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# Characterization and Expression of NAD(H)-Dependent Glutamate Dehydrogenase Genes in Arabidopsis

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**Two distinct cDNA clones encoding NAD(H)-dependent glutamate dehydrogenase (NAD[H]-GDH) in *Arabidopsis thaliana* were identified and sequenced. The genes corresponding to these cDNA clones were designated *GDH1* and *GDH2*. Analysis of the deduced amino acid sequences suggest that both gene products contain putative mitochondrial transit polypeptides and NAD(H)- and α-ketoglutarate-binding domains. Subcellular fractionation confirmed the mitochondrial location of the NAD(H)-GDH isoenzymes. In addition, a putative EF-hand loop, shown to be associated with $Ca^{2+}$ binding, was identified in the *GDH2* gene product but not in the *GDH1* gene product. *GDH1* encodes a 43.0-kD polypeptide, designated α, and *GDH2* encodes a 42.5-kD polypeptide, designated β. The two subunits combine in different ratios to form seven NAD(H)-GDH isoenzymes. The slowest-migrating isoenzyme in a native gel, GDH1, is a homohexamer composed of α subunits, and the fastest-migrating isoenzyme, GDH7, is a homohexamer composed of β subunits. GDH isoenzymes 2 through 6 are heterohexamers composed of different ratios of α and β subunits. NAD(H)-GDH isoenzyme patterns varied among different plant organs and in leaves of plants irrigated with different nitrogen sources or subjected to darkness for 4 d. Conversely, there were little or no measurable changes in isoenzyme patterns in roots of plants treated with different nitrogen sources. In most instances, changes in isoenzyme patterns were correlated with relative differences in the level of α and β subunits. Likewise, the relative difference in the level of α or β subunits was correlated with changes in the level of *GDH1* or *GDH2* transcript detected in each sample, suggesting that NAD(H)-GDH activity is controlled at least in part at the transcriptional level.**

GDH (EC 1.4.1.2) catalyzes a reversible reaction for the reductive amination of α-ketoglutarate to form Glu in the presence of the cofactor NAD(P)H. The enzyme has been physically and biochemically characterized in several plant species (for review, see Stewart et al., 1980; Srivastava and Singh, 1987). GDH has numerous isoenzymic forms that are localized in chloroplasts and mitochondria. NAD(H)-GDH isoenzymes are localized in the mitochondria, whereas NADP(H)-GDH isoenzymes are associated with chloroplasts. The number of distinct isoenzymes can vary in plant tissues during development and under different environmental conditions. NAD(H)-GDH isoenzymes have relative molecular masses ranging from 208 to 270 kD, each composed of six subunits of approximately 42 to 45 kD. Despite an extensive knowledge of the physical and biochemical characteristics of GDH, the physiological role of the enzyme has not yet been established. The lack of understanding of the physiological role of GDH in plants can be attributed to several factors, including: (a) increased interest in the enzymes GS and GOGAT due to the discovery of GOGAT in plants (Lea and Miflin, 1974); (b) apparent contradictions among results from different investigations; and (c) until recently, the lack of molecular tools (antibodies and cDNA probes) for analyses of GDH.

Prior to the isolation of GOGAT in plant leaves (Lea and Miflin, 1974), GDH was believed to be the primary route of ammonia assimilation in plants. However, biochemical, molecular, and genetic studies have shown that the enzymes GS and GOGAT function as the primary route for ammonia assimilation in plants (Miflin and Lea, 1980). Furthermore, molecular studies have shown that under photorespiratory conditions the gene encoding the chloroplast isoenzyme of GS was induced 4-fold (Edwards and Coruzzi, 1989). These results support the earlier findings of Wallsgrove et al. (1987), which suggested that GS/GOGAT played a key role in the reassimilation of ammonia released during photorespiration. However, GDH may play a complementary role to GS/GOGAT in the reassimilation of excess ammonia released during stress conditions or during specific stages of development (Yamaya et al., 1986; Rhodes et al., 1989; for review, see Stewart et al., 1980; Srivastava and Singh, 1987).

Several approaches have been used to determine the role of GDH in nitrogen metabolism. In one study, a GDH1 null mutant of *Zea mays* was fed $^{15}N$-labeled compounds (Magalhaes et al., 1990). Root samples of the GDH1 mutant had a 10- to 15-fold reduction in GDH activity and a 40 to 50% decrease in the rate of $^{15}NH_4^+$ assimilation into reduced nitrogen compared with root samples from the wild type. It appeared that the different rates of nitrogen assimilation between the mutant and wild-type plants could be attributed to variations in GDH activity. However, the authors cautioned against such an interpretation because no differences were observed between the wild type and mutants

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Abbreviations: Cat, catalase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, Gln synthetase; NAD(H)-GDH, NAD(H)-dependent glutamate dehydrogenase; NADP(H)-GDH, NADP(H)-dependent GDH.

treated with a GS inhibitor. The authors suggested that there could be other factors such as differences in shoot: root ratios that could have contributed to the different rates of nitrogen assimilation. Data from feeding studies using grape calli also suggest that NAD(H)-GDH may play a role in nitrogen metabolism. Native PAGE analyses of protein extracts from grape calli treated with ammonia or Gln contained NAD(H)-GDH isoenzymes of high electrophoretic mobility (Loulakakis and Roubelakis-Angelakis, 1991). Two-dimensional gel electrophoresis of these samples revealed that they were composed of a single type of subunit designated $\alpha$. The authors suggested that the elevated levels of ammonia or Gln favored the metabolism of Glu (amination reaction) and the synthesis of the $\alpha$ subunit. Conversely, GDH isoenzymes with low electrophoretic mobility in native polyacrylamide gels were abundant in protein extracts from grape calli treated with Glu or nitrate, conditions that favored the catabolic (deamination) reaction and the synthesis of the $\beta$ subunit.

However, the most recent data from studies in grape suggest that the in vitro metabolic and catabolic activities of each of the seven isoenzymes are similar (Loulakakis and Roubelakis-Angelakis, 1996); thus, it is unclear how a particular isoenzyme could control metabolic or catabolic NAD(H)-GDH activity. Melo-Oliveira et al. (1996) used molecular biological techniques and characterized an *Arabidopsis thaliana* GDH1 mutant (*gdh1-1*) to demonstrate that GDH1 may play a unique role in ammonia assimilation under conditions of excess inorganic nitrogen. The gene product for *GDH1* may be involved in Glu synthesis under conditions of carbon and ammonia excess, but it may be involved in Glu catabolism under carbon-limiting conditions.

Results from studies of GDH in carrot cell suspension cultures grown in limiting amounts of carbon also suggest that GDH is involved in the catabolism of Glu to form $\alpha$-ketoglutarate (Robinson et al., 1991, 1992). GDH activity increased in Suc-starved cells, and the addition of Suc resulted in a rapid decrease in GDH activity. The derepression of GDH was positively correlated with Glu concentrations. Similar findings were reported with tobacco calli. Samples from carbon-starved calli had elevated specific activities of GDH, and GDH isoenzymes with low electrophoretic mobility were observed in these samples by native PAGE (Masteri et al., 1991). These data suggest that the oxidation of Glu provides carbon skeletons for the TCA cycle and for energy production.

Although recent findings in Arabidopsis demonstrated that the *GDH1* gene product may play a dual role in the metabolism and catabolism of Glu, which are triggered by specific environmental stimuli (excess nitrogen and darkness), there are some apparent contradictions in the literature and several unknown factors, i.e. spatial and/or temporal regulation, that may control Glu metabolism and catabolism by GDH isoenzymes. However, as suggested by Maestri et al. (1991), some of the apparent contradictions and uncertainties can be explained in one of several ways: (a) the existence of multiple loci or genes encoding GDH subunits, as shown for Arabidopsis (Cammaerts and Jacobs, 1983) and maize (Pryor, 1979; Goodman et al., 1980; Sakakibara et al., 1995); (b) different intracellular localization of GDH isoenzymes (for review, see Stewart et al., 1980; Srivastava and Singh, 1987); or (c) posttranslational modifications as reported for GDH in *Candida* species (Hemmings, 1980). To gain a greater understanding of the structure and function of NAD(H)-GDH and the role of its isoenzymes in plant nitrogen and/or carbon metabolism, we initiated a study of the isoenzymes and genes in *A. thaliana*. In this study we combined molecular biological approaches with traditional isoenzyme analyses and immunological techniques to identify the cDNA clones and the protein/polypeptide components they encode. We also identified some environmental factors that altered NAD(H)-GDH isoenzyme patterns and gene expression in *A. thaliana*.

## MATERIALS AND METHODS

*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) seeds were obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus). Plants were grown in (6- × 20- × 10-cm [height × width × depth]) plastic pots either in a peat-vermiculite mix (Jiffy mix, Jiffy Products of America, Inc., Batavia, IL)[1] or in vermiculite. Plants grown in soil were watered as needed by subirrigation throughout the experiment. Plants grown in vermiculite were subirrigated with a complete mineral nutrient solution with a different sole nitrogen source. The composition of the mineral nutrient solution with nitrate (10 mM $KNO_3$) was the same as that described by Cammaerts and Jacobs (1985). After 15 d, pots containing vermiculite were flushed daily for 5 d with 25 mL of sterile, distilled water, and were then treated as described below for 4 d. Plants were irrigated daily with 25 mL of the mineral nutrient solution supplemented with either 10 mM $KNO_3$, 10 mM $NH_4Cl$, 5 mM $NH_4NO_3$, 5 mM Glu, 5 mM Gln, or 10 mM $KNO_3$ plus 3% Suc. The supplemented mineral nutrient solutions were filter-sterilized to control bacterial and fungal contamination.

