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Two Isoenzymes of NADH-dependent Glutamate Synthase in Root Nodules of *Phaseolus vulgaris* L.

PURIFICATION, PROPERTIES AND ACTIVITY CHANGES DURING NODULE DEVELOPMENT

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ABSTRACT

The specific activity of plant NADH-dependent glutamate synthase (NADH-GOGAT) in root nodules of *Phaseolus vulgaris* L. is over threefold higher than the specific activity of ferredoxin-dependent GOGAT. The NADH-GOGAT is composed of two distinct isoenzymes (NADH-GOGAT I and NADH-GOGAT II) which can be separated from crude nodule extracts by ion-exchange chromatography. Both NADH-GOGAT isoenzymes have been purified to apparent homogeneity and shown to be monomeric proteins with similar M_rs of about 200,000. They are both specific for NADH as reductant. An investigation of their kinetic characteristics show slight differences in their K_ms for L-glutamine, 2-oxoglutarate, and NADH, and they have different pH optima, with NADH-GOGAT I exhibiting a broad pH optimum centering at pH 8.0 whereas NADH-GOGAT II has a much narrower pH optimum of 8.5. The specific activity of NADH-GOGAT in roots is about 27-fold lower than in nodules and consists almost entirely of NADH-GOGAT I. During nodulation both isoenzymes increase in activity but the major increase is due to NADH-GOGAT II which increases over a time course similar to the increase in nitrogenase activity. This isoenzyme is twice as active as NADH-GOGAT I in mature nodules. The roles and regulation of these two isoenzymes in the root nodule are discussed.

GOGAT[2] catalyzes the transamidation of glutamine amido-nitrogen to the α-amino position of 2-oxoglutarate to form two moles of glutamate. Together with GS, these two enzymes form the major, and perhaps only, route of ammonia assimilation in higher plants (16). GS is responsible for the initial assimilation of ammonia into glutamine and because the two enzymes are dependent on each other for the provision of substrate, their activities constitute a cycle which has been termed the glutamate synthase cycle (see 16). Ammonia in higher plants is derived both from the plant's primary nitrogen sources (ammonia, nitrate or, for legumes, dinitrogen) and from a number of internal nitrogen-cycling pathways such as photorespiration, amino acid catabolism, and phenyl propanoid metabolism. The GOGAT cycle is therefore essential not only for primary nitrogen assimilation but also to maintain the general nitrogen economy of the plant.

In higher plants two types of GOGAT have been purified and characterized; one of these is dependent on ferredoxin as reductant (Fd-GOGAT, EC 1.4.7.1), whereas the other utilises NADH (NADH-GOGAT, EC 1.4.1.14) (26). The Fd-GOGAT has been shown to be most abundant in green tissues and is located in the chloroplast (28). It has been purified from a number of higher plant species and organs (26) and generally these reports agree that it is a monomeric protein of M_r about 140,000. Work on photorespiratory mutants of *Arabidopsis* (25) and barley (9) has shown that this enzyme is essential for the reassimilation of photorespiratory ammonia in leaves. The enzyme has also been detected in nongreen tissues although its precise role and the provision of its reductant in these tissues is unclear (18, 26). Studies on the Fd-GOGAT of rice roots and soybean root nodules suggest that the enzyme in these nongreen organs is physically and antigenically similar to the enzyme in leaves (26, 27). However, antibodies raised against the rice-leaf Fd-GOGAT fail to cross-react with NADH-GOGAT from several organs and species, thus suggesting that Fd-GOGAT and NADH-GOGAT are two distinct enzyme proteins (26, 27).

NADH-GOGAT has been purified from root nodules of lupin (3), soybean cell cultures (5), etiolated pea shoots (14), and *Chlamydomonas* (12). It appears to be an iron-sulfur flavoprotein with a single subunit of M_r about 230,000 and is specific for NADH as reductant, showing little or no activity with NADH or ferredoxin. Subcellular fractionation studies on nodules of *Phaseolus vulgaris* (1), soybean (4), cowpea (24), and lupin (23) and on leaf callus cultures of *Bouvardia ternifolia* (17) indicate that this enzyme is associated largely with the plastids.

^{15}N-Labeling experiments on several legume species have shown that ammonia produced by dinitrogen fixation is assimilated in the plant fraction of the nodule by the combined activities of GS and GOGAT (15, 22). The glutamine and glutamate produced are then used to synthesize the nitrogen transport compounds asparagine (in temperate legumes) and ureides (in tropical legumes). During root nodulation the activity of many of the enzymes in these nitrogen assimilatory pathways have been shown to increase severalfold, over a time course similar to the increase in nitrogenase in the bacteroids. For example GS, aspartate aminotransferase, asparagine synthetase, xanthine dehydrogenase, uricase, and allantoinase have all been shown to increase in activity during nodulation of various legumes (15, 22). Recently it has been shown that the increase in some of these enzymes, for example GS in *P. vulgaris* (8, 11), lupin aspartate aminotransferase (19) and soybean uricase (2) is due to the production of specific isoenzymes, not found in roots. Work on GOGAT activity in root nodules has shown that an NADH-dependent activity increases during nodulation of lupin (21) and soybean (20) over a time course similar to the increase in plant GS activity. In a study on soybean nodules, Fd-GOGAT activity has also been shown to be present and to be about twice as high as NADH-GOGAT activity (27).

