

## Dissociation by $\text{NH}_4\text{Cl}$ treatment of the enzymic activities of glutamine synthetase II from *Rhizobium leguminosarum* biovar *viciae*

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Glutamine synthetase II (GSII) was purified to homogeneity from *Rhizobium leguminosarum* biovar *viciae* and characterized. The sequence of 26 amino acid residues from the amino-terminal end of the protein showed high similarity with the sequence of GSII from *Bradyrhizobium japonicum* or from *Rhizobium meliloti*. Non-denaturing PAGE showed that GSII, either in crude extracts or in the pure state, was a mixture of an octamer and a tetramer and that under specific conditions the octamer/tetramer ratio could be modified in either direction. The pure enzyme was used to raise an antiserum which was highly specific. Addition of  $\text{NH}_4\text{Cl}$  to a bacterial culture derepressed for GSII caused a specific decrease in transferase activity, faster than the one observed when the amount of immunoreactive material was measured by different methods. On the other hand, biosynthetic activity, measured as the rate of ADP or glutamine formation, paralleled the rate of decrease in immunoreactive material. A partially purified enzyme preparation retained this dissociation of kinetic parameters, strongly suggesting a post-translational modification. These findings are discussed with respect to the possible role of GSII in the *Rhizobium*–legume symbiosis.

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### Introduction

Two forms of glutamine synthetase (GS) [L-glutamate : ammonia ligase (ADP); EC 6.3.1.2], GSI and GSII, have been demonstrated in all species tested of the family *Rhizobiaceae* (Fuchs & Keister, 1980), in *Frankia* sp. (Edmands *et al.*, 1987) and, more recently, in *Streptomyces hygroscopicus* (Kumada *et al.*, 1990). GSI is similar to the single GS of enteric bacteria. It is an oligomeric, relatively heat stable enzyme, comprising 12 identical subunits of  $M_r$  59000, which can be adenylylated (Darrow & Knotts, 1977; Rossi *et al.*, 1989). In contrast, GSII is similar to eukaryotic GS (Carlson & Chelm, 1986; De Vries *et al.*, 1983; Shatters & Kahn, 1989); it comprises 8 subunits of  $M_r$  42000 (De Vries *et al.*, 1983) and is heat labile with respect to GSI. GSI and GSII are products of different genes (Carlson *et al.*, 1985; Colonna-Romano *et al.*, 1987; Filser *et al.*, 1986; Somerville & Kahn, 1983). While GSI is constitutively expressed (Darrow, 1980; Ludwig, 1980; Rossi *et al.*,

1989), expression of GSII is controlled by the nitrogen source in the medium (Martin *et al.*, 1988; Rossi *et al.*, 1989). Although very different in size and structure, GSI and GSII have similar  $K_m$  values and similar turnover numbers for their substrates (Darrow, 1980).

It has been reported that transferase activity of GSII assayed in crude extracts, is rapidly removed after addition of  $\text{NH}_4\text{Cl}$  to a culture derepressed for GSII (Bravo & Mora, 1988; Fuchs & Keister, 1980; Howitt & Gresshoff, 1985; Ludwig, 1980). Addition of chloramphenicol prior to  $\text{NH}_4\text{Cl}$  prevents disappearance of GSII transferase activity (Rossi *et al.*, 1989). This phenomenon may be interpreted in several ways, as, for example, accumulation of an inhibitor or degradation of the enzyme; furthermore, a correlation with enzyme dilution following repression has not been reported.

Since addition of  $\text{NH}_4\text{Cl}$  to a culture may be analogous to the increase in  $\text{NH}_4^+$  concentration taking place when nitrogenase becomes derepressed in a bacteroid, we became interested in this phenomenon because it might lead to a better understanding of nitrogen flux in the *Rhizobium*–legume symbiosis. To this end, we obtained pure GSII from *Rhizobium leguminosarum* biovar *viciae* (hereafter called *R. l. viciae*) and raised an antiserum to

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Abbreviation: GSII, glutamine synthetase II.

it. In this paper we describe structural parameters of the pure enzyme and compare them with results obtained analysing crude extracts by means of the antiserum. We show that after  $\text{NH}_4\text{Cl}$  addition, transferase activity decreases at a faster rate than the decrease in concentration of immunoreactive material. Furthermore, we observed a dissociation of transferase and biosynthetic activities that can only be interpreted as a post-translational modification.

## Methods

**General.** The strain used was *R. l. viceae* LPR1105, a rifampicin-resistant derivative of strain RCR1001 (Hooykaas *et al.*, 1977). Growth conditions and preparations of cell extracts were as described previously (Rossi *et al.*, 1989). Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin (BSA fraction V, Sigma) as a standard.

