Regulation of Glutamine Synthetase Isoenzymes in *Rhizobium leguminosarum* biovar *viciae*

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Ammonia assimilation in *Rhizobium leguminosarum* biovar *viciae* strain RCR1001 (hereafter called *R. leguminosarum*) appears to take place only through the glutamine synthetase/glutamate synthase pathway since (a) no glutamate dehydrogenase was detected in crude extracts of bacteria grown in different nitrogen sources, and (b) the growth rate on glutamine as a nitrogen source was faster than that observed on NH_4Cl . In contrast to reports for other *Rhizobium* species, *R. leguminosarum* can definitely utilize NH_4Cl for growth. *R. leguminosarum* contains two glutamine synthetase isoenzymes, GSI and GSII, which can be detected in the presence of each other by differential heat stability, or separated by affinity chromatography or immunoabsorption with an antiserum raised against pure GSI. GSII does not cross-react with an anti-GSI antiserum. GSI was shown to be reversibly adenylylated and it was also shown that adenylylation inhibits the biosynthetic activity of this enzyme, in a similar way to that reported for *Escherichia coli* glutamine synthetase and in contrast to that observed for glutamine synthetase of *Rhizobium* sp. strain ANU289. The apparent adenylylation level in different growth conditions changes from 21% to 99%, indicating a physiological role of this post-translational modification in the *in vivo* regulation of GSI activity. The intracellular concentration of GSI varies very little when *R. leguminosarum* is grown on different nitrogen sources (twofold when measured by the transferase assay, or fourfold when measured by ELISA). In addition, the concentration of mRNA specific for GSI in different nitrogen sources does not show appreciable differences. The intracellular concentration of GSII varies from a specific activity value higher than 1000 when *R. leguminosarum* is grown on glutamate or nitrate, to an undetectable level when grown on NH_4Cl . When NH_4Cl is added to a culture growing in glutamate, GSII activity is rapidly diluted out, suggesting a post-translational mechanism of enzyme inhibition or inactivation. Chloramphenicol prevents the disappearance of GSII activity, thus suggesting that protein synthesis is required for this process.

INTRODUCTION

Two pathways of ammonia assimilation exist in bacteria: via glutamate dehydrogenase (GDH; EC 1.4.1.3) (reaction 1), and via glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.13) (reactions 2 and 3 respectively). The end product of both pathways is glutamate, from which nitrogen compounds of the cell are synthesized.

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Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; SVPD, snake venom phosphodiesterase.

GDH has a high K_m for ammonia and thus only operates at high ammonia concentrations. GS/GOGAT is the major route of ammonia assimilation when ammonia is limiting. In enteric bacteria the use of these two pathways is genetically controlled in response to the availability of ammonia. These enzymes form part of a general nitrogen control system (see Gussin *et al.*, 1986, for a review) which includes a number of enzymes involved in the breakdown of nitrogenous compounds. When ammonia is present at high concentrations, the genes for GS and other enzymes under nitrogen control are repressed, and GDH is induced. The converse occurs when ammonia is limiting, giving the cell the capability to use a variety of nitrogen sources. Additionally, GS activity is controlled biochemically in response to ammonia. In the presence of ammonia, adenylyl groups are reversibly attached to GS, causing inactivation.

Two forms of glutamine synthetase, GSI and GSII, have been demonstrated in all species tested of the family *Rhizobiaceae* (Fuchs & Keister, 1980). GSI has been shown to be similar to the single GS of enteric bacteria. It is an oligomeric enzyme consisting of 12 identical subunits of M_r 59000, is relatively heat stable, and can be adenylylated (Darrow & Knotts, 1977). In contrast, GSII is made of smaller subunits, of M_r 36000, which are heat labile and not known to be modified after translation. GSI and GSII are products of different genes (Carlson *et al.*, 1985; Colonna-Romano *et al.*, 1987; Filser *et al.*, 1986; Somerville & Kahn, 1983).

