Plant Physiol. (1996) 112: 1723–1733

Total Glutamine Synthetase Activity during Soybean Nodule Development Is Controlled at the Level of Transcription and Holoprotein Turnover[1]

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Gln synthetase (GS) catalyzes the ATP-dependent condensation of ammonia with glutamate to yield Gln. In higher plants GS is an octameric enzyme and the subunits are encoded by members of a small multigene family. In soybeans (*Glycine max*), following the onset of N_2 fixation there is a dramatic increase in GS activity in the root nodules. GS activity staining of native polyacrylamide gels containing nodule and root extracts showed a common band of activity (GSrs). The nodules also contained a slower-migrating, broad band of enzyme activity (GSns). The GSns activity band is a complex of many isozymes made up of different proportions of two kinds of GS subunits: GSr and GSn. Root nodules formed following inoculation with an Nif$^-$ strain of *Bradyrhizobium japonicum* showed the presence of GS isoenzymes (GSns1) with low enzyme activity, which migrated more slowly than GSns. Gsns1 is most likely made up predominantly of GSn subunits. Our data suggest that, whereas the class I GS genes encoding the GSr subunits are regulated by the availability of NH_3, the class II GS genes coding for the GSn subunits are developmentally regulated. Furthermore, we have demonstrated that the GSns1 isozymes in the Nif$^-$ nodules are relatively more labile. Our overall conclusion is that GSns activity in soybean nodules is regulated by N_2 fixation both at the level of transcription and at the level of holoprotein stability.

GS (EC 6.3.1.2) is a key enzyme in the assimilation of NH_3, catalyzing the ATP-dependent condensation of NH_3 with glutamate to yield Gln (Lea et al., 1990). The NH_3 is derived from symbiotic N_2 fixation, the reduction of NO_3^- or NO_2^-, photorespiration, or amino acid catabolism (Hirel et al., 1993). The reaction is believed to proceed via a two-step process, the first involving the formation of GS-bound glutamyl phosphate from ATP and glutamate, followed by the addition of NH_3 to form a tetrahedral adduct with the subsequent liberation of Gln, ADP, and Pi (Lea and Ridley, 1989).

Bacterial GS is a dodecamer (622 kD) that is assembled in two face-to-face hexameric rings. The 12 active sites of the GS holoenzyme are located at heterologous subunit interfaces in a side-to-side configuration within the hexameric ring. Each active site is formed by six antiparallel strands contributed by one subunit and two strands contributed by the neighboring subunit (Yamashita et al., 1989). Plant GS is an octamer (320–380 kD), and the subunits are probably assembled as two tetramers stacked one upon another, with the active site in the interface of subunits (as in the case with bacterial GS). The reaction mechanism catalyzed by bacteria and plant GS is probably similar (Meister, 1980; Meek and Villafranca, 1980), based on the fact that the amino acid residues involved in the proposed mechanism are invariant between the two enzymes, even though they share only up to 20% identity in the rest of the amino acid sequence (Sanangelantoni et al., 1990).

GS in plants occurs as a number of isoenzymes and is encoded by a small multigene family (Gebhardt et al., 1986; Tingey et al., 1987, 1988; Lightfoot et al., 1988; Bennett et al., 1989; Walker and Coruzzi, 1989; Peterman and Goodman, 1991; Roche et al., 1993; Temple et al., 1995). Based on the subcellular location, GS can be broadly categorized as GS_2 (plastid localized) or GS_1 (cytosol localized) (Cullimore and Bennett, 1992). The different isoforms of GS are found in different organs or cell types of the plant and assimilate the NH_3 produced by different physiological processes (Lea et al., 1990; Hirel et al., 1993). The different GS isoforms probably also have different physiological requirements for optimal activity.

In root nodules, the primary function of GS is the rapid assimilation of NH_3 released into the cytosol of the infected cells by N_2-fixing bacteroids (Atkins, 1987). Genes encoding the GS isoenzyme in the nodules have been characterized in many legumes (Bennett et al., 1989; Grant et al., 1989; Walker and Coruzzi, 1989, Roche et al., 1993; Stanford et al., 1993; Temple et al., 1995), and it appears that in most cases the genes are regulated by developmental cues. In soybean (*Glycine max*), there are two classes of GS_1 genes that are expressed in the nodules, a nodule-specific class that is developmentally regulated, and a second class that

[1] This work was supported by a grant from the U.S. Department of Agriculture (no. 92.37305-7941). C.S.G. also acknowledges support from the Agriculture Experiment Station at New Mexico State University.

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Abbreviations: DAI, days after inoculation; GS, Gln synthetase; Nif$^-$, nitrogenase-deficient *Bradyrhizobium* mutant; UTR, untranslated region.

is constitutively expressed (Roche et al., 1993; Marsolier et al., 1995). A member of the latter class has been shown to be up-regulated in soybean roots treated with NH_4NO_3 (Hirel et al., 1987) and in the functional nodules of transgenic *Lotus corniculatus* (Miao et al., 1991). NH_3-regulated GS_1 genes have not been reported for any other legume system. There have, however, been some reports of metabolite-regulated GS_1 genes in corn (Sukanya et al., 1994) and rice (Kozaki et al., 1991).

Very little is known about the regulatory mechanisms controlling plant GS at the translational level or at the level of holoenzyme assembly and protein turnover. In gram-negative bacteria, GS has been shown to be regulated by cumulative feedback inhibition, covalent modification, and repression/derepression (Shapiro and Stadtman, 1970; Stadtman, 1990). There are, however, few reports concerning the posttranslational regulation of GS in plant systems (Hemon et al., 1990; Swarup et al., 1990; Hoelzle et al., 1992; Temple et al., 1993). Root GS activity in French bean, pea, and soybean was found to be stimulated significantly following treatment with NH_3 and nitrate, even though equal numbers of GS subunits were present in both nitrogen-treated and untreated roots (Hoelzle et al., 1992). Similar observations have been made in *Lemna minor*, and the authors (Rhodes et al., 1978) suggested that GS protein is maintained in an inactive form unless sufficient nitrogen and carbon skeletons are available for Gln synthesis. Some recent papers have reported instances in which a specific GS_1 transcript level was found to significantly exceed the corresponding protein level, suggesting regulation at a level downstream of transcription (Hemon et al., 1990; Swarup et al., 1990; Temple et al., 1993). Chaperones related to chaperonin-60 have also been implicated in the assembly/folding of GS_2 in the leaves of some plants (Lubben et al., 1989; Tsuprun et al., 1992). Additional evidence for the involvement of chaperones in the assembly of GS comes from the recent report that in vitro renaturation of unfolded *Escherichia coli* GS subunits to native GS is enhanced in the presence of chaperonin-60 and ATP (Fischer, 1992). When bacterial cells are starved for nitrogen, the normally stable GS is one of the enzymes that is turned over (Fulks and Stadtman, 1985), suggesting that intracellular levels of the enzyme are also regulated by proteolysis. Turnover of bacterial GS takes place in two steps; in the first GS is oxidized, and in the second the oxidized GS is proteolytically degraded (Levine, 1983). Nothing is known about GS turnover in plants. However, recent work from our laboratory has shown that soybean GS can be oxidized in vitro and that the oxidized form is more prone to degradation than the nonoxidized form (D. Roche, unpublished data).

Two classes of GS_1 genes have been characterized in soybean, one constitutively expressed (class I) and the other nodule-specific (class II) (Roche et al., 1993; Marsolier et al., 1995). We have extended our initial studies on the regulation of GS_1 genes in soybean nodules to include regulation at the level of enzyme assembly and turnover. The data presented here show that GS activity in soybean nodules increases dramatically soon after the onset of N_2 fixation and that this increase in GS activity is due to the synthesis of GSns isoenzymes. The GSns isozymes are made up of both the class I and class II GS gene products. Our data also suggest that the synthesis of the GSns isozymes is regulated by NH_3 (or a product of its assimilation)-mediated enhanced expression of the class I GS genes and the stabilization of the GSns holoenzyme.