**Glutamine synthetase assays.** Transferase activity was measured by the  $\gamma$ -glutamyl transferase reaction (Ferguson & Sims, 1971). One unit of enzyme activity is defined as the formation of  $1\ \mu\text{mol}$   $\gamma$ -glutamylhydroxamate  $\text{min}^{-1}$  at  $37^\circ\text{C}$ . Biosynthetic activity was measured either according to Bender *et al.* (1977), except that cetyltrimethylammonium bromide was omitted, or with the coupled assay system described by Kingdon *et al.* (1968), or by the formation of radioactive glutamine, as described by Prusiner & Milner (1970). In the first assay,  $\gamma$ -glutamylhydroxamate is measured using glutamate in the reaction mixture and the unit of activity is defined as in the case of the transferase reaction. In the second assay, ADP formation is measured as the rate of NADH oxidation, through the combined action of lactate dehydrogenase and pyruvate kinase. The background activity obtained in the absence of  $\text{NH}_4\text{Cl}$  was subtracted. One unit of enzyme activity is defined as the oxidation of  $1\ \mu\text{mol}$  NADH  $\text{min}^{-1}$  at  $30^\circ\text{C}$ . In the radioactive assay [ $^{14}\text{C}$ ]glutamate [25 mM; specific activity  $0.565\ \text{mCi mmol}^{-1}$  ( $20.9\ \text{MBq}$ )] was converted to [ $^{14}\text{C}$ ]glutamine and the two amino acids were separated by passing through a column of Dowex-1-Cl (Bio-Rad). Units of activity are expressed as nmols [ $^{14}\text{C}$ ]glutamate converted to [ $^{14}\text{C}$ ]glutamine  $\text{min}^{-1}$  at  $37^\circ\text{C}$ .

**Electrophoresis.** SDS-PAGE was done on 2-mm-thick slab gels, using the discontinuous system described by Laemmli (1970).  $M_r$  markers (Sigma) were bovine serum albumin (67000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soybean trypsin inhibitor (20100) and  $\alpha$ -lactalbumin (14200).

**Non-denaturing PAGE** was done essentially as described by Davis (1964) on slab gels. When the gels were to be tested for GS activity, 10% (v/v) glycerol was included in the gel and the running buffer was 25 mM-imidazole/133 mM-glutamine, pH 8.3. Transferase activity was measured by incubating the gel at  $37^\circ\text{C}$  for 50 min in a 2-fold concentrated enzyme reaction mixture. After stopping the reaction the gels were photographed immediately.

The molecular sieving effect in polyacrylamide gels was used for  $M_r$  determinations as described by Hendrick & Smith (1968). Gels were prepared with acrylamide concentrations of 5, 7.5 and 9% (w/v). A plot of log mobility versus percentage acrylamide concentration in the gel gave a negative slope for each marker protein used. A calibration curve was established by plotting the negative slope values against  $M_r$ . The protein standards (Sigma) used were BSA monomer ( $M_r$  67000), urease dimer (235000) and urease tetramer (483000).

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975). The first dimension was isoelectrofocusing in the pH range 5–7, while the second dimension was SDS-PAGE.