We have previously reported (Colonna-Romano *et al.*, 1987) the cloning and nucleotide sequence of the *R. leguminosarum* biovar *viciae* *glnA* gene, the structural gene for GSI. Its deduced amino acid sequence shows 56%, 63% and 68% similarity with the deduced amino acid sequences of *Anabaena*, *Escherichia coli* and *Azospirillum brasilense* GSs respectively, and most of it is in boxes of identical amino acids. The deduced M_r of the protein is 52040, in good agreement with that obtained from pure GSI. Upstream of the *glnA* genes of *R. leguminosarum* and of *Bradyrhizobium japonicum* (Carlson *et al.*, 1987) there is no regulatory sequence similar to that reported for *glnA* of enteric bacteria (see, for example Dixon, 1984).

The gene of *B. japonicum* coding for GSII has been cloned and sequenced (Carlson & Chelm, 1986). Comparison of the deduced amino acid sequence showed limited similarity to other bacterial GSs, but extensive similarity to a GS of *Phaseolus vulgaris* roots, thus suggesting that the gene coding for GSII might be of eukaryotic origin. Upstream of this gene there is a good consensus sequence for an *ntr* promoter (Carlson *et al.*, 1987).

The role of the enzymes for ammonia assimilation with respect to the symbiotic phenotype of *Rhizobium* spp. is not well understood. GDH is missing in several wild-type strains (Howitt & Gresshoff, 1985; Bravo & Mora, 1988) and not very active in others. Strains defective for GOGAT are Nod⁺ and Fix⁺. Mutants of *Rhizobium meliloti* which lack GSI activity (Somerville & Kahn, 1983) are Gln⁺ in the free-living form, probably because GSII expression substitutes for GSI; they are Nod⁺ and Fix⁺. Mutants of *R. leguminosarum* biovar *phaseoli* lacking GSII are Nod⁺ and Fix⁺ (Morett *et al.*, 1985). Mutants of *B. japonicum* lacking either GSI or GSII are Gln⁺, Nod⁺ and Fix⁺; double mutants are Gln⁻ and Nod⁻ (Carlson *et al.*, 1987).

We started a study on the enzymes of ammonia assimilation in the free-living form of *R. leguminosarum* biovar *viciae* to understand nitrogen assimilation and its regulation. We have shown that GSI and GSII can be separated by affinity chromatography and that the former does not generate the latter, nor *vice versa*. We report evidence suggesting that adenylylation of GSI has physiological significance. Furthermore, we suggest that under certain conditions a post-translational modification of GSII takes place.

METHODS

Bacterial strain. The strain used was *R. leguminosarum* biovar *viciae* LPR1105, a Rif^r derivative of RCR1001 (Hooykaas *et al.*, 1977).

Growth conditions. *R. leguminosarum* biovar *viciae* was grown on RMM (Hooykaas *et al.*, 1977); the nitrogen sources used were glutamine, glutamate, NH₄Cl, or KNO₃, each at a concentration of 1 g l⁻¹. This medium has a pH of 6.3; when NH₄Cl is used as a nitrogen source the pH decreases to 5 and the growth rate is slower. To prevent acidification we added 100 mM-MOPS, pH 7.2.

Cultures were grown at 30 °C on a rotary shaker at 200 r.p.m.

Preparation of cell extracts. Bacteria (50 ml volumes, unless otherwise stated) were harvested in the exponential phase by centrifugation at 4 °C, washed with 0.9% NaCl and frozen as a pellet at -20 °C. The pellet was

resuspended in 1 ml of extraction buffer (Ferguson & Sims, 1971), sonicated twice for 45 s, with a 1 min interval at 0 °C, and centrifuged at 27000 g at 4 °C for 20 min to remove cell debris.

Heat treatment. Samples of cell extracts were heated at 55 °C for 30 min, rapidly cooled in ice and centrifuged; the supernatant was used for GS assays.

