The Purification and Properties of the Glutamine Synthetase from the Cytosol of Soya-Bean Root Nodules

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The major portion of glutamine synthetase activity in root nodules of soya-bean plants is associated with the cytosol rather than with *Rhizobium japonicum* bacteroids. Glutamine synthetase accounts for about 2% of the total soluble protein in nodule cytosol. Glutamine synthetase from nodule cytosol has been purified by a procedure involving fractionation with protamine sulphate, ammonium sulphate and polypropylene glycol, chromatography on DEAE-Bio-Gel A and Bio-Gel A-5m and affinity chromatography on glutamate-agarose columns. The purified preparation appeared to be homogeneous in the analytical ultracentrifuge. From sedimentation-equilibrium experiments a mol. wt. of about 376 000 was determined for the native enzyme and 47 300 for the enzyme in guanidinium chloride. From these data and measurements of electron micrographs, we have concluded that glutamine synthetase from nodule cytosol consists of eight subunits arranged in two sets of planar tetramers which form a cubical configuration with dimensions of about 10 nm (100 Å) across each side. Glutamine synthetase from nodule cytosol has a higher glycine and proline content and a lower content of phenylalanine than the glutamine synthetase that has been prepared from pea seed. The cytosol enzyme contains four half-cystine molecules per subunit, which is in contrast with two reported for the enzyme from pea seed. Enzyme activity is strikingly influenced by the relative proportion of Mg^{2+} and Mn^{2+} in the assay medium. Activity is inhibited by feedback inhibitors and is influenced by energy charge.

The routes of ammonium assimilation subsequent to dinitrogen reduction in the legume root nodule have not been clearly established. Glutamine, however, appears to be one of the early products of the ammonium assimilation process (Kennedy, 1966*a,b*) and an important component transported from nodules to the plant (Pate *et al.*, 1969; Streeter, 1972). Glutamine is an important metabolite in the biosynthesis of several amino acids, amino sugars, and pyrimidines (Ginsburg, 1972) and has been implicated as the physiological amide donor for the synthesis of asparagine in soya-bean cotyledons (Streeter, 1973). The biosynthesis of glutamine is catalysed by glutamine synthetase (EC 6.3.1.2) in a reaction formulated as follows:

In the presence of arsenate, ADP and an appropriate bivalent cation the enzyme also catalyses a reaction between glutamine and hydroxylamine, forming γ -glutamylhydroxamate and ammonia (Woolfolk *et al.*, 1966).

Since glutamine and glutamine synthetase occupy a key position in the nitrogen metabolism of organisms, intensive investigations have been directed toward an understanding of the properties and regulation of glutamine synthetases from several organisms. As pointed out in reviews by Ginsburg & Stadtman (1973) and Ginsburg (1972), glutamine synthetase is subject to control by bivalent cations, feedback inhibitors, energy charge, and, in several prokaryotes, by the extent of adenylylation. Results have indicated that the unadenylylated form of glutamine synthetase is involved in the activation of transcription of the histidine-utilization operon in *Salmonella typhimurium* (Tyler *et al.*, 1974). A similar role of unadenylylated glutamine synthetase in the transcription of the *nif* (nitrogen fixation) operon(s) has also been suggested (Tubb, 1974; Streicher *et al.*, 1974).

The properties of glutamine synthetase in pea seed have been investigated by Elliott (1953), and since then O'Neal & Joy (1973, 1974, 1975) have purified and characterized the enzyme from pea leaves. Mitchell & Stocking (1975) have presented evidence that glutamine synthetase is localized in chloroplasts of spinach and dwarf-pea leaves. Glutamine synthe-

tase activities have been measured in both the bacteroid and cytosol fractions of soya-bean nodules by Dunn & Klucas (1973), and their data and those of Brown & Dilworth (1975) show a greater proportion of activity in the cytosol of legume nodules than in extracts of nodule bacteroids.

Our investigations have revealed that soya-bean nodule cytosol contains a glutamine synthetase that is quite different from the enzyme in soya-bean nodule bacteroids in that it is unadenylylated and exhibits a different electrophoretic mobility (Guevara *et al.*, 1975). The nodule cytosol accounts for more than 90% of the total activity in soya-bean nodules. From the information available it appears that glutamine synthetase in nodule cytosol may play a major role in the metabolism of the early products of N_2 fixation. The purpose of this paper therefore is to describe the purification and some properties of the enzyme from this source.