MATERIALS AND METHODS

Plant Growth, Bacterial Culture, and Inoculation

Seeds of soybean (*Glycine max* cv Williams) were purchased from Strayer Seed Farms (Hudson, IA). Wild-type *Bradyrhizobium japonicum* strain USDA 110 was obtained from the U.S. Department of Agriculture (Beltsville, MD). Mutant strain Bj 702 was a generous gift from the late Dr. B. Chelm. Bj 702 is derived from USDA 110 and has a deletion in the *Nif*KD genes (Nif^-) and is thus unable to fix N_2. The growth of the plant tissue, bacterial cultures, and plant inoculation techniques were as described previously (Sengupta-Gopalan and Pitas, 1986). The root nodules were detached from the plants, frozen in liquid N_2, and stored at −70°C.

Determination of GS Activity and Nitrogenase Activity

Nodule tissue (1 g of liquid N_2 frozen material) was ground in a mortar with 4.0 mL of ice-cold 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM magnesium acetate, 1 mM DTT, 10% glycerol, and 5% ethylene glycol containing 1% polyvinylpolypyrrolidone, and the homogenate was clarified by centrifugation. The GS activity of the supernatant was measured using the ADP-dependent transferase assay slightly modified from that of Ferguson and Sims (1971). A 1.0-mL reaction contained 200 mM Tris-acetate (pH 6.4), 35 mM L-Gln, 8.75 mM hydroxylamine, 0.75 mM ADP, 2.25 mM $MnCl_2$, 17.5 mM sodium arsenate, and 1 mM EDTA. The reaction was terminated after 15 min of incubation at 37°C by the addition of 0.5 mL of ferric chloride reagent, followed by centrifugation at 13,000*g* for 2 min. The A_{500} of the supernatant was determined. One unit of GS activity is defined as 1 μmol of γ-glutamyl hydroxamate formed per minute. The protein concentration was determined by the dye-binding method of Bradford (1976) using BSA as a standard. Samples prepared for GS activity measurements were also used for PAGE analysis following precipitation with 10 volumes of ethanol. GS activity was also measured using the biosynthetic assay of Kim and Rhee (1987). Nitrogenase activity was measured using the acetylene reduction assay (Dart et al., 1972).

PAGE

Three different PAGE systems were utilized, all using the Bio-Rad Protean II system. SDS-PAGE according to Laemmli (1970) using a stock solution of 30:0.15 of acrylamide:bisacrylamide instead of 30:0.8 acrylamide:bisacrylamide. Two-dimensional SDS-PAGE was carried out essentially as described by O'Farrell (1975) using 1.6% of pH 5.0 to 7.0 and 0.4% of pH 3.5 to 10.0 Ampholines

(Pharmacia). Two percent 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate replaced Nonidet P-40 in all IEF solutions (Perdew et al., 1983) and the IEF was run overnight (14 h) at 400 V, followed by 1 h at 800 V. The IEF tube gel was equilibrated for 15 min in 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 5% β-mercaptoethanol, and 10% glycerol before being mounted on a 12% SDS-PAGE (30:0.8 acrylamide:bisacrylamide) slab gel. For western analysis, following electrophoresis the proteins were blotted onto nitrocellulose electrophoretically in 25 mM Tris, 192 mM Gly, and 5% methanol (pH 8.2). The nitrocellulose was blocked with 1% BSA in TBS containing 0.05% Tween 20 and probed with antibody against *Phaseolus vulgaris* nodule GS_1 (1:2000 dilution) (Cullimore and Miflin, 1984). Cross-reacting polypeptides were made visible using an alkaline phosphatase-linked second antibody using the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate according to the supplier's instructions (Promega). A native PAGE system using 7.0% slab gels was run in 25 mM Tris and 192 mM Gly overnight at 4°C and 30 mA of constant current. The GS activity on native PAGE was detected using the ADP-dependent transferase assay as described above. Following staining, the activity gels were photographed with Tech Pan film (Kodak) using a blue filter. The electrophoretic transfer and immunological detection of GS proteins on native gels was carried out as described above. The antibody used for native gel western analysis was against pea seed GS (1:1000 dilution) (Langston-Unkefer et al., 1987).

Ion-Exchange Chromatography

Crude extracts from 2 g of nodules were applied to a small column of DEAE-Sephacel (100 × 16 mm) pre-equilibrated in running buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, and 10 mM sodium glutamate). The column was washed with running buffer until no A_{280} was detected in the flow-through. Bound proteins were then eluted by a 50-mL linear, 0 to 400 mM KCl gradient. Fractions of 1.4 mL were collected at 0.8 mL/min. Fractions with activity were pooled by pairs and concentrated on Centriprep cartridges (Amicon, Beverly, MA) by spinning for 1 to 2 h at 4°C, and the protein content was measured in the concentrated fractions. The samples were either used directly for native gels or ethanol-precipitated for two-dimensional SDS-PAGE.

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated using the LiCl precipitation procedure described by De Vries et al. (1982) and was fractionated on 1% agarose/formaldehyde gels and blotted onto nitrocellulose. Hybridization was carried out in 50% formamide at 42°C using standard conditions (Sambrook et al., 1989). Probes were prepared by labeling purified DNA fragments by random priming (Feinberg and Vogelstein, 1983).

In Vitro GS Holoenzyme Stability Studies

For the enzyme stability studies (Fig. 1) soybean nodule tissue (0.5 g of liquid-N_2-frozen material) was ground in a mortar with 2.0 mL of ice-cold, 50 mM imidazole (pH 7.4) and 10 mM $MgCl_2$ containing 1% polyvinylpolypyrrolidone. After the sample was centrifuged the supernatant was assayed for GS activity using the ADP-dependent transferase assay, and protein content was determined as described above. Samples containing 150 μg of soluble protein for native PAGE analysis were removed and stabilized by the addition of glycerol to 10% (v/v). For inactivation studies the supernatant was incubated at 25°C and samples were removed for GS activity determination and native PAGE analysis at the prescribed time intervals. Most of the experiments described in this paper were performed at least three times; a representative experiment has been presented for each section.

RESULTS

Increased GS Activity in Soybean Nodules Is Due to the Synthesis of Novel GS Isozymes

N_2-fixing root nodules are associated with very high levels of GS activity (Robertson et al., 1975; Werner et al., 1980). To determine whether the increased GS activity in soybean nodules is due to novel, nodule-specific GS isoenzymes, total soluble protein extract from nodules 14 DAI and uninfected 4-d-old roots were subjected to native PAGE and then gel-stained for GS activity using the transferase assay (Fig. 1A). The samples were also subjected to native PAGE followed by western analysis using GS antibodies (Fig. 1B). A common band of activity was seen in both the root and nodule extract and we will refer to it as GSrs. In addition to GSrs, the nodule extract also showed a slower-migrating broad band of activity, which we will refer to as GSns. Western analysis showed the presence of numerous distinct immunoreactive bands in the region of GSns activity and extending up in the region with no detectable GS enzyme activity. An immunoreactive band

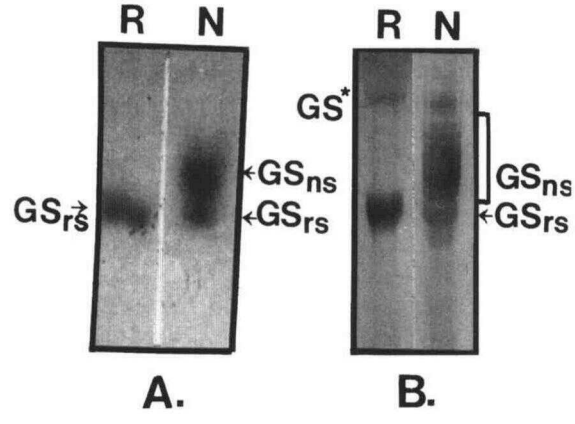

Figure 1. Fractionation of GS isozymes on native gels. A, Total soluble protein extracts (400 μg) from 4-d-old roots (R) and from wild-type nodules 14 DAI (N) were fractionated on a 7% native polyacrylamide gel and the gel was stained for GS transferase activity as described in "Materials and Methods." B, Protein extract (200 μg) for the samples described in A were fractionated by native PAGE, and the proteins were electroblotted onto nitrocellulose and subjected to western analysis using pea seed GS antibody.

migrating slower than GSns and referred to as GS* was detected in both the root and nodule lanes (Fig. 1B). This band did not show any enzymatic activity on the GS activity gel.