**Purification of GSII.** GSII was purified to homogeneity from *R. l. viceae* grown in minimal medium containing glutamate as a nitrogen source. A crude extract was prepared by sonication of 5 g (wet wt) of bacteria resuspended in 5 ml of extraction buffer (10 mM-imidazole/HCl, pH 7.2, 1 mM- $\text{MnCl}_2$ , 0.5%  $\beta$ -mercaptoethanol, 5 mM-EDTA). After centrifugation at  $12000\ g$  for 10 min at  $4^\circ\text{C}$ , the supernatant was treated with 1% (w/v) streptomycin sulphate for 30 min at  $4^\circ\text{C}$  with gentle stirring and centrifuged. The pellet, containing almost all the GSI activity, was discarded and GSII activity was precipitated with ammonium sulphate. The fraction of proteins precipitating between 33 and 60% saturation was redissolved in 5 ml of buffer A (20 mM-imidazole/HCl, pH 7.2, 1 mM- $\text{MnCl}_2$ ), dialysed against buffer A and loaded onto a column (1  $\times$  10 cm) of Red Sepharose CL-6B (Pharmacia) equilibrated in buffer A. After a period of 2 h, required for efficient adsorption, the column was washed with buffer A and GSII activity was eluted with 40 ml of buffer A containing 33 mM-glutamine and 2 mM-ADP. The most active fractions were concentrated by ultrafiltration and diafiltered with buffer A until glutamine and ADP concentrations were reduced 100-fold. The concentrated sample (4 mg in 1.5 ml) was applied onto a Mono Q Sepharose Fast Flow (Pharmacia) column for FPLC, equilibrated in buffer A. After washing, the column was eluted with a linear gradient of NaCl (0–0.5 M) in buffer A. Fractions (1 ml) were analysed for GSII activity, which resulted in two peaks, A and B. Each fraction was analysed by SDS-PAGE and was found to be >95% pure by scanning analysis. Fractions from each peak were pooled separately and stored at  $-20^\circ\text{C}$  after addition of glycerol (20%, v/v, final concentration). Their specific activity in the transferase assay was usually between 170 and 200, with no significant difference between the two peaks. The overall purification was only 30-fold, indicating a high intracellular concentration of GSII under the growth conditions used. In a typical experiment we obtained, from 5 g of bacteria and 408 mg of soluble protein, 0.41 mg of GSII in peak A and 0.37 mg in peak B with transferase specific activity values of 168 and 197, respectively, and biosynthetic specific activity values in the coupled assay system (Kingdon *et al.*, 1968) of 2.6 and 2.5, respectively. Measurement of the apparent molecular mass by SDS-PAGE showed that each peak contained a single protein of  $M_r$   $40000 \pm 2000$ , in accordance with the value reported by De Vries *et al.* (1983). We used peak A to raise an antiserum and found that it cross-reacted with peak B in an immunoblot experiment. When analysed by two-dimensional gel electrophoresis a sample of peak A gave two spots of identical molecular mass and isoelectric points of 6.0 and 5.85 (data not shown). The ratio of the two spots was analysed by means of a scanner and was found to be 1:5. An identical result was obtained with a sample of peak B, or with a mixture of the two peaks. Different preparations of GSII consistently gave, at the last step of purification, two peaks which, when analysed by two-dimensional gel electrophoresis, resulted in the two spots described above.

**Gel filtration analysis.** Pure GSII was applied to a Superose 12 (Pharmacia) column run on an FPLC apparatus. The elution buffer was 20 mM-imidazole/HCl, pH 7.2, containing 0.3 M-NaCl, at a flow rate of  $0.5\ \text{ml min}^{-1}$ .  $M_r$  markers (Sigma) were ferritin (440000), catalase (230000), aldolase (153000), bovine serum albumin (67000) and ovalbumin (45000).

**Sequence determination.** Automated sequence analysis of pure GSII was performed by Edman degradation with a gas-phase microsequencer (model 470A, Applied Biosystems) and showed homogeneity at each amino acid position, thus confirming enzyme purity. The

sequence was compared with the protein sequences present in the March 1989 EMBL databank using the Microgenie program (Queen & Korn, 1984).

**Immunology.** An anti-GSII antiserum was prepared by injecting rabbits with aliquots of GSII from peak A and, after preliminary tests to check its activity, the serum obtained from a single rabbit was heated at 56 °C for 30 min and stored in 10% (v/v, final concentration) glycerol at -20 °C. Initially this antiserum showed, in an immunoblot experiment, cross-reactivity to several proteins of a crude extract of *Escherichia coli*. Therefore we purified it by affinity chromatography on a microcolumn containing 0.5 ml of CNBr-activated Sepharose 4B (Pharmacia) cross-linked with pure GSII (200 µg) according to the manufacturer's specifications. The crude serum was diluted 10-fold in 10 mM-Tris/HCl buffer, pH 7.4, and loaded onto the column at 4 °C. The flow-through was recirculated 3-fold on the column for maximal adsorption. After washing with 5 ml 10 mM-Tris/HCl, pH 7.4, the column was eluted with 5 ml 100 mM-glycine/HCl, pH 3.0. Fractions were collected in tubes containing an equal volume of 1 M-Tris/HCl, pH 8.0. After concentration by ultrafiltration the sample was dialysed against 4 litres of PBS (1.47 mM-KH<sub>2</sub>PO<sub>4</sub>, 8.2 mM-Na<sub>2</sub>HPO<sub>4</sub>, 1.37 mM-NaCl) containing 0.02% sodium azide, then supplemented with 50% (v/v) glycerol and stored at -20 °C. The purified immunoglobulins were very specific and were used for immunoblots and ELISA experiments.

Immunodiffusion experiments (Ouchterlony, 1958) were done in 1% (w/v) agar in PBS for 1–2 d at 37 °C in a humid chamber. We obtained (not shown) a single precipitin band when using a crude extract of *R. l. viceae* or a crude extract of wild type *E. coli* carrying plasmid pBJ196A (coding for *Bradyrhizobium japonicum* GSII) and no band with a crude extract of wild type *E. coli*.

