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Appearance of a novel form of plant glutamine synthetase during nodule development in *Phaseolus vulgaris* L.

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Abstract. The activities of glutamine synthetase (GS), nitrogenase and leghaemoglobin were measured during nodule development in *Phaseolus vulgaris* infected with wild-type or two non-fixing (Fix^-) mutants of *Rhizobium phaseoli*. The large increase in GS activity which was observed during nodulation with the wild-type rhizobial strain occurred concomitantly with the detection and increase in activity of nitrogenase and the amount of leghaemoglobin. Moreover, this increase in GS was found to be due entirely to the appearance of a novel form of the enzyme (GS_{n1}) in the nodule. The activity of the form (GS_{n2}) similar to the root enzyme (GS_r) remained constant throughout the experiment. In nodules produced by infection with the two mutant strains of *Rhizobium phaseoli* (JL15 and JL19) only trace amounts of GS_{n1} and leghaemoglobin were detected.

Key words: Glutamine synthetase – Leghaemoglobin – Nitrogenase – Nitrogen fixation – *Phaseolus* – *Rhizobium*.

Introduction

During nodulation of legumes, *Rhizobium* bacteroids synthesize nitrogenase and can thus fix dinitrogen into ammonia which is then excreted into the plant fraction of the nodule (for a recent review see Robertson and Farnden 1980). Increases in the activity of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (EC 1.4.1.14) in the plant cells of the nodule occur concomitantly with nitrogenase production (Robertson et al. 1975a, b; Werner et al. 1980) and these two enzymes together are responsible for assimilating the newly fixed ammonia via the glutamate synthase cycle (Meeks et al. 1978; Ohyama and Kumazawa 1980). In nodules which lack nitrogenase activity, due to changes in either the rhizobial or plant genome, no increase in GS activity have been observed (Werner et al. 1980; Sen and Schulman 1980; Groat and Vance 1982).

We have been concerned with the regulation of plant GS in the nodule and have established that nodules of *Phaseolus vulgaris* possess a novel form of the enzyme (GS_{n1}) in addition to a form (GS_{n2}) which appears identical to that in roots (GS_r) (Cullimore et al. 1983). In this paper, changes in the activities of these two forms during nodulation using both fixing (Fix^+) and non-fixing (Fix^-) strains of *Rhizobium* have been investigated.

Materials and methods

Chemicals. Sephadex G-25 and diethylaminoethyl (DEAE)-Sephacel were obtained from Sigma Chemical Company, Poole, Dorset, UK.

Organisms. Phaseolus vulgaris L. cv. Bush Blue Lake 274. *Pisum sativum* L. cv. Feltham First. *Rhizobium phaseoli*, wild-type strains R3622 and 8002; Nod^+ Fix^- mutants JL15, JL19 (Lamb et al. 1982). *Rhizobium leguminosarum*, Fix^+ strain T83K3 (Johnston et al. 1978).

Growth. Plants were inoculated with *Rhizobium* 10 d after sowing and grown in Leonard jars (Leonard 1943) as modified by Dye (1979) under the conditions described by Cullimore et al. (1983). Those inoculated with the Fix^- mutants of *Rhizobium* were supplemented with 30 ppm KNO_3 in the nutrient solution. The nodulated root systems were harvested starting 10 d following inoculation and assayed immediately for nitrogenase activity. Nodules were then picked and stored in liquid nitrogen prior to assaying leghaemoglobin and GS.

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Abbreviations: DEAE-Sephacel = diethylaminoethyl-Sephacel; GS = glutamine synthetase

Glutamine synthetase determination. Nodules (0.5 g) were homogenised in a mortar and pestle with 0.2 g polyclar AT and 2.5 ml running buffer (10 mM 2-amino-2-(hydroxymethyl)-1,3-propandiol (Tris)-Cl buffer, pH 8.4, containing 10 mM Mg-acetate and 10% glycerol). The homogenate was centrifuged at 12,000*g* for 15 min and the supernatant was adjusted to 3 ml; 1 ml of this crude extract was desalted on a small column of Sephadex G-25 and then applied to a column (14 cm long, 1.0 cm diameter) of DEAE-Sephacel equilibrated in running buffer. The proteins were eluted at a flow rate of 7.5 ml h^{-1} with 20 ml buffer followed by 40 ml of a linear 0–300 mM KCl gradient in buffer. Fractions (1 ml) were collected and GS activity was determined by the transferase assay (Cullimore and Sims 1980).