On d 24, leaf and root samples were collected for protein and RNA extractions. Proteins were extracted from fresh tissue or from tissue frozen in liquid nitrogen and stored at −80°C. RNA was extracted from tissue frozen in liquid nitrogen and stored at −80°C. Similar experiments were conducted in the dark with plants grown in mineral nutrient solution plus 10 mM $KNO_3$ or 10 mM $KNO_3$ plus 3% Suc. Plants were grown under identical conditions, as described above, for 20 d and then placed in the darkness for 4 d prior to sample collection. Plants were maintained at 20 to 21°C, 60 to 70% RH under cool-white lights (120–140 $\mu$mol PPFD $m^{-2}$ $s^{-1}$) in a 16-h light/8-h dark cycle. The experiments to determine the effects of supplemented nitrogen and carbon, described above, were also performed without flushing the pots with water for 5 d. Instead, the

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

plants received the complete mineral nutrient solution with 10 mM $KNO_3$ as the sole nitrogen source for 20 d prior to initiating the treatments. The change in the protocol did not significantly alter the results (data not shown).

### Crude Protein Extractions

Proteins were extracted from frozen or fresh samples. Frozen samples (approximately 500 mg) were ground to a fine powder with a mortar and pestle in liquid nitrogen. The powder was transferred to 1 mL of extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5% [v/v] glycerol, 0.05% [v/v] Triton X-100, and 0.5% [w/v] PVP-40). Fresh PMSF was added to a final concentration of 1 mM in all extracts. The fresh samples (200 mg) were ground in 400 μL of extraction buffer as described above. The samples were incubated on ice for 15 to 30 min. Debris was removed from the sample by centrifugation at 13,000*g* for 10 min.

### Protein Determinations and Enzyme Assays

Protein concentrations were determined using a Bio-Rad or Pierce protein assay kit. The amination reaction was used routinely to determine activity and the amount of GDH loaded onto gels as described by Turano et al. (1996).

### Gel Electrophoresis, Gel-Staining Procedures, and Immunoblot Analysis

Proteins were separated by native-PAGE or SDS-PAGE using a Mini-Protean II system (Bio-Rad). For native-PAGE and SDS-PAGE, an equal amount of NADH-GDH activity (0.250 unit) was added per lane. To identify different GDH isoenzymes, proteins were separated in a native 5% polyacrylamide gel and stained for NAD-GDH activity as described by Turano et al. (1996). Identical gels were incubated in the GDH stain solution minus Glu as negative controls. Immunoblot analysis was conducted as described by Turano et al. (1990); proteins were resolved in a SDS-polyacrylamide (8.0%) gel (Laemmli, 1970). Rabbit serum raised against grape leaf NAD(H)-GDH was kindly provided by Loulakakis and Roubelakis-Angelakis (1990b).

### Subcellular Localization

Tissue, mainly leaves and some flower stalks, was collected from 24-d-old plants. The plant material was diced with a mechanical device equipped with 10 razor blades 2.5 mm apart in a Plexiglas trough chilled to 4°C for 10 min. Cold extraction buffer containing 50 mM Tes, pH 7.2, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM DDT, 0.4 M mannitol, 0.5% (w/v) PVP-40, 4 mM Cys, and 0.2% BSA was used to suspend the diced plant material at a ratio of 1:1.5 (w/v). Fresh PMSF was added to a final concentration of 1 mM. Cellular debris was removed by filtration through four layers of cheesecloth and one layer of Miracloth (Calbiochem), followed by centrifugation at 50*g* for 15 min. Chloroplasts were removed from the supernatant by centrifugation at 1500*g* for 30 min. The supernatant was used for mitochondria and peroxisome isolation. Chloroplasts in the 1500*g* pellet were resuspended in 8 mL of resuspension solution (50 mM Tes, pH 7.2, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM DDT, 0.4 M mannitol, and 0.2% BSA) and loaded onto a discontinuous Suc gradient (25, 34, and 51%). After centrifugation at 2000*g* for 20 min at 4°C, the chloroplast band at the 34/51% Suc interface was collected. The chloroplast suspension was diluted with 2 volumes of resuspension solution minus BSA. Chloroplasts were concentrated by centrifugation at 5000*g* for 30 min.

Pure chloroplasts were resuspended in 1 mL of resuspension solution. Mitochondria and peroxisomes in the supernatant of the 1,500*g* centrifugation described above were concentrated by centrifugation at 18,000*g* for 30 min. The mitochondrial/peroxisomal pellets were resuspended in 6.5 mL of resuspension solution and loaded onto a discontinuous Percoll (21, 26, 32, 47, and 60%) gradient. After centrifugation at 45,000*g* for 45 min at 4°C, the mitochondrial and peroxisomal bands were collected from the 32/47% and 47/60% Percoll interfaces, respectively. The peroxisomal suspension was diluted and concentrated as described above, except that the final centrifugation was at 22,000*g* for 30 min. The mitochondrial band was diluted with 2 volumes of the resuspension solution, loaded onto a second discontinuous Percoll gradient (26, 32, and 47%), and centrifuged as described above. The mitochondrial band was collected and concentrated by centrifugation, and the pure mitochondria were resuspended as described for the peroxisomes. The organelle preparations were assayed for NADH-GDH activity and specific organelle markers. Chlorophyll was determined as described by Bruinsma (1961). The mitochondrial marker enzyme fumarase and the peroxisomal/glyoxysomal marker enzyme Cat were assayed as described by Hill and Bradshaw (1969) and Beers and Sizer (1952), respectively.

### Arabidopsis cDNA Library, Clone Identification, and DNA Sequence Analysis

An Arabidopsis ecotype Columbia (Col-0) cDNA library, λPRL2 (Newman et al., 1994), and the expressed sequence tag clones 137O24T7 (T46178) and 188I23T7 (H37750) were obtained from the Arabidopsis Biological Resource Center. Rabbit serum raised against grape leaf NAD(H)-GDH (Loulakakis and Roubelakis-Angelakis, 1990b) was used to screen the cDNA expression library as described by Young and Davis (1983). Putative GDH clones were sequenced by the dideoxy chain-termination method using modified T7 DNA polymerase (Sequenase 2.0, United States Biochemical) as described by the manufacturer. The data were analyzed with the IntelliGenetics program (IntelliGenetics, Inc., Mountain View, CA) on a Sun system.

### RNA Isolation and Gel Electrophoresis

For the determination of organ-specific expression, total RNA was isolated from roots, leaves, flower stalks, flowers, and siliques of 42-d-old Arabidopsis plants. To determine the effects of nitrogen or darkness on NAD(H)-GDH, total RNA was isolated from roots (in the nitrogen study only) and leaves of 24-d-old plants. Total RNA was isolated from each organ, separated by gel electrophoresis using

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                                    aatactcaattctttcttcttcaattcatcacaacaaatcaaaaactccaagttcttacctttttcccatttacaatcagtaaatttctccgatcacg
agatacaaatttcactttctgatatcttggatcttccgatcactttgaatttttggtttgttgatttttttttttttttttgttccataaagttcgaatttttatagagaagaaaacc

atgaatgctttagctgcaacaaacagaaacttccgtcatgcatctcgaatcctgggtttggattcgaagatcgagagaagtcttatgatcccatttagagaaatcaaggttgagtgtacg
METAsnAlaLeuAlaAlaThrAsnArgAsnPheArgHisAlaSerArgIleLeuGlyLeuAspSerLysIleGluArgSerLeuMETIleProPheArgGluIleLysValGluCysThr 40

attcctaaagacgatggcactctggtttcatacatcggatttagggttcaacatgacaatgctcgtggacccatgaaaggtggaatcagatatcatcctgaggttgatccagatgaagtt
IleProLysAspAspGlyThrLeuValSerTyrIleGlyPheArgValGlnHisAspAsnAlaArgGlyProMETLysGlyGlyIleArgTyrHisProGluValAspProAspGluVal 80

aacgcactagctcagctgatgacttggaagactgctgtagcagatattccatatggtggtgctaaaggtggaattggatgtagtcctcgtgacttgagtttgagcgagcttgagaggttg
AsnAlaLeuAlaGlnLeuMETThrTrpLysThrAlaValAlaAspIleProTyrGlyGlyAlaLysGlyGlyIleGlyCysSerProArgAspLeuSerLeuSerGluLeuGluArgLeu 120

actcgtgtgtttacacagaagattcatgatcttatcggtattcataccgatgtgcctgctcctgatatgggcactaacgctcaaaccatggcttggattcttgatgagtactccaagttt
ThrArgValPheThrGlnLysIleHisAspLeuIleGlyIleHisThrAspValProAlaProAspMETGlyThrAsnAlaGlnThrMETAlaTrpIleLeuAspGluTyrSerLysPhe 160

catggtcattcccctgctgttgtcactggaaagcccattgatcttggtggttcattaggtagggaagctgccacaggtcgtggtgtagtatttgccaccgaagctcttcttgctgagtac
HisGlyHisSerProAlaValValThrGlyLysProIleAspLeuGlyGlySerLeuGlyArgGluAlaAlaThrGlyArgGlyValValPheAlaThrGluAlaLeuLeuAlaGluTyr 200

gggaaatcgattcagggtttgacatttgttattcagggttttgggaatgttggaacatgggctgcgaagctgatccacgagaaaggcggtaaagtggttgcagtaagcgacattactggt
GlyLysSerIleGlnGlyLeuThrPheValIleGlnGlyPheGlyAsnValGlyThrTrpAlaAlaLysLeuIleHisGluLysGlyGlyLysValValAlaValSerAspIleThrGly 240

gcaatcaggaaccctgaaggtatcgacatcaacgctctcataaaacacaaggacgcaactggaagtctcaatgatttcaatggtggagacgctatgaattcagatgaattgctcattcat
AlaIleArgAsnProGluGlyIleAspIleAsnAlaLeuIleLysHisLysAspAlaThrGlySerLeuAsnAspPheAsnGlyGlyAspAlaMETAsnSerAspGluLeuLeuIleHis 280

gagtgtgatgttctcattccatgcgctcttggtggtgtcctgaacaaggaaaatgctggagatgtgaaggcaaagtttatagtagaggcagctaaccatccaacagatccagatgctgat
GluCysAspValLeuIleProCysAlaLeuGlyGlyValLeuAsnLysGluAsnAlaGlyAspValLysAlaLysPheIleValGluAlaAlaAsnHisProThrAspProAspAlaAsp 320