Immunoblotting was done by transferring proteins from gels to a nitrocellulose sheet using a Bio-Rad transblot cell unit. The nitrocellulose sheets were then incubated with 20 mM-Tris/HCl, pH 7.4, 150 mM-NaCl (Tris-buffered saline, TBS) containing 3% (w/v) bovine serum albumin (BSA) for 1 h at room temperature, with gentle shaking, followed by a 1 h incubation with antiserum (diluted 1:200 in TBS). They were then rinsed three times for 15 min with 20 mM-Tris/HCl, pH 7.4, 0.5 M-NaCl, 0.05% Nonidet P-40 (washing buffer, WB) and once for 10 min with TBS, followed by incubation for 1 h with peroxidase-conjugated goat anti-rabbit antibody. After rinsing three times with WB and once with TBS, the sheets were incubated with TBS containing 0.3 mg diaminobenzidine ml<sup>-1</sup> and 0.03% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped by rinsing with tap water.

ELISA was done according to Engvall (1980), with minor modifications. Microtiter plates were coated overnight at 4 °C with 100 µl per well of a partially purified GSII preparation (100 U mg<sup>-1</sup>; 0.25 mg ml<sup>-1</sup> in 0.1 M-carbonate/bicarbonate coating buffer, pH 9.6). After five washings with 1.47 mM-KH<sub>2</sub>PO<sub>4</sub>, 8.2 mM-Na<sub>2</sub>HPO<sub>4</sub>, 0.5 M-NaCl, 0.05% Tween 20 (phosphate-buffered saline/Tween, PST), the wells were saturated with 100 µl 1% BSA in PBS and the plate was incubated for 1 h at 37 °C in a humid chamber. After washing three times with PST, 100 µl samples of PST containing 0.5% BSA, polyclonal anti-GSII antiserum (1:1600 dilution) and different dilutions of cellular extracts (or pure GSII for calibration) were added to the wells and incubated for 1 h at 37 °C in a humid chamber and then for 2 h at 4 °C. After five washings with PST, 100 µl of peroxidase-labelled goat anti-rabbit IgG at 1:500 dilution were added per well and incubated for 1 h at 37 °C. The amount of antibody bound was detected using 200 µl 0.33 mg *o*-phenylenediamine hydrochloride ml<sup>-1</sup> and 0.016% hydrogen peroxide in 63 mM-Na<sub>2</sub>HPO<sub>4</sub>, 29 mM-citric acid, pH 6.0, for 10 min at room temperature. The reaction was stopped by adding 50 µl 2 M-H<sub>2</sub>SO<sub>4</sub> and the absorbance at 420 nm was determined with an automatic ELISA reader apparatus.

## Results

### Structural analysis of pure GSII

The amino-terminal sequence of GSII is TKFKLEYIWLGDGYTPVNLRGKTQIK; a search in the March 1989 EMBL protein sequences databank revealed no significant homology, except with the amino-terminal sequence of GSII from *B. japonicum* (92.3% identity; Carlson & Chelm, 1986). The protein sequence deduced from the DNA sequence of the GSII structural gene of *R. l. viceae* (Patriarca *et al.*, 1992) is identical to that described above, except for the presence of a methionine codon at the amino-terminus.

### Oligomeric state of GSII

A sample of the pure enzyme was analysed by gel filtration on a Superose 12 column run on a FPLC apparatus. The elution profile showed a major peak corresponding to a  $M_r$  of 350 000, a shoulder at 185 000 and a small peak at 46 000, indicating the presence of an octamer, a tetramer and a monomer, the octamer being predominant. Non-denaturing PAGE (Fig. 1) of pure GSII gave two predominant bands (lane 1), which were both active when the gel was assayed for GSII activity and were present after immunoblotting with the antiserum against GSII (not shown in the Figure). Pre-incubation for 15 min at 4 °C immediately before starting the electrophoresis with either 1 mM-ATP or 1 M-urea gave a different pattern (Fig. 1, lanes 2 and 3, respectively). The slow band was preponderant when the enzyme was pre-incubated with urea, while it disappeared when the enzyme was pre-incubated with ATP and again activity assayed on gels coincided with the bands revealed by Coomassie stain. Non-denaturing PAGE in the presence of appropriate standards (see Methods) and determination of the  $M_r$  by interpolation gave values of 336 000 for the slow band and 170 000 for the fast one. These values fit with those expected for an octamer and a tetramer of an  $M_r$  42 000 monomer. These experiments indicate that under the experimental conditions used, urea has a stabilizing influence on the native conformation and this action is prevalent as compared to the denaturation which would take place at higher urea concentrations.