Assay of nitrogenase and leghaemoglobin. Nitrogenase activity was measured by acetylene reduction as described by Dart et al. (1972). Leghaemoglobin was determined by the pyridine haemochromogen method as described by Bergersen et al. (1973) in extracts prepared from 0.1 g of nodules in 2 ml 100 mM K-phosphate buffer, pH 7.0.

Results

Plants were grown in Leonard jars in order to obtain uniform development in response to inoculation with defined strains of *Rhizobium*. Samples of nodules were harvested at times following infection of *Phaseolus vulgaris* (cv. Bush Blue Lake 274) with *Rhizobium phaseoli* R3622. Nodule nitrogenase and leghaemoglobin were assayed and the contribution of each form of GS to total GS activity was determined following separation of the two forms on DEAE-Sephacel (Fig. 1). Ten days after inoculation neither nitrogenase nor leghaemoglobin were detected and only a single form of GS activity (GS_{n2}) eluted from DEAE-Sephacel at a KCl concentration identical to that of the root enzyme (Cullimore et al. 1983). At day 12, however, another peak of GS activity (GS_{n1}) was observed which eluted at a KCl concentration of 25 mM. The activity of GS_{n1} increased over the subsequent days such that by day 18 its activity was greater than GS_{n2}. Because of its greater sensitivity, the transferase activity of GS was measured in these experiments. However, it has previously been shown that GS_{n1} and GS_{n2} consistently have different transferase:synthetase-activity ratios of 30 and 90 respectively (Cullimore et al. 1983) and in Fig. 2 these ratios have been used to calculate from Fig. 1 the synthetase activities of the two forms at different times during nodulation. The results establish that by day 18 GS_{n1} represented 84% of total nodule GS activity. Moreover, the appearance and increase in activity of this form occurred simultaneously with nitrogenase and leghaemoglobin, whereas, the activity of GS_{n2} decreased slightly during the experiment and was not markedly different from the activity in non-nodulated

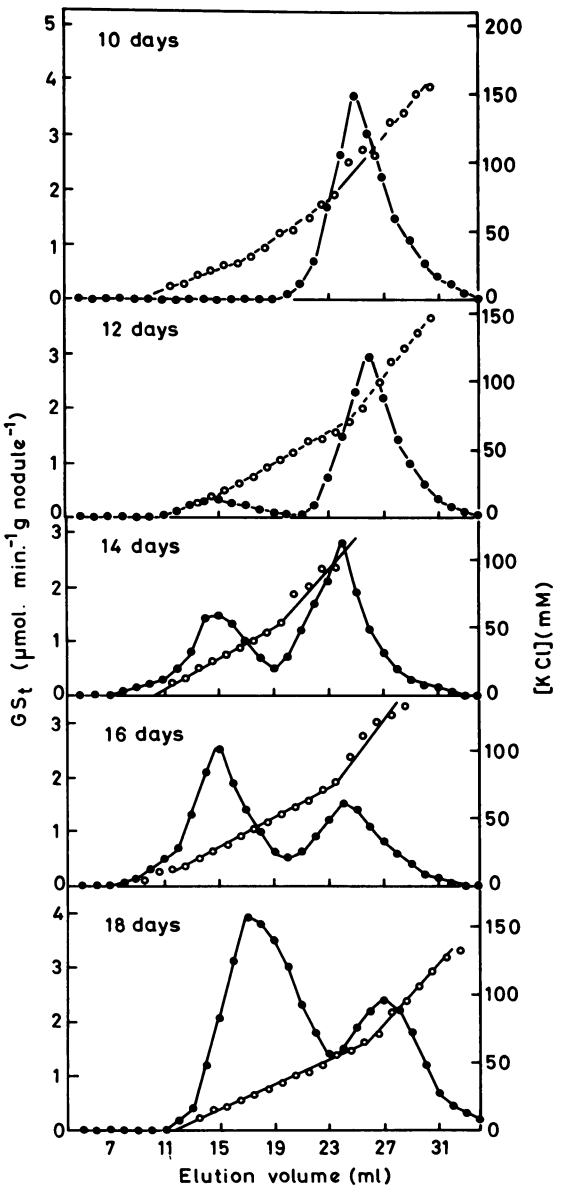

Fig. 1. Elution profiles of GS activity from DEAE-Sephacel of extracts of nodules harvested at different times following inoculation of *Phaseolus vulgaris* with *Rhizobium phaseoli* R3622. Activity of GS (●) and KCl concentration (o) were determined. GS_t = transferase activity

roots. Roots treated for 7 d with 2 mM ammonium sulphate contained only a single form of GS which eluted from DEAE-Sephacel at the same KCl concentration as the enzyme from untreated roots (data not shown).

