Universidad Nacional Autónoma de México

Unidad Académica de los Ciclos Profesionales

y de Posgrado del C.C.H.

Centro de Investigación sobre Fijación de Nitrógeno

Asimilación de amonio en Phaseolus vulgaris

T E S I S

Que para obtener el grado de

Doctor en Investigación Biomédica Básica

presenta el Maestro:

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1985

759580

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A mi esposa y a mi hijo con
todo mi amor.

A mis padres y hermanos a quienes
tanto quiero.

A Jaime por sus enseñanzas y su amistad, con profundo cariño.

A Rafael con gratitud por su amistad y confianza.

A todas las personas del CIFN.

A todas las personas del queridisimo laboratorio III.

A Paty un especial agradecimiento por su generosisima ayuda.

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OBJETIVO

Una de las principales fuentes de ntirógeno para las plantas, especialmente las leguminosas, es el amonio formado en la simbiosis con bacterias del género Rhizobium, las cuales son capaces de fijar nitrógeno. Durante la simbiosis, el N_2 es reducido hasta NH_4 por la bacteria y excretado al citoplasma de la célula vegetal, donde es asimilado por la vía glutamino sintetasa (GS) - glutamato sintasa (GOGAT). Los productos de ésta vía (glutamina y ácido glutámico) proporcionan, a través de reacciones de transaminación y transamidación, el nitrógeno para la síntesis de los compuestos nitrogenados de la célula. Durante la simbiosis entre Phaseolus vulgaris y Rhizobium phaseoli, el nitrógeno de éstos aminoácidos es derivado a la síntesis de purinas los cuales son posteriormente metabolizados hasta ácido alantóico y alantoina, que son los principales transportadores de nitrógeno en el frijol nodulado.

El objetivo de éste proyecto es conocer las características estructurales y regulatorias de las enzimas responsables de la asimilación de amonio, así como estudiar y definir los factores que participan en la regulación del metabolismo nitrogenado durante el establecimiento y desarrollo de la simbiosis entre Phaseolus vulgaris y Rhizobium phaseoli.

INTRODUCCION

1. FUENTES DE NITROGENO PARA LAS PLANTAS

Las dos fuentes de nitrógeno más importantes para las plantas, son el nitrato y el nitrógeno atmosférico. La primera (nitratos), se deriva de la oxidación del amonio (nitrificación), por bacterias nitrificantes como nitrosómonas y nitrobacterias presentes en casi todos los tipos de suelos. El amonio oxidado por éste tipo de bacterias procede de la descomposición de la materia orgánica (amonificación), ó en áreas de alto desarrollo agrícola, de los fertilizantes empleados. La otra fuente de nitrógeno, es el nitrógeno atmosférico el cual requiere ser reducido a amonio para ser utilizado por las plantas.

La reducción del nitrógeno atmosférico se lleva a cabo por bacterias fijadoras de nitrógeno, las cuales pueden dividirse en dos grupos:

1) las que fijan nitrógeno en vida libre,
2) las que lo fijan en simbiosis con la planta.

En el primer plano existen microorganismos capaces de desarrollar asociaciones con plantas que pudieran ser importantes, tales como la alga verde-azul anabaena, con el lirio acuático azolla; y las asociaciones de pastos tropicales

y subtropicales, como Paspalum notatum y Digitaria decumbens, con Azobacter y Azospirillum, respectivamente (1).

El segundo grupo y más importante, lo forman las bacterias del género Rhizobium que son microorganismos aeróbicos, gram negativos, capaces de establecer una simbiosis con plantas de la familia leguminosae y, particularmente, con la subfamilia de las papilinoidaceas la cual incluye las leguminosas más comunes para el hombre.

Este género Rhizobium está subdividido en siete grupos por su especificidad para nodular al huésped:

Bacteria	Huésped (Ej.)
R. meliloti	alfalfa
R. trifolii	trébol
R. leguminosarum	chícharo
R. phaseoli	frijol
R. lupini	lupin
R. japonicum	soya
Cowpea rhizobia	cacahuate

2. SIMBIOSIS *Rhizobium*-leguminosas

Como se mencionó previamente, una de las fuentes de nitrógeno más importantes para las plantas es el nitrógeno atmosférico, el cual es fijado en tejidos especializados denominados nódulos, los cuales se forman en la raíz, como resultado de la infección por bacterias del género *Rhizobium*.

La infección de la raíz se inicia generalmente, en un pelo radicular donde se lleva a cabo el reconocimiento bacteria-huésped, y continúa por un hilo de infección a través de las células de la corteza hasta la zona de células meristemáticas donde son liberadas las bacterias envueltas en la membrana de la punta del hilo de infección (2). Estas membranas son de origen vegetal y forman las membranas peribacteroidales (3).

Una vez que las bacterias se han liberado dentro de la célula vegetal, se dividen hasta llenar prácticamente todo el citoplasma en ésta etapa, las bacterias transformadas en bacteroides inician la fijación del nitrógeno atmosférico a través de expresar la nitrogenasa (4). El amonio formado por el bacteroide es excretado al citoplasma de la célula vegetal donde es asimilado por la planta.

Paralelo al aumento de la actividad de la nitrogenasa en el bacteroide, se presenta un incremento en la síntesis

de la leghemoglobina y en la actividad de las enzimas responsables de asimilar el amonio (5) y de sintetizar los compuestos que finalmente transportarán el nitrógeno a los demás tejidos de la planta (5,6).

3. VIAS DE ASIMILACION DE AMONIO

Dos vías de asimilación de amonio han sido descritas en plantas; la primera implica la incorporación de amonio en el grupo amido de la glutamina por la enzima glutamino sintetasa (GS), y la transferencia de este grupo a un cetoácido (α-cetoglutarato) por la glutamato sintasa (GOGAT), para la formación de dos moléculas de ácido glutámico (Fig. 1).

La segunda vía implica la aminación de α-cetoglutarato por la enzima glutamato deshidrogenasa (GDH), para la formación de una molécula de ácido glutámico (Fig. 1).

Por mucho tiempo, y debido a la gran distribución de la GDH en la naturaleza, se pensó que la única forma de incorporar amonio en grupos α-amino, era a través de ésta enzima. Sin embargo, a partir de los trabajos de Tempest y col., (7) donde se describe la actividad de GOGAT en bacterias. Dougall (8), Lea y Miflin (9) describen la actividad de GOGAT en tejidos vegetales, y plantean la existencia de dos vías de asimilación de amonio en plantas.

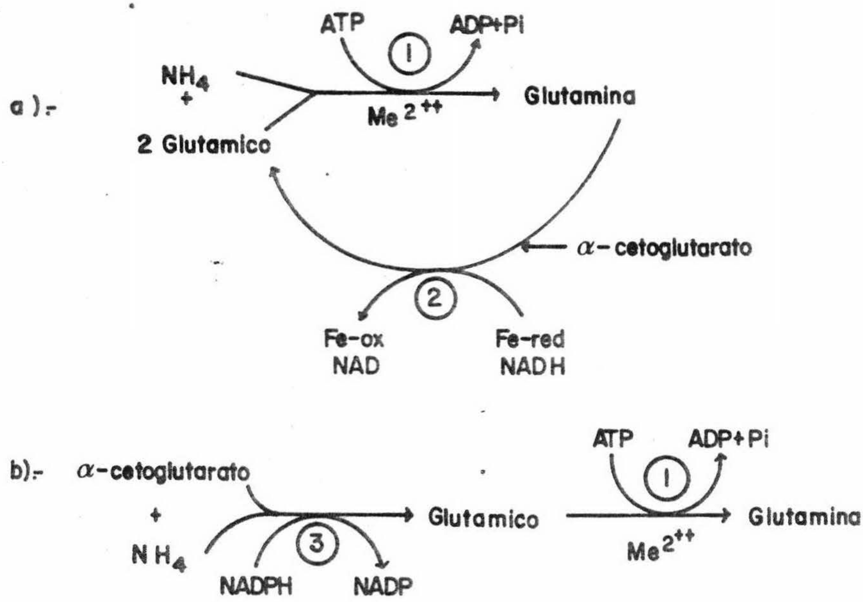

Figura I.- Vias de asimilación de amonio en plantas
1-GS, 2-GOGAT, 3-GDH.

Los estudios realizados en leguminosas muestran que durante el desarrollo del nódulo ocurre una inducción de las actividades tanto de GS (10,11) como de GOGAT (12,5), así como una baja actividad de GDH, cuya afinidad por amonio es entre 50 y 100 veces menor que la afinidad de GS por el mismo substrato (8,13).

Por otro lado, los estudios con ^{13}N y ^{15}N en nódulos de soya (14,15) así como los estudios con inhibidores específicos de la actividad de la GS y de la GOGAT (15), muestran que la cinética de marca entre glutamina y glutámico guardan una relación precursor-producto.

Todos estos resultados sugieren fuertemente que la vía de asimilación de amonio en plantas es a través de las enzimas GS y GOGAT, lo cual coloca a la glutamino sintetasa como la enzima responsable de asimilar el amonio y a la glutamina, como el principal donador de nitrógeno en la célula.

4. SINTESIS DE GLUTAMINA

a) Características de la glutamino sintetasa.

Los estudios realizados sobre la glutamino sintetasa en diferentes tejidos de la planta, muestran varias isoformas de ésta enzima, las cuales pueden ser separadas por cromatografía de intercambio iónico (16,17,18).

En tejidos verdes (tallo y hoja) se han encontrado dos formas de GS denominadas GS-1 y GS-2. La primera ha sido localizada en el citoplasma, mientras que la segunda, es una forma que se encuentra en el cloroplasto (17). Los estudios inmunológicos utilizando suero anti GS-2 indican diferencias antigénicas entre ambas formas (19).