Nodule-Specific GS Isozymes Are Made Up of Both Root GS Subunits and Nodule-Specific GS Subunits

To identify and distinguish the nodule-specific GS subunits from the root GS subunits, root and wild-type nodule (12 DAI) extracts were subjected to one-dimensional SDS-PAGE followed by western blot analysis. The one-dimensional SDS-PAGE showed two distinct GS bands (GSr1 and GSr2) in the roots, whereas the nodules showed an additional band (GSn) migrating between the two GSr bands (Fig. 2A). To further fractionate the GS polypeptides, the root and nodule extracts were subjected to two-dimensional SDS-PAGE western blot analysis. The GS subunits in roots migrated as a cluster of two major (GSr1 and GSr2) and a few minor spots (Fig. 2B). In relative terms GSr1 accumulated to higher levels than GSr2 in the roots. In the nodules, the GSn band seen on one-dimensional SDS-PAGE resolved into two major forms (GSn1 and GSn2) and two minor forms when analyzed by two-dimensional SDS-PAGE (Fig. 2B). GSr1 and GSr2, along with the minor spots seen in the root sample, appeared greatly enhanced in the nodule extract.

To determine the subunit composition of the GSns isozymes in the nodules, total soluble protein extract from the wild-type nodule (12 DAI) was fractionated by DEAE anion-exchange chromatography and the fractions with GS enzymatic activity were subjected to native gel and two-dimensional SDS-PAGE western analysis (Fig. 3). On native gels the crude extract showed both the GSrs and GSns isoenzymes and a low level of the immunoreactive band with no GS activity (GS*; Fig. 3B). GSns and GS* eluted early in the salt gradient. The first set of active fractions (nos. 12–17) contained the slowest-migrating component of the GSns subset of isoenzymes and no GSrs. On two-dimensional SDS-PAGE, these fractions showed only GSn1 and GSr2 subunits (Fig. 3, B and C). The faster-migrating components of the GSns isozymes eluted at a higher salt concentration, GSrs isozymes started eluting in fractions 20 and 21, and the last fractions (nos. 32–33) contained only the GSrs isozymes. With an increase in the salt concentration, the level of GSn1 and GSr2 subunits decreased, whereas the level of GSn2 and GSr1 subunits showed a gradual increase. Fractions 32 and 33 were exclusively made up of GSr1 subunits (Fig. 3C). The GS subunit composition of the different fractions eluting off the DEAE column, along with the multiple immunoreactive bands co-migrating with GSns activity (Fig. 1B), suggests that there are multiple GSns isoenzymes and that each of these isoenzymes is probably made up of different combinations of the GSn1, GSn2, GSr1, and GSr2 subunits. The slower-migrating GSns isozymes contained more GSn subunits relative to the faster-migrating isozymes (compare the native gel profile with the two-dimensional SDS-PAGE western profile of the different fractions, Fig. 3, B and C).

Increased GS Activity in Soybean Nodules Is Dependent on N_2 Fixation

To determine whether the increased GS activity is regulated by N_2 fixation, nodules collected at different developmental stages were analyzed for both nitrogenase and GS activity. Nitrogenase activity was first detectable between 8 and 10 DAI with the wild-type strain and rapidly increased during the subsequent 4 d of measurement (data not shown). GS activity showed a slight increase between 8 and 11 DAI, a dramatic increase starting at 11 DAI, and a peak at approximately 20 DAI in nodules formed with the wild-type strain (Fig. 4). To further investigate the relationship between the onset of N_2 fixation and the increase in GS activity, GS activity was measured in nodules formed by an ineffective strain, *Bj* 702, which contains a deletion in the structural nitrogenase gene (Nif^-). As expected, no nitrogenase activity was observed in the nodules formed by the ineffective strain (data not shown). These nodules showed only a very slight increase in GS activity during the developing stages between 9 and 24 DAI (Fig. 4). The data on GS activity presented here are based on the ADP-dependent transferase assay; however, a similar pattern was obtained when GS activity was measured using the synthetase assay (data not shown). These results strongly suggest that a

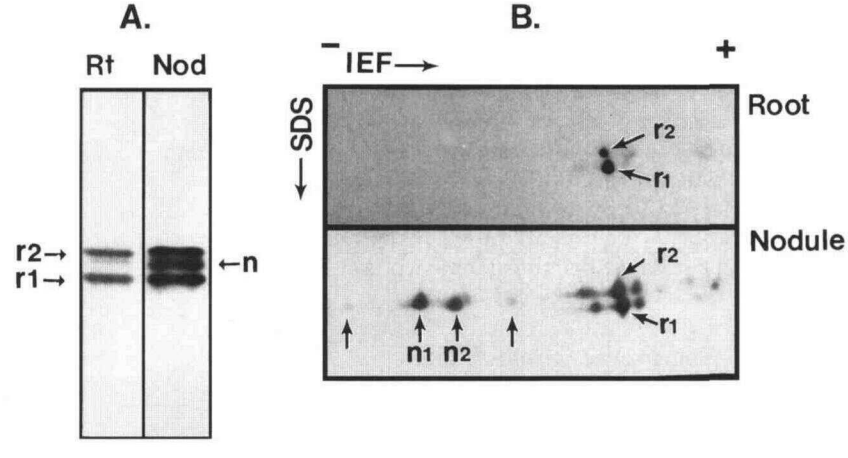

Figure 2. Fractionation of GS polypeptides in roots and nodules of soybean by one-dimensional SDS-PAGE (A) and two-dimensional SDS-PAGE (B). Soluble protein from soybean roots (Rt) and nodules 12 DAI (Nod) were subjected to one-dimensional SDS-PAGE (10 μg/lane) and two-dimensional SDS-PAGE (20 μg/gel). The fractionated proteins were electroblotted onto nitrocellulose and the filter was probed with GS antibody. r1 and r2 refer to the constitutively expressed GS subunits encoded by class I genes, and n1, n2, and n (collectively) refer to the class II GSns.