Experimental and Results

Materials

Reagents including γ -glutamylhydroxamate, 2-mercaptoethanol, dithiothreitol, imidazole, EDTA and Tes [N-tris(hydroxymethyl) methyl-2-aminoethanesulphonic acid] were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Polyvinylpyrrolidone was purchased from the GAF Corp. (New York, NY, U.S.A.) and washed with 3M-HCl as described elsewhere (Evans *et al.*, 1972). Purified $(NH_4)_2SO_4$ for enzyme fractionation was purchased from Mann Laboratories (New York, NY, U.S.A.), protamine sulphate from Eli Lilly and Co. (Indianapolis, IN, U.S.A.) and polypropylene glycol-400 from Matheson, Coleman and Bell (Los Angeles, CA, U.S.A.). Biol-Gel A-5m and DEAE-Bio-Gel A-15m were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Acrylamide was obtained from Baker Chemical Co. (Phillipsburg, NJ, U.S.A.) and *NN'*-methylenebisacrylamide from Eastman Kodak Co. (Rochester, NY, U.S.A.). All other chemicals used were reagent grade or of the highest quality commercially available.

Glutamate-agarose was prepared from agarose-aminohexane (P-L Biochemicals Inc., Milwaukee, WI, U.S.A.) as described by Lowe & Dean (1974) and Wedler & Hoffman (1974a). A 20ml portion of the agarose-aminohexane was washed thoroughly with glass-distilled water. The washed gel was treated with 1% succinic anhydride at 4°C. The pH of the mixture was adjusted immediately to 6.0 and maintained at this value with 2% NaOH until there was no change (approx. 90min). The preparation was then washed with 200ml of water and adjusted to pH4.7 with 1% HCl. The activation of the carboxylate moiety on the agarose was accomplished by the dropwise addition, at 25°C, of 5ml of a solution

containing 0.1g of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) in 5ml of water. 1M-Glutamate (21ml) was added and the mixture was stirred for 20h at 25°C. This preparation was then washed with water and stored at 4°C.

The root nodules used were obtained from 28-day-old soya-bean plants (*Glycine max* Merr., var. 'Chippewa') grown in an N-free nutrient medium as described previously (Evans *et al.*, 1972), and were stored at -80°C.

Enzyme assays

Glutamine synthetase activity was determined as a routine by use of the γ -glutamylhydroxamate assay described by Shapiro & Stadtman (1970), unless otherwise indicated (γ -glutamylhydroxamate formation is a measure of the transferase activity of glutamine synthetase). Glutamine synthetase activity is defined such that 1 unit is the amount of enzyme which catalyses the formation of 1 μ mol of γ -glutamylhydroxamate/min at 25°C.

The biosynthetic assay system of Shapiro & Stadtman (1970) was modified for use in experiments described in Table 3 and Figs. 7, 9, 10. Each reaction mixture, in a final volume of 0.2ml, was incubated at 25°C. Reactions were initiated by the addition of the enzyme and terminated by the addition of 1.8ml of 0.8% $FeSO_4$ in 7.5mM- H_2SO_4 followed by 0.15ml of 6.6% $(NH_4)_6Mo_7O_{27} \cdot 4H_2O$ in 3.75M- H_2SO_4 .

Protein determination

Protein content was determined by use of the E_{280}/E_{260} ratio method of Warburg & Christian (1942) or the Lowry method of Layne (1957). The specific activity of glutamine synthetase preparations is defined as units of activity/mg of protein.

Sedimentation analysis

The molecular weights of the enzyme and its subunits were determined by high-speed sedimentation-equilibrium experiments (Yphantis, 1964) with a Spinco model E ultracentrifuge equipped with Rayleigh interference optics. An An-F or an An-D rotor and cells with Yphantis or double-sector centrepieces (Beckman Instruments, Palo Alto, CA, U.S.A.) were used. Interference patterns were recorded on Kodak type IIG spectroscopic plates and the fringe patterns were measured with a Nikon model 6C micro-comparator (Tokyo, Japan) equipped with a digitizer (L and W Electronics, Houston, TX, U.S.A.) and computer punch tape.

Sedimentation-velocity experiments were performed with a Spinco model E ultracentrifuge equipped with an electronic scanner and monochromator. Samples of enzyme (0.2, 0.3 and 0.4mg/ml) were equilibrated with a buffer solution containing 50mM-KCl, 1mM-MnCl₂ and 10mM-imidazole

(pH 7.3) and centrifuged at 4°C at 52000 rev./min (r_{av} , 6.64 cm; 42 min). Changes in concentration distribution at 280 nm were recorded with the scanner attachment. Data were corrected for viscosity differences and reported as $s_{20,w}$ values.

Polyacrylamide-gel electrophoresis

Gels for electrophoresis at pH 8.3 were prepared as described by Davis (1964) except that the polyacrylamide concentration was decreased from 7 to 5%. Sufficient 2-mercaptoethanol was added to the electrode buffers to obtain a concentration of 1 mM. Gels were subjected to electrophoresis for 1 h at 100 V before protein samples were applied.

