Molecular Identification and Characterization of Cytosolic Isoforms of Glutamine Synthetase in Maize Roots*

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In maize, a small multigene family encodes the cytosolic isoforms of glutamine synthetase (GS), and five cDNAs, designated pGS1a, pGS1b, pGS1c, pGS1d, and pGS1e, have been cloned (Sakakibara, H., Kawabata, S., Takahashi, H., Hase, T., and Sugiyama, T. (1992) Plant Cell Physiol. 33, 49–58; Li, M., Villemur, R., Hussey, P. J., Sillflow, C. D., Gantt, J. S., and Snustad, D. P. (1993) Plant Mol. Biol. 23, 401–407). This report describes the identification and enzymatic characterization of the cytosolic isoforms of GS in maize roots, namely GS1 and GSr. The purified isoforms, as well as recombinant enzymes that had been overexpressed in Escherichia coli, were analyzed by capillary liquid chromatography/electrospray ionization-mass spectrometry, and GS1 and GSr were identified as the products of the GS1a/GS1b and GS1c/GS1d genes, respectively. Upon the addition of ammonia to the culture medium, significant amounts of GSr accumulated and a preferential increase in GS synthetase activity, as compared to GS transferase activity, was found in the root extract. Assays with the purified recombinant enzymes confirmed that the specific biosynthetic and synthetase activities of GSr were 1.6-fold higher than those of GS1. Marked differences in stability were also found between the two isoforms: GSr was more sensitive to heat than GS1 and octameric aggregates of the subunits of GSr were easily dissociated to monomers than those of GS1 at low concentrations of Mn2+ and Mg2+ ions. These characteristics of the ammonia-induced isoform of GS seem to be physiologically important for the primary assimilation of external ammonia by roots.

Glutamine synthetases (GS; EC 6.3.1.2) of higher plants are octameric proteins composed of subunits with molecular weights of about 40,000. They have important roles in the assimilation of nitrogen into glutamine (1–3). There are two types of GS and they are localized in distinct subcellular compartments, namely the cytosol and the plastids (4). In leaves, plastidic GS functions to assimilate the ammonia that is produced from the reduction of nitrate and also to reassimilate ammonia that is released during photorespiration. Ammonia from the soil and ammonia derived from the fixation of dinitrogen in legumes is assimilated into glutamine by cytosolic GS in roots and nodules, respectively.

In most species of plants examined to date there appears to be a single gene for plastidic GS and a small multigene family for the cytosolic forms of GS. The presence of multiple polypeptides obviously complicates the characterization of cytosolic isoform of GS. The pattern of distribution of cytosolic isoforms of GS and the expression of their genes vary among tissues and organs of individual plant species in response to the source of nitrogen and with the stage of development. For example, Phaseolus vulgaris has at least three genes for cytosolic GS that encode, respectively, the α-subunit found in leaves and roots, the β-subunit found in leaves, roots, and nodules, and the γ-subunit, which is specific to nodules (5). Multiple forms of GS and the differential distribution of the corresponding mRNAs have also been reported in other species, including species of nonleguminous plants (6–8).

We have cloned four cDNAs that encode cytosolic isoforms of GS in maize. These clones were originally designated pGS307, pGS112, pGS117, and pGS122 (9) but they were recently renamed pGS1a, pGS1b, pGS1c, and pGS1d, respectively. A fifth cDNA for a cytosolic GS, ZMGS12 (pGS1e), has also been reported (10). Studies with gene-specific probes have revealed clear differences among the patterns of expression of the various genes at the mRNA level (9–11). The mRNAs corresponding to GS1a and GS1b are abundantly and constitutively expressed in all organs. By contrast, mRNAs corresponding to GS1c and GS1d are expressed predominantly in etiolated leaves and roots, and their levels increase upon the addition of ammonia and/or nitrate to the growth medium. At the protein level, two cytosolic GS polypeptides, with different mobilities distinguished in SDS-PAGE, can be detected by Western blotting in extracts from nitrate- and/or ammonia-treated roots (11). These polypeptides have not yet been related to specific cDNAs.

