Glutamine synthetase from the plant fraction of *Phaseolus* root nodules

Purification of the mRNA and in vitro synthesis of the enzyme

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Received 11 May 1983

Antiserum raised to glutamine synthetase (GS) from the plant fraction of *Phaseolus vulgaris* root nodules was used to immunoprecipitate in vitro translation products of nodule and root poly(A)$^+$ RNA. The results showed that the large increase in GS activity which occurs during nodulation is due to an increased supply of GS mRNA. A 3-fold enrichment of GS mRNA was obtained by centrifugation of total nodule poly. (A)$^+$ RNA on dimethylsulphoxide–formamide-containing sucrose density gradients and purification to near homogeneity was achieved by immunopurification of nodule polysomes.

Phaseolus vulgaris *Glutamine synthetase* *In vitro translation* *mRNA purification* *Nodulin* *Legume*/Rhizobium *symbiosis*

1. INTRODUCTION

Glutamine synthetase (GS) (EC 6.3.1.2) has a universal role in the nitrogen metabolism of all groups of living organisms [1–3]. In higher plants it is of particular importance as apparently it represents the only route utilized for the assimilation of inorganic nitrogen whereas in some prokaryotes, lower plants and mammals, alternative pathways may also operate [3].

In legume root nodules, plant GS activity may represent up to 2% of the total soluble protein [4,5] and is responsible for the assimilation of ammonia derived from dinitrogen fixation in the bacteroids (see [6]). During nodulation a large increase in GS plant activity occurs [7,8] and in *Phaseolus* this is due to the production of a 'nodule specific'* form of the enzyme (GS_{n1}) different from the form (GS_r) present in the roots; nodules also contain a second form of GS (GS_{n2}), apparently identical to GS_r, which changes little in activity during nodulation [9,10].

Despite the importance of GS in nodules, little is known of the mechanism of the regulation of its expression. Messenger RNA for this enzyme has been identified and quantified in some animal tissues [11,12], prokaryotes [13,14] and *Neurospora crassa* [15–18] and GS genes have been isolated from several prokaryotes [19–22]. However, to date there appears to be no published reports on GS mRNA from higher plants where the enzyme is of the most importance. Here, we report the identification and purification of GS mRNA isolated from roots and nodules of *Phaseolus vulgaris*.

* In this paper 'nodule specific' signifies proteins not detectable in control root tissue

2. MATERIALS AND METHODS

2.1. *Plant material*

Seedlings of *Phaseolus vulgaris* var. Bush Blue Lake 274 were inoculated with *Rhizobium phaseoli* R3622 and grown as in [9]. Nodules were harvested 3 weeks after inoculation and stored in liquid N_2.

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00145793/83/$ 3.00

Roots were obtained from non-inoculated plants grown for 2 weeks.

2.2. *Isolation of polyadenylated RNA*

Poly(A)$^+$ RNA was isolated from total polysomes obtained from nodules and roots [23].

2.3. *Size fractionation of polyadenylated RNA*

Poly(A)$^+$ RNA, prepared as above but passed twice through the oligo(dT)-cellulose column, was boiled for 1 min, cooled rapidly and loaded (33 μg/gradient) onto 3 dimethyl sulphoxide–formamide containing sucrose gradients (5–20%, w/v) [24]. A marker gradient containing 30 μg calf liver tRNA and 60 μg nodule rRNA was also run. Centrifugation was at 40000 rev./min at 28°C for 37 h on a Beckman L5-65 ultracentrifuge using rotor SW40. The gradients were fractionated (0.2 ml fractions) and the RNA recovered with 5 μg/fraction of tRNA by ethanol precipitation. Fractions 14–17 which contained mRNA coding for GS (fig.2b,c) were bulked, rerun on similar gradients and the RNA recovered from the fractions as before.

2.4. *Immunopurification of GS mRNA*

Essentially the procedures in [25] were followed. Total polysomes freshly prepared from 58 g nodules in solutions containing 100 μg sodium heparin/ml and 1 μg trichodermin/ml (a kind gift from Leo Pharmaceutical Products, Ballerup) were resuspended in polysome buffer [50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 100 μg sodium heparin/ml and 1 μg trichodermin/ml], centrifuged at 12000 × g for 10 min and the supernatant adjusted to A_{260} of 6 units/ml. The IgG fraction of antiserum prepared against GS_{n1} [26] was made RNase free [27] and 3 mg was added to the polysomes. Incubation was allowed to proceed at 0°C for 16 h. The immunoprecipitated polysomes were isolated on a 2 ml column of protein A–Sepharose and the mRNA was obtained from them by oligo(dT)-cellulose chromatography and recovered by ethanol precipitation.