gagattctgtcgaagaaaggagtgattatacttccagatatctacgcaaacgcaggaggagtgacagtgagttacttcgagtgggtgcagaacattcaaggattcatgtgggaagaggaa
GluIleLeuSerLysLysGlyValIleIleLeuProAspIleTyrAlaAsnAlaGlyGlyValThrValSerTyrPheGluTrpValGlnAsnIleGlnGlyPheMETTrpGluGluGlu 360

aaagtgaacttggagctgcaaaaatacatgactcgagcctttcacaacatcaagacaatgtgccatactcattcttgcaacctccgtatgggagctttcactcttggagttaaccgagtc
LysValAsnLeuGluLeuGlnLysTyrMETThrArgAlaPheHisAsnIleLysThrMETCysHisThrHisSerCysAsnLeuArgMETGlyAlaPheThrLeuGlyValAsnArgVal 400

gctcgagccacccagttgcgtggttgggaagcttaattcagttgaatccccttatatactctgtttcttgcttgctctgttttccatttttttttttttggtttcattgttactgaaaa
AlaArgAlaThrGlnLeuArgGlyTrpGluAlaxxx

tcattgagctttccgaaatggaaaaggaaacaaatggattgaataaaaactaacaaattggaataaaaaaaaaaaaaaaaaa
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**Figure 1.** The nucleotide sequence of the full-length cDNA clone corresponding to *GDH2* is shown in the sense strand. Nucleotides are numbered from the putative start codon.

formaldehyde-formamide gels, and blotted onto nitrocellulose as described by Turano et al. (1992).

### Amplification and Radioactive Labeling of GDH Probes and Hybridization

Individual Arabidopsis cDNA clones (full-length) were used as a template in a PCR using a gene amplification kit (Perkin-Elmer) and M13-forward and M13-reverse primers. Gene amplification reactions were conducted as follows: 94°C for 30 s, 45°C for 30 s, and 72°C for 2 min, for 40 cycles. The amplified DNA fragments were gel-purified, amplified a second time, and used as gene-specific probes for RNA blot analyses. Full-length cDNA probes were radioactively labeled with [$\alpha$-$^{32}$P]dCTP (New England Nuclear) using the random-primer method (Pharmacia). The gene-specific probes were hybridized against RNA blots. The filters were prehybridized in 430 mM $NaPO_4$, pH 7.2, 7% SDS, 0.5% BSA, and 20 mM EDTA at 50°C for 1 to 4 h. Labeled probe was added and the filters were hybridized at 50°C for 18 h. Final washes were in 0.2× SSC and 0.1% SDS at 55°C. A probe encoding for the 26S rRNA gene (F.J. Turano, unpublished data) was used as an internal control on RNA blots containing total RNA to ensure equal loading of RNA per lane. Data were quantified on a beta scanner (Betagen, IntelliGenetics, Mountain View, CA).

## RESULTS

### Clone Identification and Sequence Analyses

Two full-length cDNA clones were identified and sequenced. The initial clone, *GDH1*, was a single clone identified from a screen of $3.0 \times 10^5$ plaques from an Arabidopsis cDNA expression library using immunodetection with rabbit antiserum against grape NAD(H)-GDH. The cDNA clone is 1539 bp long and encodes a 411-amino acid peptide. This clone was nearly identical to the cDNA clone encoding *GDH1* described by Melo-Oliveira et al. (1996). There are two differences among the sequences, a G to A transition at nucleotide 13 and an additional 23 nucleotides prior to the poly(A) tail in the GDH1 sequence reported here (data not shown). The other cDNA clone, designated GDH2, was identified in the expressed sequence tag databank by its homology with GDH1. The cDNA clone was obtained from the Arabidopsis Biological Resource Center and sequenced in both directions. The cDNA clone for GDH2 is 1620 bp long and encodes a 411-amino acid peptide (Fig. 1). The nucleotide identity between GDH1 and

GDH2 is 31% in the 5′ noncoding region, 75% in the coding region, and 46% in the 3′ noncoding region. The amino acid identity between the two Arabidopsis clones is 81%. The deduced amino acid sequences of the two Arabidopsis NAD(H)-GDH cDNA clones were aligned with the deduced amino acid sequences of grape (Syntichaki et al., 1996) and maize (Sakakibara et al., 1995) NAD(H)-GDH cDNA clones (Fig. 2). GDH1 has 79 and 84% amino acid identity with the grape and maize NAD(H)-GDH sequences, respectively. GDH2 has 86 and 81% amino acid identity with the grape and maize NAD(H)-GDH sequences, respectively. Both Arabidopsis peptides appear to contain putative mitochondrial target sequences. The properties of mitochondrial target sequences as described by Hartl et al. (1989) are: (a) they are rich in positively charged

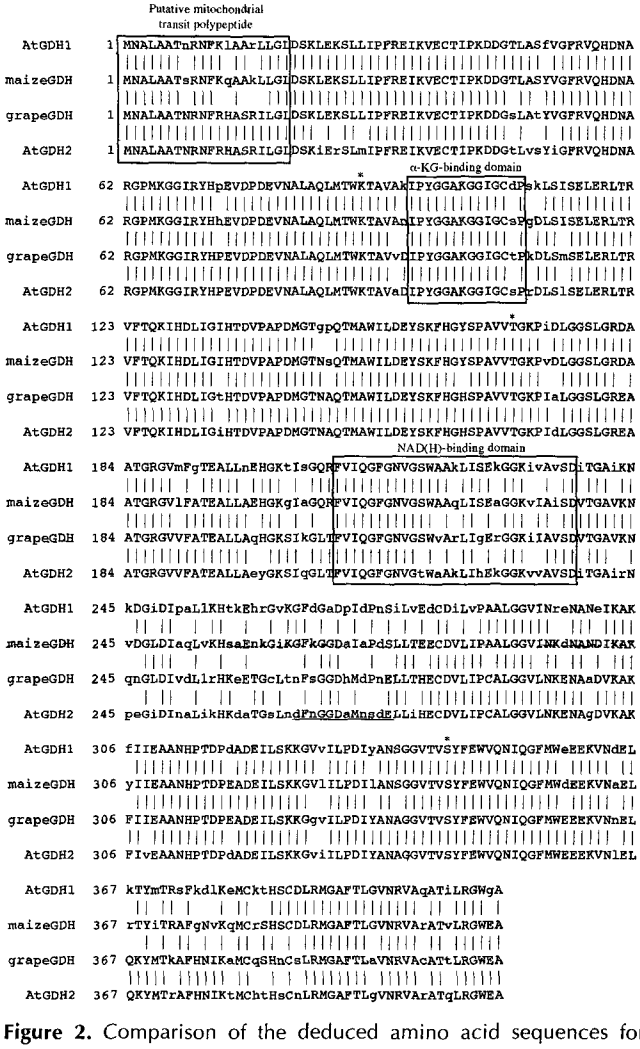


**Figure 2.** Comparison of the deduced amino acid sequences for GDH1 (AtGDH1) and GDH2 (AtGDH2) with NAD(H)-GDH for maize (Sakakibara et al., 1995; accession no. D49475) and grape (Syntichaki et al., 1996; accession no. S54797). The mitochondrial transit sequence, α-ketoglutarate (α-KG)-binding domain, and NAD(H)-binding domain are labeled and boxed. Amino acid residues associated with Glu binding are indicated with an asterisk above. The region homologous to an EF-hand motif in the *GDH2* gene product is underlined.

amino acids; (b) they generally lack acidic amino acids; (c) in most cases they have several hydroxylated amino acids; and (d) they can fold into an amphiphilic α helix. The first 20 amino acids of the N termini of GDH1 and GDH2 fit these criteria well. In GDH1 there are 3 positively charged amino acid residues (1 Lys and 2 Arg), no acidic residues, and 1 hydroxylated residue (Thr) in the N-terminal region. In addition, the α helix formed by the N-terminal 20 residues has positively charged amino acids on one face and hydrophobic residues on the other. These features are also preserved in GDH2. There is a conservative change of Lys-12 to Arg and the addition of 1 Ser at position 15. In both cases the sequence RXLG at positions 16 to 19 could fit the conserved sequence motif for cleavage of the target peptide between residues 25 and 26 (Gavel and von Heijne, 1990; von Heijne, 1992).

Several regions and amino acids associated with metabolic function of NAD(H)-GDH are conserved among the amino acid sequences deduced from plant cDNA clones. As discussed by Sakakibara et al. (1995) and Melo-Oliveira et al. (1996), the regions include the putative α-ketoglutarate-binding domain from Ile-96 to Pro-109, which includes the Lys-102 that has been shown to be essential for enzyme function in *Clostridium* (Lilly and Engel, 1992), and the putative NAD(H)-binding domain, from Phe-209 to Asp-237, which contains the nucleotide-binding motif G-X-G-X-X-G(A) (Britton et al., 1992). In addition, the amino acid residues Lys-90, Thr-169, and Ser-344, which are involved in Glu binding according to Britton et al. (1992), are conserved. In the gene product for *GDH2*, the region Asp-265 to Glu-276 has similarity to an EF-hand loop motif, and these regions have been shown to be associated with $Ca^{2+}$ binding in other proteins (Haiech and Sallantin, 1985).

## Subcellular Localization

Subcellular components from leaf tissue were fractionated in discontinuous Suc or Percoll gradients to determine the location of NAD(H)-GDH isoenzymes (Table I). Mitochondrial, peroxisomal, and chloroplast preparations were assayed for NADH-GDH, Cat, and fumarase activities, and for chlorophyll (Table I). Consistent with our sequence data, NADH-GDH activity was observed only in mitochondrial preparations. The mitochondrial preparations had no detectable levels of Cat activity or chlorophyll and contained nearly 3-fold more fumarase activity than the peroxisomal preparation. A high percentage (92%) of the mitochondria were intact. These data suggest that the NADH-GDH is associated with purified mitochondria. Protein from crude cell lysates or purified mitochondria were separated on a 5% native polyacrylamide gel and stained for NAD-GDH activity (Fig. 3). All seven NAD(H)-GDH isoenzymes from Arabidopsis that were apparent in crude cell lysates were also present in intact mitochondrial preparations.