Prokaryotic GS is a well-characterized protein not only in terms of the regulation of gene expression but also in terms of the regulation of enzymatic activity (12). In Escherichia coli, GS is a dodecameric enzyme composed of identical subunits whose primary structure is distinct from that of the subunits of plant GS (13). The enzymatic activity in vivo is regulated in

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§The abbreviations and trivial names used are: GS, glutamine synthetase; GSα, the biosynthetic activity of GS; GSγ, the synthetase activity of GS; GSγ, the synthetase activity of GS; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

In plants, a nomenclature, GS1 and GS2, has been generally used to classify the cytosolic and plastidic types of GS, respectively, although another nomenclature, GS1 and GSII, was also used to classify prokaryotic and eukaryotic types of GS, respectively.
response to the intracellular nitrogen status at both the transcriptional and the post-translational level, where modification involves adenylylation of a specific amino acid residue (12, 14). The bacterial GS requires divalent cations, such as Mg$^{2+}$ and Mn$^{2+}$ ions, not only for expression of its activity but also for maintenance of its quaternary structure (15–17). Although GS from plants has been well studied in terms of the regulation of gene expression, the biochemical properties of the enzyme are poorly understood as compared to those of the bacterial GS. If we are to understand the physiological significance of the existence of multiple cytosolic isoforms of GS, both types of information are obviously required.

In this study, we focused on the biochemical characterization of the cytosolic isoforms of GS in maize roots. Using authentic enzymes from root tissues and recombinant enzymes expressed in E. coli cells, we have been able to relate individual GS polypeptides to the cloned cDNAs on a chemical basis. We present here the first results, to our knowledge, that reveal significant differences between ammonia-induced and noninduced enzymes in terms of their enzymatic and physicochemical properties.

EXPERIMENTAL PROCEDURES

Plant Materials—Maize (Zea mays L. cv. Golden Cross Bantam T51) seedlings were grown initially in an aerated hydroponic system in 0.1 Y Hoagland’s nutrients (18), without a nitrogen source, at 28 °C for 3 to 4 days under continuous fluorescent light at an intensity of about 300 μE m$^{-2}$ s$^{-1}$. The young seedlings were then transferred to fresh medium supplemented with 10 mM NH$_4$Cl (treatment with ammonia) and allowed to grow under the same conditions for the indicated times.

Extraction of Proteins from Root Tissues—Approximately 1.5 g of frozen root tissues were ground with a chilled mortar and pestle with 5% (w/w) polyacrylamide gel and a small amount of quartz sand in an equal volume of extraction buffer (50 mM Tris-HCl (pH 7.5), 20 mM MgCl$_2$, 15% (w/w) polyclar AT and 2% (v/v) methanol, 20 mM sodium phosphate buffer (pH 7.4), and 2% (v/v) potassium phosphate buffer (pH 7.5), 10 mM ATP, 20 mM MgCl$_2$, 15 mM L-glutamate, and 10 mM NH$_4$Cl at 30 °C for an appropriate time. The reaction was terminated by boiling for 2 min and then insoluble materials were removed by centrifugation. Twenty μl of the supernatant were reacted with a solution of o-phthalaldehyde (350 μM borate buffer (pH 9.5), 20 mM o-phthalaldehyde, 10% (v/v) methanol, 300 mM 2-mercaptoethanol) for 20 min, and the resulting derivative of glutamine was purified by HPLC on a reverse-phase column (Synchron C18; 2.1 mm inside diameter × 100 mm; SynChrom Inc., Tokyo, Japan) with 22% (v/v) methanol, 20 mM sodium phosphate buffer (pH 7.4), and 2% (v/v) tetrahydrofuran as an eluent at a flow rate of 50 μl/min. The glutamine derivative in the eluate was detected by monitoring absorbance at 340 nm. One unit of GS activity was defined as the amount of enzyme that produced 1 μmol of L-glutamine per min under the conditions of the reaction for assays of GS synthetase and GS transferase activities or of GS biosynthetic activity, respectively. Protein was quantitated by Bradford’s method (21) with bovine serum albumin as the protein standard.

Extraction of Total RNA and Dot-Blot Analysis—Total RNA was extracted from 1 g of root tissue, harvested at various times during treatment with ammonia, by the method of Wadsworth et al. (22). The RNA (5 μg per dot) was blotted onto a set of nylon membranes (Hybond N’; Amersham, Buckinghamshire, UK) with an RNA dot-blot apparatus (MilliBlot-D; Millipore). Each blot was probed with a $^{32}$P-labeled subfragment of a cDNA that included the 3′-untranslated region of the cDNA (9). Hybridization and washing of the membranes were performed as described previously (23). Changes in the relative levels of hybridizable RNAs were determined with a Bio Imaging Analyzer (BAS2000; Fuji Photo Film, Kanagawa, Japan).