Enzyme assays. For the γ -glutamyltransferase activity of GSII, we used the method described by Ferguson & Sims (1971) and measured activity in samples of cell extract before and after heat treatment to assay the heat-stable fraction relative to total activity. The γ -glutamyltransferase activity of GSI was assayed according to the protocol of Shapiro & Stadtman (1970) at pH 7.43 (the isoactivity point of the adenylylated and non-adenylylated form of GSI; see below) on cell extracts subjected to heat treatment. For the detection of GSI activity on gels (Fig. 1) the concentration of the substrates was doubled. The degree of adenylylation of GSI is given by the ratio of transferase activity at pH 7.43 in the presence and absence of 60 mM-MgCl₂. The biosynthetic activity of GSI was determined by the method of Bender *et al.* (1977) except that cetyltrimethylammonium bromide was omitted. Units of activity are given as nmol γ -glutamyl-hydroxamate formed min⁻¹. Specific activity is expressed as units (mg protein)⁻¹. Treatment of purified GSI with snake-venom phosphodiesterase (SVPD) was performed with 500 μ g ml⁻¹ SVPD in 0.1 M-Tris/HCl pH 9.0 containing 5 mM-MgCl₂ for 3 h at 37 °C. Glutamate dehydrogenase (GDH) activity was determined according to Prusiner *et al.* (1972).

Purification of GSI from *R. leguminosarum* biovar *viciae*. GSI was partially purified from *R. leguminosarum* biovar *viciae* grown in RMM plus NH₄Cl. A cell extract of an exponential-phase culture was adsorbed on a Blue-Sepharose column equilibrated in extraction buffer. After washing, GSI was eluted with 5 mM-AMP. Fractions containing activity were pooled, concentrated and dialysed. The specific activity increased 80-fold.

Purification of *R. leguminosarum* biovar *viciae* GSI from *Klebsiella pneumoniae* and preparation of an antiserum. GSI was purified to homogeneity from *K. pneumoniae* strain UNF1827(p7D9) (Filser *et al.*, 1986). This strain is *glnA* and thus devoid of *K. pneumoniae* GS, while p7D9 codes for *R. leguminosarum* GSI. The following purification protocol was used. A crude extract in 10 mM-Tris/HCl pH 7.2, 50 mM-K₂SO₄, 2.5 mM-MgSO₄, 0.5 mM-EDTA, 0.5 mM-mercaptoethanol was treated with 2% (w/v) streptomycin sulphate; after centrifugation for 10 min at 12000 g, the supernatant was adsorbed on a Blue-Sepharose column equilibrated in extraction buffer and eluted with a gradient of AMP. The enzyme appeared at about 2 mM-AMP and was >95% pure when analysed by SDS polyacrylamide gel electrophoresis (PAGE).

This preparation was used to obtain a polyclonal antiserum from rabbit. After non-denaturing PAGE with pure GSI or with cell extracts of *R. leguminosarum* biovar *viciae*, an immunoblot was made according to Towbin *et al.* (1979) except that the nitrocellulose filter was washed extensively in cream-free milk containing 10 mM-phosphate buffer pH 7.4, 0.15 M-NaCl and 0.2% Tween 20. After development, the filter showed a single band coincident with the band obtained when a twin portion of the gel was assayed for GS activity as described above (Fig. 1). On this gel, GSII activity is not detected, probably due to inactivation. Fig. 1 also shows that *K. pneumoniae* GS migrates faster than *R. leguminosarum* GSI on non-denaturing PAGE and that the anti-GSI antiserum does not react with *K. pneumoniae* GS, at least under the conditions used.

Immunoabsorption test. A 30 μ l volume of anti-GSI antiserum was mixed with 0.5 ml Protein A-Sepharose (0.1 g ml⁻¹) in 20 mM-imidazol/HCl pH 7.0 containing 1 mM-MnCl₂, kept at 4 °C overnight for absorption of the IgG fraction, packed into a column, washed and eluted with the same buffer. Cell extract (150 μ l) was absorbed and 150 μ l fractions were collected and assayed for transferase activity.