## Organ Specificity

Specific activity, isoenzyme patterns, and immunoblot and RNA blot analyses were used to decipher the level of

**Table I.** *Subcellular localization of NAD(H)-GDH isoenzymes*

Subcellular components from leaf tissue were fractionated in discontinuous Suc or Percoll gradients to determine the location of NAD(H)-GDH isoenzymes. Mitochondrial, chloroplast, and peroxisomal preparations were assayed for NADH-GDH, Cat, and fumurase activities, and for chlorophyll.

| Location | Markers | | | | |
|---|---|---|---|---|---|
| | NADH-GDH | Cat | Chlorophyll | Fumarase | Percent intact |
| | $\mu mol\ mg^{-1}$ protein | $\mu mol\ mg^{-1}$ protein | $\mu g\ mg^{-1}$ protein | $\mu mol\ mg^{-1}$ protein | |
| Mitochondria | 31.5 | 0 | 0 | 36.2 | 92 |
| Chloroplasts | 0 | 0 | 60.5 | 0 | nd[a] |
| Peroxisomes | 0 | 72.6 | 0 | 13.8 | 99 |

[a] nd, Not determined.

activity and/or transcript(s) of each NAD(H)-GDH isoenzyme in different plant organs (Fig. 4). The specific activity of NADH-GDH was highest in flowers and lowest in older leaves (from 42-d-old plants). Each organ contained different amounts of the seven NAD(H)-GDH isoenzymes that were readily separated by native-PAGE. The isoenzymes were numbered GDH1 (lowest electrophoretic mobility or cathodal-most isoenzyme) through GDH7 (highest electrophoretic mobility or anodal-most isoenzyme), following the nomenclature of Loulakakis and Roubelakis-Angelakis (1990a), which is based on their migration in a native 5% polyacrylamide gel. The roots, flower stalks, flowers, and siliques contained mainly anodal (fast-migrating) isoenzymes (GDH5–GDH7), but GDH7 was abundant in flowers. Young leaves (from 24-d-old plants) contained all seven isoenzymes, with an abundance of cathodal isoenzymes (GDH1–GDH3). However, protein extracts from leaves of 42-d-old plants contained mainly cathodal isoenzymes, with little or no detectable anodal isoenzymes. Protein extracts from different organs were separated by SDS-PAGE (8.0% polyacrylamide). Immunoblot analysis was used to determine the subunit composition of the NAD(H)-GDH isoenzymes.

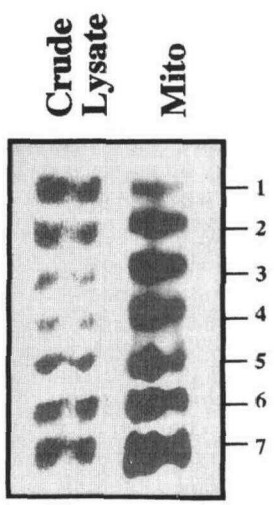


**Figure 3.** Subcellular localization of NAD(H)-GDH isoenzymes. Subcellular components from leaf tissue were fractionated in discontinuous Suc or Percoll gradients to determine the location of NAD(H)-GDH isoenzymes. Protein extracts from crude lysate (Crude Lysate) or purified mitochondria (Mito) were separated in a 5% polyacrylamide gel and stained for NAD-GDH activity. An equal amount of NADH-GDH activity (0.250 unit) was added per lane.

A 42.5-kD polypeptide was identified in protein samples from all organs, based on the polypeptide migration in the gel (highest electrophoretic mobility) and following the nomenclature system of Loulakakis and Roubelakis-Angelakis (1991). This subunit was designated as $\beta$. A 43.0-kD polypeptide (lowest electrophoretic mobility), designated $\alpha$, was identified in all of the samples except those from the roots. A unique 40.0-kD polypeptide was observed in roots (from 42-d-old plants) and, due to its transient appearance in gels throughout the study (see below), remained undesignated. The $\beta$ subunit was more abundant than the $\alpha$ subunit in protein extracts from roots, flowers, and siliques (Fig. 4B) in SDS gels, and the anodal isoenzymes were more abundant in these samples (Fig. 4A). The $\beta$ subunit was readily apparent in roots, but the $\alpha$ subunit was not detectable. The relative difference in the levels of $\alpha$ and $\beta$ subunits can be conveniently expressed as a ratio, i.e. the $\alpha$:$\beta$ subunit ratio in roots was $<$1:100, whereas the ratios of $\alpha$:$\beta$ subunits in flowers and siliques were 1:3 and 1:2, respectively. Protein extracts from organs with a predominance of cathodal isoenzymes had a high $\alpha$:$\beta$ subunit ratio; in general, the ratio was 1:1 or greater. The $\alpha$:$\beta$ subunit ratios in 24- and 42-d-old leaves were 1:1 and 2:1, respectively (Table II).

The data show that protein extracts containing an abundance of anodal isoenzymes had a low $\alpha$:$\beta$ subunit ratio, whereas extracts with an abundance of cathodal isoenzymes had a high ratio. However, protein extracts from flower stalks were an exception to this trend. Anodal isoenzymes were predominant in flower stalks, but the $\alpha$:$\beta$ subunit ratio was nearly 1.5:1. A possible explanation for the deviation of flower stalk protein extracts and other organs may be the relatively high occurrence of isoenzymes 4 to 6 in flower stalks, which have higher numbers of $\alpha$ subunits compared with samples rich in anodal isoenzymes, such as roots, flowers, and siliques. Transcript levels for *GDH1* were highest in young leaves, and there were significant levels in roots and flowers. Levels of *GDH1* transcript were low in flower stalks, siliques, and older leaves. Transcript levels for *GDH2* were highest in flowers and siliques, and there were significant levels in roots and

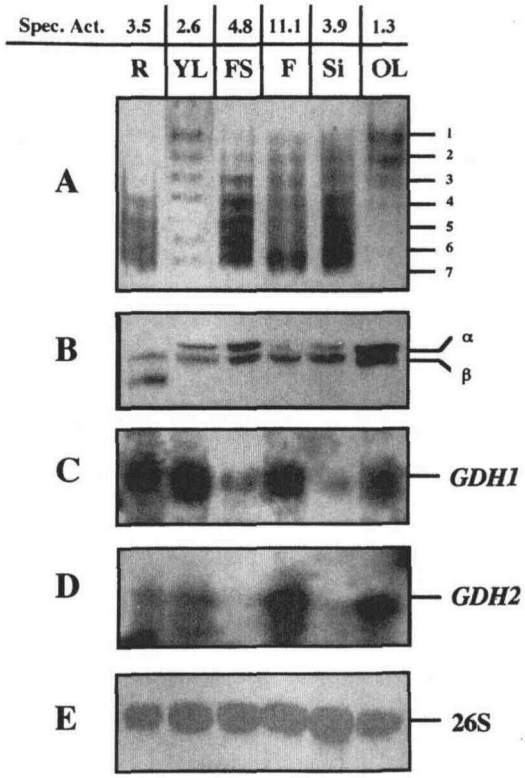


**Figure 4.** Specific NADH-GDH activity, isoenzyme patterns, immunoblot analysis, and expression of *GDH1* and *GDH2* in different organs of Arabidopsis. Protein or total RNA extractions were performed on roots (R), flower stalks (FS), flowers (F), siliques (Si), and leaves of 42-d-old plants (OL), and the leaves of 24-d-old plants (YL). Proteins were separated in 5% native polyacrylamide gels and stained for NAD-GDH activity (A). The positions of the seven NAD(H)-GDH isoenzymes are indicated. For immunoblot analysis (B), proteins were resolved in a SDS (8.0%) polyacrylamide gel, blotted onto nitrocellulose, and incubated with antiserum raised against grape leaf NAD(H)-GDH (Loulakakis and Roubelakis-Angelakis, 1990b). The positions of the $\alpha$ and $\beta$ subunits are indicated. For native-PAGE and SDS-PAGE, an equal amount of NADH-GDH activity (0.250 unit) was added per lane. Total RNA (10 $\mu$g) was separated by gel electrophoresis, blotted onto nitrocellulose, and hybridized with a full-length probe for either *GDH1* (C) or *GDH2* (D). A portion of the 26S rRNA probe was used as a control to ensure equal loading of total RNA into the lanes (E). The results are representative of two separate experiments.

young leaves. Levels of *GDH2* transcript were lowest in flower stalks and siliques.

### The Effect of Different Nitrogen Sources on NAD(H)-GDH

Plants were maintained in soil or vermiculite with complete nutrient solution and 10 mM $KNO_3$ as the sole nitrogen source. After 20 d (see "Materials and Methods"), the pots containing vermiculite were treated with complete mineral nutrient solution containing either 10 mM $KNO_3$, 10 mM $NH_4Cl$, 5 mM $NH_4NO_3$, 5 mM Glu, or 5 mM Gln as the sole nitrogen source. Determination of specific NADH-GDH activity and isoenzyme patterns, and immunoblot and RNA blot analyses were performed on protein and total RNA extracts from leaves (Fig. 5) and roots (Fig. 6).