Dado el papel primordial de la GS en la reasimilación del amonio procedente de la fotorespiración en hojas de plantas tipo C-3 (20), se pretendió establecer cuál de las dos formas enzimáticas (GS-1 ó GS-2) era la responsable de ésta reasimilación, a través de un estudio comparativo de la GS en hojas de diversas plantas. A partir de éste estudio se establecieron cuatro grupos de plantas basados en la diferente proporción de las formas de GS que presentan (20).

Grupo 1 - sólo presentan GS-1
Grupo 2 - mayor proporción de GS-1
Grupo 3 - mayor proporción de GS-2
Grupo 4 - sólo presentan GS-2

Sin embargo, este estudio no mostró ninguna relación entre la forma de GS existente y la actividad fotorespiratoria de la planta. Cabe señalar que la presencia de luz y el estado de desarrollo de la hoja modifican la proporción entre GS-1 y GS-2, por lo que dicha clasificación debe ser considerada con cautela.

En raíces solo se ha encontrado una forma de GS, la cual en columnas de hidroxilapatita se comporta de manera diferente a las dos formas de GS en hoja, no así en cromatografía por DEAE celulosa, donde su perfil de elución es similar a la GS-1 citoplásmica (21).

Neurospora crassa es uno de los organismos eucarióticos donde mayor conocimiento se tiene de las características de GS. En este hongo la GS se encuentra en una forma tetramérica cuando la fuente de nitrógeno es limitada y en una forma octamérica cuando en el medio el nitrógeno está en altas concentraciones (22,23).

El análisis polipeptídico de éstas dos formas oligoméricas en éste hongo mostró que el tetrámero se compone principalmente de un polipéptido denominado α y el octámero de uno denominado β siendo éste último polipéptido de mayor peso molecular (23).

Por otro lado, se ha demostrado que en condiciones de limitación de nitrógeno la actividad de GOGAT se encuentra elevada, mientras que la de GDH está reprimida (24,25), siendo el fenómeno inverso en altas concentraciones de amonio. Estos datos sugieren que la asimilación de nitrógeno en Neurospora es a través de la forma tetramérica de GS (α) y la GOGAT en condiciones de limitación de nitrógeno, mientras que en exceso de nitrógeno la vía de asimilación es GDH-GS octamérica (β) (25).

5. SINTESIS DE TRANSPORTADORES DE NITROGENO

En las plantas, el amonio incorporado en glutamina y ácido glutámico por la vía GS-GOGAT, es distribuido por reacciones de transamidación y transaminación a otros compuestos, los cuales son los responsables de transportar el nitrógeno a las diferentes partes de la planta.

Los principales transportadores de nitrógeno son de dos tipos: a) amidas: asparagina y glutamina, b) ureidos: ácido alantoico y alantoina.

La síntesis de asparagina en plantas es el resultado de la incorporación del nitrógeno del ácido glutámico en ácido oxalacético para formar aspártico y la incorporación del grupo amido de glutamina en el ácido aspártico para formar asparagina (26).

Por otro lado, la síntesis de ureidos en plantas resulta de la incorporación del grupo amino de glutámico y el amido de la glutamina en el anillo de purinas, las cuales son posteriormente metabolizadas hasta ácido alantóico y alantoina (27,28).

En particular, en las leguminosas los principales transportadores de nitrógeno son asparagina y glutamina, tanto si el nitrógeno proviene del medio externo ó de la simbiosis

con Rhizobium. Sin embargo, en plantas como soya y frijol, cuando han sido infectadas con Rhizobium y desarrollan una simbiosis efectiva, los principales transportadores de nitrógeno son los ureidos. Los estudios en éstas leguminosas durante la simbiosis, muestran un incremento paralelo entre la actividad de nitrogenasa y la actividad de las enzimas responsables de la síntesis de purinas y ureidos, mientras que la síntesis de asparagina se reduce hasta en un 80% (29).

Estos cambios en el manejo del nitrógeno durante la simbiosis tanto en la soya como en el frijol, tienen una estrecha correlación con las características de las células que componen el tejido nodular. Por un lado, las células infectadas del nódulo además de contener los bacteroides presentan un gran número de proplástidos, mientras que las células no infectadas, se caracterizan por un mayor número y tamaño de los peroxisomas y abundancia de retículo endoplásmico (29).

Recientemente se ha descrito que las enzimas de la vía de biosíntesis de purinas se localizan en los proplástidos de las células infectadas; mientras que en las células no infectadas la uricasa, que promueve la síntesis de alantoina a partir del ácido úrico, se encuentra en los peroxisomas y la alantoinasa que cataliza la síntesis de ácido alantóico, a partir de alantoina está en el retículo endoplásmico (30).

A N T E C E D E N T E S

Planta (1983) 157:245 253

Planta
© Springer-Verlag 1983

Purification and properties of two forms of glutamine synthetase from the plant fraction of *Phaseolus* root nodules

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Abstract. Two forms of glutamine synthetase (GS) have been purified to apparent homogeneity from the plant fraction of *Phaseolus vulgaris* root nodules. One of these forms appears identical to the form of the enzyme found in roots but the other is probably specifically associated with the nodule. Free-living *Rhizobium phaseoli* also contain two forms of GS both of which have different molecular weights from the plant enzymes. Bacteroids contain solely the higher-molecular-weight form of rhizobial GS. There are only minor differences between the plant enzymes in K_m or $S_{0.5}$ values for the synthetase-reaction substrates and both forms have identical molecular weights of the holoenzyme (380.000 daltons) and its sub-units (41,000 daltons). They can be separated by ion-exchange chromatography on diethylaminoethyl-Sephacel and by native polyacrylamide-gel electrophoresis. The only other distinguishing feature observed is that the ratio of transferase:synthetase activity of the root form is threefold greater than that of the nodule-specific GS.

Key words: Glutamine synthetase – Legume-*Rhizobium* symbiosis – Nitrogen assimilation – *Phaseolus* (glutamine synthetase) – *Rhizobium* – Root nodule.

Introduction

Labelling studies using ^{15}N and ^{13}N have shown that ammonia produced by dinitrogen fixation in the bacteroids is excreted into the plant fraction of the nodules and assimilated there by the glutamate-synthase cycle i.e. the combined action of GS (EC 6.3.1.2) and glutamate synthase (EC 1.4.1.14, EC 1.4.7.1) (for a review see Robertson and Farnden 1980). The amount of GS activity found in bacteroids is considered too low to perform this function as it represents only about 2% of the total GS activity of the nodule (Dunn and Klucas 1973; Brown and Dilworth 1975; Planqué et al. 1977; Streeter 1979; Cullimore et al. 1982). Furthermore, although free-living rhizobia possess two forms of GS with differing molecular weights and properties (Darrow and Knotts 1977; Fuchs and Keister 1980a), under nitrogen-fixing conditions, only the high-molecular-weight form is present (Rao et al. 1978). In contrast, the synthetase activity of the plant GS in the nodule was found to be approximately 14-fold higher than in the root (Robertson et al. 1975; Cullimore et al. 1982), the large increase in activity occurring coincidently with the appearance of nitrogenase and leghaemoglobin (Robertson et al. 1975).

Glutamine synthetase has been purified from the plant fraction of soybean nodules (McParland et al. 1976) and shown to have a molecular weight of 376,000 daltons and properties similar to the enzyme of many higher plants (see Stewart et al. 1980). Recently, however, it has been established that the plant portion of *Phaseolus* root nodules, like rhizobia and many other higher plant tissues (Stasiewicz and Dunham 1979; Mann et al. 1979; Guiz et al. 1979; Hirel and Gadal 1980), contain multiple forms of GS (Cullimore et al. 1982). In this paper the properties and purification of two forms of the enzyme from *Phaseolus* root nodules are described.

Materials and methods

Chemicals

Sephadex G-25, diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-300, Phenyl-Sepharose, protamine sulphate, *E. coli* β-galactosidase, bovine liver catalase, yeast alcohol dehydrogenase and horse heart myoglobin were all obtained from Sigma

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

Chemical Company, Poole, Dorset, UK. Polyclar AT was obtained from BDH (Poole, Dorset, UK) and hydroxyapatite from Bio-rad Laboratories (Richmond, Cal., USA). All chemicals were of the highest analytical grade available.

Growth and preparation of cell-free extracts of Rhizobium

Free-living *Rhizobium* (*Rhizobium phaseoli* R3622) were grown in yeast extract-mannitol medium as described by Dye (1979). The cells were collected by centrifugation, washed in buffer and stored at −20° C. Bacteroids were isolated from the pellet obtained from centrifugation of the nodule extract (see below) and extracted as described for free-living rhizobia. Extracts were prepared using an LKB X-press (LKB, Bromma, Sweden). The broken cells were resuspended in 5 ml of 10 mM imidazole-HCl buffer, pH 7.6, containing 20 mM $MgSO_4$ and the brei was centrifuged at 20,000 *g* for 30 min. The resulting supernatant was desalted on a small column of Sephadex G-25.

Growth and preparation of cell-free extracts of Phaseolus

Phaseolus vulgaris L. var. Bush Blue Lake 274 was inoculated with *Rhizobium phaseoli* R3622 and grown under 18 h light (22° C) – 6 h dark (16° C) cycles. Natural daylight was supplemented when necessary with 400-W SonT lamps (Camplex, Cambridge, UK) to maintain a light intensity at pot level of 200 μmol m^{-2} s^{-1}. The plants were watered with nutrient solution lacking a nitrogen source (Eaglesham et al. 1977). Nodules were harvested after 5 weeks growth and leaves after 3 weeks. Roots (2 weeks old) were obtained from non-inoculated plants.

Extracts of approx. 10 g of frozen plant material were made by grinding the tissue with 20% (w/v) polyclar AT in a mortar and pestle together with 10 ml running buffer (10 mM 2 amino-2(hydroxymethyl)-1,3-propandiol (Tris)-Cl buffer, pH 7.8, containing 5 mM Na-glutamate, 10 mM $MgSO_4$, 10% glycerol). The brei was filtered through four layers of muslin and centrifuged at 20,000 *g* for 20 min. The supernatant was desalted on a small Sephadex G-25 column.