Amino acid analyses

Amino acid analyses were performed on a modified Beckman model 120B amino acid analyser by using procedures described by Spackman *et al.* (1958). The enzyme was dialysed exhaustively against water. Portions of the dialysed enzyme were then dried by rotary evaporation. Five 250 μ g portions were hydrolysed in 0.5 ml of constant-boiling HCl for 18, 20, 24, 48 or 72 h at 110°C. The serine and threonine contents were then obtained by extrapolating to zero time of hydrolysis. Valine, isoleucine and phenylalanine contents were determined from the analysis of the 72 h hydrolysate or from an average of analyses of the 48 and 72 h hydrolysates. Portions (1 mg) of enzyme were used to determine half-cystine as cysteic acid after performic acid oxidation (Moore, 1963) and tryptophan by NaOH hydrolysis (Hugli & Moore, 1972) by using the buffer system described by Knox *et al.* (1970). The contents of all other amino acids were determined on a 20 h hydrolysate.

Experiments involving cations

Metal cations were removed from the purified enzyme used in the experiment described in Figs. 7, 8 and 9 by mixing 2.7 ml of the enzyme preparation with 0.15 ml of 0.1 M-EDTA (pH 7.3) and 0.15 ml of 0.2 M-2-mercaptoethanol followed by dialysis for

16 h against two changes of 100 ml of 50 mM-KCl/2 mM-imidazole, pH 7.3. Just before use the enzyme was diluted as necessary with cold water. Enzyme for use in experiments involving univalent cations was prepared as described for removal of bivalent metal cation except that dialysis was carried out against 10 mM-Tes/dimethylamine, pH 7.3, instead of the buffer solution containing KCl and imidazole.

Univalent cation effects on the Mn^{2+} -promoted transferase activity and on the Mg^{2+} -promoted biosynthetic activity were determined by using the following assays. The transferase assay mixture contained 5 mM-glutamine, 5 mM-hydroxylamine hydrochloride (neutralized with dimethylamine), 5 mM- H_3AsO_4 , 0.4 mM-ADP (sodium salt), 0.3 mM- $MnCl_2$, 10 mM-Tes/dimethylamine (pH 6.5), and 0 or 100 mM-KCl, -NaCl or -dimethylamine hydrochloride in a total volume of 1 ml. The reaction was initiated by the addition of 0.5 unit of enzyme in 0.1 ml. The assay mixture for biosynthetic activity contained 5 mM-sodium glutamate, 5 mM-ATP (sodium salt), 5 mM- $MgCl_2$, 5 mM- NH_4Cl , 10 mM-Tes/dimethylamine (pH 6.5 or 8.0) and 100 mM-KCl, -NaCl or -dimethylamine hydrochloride in a total volume of 0.2 ml. Each reaction was initiated by the addition of 2 units (pH 6.5) or 1 unit (pH 8) of enzyme in 0.05 ml.

Purification of glutamine synthetase

All purification procedures were performed at 0–4°C and all centrifugations were at 15000g for 10 min unless otherwise stated. A summary of the purification procedure is presented in Table 1.

Preparation of extract. A crude cytosol extract of soya-bean root nodules was prepared under anaerobic conditions as described by Evans *et al.* (1972). Nodules (100 g) and polyvinylpyrrolidone (33 g) were placed into a 900 ml Omni-Mixer vessel (Ivan Sorvall Inc., Norwalk, CT, U.S.A.) equipped with gassing vents. After flushing with purified N_2 for 10 min while shaking, ice-cold 0.025 M- KH_2PO_4 / K_2HPO_4 buffer, pH 7.3, containing 1 mM-dithiothreitol and previously sparged with purified N_2 for

Table 1. *Purification of glutamine synthetase*

The enzyme was purified from 100 g of soya-bean root nodules that had been stored for 6 months at –80°C. Protein was measured by E_{280}/E_{260} ratios, and 1 unit of enzyme activity is that amount of enzyme required to produce 1 μ mol of γ -glutamyl-hydroxamate/min in the standard assay procedure.

Preparation	Total protein (mg)	Sp. activity (units/mg of protein)	Recovery (%)
Crude extract	3077	1.3	—
Protamine sulphate supernatant solution	2350	1.8	100
35–50%-satd.-(NH_4) ₂ SO ₄ ppt.	516	8	98
35–50%-(v/v)-polypropylene glycol ppt.	294	11	81
DEAE-Bio-Gel A-15m eluate	96	24	58
Bio-Gel A-5m eluate	40	46	46
Affinity-gel eluate	15	75	27

10 min, was added. The mixture was then macerated for 3 min at 70 V. The Omni-Mixer unit was opened in air and the contents were pressed through four layers of cheesecloth and then filtered through bolting silk. The filtered macerate was centrifuged at 40000g for 30 min and the supernatant fluid (referred to as cytosol) was collected. An examination of the cytosol and bacteroids for 2-hydroxybutyrate dehydrogenase showed that over 99% of the activity of this enzyme in the nodules was associated with bacteroids. Since 2-hydroxybutyrate dehydrogenase is known to be associated with bacteroids and not with plant cytosol, contamination of the cytosol extract with bacteroid proteins appeared to be minimal. The specific activity of the enzyme in crude extracts ranged from 1 to 2 units/mg of protein when E_{280}/E_{260} ratios were used to determine protein concentration.