### Analysis of GSII in cell-free extracts

The anti-GSII antiserum described in Methods was used to analyse cell-free extracts of bacteria grown in minimal medium containing different nitrogen sources. A sample of a crude extract of *R. l. viceae* grown in glutamate

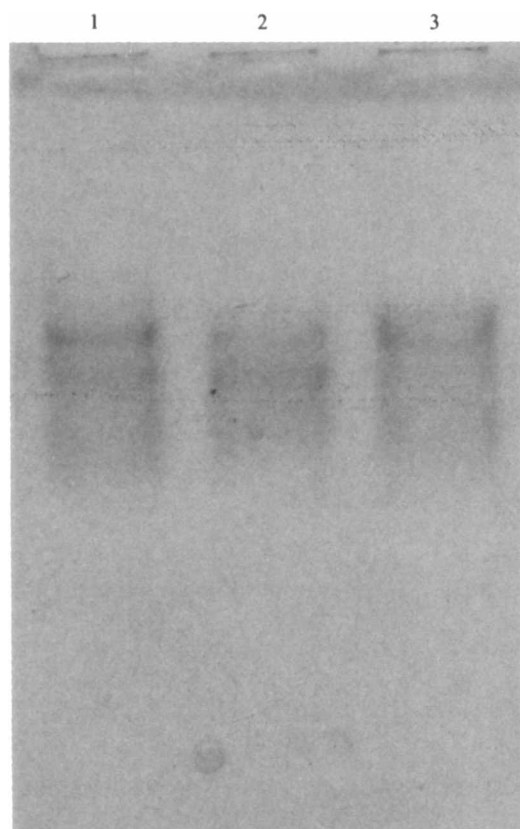


Fig. 1. Non-denaturing PAGE of pure GSII. Samples (10  $\mu\text{g}$ ) were analysed by non-denaturing 5% PAGE. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, untreated enzyme. In lanes 2 and 3 the enzyme was preincubated for 15 min at 4  $^{\circ}\text{C}$  with 1 mM-ATP (lane 2) or with 1 M-urea (lane 3), immediately before starting electrophoresis.

showed a band, after SDS-PAGE and immunoblotting, migrating at the same distance as the pure enzyme. Determination of the  $M_r$  using appropriate markers, gave a value of 40000. No band was detected with a crude extract of *R. l. viceae* grown with  $\text{NH}_4\text{Cl}$ , as nitrogen source, not even when 7.5 times more protein was loaded on the gel, and analysis with a scanner showed that in this crude extract the enzyme concentration was <4% that for bacteria grown with glutamate as nitrogen source. Thus, this experiment shows a correlation between the presence of GSII activity as reported by Rossi *et al.* (1989) and immunoreactive material.

When a culture of *R. l. viceae* grown in glutamate is centrifuged and resuspended in  $\text{NH}_4\text{Cl}$  a decrease in transferase activity is observed (Rossi *et al.*, 1989). We analysed this phenomenon further, and in Fig. 2(a) we report the theoretical rate of disappearance of GSII, assuming that complete repression and no inactivation takes place after  $\text{NH}_4\text{Cl}$  addition. The doubling time of *R. l. viceae* grown under these conditions was 9.5 h and

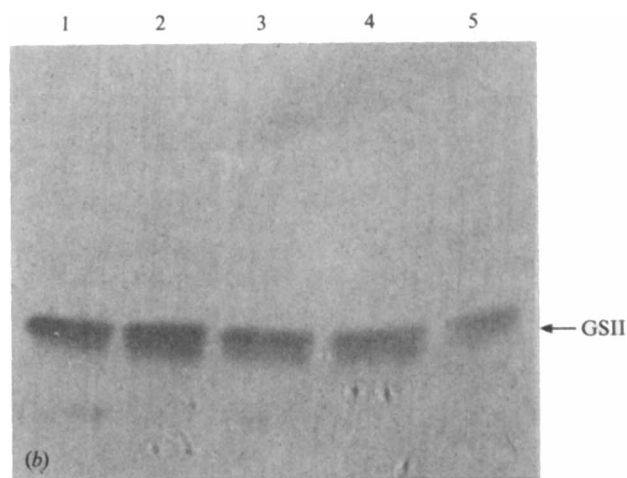
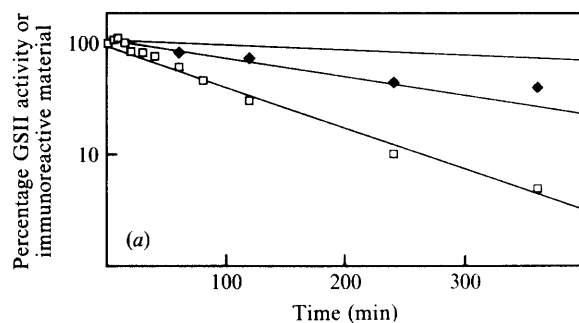