The above experiment was repeated using two Fix^- mutants derived from the wild-type *Phaseolus* strain 8002. These were obtained by insertion of transposon Tn5 into pRP2JI, the plasmid carrying the genes for nodulation and nitrogen fixation (Lamb et al. 1982). Nodules were harvested 19 d

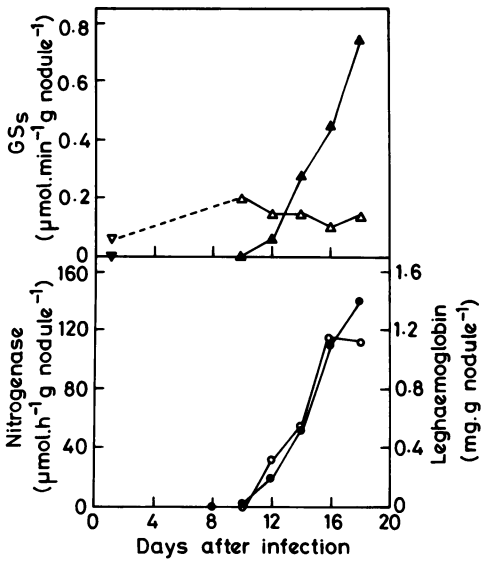

Fig. 2. Time course of changes in the activity of GS_{n1} (▲), GS_{n2} (△) and nitrogenase (●) and amount of leghaemoglobin (○) during nodule development of *Phaseolus vulgaris* infected with *Rhizobium phaseoli* R3622. Activity of GS_{n1} (▼) and GS_{n2} (▽) in non-nodulated roots. GS_s = synthetase activity

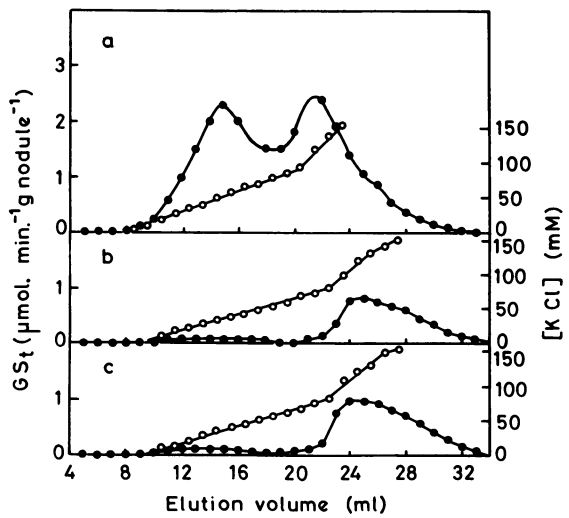

Fig. 3a–c. Elution profiles of GS activity from DEAE-Sephacel of extracts of nodules of *Phaseolus vulgaris* harvested 19 d following inoculation with *Rhizobium phaseoli* strains **a** Fix^+, 8002, **b** Nod^+ Fix^- mutant JL15 and **c** Nod^+ Fix^- mutant JL19. Activity of GS (●) and KCl concentration (○) were determined

after infection and analysed for GS activity (Fig. 3). Although nodules, formed after inoculation with the wild-type strain, contained both forms of GS as before, those formed after inoculation with the mutants had only trace amounts of GS_{n1} and only 22% and 24% of normal GS activity (Table 1). Small amounts of leghaemoglobin

Table 1. Activities of GS and nitrogenase and amount of leghaemoglobin in nodules of *Phaseolus vulgaris* infected with wild-type or Nod^+ Fix^- mutants of *Rhizobium phaseoli*. Nodules were harvested 19 d after infection

Rhizobium strain	GS_t	Nitrogenase	Leghaemoglobin
	(% of that with 8002)		
8002	100[a]	100[a]	100[a]
JL15	22	—[b]	6
JL19	24	—[b]	3

[a] 100% for GS, nitrogenase and leghaemoglobin represented respectively 72 μmol min^{-1} g $nodule^{-1}$, 120 μmol h^{-1} g $nodule^{-1}$ and 1.9 mg g $nodule^{-1}$