Protamine sulphate fractionation. Freshly prepared aq. 2% protamine sulphate was added dropwise to the extract (5 ml to 150 ml). After 15 min, the precipitate was removed by centrifugation and was discarded.

Ammonium sulphate fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant fluid to 35% saturation. The pH was adjusted to pH 7.3 with 6M- NH_3 , and after 15 min the precipitate was removed by centrifugation and discarded. The $(\text{NH}_4)_2\text{SO}_4$ concentration of the supernatant fluid was increased to 50% saturation, and after 15 min the precipitate was collected by centrifugation and dissolved in 40 ml of 10 mM-imidazole buffer, pH 7.3, containing 10 mM-2-mercaptoethanol.

Polypropylene glycol fractionation. Sufficient polypropylene glycol-400 was added dropwise to the 35–50% ammonium sulphate fraction to obtain a 35% (v/v) solution. The precipitated protein was removed by centrifugation for 5 min and was discarded. The supernatant fluid was collected and sufficient polypropylene glycol was added dropwise with stirring to obtain a 50% (v/v) solution. The precipitate was collected by centrifugation and dissolved in 20 ml of 10 mM-imidazole buffer, pH 7.3, containing 10 mM-2-mercaptoethanol.

DEAE-Bio-Gel chromatography. The 35–50% polypropylene glycol fraction was adsorbed to a column of DEAE-Bio-Gel A that had been previously equilibrated with 10 mM-imidazole buffer, pH 7.3, and eluted at 4°C as described in the legend to Fig. 1. Fractions (3 ml) were collected and those containing 50% or more of the activity of the peak fraction were combined. 2-Mercaptoethanol was added to 5 mM and the solution transferred to a 50 ml Diaflo concentration cell equipped with a XM-100A membrane (Amicon Corp., Lexington, MA, U.S.A.). Purified N_2 was flushed through the cell with stirring for about 10 min to remove most of the O_2 and the enzyme was concentrated to 3–4 ml at a pressure of 345 kPa (50 lb/in²). The concentrated enzyme was withdrawn

Fig. 1. Chromatography of glutamine synthetase on DEAE-Bio-Gel A

The 35–50% (v/v) polypropylene glycol precipitate was dissolved in 20 ml of 10 mM-imidazole buffer, pH 7.3, containing 10 mM-2-mercaptoethanol and adsorbed on a column (2.2 cm × 8 cm) of DEAE-Bio-Gel A-15m equilibrated with 10 mM-imidazole buffers, pH 7.3. The column was washed with 50 ml of 10 mM-imidazole buffer, pH 7.3, containing 50 mM-KCl and then eluted at 45 ml/h with a linear 0.05–0.40 M-KCl gradient, also containing 10 mM-imidazole buffer, pH 7.3, and 1 mM- MnCl_2 in a total volume of 200 ml. ○, E_{280} ; ●, glutamine synthetase activity.

Fig. 2. Chromatography of glutamine synthetase through Bio-Gel A-5m

The enzyme fractions from the DEAE-Bio-Gel A were concentrated to 3.5 ml and chromatographed at 5 ml/h through a column (1.5 cm × 80 cm) of Bio-Gel A-5m previously equilibrated and eluted with 50 mM-KCl containing 10 mM-imidazole buffer, pH 7.3, and 1 mM- MnCl_2 . ○, E_{280} ; ●, glutamine synthetase activity.

and the precipitated material was removed by centrifugation and discarded.

Bio-Gel A-5m molecular sieving. The concentrated enzyme from the DEAE-Bio-Gel eluate was chro-

Fig. 3. Affinity chromatography of glutamine synthetase

Enzyme from the Bio-Gel column was adsorbed directly on to a column (1.2 cm $\times$ 9 cm) of affinity gel prepared as described in the Experimental and Results section and equilibrated with 10 mM-imidazole buffer, pH 7.3, containing 1 mM-MnCl₂. The enzyme was eluted at 15 ml/h with a linear 0–0.50 M-sodium glutamate gradient also containing 10 mM-imidazole buffer, pH 7.3, and 1 mM-MnCl₂ in a total volume of 100 ml. $\circ$, E_{280} ; $\bullet$, glutamate synthetase activity.

matographed on Bio-Gel A-5m as described in the legend to Fig. 2. Fractions (3 ml) were collected and those with at least 50% of the activity of the peak fraction were combined.