2.5. *In vitro protein synthesis and immunoprecipitation of translation products*

Cell-free translations used the rabbit reticulocyte lysate system [28] and [^{35}S]methionine. Translation products were immunoprecipitated using 50 μg anti-GS-IgG [26] exactly as in [29].

2.6. *SDS–polyacrylamide gel electrophoresis (SDS–PAGE)*

Acrylamide gels (12.5%) were run according to [30]. Protein standards: β-lactoglobin (M_r 18400), trypsinogen (M_r 24000), ovalbumin (M_r 45000) and bovine plasma albumin (M_r 66000) were labelled using [^{14}C]formaldehyde [31]. GS_{n1} was purified as in [9] and was also labelled. The gels were subjected to fluorography [32], dried and ex-

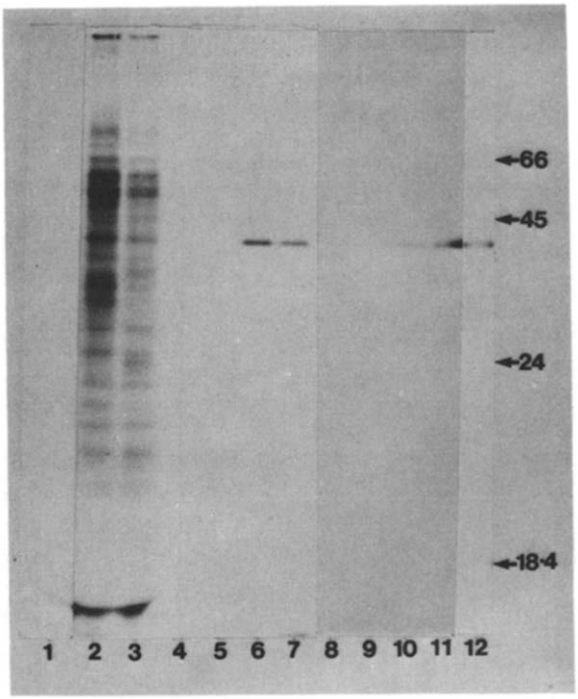

Fig.1. SDS–PAGE of the in vitro translation products of nodule and root poly(A)$^+$ RNA and immunoprecipitates of these products using anti-GS-IgG. Translations using: (1) no added RNA; (2) nodule poly(A)$^+$ RNA; (3) root poly(A)$^+$ RNA; (4–7) immunoprecipitates of nodule poly(A)$^+$ RNA translation products using (4) no IgG, (5) control (pre-immune) IgG, (6) anti-GS_{n1}-IgG, (7) anti-GS_{n2}-IgG; (8–11) as (4–7) but immunoprecipitates of root poly(A)$^+$ RNA translation products; (12) GS marker. Tracks 2 and 3 contained 41000 trichloroacetic acid-precipitable cpm. Three-fold this amount of translation products was used for immunoprecipitation and this part of the gel was exposed to X-ray film for 3-fold longer.

posed to Fuji RX X-ray film. Relative amounts of polypeptides were determined by measuring and integrating the densities and areas using a Quantimet 720 Image Analysing Computer (Cambridge Instruments).

3. RESULTS

Poly(A)$^+$ RNA, isolated from polysomes of nodules and roots of *Phaseolus vulgaris* was translated in vitro and the products characterized by SDS–PAGE (fig.1). Many polypeptides were present in both sets of translation products although some were more abundant or exclusive to either the nodule or root mRNA translations. Leghaemoglobin, which constitutes 20–30% of the total soluble protein and which is specific to nodules [33] could not be identified as it lacks methionine [34].

The translation products were immunoprecipitated with antisera raised to either GS_{n1} or GS_{n2} and the immunoprecipitates were run on the same SDS–polyacrylamide gels (fig.1). Control (pre-immune) serum or buffer alone did not produce an immunoprecipitate. However, the two antisera, both of which precipitate GS_{n1} and GS_{n2} [26], immunoprecipitated a single polypeptide from both the nodule and root mRNA translations which comigrated with authentic labelled GS. This immunoprecipitated polypeptide (GS) was more abundant in the nodule than the root mRNA translations.