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[2] Abbreviations: GOGAT, glutamate synthase; GS, glutamine synthetase; PMSF, phenylmethylsulfonyl fluoride.

In this paper we describe work on the structure and activity of NADH-GOGAT in root nodules of *P. vulgaris*. Two isoenzymes have been identified and purified, and their kinetic and physical properties have been examined. We have also measured the changes in the activity of these two isoenzymes during nodule development. The results of these experiments are described.

MATERIALS AND METHODS

Plant Materials. *Phaseolus vulgaris* L. cv Tendergreen was generally grown in growth rooms in 6 inch pots with perlite under 18 h light/6 h dark cycles (22–24°C). The light intensity was 100 μmol photons m^{-2} s^{-1}. The plants were watered with nutrient solution lacking a nitrogen source made up in tap water. Nodules were formed by inoculating 5-d-old seedlings (d 0) with *Rhizobium leguminosarum bv phaseoli* R3622 previously grown for 2 d at 30°C in yeast extract/mannitol medium (8). Unless otherwise stated nodules were picked, free of the root system, and stored in liquid nitrogen. For the nodule development experiment, samples harvested at d 5, 8, and 10 consisted of the top 5 cm of the nodulating root system. For purification of NADH-GOGAT, nodules were also grown in green houses (21–24°C) with natural daylight supplemented to 14 h d length with artificial lighting. Soybean nodules were harvested from *Glycine max* cv Prize grown in gravel in growth rooms as described above except that the daylength was 12 h. The rhizobial strain used was *Bradyrhizobium japonicum* USDA 110.

Preparation of Plant Cell-Free Extracts. Frozen nodules (0.5 g) or root systems (2 g) were ground in a mortar and pestle with 2.5 mL extraction buffer (50 mM Hepes [pH 7.5] containing 0.5 M sucrose, 10 mM DTT, 1 mM EDTA, and 1 mM PMSF). The homogenate was transferred to microfuge tubes and centrifuged at 11,600 *g* for 10 min. The supernatant (about 2.5 mL) was desalted on a 5 mL Sephadex G-50 column and either used directly or prepared for the HPLC columns as follows. The extract was diluted to 6 mL with HPLC running buffer (50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT) and filtered through a 0.2 μm filter and used immediately for enzyme activity measurements and chromatography on HPLC columns. All procedures were carried out at 0 to 4°C.

Chromatography on HPLC Ion-Exchange Column. Five mL of desalted extract was loaded onto a DEAE TSK 5PW (7.5 × 75 mm) column preequilibrated with HPLC running buffer at room temperature. The proteins were then eluted at a flow rate of 0.5 mL min^{-1} with 5 mL HPLC running buffer followed by 20 mL of a 0 to 0.4 M KCl gradient and then 5 mL of 0.6 M KCl all made up in HPLC running buffer. Fractions of 0.5 mL were collected, placed on ice, and then assayed for NADH-GOGAT activity.

Chromatography on HPLC Gel Filtration Column. An extract of 0.5 mL was loaded onto an analytical HPLC gel filtration column (TSK G4000SW 7.5 × 600 mm) preequilibrated and then run with the buffer described above; 0.5 mL fractions were collected at a flow rate of 0.5 mL min^{-1}. Marker proteins dissolved in the same buffer were run under identical conditions; the proteins used were β-galactosidase (M_r 520,000), glutamate dehydrogenase (M_r 340,000), catalase (M_r 232,000), alcohol dehydrogenase (M_r 150,000), haemoglobin (M_r 64,000), Cyt *c* (13,000).

Table I. *Activities of NADH and Ferredoxin Dependent GOGAT in Root Nodules of P. vulgaris*

Assay Conditions	GOGAT Activity[a]
	nmol min^{-1} mg $protein^{-1}$
Complete with NADH	12.3
−2-oxoglutarate	0.2
+ azaserine	0
Complete with ferredoxin	3.9
−2-oxoglutarate	0.6
+ azaserine	0
Complete − reductant	0.6

[a] The activities were measured by the radiochemical assay.