ELISA assay. The competitive assay method described by Engvall (1980) was used. Microtitre plates were coated overnight at 4 °C with 100 μ l per well of pure GSI (0.9 μ g ml⁻¹ in 0.1 M-carbonate/bicarbonate coating buffer pH 9.6). After three washings with PBS (1.47 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄, 137 mM-NaCl, pH 7.4) containing 0.05% Tween 20, the wells were saturated with 0.5% BSA in PBS (100 μ l per well for 1 h at 37 °C in a humid chamber); after washing with PBS, 100 μ l samples containing polyclonal anti-GSI antiserum (1/800 dilution) and different dilutions of cellular extracts (or pure GSI for calibration) were added to the wells and incubated for 1 h at 37 °C, followed by washing and 1 h incubation with peroxidase-labelled goat anti-rabbit IgG at 1/200 dilution; the amount of antibody bound was detected using 200 μ l of OPD (0.33 mg ml⁻¹ *o*-phenylenediamine hydrochloride and 0.016% hydrogen peroxide in 63 mM-Na₂HPO₄, 29 mM-citric acid, pH 6.0) for 15 min at 37 °C; the reaction was stopped by addition of 50 μ l of 2 M-H₂SO₄ and the absorbance at 420 nm was determined.

Extraction of RNA and slot-blot hybridization. Cells from a 250 ml early-exponential-phase culture of *R. leguminosarum* biovar *viciae* LPR1105 in RMM medium plus 100 μ g rifampicin ml⁻¹ were spun down and resuspended in 2.5 ml sterile 25% (w/v) sucrose. This solution was transferred to a 50 ml Falcon tube and 16.5 ml M-STET [4% (w/v) sucrose, 6% (v/v) Triton X-100, 60 mM-Tris/HCl pH 8.0], 1.5 ml lysozyme (10 mg ml⁻¹, freshly prepared in sterile distilled water) and 0.15 ml diethyl pyrocarbonate were added. The mixture was incubated on ice for 5 min. SDS (1 ml; 20%, w/v) was added and boiled for 2 min. After boiling, 0.15 ml diethyl pyrocarbonate was added and the viscous solution was cooled immediately in a bath of ethanol and dry ice. This solution was treated four times with phenol/chloroform/isoamyl alcohol (50:50:1, by vol.) at 65 °C and precipitated with 0.7 vol. of 2-propanol at room temperature. The pellet was redissolved in 40 ml 2 M-LiCl and precipitated overnight at 4 °C.

Fig. 1. Analysis of *R. leguminosarum* biovar *viceae* GSI by PAGE and immunoblotting. (a) Non-denaturing PAGE stained for transferase activity. Lanes: A, cell extract from *R. leguminosarum* grown in RMM plus glutamate; B, cell extract from *K. pneumoniae*, wild-type strain; C, *R. leguminosarum* GSI purified from *K. pneumoniae* strain UNF1827(p7D9); D, cell extract from *K. pneumoniae* strain UNF1827(p7D9). (b) Immunoblot, with an antiserum to *R. leguminosarum* GSI. Lanes: 1, cell extract from *K. pneumoniae* strain UNF1827(p7D9); 2, *R. leguminosarum* GSI purified from *K. pneumoniae* strain UNF1827(p7D9); 3, cell extract from *R. leguminosarum* grown in RMM plus glutamate; 4, cell extract from *K. pneumoniae*, wild-type strain.

After centrifugation RNA was redissolved in 40 mM-Tris/HCl, pH 7.9, 10 mM-NaCl, 6 mM-MgCl₂ and digested with pancreatic deoxyribonuclease. After precipitation, the RNA samples were resuspended in H₂O containing 0.05% diethyl pyrocarbonate, boiled for 3 min and cooled on ice. Then 350 µl 20 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) was added to each 50 µl RNA.

The samples were slot-blotted on nitrocellulose using a Schleicher & Schuell Hybri-slot apparatus. The filters were hybridized with a riboprobe made of the *Eco*RI-*Sma*I fragment internal to the structural gene, cloned in the pGem3 vector, prepared as described by Melton *et al.* (1984). Hybridization was carried out in: 50% (v/v) formamide, 50 mM-sodium pyrophosphate pH 6.5, 0.8 M-NaCl, 1 mM-EDTA, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) and denatured salmon sperm DNA (100 µg ml⁻¹) at 55 °C. Filters were washed in 0.1 × SSC, 0.1% SDS at 65 °C.