Chromatography on DEAE-Sephacel

The desalted extract (3 ml) was applied to a column (8 cm long, 1 cm diameter) of DEAE-Sephacel equilibrated in running buffer. The proteins were eluted at 0.4 ml min^{-1} with 20 ml buffer followed by 150 ml of a 0–0.6 M KCl gradient in buffer. Fractions of approx. 2 ml were collected and assayed for GS activity.

Sucrose-density-gradient centrifugation

A desalted extract (1 ml) containing marker proteins (β-galactosidase, EC 3.2.1.23. from *Escherichia coli*; catalase, EC 1.11.1.6. from bovine liver; alcohol dehydrogenase, EC 1.1.1.1. from yeast; myoglobin from horse heart) was applied to a 38-ml, linear, 5–20% (w/v) sucrose gradient made up in 10 mM Tris-Cl, pH 7.8, containing 5 mM Na-glutamate and 10 mM $MgSO_4$. Centrifugation was performed at 27,000 *g* on a Beckman L2-65B ultracentrifuge (rotor SW27) (Beckman, Palo Alto, Cal., USA) for 24 h at 3° C. The gradients were fractionated into 1.2-ml aliquots and GS activity and marker proteins determined.

Determination of Stokes' radii, molecular weight and frictional ratio

Stokes' radii were determined on a Sephacryl S-300 column exactly as described by Cullimore et al. (1982). Molecular weights and frictional ratios of GS were calculated from their Stokes' radii and $S_{20,w}$ values by the equations of Siegel and Monty (1966).

Purification of GS

Preparation of extract. Nodules (100 g) from 4-week-old plants were extracted in a mortar and pestle with 20 g polyclar AT and 150 ml of 25 mM Tris-Cl buffer, pH 7.8, containing 1 mM dithiothreitol. The brei was filtered through four layers of muslin and centrifuged at 20,000 *g* for 40 min. All procedures were carried out at 4° C.

Protamine-sulphate fractionation. A freshly prepared 2% aqueous solution (5 ml) of protamine sulphate was added dropwise to the extract and the precipitate was removed by centrifugation.

Ammonium-sulphate fractionation. The precipitate obtained from a 35–55%-saturated $(NH_4)_2SO_4$ solution was taken and dissolved with 3 ml running buffer.

Sephacryl S-300 chromatography. The extract (5 ml) was applied to a column (55 cm long, 1.6 cm diameter) of Sephacryl S-300 pre-equilibrated in running buffer and the proteins were eluted at 0.38 ml min^{-1}. Fractions of approx. 2.2 ml were collected and those containing GS activity were bulked (approx. 20 ml).

DEAE-Sephacel chromatography. A column (12 cm long, 2.5 cm diameter) of DEAE-Sephacel was used to separate the two forms of nodule GS (Fig. 1). The extract was applied and the column then washed with 65 ml of running buffer followed by a 300-ml, linear, 0–0.25 M KCl gradient in buffer. Fractions (3 ml) were collected at 0.29 ml min^{-1} and the two peaks of GS activity were bulked separately.

Phenyl-Sepharose chromatography. The extracts were applied to columns (7 cm long, 1.0 cm diameter) of Phenyl-Sepharose and the proteins eluted at 0.33 ml min^{-1} with successive 15-ml steps of buffer containing 0, 10 and 40% ethylene glycol; 1.6-ml fractions were collected and the single peaks of GS activity were bulked.

Hydroxyapatite chromatography. Finally the GS extracts were applied to 3-ml columns of hydroxyapatite equilibrated in running buffer lacking $MgSO_4$. The proteins were eluted at 0.24 ml min^{-1} with steps containing 0, 100 and 400 mM Na_2HPO_4 and 1.2-ml fractions were collected.

Native polyacrylamide-gel electrophoresis

Polyacrylamide (8.5%) gels were run at 20 mA for 7 h according to Davis (1964). Glutamine synthetase activity was detected by the transferase assay as described by Barratt (1980) and protein by staining with Coomassie Blue R.

Sodium dodecyl sulphate (SDS) – polyacrylamide-gel electrophoresis

Denaturing, SDS-urea, 15% polyacrylamide gels (according to Laemmli 1970, but with the modifications of Forde et al. 1981) were run at 20 mA for 6 h. The proteins were fixed and then stained with Coomassie Blue R.

Protein determination

In cell-free extracts, protein was determined according to Lowry et al. (1951).

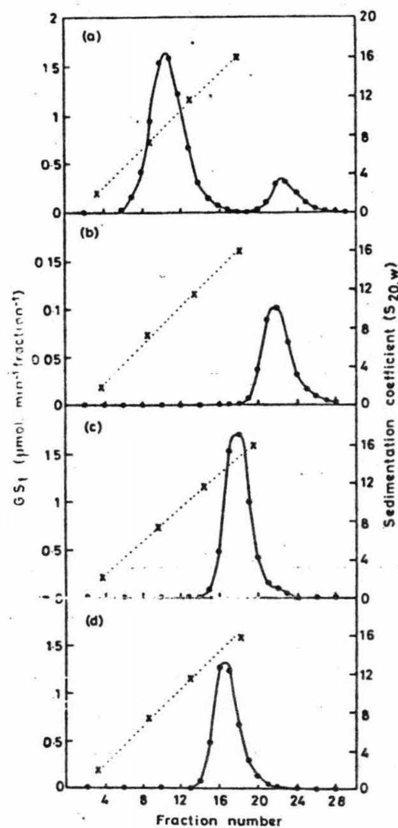


Fig. 2. Glutamine-synthetase-activity profiles following sucrose-density-gradient centrifugation of extracts of a free-living *Rhizobium phaseoli* and b bacteroids and partially purified preparations of c GS_{n1} and d GS_{n2} of nodules. Activity of GS transferase was determined (•). Position of marker proteins (x)

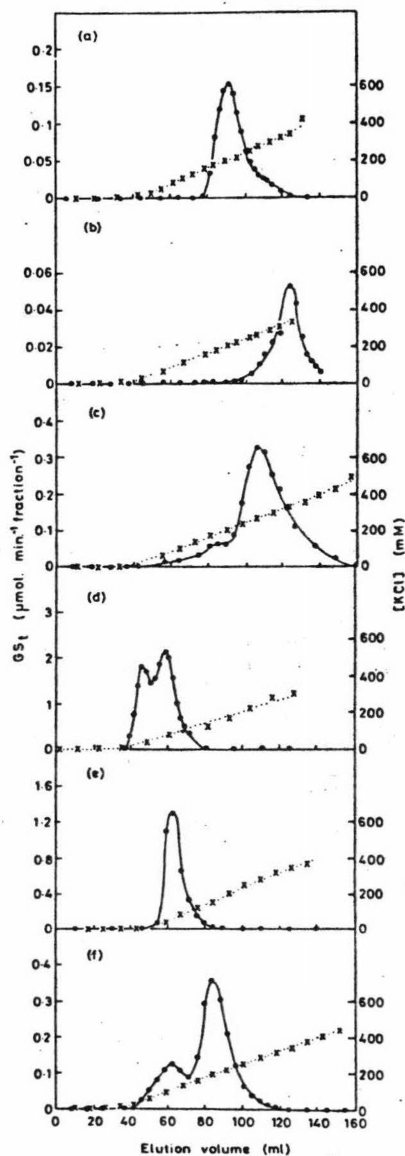


Fig. 3a–f. Elution profiles of various *Phaseolus* and *Rhizobium phaseoli* GS forms on DEAE-Sephacel. Partially purified extracts of a the low- and b the high-molecular-weight forms of rhizobial GS and c bacteroid GS. Extracts of d nodules, e roots and f leaves of *Phaseolus*. Activity of GS transferase (•) and the KCl concentration (x) were determined

Assay of glutamine synthetase

The transferase assay (Cullimore and Sims 1980), semi-biosynthetic hydroxylamine assay (Cullimore et al. 1982) and the biosynthetic assay using [^{14}C]glutamate as labelled substrate (Cullimore and Sims 1981) have all been previously described.

Assay of β-hydroxybutyrate dehydrogenase

The enzyme was assayed by a modification of the method of Wong and Evans (1971) as described by Awonaike et al. (1981).

Results

In these studies GS activity has been determined both by a semi-biosynthetic assay in which hydroxylamine replaced the physiological nitrogen donor, ammonia, (Miflin and Lea 1977), and also by the transferase reaction (Woolfolk et al. 1966). The semi-biosynthetic assay can be used as a measure of the in-vivo enzyme activity since rates obtained by this method were found to be almost identical to those obtained when ammonia and [^{14}C]glutamate were used in a true biosynthetic assay (data not shown). The transferase assay was used because of its greater sensitivity.

Using these assays the elution profile of GS activity was determined following chromatography of an extract of *Phaseolus* root nodules on DEAE-Sephacel (Fig. 1a). The increasing KCl gradient eluted two distinct peaks of GS activity at concentrations of 25 mM and 65 mM (designated GS_{n1} and GS_{n2} respectively). On rechromatography each form eluted as a single peak at their same respective positions on the KCl gradient (Fig. 1b, c). The two forms showed quite different ratios of transferase:synthetase activity. Values, over the whole peak, of 30 and 90 were observed for GS_{n1} and GS_{n2} respectively. A small proportion of GS activity, with a ratio of transferase:synthetase activity of 20, did not bind to the column but eluted during the buffer wash (Fig. 1a). On subsequent rechromatography, only 20% of this activity remained unbound to the column and the remaining activity eluted at the same position as GS_{n1} (data not shown).