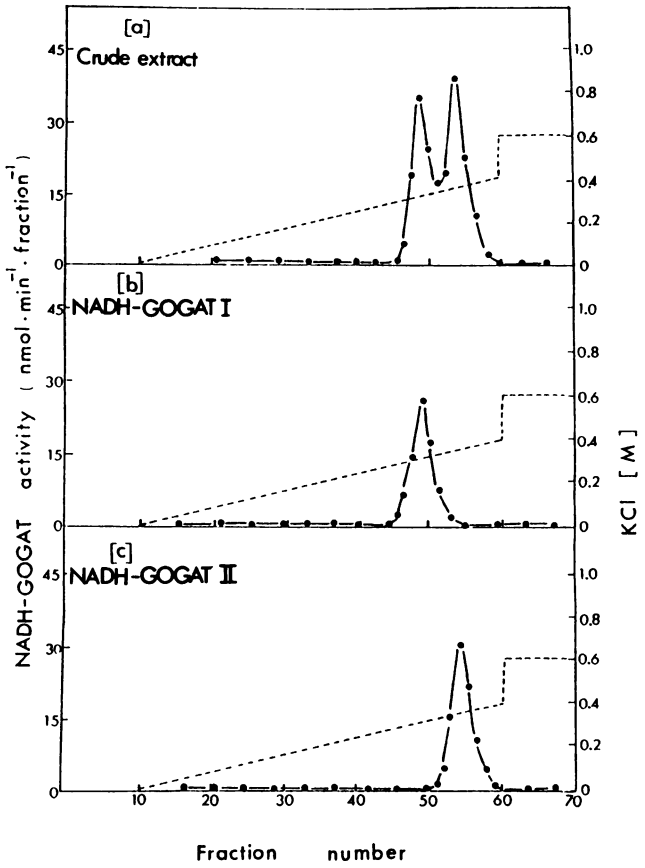

FIG. 1. Elution profile of nodule NADH-GOGAT activity from an HPLC ion-exchange column. [a], Crude nodule extract. The two activity peaks were then diluted and rerun separately on the column. [b], Rerun of activity peak I; [c], rerun of activity peak II. Hatched line, [KCl].

Purification of NADH-GOGAT Isoenzymes. *a. Crude Extract.* Frozen nodules (100 g) were ground in a coffee grinder for 2 min then transferred to a mortar and pestle and ground with about 200 mL extraction buffer. The brei was then diluted to 500 mL with extraction buffer, filtered through four layers of muslin, and centrifuged at 11,000*g* for 20 min. The supernatant was then subjected to stepwise ammonium sulphate precipitation and the pellet, precipitated by 35 to 60% saturation with ammonium sulphate, was redissolved in 20 mL of potassium phosphate buffer (pH 7.5) containing 1 mM PMSF.

b. Gel Filtration on Sephacryl S-300. The extract was loaded onto a 100 × 3.5 cm Sephacryl S-300 column. The column was run overnight with 50 mM potassium phosphate buffer containing 1 mM EDTA and 0.5% β-mercaptoethanol (running buffer) at 24 mL h^{-1} and 8 mL fractions were collected. Fractions showing highest activity of NADH-GOGAT were pooled and glutamic acid (Na salt) was added to 5 mM.

c. Chromatography on Blue Sepharose Column. The partially purified extract was then loaded onto a 50 × 1.5 cm Blue Sepharose column previously equilibrated with running buffer. The column was washed overnight with about 400 mL of the same buffer and then with 100 mL of the same buffer but containing 0.1 M NaCl. NADH-GOGAT activity was then eluted with running buffer containing 0.3 M NaCl, 50 mM 2-oxoglutarate, and 50 mM L-glutamine. The fractions containing NADH-

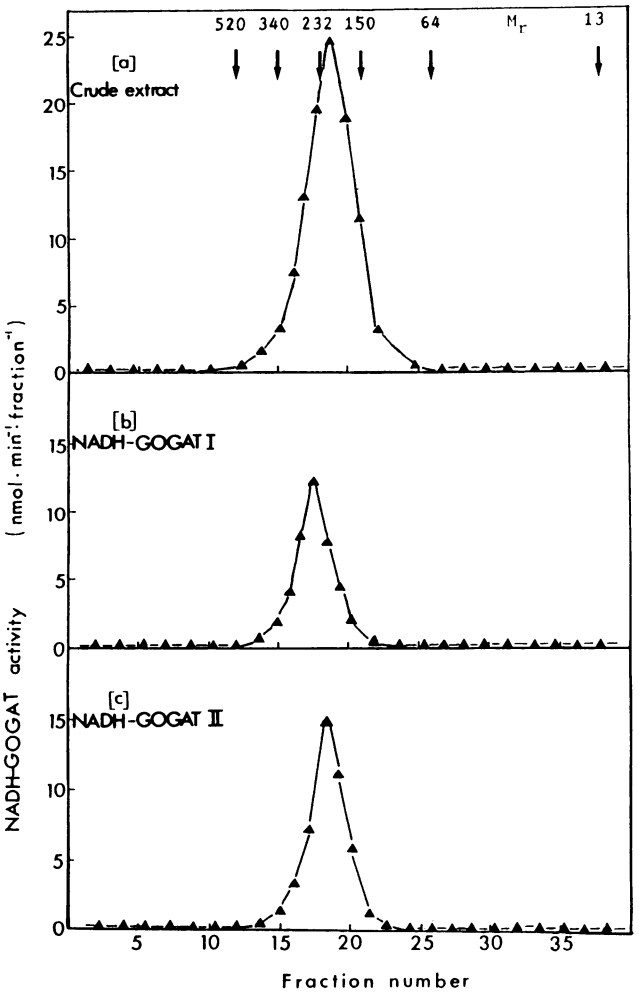

FIG. 2. Elution profile of NADH-GOGAT activity of [a] a crude nodule extract, [b] NADH-GOGAT I, and [c] NADH-GOGAT II, from an HPLC gel-filtration column. The elution positions of protein markers of the following M_rs are shown: (a) 520,000; (b) 340,000; (c) 232,000; (d) 150,000; (e) 64,000; (f) 13,000.