The specific activity of NADH-GDH increased in protein extracts from leaves of plants treated with different nitrogen sources compared with the 10 mM $KNO_3$ (control) treatment (Fig. 5). The greatest increase was 2.9-fold in leaves of plants treated with 5 mM $NH_4NO_3$ (Table III). NAD-GDH stain of a 5% polyacrylamide gel shows that all seven isoenzymes were present, and the cathodal isoenzymes were readily detected in each sample. However, the cathodal isoenzymes were most abundant in samples treated with 10 mM $KNO_3$, 5 mM $NH_4NO_3$, and 5 mM Glu. Both $\alpha$ and $\beta$ subunits were detected in all treatments. The level of $\alpha$ subunit varied among nitrogen treatments, but the level of $\beta$ subunit remained constant. The $\alpha$ subunit was most abundant in 5 mM $NH_4NO_3$- and 5 mM Gln-treated samples, and the $\alpha$:$\beta$ ratios in those samples were 4:1 and 3:1, respectively. When compared with controls (10 mM $KNO_3$), transcripts corresponding to *GDH1* and *GDH2* increased approximately 3- and 5-fold, respectively, in leaves treated with either 10 mM $NH_4Cl$ or 5 mM $NH_4NO_3$. The level of *GDH1* transcript increased in leaves treated with Glu but not in leaves of Gln-treated plants. Conversely, the level of *GDH2* transcript increased in leaves treated with Gln but not in leaves of Glu-treated plants. The level of *GDH1* and *GDH2* transcripts corresponded to the estimated specific activity for each treatment. Samples with specific activities of 2.7 or lower had low levels of *GDH1* and/or *GDH2* transcript. Conversely, samples with high specific activities, 3.8 or greater, had high levels of *GDH1* and *GDH2*. The relative levels of *GDH1* and *GDH2* transcript appeared to correlate with the relative levels of the $\alpha$ and $\beta$ polypeptides. In leaves of plants grown in soil, the ratio of *GDH1*:*GDH2* transcripts was approximately 2:1, which is very similar to the ratio of $\alpha$:$\beta$ polypeptides at 3:1. However, it is risky to base definitive conclusions on such observations, even though all of the parameters associated with the hybridization experiments were identical.

The specific activity of NADH-GDH in protein extracts from roots treated with different nitrogen sources was relatively unchanged except in samples from plants treated with 5 mM Gln (Fig. 6). In addition, the specific activity in extracts from roots of plants treated with different nitrogen sources was 2 to 7 times greater than in leaf extracts from the same plants (Fig. 5). NAD-GDH stain of a 5% polyacrylamide gel shows that anodal isoenzymes, especially GDH7, were abundant in all of the root samples (Fig. 6A). However, samples from plants treated with 5 mM Gln had more GDH5 and GDH6 compared with samples from other nitrogen treatments. Likewise, the $\beta$ subunit was visualized in all samples, but the $\alpha$ subunit was not detected in any root sample (Fig. 6B). Root tissue from 24-d-old plants grown in soil or vermiculite treated with $NH_4Cl$ or Gln contained a 40.0-kD polypeptide. The level of 40.0-kD polypeptide in $NH_4Cl$-treated tissues was equal to that of the $\beta$ subunit, whereas the level of 40.0-kD polypeptide was negligible in roots of plants grown in soil or treated with Gln. Analysis of RNA blots show no detectable level of *GDH1* transcript in any of the root samples. After nor-

**Table II.** *The relative abundance of cathodal and anodal NAD(H)-GDH isoenzymes and the ratio of α and β subunits from various organs of A. thaliana*

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

| NAD(H)-GDH | Organ | | | | | |
|---|---|---|---|---|---|---|
| | Roots | Young leaves | Flower stalks | Flowers | Siliques | Old leaves |
| Cathodal isoenzymes (GDH1–GDH3) | −[a] | +[b] | +/− | +/− | +/− | + |
| Anodal isoenzymes (GDH5–GDH7) | + | +/− | + | + | + | − |
| α:β Subunit (ratio) | <1:100 | 1:1 | 1.5:1 | 1:3 | 1:2 | 2:1 |

[a] −, Absent. [b] +, Present.

malization of the data to the 26S probe (data not shown), the level of *GDH2* transcript in each sample remained unchanged except in Glu-treated roots, in which the level decreased 70% (Table IV). The results from this experiment strongly suggest that *GDH2* encodes for the β subunit and *GDH1* encodes for the α subunit.

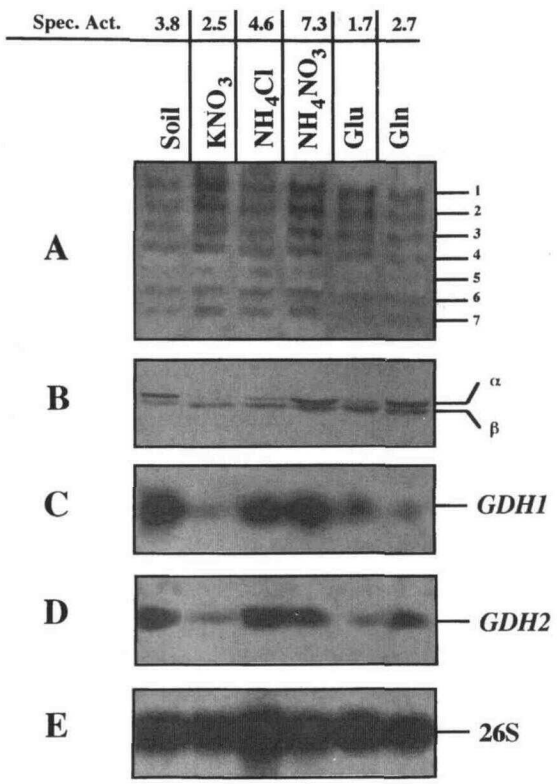


**Figure 5.** Effect of various nitrogen treatments on NAD(H)-GDH in leaves of Arabidopsis. Plants were grown in vermiculite and maintained in the environmental conditions described in Figure 4. Plants were subirrigated with a mineral nutrient solution (see "Materials and Methods") containing nitrate (10 mM $KNO_3$) for 20 d. The plants were treated with either 10 mM $KNO_3$, 10 mM $NH_4Cl$, 5 mM $NH_4NO_3$, 5 mM Glu, or 5 mM Gln for 4 d prior to the harvest of the leaves on d 24. One set of plants was grown in soil and maintained as described in Figure 4. Protein and RNA extractions were collected and analyzed as described in Figure 4. The specific activity, seven NAD(H)-GDH isoenzymes (A), α and β subunits (B), and the *GDH1* (C), *GDH2* (D), and 26S (E) transcripts are indicated. The results are representative of two separate experiments.

### The Effect of Light, Darkness, and Suc on NAD(H)-GDH

Plants were grown in the light (see "Materials and Methods" for conditions) for 20 d in soil given deionized water or in vermiculite watered with complete nutrient solution and 10 mM $KNO_3$ as the sole nitrogen source. After 20 d, one-half of the plants were transferred to complete darkness for 4 d and the other half were maintained in the light. One set of plants grown in vermiculite was treated with 10 mM $KNO_3$ plus 3% Suc for 4 d either in the light or in the dark. The other two sets of plants were maintained in either soil or 10 mM $KNO_3$ for the 4-d light or dark treat-

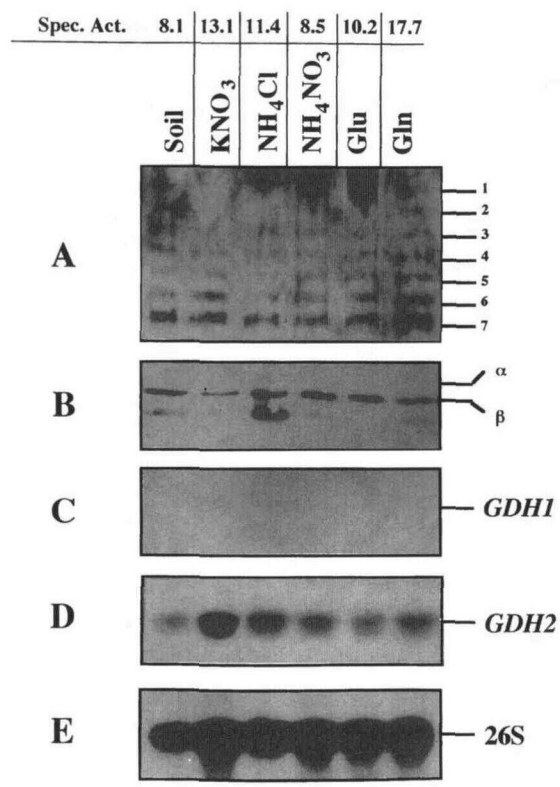


**Figure 6.** Effect of various nitrogen treatments on NAD(H)-GDH in roots of Arabidopsis. Plants were grown and maintained as described in Figure 5. Protein and RNA extractions were collected and analyzed as described in Figure 4. The specific activity, seven NAD(H)-GDH isoenzymes (A), α and β subunits (B), and the *GDH1* (C), *GDH2* (D), and 26S (E) transcripts are indicated. The results are representative of two separate experiments.

**Table III.** *The effects of different nitrogen treatments on NAD(H)-GDH specific activity, the presence of cathodal and anodal NAD(H)-GDH isoenzymes, the ratio of α and β subunits, and the levels of GDH1 and GDH2 transcripts in leaves of A. thaliana*

The changes in specific activity and the levels of *GDH1* and *GDH2* transcripts are presented as an increase (−fold) in the samples from various nitrogen treatments compared with the control, 10 mM $KNO_3$ treatment. All samples were obtained from 24-d-old plants. See Figure 5 or "Materials and Methods" for the experimental procedure. The results presented in this table are representative of two separate experiments.

| NAD(H)-GDH | Nitrogen Treatment | | | | |
|---|---|---|---|---|---|
| | $KNO_3$ (10 mM; control) | $NH_4Cl$ (10 mM) | $NH_4NO_3$ (5 mM) | Glu (5 mM) | Gln (5 mM) |
| Increase (−fold) in specific activity NADH-GDH | x[a] | 1.8 | 2.9 | 0.7 | 1.0 |
| Cathodal isoenzymes (GDH1–GDH3) | ++[b] | +[c] | ++ | ++ | + |
| Anodal isoenzymes (GDH5–GDH7) | + | + | ++ | + | + |
| α:β Subunit (ratio) | 1:4 | 1:1 | 4:1 | 1:3 | 3:1 |
| Increase (−fold) in *GDH1* transcript | x | 2.8 | 3.3 | 2.3 | 1.3 |
| Increase (−fold) in *GDH2* transcript | x | 5.1 | 4.6 | 1.0 | 2.9 |

[a] x, Control. [b] ++, More abundant. [c] +, Present.

ment. On d 24, protein and total RNA extractions were conducted on leaf samples from light- and dark-grown plants for various analyses (Fig. 7).