RESULTS AND DISCUSSION

Glutamate dehydrogenase activity

Cell extracts of *R. leguminosarum* biovar *viceae* grown in NH₄Cl, glutamate, glutamine or KNO₃ as nitrogen sources had undetectable [$<3 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] GDH activity when either NADH or NADPH was used as cofactor, whereas a wild-type strain of *E. coli* grown in NH₄Cl gave an activity of 12 (with NADH) and 257 (with NADPH) units. This result suggests, as was found with other *Rhizobium* spp. (Howitt & Gresshof, 1985; Ludwig, 1978; Darrow, 1980; Bravo & Mora, 1988), that GDH activity is absent in *R. leguminosarum* biovar *viceae* under the conditions tested. In agreement with this hypothesis, *R. leguminosarum* biovar *viceae* grew slightly better on glutamine or glutamate than on NH₄Cl (see Table 1), but *E. coli* grown in the same medium showed a marked preference for NH₄Cl (the growth rate observed at 37 °C in RMM was 138 min when glutamine was the nitrogen source, and 45 min with NH₄Cl).

GSI and GSII activities

After 5 min of preincubation at 55 °C all the labile activity of a crude extract of *R. leguminosarum* biovar *viceae* disappeared, while a heat-stable fraction persisted, even after 90 min incubation. In agreement with previously published evidence (Darrow & Knotts, 1977; Filser *et al.*, 1986), we call GSI the heat-stable fraction and GSII the heat-labile one.

We separated GSI and GSII activities in the presence of each other by different methods.

(a) *Affinity chromatography.* A crude extract of *R. leguminosarum* biovar *viceae* grown in glutamate and containing both GSI and GSII was adsorbed on a Blue-Sepharose column previously equilibrated with extraction buffer containing 1 mM-MgCl₂. As shown in Fig. 2, part of the activity was not adsorbed and it was found to be heat labile; more activity was eluted with 5 mM-AMP and was found to be heat stable. Repeated adsorption-elution experiments of the

Fig. 2. Separation of GSI and GSII activities. A cell extract from *R. leguminosarum* biovar *viceae* grown in RMM plus glutamate was applied on a Blue-Sepharose column (1.6 × 10 cm) previously equilibrated with extraction buffer containing 1 mM-MgCl₂. After washing, the column was eluted with 5 mM-AMP. Samples from each fraction were assayed for transferase activity before (●) and after (○) heat treatment at 55 °C for 30 min.

Table 1. Influence of nitrogen sources on cell-doubling time and GSI and GSII transferase activities

Growth medium	Doubling time (min)	GSI			GSII Specific activity
		Specific activity	Degree of adenylation (%)	Concentration µg (mg protein) ⁻¹ *	
RMM + glutamate	276	578	22	32.5	1023
RMM + KNO ₃	300	394	51	18.93	1120
RMM + glutamine	220	226	81	14.93	43
RMM + NH ₄ Cl†	318	228	86	8.0	<5

*These values were determined by ELISA and are the mean of at least three determinations. The standard error is less than 10%.

†When NH₄Cl was the nitrogen source 100 mM-MOPS was added (see Methods). In its absence the doubling time is higher than that reported here.

two fractions showed that either peak of activity did not generate the other. We conclude that GSI and GSII can be separated by Blue-Sepharose and behave as true isoenzymes.

(b) *Immunoabsorption of GSI*. A sample of a cell extract of *R. leguminosarum* biovar *viceae* grown in RMM with glutamate as the nitrogen source, containing 1316 units of heat-stable activity and 12639 units of heat-labile activity, was applied to a 0.5 ml column of Protein-A Sepharose pretreated with anti-GSI antiserum. This column retained all (>98%) heat-stable activity, and the heat-labile activity was recovered in the flow-through fractions. When Protein-A Sepharose was pretreated with non-immune serum the heat-stable activity was not absorbed. We conclude that the heat-stable fraction of GSI from crude cell extracts specifically cross-reacts with the antiserum obtained against pure GSI and that under these conditions there is no cross-reactivity between GSI and GSII. Therefore, the activity detected in the immunoblot shown in Fig. 1(b), lane 3, is GSI.