Nodule poly(A)$^+$ RNA was size fractionated by centrifugation on denaturing sucrose density gra-

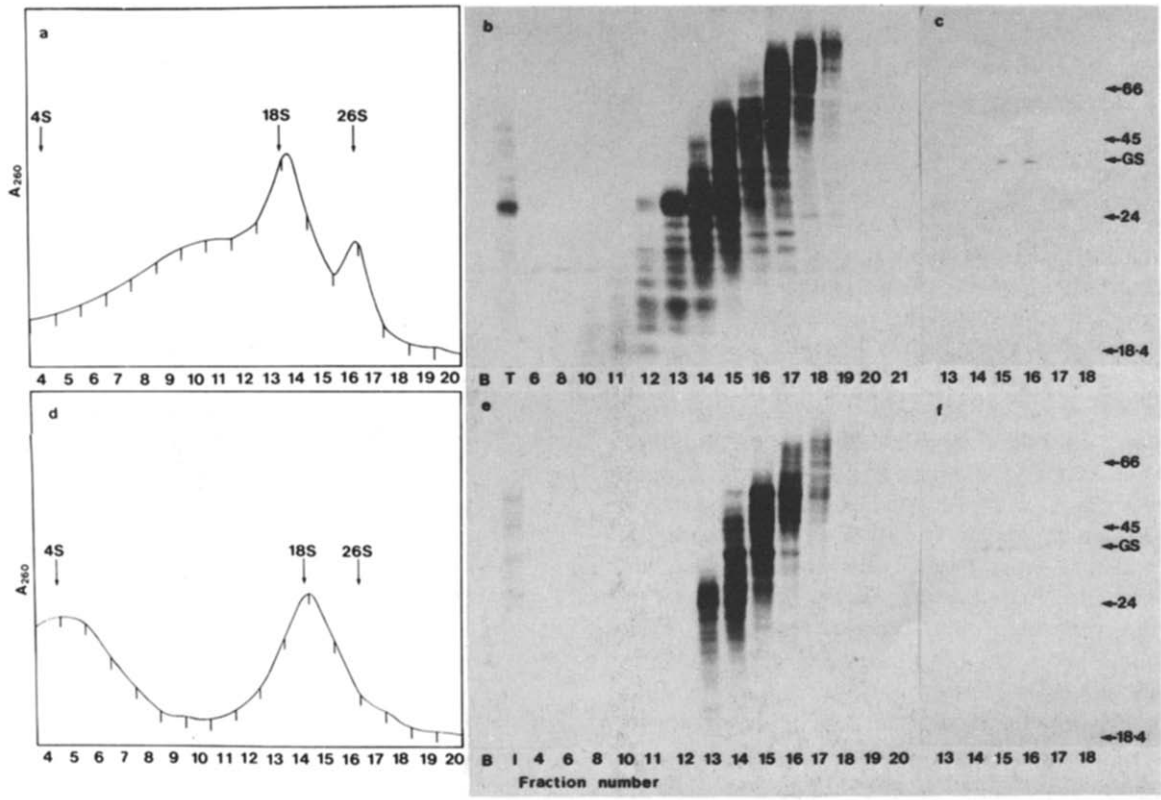

Fig.2. Partial purification of GS mRNA by dimethyl sulphoxide–formamide sucrose density-gradient centrifugation of nodule poly(A)$^+$ RNA: (a) profile of A_{260} during fractionation of the gradient; (⟶) position of RNA markers. SDS–PAGE of (b) in vitro translation products of the fractions and (c) immunoprecipitates of the translation products using anti-GS_{n1}-IgG. (d–f) Fractions 14–17 of the first gradient were bulked, rerun on similar gradients and analyzed as (a–c). B, translation using no added RNA; T, using nodule poly(A)$^+$ RNA; I, using RNA bulked from fraction 14–17 of first gradient.

dients. The UV absorbance of the fractions (fig.2a) revealed major peaks at 18 S and 26 S suggesting that substantial amounts of rRNA were still present in the mRNA sample. The broad peak at 9 S probably represents leghaemoglobin mRNA [35]. The fractions were translated in vitro (fig.2b) and the products immunoprecipitated (fig.2c) and analyzed by SDS–PAGE. Partial separation of the mRNAs had been achieved as judged by the increasing size of the translation products in the consecutive fractions (fig.2b). GS mRNA was identified in fractions 14–17 covering a range of ~16–26 S (fig.2c). These fractions were bulked and rerun on similar gradients in order to achieve a greater enrichment of GS mRNA (fig.2d–f). A single UV absorbance peak at about 18 S was observed in these second gradients (fig.2d) apart from the peak at 4 S which corresponded to tRNA previously added to the sample. GS mRNA was again identified in fractions 14–17 (fig.2e,f). Quantimet analysis of the fluorographs showed that GS represented about 2.4% of the total translation products of nodule poly(A)$^+$ RNA, 3.5% in fraction 15 of the first sucrose density gradients and 5.8% in fraction 15 of the second gradients.

GS mRNA was also purified by immunoprecipitating polysomes with anti-GS_{n1}-IgG followed by isolation of the poly(A)$^+$ RNA (fig.3). When translated in vitro the mRNA obtained by this method produced a single major band on SDS–polyacrylamide gels (track 5) and this comigrated with authentic labelled GS (track 6). The two other bands in this track at M_r ~52000 and 22000 were also apparent when no RNA was added to the reticulocyte lysate system (track 1 and 2).