Fig. 2. Effect of  $\text{NH}_4\text{Cl}$ . (a) Specific activity and ELISA of GSII in extracts from  $\text{NH}_4\text{Cl}$ -treated cells. To a culture growing exponentially at 30  $^{\circ}\text{C}$  in minimal medium containing glutamate as sole nitrogen source 1 g  $\text{NH}_4\text{Cl l}^{-1}$  was added at zero time. Samples were removed at the times indicated and cell-free extracts were assayed for transferase ( $\square$ ) activity or for GSII immunoreactive material by Elisa ( $\blacklozenge$ ). Values represent the means of duplicate or triplicate determinations. The extracts were also assayed for biosynthetic activity, either measuring the formation of  $\gamma$ -glutamylhydroxamate formation (Bender *et al.*, 1977) or ADP formation (Kingdon *et al.*, 1968). Data (not shown) superimpose the transferase and ELISA curves, respectively. Curves are linear regression fitting of data points except the upper line which represents the theoretical decrease in specific activity assuming *in vivo* stability of GSII and complete repression. Correlation coefficients were 0.99 for transferase activity, 0.94 for biosynthetic activity ( $\gamma$ -glutamylhydroxamate formation), 0.97 for biosynthetic activity (ADP formation) and 0.94 for the ELISA assay. 100% specific activity corresponds to 4.5 U  $\text{mg}^{-1}$  in the transferase assay, 0.026 U  $\text{mg}^{-1}$  in the biosynthetic assay ( $\gamma$ -glutamylhydroxamate formation) and 0.032 U  $\text{mg}^{-1}$  in the biosynthetic assay (ADP formation); 100% immunoreactive material corresponds to 20  $\mu\text{g}$  (mg proteins) $^{-1}$ . (b) SDS-PAGE and immunoblot of extracts of  $\text{NH}_4\text{Cl}$ -treated cells. Samples were removed at times 0, 1, 3 and 6 h (lanes 1, 2, 3 and 4, respectively). Cell-free extracts (50  $\mu\text{g}$ ) were separated by 10% SDS-PAGE, transferred to a nitrocellulose sheet and treated with the immunostaining procedure. Pure GSII (2  $\mu\text{g}$ ) was run as a control (lane 5). Determination of the  $M_r$ , using appropriate markers (not shown in the figure), gave a value of 40000.

the theoretical rate of disappearance was drawn to give a half-life of 9.5 h. When analysed by ELISA the half-life of GSII disappearance was 4 h. Some of the samples

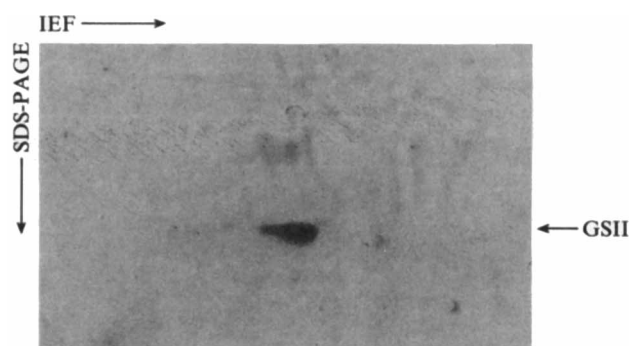


Fig. 3. Two-dimensional PAGE and immunoblot of a cell-free extract after 2 h  $\text{NH}_4\text{Cl}$  treatment. The first dimension was isoelectrofocusing (pH range 7–5, left to right); the second dimension was SDS-PAGE (5–20% acrylamide gradient). A sample (150  $\mu\text{g}$ ) of an extract from cells grown in minimal medium with glutamate as nitrogen source and then treated with 1 g  $\text{NH}_4\text{Cl l}^{-1}$  for 2 h was separated on two-dimensional PAGE, transferred to a nitrocellulose sheet and treated with the immunostaining procedure.

were separated by SDS-PAGE, blotted onto a nitrocellulose sheet and treated with anti-GSII antiserum. As shown in Fig. 2(b), the band corresponding to pure GSII (lane 5) was present in all samples (lanes 1–4) and no extra bands were detected. Scanning analysis of these bands gave a rate of disappearance very similar to that found by ELISA. A sample of the extract from bacteria treated for 2 h with  $\text{NH}_4\text{Cl}$  was analysed by two-dimensional PAGE and immunoblot (Fig. 3); it gave two spots similar to those revealed when the pure enzyme was used (see Methods). The half-lives of disappearance of transferase and biosynthetic activities measured according to Bender (1977) were 90 and 60 min, respectively. On the other hand, biosynthetic activity measured as ADP formation in the coupled assay of Kingdon *et al.* (1968) decreased with a half-life of 4 h.