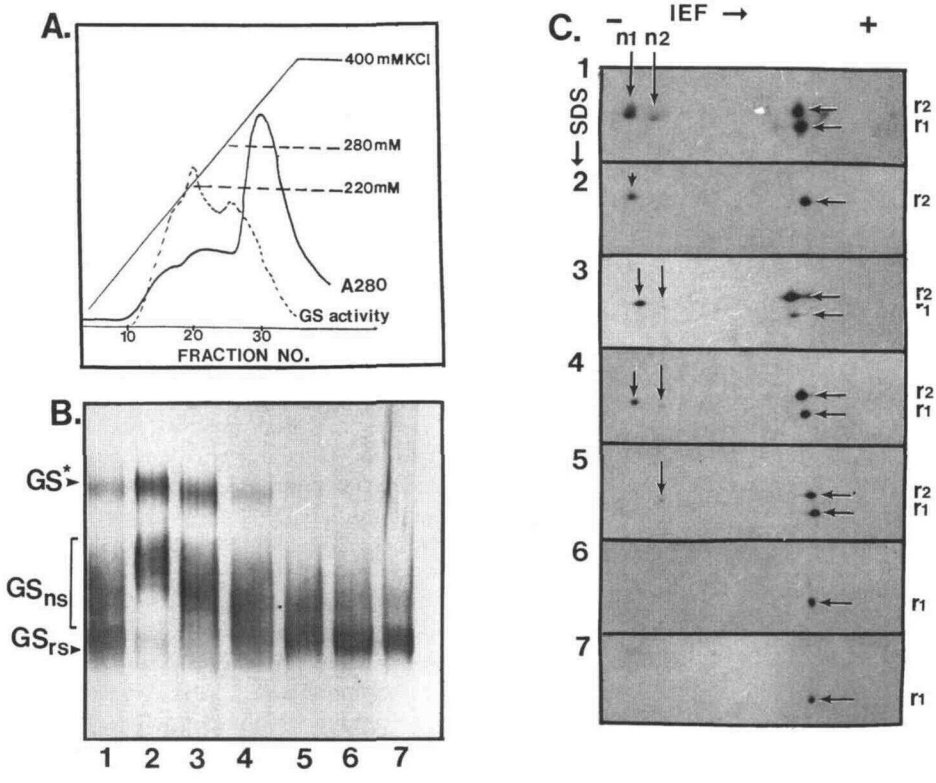

Figure 3. Fractionation of nodule GS isoenzyme by anion-exchange chromatography and analysis of the subunit composition of the different fractions. A, Crude extract of wild-type nodules (12 DAI) was loaded on a DEAE-Sephacel column and the bound proteins eluted with a 50-mL linear KCl gradient (0–400 mM). Fractions (1.4 mL) were collected and assayed for GS activity and protein concentration. The KCl concentration at relative points in the gradient is indicated. GS activity in the fractions is represented by a dashed line, and the protein concentration (A_{280}) is represented by the solid tracing. The fractions containing GS activity were then subjected to native PAGE (B) and two-dimensional SDS-PAGE (C), followed by western analysis using GS antibody. B, Crude extract (CE, 100 μg; lane 1), 25 μg of fractions 12 and 13 (lane 2), and 50 μg of fractions 16 and 17, 20 and 21, 24 and 25, 28 and 29, and 32 and 33 in lanes 3, 4, 5, 6 and 7, respectively, were subjected to native PAGE followed by western analysis. C, The same samples and loading as described in B were subjected to two-dimensional SDS-PAGE followed by western analysis with GS antibody. GSrs and GSns refer to the isoenzymes, whereas r1, r2, n1, and n2 refer to the subunits.

major determinant for the increased GS activity in soybean nodules is active N_2 fixation or a product of the reaction.

Transcripts for the Nodule-Specific GS Genes Accumulate Independently of Nitrogenase Activity, whereas Transcripts for Root GS Genes Exhibit a Nitrogenase-Dependent Increase in Transcript Level

To determine whether the N_2-fixation-dependent increase in GS activity is due to the increased availability of GS transcripts, developing nodules formed by either the wild-type or the Nif$^-$ strains of *B. japonicum* were analyzed for GS transcript levels (Fig. 5). We had shown earlier that there are at least two classes of differentially regulated GS_1 genes that are expressed in the soybean nodules (Roche et al., 1993), one constitutively expressed (class I) and the other expressed in a nodule-specific manner (class II). By analyzing the hybrid-select translation products corresponding to the two classes of GS_1 genes, we also showed that whereas the class I genes encode the GSr subunits, the class II codes for some of the GSn subunits (Roche et al., 1993). To check the expression pattern of these two classes of GS_1 genes, RNA from developing nodules formed by the wild-type and Nif$^-$ strain were subjected to northern analysis using the GS_1 class-specific probes (3′ UTR of a class I and class II representative GS_1 genes). The gels with ethidium-bromide-stained RNA samples were photographed to check the RNA loads (Fig. 5C). The class I probe showed a dramatic increase in hybridization to RNA from wild-type nodules between 10 and 14 DAI, and the high levels were maintained until 28 d postinfection (Fig. 5A). Densitometric scans of the autoradiograms indicated a 2.5-fold increase in class I transcripts in wild-type nodules between 10 and 28 DAI. In the nitrogenase-deficient nodules (9–10 DAI), the level of hybridization with the class I probe was maintained at levels comparable to the levels in wild-type nodules of the same age (Fig. 5A). Starting at 10 DAI, the Nif$^-$ nodules showed a dramatic decrease in hybridization signal (the slightly higher hybridization signal at 16 DAI is due to a slightly higher load of RNA). The hybridization signal in the Nif$^-$ nodules at 21 and 28 DAI was 9-fold lower than in wild-type nodules of the same age.

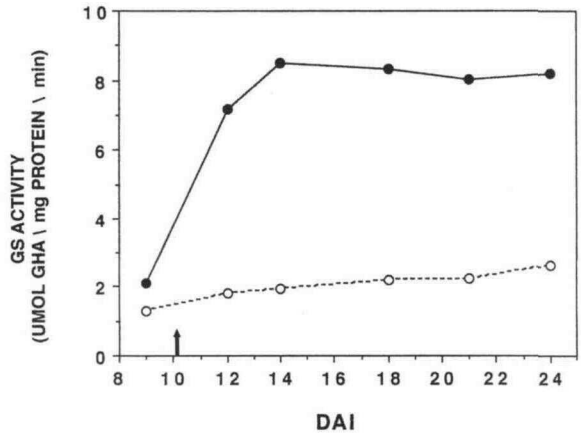

Figure 4. GS activity in relation to nitrogenase activity. Nodules formed by the wild strain of *B. japonicum*, USDA 110 (wild type), and Bj 702, a mutant strain deficient in the *Nif*KD genes (Nif$^-$), at different DAI were analyzed for GS activity. GS transferase activity for the wild-type (●) and Nif$^-$ (○) nodules, expressed as μmol γ-glutamyl hydroxamate formed mg^{-1} protein min^{-1}. The arrow indicates the time when nitrogenase activity was induced under our growth conditions.

The class II-specific probe showed a developmental increase in hybridization between 9 and 16 DAI, after which steady-state levels were maintained in the wild-type nodules. The nitrogenase-deficient nodules showed a lower level of class II transcripts between 9 and 12 DAI compared with the wild-type nodules of the same age, after which the level increased to the same level as in the wild-type nodules and was maintained (Fig. 5B). The hybridizing band with the class II-specific probe is fairly broad, suggesting heterogeneity in the size of the transcripts in this subclass.

Taken together our data suggest that, although the expression of class I GS_1 genes is enhanced by N_2 fixation, transcription of the class II genes is developmentally regulated in the nodules and is independent of N_2 fixation.

Nif$^-$ Nodules Accumulate GSn Subunits but Not GSr Subunits at Levels Comparable to That in the Wild-Type Nodules

To determine whether the GS class I and II transcript accumulation pattern reflects the GS_1 polypeptide levels in the two nodule types, protein extracts from nodules at different developmental stages (9, 12, 14, 18, 21, and 24 DAI) were subjected to one-dimensional SDS-PAGE followed by western analysis. The GS polypeptides in all lanes resolved into the two GSr bands (GSr1 and GSr2) and a GSn band that migrated between them (Fig. 6). All three bands showed a dramatic increase between 9 and 12 DAI in the wild-type strain, after which steady-state levels were maintained (Fig. 6A). With nodules formed by the Nif$^-$ *B. japonicum* strain, the GS level of all of the subunits at 9 to 12 DAI was significantly lower than in wild-type nodules of the same age (Fig. 6B). Although the level of both GSr1 and GSr2 polypeptides showed no increase in developing nodules formed by the Nif$^-$ strain, the GSn band showed a developmental increase in level and maintained levels comparable to that of wild-type nodules (Fig. 6). Equal amounts of protein extracts (12 μg/lane) from developing nodules formed by the wild-type and Nif$^-$ strain were fractionated on the same gel, so the samples could be compared across the gel (Fig. 6, A and B). Figure 6C is a photographic enhancement of Figure 6B that shows the immunoreactive bands in the younger nodules.

To ensure that the GS subunit profile was identical between the two nodule types, protein extracts from wild-type and Nif$^-$ nodules at 12 DAI were also subjected to two-dimensional gel electrophoresis followed by western analysis. As seen in Figure 7, except for the levels, the

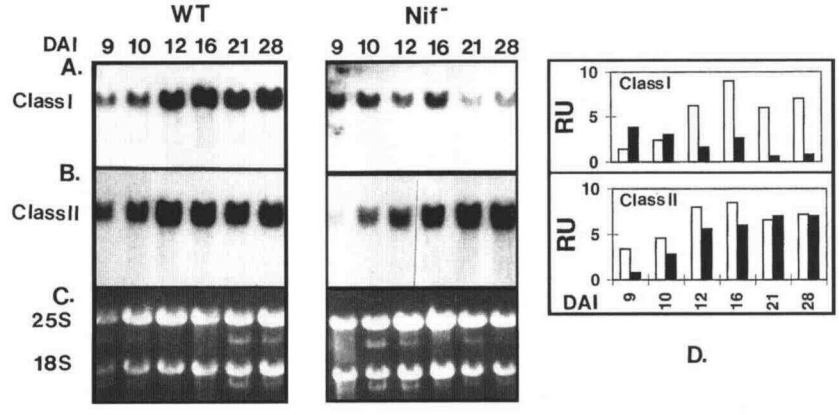

Figure 5. Analysis of GS_1 transcripts in developing nodules of soybean. Total RNA (20 μg), isolated from developing nodules (9, 10, 12, 16, 21, and 28 DAI) formed by either the wild-type (WT) strain or a Nif$^-$ mutant of *B. japonicum* fractionated on duplicate formaldehyde-agarose gels. The gels were blotted onto nitrocellulose and hybridized with ^{32}P-labeled inserts of the 3′ UTR of GS cDNAs for the constitutively expressed class I form (A) or the nodule-specific class II form (B). Representative gels were treated with ethidium bromide and photographed under UV light (C). The 25S and 18S in C refer to the very abundant rRNA. The autoradiograms (A and B) were subjected to densitometric scanning and the data are represented as relative units (RU). The values for the wild-type nodule are represented as open boxes and those for the Nif$^-$ nodules as solid boxes (D).