Adenylation of GSI

Since it has been reported that GSI activity of *Rhizobium* is subject to adenylation (Darrow & Knotts, 1977), we characterized GSI of *R. leguminosarum* biovar *viceae* from this point of view. A completely adenylylated GS from enteric bacteria is inactive in the transferase assay when 60 mM-MgCl₂ is added to the reaction mixture (Stadtman & Ginsburg, 1974) and therefore the degree of adenylation is measured as the ratio of transferase activity in the presence or

Fig. 3. Determination of the isoactivity point for GSI. A culture grown in RMM plus glutamate was divided in two aliquots and NH_4Cl was added to one of them. After 20 min, both cultures were collected and centrifuged. Cell extracts were prepared, heated at 55°C for 30 min and transferase activity determined at different pH values. ●, Activity of cell extracts from RMM + glutamate; ○, activity of cell extracts from RMM plus glutamate, NH_4Cl shocked.

absence of 60 mM-MgCl_2 . However, a preliminary experiment should be performed with GSI to determine the activity as a function of pH for the adenylylated and non-adenylylated forms of the enzyme. As a source of relatively non-adenylylated GSI we used *R. leguminosarum* biovar *viceae* grown in RMM plus glutamate. As a source of adenylylated GSI we used the same culture incubated for 20 min with $1\text{ mg NH}_4\text{Cl ml}^{-1}$. We prepared extracts from these two cultures and assayed GSI concentration by ELISA. Transferase activity values at different pH values are reported in Fig. 3. They are strikingly different and pH 7.43 is the isoactivity point, namely the pH at which the same concentration of enzyme gives the same activity. When 60 mM-MgCl_2 was added to the reaction mixture at pH 7.43, the activity of the extract from *R. leguminosarum* biovar *viceae* grown in glutamate decreased to 78% of the starting value, while the activity of the extract treated with NH_4Cl decreased to 14%. Assuming that adenylation of GSI completely inhibits transferase activity in the protocol used, we conclude that GSI of these two cultures is 22% and 86% adenylylated respectively.

We showed that treatment with SVPD, which removes adenylyl residues from *E. coli* GS, causes an increase in biosynthetic activity. We used a GSI preparation enriched 80-fold from *R. leguminosarum* biovar *viceae* grown in RMM plus NH_4Cl . Treatment of GSI with SVPD for 3 h as described in Methods caused a decrease in adenylylation, from 87% to 20%, and a doubling of biosynthetic activity, from 2290 to 4640 units $(\text{mg protein})^{-1}$. Therefore, contrary to what is observed with GSI from the slow-growing *Rhizobium* sp. strain ANU289 (Howitt & Gresshoff, 1985), adenylation of GSI inhibits biosynthetic activity and therefore has a physiological role analogous to that observed with *E. coli* GS. SVPD treatment caused a decrease (23%) in transferase specific activity, from a value of 38 500 to 29 900 units $(\text{mg protein})^{-1}$; this may be due to a change in the kinetic constants of GSI related to the degree of adenylylation or to impurities in SVPD affecting the activity. We prefer the first hypothesis since, in a different protocol, when NH_4Cl is added to a culture growing in glutamate, concomitant with an increase in the apparent degree of adenylylation of GSI from 21% to 100%, there is a 50% increase in transferase specific activity, even if chloramphenicol ($300\text{ }\mu\text{g ml}^{-1}$) is added to the culture 1 h before NH_4Cl addition.

Transcriptional analysis of the *glnA* gene

The *R. leguminosarum* biovar *viceae* gene coding for GSI activity has been cloned and sequenced (Colonna-Romano *et al.*, 1987). Regulatory sequences similar to those found

Fig. 4. Slot-blot hybridization experiment. Lanes A, B and C were loaded with: 1.5 (A), 3 (B) and 6 μ g (C) of RNA extracted from *R. leguminosarum* biovar *viceae* grown on RMM plus NH_4Cl (1), KNO_3 (2), glutamine (3) or glutamate (4) as the sole nitrogen source. In the lower part of the figure a restriction map of the GSI region is shown. The hatched bar indicates the GSI coding region, while the black bar indicates the probe used in the slot-blot hybridization experiment.

upstream of *glnA* in other enteric bacteria (Dixon, 1984) are not present upstream of the *R. leguminosarum glnA* gene.