GOGAT activity were pooled and solid ammonium sulfate was then added to 80% saturation. The precipitated proteins were collected by centrifugation and the pellet was dissolved with 2 mL HPLC running buffer and desalted on a Sephadex G-50 column (as above).

d. Chromatography on HPLC Ion-Exchange Column. The desalted extract was made up to 5mL and chromatographed on the HPLC ion-exchange column as described above.

e. Chromatography on HPLC Gel Filtration Column. The separated two isoenzymes were purified to homogeneity by passage through an HPLC gel filtration column (as above).

Denaturing Polyacrylamide Gel Electrophoresis. The proteins after each purification step were run on a 10% polyacrylamide SDS gel (10). Mol wt markers were myosin (M_r 200,000) β-galactosidase (M_r 116,250), phosphorylase B (M_r 92,500), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000). The gels were stained either with Coomassie brilliant blue or by silver staining.

Assay of NADH-GOGAT Activity. *a. Spectrophotometric Assay.* The activity of NADH-dependent GOGAT was assayed at 18 to 22°C by measuring the oxidation of NADH at 340 nm. The reaction mixture routinely contained 25 mM HEPES buffer (pH 7.5), 1 mM 2-oxoglutarate, 2.5 mM L-glutamine, 0.16 mM NADH, 1% β-mercaptoethanol, and 100 to 200 μl enzyme solution in a total volume of 1.2 mL. The reaction was initiated by the addition of the enzyme and the change in A_{340} was monitored over a period of at least 2 min and was found to be linear over this time. An assay without 2-oxoglutarate and L-glutamine was used as control. The activities are expressed in μmol NADH oxidised min^{-1}. In the determination of the K_m values of NADH-GOGAT for the various substrates, the concentration of the substrates were varied in the reaction mixture. For pH optima determinations the pH of the HEPES buffer was varied. Partially purified extracts prepared as in the purification procedures but lacking the Blue Sepharose and final gel filtration steps were used in these determinations.

b. Radiochemical Assay. NADH-GOGAT activity was measured by the production of ^{14}C-labeled glutamate from ^{14}C-glutamine essentially as described earlier (29) but with the following modifications. ^{14}C-Glutamine, obtained from Amersham International, was purified on a column of Dowex-1-chloride and was used at a specific activity of 109 μCi/mmol in the reaction mixture. The separation of glutamate from glutamine by paper electrophoresis was achieved at about 90 V for about 4 h.

Assay of Fd-GOGAT Activity. Fd-GOGAT activity was measured according to Wallsgrove *et al.* (29) but with the modifications described above.

Assay of Nitrogenase Activity. Nitrogenase activity of nodulated root systems was measured by acetylene reduction as described previously (11). At the end of the assay the nodules were picked and weighed.

Protein Determination. Protein, in cell-free extracts, was deter-

Table II. *Purification of Two Isoenzymes of NADH-GOGAT from Root Nodules*

Purification Step	Total Activity	Total Protein	Specific Activity	Purification[a]	Recovery
	μmol min⁻¹	*mg*	*μmol min⁻¹ mg protein⁻¹*	*-fold*	*%*
Crude extract	21.50	1,248	0.017	1	100
Sephacryl S-300 column	12.33	144	0.084	4.9	57.3
Blue Sepharose column	10.58	11.8	0.87	51.4	49.2
HPLC ion-exchange column					
NADH-GOGAT I	1.37	1.48	0.92	216	6.3
NADH-GOGAT II	3.36	1.94	1.73	136	15.6
HPLC gel filtration column					
NADH-GOGAT I	0.47	0.13	3.77	887	2.1
NADH-GOGAT II	1.26	0.16	8.05	631	5.8

[a] The -fold purification of each isoenzyme was based on a determined activity ratio of NADH-GOGAT I: NADH-GOGAT II of 1:3 in crude nodule extracts.