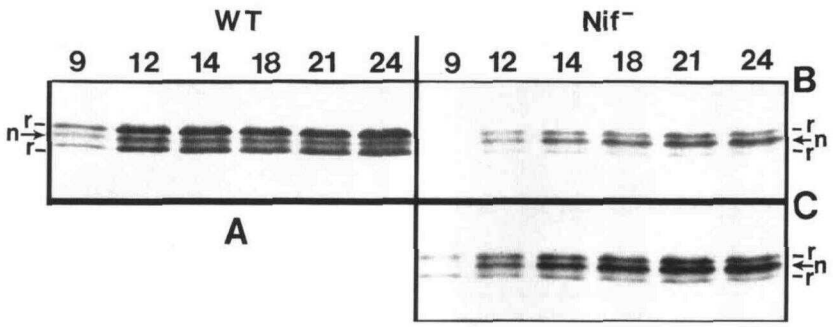

Figure 6. Analysis of the GS polypeptides in developing nodules of soybean. Soluble proteins from developing nodules (9, 12, 14, 18, 21, and 24 DAI) formed following inoculation with either the wild-type (WT) strain (12 μg/lane, A) or the Nif⁻ mutant of *B. japonicum* (12 μg/lane, B) were fractionated by SDS-PAGE, transferred to nitrocellulose, and the filter probed with GS antibody. C is a photographic enhancement of B. The root-expressed GS_1 polypeptides and the nodule specific GS_1 polypeptides are labeled as r and n, respectively.

two-dimensional profile of GS is identical between the two nodule types, with two major and several minor GSr subunits and two major GSn isoforms. The level of the GSn1 and GSn2 polypeptides at 12 DAI, however, was significantly lower in the Nif⁻ nodules compared with the wild-type nodules.

The GSns Isozyme Profile in Wild-Type Nodules Is Different from That in the Nif⁻ Nodules

The GS activity measurements indicated that in the absence of N_2 fixation, GS activity in developing nodules formed by the Nif⁻ strain of *B. japonicum* showed a lower maximal level of activity compared with the wild-type nodules (Fig. 4). This could imply that GSns is not made in the Nif⁻ nodules or that the nodule-specific GS isozymes have no activity. To test this possibility, the GS isozyme profile in the two kinds of nodules at different developmental stages was analyzed by subjecting nodule extracts to native PAGE followed by GS activity staining (Fig. 8) or immunostaining (Fig. 9). While the GSrs activity band remained constant in both nodules at all developmental stages, the broad band of activity that we refer to as GSns first appeared between 9 and 12 DAI in the wild-type nodules and showed a developmental increase until 14 DAI, after which the levels were maintained until 24 DAI (Fig. 8A). In the Nif⁻ nodules, the major band of GS activity was the GSrs band. Between 9 and 12 DAI, several other bands of activity appeared in the Nif⁻ nodules and were maintained until 24 DAI. These included a band of activity (GSns1) with slower migration than GSns and a faster-migrating band of activity (GSns2) (Fig. 8B).

Immunostaining of the native gels showed a profile very similar to that of GS activity staining except that several distinct bands in the region of GS activity were observed (Fig. 9). The wild-type nodules at 9 DAI showed faint immunoreactive bands co-migrating with GSns1, and as the nodules developed, these immunoreactive bands showed a gradual shift toward GSns (Fig. 9A). The GSns1 band of activity in the Nif⁻ nodules was resolved into five distinct immunoreactive bands at all developmental stages between 12 and 24 DAI. At 9 DAI, the Nif⁻ nodules showed the slow-migrating immunoreactive band that we have referred to as GS*. The Nif⁻ nodules (12–24 DAI) also showed immunoreactive material migrating faster than GSrs, which was not observed in the wild-type nodules (Fig. 9B). We postulate that the faster-migrating immunoreactive band (GSns2) represents a tetrameric form of GS.

The GSns1 Isozyme Complex in the Nif⁻ Nodules Is Unstable Compared with GSrs and GSns Isozyme Complexes

Our data suggest that both classes of GS_1 transcripts are made in the Nif⁻ nodules, although the level of the class I transcripts is 4- to 10-fold lower than in the wild-type nodules. The class II transcript level at older developmental stages appears similar between the two nodule types, and the GS_1 polypeptide profile is qualitatively identical. It would thus follow that the absence of an increase in GS activity in the Nif⁻ nodules is not entirely due to the nonavailability of subunits but rather to nonassembly or incorrect assembly into a holoprotein or to rapid turnover of an assembled holoprotein. Under the extraction conditions optimized for maximum GS stability (see "Materials and Methods"), low levels of GSns1 are detected in the Nif⁻ nodules, suggesting that the GS subunits in the absence of N_2 fixation do assemble into active holoenzyme.

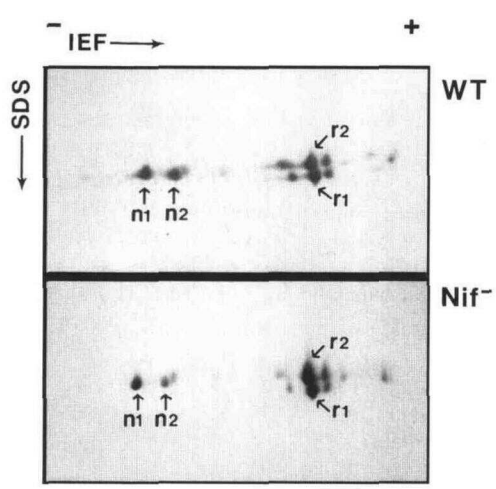

Figure 7. Two-dimensional gel electrophoretic pattern of the GS polypeptides from nodules formed by the wild-type (WT) and Nif⁻ strains. Total soluble protein extracts (30 μg/gel) from wild-type and Nif⁻ nodules 12 DAI were fractionated by two-dimensional SDS-PAGE and transferred to nitrocellulose, and the filter was probed with GS antibody. Only the relevant part of the gel is shown. r1, r2, n1, and n2 refer to the root and nodule GS subunits, respectively.

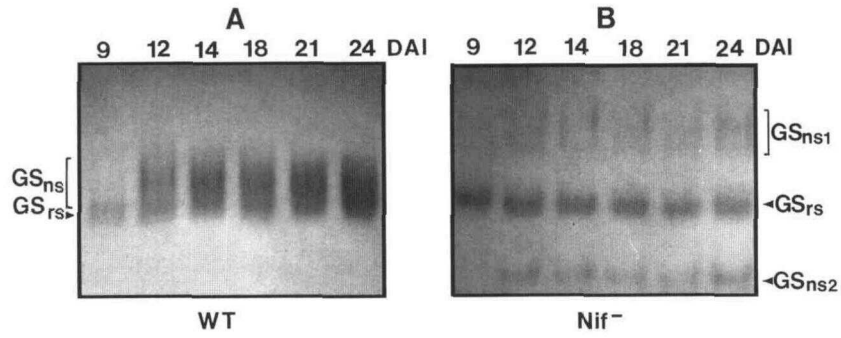

Figure 8. GS activity staining of GS isozymes following fractionation by native PAGE. Total soluble protein (200 μg/lane) from developing soybean root nodules (9, 12, 14, 18, 21, and 24 DAI) formed by the wild-type (WT) strain (A) and the Nif⁻ mutant of *B. japonicum* (B) were fractionated on 7% native PAGE gels and the gels were stained for GS transferase activity (see "Materials and Methods"). GSns1 and GSns2 refer to the slower- and faster-migrating species of nodule-specific GS isozymes found in the Nif⁻ nodules.

