots and root nodules. Immunological studies. *Physiol Plant* **67**: 685–689
  23. Roubelakis-Angelakis KA, Zivanovitch SA (1991) A new culture medium for *in vitro* rhizogenesis of grapevine (*Vitis* spp.) genotypes. *HortScience* (in press)
  24. Scheid HW, Ehmke A, Hartman T (1980) Plant NAD-dependent glutamate dehydrogenase. Purification, molecular properties and metal ion activation of the enzymes from *Lemna minor* and *Pisum sativum*. *Z Naturforsch* **35**: 213–221
  25. Singh RP, Srivastava HS (1982) Glutamate dehydrogenase activity and assimilation of inorganic nitrogen in maize seedlings. *Biochem Physiol Pflanzen* **177**: 633–642
  26. Srivastava HS, Singh RP (1987) Role and regulation of L-glutamate dehydrogenase activity in higher plants. *Phytochemistry* **26**: 597–610
  27. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Nat Acad Sci USA* **76**: 4350–4354
  28. Wray W, Boulicas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. *Anal Biochem* **118**: 197–203
  29. Yamaya T, Oaks A, Matsumoto H (1984) Characteristics of glutamate dehydrogenase in mitochondria prepared from corn shoots. *Plant Physiol* **76**: 1009–1013