The specific activity of NADH-GDH in protein extracts from leaf samples of dark-treated plants was 2.2- to 2.5-fold greater than that from light-grown plants (Table V). Protein samples from plants grown in the light contained more cathodal than anodal isoenzymes compared with leaf samples from dark-treated plants, which contained more anodal isoenzymes. The α:β subunit ratios in leaves from light-grown plants were higher than the corresponding samples from dark-treated plants. The ratios were 4:1 and 1:4, 1:4 and 1:>20, and 1:4 and 1:10 in samples from light- and dark-treated plants maintained in either soil, 10 mM $KNO_3$, or 10 mM $KNO_3$ plus 3% Suc, respectively. The level of *GDH1* transcript increased 2- to 3-fold in dark-treated plants maintained in either soil or 10 mM $KNO_3$ compared with similar samples from plants grown in the light. However, the level of *GDH1* transcript remained unchanged among light- and dark-treated plants maintained in 10 mM $KNO_3$ plus 3% Suc for 4 d. The level of *GDH2* transcript increased 18- to 25-fold in dark-treated plants maintained in either soil, 10 mM $KNO_3$, or 10 mM $KNO_3$ plus 3% Suc, compared with similar samples from plants grown in the light. These data suggest that GDH1 and GDH2 are not always coordinately expressed. Furthermore, these data are consistent with earlier observations (Figs. 4 and 5) in which the abundance of cathodal isoenzymes in a protein extract corresponded to the detection of the α subunit by immunoblot analysis. In addition, there was a strong correlation with the increased expression of *GDH2* and the increase in the specific enzyme activity from dark-treated samples. The different ratios of the α:β subunits in dark- compared with light-treated leaves can be partially explained by the relative differences in the levels of *GDH1* and *GDH2* transcript. In dark-treated plants, *GDH1* increased only 2- to 3-fold and *GDH2* increased approximately 18- to 25-fold, and therefore the ratio of α:β subunits decreased.

## DISCUSSION

Two full-length cDNAs encoding NAD(H)-GDH in *A. thaliana* were identified and characterized. Our findings are consistent with earlier genetic analyses conducted between two Arabidopsis ecotypes containing electrophoretic variants of NAD(H)-GDH that suggested that the isoenzymes were controlled by two nonallelic genes (Cammaerts and Jacobs, 1983), and with molecular studies by Melo-Oliveira et al. (1996) that suggested that there was more than one GDH gene. The presence of a small NAD(H)-GDH family appears to be common among plants. Genetic (Pryor, 1979; Goodman et al., 1980) and molecular (Sakakibara et al.,

**Table IV.** *The effects of different nitrogen treatments on NAD(H)-GDH specific activity, the presence of cathodal and anodal NAD(H)-GDH isoenzymes, and the level of GDH2 transcript in roots of A. thaliana*

The changes in specific activity and the level of GDH2 transcript are presented as an increase (−fold) in the samples from various nitrogen treatments compared with the control, 10 mM $KNO_3$ treatment. All samples were obtained from 24-d-old plants. See Figure 6 or "Materials and Methods" for the experimental procedure. The results presented in this table are representative of two separate experiments. The ratio of α and β subunits was not determined because the α subunit was not detected by immunoblot analysis (see Fig. 6). Likewise, the *GDH1* transcript was not detected by RNA blot analysis.

| NAD(H)-GDH | Nitrogen Treatment | | | | |
|---|---|---|---|---|---|
| | $KNO_3$ (10 mM; control) | $NH_4Cl$ (10 mM) | $NH_4NO_3$ (5 mM) | Glu (5 mM) | Gln (5 mM) |
| Increase (−fold) in specific activity NADH-GDH | x[a] | 0.9 | 0.6 | 0.8 | 1.4 |
| Cathodal isoenzymes (GDH1–GDH3) | −[b] | − | − | − | − |
| Anodal isoenzymes (GDH5–GDH7) | +[c] | + | + | + | ++ |
| Increase (−fold) in *GDH2* transcript | x | 1.0 | 1.0 | 0.7 | 1.0 |

[a] x, Control. [b] −, Absent. [c] +, Present.

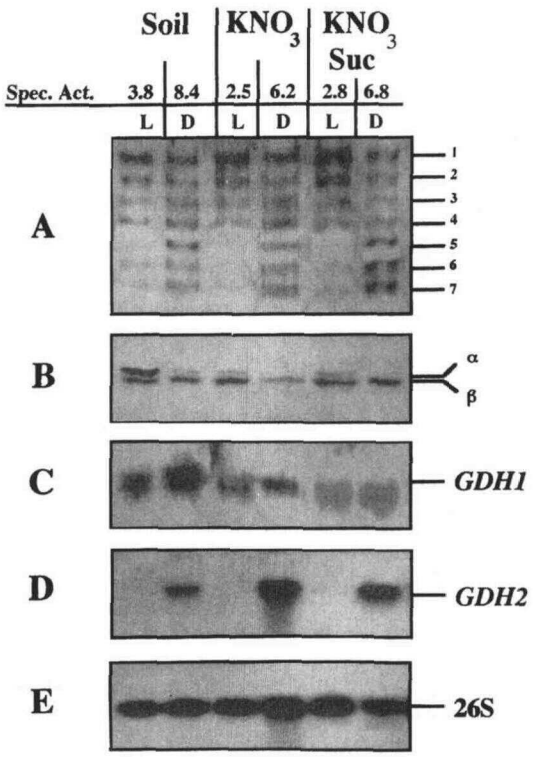


**Figure 7.** Effect of darkness and Suc treatments on NAD(H)-GDH in leaves of Arabidopsis. Plants were maintained in the environmental conditions described in Figure 4. Plants were grown in vermiculite and irrigated with a mineral nutrient solution (see "Materials and Methods") containing nitrate (10 mM $KNO_3$) for 20 d. After 20 d, the vermiculite-grown plants were treated with either 10 mM $KNO_3$ or 10 mM $KNO_3$ plus 3% Suc for 4 d, and one-half of each set of plants was maintained in the light and the other half was transferred into the dark for 4 d. One set of plants was grown in soil; one-half of the set was maintained in the light and the other half was transferred into the dark for 4 d. All protein and RNA extractions were collected on d 24 and analyzed as described in Figure 4. The specific activity, seven NAD(H)-GDH isoenzymes (A), α and β subunits (B), and the *GDH1* (C), *GDH2* (D), and 26S (E) transcripts are indicated. The results are representative of two separate experiments.

1995) findings in maize suggest that NAD(H)-GDH isoenzymes are encoded by more than one gene. Furthermore, the presence of multiple genes encoding for enzymes involved in nitrogen metabolism appears to be a common occurrence in *A. thaliana*, and small multigene families encoding for GS (Peterman and Goodman, 1991), GOGAT (Lam et al., 1995a), Asn synthetase (Lam et al., 1995a, 1995b), and aspartate aminotransferase (Schultz and Coruzzi, 1995; Wilkie et al., 1995) have been reported. Our findings confirm the existence of a small gene family encoding NAD(H)-GDH. In addition, there is a distinct NADP(H)-GDH gene in Arabidopsis, which brings the total number of members in the NAD(P)-GDH family to at least three (F.J. Turano and S.S. Thakkar, unpublished results).

The mitochondrial location of the two NAD(H)-GDH gene products in Arabidopsis was suggested by the presence of putative mitochondrial target sequences at the N termini of each gene product, as described by Hartl et al. (1989), and the subcellular location of the NAD(H)-GDH isoenzymes was demonstrated by fractionation of cellular components in discontinuous Suc and Percoll gradients. All seven NAD(H)-GDH isoenzymes from Arabidopsis were present in intact mitochondrial preparations. The mitochondrial location of the NAD(H)-GDH isoenzymes is consistent with the subcellular location reported for most plant NAD(H)-GDH isoenzymes (for review, see Stewart et al., 1980; Srivastava and Singh, 1987).

The seven NAD(H)-GDH isoenzymes in Arabidopsis are composed of two subunits that combine in different ratios in a manner similar to those of grape (Loulakakis and Roubelakis-Angelakis, 1991) and avocado (Loulakakis et al., 1994) NAD(H)-GDH isoenzymes; however, the subunit makeup of the anodal and cathodal isoenzymes in Arabidopsis are the reverse of those of grape and avocado. In Arabidopsis the slowest-migrating isoenzyme in a native gel, GDH1, is a homohexamer composed of α subunits, and the fastest-migrating isoenzyme, GDH7, is a homohexamer composed of β subunits. GDH isoenzymes 2 through 6 are heterohexamers composed of decreasing to increasing numbers of α and β subunits, respectively. Conversely, in grape and avocado the slowest-migrating isoenzymes in native gels, GDH1, are homohexamers composed of β subunits, and the fastest-migrating isoenzymes, GDH7, are homohexamers composed of α subunits. One plausible explanation for the different subunit/isoenzyme arrangements could be differential processing of the subunits in such a way as to alter the molecular weight of the polypeptide upon transport into the mitochondria in the various plant species. Because cDNAs that encode grape and/or avocado NAD(H)-GDH subunits have not been characterized and the gene/subunit assignments have not been completed, it is not possible to determine why there are differences among plant species.