[b] Not detected

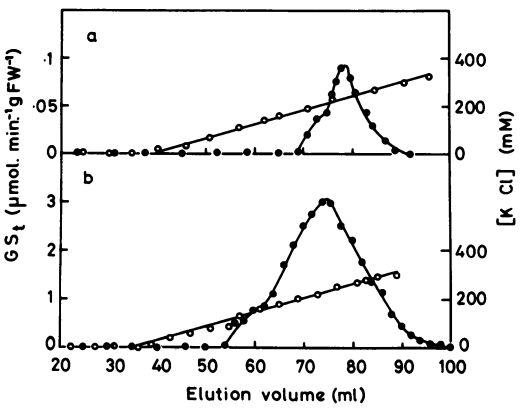

Fig. 4a, b. Elution profiles of GS activity from DEAE-Sephacel of extracts of **a** roots of *Pisum sativum* and **b** nodules harvested 18 d after inoculation with *Rhizobium leguminosarum* Fix^+ strain T83K3. Activity of GS (●) and KCl concentration (○) were determined

were detectable but the nodules completely lacked nitrogenase activity.

In pea nodules the activity of GS (synthetase activity g^{-1} fresh weight) was 38-fold higher than in roots (Fig. 4). However, multiple forms of the enzyme could not be separated on DEAE-Sephacel, although the GS elution profile was broader than that from roots. Also the transferase:synthetase-activity ratios of the most active fractions were found to differ with values of 40 and 95 for the nodule and root activities respectively.

Discussion

The results reported here demonstrate that the 16-fold increase in GS activity that occurs during nodulation of *Phaseolus vulgaris* is the result of the production of a new, nodule-specific, form of the enzyme. The presence of multiple forms of GS has not previously been demonstrated in nodules

during either the purification of soybean nodule GS (McParland et al. 1976), polyacrylamide-gel electrophoresis of lupin nodule GS (Robertson et al. 1975a) or DEAE-Sephacel chromatography of extracts of nodules of a number of different legume species (including *Pisum sativum, Glycine max, Lupinus angustifolius, Vigna unguiculata, Vicia faba, Cajanus cajan* and *Cicer arietinum,* data not shown). However, although only one peak of GS activity was observed in nodules of *Pisum* (Fig. 4), its increased width and lower transferase: synthetase-activity ratio in comparison with the root enzyme indicates that multiple forms may also be present in this species but difficult to separate under the conditions employed. It is possible, therefore, that the production of nodule-specific forms of GS may be a widespread strategy among the legumes for assimilating ammonia produced by nitrogen fixation.

During nodule development of *Phaseolus*, the appearance of GS_{n1} occurred concomitantly with the production of nitrogenase and leghaemoglobin (Fig. 2). Similar concurrent increases in these three proteins occur in lupin (Robertson et al. 1975a). These observations could indicate that bacterial nitrogenase and the plant proteins, leghaemoglobin and GS_{n1}, are under a common control mechanism and that their synthesis is interdependent. However, using specific antibodies to leghaemoglobin, a much more sensitive method of detecting this protein than the haemochromogen method used here, leghaemoglobin was found in nodules of soybean (Verma et al. 1979) and pea (Bisseling et al. 1979) several days before nitrogenase. Furthermore, in both soybean (Werner et al. 1980; Sen and Schulman 1980) and *Phaseolus* nodules infected with Fix^- mutants of *Rhizobium* no increase in GS activity was observed during nodule development. In *Phaseolus* this was because of the almost complete lack of production of GS_{n1} (Fig. 3). However, very small amounts of GS_{n1} and also leghaemoglobin could be detected in these nodules, although nitrogenase activity was completely absent (Table 1; Fig. 3). These results are in agreement with work on soybeans where leghaemoglobin and its mRNA were detected, although at much reduced levels, in nodules produced using several non-fixing strains of *R. japonicum* (Verma et al. 1981). Verma (1980) has suggested that induction of leghaemoglobin is independent of nitrogenase but that factors from *Rhizobium* may modify the magnitude of the induction. It appears, therefore, that regulation of the host-nodule proteins cannot be explained by a simple control mechanism. This conclusion has been supported by recent work which has demonstrated that a large number of nodule-specific proteins (nodulins), in addition to leghaemoglobin, are involved in an effective symbiosis (Legocki and Verma 1980) and that in nodules developed with several ineffective strains of *Rhizobium*, the abundance of these proteins as well as the amounts of mRNA associated with them are differentially affected (Auger and Verma 1981).

The work reported in this paper could indicate that GS_{n1} is a nodulin. Although activity of this enzyme appears to be ubiquitous in higher plant tissues, different forms of the enzyme are produced to assimilate ammonia from different sources at different locations (see Miflin and Lea 1982). In nodules, the primary role of GS_{n1} must be to assimilate the ammonia produced by nitrogen fixation since the activity of GS_{n2} alone is too low to perform this function; the regulation of the appearance of GS_{n1} is being investigated.

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