With the extraction procedures employed, about 25% of the bacteroids were broken, as judged by the proportion of β-hydroxybutyrate dehydrogenase activity released into the crude nodule extract. Experiments were carried out to ensure that the two forms of GS observed in these extracts were not of bacterial origin. *Rhizobium phaseoli* was grown axenically in yeast extract-mannitol medium, extracted and the crude desalted extracts were subjected to sucrose-density-gradient centrifugation (Fig. 2a). Two forms of GS were observed

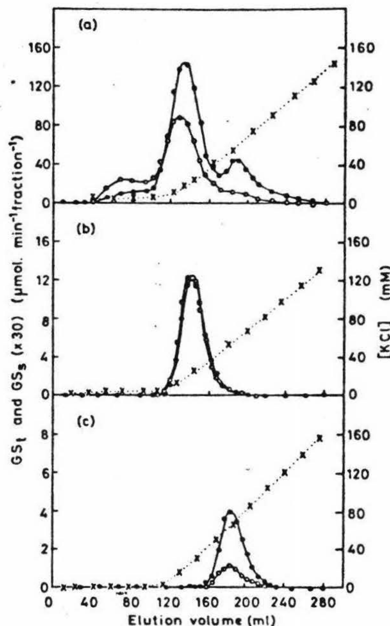


Fig. 1. a Elution profile of nodule GS activity from DEAE-Sephacel. The extract was prepared and partially purified as described in Materials and methods (purification of GS). Rechromatography of b GS_{n1} and c GS_{n2}. Glutamine synthetase transferase (GS_t) (•) and synthetase (GS_s) (o) activities and KCl concentration (x) were determined

with $S_{20,w}$ values of 9.0 and 20.2. Bacteroids had only the high-molecular-weight form of the enzyme (Fig. 2b). The two nodule forms of GS, however, sedimented mid-way between the rhizobial forms, at identical positions, corresponding to $S_{20,w}$ values of 14.6 (Fig. 2c, d). On DEAE-Sephacel the two rhizobial GS enzymes eluted at much higher KCl concentrations than either of the nodule forms (Fig. 3); these positions were 220 and 300 mM KCl for the low- and high-molecular-weight rhizobial GS enzymes respectively and 280 mM for bacteroid GS.

Extracts of roots and leaves of *Phaseolus* were also run on DEAE-Sephacel columns (Fig. 3e, f). The results established that only a single form of GS occurred in roots and this activity eluted at an identical KCl concentration (65 mM) to GS_{n2} of nodules (Fig. 3d). Leaves however, were found to contain two forms of the enzyme; a cytosolic

Table 1. Purification of two forms of glutamine synthetase from *Phaseolus* root nodules

	Synthetase activity (μmol min^{-1})	Total protein (mg)	Specific activity (μmol min^{-1} mg protein^{-1})	Fold purification	Recovery [%]	Transferase: synthetase ratio
Crude extract	76.9	977	0.08	1.0	100	57
Protamine-sulphate supernatant	80.0	777	0.10	1.3	104	54
35–55% saturated $(NH_4)_2SO_4$ precipitate	–	227	–	–	–	–
Sephacryl S-300	70.0	90.0	0.78	9.9	91	62
GS_{n1} DEAE-Sephacel	34.6	4.4	7.9	100	45	30
Phenyl-Sepharose	32.6	2.7	11.9	152	43	34
Hydroxyapatite	19.1	1.7	11.3	144	25	36
GS_{n2} DEAE-Sephacel	9.2	12.2	0.75	9.6	12	67
Phenyl-Sepharose	5.3	1.4	3.7	48	7	87
Hydroxyapatite	3.7	1.0	3.6	46	5	88

form which eluted at 90 mM KCl and a chloroplast-located enzyme which eluted at 190 mM KCl. [The localization of these forms was established by differentially extracting leaf tissue in the presence or absence of 0.05% Triton X-100 in order to disrupt or keep intact the organelle membranes, followed in the latter case by removal of the intact chloroplasts by centrifugation. The absence of GS in organelles other than chloroplasts has previously been established (Wallsgrove et al. 1980)].

The two plant-nodule forms of GS were purified to apparent homogeneity (140- and 50-fold respectively) by a procedure involving gel filtration, ion-exchange chromatography and affinity chromatography on Phenyl-Sepharose and hydroxyapatite (Table 1). The final specific synthetase activities of GS_{n1} and GS_{n2} were 11.3 and 3.6 μmol min^{-1} mg protein^{-1} respectively. A total recovery of GS activity of 30% was obtained after the six purification steps. On SDS polyacrylamide gels each final preparation of the two forms produced only a single protein band of identical mobilities corresponding to subunit molecular weights of approx. 41,000 daltons (Fig. 4). In crude extracts leghaemoglobin was the predominant protein but following gel filtration the 41,000-dalton band was of major importance (Fig. 4).

On native polyacrylamide gels several bands of GS activity were observed in the crude extract (Fig. 5). Purified GS_{n1} and GS_{n2} however, behaved as single entities but with slightly different mobilities and their positions of activity corresponded to the only protein bands observed in these tracks. The GS activity which did not bind to the DEAE-Sephacel column in Fig. 1. had a slower mobility than either GS_{n1} or GS_{n2} confirming that its determination was not an artifact of the column chromatography.

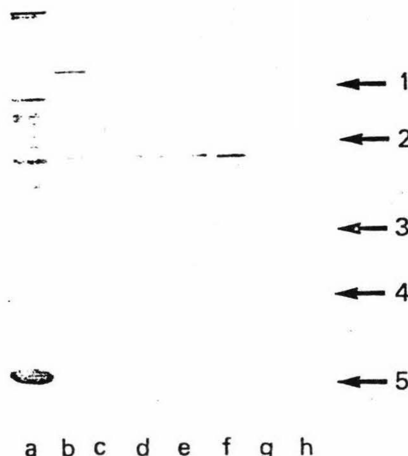
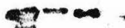


Fig. 4. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of nodule extracts at different stages of the purification of GS. Crude nodule extract (*a*) and following gel filtration on Sephacryl S-300 (*b*). Extracts of GS_{n1} following chromatography on DEAE-Sephacel (*c*), Phenyl-Sepharose (*d*) and hydroxyapatite (*e*). Extracts of GS_{n2} after DEAE-Sephacel (*f*), Phenyl-Sepharose (*g*) and hydroxyapatite (*h*) chromatography. Position of the molecular-weight markers, bovine plasma albumin, 66,000 (*1*); ovalbumin, 45,000 (*2*), trypsinogen, 24,000 (*3*), β-lactoglobin, 18,400 (*4*) and lysozyme, 14,300 (*5*). Protein was determined by staining with Coomassie Blue R

It was reported in an earlier paper (Cullimore et al. 1982) that GS activity in crude extracts of nodules was very unstable in Tris-Cl buffer alone



a b c d

Fig. 5. Native polyacrylamide-gel electrophoresis of crude and purified GS extracts from nodules. Crude extract (*a*), GS activity which did not bind to DEAE-Sephacel (see Fig. 1) (*b*), purified GS_{n1} (*c*) and purified GS_{n2} (*d*). Activity of GS transferase was determined

but that substrates of the synthetase reaction or dithiothreitol could prevent activity losses. It can be seen from Table 2, however, that following purification, both forms of the enzyme were stable in buffer alone. Moreover, dithiothreitol or ATP were found to promote the almost complete deactivation of both forms, although this could be prevented by the inclusion of 5 mM glutamate and 10 mM $MgSO_4$ in the buffer. A differential effect of the thiol reactive reagents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and N-ethyl maleimide (NEM) on GS_{n1} and GS_{n2} was observed such that only 8% of GS_{n1}, but 36% of GS_{n2}, activity remained in the presence of DTNB under the conditions employed. The corresponding values with NEM were 66% and 100% respectively.

An examination of the properties of GS_{n1}, GS_{n2} and root GS (GS_r) (Table 3) revealed that all three enzymes possessed identical molecular weights (of 380,000 daltons) and frictional ratios, as determined from their Stokes' radii and sedimentation coefficients. Their kinetic properties were also remarkably similar; although slight differences were noted for ammonia, the K_m or $S_{0.5}$ values for hydroxylamine, glutamate, ATP and Mg^{2+} did not vary between the forms by factors greater than 1.6. The properties of the root enzyme were found to more closely resemble those of GS_{n2} than GS_{n1}. The pH optima of all three enzymes were broad but centred around a value of 7.7. The only noticable difference between the enzymes, from these data, were the ratios of transferase: synthetase activity which were reproducibly determined to be 30 and 90 for GS_{n1} and GS_{n2} respectively (see also Table 1) and 100 for the root enzyme.

Table 2. Effect of various ligands on the stability of purified forms of glutamine synthetase. Preparations of GS purified to apparent homogeneity were desalted into 50 mM Tris-Cl buffer, pH 7.8 and activity determined initially and after 17 h at 24° C

	Synthetase activity (% initial activity)	
	GS_{n1}	GS_{n2}
Buffer only	99	115
+ $MgSO_4$ (20 mM)	115	119
+ Glutamate (10 mM)	89	116
+ ATP (3 mM)	3	18
+ DTT (3 mM)	0	2
+ $MgSO_4$ + glutamate + ATP	128	123
+ $MgSO_4$ + glutamate + ATP + dithiothreitol	78	77
+ $MgSO_4$ + glutamate + ATP + N-ethyl maleimide (0.1 mM)	66	100
+ MgSO + glutamate + ATP + 5,5'-dithiobis(2-nitrobenzoic acid) (0.1 mM)	8	36

Table 3. Properties of the root and the two nodule forms of glutamine synthetase of *Phaseolus*. Preparations of the enzymes purified to apparent homogeneity were used in these determinations

Property	GS_{n1}	GS_{n2}	GS_r
Stokes' radius (nm)	6.5	6.4	6.4
$S_{20,w}$	14.6	14.6	14.6
Molecular weight	380,000	380,000	380,000
Frictional ratio	1.35	1.34	1.34
K_m for glutamate (mM)	2.1	1.4	1.1
$S_{0.5}$ for ammonia (mM)	0.55	0.21	–[a]
Hill coefficient for ammonia	1.7	1.8	–[a]
$S_{0.5}$ for hydroxylamine (mM)	1.1	0.6	0.4
Hill coefficient for hydroxylamine	1.6	1.7	2.0
K_m for ATP	1.5	2.4($S_{0.5}$)	2.0($S_{0.5}$)
$S_{0.5}$ for Mg^{2+}	4.8	2.6	4.4
pH optimum	7.7	7.7	7.7
Transferase:synthetase ratio	35	88	100

[a] Not determined

Discussion

The two forms of GS isolated from *Phaseolus* root nodules had remarkably similar kinetic and physical properties and identical native and sub-unit molecular weights (Table 3, Fig. 4) but could be separated, because of slight differences in charge, by ion-exchange chromatography (Fig. 1), starch-gel electrophoresis (Cullimore et al. 1982), and native polyacrylamide-gel electrophoresis (Fig. 5). Multiple forms of this enzyme separated from other higher plant tissues and species also have these characteristics (Stasiewicz and Dunham 1979; Mann et al. 1979; Hirel and Gadal 1980).