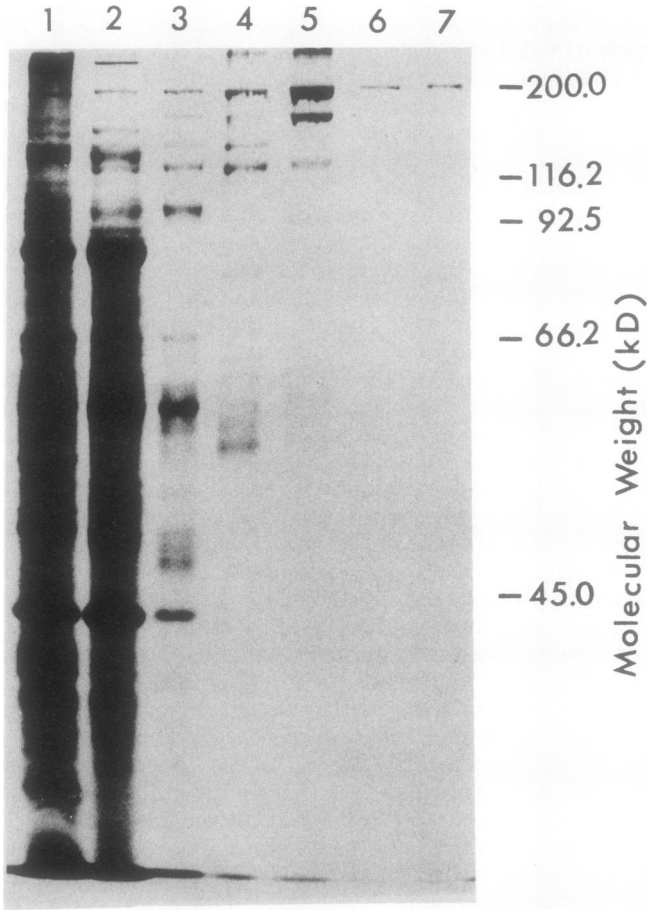

FIG. 3. SDS-polyacrylamide gel of proteins from different stages in the purification of NADH-GOGAT I and NADH-GOGAT II from root nodules. 1, Crude nodule extract; 2, Sephacryl S300 column; 3, Blue Sepharose column; 4, HPLC ion-exchange column; NADH-GOGAT I; 5, NADH-GOGAT II; 6, HPLC gel filtration column, NADH-GOGAT I; 7, NADH-GOGAT II. The position, and sizes in M_r, of protein markers are indicated.

mined using the BioRad Protein Assay Dye-Binding reagent, and bovine γ-globulin as standard.

RESULTS

Comparison of NADH-GOGAT and Fd-GOGAT Activities in *Phaseolus vulgaris* Root Nodules. A comparison was made of NADH-dependent and Fd-dependent GOGAT activities in crude nodule extracts of *P. vulgaris* cv Tendergreen, using a radiochemical assay (Table I). Care was taken in the extraction procedures to minimize proteolysis and inactivation of the enzymes by including a protease inhibitor and activity stabilizers in the buffers and by preparing and assaying the extracts rapidly. Both activities were found to be largely dependent on 2-oxoglutarate and reductant, and were abolished by azaserine (an inhibitor of glutamine-amido transferases [16]). The activity of NADH-GOGAT was found to be over threefold higher than the Fd-GOGAT activity. Measurements of NADH-GOGAT in these extracts using a spectrophotometric assay were almost identical to the radiochemical method and therefore this simpler assay was used in all further experiments.

Identification of Two Isoenzymes of NADH-GOGAT in Root Nodules. A crude extract of *P. vulgaris* root nodules was run on an HPLC ion-exchange column and the eluted fractions were assayed for NADH-GOGAT activity (Fig. 1a). Two peaks of activity eluted from the column at [KCl] of about 0.35 and 0.39 M. The two peak fractions were then diluted about 10-fold to reduce the [KCl] to less than 40 mM and were then rerun separately on the HPLC column (Fig. 1, b and c); each activity peak eluted at its original position on the [KCl] gradient. The two separated activity peaks were assayed for ferredoxin-dependent GOGAT activity and neither showed any activity with this reductant. We propose that these two activity peaks represent two isoenzymes of NADH-GOGAT which we have called NADH-GOGAT I and NADH-GOGAT II, respectively.

The crude nodule extract was also run on an HPLC gel-filtration column and the fractions were assayed for NADH-GOGAT activity (Fig. 2a). Only a single peak of activity eluted from the column and in comparison to protein markers of known M_rs, the native M_r of NADH-GOGAT in crude nodule extracts appears to be about 200,000. The two nodule NADH-GOGAT isoenzymes, which had been separated on the ion-exchange column, were then run separately on the gel-filtration column and were reproducibly found to elute at slightly different positions corresponding to M_rs of 210,000 and 200,000 for NADH-GOGAT I and NADH-GOGAT II, respectively (Fig. 2, b and c).

Purification of the Two NADH-GOGAT Isoenzymes. The two isoenzymes of NADH-GOGAT were purified from root nodules as shown in Table II. The proteins at different stages during the purification procedures were run on SDS-polyacrylamide gels (Fig. 3). The total NADH-GOGAT activity recovered in the purification was about 8%, which was represented by about 2% and 6% for NADH-GOGAT I and NADH-GOGAT II, respectively. The activities of both isoenzymes, but particularly of

Table III. *Substrate Specificity of the Two Nodule NADH-GOGAT Isoenzymes*

Assay Conditions	NADH-GOGAT Activity	
	NADH-GOGAT I	NADH-GOGAT II
	% of complete assay	
Complete assay[a]	100	100
+ 0.4 mM azaserine	0	0
− Glutamine	7.0	1.8
− Glutamine + 1 mM asparagine	7.0	0
− Glutamine + 1 mM NH_4Cl	3.0	0
− 2-Oxoglutarate	3.6	3.6
− 2-Oxoglutarate + 0.8 mM pyruvate	2.8	1.6
− 2-Oxoglutarate + 0.8 mM oxaloacetate	3.2	2.6
− NADH	0	0
− NADH + 160 μM NADPH	2.6	0.8

[a] Complete assay includes 50 mM HEPES (pH 7.5), 1% β-mercaptoethanol, 1 mM 2-oxoglutarate, 2.5 mM L-glutamine, 0.16 mM NADH.

Table IV. *Kinetic Properties of the Two NADH-GOGAT Isoenzymes*[a]

Property	NADH-GOGAT I	NADH-GOGAT II
K_m for L-glutamine	770 μM	240 μM
K_m for 2-oxoglutarate	22 μM	87 μM
K_m for NADH	14 μM	5.2 μM
pH optimum	8.0	8.5
Temperature optimum	30°C	30–32°C

[a] The correlation coefficients obtained for the linear regression analyses of the K_ms were greater than 0.97.