Incubation of the extracts under these conditions led to no significant loss of GS enzyme activity; therefore, we tested the stability of the different GS isoenzymes under nonstabilizing conditions. Protein extracts from the two nodule types (14 DAI) were incubated at 25°C (see "Materials and Methods") and at regular intervals aliquots were tested for GS activity and isozyme profile by electrophoretic separation by native PAGE (Fig. 10). Based on the initial GS activity, the Nif⁻ nodule extract showed a significant decrease in activity compared with the wild-type nodule extract (10% decrease in the wild-type nodules versus 25% decrease in the Nif⁻ nodules after 2 h of incubation, data not shown). Native gel western blots of the same extracts showed a dramatic loss in GSns1 isozymes by 1 to 2 h, and they were almost abolished by 6 h (Fig. 10B). Under the same incubation conditions, the GSrs band showed no significant reduction, even after 6 h. In the extracts of the wild-type nodule, there was little change in the levels of GSns and GSrs isozyme complexes during the first 4 h, and at 6 h only a small reduction in the GSns isozyme complex was detected (Fig. 10A). Taken together, these results suggest that the GSns1 isozyme complex isolated from the Nif⁻ nodules is relatively more unstable than either the GSns or the GSrs isozyme complex.

DISCUSSION

The data presented in this paper demonstrate a direct correlation between increased GS activity and nitrogenase function in soybean nodules. Our data further suggest that the increase in GS activity following the onset of N_2 fixation is due to a whole array of novel GS isozymes, which we collectively refer to as GSns. GS isozymes have been previously characterized in *P. vulgaris* (Lara et al., 1983). Analysis of the soybean GSns isozyme complex showed that it is made up of two kinds of GS_1 subunits, GSn and GSr. The GSn subunits are made specifically in the nodules, whereas the GSr subunits are also made in the roots. The GSns in *P. vulgaris* have also been shown to be made up of two kinds of GS_1 subunits: the nodule-specific γ subunits and the root-specific β subunits (Lara et al., 1983). Cai and Wong (1989) showed nine different GS_1 isozymes in the root nodules of *P. vulgaris*, each containing different proportions of the γ and β subunits. The profile of the GSns isoenzymes of soybean nodules appeared more complex when displayed on native gels, probably because of multiple GSn and GSr subunits combining in different proportions to form the holoenzymes.

Two classes of GS_1 genes have been characterized in soybean (Roche et al., 1993). While class I is more constitutive in its expression pattern, class II genes are expressed in a nodule-specific manner. Based on hybrid select translation experiments using the unique 3′ UTR of a representative gene member from each class, we showed previously that the class I genes encode GSr subunits, whereas the class II code for GSn subunits (Roche et al., 1993). The data presented here show that the class I transcript is maintained at a steady-state level until the onset of N_2 fixation (10 DAI), after which there is a dramatic increase in the transcript level. The class II genes, however, showed a developmental increase in expression starting as early as 9 DAI. The class II transcript level, showing a lower increment between 9 and 16 DAI in the Nif⁻ nodules, maintained levels comparable to the wild-type nodules at later stages. It is not clear as to how N_2 fixation affects the transcript levels of the nodule-specific GS genes, which are otherwise developmentally regulated. The class I tran-

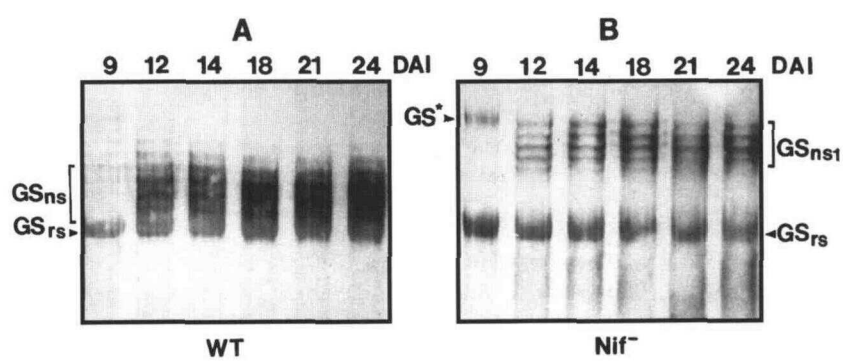

Figure 9. GS immunostaining of GS isozymes following fractionation by native PAGE. Total soluble protein (150 μg/lane) from developing soybean root nodules (9, 12, 14, 18, 21, and 24 DAI) formed by the wild-type (WT) strain (A) and the Nif⁻ mutant of *B. japonicum* (B) were fractionated on 7% native PAGE, electroblotted onto nitrocellulose, and subjected to western analysis using pea seed GS antibody.

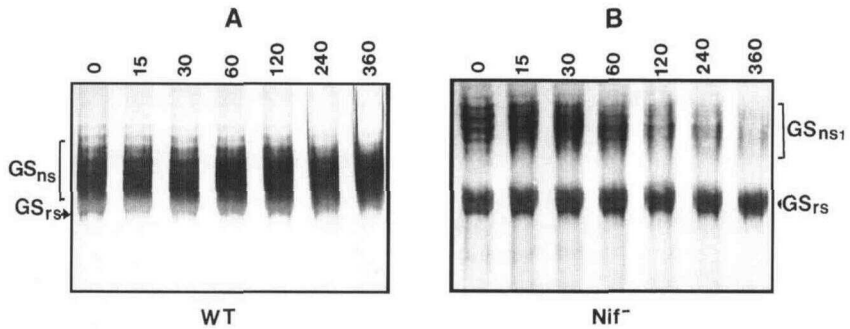

Figure 10. Analysis of the stability of the GSrs and GSns isozyme complexes in wild-type (WT) and Nif$^-$ mutants. Total soluble protein was extracted from wild-type and Nif$^-$ nodules (14 DAI) with a buffer containing imidazole and $MgCl_2$. The extracts were incubated at 25°C, and 150-μg aliquots were removed at regular intervals (0, 15, 30, 60, 120, 240, and 360 min). To each aliquot glycerol was added to 10% (v/v) and the samples were stored on ice. The proteins were fractionated on 7% native PAGE gels, electroblotted onto nitrocellulose, and subjected to western analysis using pea seed GS antibody.

scripts in the Nif$^-$ nodules did not show the dramatic increase between 10 and 12 DAI. The transcript data corroborate very well with the accumulation pattern of GSr and GSn polypeptides. Taken together, the data suggest that the transcriptional activation of the class I GS_1 genes in soybean nodules is triggered by NH_3 or a product of NH_3 assimilation.

The GSns isozyme complex is made up of both the GSr and GSn subunits. Native PAGE analysis, along with the two-dimensional SDS-PAGE western blots of the fractions of GS activity eluted off a DEAE-Sepharose column loaded with nodule extract (Fig. 3), suggests that with an increase in the GSr component the mobility of the GSns isozymes increases. Thus, the gradual increase in the mobility of the GSns isozymes in the developing wild-type nodules between 9 and 12 DAI might imply that more GSr subunits are recruited into the GSns complex. In the same context, it would follow that the slower-migrating GS activity band, GSns1 in the Nif$^-$ nodules, is made up predominantly of GSn subunits.