A number of observations indicate that these forms are unlikely to be of bacterial origin. Firstly, bacteroids possess very little GS activity (only 2% of the total nodule activity in *Phaseolus* nodules-Cullimore et al. 1982) and the single form of the enzyme they contain has a very different molecular weight (Fig. 2) and elution characteristics from DEAE-Sephacel (Fig. 3) from both of the nodule forms. Furthermore, although free-living *Rhizobium phaseoli*, in common with other members of the Rhizobiaceae and Agrobacteriaceae (Darrow and Knotts 1977; Fuchs and Keister 1980a, b), possess a second form of the enzyme, it too behaves differently from the nodule forms in these respects. Data presented here on the occurrence and characteristics of the two bacterial GS forms in free-living and bacteroid *Rhizobium phaseoli* agree closely with those described for GS of *R. japonicum* (Darrow and Knotts 1977). Finally, the striking similarities between the two nodule forms and the enzyme studied from a whole range of higher-plant tissues (see Stewart et al. 1980) further indicate that the two forms are of plant origin. This conclusion is supported by the observation that antisera raised to purified GS_{n1} or GS_{n2} cross react with other higher-plant GS enzymes but not with either of the rhizobial forms (data not shown).

Although only minor differences have been observed in the kinetic properties of the two nodule forms for both the synthetase (Table 3) and transferase activities (data not shown), one distinguishing property of these forms was the differences in their ratios of transferase:synthetase activity. The values obtained (30 and 90 for GS_{n1} and GS_{n2} respectively) appear to be an intrinsic characteristic of each form and did not vary during purification of the enzymes (Table 1). The significance of this ratio is unclear since only the synthetase activity is thought to be of physiological importance. Changes in this ratio, however, have been shown to occur during modulation of GS activity in both *Escherichia coli* (Shapiro and Stadtman 1970) and *Candida utilis* (Sims et al. 1974), processes which appear to affect rapidly the in-vivo rate of ammonia assimilation.

It can be seen from Table 2 that the requirements for maintaining the stability of GS are different when it is purified than in crude extracts (Cullimore et al. 1982), an observation also reported for pea leaf GS (O'Neal and Joy 1973). The most noticeable effect was that of dithiothreitol, which in crude extracts stabilized nodule GS but which led to the almost complete loss of activity of the purified forms. The thiol probes 5,5'-dithiobis(2-nitro-benzoic acid) and, to a lesser extent, N-ethyl maleimide also promoted a loss of activity indicating that maintaining thiol groups in the reduced form could still be important to the activity of the purified enzyme.

In Fig. 1 it is apparent that 16% of GS activity in these nodules did not bind to DEAE-Sephacel but eluted during the buffer wash. In an earlier study, using nodules grown during the winter at a lower light intensity and temperature, the proportion of this unbound activity was much greater (Cullimore et al. 1982). When rerun on the column this activity did not behave as a single peak but also partly eluted in a position similar to GS_{n1}. However, it is unlikely that this first peak is solely the result of overloading or incomplete equilibration of the column, because on native polyacrylamide gels (Fig. 5) and on starch gels (Cullimore et al. 1982) it had a lower mobility than either of the other two nodule forms. It might be that the presence of this form in the nodules is dependent on the growth conditions of the plant. Alternatively, it is possible that this form only becomes apparent in-vitro under certain undefined extraction and purification procedures. Clearly this problem needs further investigation.

Finally, the results show that *Phaseolus* contains a number of different forms of GS which are tissue-specific. Roots contain only a single form of the enzyme (different from either of the two leaf forms) which appears to be identical to nodule GS_{n2}. Both GS_{n2} and root GS elute from DEAE-Sephacel at the same KCl concentration (Fig. 3), behave similarly on starch (Cullimore et al. 1982) and polyacrylamide gels (data not shown), and have almost identical kinetic properties (Table 3). Although the determined ratios of transferase:synthetase activity of the purified enzymes were slightly different (Table 3), both were much higher than that of GS_{n1}. Moreover, root GS and GS_{n2} appear antigenically identical to antiserum prepared against either GS_{n1} or GS_{n2} (data

not shown). During nodulation of *Phaseolus* with fixing strains of *Rhizobium* a distinct nodule-specific form of GS (GS_{n1}), different to the root enzyme, is produced (see also Lara et al. 1983). Whether the various forms are the products of separate genes or represent post-transcriptional or post-translational modifications of a single gene product has yet to be established. It is clear however that *Rhizobium* directly or indirectly promotes the appearance of GS_{n1} and in this respect regulation of this enzyme resembles that of leghaemoglobin (Verma 1980) and a number of other nodule-specific proteins (nodulins) of plant origin (Legocki and Verma 1979). The activity of the two GS forms during nodulation using fixing and non-fixing strains of *Rhizobium* has been investigated in a following paper (Lara et al. 1983).

One of us (JVC) gratefully acknowledges the receipt of a Pickering Research Fellowship from the Royal Society.

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Received 3 August; accepted 21 September 1982

Planta (1983) 157:254–258

Planta
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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.

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

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.

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

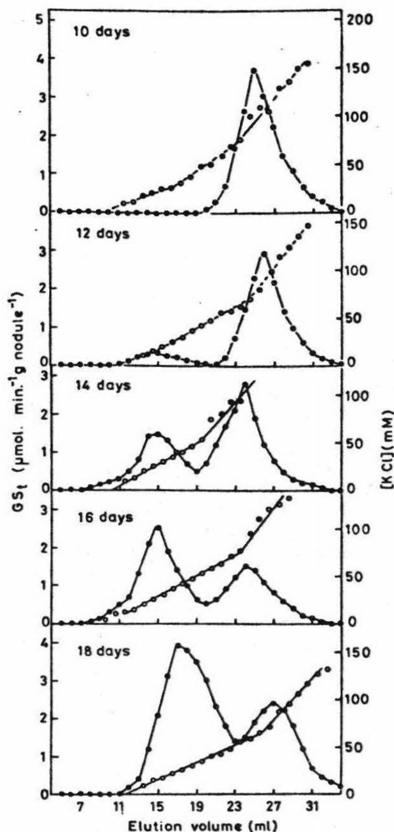

Fig. 1. Elution profiles of GS activity from DEAE-Sephacel of extracts of nodules harvested at different times following inoculation of *Phaseolus vulgaris* with *Rhizobium phaseoli* R3622. Activity of GS (●) and KCl concentration (o) were determined. GS_t = transferase activity

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

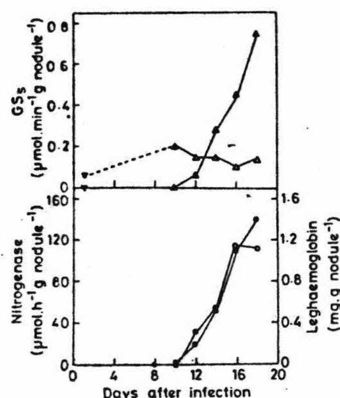

Fig. 2. Time course of changes in the activity of GS_{n1} (▲), GS_{n2} (△) and nitrogenase (●) and amount of leghaemoglobin (o) during nodule development of *Phaseolus vulgaris* infected with *Rhizobium phaseoli* R3622. Activity of GS_{n1} (▼) and GS_{n2} (▽) in non-nodulated roots. GS_s = synthetase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

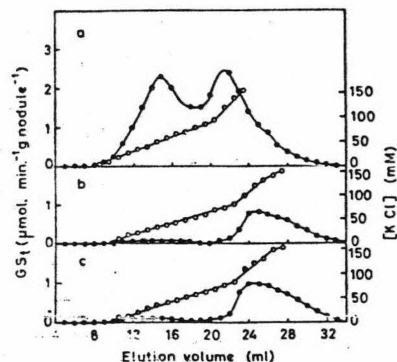

Fig. 3a–c. Elution profiles of GS activity from DEAE-Sephacel of extracts of nodules of *Phaseolus vulgaris* harvested 19 d following inoculation with *Rhizobium phaseoli* strains **a** Fix⁺, 8002. **b** Nod⁺ Fix⁻ mutant JL15 and **c** Nod⁺ Fix⁻ mutant JL19. Activity of GS (●) and KCl concentration (o)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

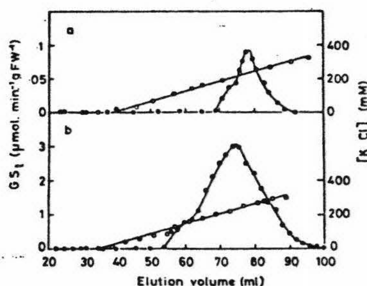

Fig. 4a, b. Elution profiles of GS activity from DEAE-Sephacel of extracts of **a** roots of *Pisum sativum* and **b** nodules harvested 18 d after inoculation with *Rhizobium leguminosarum* Fix⁺ strain T83K3. Activity of GS (●) and KCl concentration (o) were determined

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.