By comparison to the labelling obtained when 0.5 μg nodule poly(A)$^+$ RNA was translated in this system, and taking into account that 25% of the products (represented by leghaemoglobin) were not labelled with methionine, it was calculated that fraction 15 of the second sucrose density gradient and the sample obtained by polysome immunoprecipitation contained 2.6 μg and 170 ng of RNA, respectively.

4. DISCUSSION

This paper represents the first report on the study and partial purification of GS mRNA from higher plants. In nodules and roots of *Phaseolus vulgaris* GS mRNA could be demonstrated in preparations of poly(A)$^+$ RNA by in vitro translation and immunoprecipitation of the products with anti-GS-IgG (fig.1). GS produced in this way had the same subunit M_r as the enzyme isolated from extracts of nodules and this suggests that the newly synthesized polypeptide is not processed by proteolysis before the active octomeric enzyme is formed. This result coupled with the observation that GS is mostly synthesized on free polysomes (unpublished) lends support to the cytosolic localization of GS in these tissues [3,36] since many organelle located proteins encoded in the nucleus are synthesized either on membrane-bound polysomes and/or with peptide leader sequences which enable transport across the organelle membranes [37].

GS mRNA behaved on sucrose density gradients as a single entity and corresponded to a size of about 16–26 S, sufficient to code for the M_r 41000 polypeptide [9] and to contain a substantial

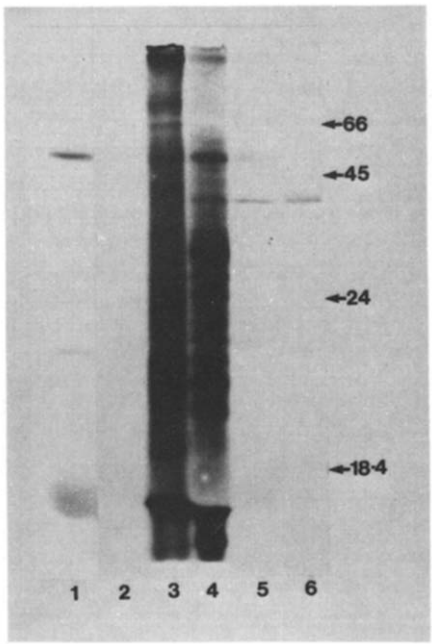

Fig.3. Immunopurification of GS mRNA. SDS–PAGE of translations using: (1,2) no added RNA; (3) total nodule polysomes; (4) total nodule poly(A)$^+$ RNA; (5) poly(A)$^+$ RNA extracted from immunopurified polysomes; (6) GS marker. Track 1 was exposed to X-ray film 3-fold longer than rest of gel.

5′-untranslated region and poly(A) tail. GS mRNA had not been purified, although some separation of the mRNA species coding for the α- and β-polypeptides of *Neurospora* GS was obtained by electrophoresis on methyl mercury containing agarose gels [18]. In fig.3, GS mRNA was purified to near homogeneity by immunopurification of nodule polysomes, a method previously used to isolate several low abundance mRNA species from a human β-lymphoblastoid cell line [25] and also from rat liver [38]. GS mRNA was also partially purified (~3-fold) by centrifugation of nodule poly(A)$^+$ RNA on sucrose density gradients containing dimethyl sulphoxide and formamide (fig.2).

In vitro translation of poly(A)$^+$ RNA followed by immunoprecipitation of the products with anti-GS-IgG had been used to estimate the relative abundance of GS mRNA during development processes [11,12,16,18]. Using the same approach (fig.1) the proportion of GS mRNA appears to be much greater in nodules, harvested 18 days after *Rhizobium* infection, than in non-nodulated roots. These data correlate with the large increase in GS activity occurring at this time [10]. Moreover, since this increase in activity is due almost entirely to the production of GS_{n1}, the nodule specific form of the enzyme, it follows that this form is synthesized de novo during nodulation. Thus the regulation of GS_{n1} appears to resemble that of leghaemoglobin [33], nodulin 35 [39] and other nodule-specific proteins [40], in which the synthesis of the mRNA and the proteins is initiated during nodulation with fixing strains of *Rhizobium*. However, as with GS_{n1} [10], the synthesis of these proteins [40,41] may be affected in nodules produced with mutant, non-fixing strains of *Rhizobium*.

ACKNOWLEDGEMENTS

J.V.C. would like to thank the Royal Society for receipt of a Pickering Research Fellowship. We are grateful to Mr J. Franklin for help with plant growth.

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