NADH-GOGAT I, were found to be very unstable and sensitive to low temperatures and it was found necessary to include either DTT or β-mercaptoethanol, and EDTA and PMSF in the extraction and purification buffers.

The specific activities of NADH-GOGAT I and NADH-GOGAT II at the end of the purification procedures were 3.8 μmol min^{-1} mg $protein^{-1}$ and 8.1 μmol min^{-1} mg $protein^{-1}$, respectively. Both proteins appeared to be homogeneous on SDS-polyacrylamide gels and revealed denatured M_rs of each of the two isoenzymes of about 200,000 (Fig. 3).

Activity Characteristics of the Two NADH-GOGAT Isoenzymes. Partially purified preparations were used to study the specificity of the two isoenzymes for their substrates (Table III). Both NADH-GOGAT I and NADH-GOGAT II activities were totally abolished by adding azaserine to the reaction mixtures. The activities of both isoenzymes were largely dependent on L-glutamine, 2-oxoglutarate, and NADH. Neither L-asparagine nor NH_4Cl could substitute for L-glutamine in the assay and neither pyruvate nor oxaloacetate could substitute for 2-oxoglutarate. Very little activity was measureable with either isoenzyme when NADH was replaced by NADPH.

The K_m for each substrate was determined for each isoenzyme (Table IV) by linear regression analysis of Lineweaver and Burk double reciprocal data. NADH-GOGAT II had higher affinities for L-glutamine and NADH, but a lower affinity for 2-oxoglutarate when compared to the corresponding affinities for these substrates of NADH-GOGAT I. The pH optimum of the two isoenzymes were also different: NADH-GOGAT I had a broad pH optimum centering at pH 8.0 whereas NADH-GOGAT II had a much narrower pH optimum of 8.5. Both isoenzymes had a temperature optimum of about 30°C.

Changes in Activity of the Two NADH-GOGAT Isoenzymes during Nodule Development. Five-d-old *P. vulgaris* seedlings were inoculated with *R. leguminosarum bv phaseoli* (d 0) and nodulating roots or nodules were harvested at various times during development and assayed for nitrogenase and NADH-GOGAT activity (Fig. 4). Nitrogenase activity was first detectable on d 10 following inoculation and increased dramatically from d 10 to d 20. NADH-GOGAT activity was detectable even in the youngest samples but increased about 27-fold in specific activity up to d 18 over a time course similar to the increase in nitrogenase activity. The proportion of NADH-GOGAT activity attributable to NADH-GOGAT I and NADH-GOGAT II was determined by separating the two isoenzymes by ion-exchange chromatography (Fig. 5). A 70 to 80% recovery of NADH-GOGAT activity was routinely obtained in these procedures. Uninoculated roots contained very low NADH-GOGAT activities (about 0.63 nmol min^{-1} mg $protein^{-1}$) and was comprised largely of NADH-GOGAT I. This isoenzyme was detectable in nodules throughout the development period and was the only isoenzyme present in the nodulating root systems at d 5. NADH-GOGAT II was detectable at d 8 (at a time when nodules were clearly visible) and its activity increased in proportion throughout nodule development and at d 20 was about twice as active as