We are grateful to Dr. J. Witty, Dr. J. Day and Mr. J. Franklin for help with plant growth. One of us (JVC) would like to thank the Royal Society for receipt of a Pickering Research Fellowship.

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Received 16 August; accepted 25 October 1982

R E S U L T A D O S

Plant Physiol. (1984) 76, 1019–1023
0032-0889/84/76/1019/05/$01.00/0

Heterogeneity of Glutamine Synthetase Polypeptides in *Phaseolus vulgaris* L.[1]

Received for publication March 31, 1984 and in revised form August 6, 1984

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ABSTRACT

Glutamine synthetases from roots, nodules, and leaves of *Phaseolus vulgaris* L. have been purified to homogeneity and their polypeptide composition determined.

The leaf enzyme is composed of six polypeptides. The cytosolic fraction contains two 43,000 dalton polypeptides and the chloroplastic enzyme is formed by four 45,000 dalton polypeptides. Root glutamine synthetase consists only of the same two polypeptides of 43,000 dalton that are present in the leaf enzyme. The nodule enzyme is formed by two polypeptides of 43,000 dalton, one is common to the leaf and root enzyme but the other is specific for N_2-fixing nodule tissue. The two glutamine synthetase forms of the nodule contain a different proportion of the 43,000 dalton polypeptides.

Glutamine synthetase (EC 6.3.1.2.) is the major enzyme for ammonia assimilation in higher plants (15). GS[2] from different plant tissues has a mol wt in the range of 350,000 to 400,000 (14, 17). The enzyme consists of eight subunit of 43,000 to 47,000 D. Multiple forms of GS have been separated by ion exchange chromatography from a variety of plant tissues. In green tissue of barley (12) and rice (7, 9), cytosolic and chloroplastic GSs (called GS_1 and GS_2) have been described. In root tissue only one form of GS has been found (12, 17). Two forms of GS, GSn-1 and GSn-2 have been observed in *Phaseolus* root nodules (3). GSn-1 is a new form which appears during nodule development (10), whereas GSn-2 is undistinguishable from the one found in roots (3). Recently, two GS polypeptides have also been identified in alfalfa root nodules (6). One of the best characterized eucaryotic GS is the one from *Neurospora crassa*, in which two nonidentical polypeptides are structured in different oligomeric forms (4, 11, 21). This paper reports the purification and polypeptide composition of GS from leaves, roots, and nodules of *Phaseolus vulgaris*.

MATERIALS AND METHODS

Plant Material. *Phaseolus vulgaris* L. cv Ojo de cabra was inoculated with the wild type *Rhizobium phaseoli* strain CFN-42 (20) and grown for 4 weeks in a glasshouse under natural daylight (14 h) (mean day temperature, 25 to 30°C; RH, 45%). The plants were watered every other day with nitrogen-free nutrient solution (2), and with water alternatively. Nodules and leaves were harvested after 4 weeks, roots from noninoculated plants after 2 weeks. Etiolated leaves were obtained after 10 d from noninoculated plants grown at 30°C in the dark and watered only with distilled H_2O.

Root GS Purification. All purification steps were carried out at 4°C. Root tissue, frozen in dry ice, (100 g) was ground in a mortar with 100 ml of 2-fold concentrated RB (10 mM Tris-HCl (pH 8.4), 10 mM Mg-acetate, 10% (v/v) glycerol). The brei was filtered through four layers of muslin and centrifuged at 20,000*g* for 45 min. The supernatant was brought to 70% $(NH_4)_2SO_4$, centrifuged at 15,000*g* for 15 min. The pellet was resuspended in 10 ml RB, desalted on a Sephadex G25 column (2.5 × 40 cm), and loaded on a DEAE cellulose column (2.5 × 30 cm). Both columns were previously equilibrated in RB. After washing with 400 ml RB, proteins were eluted with 400 ml of a 0 to 0.4 M KCl linear gradient in RB. Four-ml fractions were collected and those showing GS activity were pooled and chromatographed on a column (1 × 25 cm) of Sepharose-anthranilic acid as described previously (18), omitting $MnCl_2$ in the elution gradient. The fractions showing GS activity were concentrated 5- to 10-fold in a dialysis tube immerged in crystalline sucrose and then dialyzed overnight in 10-fold diluted RB containing 10% (v/v) glycerol. The purified enzyme solution was brought to 50% (v/v) with glycerol and stored at −20°C.

GS Purification from Nodules. Nodule extract was prepared by grinding 20 g of nodule tissue in a mortar with 100 ml of extraction buffer, 100 mM Tris-HCl (pH 8.4), 10 mM Mg-acetate, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100. The brei was centrifuged at 20,000*g* for 45 min. The supernatant (100 ml), was precipitated by adding 1 volume of acetone at −20°C and immediately centrifuged at 16,000*g* for 15 min. The collected pellet was dried with filtered air, resuspended in RB, and loaded on a column (2.5 × 40 cm) of DEAE cellulose. Further purification was done following the same steps described for root GS.

GS Purification from Green and Etiolated Leaves. Green (50 g) or etiolated (12 g) leaves were ground in a mortar with dry ice to yield a fine powder. Two volumes (w/v) of extraction buffer, 100 mM (Tris-HCl (pH 8.4), 10 mM Mg-acetate, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 1 mg/ml of BSA (w/v), were added. The extract was filtered through four layers of muslin and centrifuged at 20,000*g* for 45 min. The supernatant was fractionated with $(NH_4)_2SO_4$. The precipitate obtained from 35 to 70% saturation was dissolved in a minimal volume of RB containing 10 mM β-mercaptoethanol. The sample was applied on a column (2.5 × 60 cm for green and 1 × 68 cm for etiolated leaves) of Sephacryl S-300 equilibrated in the same buffer. The volume of the sample applied did not exceed 2.5% of the volume of the

[1] Supported in part by grants from Consejo Nacional de Ciencia y Tecnología and from Fondo de Estudios e Investigaciones Ricardo J. Zevada.

[2] Abbreviations: GS, glutamine synthetase; GS_1, cytosolic glutamine synthetase; GS_2, chloroplastic glutamine synthetase; GSn-1 and GSn-2, nodule glutamine synthetases; RB, running buffer; PBS, phosphate buffered saline; IP, isolectric point.

column. Proteins were eluted with RB and fractions containing GS activity were pooled and loaded on a column of Sepharose-anthranilic acid as described above.

Glutamine Synthetase Determination. GS activity was measured by the transferase assay of Ferguson and Sims (5). One unit of activity represents 1 μmol of γ-glutamyl-hydroxamate formed per minute at 30°C.

Polypeptide Analysis of the GS Forms. GS forms were isolated by ion exchange chromatography from crude extracts of nodule and etiolated leaf, following the procedure described by Lara *et al.* (10). The fractions containing the highest activity of each peak were pooled and immunoprecipitated as reported previously (11) using anti-GS serum raised against the purified enzyme from nodule for the nodule GS forms and from green leaf for the leaf GS forms. These immune complexes were analyzed by two-dimensional gel electrophoresis.

Electrophoresis. SDS-PAGE was performed in 7.5% (w/v) polyacrylamide gel according to Palmiter *et al.* (19). Two-dimensional gel electrophoresis was done according to O'Farrell (16), except that electrofocusing gels were run at 400 v for 20 h omitting the final high voltage pulse. Electrophoresis in the second dimension was carried out as previously reported (21) without urea. Proteins were stained with Coomassie blue R (13).

Protein Determination. Protein was measured colorimetrically by the Bradford procedure (1).

RESULTS

The GS from roots, nodules, and leaves was purified using a column of anthranilic acid bound to Sepharose (Table I). This procedure of affinity chromatography was reported previously for *N. crassa* GS (18). SDS-PAGE shows that the enzyme from roots or nodules migrates as a single protein band of 43,000 D (Fig. 1, lanes 1 and 2). GSs from green and etiolated leaves present two protein bands of 45,000 and 43,000 D (Fig. 1, lanes 3 and 4). The 45,000 D band is the major component in the green leaf enzyme, while in etiolated leaves both proteins are present in approximately equal amounts. A band with higher mol wt is still observed in the etiolated leaf (Fig. 1, lane 4).

Subsequently, the polypeptide composition of the purified

Table I. *Purification of GS from Roots, Nodules, Green, and Etiolated Leaves of Phaseolus vulgaris*

Purification Step	Total Protein	Total Units	Specific Activity	Purification Factor
			units/mg protein	*-fold*
Roots				
Crude extract				
$(NH_4)_2SO_4$	330.0	284.0	0.86	
Sephadex G-25	193.0	240.0	1.24	1.44
DEAE-cellulose	33.0	101.0	3.06	3.55
Sepharose-anthranilic	0.5	25.8	51.6	60.0
Nodules				
Crude extract	315.0	302.4	0.96	
Acetone precipitation	74.7	349.7	4.68	4.87
DEAE-cellulose	24.4	130.5	5.34	5.56
Sepharose-anthranilic	0.5	98.1	196.2	204.37
Green leaves				
Crude extracts				
$(NH_4)_2SO_4$	983.4	1326.0	1.34	
Sephacryl S-300	18.7	501.5	26.81	20.0
Sepharose-anthranilic	1.9	181.9	95.73	71.44
Etiolated leaves				
Crude extracts				
$(NH_4)_2SO_4$	175.0	167.5	0.95	
Sephacryl S-300	27.2	76.6	2.81	2.95
Sepharose-anthranilic	0.2	33.23	166.15	174.89