The appearance of the GSns isozyme complex coincided with the induction of the class I GS_1 genes. There was no induction of the class I genes, nor was the GSns isozyme complex made in the Nif$^-$ nodules. Transcripts for the class I genes, however, were maintained at or below a basal level (the level at 9–10 DAI) throughout the development of the Nif$^-$ nodules. Taken together, these results would suggest that the GSr subunits made in the Nif$^-$ nodules at all developmental stages, or in the wild-type nodules prior to the onset of N_2 fixation, are not available for assembly into GSns but are available for GSrs assembly. Histochemical analysis of transgenic *L. corniculatus* plants containing the *P. vulgaris* Gln β and Gln γ promoter-GUS fusion showed that, whereas the Gln γ gene fusion is expressed only in the infected cells of the nodule, the Gln β gene fusion is expressed in the vascular tissues and in the infected zone of young nodules (Forde et al., 1989). We could then postulate that the soybean homolog of the *P. vulgaris* Gln γ gene, the class II gene, is expressed exclusively in the infected cells, whereas class I genes like the Gln β gene are expressed in all cell types but mainly in the vascular tissue. The class I GS_1 genes of soybean, however, are induced by NH_3 (or a product of NH_3 assimilation) (Hirel et al., 1987; Miao et al., 1991), suggesting that following the onset of N_2 fixation, this gene class is induced in the infected cells of the wild-type nodules. This would explain the gradual recruitment of the GSr subunits into GSns isozymes and account for the shift in the migration of GSns isozymes on native gels with extracts of wild-type nodules between 9 and 12 DAI. The absence of class I GS_1 induction in the infected cells of Nif$^-$ nodules would also explain why the GSns in these nodules (GSns1) are made up predominantly of GSn subunits.

The fact that GSr subunits are present in the Nif$^-$ nodules at all stages but are not recruited into GSns1 strongly suggests that the GSns/GSns1 isozymes are made in cells distinct from those in which the GSrs isozyme is made. This would imply that the two classes of GS genes show expression in different cell types prior to the onset of N_2 fixation. Following the onset of N_2 fixation the class I genes are induced in cells in which the class II genes are already being expressed. Experiments are in progress to localize the two classes of GS_1 gene transcripts in the two nodule types: wild-type and Nif$^-$.

It is interesting to note that, in spite of the fact that GSn subunits in the Nif$^-$ nodules are maintained at levels equivalent to that in the wild-type nodules, no significant increase in GS activity was observed during the development of Nif$^-$ nodules. In vitro studies looking at the stability of the different isozymes of GS, GSns, GSns1, and GSrs have shown that GSns1 is the most unstable. In fact, the GSns1 isoenzymes are not always detected in all of the preparations of Nif$^-$ nodule extracts, whereas GSrs and GSns are consistently detectable. The instability of the GSns1 isoenzymes in vitro probably reflects the in vivo situation. The faster-migrating band of activity in the Nif$^-$ nodules (GSns2) probably represents a tetramer resulting from the disassembly of the GSns1 octamer. Since the GSn subunit levels in the Nif$^-$ nodules are maintained at levels similar to those in the wild-type nodules, it would follow that the GSns1 isozyme complex in the Nif$^-$ nodules is not more prone to proteolytic degradation but rather to disassembly. The presence of a proteolytic system in the Nif$^-$ nodules that specifically degrades GS is not likely because incubation of extracts from the two nodule types did not

cause degradation of GSns from the wild-type nodules (data not shown). In *P. vulgaris* nodules, in which the γ and β GS_1 genes are expressed independently of N_2 fixation, no increase in GS activity is observed in developing nodules formed by the Nif^- strain, suggesting that the GSns in the Nif^- nodules of *P. vulgaris* is just as unstable as it is in soybean Nif^- nodules. (Lara et al., 1983). The nature of the slow-migrating immunoreactive band (referred to as GS*) is not known at this time. We speculate that this band might represent an aggregate of denatured GS protein or a multicatalytic proteinase complex (Rivett, 1989).

Our interpretation of the data is that NH_3 or a product of NH_3 assimilation stabilizes the GS holoprotein. The faster-migrating GS activity band in the Nif^- nodules (GSns2) most likely is a tetramer made up of GSn subunits. In higher plants, a catalytically active tetrameric GS has to our knowledge been reported only for mustard (Hopfner et al., 1988) and sugar beet (Mack and Tischner, 1990, 1994). At this stage, we think that GSns2 is a product of GSns1 disassembly, but we cannot rule out the possibility that it is an intermediate in the assembly of GSns1. GS in bacteria is normally stable, but under conditions of N_2 starvation, the enzyme is turned over (Fulks and Stadtman, 1985). Limon-Lason et al. (1977) found that the α and β subunits of *Neurospora* GS are organized in tetrameric or octameric forms according to the culture conditions of the organism. Lara et al. (1983) showed that excess ammonium leads to NH_3 assimilation via an octameric GS composed of only β-polypeptides, whereas in ammonium-limited cultures, a tetrameric GS composed of only α-polypeptides is synthesized by the fungus. Hoelzle et al. (1992) showed that nitrate or ammonium fertilization significantly increased GS activity in nonnodulated roots of French bean, soybean, and pea. However, in all cases the number of GS subunits that accumulated in the presence or absence of nitrate or ammonium was the same. We would postulate that activation of GS with NH_3 treatment in these roots was a result of GS holoenzyme stabilization. The questions that we need to address now are what stabilizes GSrs in the Nif^- nodules? If they are primarily located in the vascular tissue, is the NH_3 released as a byproduct in the synthesis of lignin enough to maintain GSrs? Since turnover of GS involves an oxidative modification step (D. Roche, unpublished data), can we ascribe the instability of the GSns1 isozymes in the Nif^- nodules to the concentration of the active oxygen radicals in the infected cells? Work is in progress to understand mechanistically the process of GS assembly and turnover in soybean nodules and what role the substrates have on this process.

ACKNOWLEDGMENTS

The authors thank Drs. J.V. Cullimore and P.J. Unkefer for very generously providing GS antibody and Dr. Jose Luis Ortega for his critical reading of the manuscript.

Received June 6, 1996; accepted August 28, 1996.

LITERATURE CITED

Atkins CA (1987) Metabolism and translocation of fixed nitrogen in the nodulated legume. Plant Sci **100:** 157–169

Bennett MJ, Lightfoot DA, Cullimore JV (1989) cDNA sequence and differential expression of the gene encoding the glutamine synthetase γ polypeptide of *Phaseolus vulgaris* L. Plant Mol Biol **12:** 553–565

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–252

Cai X, Wong PP (1989) Subunit composition of glutamine synthetase isozymes from root nodules of bean (*Phaseolus vulgaris* L.). Plant Physiol **91:** 1056–1062

Cullimore JV, Bennett MJ (1992) Nitrogen assimilation in the legume root nodule: current status of the molecular biology of the plant enzymes. Can J Microbiol **38:** 461–466

Cullimore JV, Miflin BJ (1984) Immunological studies on glutamine synthetase using antisera raised to the two plant forms of the enzyme from *Phaseolus* root nodules. J Exp Bot **35:** 581–587

Dart PJ, Day JM, Harris D (1972) Assay of nitrogenase activity by acetylene reduction: proceedings of a panel on the use of isotopes for study of fertilizer utilization by legume crops. International Atomic Energy Agency Tech Rep Ser **149:** 85–97

De Vries SC, Springer J, Wessels JGH (1982) Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta **156:** 129–135

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Ferguson AR, Sims AP (1971) Inactivation *in vivo* of glutamine synthetase and NAD-specific glutamate dehydrogenase. Its role in the regulation of glutamine synthesis in yeast. J Gen Microbiol **69:** 423–427

Fischer MT (1992) Promotion of the *in vitro* renaturation of dodecameric glutamine synthetase from *Escherichia coli* in the presence of GroEL (chaperonin-60) and ATP. Biochemistry **31:** 3955–3963

Forde BG, Day HM, Turton JF, Shen W-J, Cullimore JV, Oliver JE (1989) Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. Plant Cell **1:** 391–401

Fulks RM, Stadtman ER (1985) Regulation of glutamine synthetase, aspartokinase and total protein turnover in *Klebsiella arogenes*. Biochim Biophys Acta **843:** 214–229

Gebhardt C, Oliver JE, Forde BG, Saarelainen R, Miflin BJ (1986) Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of *Phaseolus vulgaris*. EMBO J **5:** 1429–1435

Grant MR, Carne A, Hill DF, Farnden KJF (1989) The isolation and characterization of a cDNA clone encoding *Lupinus angustifolius* root nodule glutamine synthetase. Plant Mol Biol **13:** 481–490

Hemon P, Robbins MP, Cullimore JV (1990) Targeting of glutamine synthetase to the mitochondria of transgenic tobacco. Plant Mol Biol **15:** 895–904

Hirel B, Bouet C, King B, Layzell D, Jacobs F, Verma DPS (1987) Glutamine synthetase genes are regulated by ammonia provided externally or by symbiotic nitrogen fixation. EMBO J **6:** 1167–1171