GSII was partially purified from cells harvested at 0 and 3 h after  $\text{NH}_4\text{Cl}$  treatment. The purification was as described in Methods except that the  $(\text{NH}_4)_2\text{SO}_4$  precipitation and the final Mono Q Sepharose step were omitted. After affinity chromatography on Red Sepharose CL-6B the specific activity values for GSII purified from untreated bacteria were 29 for transferase and 147 for biosynthetic activity (measured as the rate of  $[^{14}\text{C}]$ -glutamine synthesis), with a ratio of 0.2. In the case of the enzyme purified from the sample treated with  $\text{NH}_4\text{Cl}$  the specific activity values were 6 for transferase and 47 for biosynthetic activity, with a ratio of 0.13.

In conclusion, the ratio of transferase to biosynthetic activity was shown to be altered in the partially purified GSII to about the same extent as it is in crude extracts (Fig. 2a). This result indicates that the presumed post-translational modification of GSII is stable, being

present also after purification and that, under our experimental conditions, the radioactive assay gives the same result as obtained with extracts in the coupled assay system (Fig. 2a).

Since  $\text{NH}_4\text{Cl}$  treatment has been reported to cause a dissociation of active octamers in the case of GS activity of *Candida utilis* (Sims *et al.*, 1974) and *Neurospora crassa* (Lara *et al.*, 1982) we investigated this phenomenon with *R. l. viceae* GSII. Crude extracts of *R. l. viceae* grown with glutamate were separated on non-denaturing PAGE and analysed both by immunoblotting and as enzyme activity using a sample of pure enzyme as a control. With both methods we detected two bands in the same position as those shown for pure enzyme in Fig. 1. When the experiment was done with bacteria treated for 6 h with  $\text{NH}_4\text{Cl}$ , the ratio of the two bands did not change.

## Discussion

The antiserum we obtained was highly specific since it gave a single band when a crude extract of *R. l. viceae* was run on SDS-PAGE (Fig. 2b). The availability of this antiserum permits correlation of the measurement of intracellular enzyme activity (Rossi *et al.*, 1989) with that of immunoreactive material.

Pure GSII from *R. l. viceae* is an octamer made of identical subunits of  $M_r 40000 \pm 2000$  and under specific experimental conditions the octamer dissociates into a tetramer (Fig. 1). Pre-incubation of the enzyme in 1 M-urea for 15 min at 4 °C immediately before starting electrophoresis causes association of the tetramer into an octamer (Fig. 1). We believe that under these conditions urea enhances hydrophobic interactions, thus favouring association, or, alternatively, induces a conformational change, which in turn favours association. A temperature-dependent reversible dissociation of GS from bovine brain incubated in 1 M-urea was reported by Wilks *et al.* (1969). *E. coli* GS, dissociated and inactivated with urea and EDTA, was reported (Woolfolk & Stadtman, 1967) to need 1.5 M-urea for maximal reassociation and reactivation. In Fig. 1 we also show that pre-incubation of the pure enzyme with 1 mM-ATP causes dissociation of the octamer into tetramers. An analogous destabilizing effect of ATP was reported for GS of *Candida utilis* (Sims *et al.*, 1974). However, these authors and others (Lara *et al.*, 1982), working on GS from *Neurospora crassa*, found evidence that GS activity may be controlled *in vivo* when the culture is transferred from glutamate to ammonium by a shift from active octamers to tetramers with decreased catalytic activity. We did not observe evidence for a change in the octamer/tetramer ratio in the experimental conditions of Fig. 2.

Pure GSII obtained with the procedure described in this paper has two isoforms of identical  $M_r$  but different isoelectric points (5.85 and 6.0) (data not shown). Although it is possible that one of these forms is generated from the other during the purification procedure, a comparison with results obtained with the crude extract (Fig. 3) makes this interpretation unlikely. We believe that this microheterogeneity is either produced during the two-dimensional gel electrophoresis or, more likely, is present *in vivo* as reported previously for other proteins (for a review see Stadtman, 1990).