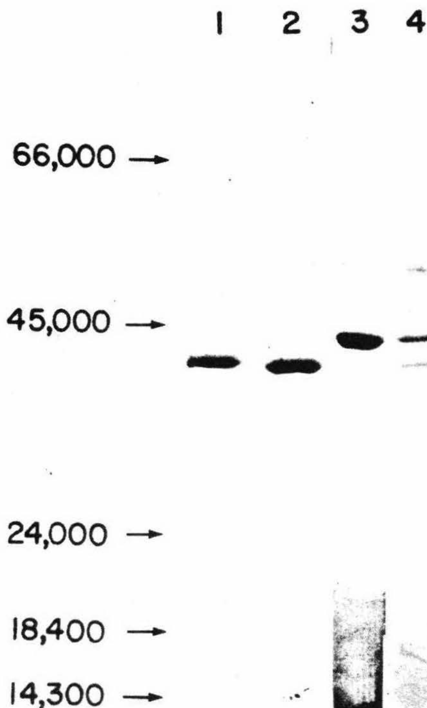

FIG. 1. SDS-PAGE of the purified GS from different tissues of *P. vulgaris*. SDS-PAGE of the GS purified from (1) root (8 μg), (2) nodule (10 μg), (3) green leaf (12 μg), and (4) etiolated leaf (4 μg) of *P. vulgaris*. Mol wt markers: bovine plasma albumin, 66,000; ovalbumin, 45,000; trypsinogen, 24,000; β-lactoglobin, 18,400; and lysozyme, 14,300. Proteins were stained with Coomassie blue R.

enzymes was analyzed by two-dimensional gel electrophoresis. Root GS is composed of two polypeptides, called α and β, IP-5.8 and 6.2, respectively (Fig. 2, A). The nodule GS is formed by two polypeptides. The first one (γ) is a specific nodule component and the second one is similar to the β polypeptide observed in roots. (Fig. 2, B). Root and nodule enzymes were co-electrophoresed and only three polypeptides were observed indicating that β is the same in both tissues (Fig. 2, C). The 43,000 D GS band in the leaf enzymes is composed of the same α and β polypeptides as present in the root GS (Fig. 2, D and E). This was confirmed when both root and leaf enzymes were co-electrophoresed (Fig. 2, F). The 45,000 D protein band observed in the enzyme of green leaves is composed of four polypeptides, called a, b, c, and d. In the etiolated leaf component 'a' is not observed (Fig. 2, D and E).

To demonstrate if the native GS forms contain the same polypeptides found in the pure enzymes, GSn-1 and GSn-2 from nodule extract and GS_1 and GS_2 from etiolated leaf extract were separated using a DEAE-Sephacel column (Fig. 3, A and B).

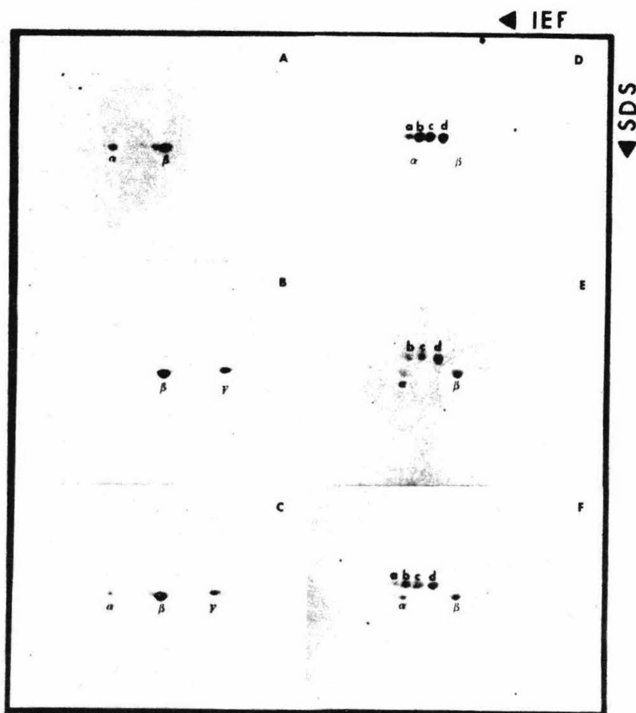

FIG. 2. Two-dimensional gel electrophoresis of the purified GS from different tissues of *P. vulgaris*. Two-dimensional gel analysis of the GS purified from: A, root (8 μg); B, nodule (10 μg); C, root plus nodule (4 μg + 5 μg); D, green leaf (12 μg); E, etiolated leaf (8 μg); F, root plus green leaf (4 μg + 6 μg). α, β, γ, a, b, c, and d are GS polypeptides.

Fractions with the highest enzyme activity from each peak were immunoprecipitated and their polypeptide composition was analyzed. The γ polypeptide from nodules is the main component in the GSn-1 form (Fig. 4, A), while the β polypeptide is present in a high proportion in the GSn-2 form (Fig. 4, B). Analysis of the leaf GS forms indicated that the chloroplastic GS_2 form is composed of b, c, and d polypeptides (Fig. 4, D). The GS_1 form is mainly composed of α and β polypeptides (Fig. 4, C), but a third component appears weakly between α and β.

Furthermore, the component 'd' is still observed in the GS_1 fraction. Studies are in progress to clarify whether 'd' is a real component of the cytosolic GS_1 or a cross-contamination between the GS_1 and GS_2 peaks.

DISCUSSION

The results reported here demonstrate that the multiple forms of GS are related to the presence of different polypeptides of this enzyme in *P. vulgaris*.

The polypeptide analysis of the two nodule forms of GS (3) shows that GSn-1 has a higher proportion of the γ and GSn-2 of the β polypeptide (Fig. 4, A and B). Improved chromatographic methods for the separation of the two GS forms are required to elucidate if the presence of β in GSn-1 and of γ in GSn-2 is not an artifact. GSn-1 form, which is expressed only in effective nodules (10), is composed mainly of the nodule specific polypeptide γ. This suggests that this polypeptide is responsible for the assimilation of ammonium produced by the bacteroids during symbiosis.

In the root tissue, the GS is composed of two polypeptides which comigrate with the α and β polypeptides of the leaf GS_1 form (Fig. 2, F), indicating that the root GS and the cytosolic GS_1 form are identical. Considering our polypeptide analysis of leaf and root extracts, we presume that the component observed between α and β in the GS_1 peak (Fig. 4, C) has no relation with the GS polypeptides.

The GS_2 form is composed mainly of the 45,000 D polypeptides b, c, and d (Fig. 4, D). The chloroplastic localization of this form (22) and the absence of these higher mol wt polypeptides in the root and nodule GS and in embryo leaves (data not shown), imply that this GS_2 form could be encoded by a gene that is only expressed in mature leaves. During the preparation of this paper, the multiple polypeptide composition in the chloroplastic GS_2 has been described (8). It is not yet known whether these polypeptides are encoded by one or more different genes or whether they arise by post-transcriptional modification. The a, b, c, and d GS polypeptides are specific for mature leaves and the γ polypeptide is specific for nodules. The physiological and developmental importance of these observations is currently under study.

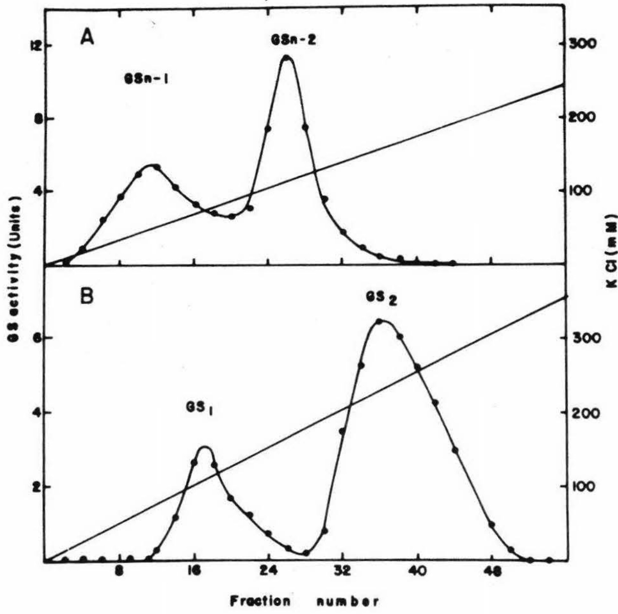

FIG. 3. Elution profile after chromatography on DEAE-Sephacel columns of the different GS forms from nodule and etiolated leaf extracts. A, Nodule glutamine synthetase forms, GSn-1 and GSn-2; B, etiolated leaf glutamine synthetase forms GS_1 and GS_2.

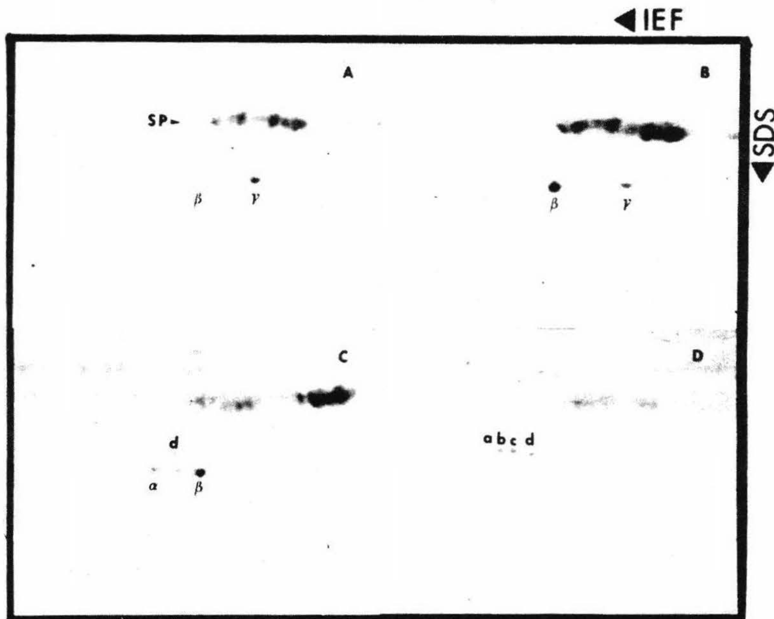

FIG. 4. Two-dimensional gel electrophoresis analysis of the GS forms from nodule and etiolated leaf. Two-dimensional gel electrophoresis of the immunoprecipitated GS. A, GSn-1; B, GSn-2; C, GS_1; D, GS_2. α, β, γ, a, b, c, and d are GS polypeptides; SP, serum proteins.