Affinity chromatography. The partially purified enzyme was then adsorbed to an affinity column equilibrated with 10 mM-imidazole buffer (pH 7.3). Conditions used in the elution are described in the legend to Fig. 3. Fractions (3 ml) were collected and those having an approximately constant specific activity of glutamine synthetase were pooled. The specific activities of the purified preparation were 75, 110 or 120 for protein determinations based on the E_{280}/E_{260} ratio (Warburg & Christian, 1942), the Lowry method (Layne, 1957) or the results of amino acid analysis respectively. Specific activities of the crude extract and the purified enzyme indicated a 50–60-fold purification. This suggests that the glutamine synthetase represents about 2% of the total protein in the nodule cytosol.

Properties

Sedimentation analyses. The results of the high-speed sedimentation-equilibrium analysis are shown in Fig. 4(a) (intact enzyme) and Fig. 4(b) (dissociated enzyme). Both plots show apparent linearity, which suggests a high degree of purity for the enzyme preparation. From the data of Fig. 4(a) the mol.wt. of the native enzyme was calculated to be 376 000. From the data of Fig. 4(b) and a second analysis, the mol.wt. of the subunit was determined to be 47 300 ($\pm 7\%$). The native enzyme therefore appears to consist of

eight subunits of equal size. Based on sedimentation-velocity experiments the $s_{20,w}$ value for the enzyme was determined to be 14.5×10^{-13} , 14.5×10^{-13} and 14.3×10^{-13} s at protein concentrations of 0.2, 0.3 and 0.4 mg/ml respectively.

Electrophoretic analysis. Glutamine synthetase was subjected to electrophoretic analysis in the presence and absence of EDTA (Plate 1). The lightly stained region beneath the major protein band of the gel (b) appears to have resulted from the partial removal of the Mn²⁺ from the enzyme. When the

Fig. 4. Results obtained from high-speed equilibrium sedimentation of the purified glutamine synthetase

The data are plotted as the natural logarithm of the net fringe displacement ($\ln C$) as a function of $R^2 - R_a^2$, where R_a is the radial position of the meniscus (in cm) and R is the radial position of the indicated points. In (a), 0.5 mg of purified enzyme/ml was equilibrated by dialysis for 18 h against a solution of 10 mM-imidazole, pH 7.3, 50 mM-KCl and 1 mM-MnCl₂. In (b), 0.5 mg of purified enzyme/ml was equilibrated by dialysis for 48 h against a solution containing 10 mM-imidazole, pH 7.3, 6 M-guanidinium chloride, 5 mM-dithiothreitol and 5 mM-EDTA. In the sedimentation-equilibrium experiment with the native enzyme (a), centrifugation was conducted at 11 000 rev./min at 4°C, and in the experiment for the determination of subunit molecular weight (b), the centrifugation was at 36 000 rev./min at 25°C. The data were analysed by a computer program kindly provided by Professor Robert Dyson, Department of Biochemistry and Biophysics, Oregon State University.

Table 2. *Amino acid composition of glutamine synthetase*

For details of the methods of preparation and analysis, see the Experimental and Results section.

Amino acid	Content (mol %)	Amino acid content [mol/mol of subunit (mol.wt. 47300)]
Lysine	5.9	26.9
Histidine	2.2	10.2
Arginine	5.6	25.5
Tryptophan	1.5	6.8
Aspartic acid	10.4	47.4
Threonine	5.1	23.0
Serine	5.4	24.6
Glutamic acid	10.0	45.7
Proline	6.1	27.8
Glycine	10.6	48.4
Alanine	8.4	38.0
Half-cystine*	0.8	3.7
Valine	6.3	28.5
Methionine	1.6	7.4
Isoleucine	6.6	30.0
Leucine	6.7	30.4
Tyrosine	3.9	17.6
Phenylalanine	2.8	12.8

* As cysteic acid.

Fig. 5. *pH optimum of the biosynthetic activity of glutamine synthetase with either Mn^{2+} or Mg^{2+}*

Reaction mixtures contained 50mm-sodium glutamate, 50mm- NH_4Cl , 5mm-ATP (sodium salt), 50mm-Tes, 50mm-acetic acid, 5mm- $MgCl_2$ (Δ) or 5mm- $MnCl_2$ ($\circ$) as indicated, and 1 unit of enzyme in a total of 0.2ml. The pH was adjusted with NaOH and the reaction mixture was incubated for 10min at 25°C.

enzyme was treated with 10mm-EDTA before electrophoresis the intensity of the protein band at the top of the gel (c) is decreased and the lower band on the same gel is exceptionally prominent.