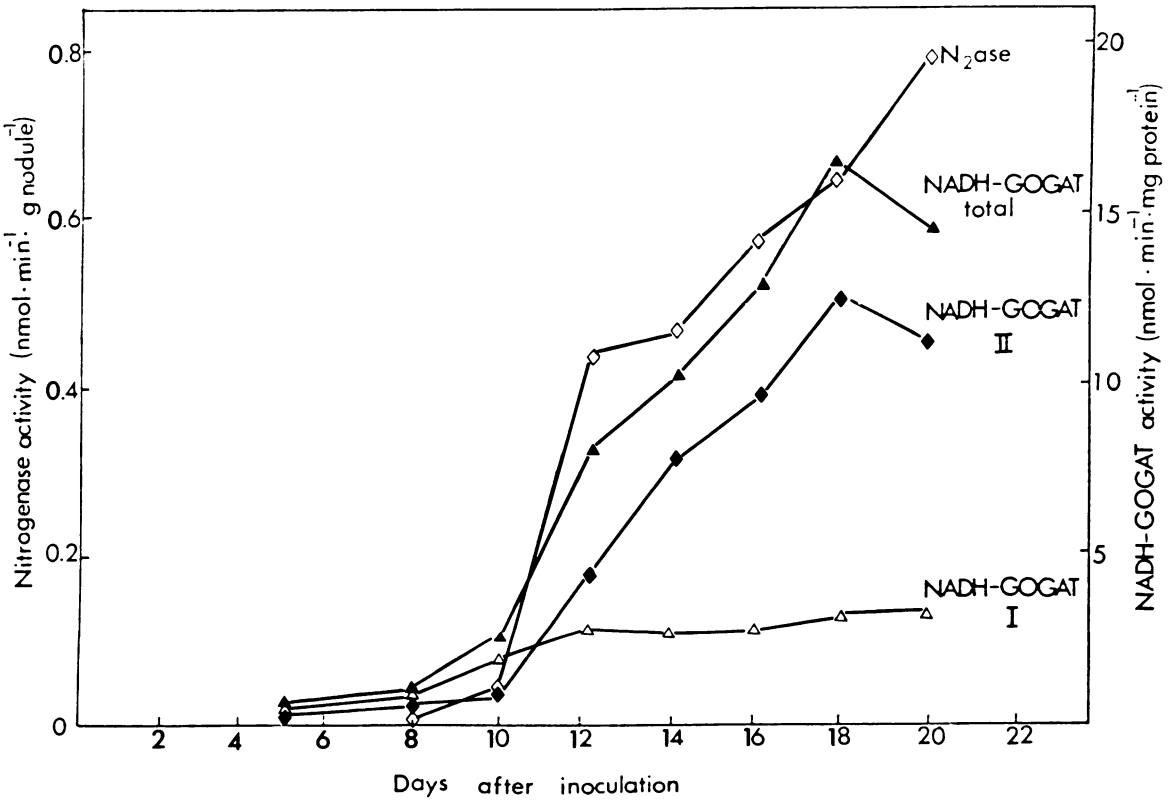

FIG. 4. Changes in the activities of nitrogenase and the two isoenzymes of NADH-GOGAT during nodule development. The total NADH-GOGAT specific activities and the relative proportions of the two isoenzymes determined in Figure 5, were used to calculate the separate specific activities of NADH-GOGAT I and NADH-GOGAT II at each time point.

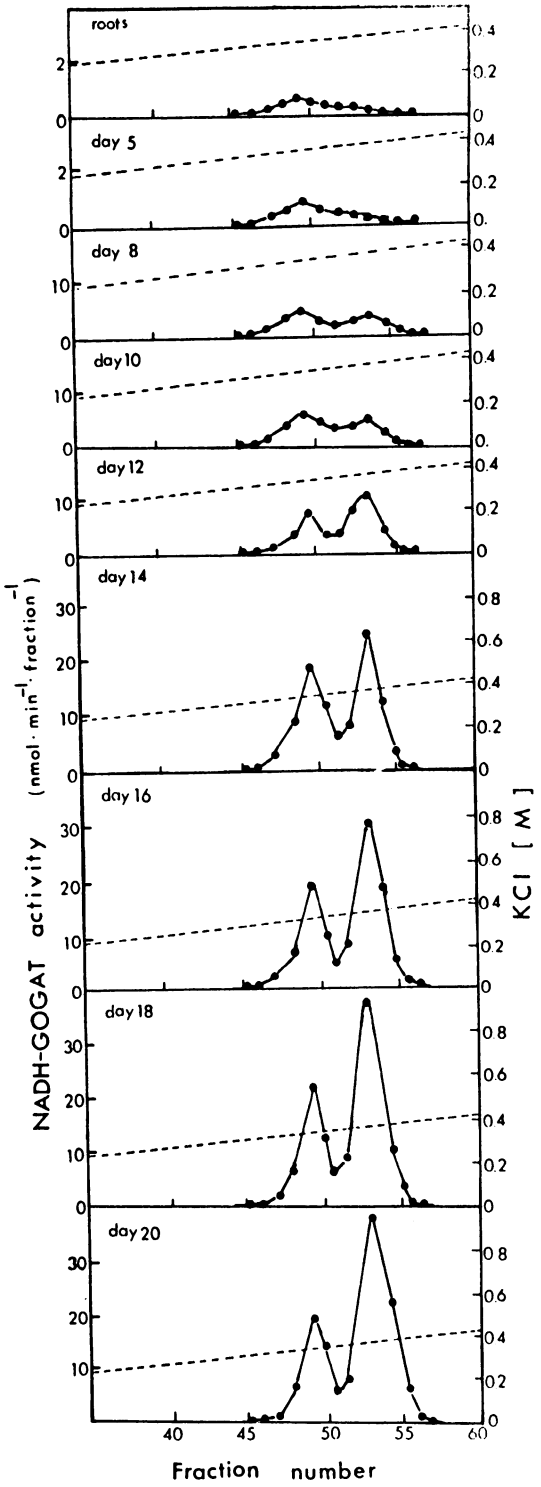

FIG. 5. Changes in the relative activities of NADH-GOGAT I and NADH-GOGAT II during nodule development, determined by activity elution profiles from an HPLC ion-exchange column.

NADH-GOGAT I. When the activities of each isoenzyme were expressed as specific activities (Fig. 4) it can be seen that NADH-GOGAT I activity increased about threefold until about d 14 and then remained constant. However, a much greater increase in specific activity was observed for NADH-GOGAT II and this isoenzyme accounted largely for the total increase in NADH-GOGAT activity during nodule development.

An examination of the protein profiles of nodules at different stages of development by SDS-polyacrylamide gel electrophoresis, revealed that the two most abundant nodule-specific proteins, leghaemoglobin and uricase, were both initially detectable at d 10 and then increased in abundance up to d 20 and hence they show a time course of appearance similar to nitrogenase and NADH-GOGAT II (data not shown).