Construction of Expression Plasmids—cDNA fragments encoding the GS1a, GS1b, GS1c, and GS1d proteins (9) were inserted at the NcoI site of an expression plasmid, pTrc99A (Pharmacia), which contained an IPTG-inducible strong trc promoter. For the construction of pTrcGS1c and pTrcGS1d, an NcoI site was created at the position of an ATG initiation codon by site-directed mutagenesis and PCR. Sequences of the forward and reverse primers for PCR were 5′-TACCATG-GCTAGTTAAGCAGCT-3′ and 5′-GATCTCTCGAGAATTGATG-3′, respectively. The forward primer was designed to have an NcoI site at the ATG initiation codon and to have an AT-rich structure without any changes in the encoded amino acid sequence for higher level expression (24), and the reverse primer included an XhoI site. PCR was carried out for 30 cycles at 94 °C for 1 min, 37 °C for 2 min, and 72 °C for 2 min in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT) with Tth DNA polymerase (TOYOBO, Osaka, Japan). The amplified DNA fragment was digested with NcoI and XhoI and then ligated into the expression plasmid.

Expression of Maize GS Proteins in E. coli—The JM105 strain of E. coli was used as the host for the manipulation of genes and the expression of GS. Transformants were grown in Luria-Bertani medium, which was supplemented with 50 μg/mL ampicillin to stabilize pTrc99A, and the expression of maize genes for GS was induced by incubation with 1 mM IPTG at 37 °C for 12 h. These procedures and the preparation of crude extracts of E. coli cells were carried out as described previously (25) with the exception that all buffers contained 1 mM MnCl$_2$ in place of EDTA.

Purification of Maize GS from Roots and E. coli Cells—A crude extract of 60 g of root tissues or of the bacterial cells obtained from 400 ml of culture was fractionated by precipitation with ammonium sulfate between 30% and 70% saturation. The precipitate was suspended in a small volume of buffer A (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM MnCl$_2$, and 7 mM 2-mercaptoethanol) and dialyzed overnight against the same buffer. The dialyzed solution was centrifuged to remove undissolved material and loaded onto a column of Mono Q (FPLC system; Pharmacia) that had been equilibrated with buffer A, and the column was eluted with a linear gradient of NaCl from 50 to 400 mM. Pooled fractions containing GS activity were loaded on an anion exchange affinity column that had been prepared as described by Palacios (26). After the column had been washed with buffer A, GS was eluted with buffer B that contained 20 mM AMP. The GS fraction was further purified on Superose 12 (FPLC system) in buffer A that contained 150 mM NaCl.

Reduction and Carbamylation of GS and Analysis of Proteolytic Digests by Capillary LC/ESI-MS—About 50 μg of purified GS were dialyzed against distilled water and then desiccated by vacuum centrifugation. A ρmol of a reduced and carbamylated protein was prepared by the method of Crestfield et al. (27). The carbamylation protein was loaded on a reverse-phase column (Develosil 300 C4-HG-5; 4.6 mm inside diameter × 150 mm; Nomura Chemical, Aichi, Japan) that formed part of an HPLC system (model LC204 gradient system; Waters, Bedford, MA) and eluted with a linear gradient of 20–80% acetonitrile (1%/min) in 0.1% trifluoroacetic acid at a flow rate of 1 μl/min. The main peak fractions were collected and evaporated to dryness. The dried protein was dissolved in 15 μl of 8 M urea. The solution was supplemented with an equal volume of 100 mM Tris-HCl (pH 9.5) and 1 μl of a solution of 20 pmol/μl lysyl endopeptidase, and then it was incubated for 12 h at 37 °C. The resultant digest was loaded on a capillary column of C$_{9}$ (Develosil 300 ODS-5; 300 μm inside diameter × 150 mm; Nomura Chemical) and fractionated by elution with a linear gradient of 10%–60% acetonitrile. The eluant was monitored at 214 nm with a FOCUS UV detector (Spectra Physics Analytical, San Jose, CA). LC/ESI mass spectra were obtained with a double-focusing mass spectrometer (JMS-HX/HX110A; JEOL, Tokyo, Japan) equipped with an ESI source (Analytica of Branford, Branford, CT) by directing the effluent from the UV detector to the ESI source. All other details have been described elsewhere (28, 29).