Acknowledgments—We are grateful to Drs. Jaime Mora, Rafael Palacios, Guadalupe Espin, and Jan Leemans for the critical reading of the manuscript and Patricia Vázquez for secretarial assistance. We also thank Dr. Jesús Calderón for the preparation of the antiserum used in this work.

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DISCUSION Y PERSPECTIVAS

Los resultados obtendios a través de la purificación y el análisis de los polipéptidos de la GS en los distintos tejidos de frijol, muestran que las diferentes formas de ésta enzima, tienen una composición polipeptídica difetente y que algunos de éstos polipéptidos son tejido específico.

La forma única de GS presente en raíces y la GS-1 del citoplasma de la hoja, presentan la misma composición, lo cual sugiere que sean la misma forma de GS. Es importante señalar que durante el desarrollo de la raíz, la proporción entre el polipéptido α y β se modifica, siendo α el principal componente en la raíz primaria y β el único componente de la raíz madura (datos no mostrados). Este hecho plantea dos alternativas: ó un polipéptido es precursor del otro ó son dos componentes que se expresan en diferentes estadios del desarrollo. La primera posibilidad indicaría que en la raíz y durante su desarrollo está presente un mecanismo específico que permite la conversión de un polipéptido a otro, mientras que en hojas este mecanismo no está presente. La segunda posibilidad indicaría que la expresión de ambos polipéptidos (α y β) está regulada de manera diferente en hoja que en la raíz.

La mayor abundancia del polipéptido α en las etapas tempranas del desarrollo de la raíz lo relacionan a éste con estadios de crecimiento y de una alta actividad biosintética del

tejido. Por lo que es interesante explorar la distribución tanto del polipéptido α como del β en las diferentes partes del sistema radicular y analizar la proporción entre ambos polipéptidos en los diferentes estadios de desarrollo de la hoja.

En el tejido nodular, se encontró que la glutamino sintetasa analizada por cromatografía de intercambio iónico, se presenta en dos formas. Una de éstas formas la cual se eluye con menor fuerza iónica denominada GSn-1 se induce conforme se desarrolla el nódulo, y su actividad se elevaba paralelamente al aumento en la actividad de la nitrogenasa. La segunda forma de ésta enzima, presente en el nódulo, se comporta de una manera similar a la forma de raíz en cromatografía de intercambio iónico.

Es importante recalcar que la forma GSn-1 es específica del tejido nodular, y que sólo se encuentra en nódulos efectivos y no en nódulos formados por cepas mutantes de *Rhizobium* incapaces de fijar nitrógeno.

El análisis polipeptídico de la enzima purificada de los nódulos, muestra dos componentes denominados β y γ de igual peso molecular pero diferente punto isoeléctrico.

El análisis en geles de dos dimensiones de los inmunoprecipitados de las dos formas de la GS del nódulo mostró que el polipéptido γ era el principal componente de la forma GSn-1, y el polipéptido β de la forma GSn-2.

Estos datos indican que durante la simbiosis, la planta expresa una forma específica de GS la cual está compuesta de un polipéptido específico del nódulo denominado γ. La ausencia de ésta forma en nódulos ineficientes sugiere que esta es la forma DEAE responsable de asimilar el amonio formado por la nitrogenasa en el bacteroide. Sin embargo, la ausencia del polipéptido γ en nódulos inefectivos no ha sido demostrada.

Dada la distribución específica de las enzimas en los organelos de las células infectadas y de las no infectadas, que participan en la síntesis de los transportadores de nitrógeno como los ureidos, se están tratando de definir si existe una distribución específica de los polipéptidos β y γ en ambos tipos de células. La localización de éstos polipéptidos, establecería con mayor precisión cual de ellos es el responsable de asimilar el amonio en la simbiosis.

Si bien el polipéptido β se localiza tanto en raíz como en nódulo, el componente α de la enzima de raíz no se encuentra en el tejido nodular. Como se mencionó anteriormente, este

componente desaparece conforme se desarrolla la raíz por lo que su ausencia en el nódulo se explicaría debido a que la maduración del nódulo se logra en las raíces bien desarrolladas en donde sólo está presente el polipéptido β. Esto de ninguna manera invalida la posibilidad de que la ausencia del polipéptido α en el nódulo no se relacione con el proceso simbiótico y las condiciones metabólicas en este tejido.

Al igual que se mencionó en el caso de la raíz, la posible relación precursor-producto entre los polipéptidos β y γ de nódulo no ha sido completamente descartada, sin embargo los resultados de la síntesis <u>in vitro</u>, a través de la traducción y la inmunoprecipitación del mRNA polisomal del nódulo, indican fuertemente que estos polipéptidos son producto de dos diferentes mensajeros.

Una de las características de la simbiosis en el frijol es que el amonio asimilado en glutamina es transportado en forma de ureidos al resto de la planta y no en forma de amidas como sucede en condiciones de no infección. Este hecho, junto con el resultado obtenido en nódulos de chícharo donde no se encontró la forma específica de nódulo GSn-1 y donde los transportadores de nitrógeno siempre son amidas, plantea la posible relación entre esta forma nueva de GS, el polipéptido γ (nódulo específico) y la síntesis

de ureidos en la simbiosis R. phaseoli y frijol. Un análisis comparativo de los polipéptidos de GS aislada de nódulos de plantas que transportan ureidos como de plantas que transportadoras de amidas, se están llevando a cabo para definir si existe ó no dicha relación.

Un punto a considerar dada su posible reelevancia en el estudio de las interacciones planta-bacteria, es que la expresión de un polipéptido específico de GS, como es el γ, esté mediado por la bacteria directa o indirectamente. Hasta el momento parece ser claro que ni la presencia de amonio en la célula vegetal, ni de compuestos como glutámico y glutamina, promueven la expresión de éste polipéptido en raíces no infectadas; esto sugiere que la condición nitrogenada en el nódulo no es la responsable directa de la expresión de ésta enzima GSn-1.

En hojas de plantas tipo C_3 existen dos procesos íntimamente relacionados con el manejo del nitrógeno en la planta; uno es la asimilación del nitrógeno que procede de la raíz, ó el que no ha sido incorporado y llega a la hoja como nitrógeno inorgánico (NO_3). El otro proceso es la reasimilación del amonio durante la fotorespiración, el cual es de una gran trascendencia dada la magnitud de éste proceso.

En ambos casos la asimilación o reasimilación del amonio se lleva a cabo a través de la actividad de la GS. Dada la

presencia de la GS-1 citoplásmica y la GS-2 cloroplástica en las hojas de frijol, queda por discernir cuál de éstas formas de la enzima participa en el manejo del nitrógeno en éste tejido. La alta actividad de GS-2 y la muy baja actividad de GS-1 en hojas maduras y, dada la magnitud de la fotorespiración en éstas plantas, permite pensar que es la forma cloroplástica (GS-2) la encargada de manejar el nitrógeno de la fotorrespiración. Sin embargo, dada la localización cloroplástica de la GS-2 y el hecho de que el amonio es liberado dentro de la mitocondria, se tendría que presuponer que este amonio pasa libremente hacia el cloroplasto de una u otra manera ó que la enzima citoplásmica es una enzima con una gran capacidad para reasimilar el amonio.

En el caso de que alguna de las formas de ésta enzima fuese la responsable de asimilar el amonio de la fotorrespiración y/o el procedente de la raíz, la presencia y función de dos formas de GS, las cuales presentan una composición polipeptídica diferente, sigue siendo una pregunta a responder.

En cuanto a la composición de las dos formas de GS en hoja, se está estudiando si la forma cloroplástica, GS-2, es sintetizada como un precursor en el citoplasma y procesada en su entrada al cloroplasto, ó bien que sin ser modificada en su entrada al organelo, alguno de los cuatro componentes identificados (a, b, c y d) es el que se sintetiza en el

citoplasma. Esto último se apoya en el hecho de que la diferencia entre estos 4 polipéptidos, parece radicar en que están glucosilados (32) por lo que es difícil pensar que los cuatro sean productos de diferentes mensajeros, no así que uno sea el precursor y/o los cuatro procedan de un precursor común. Estudios de síntesis in vitro e inmunoprecipitación con anticuerpos específicos anti-GS no han dado datos relevantes al respecto. Sin embargo, en trabajos recientes, diversos grupos han podido aislar una clona con el gene específico para GS a partir de un banco de genes de frijol. Sus estudios de hibridización utilizando mRNA de diferentes tejidos indican por un lado que existe una familia de genes para GS en frijol y por otro, que un sólo gene parecería ser el responsable de la GS-2 de cloroplasto en hoja (31).

CONCLUSIONES

Con base en los resultados obtenidos de la purificación y caracterización de la GS en los diferentes tejidos de frijol, se puede concluir:

a) Las diferentes formas de GS identificadas por cromatografía de intercambio iónico en nódulo, raíz y hoja de frijol son el resultado de diversos polipéptidos de GS existentes en esta planta.

b) En nódulos de frijol existe una forma de GS compuesta por un polipéptido específico de este tejido y el cual se propone como el responsable de asimilar el amonio durante la simbiosis.

c) La forma de la GS descrita en raíz de frijol, presenta la misma composición polipeptídica que la forma citoplásmica de la GS en la hoja por lo que se sugiere que son formas idénticas.

d) La forma cloroplástica de GS (GS-2) en frijol se compone de 4 polipéptidos específicos de éste tejido y su peso molecular es mayor que los otros polipéptidos encontrados en raíz y nódulo.

e) La presencia de polipéptidos de GS tejido específicos en frijol, su expresión durante el desarrollo de los diferentes tejidos y los datos de la síntesis *in vitro* de GS abren la posibilidad de que exista una familia de genes de GS en frijol.

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