DISCUSSION

In this paper we have shown that NADH-GOGAT occurs as two distinct isoenzymes in the plant fraction of root nodules of *Phaseolus vulgaris*. Evidence for this statement are (a) two NADH-GOGAT activity peaks can reproducibly be separated from crude nodule extracts by ion-exchange chromatography and as each peak reruns in its original position on the column (Fig. 1) it appears that the two activities are separate entities and are not interconvertible in cell-free extracts, (b) the kinetic properties of the two activities show slight differences (Table IV), and (c) the two activities are regulated differently in roots and during nodule development (Figs. 4 and 5). It is extremely unlikely that either of these isoenzymes arise from the bacteroid fraction of the nodule as care was taken not to extract the bacteroids, which pellet in the centrifugation of the crude nodule extracts (1, 27), and it has previously been shown that most activity of NADH-GOGAT in *P. vulgaris* nodules occurs in the plant cell cytoplasm and very little activity is associated with the bacteroids (1). Moreover, the structure and coenzyme specificities of the two isoenzymes are similar to other plant NADH-GOGATs and not to most bacterial GOGATs (26).

Studies on the two purified isoenzymes have shown that they have fairly similar kinetic and physical properties. Both isoenzymes are monomeric with M_rs of about 200,000, similar to the M_rs determined for NADH-GOGATs isolated from other higher plants and *Chlamydomonas* (26). A slight difference in the elution position of the two isoenzymes was observed from the HPLC gel filtration column (Fig. 2) but whether this reflects actual differences in their M_rs is uncertain; no difference could be detected in their positions on SDS-polyacrylamide gels (Fig. 3). A comparison of the kinetic characteristics of the two *P. vulgaris* NADH-GOGATs have revealed several differences, notably in their respective K_ms for L-glutamine, 2-oxoglutarate, and NADH and in their pH optima (Table IV). Generally, the values determined fall within the range of values obtained for the kinetic properties of other plant NADH-GOGATs (26).

The activities of both isoenzymes were found to be strongly dependent on NADH as reductant; only a very low activity could be supported by NADPH (Table III) and neither isoenzyme showed any activity with ferredoxin. The reductant specificities of these two isoenzymes are therefore similar to the specificities of other pyridine nucleotide dependent GOGATs that have been purified from higher plants and *Chlamydomonas* (7, 12–14, 21). Fd-GOGAT activity was also detected in *P. vulgaris* nodules but this activity was found to be over threefold lower than the NADH-GOGAT activity (Table I). This observation is in contrast to work on soybean nodules where the Fd-GOGAT activity has been shown to be twice as high as the NADH-GOGAT activity (27). However, soybean nodules grown under our conditions also have over a threefold lower Fd-GOGAT activity compared to the activity of NADH-GOGAT (data not shown).

During nodulation of *P. vulgaris* the specific activity of NADH-GOGAT has been shown to increase about 27-fold and from Figure 4 it can be seen that this increase is due largely to the production of NADH-GOGAT II. Moreover, in *P. vulgaris* the increase in activity of GS during nodulation occurs via the production of a nodule-specific isoenzyme (11). Thus, both enzymes of the ammonia-assimilatory glutamate synthase cycle in *P. vulgaris*, appear to be regulated to increase their activities in root nodules by the increase in activity of specific isoenzymes. For GS, the appearance of this isoenzyme has been shown to be

the result of the nodule-specific induction of one gene from the four-member GS multigene family in this species (6). Whether the appearance of NADH-GOGAT II during nodulation is also the result of a nodule-specific gene induction remains to be determined. At present, we are unsure whether NADH-GOGAT II is truly nodule-specific as the total NADH-GOGAT activity in roots and other organs appears to be very low and to be mainly composed of NADH-GOGAT I (Fig. 5); we cannot exclude the possibility, however, that NADH-GOGAT II is also present but at levels difficult to detect in our assays.

The presence of two NADH-GOGAT isoenzymes in root nodules poses the question of their physiological functions in this organ. Undoubtedly, the main role of GOGAT in nodules is to work in conjunction with GS to assimilate ammonia produced from dinitrogen fixation and released into the cytosol of infected cells. However, the glutamate synthase cycle may also be involved, in nodules, in assimilating ammonia produced from other cellular reactions and in other cell types and subcellular compartments. Clearly, studies on the cellular and subcellular localization of the two NADH-GOGAT isoenzymes in nodules will help to clarify their roles in this organ.

Whether the presence of two isoenzymes of NADH-GOGAT in root nodules is common to other legume species in addition to *P. vulgaris* has not yet been determined. The instability of the enzyme in cell-free extracts was most probably responsible for the unreliable reports on this enzyme in earlier papers (16), and we have found it essential to include thiol reagents and protease inhibitors in the buffers, and not to freeze the enzyme, in order to minimize these problems. It will be interesting to examine, for example, whether ureide and asparagine-transporting legumes differ in the possession of more than one nodule NADH-GOGAT. With GS there is no such clear division; *P. vulgaris* and *Medicago sativa* which transport ureides and asparagine, respectively, both possess a nodule-specifically expressed GS whereas soybean (a ureide-transporter) and pea (an asparagine-transporter) appear not to contain a GS gene regulated in this way (see Ref. 6, for a review).

Further studies are now in progress to study the regulation and localisation of the two NADH-GOGAT isoenzymes in *P. vulgaris*.

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