Other Techniques—The conventional techniques for manipulation of DNA were those described by Sambrook et al. (30). SDS-polyacrylamide gel electrophoresis and Western blotting were performed by the methods of Laemmli (31) and Towbin et al. (32), respectively. Details of conditions for detection of GS polypeptides were described previously (9).
GS biosynth is reflected by the results of the assay of GS synth. GS r has a higher ratio of GS synth to GS trans than GS 1. Since GS 1 remained almost constant during the 35-h period (Fig. 1), a relative activity of 1 corresponds to a synthetase activity (●) and a transferase activity (○) of 1.4 × 10⁻² and 3.6 units/g fresh weight, respectively. In B, proteins equal to 1.5 mg fresh weight of roots were loaded onto each lane of an SDS-polyacrylamide gel. The isoforms of GS were detected immunologically as described previously (9).

RESULTS

Induction of GS and Changes in Enzymatic Activities of the Isoforms during Treatment of Roots with Ammonia—We demonstrated previously that, in maize roots, treatment with either nitrate or ammonia induces a novel cytosolic isoform of GS, GS r, which can be distinguished from the constitutively expressed isoform, GS 1, by SDS-PAGE (11). Two different GS activities, GS synth and GS trans, were measured, with simultaneous monitoring of the levels of the polypeptides of the GS isoforms in crude extracts of root tissues that had been harvested at various times after the start of feeding with ammonia. As shown in Fig. 1A, GS synth and GS trans increased up to 5.1-fold and 1.9-fold, respectively, after 35 h. The difference in the extent of the increase in the respective activities seemed to be attributable to the accumulation of GS r because the level of GS 1 remained almost constant during the 35-h period (Fig. 1B). With respect to catalytic activity, these results imply that GS r has a higher ratio of GS synth to GS trans than GS 1. Since GS biosynth is reflected by the results of the assay of GS synth, rather than of GS trans, the preferential increase in GS synth would seem to favor the efficient assimilation of ammonia in roots.

Differential Responses of the Genes for Cytosolic Isoforms of GS to the Presence of Ammonia—Five cDNAs that potentially encode the cytosolic isoforms of GS have been isolated, namely, pGS 1a, pGS 1b, pGS 1c, pGS 1d, and pGS 1e. The effects of the presence of GS biosynth on the levels of the corresponding mRNAs were investigated by dot-blot analysis of total RNA extracted from roots (Fig. 2). GS 1c and GS 1d mRNAs began to accumulate within 1 h after the addition of ammonia, reaching their highest levels in 6 h and then gradually decreasing. This result is consistent with the previous result that no significant accumulation of GS 1c mRNA was observed after 3 days (11). The levels of the mRNAs for GS 1a and GS 1b decreased, and that of mRNA for GS 1e was very low or undetectable. A good correlation in terms of the inducibility by ammonia was found between the level of the GS r polypeptide and the levels of the transcripts that corresponded to the GS 1c and GS 1d genes.

Expression of cDNAs for Isoforms of GS in E. coli—The four cDNAs, pGS 1a, pGS 1b, pGS 1c, and pGS 1d, were expressed in E. coli under the control of the pTrc promoter. As shown in Fig. 3, high levels of GS polypeptides were obtained. All polypeptides were enzymatically active (data not shown). The mobilities of GS 1a/GS 1b and GS 1c/GS 1d on SDS-PAGE corresponded closely to those of authentic GS 1 and GS r, respectively, from maize roots. This result suggests that GS r is encoded by the GS 1c gene and/or the GS 1d gene, as do the patterns of induction described above. However, identification of the genes for the isoforms of GS on a chemical basis is necessary for an unequivocal conclusion.

Chemical Analysis of the Isoforms of GS from Maize Roots and from E. coli Cells—GS 1 and GS r were the major isoforms from control and ammonia-treated roots, respectively (Fig. 4). The amino-terminal sequences of GS 1 and GS r could not be determined by Edman degradation, probably because of blockage of the respective amino termini. We then attempted to analyze the GS polypeptides by peptide mapping in combination with mass spectrometry. This analysis enabled us to relate GS 1 and GS r to the corresponding gene products on the basis of the molecular weights of the various proteolytic peptides.