We quantified the GSI RNA transcript present in *R. leguminosarum* biovar *viceae* grown on different nitrogen sources by slot-blot hybridization, using a riboprobe of a fragment internal to *glnA* (see Methods). By scanning analysis and using arbitrary units we found that the concentration of the GSI transcript present in *R. leguminosarum* biovar *viceae* with glutamine, glutamate, KNO_3 or NH_4Cl was 1.0, 1.25, 1.8 or 1.85, respectively. These results are shown in Fig. 4 and indicate that *glnA* is regulated very little if at all at the transcriptional level. Similar results were reported for the *B. japonicum glnA* gene (Carlson *et al.*, 1987).

Intracellular concentrations of GSI and GSII

We measured the levels of GSI and GSII activities as well as the GSI concentration by ELISA in *R. leguminosarum* biovar *viceae* grown with different nitrogen sources. The intracellular concentration of GSI changes very little when the cells are grown in different nitrogen sources (Table 1). The apparent ratio of GSI present in bacteria grown with glutamate versus glutamine or NH_4Cl is 2.5 as measured by the transferase assay, but is 4 when measured by ELISA. On the other hand, the degree of adenylation changes from 22% with glutamate to >80% with glutamine or NH_4Cl . Since under specific conditions (see below) the apparent degree of adenylation approaches 100%, it appears that regulation of GSI activity takes place mainly at the post-translational level.

In contrast, GSII activity, as measured by the transferase assay, varied by >100-fold with the different nitrogen sources, being high when *R. leguminosarum* biovar *viceae* is grown with glutamate or KNO_3 , and low with glutamine or NH_4Cl . This observation suggests that GSII activity is regulated at the transcriptional level. Moreover, it appears that GSII activity is not needed when NH_4Cl is the nitrogen source and that it is instead needed to scavenge NH_3 when glutamate or KNO_3 are the nitrogen sources and the intracellular concentration of NH_3 is low. On the other hand, it is not easy to understand how NH_4Cl is utilized when it is the only nitrogen source, since GDH and GSII are undetectable, while GSI is almost completely adenylylated. It is possible that the *in vivo* activity of these enzymes is different from that extrapolated by the

Fig. 5. Time course of GSII activity in crude extracts of *R. leguminosarum* grown in minimal medium, washed and resuspended in different nitrogen sources. ■, Culture grown in NH_4Cl and resuspended in KNO_3 ; ○, culture grown in glutamate and resuspended in NH_4Cl ; ●, culture grown in glutamate, resuspended for 1 h in chloramphenicol ($300 \mu\text{g ml}^{-1}$) prior to the addition of NH_4Cl .

in vitro experiments, or that another enzyme(s) for NH_3 utilization is present under these conditions.

Metabolic shock

We studied the kinetic changes of GSII levels when the nitrogen source in the medium was changed. When *R. leguminosarum* biovar *viciae* was grown in NH_4Cl , centrifuged while in exponential growth and resuspended in minimal medium containing KNO_3 , an increase in specific activity was observed (Fig. 5). When a culture growing in glutamine was centrifuged and resuspended in KNO_3 analogous changes were observed (not reported in the figure).

If *R. leguminosarum* biovar *viciae* was grown with glutamate, centrifuged while in exponential growth and resuspended in RMM containing NH_4Cl , we observed (Fig. 5) a striking decrease in GSII transferase activity after 6 h incubation, from a value of 1210 to 65, namely to 5% of the starting value. Since in these 6 h of incubation the turbidity of the culture increased by a factor of 16%, if the decrease in GSII activity was due to repression then its value should have decreased to about 80%. The high rate of disappearance suggests that a post-translational modification of GSII is taking place. This evidence is in agreement with results obtained with other *Rhizobium* and *Bradyrhizobium* spp.: *Rhizobium* 32H1 (Ludwig, 1980), *B. japonicum* and *R. trifolii* (Fuchs & Keister, 1980), *Rhizobium* sp. ANU289 (Howitt & Gresshoff, 1985) and *R. phaseoli* (Bravo & Mora, 1980). When chloramphenicol was added prior to NH_4Cl the activity decreased only to 60%, thus suggesting that the presumed post-translational modification requires protein synthesis.

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