Examination of electron micrographs for quaternary structure. The structure of the purified enzyme indicates that glutamine synthetase has a cubic symmetry with a subunit at each corner and a distance of about 10nm (100 Å) across each side (Plate 2). There appears

Fig. 6. *pH optimum of the transferase activity of glutamine synthetase as influenced by Mn^{2+} or Mg^{2+}*

Reaction mixtures contained 30mm-glutamine, 60mm-hydroxylamine/NaCl, 0.4mm-ADP (sodium salt), 20mm- Na_2HAsO_4 , 50mm-Tes and 0.3mm- $MnCl_2$ ($\circ$) or 5mm- $MgCl_2$ (Δ) as indicated, and 0.2 unit of enzyme in a total of 1ml. The pH was adjusted with 6M-HCl or saturated NaOH. Reaction mixtures were incubated for 10min at 25°C and terminated by addition of 2ml of acidic $FeCl_3$ reagent (Shapiro & Stadtman, 1970).

Fig. 7. *Influence of bivalent cation concentration and pH on the biosynthetic activity of glutamine synthetase*

The reaction mixture contained 50mm-sodium glutamate, 50mm- NH_4Cl , 5mm-ATP (sodium salt), 5mm-bivalent cation mixture as indicated, 50mm-Tes and 1 unit of enzyme in a final volume of 0.2ml. The pH was adjusted with saturated NaOH. The concentrations of Mg^{2+} and Mn^{2+} were varied as indicated while the total bivalent cation concentration was maintained at 5mm in each reaction mixture. The reaction period was 10min at 25°C. $\circ$, pH 6.0; $\square$, pH 6.5; Δ , pH 7.0.

EXPLANATION OF PLATE I

Illustration of the results of polyacrylamide-gel electrophoresis of purified glutamine synthetase

Gel (a) contained 20 μ g and gel (b) contained 40 μ g of purified enzyme in 0.1 ml of a solution containing 10 mM-2-mercaptoethanol, 5% sucrose and 10 mM-imidazole buffer, pH 7.3. Gel (c) contained 40 μ g of enzyme in 0.1 ml of 10 mM-2-mercaptoethanol, 5% sucrose, 10 mM-EDTA and 10 mM-imidazole buffer, pH 7.3. The enzyme samples were layered on the surface of 5% polyacrylamide gels (0.5 cm $\times$ 8 cm). Electrophoresis was conducted at pH 8.3 and at 100 V until the Bromophenol Blue marker reached the bottom of gels. Gels were stained for 15 h with 0.05% Coomassie Brilliant Blue and destained with a solution of 5% (v/v) methanol and 7% (v/v) acetic acid.

EXPLANATION OF PLATE 2

Electron microscopy of glutamine synthetase

Purified enzyme (0.5 mg/ml) was dialysed for 8 h against 10 mM-imidazole buffer, pH 7.3, containing 1 mM-MnCl₂ and diluted to 50 µg/ml with the dialysis buffer just before use. Enzyme (1 drop) was mixed with 2 drops of 4% (w/v) tungstosilicic acid on a 300-mesh copper grid covered with a vaporized carbon film. The Philips 300 microscope was set at 60 kV with a 30 µm objective aperture. The enzyme was photographed at a magnification of $\times 74\,500$ by using Kodak (Rochester, NY, U.S.A.) electron image plates. Further magnifications by photographic enlargements are as follows: A, $\times 150\,000$; B, $\times 440\,000$; and C, $\times 330\,000$.

to be a 0.1–0.15 nm (10–15 Å) space separating the front and back component of the cube.

Amino acid analysis. The amino acid composition of glutamine synthetase from soya-bean root-nodule cytosol is presented in Table 2. The partial specific volume of the enzyme was calculated to be 0.74 ml/g by using the partial specific volume of each amino acid compiled by Cohn & Edsall (1943) and the amino acid composition of the enzyme. The E_{280} of a 1 mg/ml enzyme solution, based on amino acid content, would be 1.64.

pH optima. The biosynthetic activity of the enzyme has a pH optimum of 4.9 with 5 mM-MnCl₂ and 8.0 in an assay medium with 5 mM-MgCl₂ (Fig. 5). The activity at the pH optimum with Mn²⁺ was about the same as that at the pH optimum with Mg²⁺. The transferase activity of glutamine synthetase is optimum at pH 6.5 in the presence of 0.3 mM-MnCl₂, and at pH 7.5 with 5 mM-MgCl₂ (Fig. 6). Transferase activity however is sixfold greater with Mn²⁺ than with Mg²⁺ at their respective pH optima.

Cation specificity. The transferase activity of glutamine synthetase is activated 30% by 100 mM-K⁺ and -Na⁺ and as much as 100% with 100 mM-dimethylamine at pH 6.5. Biosynthetic activity, however, was not activated by univalent cations at pH 6.5, but was activated about 10–15% at pH 8; however, such activation by univalent cations was observed only at low substrate concentrations (5 mM).