Since we failed to separate the isoforms of GS in their native forms, these enzymes were reduced and carboxymethylated, and then they were separated by reverse-phase liquid chromatography. As shown in Fig. 5, the preparation of GS from noninduced roots gave three peaks, and the last peak was clearly elevated in the preparation of GS from ammonia-treated roots. The polypeptides in the first two peaks and the last peak were purified and subjected to peptide mapping. The primary structures of GS 1a and GS 1d were found to be 99% and 96% identical to those of GS 1b and GS 1c, respectively (9).

Digests of Fractions I and II were each separated into about 15 peaks with distinct patterns (Fig. 6, A and B). The peptide maps of Fraction I and Fraction II were identical, for the most
part, to those of GS1a and GS1d, respectively (Fig. 6, C and D). All peptides whose molecular weights were determined could be equated to segments of the deduced amino acid sequences of GS1a and GS1d (Tables I and II), confirming the correspondence between GS1a and GS1 and between GS1d and GSr. Two peptides, marked with an asterisk in Fig. 6B, were located in sequences unique to GS1c, which exhibits 96% sequence identity to GS1d (Fig. 6B and Table II). Therefore, we concluded that GSr consisted of GS1c and GS1d. We presume, similarly, that GS1 consisted of GS1a and GS1b.

During the feeding of roots with ammonia, the levels of mRNAs for GS1a and GS1b decreased substantially whereas the level of the GS1 polypeptide remained almost constant (Fig. 2). The absence of a correlation between the level of GS1 and the levels of mRNAs for GS1a and GS1b (Figs. 1B and 2) was probably due to differences in the stabilities of the protein and the mRNAs.

Comparison of the Enzymatic Properties of the Isoforms of GS—The recombinant isoforms of GS allowed us to characterize the enzymatic properties of the authentic isoforms of GS from roots, which were hardly separable from each other in their native forms. The two recombinant enzymes were assayed for $K_m$ values for $\text{NH}_4^+$ and L-glutamate.
mate in \( G_{\text{biosynth}} \) were found between GS1a and GS1d. ATP is known to be an allosteric effector of plant GS (33). Therefore, the \( K_m \) value for ATP could not be determined. However, the saturation curves for the activities of GS1a and GS1d at various concentrations of ATP were essentially identical (data not shown).

In contrast to the above-described similarities, significant differences in specific activity were found: the \( G_{\text{trans}} \) of GS1d was 0.77-fold lower than that of GS1a, but both the \( G_{\text{biosynth}} \) and \( G_{\text{synth}} \) of GS1d were 1.6-fold higher than those of GS1a. Authentic GSs purified from ammonia-treated and from nontreated roots were also assayed. The specific \( G_{\text{biosynth}} \) and \( G_{\text{synth}} \) of the GS fraction from ammonia-treated roots was 1.5-fold higher than that of the GS fraction from nontreated roots, whereas the specific \( G_{\text{trans}} \) of each of the two fractions was almost identical (data not shown). These results confirm our initial hypothesis that the increase in synthetase activity that occurs during treatment of roots with ammonia is most probably due to the induction of GSR.

Fig. 7 shows the effect of the concentration of \( \text{Mg}^{2+} \) ions on the activity of the isoforms of GS. When the concentration of \( \text{Mg}^{2+} \) ions was varied, each isoform generated a sigmoidal curve of activity, as observed in the case of GS from rice (34). The maximum activities were observed at about 15 mM \( \text{Mg}^{2+} \), and no significant difference was found between the two isoforms. The \( \text{Mn}^{2+} \)-dependent activities could not be measured at neutral pH because the optimum pH for the \( G_{\text{synth}} \) of plant GS in the presence of \( \text{Mn}^{2+} \) is shifted to an acidic pH (34). The cytosolic isoform of maize GS was almost inactive in the presence of \( \text{Mn}^{2+} \) ions at pH values above 6.5 (data not shown). This result suggests that plant GS utilizes \( \text{Mg}^{2+} \) ions preferentially for its catalytic activity and requires \( \text{Mn}^{2+} \) ions for other phenomena, such as the formation of the appropriate conformation within the physiological range of pH values.