In all protein extracts subjected to immunoblot analyses, the samples either contained a 43.0-kD (α subunit) or a 42.5-kD (β subunit) polypeptide. Occasionally, a 40.0-kD polypeptide was observed, but only in root samples. Similar results were reported by Loulakakis and Roubelakis-Angelakis (1991) in protein extracts from grape. They reported the transient appearance of minor bands (at 30 and 35 kD) on immunoblots that cross-reacted with the anti-GDH serum. As explained by Loulakakis and Roubelakis-Angelakis (1991), the minor or transient bands may represent breakdown products of NAD(H)-GDH or nonspecific polypeptides that cross-reacted with the anti-GDH serum; alternatively, these polypeptides may be encoded by unique polypeptide. The 40.0-kD peptide in Arabidopsis does not appear to be a breakdown product of the α subunit, since it was visible in some root samples from 24-d-old plants that did not have detectable levels of *GDH1* transcript (Fig. 6). The results from experiments on roots from 24-d-old plants were contrary to results observed in roots of 42-d-old plants (Fig. 4), in which a 40.0-kD peptide was abundant and the *GDH1* transcript was detected; therefore, we cannot definitively state that the 40.0-kD peptide was not a breakdown product of the α subunit. In

**Table V.** *The effect of dark and Suc treatments on NAD(H)-GDH specific activity, the presence of cathodal and anodal NAD(H)-GDH isoenzymes, the ratio of α and β subunits, and the levels of GDH1 and GDH2 transcripts in leaves of A. thaliana*

The changes in specific activity and the levels of *GDH1* and *GDH2* transcripts are presented as an increase (−fold) in samples from plants after treatment in the dark compared with control plants maintained in the light with the corresponding soil or nutrient treatment. All samples were obtained from 24-d-old plants. See Figure 7 or "Materials and Methods" for the experimental procedure. The results presented in this table are representative of two separate experiments.

| NAD(H)-GDH | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Soil | | 10 mM $KNO_3$ | | 10 mM $KNO_3$ plus 3% Suc | |
| | Light | Dark | Light | Dark | Light | Dark |
| Increase (−fold) in specific activity NADH-GDH | x[a] | 2.2 | x | 2.5 | x | 2.4 |
| Cathodal isoenzymes (GDH1–GDH3) | +[b] | + | + | + | + | + |
| Anodal isoenzymes (GDH5–GDH7) | +/−[c] | + | +/− | + | +/− | + |
| α:β Subunit (ratio) | 4:1 | 1:4 | 1:4 | 1:>20 | 1:4 | 1:10 |
| Increase (−fold) in *GDH1* transcript | x | 3 | x | 2 | x | 1 |
| Increase (−fold) in *GDH2* transcript | x | 18 | x | 25 | x | 23 |

[a] x, Control. [b] +, Present. [c] −, Absent.

addition, the possibility that the 40.0-kD peptide was a breakdown product of the β subunit cannot be ruled out at this time. In the future, microsequence amino acid analysis of the 40.0-kD peptide can be used to determine its identity.

Our data demonstrate that *GDH1* encodes a 43.0-kD polypeptide (the α subunit) and *GDH2* encodes a 42.5-kD polypeptide (the β subunit). The clearest example of this gene/subunit assignment are the data from the roots of plants treated with different nitrogen sources (Fig. 6), in which both *GDH2* and the β subunit were readily detected, and *GDH1* and the α subunit were not. Equally convincing data for the gene/subunit assignments were apparent in leaf samples from light- versus dark-treated plants (Fig. 7). In the light, both α and β subunits were abundant and, upon longer exposure, *GDH1* and *GDH2* transcripts were detectable. In the dark, the β subunit was abundant and in most cases the α subunit was undetectable, whereas the level of *GDH1* transcript increased slightly (approximately 2-fold in plants maintained in soil and 10 mM $KNO_3$) compared with corresponding samples from light-grown plants. However, similar comparisons of *GDH2* show that the level of transcript increased nearly 25-fold. These data demonstrate that the abundance of anodal isoenzymes in a native gel were associated with increased detection of the β subunit and the detection of *GDH2* transcript on immunoblots and RNA blots, respectively.

The changes in the specific activity of NAD(H)-GDH among the different organs and/or treatments were correlated with levels of *GDH1* and/or *GDH2* transcript(s). In general, as the levels of *GDH1* and/or *GDH2* transcript(s) increased 5-fold or more, the specific activity in that sample increased 3- to 5-fold. These data suggest that the specific NAD(H)-GDH activity is controlled at least in part by either increased stability of the transcript(s) and/or increased expression of the gene(s). In most instances, the changes in isoenzyme patterns were correlated with relative differences in the levels of α and β subunits and the relative levels of *GDH1* and *GDH2* transcripts. Since the amount of total RNA loaded per lane, the size of the GDH probes, the amount of probe added to each hybridization reaction, and the hybridization conditions were constant, it is tempting to speculate that the relative differences in the number of α and β subunits were regulated solely by RNA abundance, but translational modification cannot be ruled out at this point.

Because the *GDH2* transcript was readily detected in all tissues tested, it appears that the gene is constitutively expressed. These data suggest that *GDH2* may play a vital role in carbon and/or nitrogen metabolism. Melo-Oliveira et al. (1996) also suggested that *GDH2* may play a vital role in plant metabolism, since they were not able to identify an Arabidopsis mutant deficient in the *GDH2* product. The expression of *GDH2* was inducible, i.e. the level of transcript increased 5- or 25-fold in leaf samples from plants treated with ammonia or dark treatments, respectively. Like *GDH2* levels, levels of *GDH1* transcript increased with ammonia or dark treatments, but the increase was only 3- and 2-fold, respectively. The two genes were not always coordinately expressed. In the present study, *GDH2* transcript was readily detected in root samples but the *GDH1* transcript was not, although Melo-Oliveira et al. (1996) were able to detect a very low level of *GDH1* transcript in 21-d-old roots. Furthermore, the level of *GDH1* transcript increased in leaves treated with Glu but not in leaves of Gln-treated plants. Conversely, the level of *GDH2* transcript increased in leaves treated with Gln but not in leaves of Glu-treated plants. However, the most striking difference between the expression of *GDH1* and *GDH2* was observed in leaf samples of dark-treated plants maintained on 10 mM $KNO_3$ plus 3% Suc for 4 d. In these samples *GDH1* transcript decreased to a level similar to that of the corresponding light-grown treatment, suggesting that *GDH1* was suppressed by carbon. Similar observations were documented by Melo-Oliveira et al. (1996). Conversely, the level of *GDH2* transcript did not decrease when plants were maintained on 3% Suc in the dark for 4 d. In summary, the data suggest that *GDH1* and *GDH2* play distinct roles in carbon and nitrogen metabolism, since *GDH1* and *GDH2* are not coordinately expressed, and in some instances, i.e. in plants maintained on ammonia for 4 d, the two genes are induced to different levels in samples subjected to the same treatment.

The presence of a putative EF-hand loop motif in the *GDH2* gene product and the absence of one in the *GDH1* gene product suggest that NAD(H)-GDH isoenzymes may differentially bind $Ca^{2+}$ and therefore could be differentially stimulated by the ligand. If the $Ca^{2+}$ site were functional and involved in the stimulation of NAD(H)-GDH in Arabidopsis, the GDH7 isoenzyme, which is composed of $\beta$ subunits and is encoded by *GDH2*, would be expected to be sensitive to $Ca^{2+}$ stimulation. Conversely, the GDH1 isoenzyme, which is composed of $\alpha$ subunits and is encoded by *GDH1*, would be expected to be insensitive to $Ca^{2+}$ stimulation.

To our knowledge, there are not yet any experimental data on NAD(H)-GDH isoenzymes from Arabidopsis, but there are data from other plant systems to support this hypothesis. The activity of several NAD(H)-GDH isoenzymes has been shown to be stimulated by divalent cations, and is particularly sensitive to $Ca^{2+}$ (Chou and Splittstoesser, 1972; Joy, 1973; Garland and Dennis, 1977; Yamaya et al., 1984; Itagaki et al., 1988; Loulakakis and Roubelakis-Angelakis, 1990a; F.J. Turano, unpublished results). In most cases, e.g. pea (Joy, 1973; Garland and Dennis, 1977), corn (Yamaya et al., 1984), isoenzyme 1 of grape (Loulakakis and Roubelakis-Angelakis, 1990a), and GDH3 of soybean (F.J. Turano, unpublished results), $Ca^{2+}$ stimulation was observed only in the direction of the amination reaction. Furthermore, there appears to be an interaction between the level of $Ca^{2+}$ stimulation and NADH concentration, i.e. maximal $Ca^{2+}$ stimulation was observed with increased (from 50–160 mM) NADH concentrations in GDH isoenzymes from corn (Yamaya et al., 1984), grape (Loulakakis and Roubelakis-Angelakis, 1990a), and soybean (F.J. Turano, unpublished results). However, Loulakakis and Roubelakis-Angelakis (1990a) demonstrated that $Ca^{2+}$ stimulation at low (10 mM) NADH concentrations varied among the grape NAD(H)-GDH isoenzymes. GDH isoenzyme 1 was stimulated, whereas isoenzyme 7 was not, by $Ca^{2+}$ at low NADH concentrations. In addition, each of the seven grape NADH-GDH isoenzymes were shown to have different degrees of $Ca^{2+}$ stimulation at low NADH concentrations. Similarly, different $Ca^{2+}$-dependent stimulatory effects were observed in corn, in which activity was not stimulated at low NADH concentrations (Yamaya et al., 1984), and in soybean, in which GDH3 was stimulated at low NADH concentrations (F.J. Turano, unpublished results).