The relative proportions of Mg²⁺ and Mn²⁺ markedly influence the biosynthetic activity of the enzyme (Fig. 7). At pH 6.5 maximum activity was observed in a medium containing 5 mM-Mg²⁺ plus Mn²⁺ when the molar ratio Mg²⁺/Mn²⁺ was 4:1. By contrast, maximum activity at pH 7 was observed when the total bivalent cation concentration (5 mM) was entirely Mg²⁺. When the ratio Mg²⁺/Mn²⁺ in the assay medium was 10:1, biosynthetic activity was not appreciably influenced by pH from 6.0 to 7.0.

Inhibition. The enzyme was inhibited to varying extents by the most common glutamine synthetase feedback inhibitors and also by ADP and AMP

Fig. 8. Modulation of glutamine synthetase by energy charge

The reaction mixture contained 5 mM-sodium glutamate, 5 mM-NH₄Cl, 20 mM-MgCl₂, 50 mM-Tes/NaOH at pH 6.5 and 2 units of enzyme in 0.2 ml. The total adenylate concentration in each reaction mixture was maintained at 5 mM. Energy charge was calculated by the method of Atkinson (1968). Each reaction period was 10 min at 25°C. ○, AMP+ATP; ●, AMP+ATP+5 mM-glycine; △, ADP+ATP; ▲, ADP+ATP+5 mM-glycine. In the inset graph the effect of concentration of ATP on the biosynthetic activity of glutamine synthetase (■) is presented. The conditions were the same as those described above except that combinations of nucleotides were omitted and the concentration of ATP was varied as indicated.

Table 3. *Inhibition of glutamine synthetase by selected nucleotides and amino acids*

The reaction mixture contained 5mM-sodium glutamate, 5mM-NH₄Cl, 5mM-ATP, inhibitor (5mM), 20mM-MgCl₂, 50mM-Tes/NaOH (pH 6.5) and 2 units of enzyme in a total volume of 0.2ml. The reaction mixtures were incubated for 10min at 25°C. Each value is the mean $\pm$ S.E.M. of triplicate determinations.

Inhibitor	Inhibition (%)*
CTP	42 $\pm$ 1
ADP	61 $\pm$ 16
AMP	29 $\pm$ 10
Histidine	0
Glycine	13 $\pm$ 1
Alanine	9 $\pm$ 3
Glutamine	7 $\pm$ 3
Tryptophan	4 $\pm$ 4

* Compared with complete system without added inhibitor.

(Table 3). The ADP and AMP inhibition suggested that the enzyme was subject to energy-charge control (Atkinson, 1968). Results shown in Fig. 8 revealed an effect of energy charge on glutamine synthetase activity at a nucleotide concentration of 5mM. Energy-charge control was further modified in the presence of 5mM-glycine.

Discussion

The nitrogen-fixation process in legume root nodules produces ammonia, which is used in the biosynthesis of glutamine and other metabolites that are exported from the nodule into the conductive elements of the plant. An understanding of the properties of glutamine synthetase and other enzymes that participate in ammonium utilization is essential for an assessment of factors that control nitrogen fixation in legumes.

Glutamine synthetase from nodule cytosol has been purified, and the factors that regulate its activity were examined. Conventional purification methods were used, including ion exchange, molecular sieving and affinity chromatography. An especially critical step in the procedure is the concentration of the enzyme by pressure dialysis. During this process, oxygen must be excluded and great care taken in gradual pressurizing and depressurizing of the cell. The overall purification obtained is about 50-fold over the crude extract and the yield is between 25 and 30%. The cytosol enzyme appears to represent about 2% of the total cytosol protein. Nodule cytosol therefore contains a considerably greater concentration of glutamine synthetase than do either pea leaves (O'Neal & Joy, 1973) or pea seed (Webster, 1964). In addition, the specific activity of the enzyme in crude soya-bean

nodule cytosol is about threefold greater than that of the glutamine synthetase in crude extracts of young (1-week-old) soya-bean roots (R. McParland, unpublished work).

The sedimentation analysis of the cytosol glutamine synthetase indicated a high degree of purity. Also the specific activity of the most highly purified cytosol preparation is comparable with that of the purified enzyme from pea leaves (O'Neal & Joy, 1973). Further assessment of purity of the cytosol enzyme by polyacrylamide-gel electrophoresis, however, was complicated by the apparent loss of Mn²⁺ from the enzyme complex, resulting in a second, faster-migrating, component.