Differences between the Isoforms of GS in Heat Stability and in the Maintenance of the Quaternary Structure at the Low Concentrations of \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) Ions—In addition to the kinetic analysis, we compared the physicochemical properties of the two GS isoforms. We examined the thermal stability of GS1a and GS1d (Fig. 8). GS1d was clearly more heat-labile than GS1a; GS1d was rapidly inactivated above 42°C whereas GS1a remained active even after a 60-min incubation at 42°C. Differences in the stability of the isoforms of GS were also found at low concentrations of divalent cations (Fig. 9). In the presence of 2 mM \( \text{Mg}^{2+} \) or 1 mM \( \text{Mn}^{2+} \) ions, GS1a and GS1d

![Fig. 5. Elution of reduced and carboxymethylated GS polypeptides from untreated (A) and ammonia-treated (B) roots during reverse-phase liquid chromatography.](image1)

![Fig. 6. Chemical analysis for the assignment of cytosolic isoforms of GS to the cloned cDNAs for GS by peptide mapping in combination with capillary LC/ESI-MS. Patterns of elution of lysyl endopeptidase digests of Fraction I (A), Fraction II (B), GS1a (C), and GS1d (D) are shown. The material in each peak was analyzed by ESI-MS. Peaks marked L (A and C) and K (B and D) contained peptides whose sequences were located within the amino acid sequences of GS1a and GS1d, respectively. Peaks marked with an asterisk in panel B contained peptides whose sequences were located within the sequence of GS1c (see Table II).](image2)
TABLE I
Theoretical molecular weights of proteolytic fragments of GS1a and the measured values for those in peaks of GS1a and Fraction I

| Peptide                   | Observed | Theoretical |
|---------------------------|----------|-------------|
|                           | GS1a     | Fraction I  | GS1a     |
| L7 (Arg107-Lys112)        | 694.7    | ND          | 694.4    |
| L13 (Ala204-Lys227)       | 539.3    | ND          | 530.3    |
| L4 (Leu50-Lys52)          | 356.2    | ND          | 356.2    |
| L3 (Ala57-Lys69)          | 1338.0   | 1338.0      | 1337.7   |
| L12 (Glu260-Lys267)       | 893.5    | 893.5       | 893.5    |
| L5 (Trp53-Lys79)          | 2972.4   | 2973.0      | 2973.2   |
| L3 (Ala37-Lys49)          | 1338.1   | 1338.6      | 1338.6   |
| L13 (Ala268-Lys272)       | 530.3    | ND          | 530.3    |
| L10 (Ser166-Lys177)       | 1406.6   | 1406.8      | 1406.7   |
| L2 (Ile113-Lys137)        | 2928.4   | 2928.2      | 2938.6   |
| L16 (Ala178-Lys237)       | ND       | 6497.4      | 6497.4   |
| L8 (Ile19-Lys36)          | 2009.6   | 2009.2      | 2009.4   |
| L14 (Leu273-Lys326)       | 6072.8   | 6074.7      | 6074.7   |
| L11 (Ala178-Lys259)       | 8869.3   | 8869.7      | 8869.0   |
| L17 (Pro238-Lys259)       | ND       | 2388.8      | 2389.6   |
| L15 (Glu327-Asn357)       | 3473.4   | 3473.4      | 3475.0   |
| L12 (Glu75-Lys106)        | 2546.5   | 2546.3      | 2546.9   |
| L8 (Arg107-Lys112)        | 694.7    | ND          | 694.4    |
| L5 (Val113-Lys177)        | 2972.4   | 2973.0      | 2973.2   |
| K10 (Asp138-Lys165)       | 2967.0   | 2966.4      | 2967.3   |
| K11 (Ala166-Lys177)       | 1376.8   | 1376.8      | 1376.7   |
| K19 (Glu75-Lys106)        | 3085.2   | 3086.1      | 3086.3   |
| K7 (Gly42-Lys52)          | 1151.6   | 1152.4      | 1151.6   |
| K5 (Trp32-Lys79)          | 1338.6   | 1338.6      | 1338.7   |
| K12 (Ala110-Lys137)       | 6832.2   | 6833.7      | 6832.9   |
| K13 (Ala178-Lys237)       | 6494.4   | 6493.9      | 6494.5   |
| K6 (Asp80-Lys84)          | 661.4    | 661.4       | 661.4    |
| K11 (Gly85-Lys106)        | 2509.8   | 2509.7      | 2509.8   |
| K12 (Ala178-Lys240)       | 6832.2   | 6833.7      | 6832.9   |
| K14 (Gly42-Lys52)         | 1579.4   | 1580.0      | 1579.6   |
| K21 (Gly327-Asn357)       | 3518.7   | 3519.6      | 3518.9   |
| K15 (Sev305-Asn326)       | 2935.4   | 2936.0      | 2936.2   |
| K13 (Ala178-Lys237)       | 6494.4   | 6493.9      | 6494.5   |
| K14 (Gly42-Lys52)         | 1579.4   | 1580.0      | 1579.6   |
| K15 (Sev305-Asn326)       | 2935.4   | 2936.0      | 2936.2   |
| K16 (Ala178-Lys237)       | 6494.4   | 6493.9      | 6494.5   |