Hirel B, Miao G-H, Verma DPS (1993) Metabolic and developmental control of glutamine synthetase genes in legume and non-legume plants. *In* DPS Verma, ed, Control of Plant Gene Expression. CRC, Boca Raton, FL, pp 443–458

Hoelzle I, Finer JJ, McMullen MD, Streeter JG (1992) Induction of glutamine synthetase activity in nonnodulated roots of *Glycine max, Phaseolus vulgaris* and *Pisum sativum*. Plant Physiol **100:** 525–528

Hopfner M, Reifferscheid G, Wild A (1988) Molecular composition of glutamine synthetase of *Sinapis alba* L. Z Naturforsch **43C:** 194–198

Kim KH, Rhee SG (1987) Subunit interaction elicited by partial inactivation with L-methionine sulfoximine and ATP differently affects the biosynthetic and gamma-glutamyltransferase reactions catalyzed by yeast glutamine synthetase. J Biol Chem **262:** 13050–13054

Kozaki A, Sakamoto A, Tanaka K, Takeba G (1991) The promoter of the gene for glutamine synthetase from rice shows organ-specific and substrate-induced expression in transgenic tobacco plants. Plant Cell Physiol **32:** 353–358

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **27:** 680–685

Langston-Unkefer PJ, Robinson AC, Knight TJ, Durbin RD (1987) Inactivation of pea seed glutamine synthetase by the toxin, tabtoxinine-β-lactam. J Biol Chem **262:** 1608–1613

Lara M, Cullimore JV, Lea PJ, Miflin BJ, Johnston AWB, Lamb JW (1983) Appearance of a novel form of plant glutamine synthetase during nodule development in *Phaseolus vulgaris* L. Planta **157:** 254–258

Lea PJ, Ridley S (1989) Glutamine synthetase and its inhibition. *In* AD Dodge, ed, Herbicides and Plant Metabolism. Cambridge University, Cambridge, UK, pp. 137–170

Lea PJ, Robinson SA, Stewart GR (1990) The enzymology and metabolism of glutamine, glutamate and asparagine. *In* BJ Miflin, PL Lea, ed, The Biochemistry of Plants, Vol 16: Intermediary Nitrogen Metabolism. Academic Press, New York, pp 121–159

Levine R (1983) Oxidative modification of glutamine synthetase. J Biol Chem **258:** 11823–11827

Lightfoot DA, Green NK, Cullimore JV (1988) The chloroplast-located glutamine synthetase of *Phaseolus vulgaris* L.: nucleotide sequence, expression in different organs and uptake into isolated chloroplasts. Plant Mol Biol **11:** 191–202

Limon-Lason J, Lara M, Resendiz B, Mora J (1977) Regulation of glutamine synthetase in fed-batch cultures of *Neurospora crassa*. Biochem Biophys Res Commun **78:** 1234–1240

Lubben TH, Donaldson GH, Viitanen PV, Gatenby AA (1989) Several proteins imported into chloroplasts form stable complexes with the GroEL related chloroplast molecular chaperone. Plant Cell **1:** 1223–1230

Mack G, Tischner R (1990) Glutamine synthetase oligomers and isoforms in sugarbeet (*Beta vulgaris* L.). Planta **181:** 10–17

Mack G, Tischner R (1994) Activity of the tetramer and octamer of glutamine synthetase isoforms during primary leaf ontogeny of sugar beet (*Beta vulgaris* L.). Planta **194:** 353–359

Marsolier M-C, Debrosses G, Hirel B (1995) Identification of several soybean cytosolic glutamine synthetase transcripts highly or specifically expressed in nodules: expression studies using one of the corresponding genes in transgenic *Lotus corniculatus*. Plant Mol Biol **27:** 1–15

Meek TD, Villafranca JJ (1980) Kinetic mechanism of *Escherichia coli* glutamine synthetase. Biochemistry **19:** 5513–5519

Meister A (1980) Glutamine synthetase. *In* J Mora, R Palacios, eds, Glutamine: Metabolism, Enzymology and Regulation. Academic Press, New York, pp 1–40

Miao G-H, Hirel B, Marsolier M-C, Redge RW, Verma DPS (1991) Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. Plant Cell **3:** 11–22

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021

Perdew GH, Schaup HW, Selivonchic DP (1983) The use of a zwitteronic detergent in two-dimensional gel electrophoresis of trout liver microsomes. Anal Biochem **135:** 453–455

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

Rhodes D, Sims AP, Stewart GR (1978) Glutamine synthetase and the control of nitrogen assimilation in *Lemna minor* L. *In* EJ Hewitt, CV Cutting, eds, Nitrogen Assimilation of Plants. Proceedings of the Long Ashton Research Station Symposium. Academic Press, New York, pp 501–520

Rivett AJ (1989) The multicatalytic proteinase of mammalian cells. Arch Biochem Biophys **278:** 1–8

Robertson JG, Farnden KJF, Banks JM (1975) Induction of glutamine synthetase during nodule development in lupin. Aust J Plant Physiol **2:** 265–272

Roche D, Temple SJ, Sengupta-Gopalan C (1993) Two classes of differentially regulated glutamine synthetase genes are expressed in the soybean nodule: a nodule-specific class and a constitutively expressed class. Plant Mol Biol **22:** 971–983

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanangelantoni AM, Barbarini D, Di Pasquale G, Cammarano P, Tiboni O (1990) Cloning and nucleotide sequence of an archaebacterial glutamine synthetase gene. Phylogenetic implications. Mol Gen Genet **221:** 187–194

Sengupta-Gopalan C, Pitas JW (1986) Expression of nodule-specific glutamine synthetase genes during nodule development in soybeans. Plant Mol Biol **7:** 189–199

Shapiro BM, Stadtman ER (1970) Glutamine synthetase (*Escherichia coli*). Methods Enzymol **17A:** 910–922

Stadtman ER (1990) Covalent modification reactions are marking steps in protein turnover. Biochemistry **29:** 6323–6331

Stanford AC, Larsen K, Barker DG, Cullimore JV (1993) Differential expression within the glutamine synthetase gene family of the model legume *Medicago truncatula*. Plant Physiol **103:** 73–81

Sukanya R, Li M-G, Snustad DP (1994) Root- and shoot-specific responses of individual glutamine synthetase genes of maize to nitrate and ammonium. Plant Mol Biol **26:** 1935–1946

Swarup R, Bennett MJ, Cullimore JV (1990) Expression of glutamine-synthetase genes in cotyledons of germinating *Phaseolus vulgaris* L. Planta **183:** 51–56

Temple SJ, Heard J, Ganter G, Dunn K, Sengupta-Gopalan C (1995) Characterization of a nodule-enhanced glutamine synthetase from alfalfa: nucleotide sequence, *in situ* localization and transcript analysis. Mol Plant-Microbe Interact **8:** 218–227

Temple SJ, Knight TJ, Unkefer PJ, Sengupta-Gopalan C (1993) Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis. Mol Gen Genet **236:** 315–325

Tingey SV, Tsai F-Y, Edwards JW, Walker EL, Coruzzi GM (1988) Chloroplast and cytosolic glutamine synthetase are encoded by homologous nuclear genes which are differentially expressed *in vivo*. J Biol Chem **263:** 9651–9657

Tingey SV, Walker EL, Coruzzi GM (1987) Glutamine synthetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots and nodules. EMBO J **6:** 1–9

Tsuprun VL, Boekema EJ, Pushkin AV, Tagunova IV (1992) Electon microscopy and image analysis of the GroEL-like protein and its complexes with glutamine synthetase from pea leaves. Biochim Biophys Acta **1099:** 67–73

Walker EL, Coruzzi GM (1989) Developmentally regulated expression of the gene family for cytosolic glutamine synthetase in *Pisum sativum*. Plant Physiol **91:** 702–708

Werner D, Morschel E, Stripf R, Winchenbach B (1980) Development of nodules of *Glycine max* infected with an ineffective strain of *Rhizobium japonicum*. Planta **147:** 320–329

Yamashita MM, Almassey RJ, Janson CA, Cascio D, Eisenberg D (1989) Refined atomic model of glutamine synthase at 3.5 A resolution. J Biol Chem **264:** 17681–17690