On the basis of sedimentation-equilibrium experiments, the mol.wt. of the cytosol glutamine synthetase is 376000. This is comparable with the values of 330000–370000 which O'Neal & Joy (1973) obtained for the pea leaf enzyme by use of molecular-sieving techniques. It also is in the same range as the mol.wt. of 370000 which Stahl & Jaenicke (1972) determined for the enzyme from pig brain. By contrast, Wilk *et al.* (1969) obtained a mol.wt. of 525000 from sedimentation analysis of the glutamine synthetase from sheep brain. These enzymes are all smaller than their prokaryotic counterparts, which have mol.wts. near 600000 (Ginsburg, 1972). The subunit mol.wt. of the nodule glutamine synthetase in guanidinium chloride is 47300. The enzyme appears therefore to be composed of eight subunits of identical size. Results from electron-microscopic analyses suggest that these subunits are arranged in two sets of planar tetramers which are parallel to each other and about 1 nm (10 Å) apart. The dimensions of the intact enzyme are approximately 10 nm (100 Å) along each side and the resulting configuration is apparently cubical. The structural features of this enzyme appear to be very similar to those of the glutamine synthetase from sheep brain (Haschemeyer, 1968) and from pig brain (Stahl & Jaenicke, 1972). An interesting aspect of the two-plane structure is that it is conserved in all prokaryotic and eukaryotic glutamine synthetases that have been characterized to date [e.g., see Valentine *et al.* (1968)]. The physiological significance of the two-plane arrangement is unclear. It has been shown (Stahl & Jaenicke, 1972) that separation of the two tetramers of the pig brain enzyme by use of 2M-urea resulted in a complete loss of activity.

The $s_{20,w}$ value of the nodule-cytosol glutamine synthetase was determined by sedimentation-velocity experiments to be 14.5×10^{-13} s. This compares well with that of the sheep and pig brain enzymes, of 15×10^{-13} s. The $s_{20,w}$ values of both enzymes are considerably lower than that of the enzyme from *Escherichia coli*, which is 20×10^{-13} s (Ginsburg, 1972).

The amino acid composition of the cytosol glutamine synthetase differs from that of the enzyme from

pea seed (Tate & Meister, 1973) but is within the range of variability for the glutamine synthetases that have been prepared from a variety of other sources [for a compilation, see Wedler & Hoffman (1974a)]. The nodule enzyme has higher glycine and proline contents and a lower phenylalanine content than the glutamine synthetase from pea seed. The cytosol enzyme appears to have four half-cystine residues per subunit, in contrast with the two that were reported for the pea seed enzyme. Since the nodule represents a unique organ in the development of the leguminous plant, the possibility exists that the amino acid composition of glutamine synthetase from this source may be different from that of the enzyme from other organs of legumes.

The regulatory properties of the cytosol glutamine synthetase from soya-bean root nodules are similar to those of glutamine synthetase from pea leaves (O'Neal & Joy, 1974, 1975), pea seed (Kingdon, 1974) and other eukaryotic organisms (Schnackerz & Jaenicke, 1966; Deuel *et al.*, 1972). The cytosol enzyme responds to changes in the relative proportion of Mg^{2+} and Mn^{2+} , is inhibited by feedback inhibitors, and is apparently controlled by the magnitude of the energy charge.

The pH optimum for Mg^{2+} -promoted biosynthetic activity is 8.0 and that for Mn^{2+} -promoted activity is 4.9. Both optima are near those reported for the pea leaf glutamine synthetase, which are 8.2 and 5.2 in the presence of Mg^{2+} and Mn^{2+} respectively. The biosynthetic activity of the cytosol enzyme at its pH optimum with Mg^{2+} is about the same as that with Mn^{2+} . By contrast, the transferase activity of the enzyme is sixfold greater in the presence of Mn^{2+} than with Mg^{2+} at their respective pH optima of 6.5 and 7.5. The molar ratio Mg^{2+}/Mn^{2+} has a strong influence on the biosynthetic activity of the cytosol glutamine synthetase. This apparently results from an inhibitory effect of Mn^{2+} on the activity of the enzyme at pH values above 4.9. When the molar ratio Mg^{2+}/Mn^{2+} is maintained at 10:1 the activity of the enzyme is relatively constant between pH values of 6.0 to 7.0, which is the range where the nodule would be expected to function.

The biosynthetic activity of the nodule glutamine synthetase is inhibited by CTP, glycine, alanine and glutamine, and is influenced by the magnitude of the energy charge. Such regulatory control mechanisms are common to prokaryotic (Ginsburg & Stadtman, 1973; Wedler & Hoffman, 1974b) and eukaryotic glutamine synthetases (O'Neal & Joy, 1974, 1975).

Insufficient information is available to assess the importance of different control mechanisms in the function of glutamine synthetase in nitrogen assimilation by nodules. Presuming that the physiological pH of the environment where cytosol glutamine synthetase functions is about 6.5, the enzyme would be expected to function most efficiently in ammonium

assimilation where the ratio Mg^{2+}/Mn^{2+} is no lower than 10:1 and where a relatively high energy charge is maintained. Obviously some of the products of ammonium assimilation would need to be actively transported out of the nodule environment to allow glutamine synthetase to function optimally.

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