* Numbering of amino acid residues is taken from the work of Sakakibara et al. (9).
ND, not determined.

Effects of the concentration of Mn2⁺ ions on the activities of GS1a and GS1d. The synthetase activity of GS of the purified recombinant forms of GS, namely, GS1a (●) and GS1d (○), was assayed at various concentrations of Mn⁵⁺ ions. The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 20 mM ATP, 50 mM l-glutamate, 6 mM NH₄OH, and various concentrations (0.5–40 mM) of MgSO₄, EDTA. The activity of GS1d decreased at concentrations of Mn⁵⁺ ions below 2 mM and of Mn⁵⁺ ions below 0.5 mM, and less than 20% of the activity remained in the presence of EDTA. To identify the cause of the decrease in the activity of GS1d at lower concentrations of divalent cations, we compared the patterns of elution of GS1a and GS1d from a gel filtration column (Fig. 10). In the presence of 1 mM Mn⁵⁺ ions, both GS1a and GS1d were eluted as octamers with molecular weights of 300,000 to 350,000 at pH 7.6. After the addition of 5 mM EDTA, the assembly of the subunits of GS1d into octamers was less effective at pH 7.6, and the octamers were completely dissociated into monomers, with a molecular weight of about 40,000, at pH 8.6. The dissociated form of GS1d had no significant enzymatic activity. By contrast, the octameric structure of GS1a was maintained, without loss of enzymatic activity, under these conditions. These results suggest that the decrease in the activities of GS1d at lower concentrations of Mn⁵⁺ and Mg⁵⁺ ions was caused by dissociation of the octameric aggregates of the subunits. The two GS isoforms were thus demonstrated to be remarkably different in terms of the stability of their respective quaternary structures.
DISCUSSION

Glutamine synthetase is encoded by a small multigene family, as are many other plant proteins. In most cases, the mode of expression of members of such gene families varies among organs and tissues and in response to environmental changes (5–9, 11). Previous studies of the GS multigene family have predominantly involved the analysis of gene-regulatory systems, and little is known about the physiological importance of the gene products in plant metabolism. In particular, it has been difficult to study the biochemical characteristics of the isoforms since they cannot easily be purified separately from one another. In this study, assignment of the GS isoforms to their genes by LC/ESI-MS analysis and the preparation of recombinant enzymes enabled us to analyze the isoforms individually. We found that the increase in GS activity in maize roots in response to treatment with ammonia was due to the accumulation of a root-specific form, GSr, which had higher GS biosynth than GS1, which was expressed constitutively.

During the development of nodules in P. vulgaris, the γ-polypeptide of GS accumulates preferentially, and heterooligomers of GS that contain the γ-polypeptide yield higher ratios of GSsynth to GStrans than the GS in non-nodulated roots (35). Induction by ammonia of isoforms of GS has been reported in some other leguminous plants, namely, Glycine max (36) and Pisum sativum (37), although the inducibility is still a subject of some controversy (38, 39). The cited studies do not mention enzymatic parameters, such as changes in the ratio of GSsynth to GStrans, $K_m$ values for the substrates, or physicochemical stability. Changes in the ratio of GSsynth to GStrans have been reported in other eukaryotic GSs. In yeast, the ratio of GSsynth to GStrans is affected by the nitrogen source (40); ammonia or glutamine in the growth medium decreases the ratio of GS synth to GStrans. In this case, the change has been attributed to regulation at both the transcriptional and the post-translational level. In Rhizobium leguminosarum biovar viceae, the addition of ammonia to the culture medium decreases the transferase activity of GSII, a eukaryotic type of GS, by a post-translational manner (41).