Comparison of the amino-terminal sequence of *R. l. viceae* GSII with that of *B. japonicum* and *R. meliloti* GSII (Shatters *et al.*, 1989) shows a high degree of similarity, suggesting that, at least in the amino-terminal domain of GSII, the function is important for the evolutionary fitness of these bacteria. An analysis of the location of the differences found shows that most of them are at positions 13 to 19 of *R. meliloti* GSII as compared to *R. l. viceae* and *B. japonicum* GSIIs (Carlson & Chelm, 1986).

We previously reported (Rossi *et al.*, 1989) that  $\text{NH}_4\text{Cl}$  treatment of a *R. l. viceae* culture derepressed for GSII resulted in rapid removal of transferase activity. Results reported in this paper show that the GSII protein during this treatment was neither destroyed nor grossly modified, since its  $M_r$  and isoelectric point (Fig. 3) did not change while, at the same time, transferase activity decreased more rapidly than antigenicity, assayed by ELISA (Fig. 2a) or scanning of immunoblots (Fig. 2b). These results can only be interpreted as being due to a post-translational modification. We also measured biosynthetic activity with hydroxylamine according to Bender *et al.* (1977) and the rate of ADP formation according to Kingdon *et al.* (1968). Surprisingly, we found a dissociation of the activities, in that both assays performed in the presence of hydroxylamine showed a faster rate of decrease as compared to the decrease of biosynthetic activity measured in the coupled assay of Kingdon *et al.* (1968), which paralleled the decrease in immunoreactive material. In order to check if this dissociation of activities was stable and independent of the reversible binding of some inhibitors, we partially purified GSII after  $\text{NH}_4\text{Cl}$  inactivation. The ratio of transferase to biosynthetic activity changed in the enzyme purified after  $\text{NH}_4\text{Cl}$  treatment, similar to what happens with crude extracts. It appears, therefore, that upon  $\text{NH}_4\text{Cl}$  treatment GSII is modified in such a way that glutamate activated on the enzyme surface is less susceptible to reaction with hydroxylamine. This modification can affect the activities of GSII by changing either the  $K_m$  or the  $V_{\max}$ . A preliminary experiment, in which the concentration of hydroxylamine in the

standard transferase assay was increased 10-fold, suggests that the observed effect is not due to a change in  $K_m$ .

An analogous dissociation between transferase and biosynthetic activity (measured either as ADP or  $\gamma$ -glutamylhydroxamate formation) was reported by Kim & Rhee (1987) for *Saccharomyces cerevisiae* GS treated with ATP and L-methionine sulphoximine. These authors propose that a modified subunit with decreased transferase activity transmits to the neighbouring subunits a signal enhancing their biosynthetic activity. An apparent dissociation between transferase and biosynthetic activity may be present also in the data reported by Bravo & Mora (1988).

Post-translational modifications of GSs other than adenylation/deadenylation can exist. A post-translational oxidation of *Klebsiella aerogenes* and *E. coli* GS was described by Levine *et al.* (1981) as a marking step for protein degradation. In *Rhodospirillum rubrum*, Woehle *et al.* (1990) reported that GS was the target of both ATP- and NAD-dependent modification. Moreover, GS from the cyanobacterium *Synechocystis* sp. strain PCC 6803 was modified to an inactive form when grown in  $\text{NH}_4\text{Cl}$  (Merida *et al.*, 1990) by an unidentified mechanism.

The experiments reported in this paper indicate that the reported rapid removal of GSII activity by  $\text{NH}_4\text{Cl}$  treatment in crude extracts (Bravo & Mora, 1988; Fuchs & Keister, 1980; Howitt & Gresshoff, 1985; Ludwig, 1980; Rossi *et al.*, 1989) may be explained as a post-translational modification, thus excluding other hypotheses such as accumulation of an inhibitor, change in the octamer/tetramer ratio or protein degradation. A post-transcriptional control was suggested by Adams & Chelm (1988) to explain the finding of the presence of GSII transcript in the absence of GSII transferase activity in  $\text{N}_2$ -fixing bacteroids or free living microaerobic *B. japonicum* (Rao *et al.*, 1978). We believe it is likely that GSII activity in bacteroids is modified by a mechanism similar to that reported in Fig. 2, and therefore the measurement of transferase activity of GSII in bacteroids would cause misleading interpretations. Indeed, work in our laboratory (C. Lopes, unpublished) using immunogold detection has shown that GSII is present in bacteroids of *R. l. viceae*.

It is tempting to speculate that the presence of GSII in all *Rhizobiaceae* and its strongly conserved structure both at the level of amino acid sequence and cross-hybridization (our unpublished results) are correlated with an as-yet unknown function in the symbiosis. Elucidation of the post-translational modification inferred from results presented here will help to clarify this problem.

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