In summary, the results from these studies suggest that the different stimulatory effects of $Ca^{2+}$ on NADH-GDH isoenzymes are consistent with the existence of two GDH gene products with distinct and different affinities for $Ca^{2+}$. The in vitro $Ca^{2+}$ stimulation of NADH-GDH may not be biologically significant, since estimates of $Ca^{2+}$ concentrations in the matrix of plant mitochondria range from 60 to 100 nM in pea stem and from 400 to 600 nM in Jerusalem artichoke tubers (Zottini and Zannoni, 1993), but maximal stimulation of plant NADH-GDH isoenzymes by $Ca^{2+}$ occurs at 50 to 100 $\mu$M (Yamaya et al., 1984; Itagaki et al., 1988; Loulakakis and Roubelakis-Angelakis, 1990a; F.J. Turano, unpublished results). However, there was a 2.3-fold stimulation of soybean NADH-GDH activity in the presence of 250 to 500 nM $Ca^{2+}$ (F.J. Turano, unpublished results). In addition, a 2-fold increase in NADH-GDH activity by 1000 nM $Ca^{2+}$ was reported in corn (Yamaya et al., 1984). Based on these findings, it is possible that $Ca^{2+}$ could stimulate the amination reaction and thus play a role in the regulation of Glu catabolism and metabolism in plant mitochondria.

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## LITERATURE CITED

**Beers PF, Sizer IW** (1952) A spectrophotometric assay for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem **195:** 133–140

**Britton KL, Baker PJ, Rice DW, Stillman TJ** (1992) Structural relationship between the hexameric and tetrameric family of glutamate dehydrogenase. Eur J Biochem **209:** 851–859

**Bruinsma J** (1961) A comment on the spectrophotometric determination of chlorophyll. Biochim Biophys Acta **52:** 576–578

**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

**Cammaerts D, Jacobs M** (1985) A study of the role of glutamate dehydrogenase in nitrogen metabolism of *Arabidopsis thaliana*. Planta **163:** 517–526

**Chou KH, Splittstoesser WE** (1972) Glutamate dehydrogenase from pumpkin cotyledons. Characterization and isozymes. Plant Physiol **49:** 550–554

**Edwards JW, Coruzzi GM** (1989) Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. Plant Cell **1:** 241–248

**Garland W, Dennis DT** (1977) Steady-state kinetics of glutamate dehydrogenase from *Pisum sativa* L. mitochondria. Arch Biochem Biophys **182:** 614–625

**Gavel Y, von Heijne G** (1990) Cleavage-site motifs in mitochondrial targeting peptides. Protein Eng **4:** 33–37

**Goodman MM, Stuber CA, Newton K, Weissinger HH** (1980) Linkage relationships of 19 loci in maize. Genetics **96:** 697–710

**Haiech J, Sallantin J** (1985) Computer search of calcium binding sites in a gene data bank: use of learning techniques to build an expert system. Biochimie **67:** 555–560

**Hartl F, Smith BB, James CC** (1989) Structure and function of mitochondrial transit polypeptide sequences. Biochim Biophys Acta **988:** 1–45

**Hemmings BA** (1980) Purification and properties of the phospho and dephospho forms of yeast NAD-dependent glutamate dehydrogenase. J Biol Chem **255:** 7925–7932

**Hill RA, Bradshaw RA** (1969) Fumarase. Methods Enzymol **13:** 91–99

**Itagaki T, Dry IB, Wiskich JT** (1988) Purification and properties of NAD-glutamate dehydrogenase from turnip mitochondria. Phytochemistry **27:** 3373–3378

**Joy KW** (1973) Control of glutamate dehydrogenase from *Pisum sativum* roots. Phytochemistry **12:** 1031–1040

**Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

**Lam H-M, Coschigano K, Schultz C, Melo-Oliveira R, Tjaden G, Oliveira I, Ngai N, Coruzzi GM** (1995a) Use of *Arabidopsis* mutants and genes to study amide amino acid biosynthesis. Plant Cell **7:** 887–898

**Lam H-M, Peng SS-Y, Coruzzi GM** (1995b) Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. Plant Physiol **106:** 1347–1357

**Lea PJ, Miflin BJ** (1974) Alternative route for nitrogen assimilation in higher plants. Nature **251:** 614–616

**Lilly KS, Engel PC** (1992) The essential active-site lysines of clostridial glutamate dehydrogenase: a study with pyridoxal-5′-phosphate. Eur J Biochem **207:** 533–540

**Loulakakis KA, Roubelakis-Angelakis KA** (1990a) Intracellular localization and properties of NAD(H)-glutamate dehydrogenase from *Vitis vinifera* L.: purification and characterization of the major leaf isoenzyme. J Exp Bot **41:** 1223–1230

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

**Loulakakis KA, Roubelakis-Angelakis KA** (1991) Plant NAD(H)-glutamate dehydrogenase consists of two subunit polypeptides and their participation in the seven isoenzymes occurs in an ordered ratio. Plant Physiol **97:** 104–111

**Loulakakis KA, Roubelakis-Angelakis KA** (1996) The seven NAD(H)-glutamate dehydrogenase isoenzymes exhibit similar anabolic and catabolic activities. Physiol Plant **96:** 29–35

**Loulakakis KA, Roubelakis-Angelakis KA, Kanellis KA** (1994) Regulation of glutamate dehydrogenase and glutamine synthetase in avocado fruit during development and ripening. Plant Physiol **106:** 217–222

**Maestri E, Restivo FM, Gulli M, Tassi F** (1991) Glutamate dehydrogenase regulation in callus cultures of *Nicotiana plumbaginifolia*: effect of glucose feeding and carbon starvation on the isoenzymatic pattern. Plant Cell Environ **14:** 613–618

**Magalhaes JR, Ju GC, Rich PJ, Rhodes D** (1990) Kinetics of a $^{15}NH_4^+$ assimilation in *Zea mays*. Preliminary studies with glutamate dehydrogenase (GDH1) null mutant. Plant Physiol **94:** 647–656

**Melo-Oliveira R, Oliveira IC, Coruzzi GM** (1996) *Arabidopsis* mutant analysis and regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc Natl Acad Sci USA **93:** 4718–4723

**Miflin BJ, Lea PJ** (1980) Ammonia assimilation. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 169–202

**Newman T, de Bruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikhel N, Somerville S, Thomashow M, Rezel E, Somerville C** (1994) Genes galore. A summary of the methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol **106:** 1241–1255

**Peterman TK, Goodman HM** (1991) The glutamine synthetase gene family of *Arabidopsis thaliana*: light-regulation and differential expression in leaves, roots and seeds. Mol Gen Genet **230:** 145–154

**Pryor AJ** (1979) Mapping of glutamate dehydrogenase (*Gdh*) on chromosome 1,20.1 recombination units distal to *Adh*1. Maize Genet Coop News Lett **53:** 25–26

**Rhodes D, Brunk DG, Magalhaes JR** (1989) Assimilation of ammonia by glutamate dehydrogenase? *In* JE Poulton, JT Romeo, EE Conn, eds, Plant Nitrogen Metabolism, Vol 5. Plenum Press, New York, pp 191–206

**Robinson SA, Slade AP, Fox GG, Phillips R, Ratcliffe G, Stewart GR** (1991) The role of glutamate dehydrogenase in plant nitrogen metabolism. Plant Physiol **95:** 509–516

**Robinson SA, Stewart GR, Phillips R** (1992) Regulation of glutamate dehydrogenase activity in relation to carbon limitation and protein catabolism in carrot cell cultures. Plant Physiol **98:** 1190–1195

**Sakakibara H, Fujii K, Sugiyama T** (1995) Isolation and characterization of a cDNA that encodes maize glutamate dehydrogenase. Plant Cell Physiol **36:** 789–797

**Schultz CL, Coruzzi GM** (1995) The aspartate aminotransferase family of Arabidopsis encodes isoenzymes localized to three distinct subcellular compartments. Plant J **7:** 61–75

**Srivastava HS, Singh RP** (1987) The role and regulation of L-glutamate dehydrogenase activity in higher plants. Phytochemistry **26:** 597–610

**Stewart GR, Mann AF, Fentem PA** (1980) Enzymes of glutamate formation: glutamate dehydrogenase, glutamine synthetase and glutamate synthase. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 271–327

**Syntichaki KM, Loulakakis KA, Roubelakis-Angelakis KA** (1996) The amino-acid sequence similarity of plant glutamate dehydrogenase to the extremophilic archaeal enzyme conforms to its stress-related function. Gene **168:** 87–92

**Turano FJ, Dashner R, Upadhyaya A, Caldwell CR** (1996) Purification of mitochondrial glutamate dehydrogenase from dark-grown soybean seedlings. Plant Physiol **112:** 1357–1364

**Turano FJ, Jordan RL, Matthews BF** (1990) Immunological characterization of *in vitro* forms of homoserine dehydrogenase from carrot suspension cultures. Plant Physiol **92:** 395–400

**Turano FJ, Weisemann JM, Matthews BF** (1992) Identification and expression of a cDNA clone encoding aspartate aminotransferase in carrot. Plant Physiol **100:** 374–381

**von Heijne G** (1992) Cleavage-site motifs in protein targeting sequences. Genet Eng **14:** 1–11

**Wallsgrove RM, Turner JC, Hall NP, Kendally AC, Bright SWJ** (1987) Barley mutants lacking chloroplast glutamine synthetase. Biochemical and genetic analysis. Plant Physiol **83:** 155–158

**Wilkie SE, Roper JM, Smith AG, Warren MJ** (1995) Isolation, characterization and expression of a cDNA encoding plastid aspartate aminotransferase from *Arabidopsis thaliana*. Plant Mol Biol **27:** 1227–1233

**Yamaya T, Oaks A, Matsumoto H** (1984) Characterization of glutamate dehydrogenase in mitochondria prepared from corn shoots. Plant Physiol **76:** 1009–1013

**Yamaya T, Oaks A, Rhodes D, Matsumoto H** (1986) Synthesis of [$^{15}$N]glutamate from [$^{15}$N]$H_4$ and [$^{15}$N]glycine by mitochondria isolated from pea and corn shoots. Plant Physiol **81:** 754–757

**Young RA, Davis RW** (1983) Efficient isolation of genes by using antibody probes. Proc Natl Acad Sci USA **80:** 1194–1198

**Zottini M, Zannoni D** (1993) The use of fura-2 fluorescence to monitor the movement of free calcium ions into the matrix of plant mitochondria (*Pisum sativum* and *Helianthus tuberosus*). Plant Physiol **102:** 573–578