In this study, we found that GSr was more heat-labile than GS1 (Fig. 8). The sensitivity to heat of the GS activity purified from roots has also been reported in rice (34), suggesting that such lability might be a common property of the root-specific isoform of GS. We also found that the octameric structure of GSr was less stable than that of GS1 at lower concentrations of divalent cations (Figs. 9 and 10). This report is, to our knowledge, the first to describe differences in the structural stability of the isoforms of GS in the presence and in the absence of divalent cations and at various pH values. GS1a (upper panel) and GS1d (lower panel) were injected onto a gel filtration column of Superose 12 that had been equilibrated with 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM MnCl$_2$ (A); 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA (B); and 20 mM Tris-HCl (pH 8.6), 150 mM NaCl, 5 mM EDTA (C). Elution was monitored in terms of the absorption at 280 nm (solid line). Fractions (0.5 ml) were collected and assayed for the transferase activity of GS. The activity is indicated by solid bars.

FIG. 9. Stability of the isoforms of GS in the presence and in the absence of Mg$^{2+}$ and Mn$^{2+}$ ions. A, GS1a (left panel) and GS1d (right panel) were incubated in 25 mM Tris-HCl, 150 mM NaCl with 2 mM MgCl$_2$ at pH 7.4 (●) or pH 8.2 (○), with 1 mM MnCl$_2$ at pH 7.4 (■) or pH 8.2 (□), and with 5 mM EDTA at pH 7.4 (▲) or pH 8.2 (△) for the indicated times. To complete the incubation, 20 volumes of the reaction mixture for the GS transferase assay (100 mM Tris acetate (pH 6.4), 100 mM l-glutamine, 0.5 mM ADP, 1 mM MnCl$_2$, 20 mM sodium arsenate) were added to the incubated solution, and then the GS transferase activity was assayed. B, GS1a and GS1d were incubated in 25 mM Tris-HCl (pH 7.4), with 150 mM NaCl and various concentrations of MgCl$_2$ (●, GS1a; □, GS1d) or MnCl$_2$ (○, GS1a; ◆, GS1d) for 40 min and assayed for the transferase activity of GS. EDTA of 5 mM was added to the incubation mixture without the divalent cations (0 mM).

FIG. 10. Dissociation of the quaternary structure of the isoforms of GS in the presence and in the absence of divalent cations and at various pH values. GS1a (upper panel) and GS1d (lower panel) were incubated in 25 mM Tris-HCl, 150 mM NaCl, 1 mM MnCl$_2$ (A); 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA (B); and 20 mM Tris-HCl (pH 8.6), 150 mM NaCl, 5 mM EDTA (C). Elution was monitored in terms of the absorption at 280 nm (solid line). Fractions (0.5 ml) were collected and assayed for the transferase activity of GS. The activity is indicated by solid bars.
of isoforms of plant GS. Octameric aggregates of the subunits of GSr (GS1d) were easily dissociated to monomers below 2 mM Mg$^{2+}$ or 0.5 mM Mn$^{2+}$ ions in vitro (Fig. 9B). The difference in the concentrations of the cations needed for maintenance of the quaternary structures might be physiologically significant with respect to the difference in stability between GS1 and GSr in vivo. If the lower stability of GSr were reflected by rapid turnover in vivo, GSr could be considered to be a superior isoform in terms of its ability to participate in the efficient assimilation of ammonia only when it is needed. The physiological significance of this instability awaits the results of future investigations. It will be of interest to identify the structural determinant(s) responsible for the kinetic and physicochemical differences between the two isoforms of maize GS, the amino acid sequences of which are 86% identical.

Glutamine synthetase occupies an important position in nitrogen metabolism in various organisms, and, therefore, a complicated regulatory system, which includes a variety of controls of gene expression and enzymatic properties, has evolved to allow plants to adapt to environmental changes related to nitrogen metabolism. Further studies focusing on the protein structure of plant GS should result in a better understanding of the physiological significance of the diversity of genes for cytosolic GS.

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