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Purification and Characterization of NADH-Glutamate Synthase from Alfalfa Root Nodules[1]

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ABSTRACT

Glutamate synthase (GOGAT), a key enzyme in the pathway for the assimilation of symbiotically fixed dinitrogen (N_2) into amino acids in alfalfa (*Medicago sativa* L.) root nodules, was purified and used to produce high titer polyclonal antibodies. Purification resulted in a 208-fold increase in specific activity to 13 micromole per minute per milligram of protein and an activity yield of 37%. Further purification to near homogeneity was achieved by fast protein liquid chromatography, but with substantial loss of activity. Enzymic activity was highly labile, losing 3% per hour even when substrates, stabilizers, and reducing agents were included in buffers. However, activity could be partially stabilized for up to 1 month by storing GOGAT at −80°C in 50% glycerol. The subunit molecular weight of GOGAT was estimated at 200 ± 7 kilodaltons with a native molecular weight of 235 ± 16 kilodaltons, which suggested that GOGAT is a monomer of unusually high molecular weight. The pI was estimated to be 6.6. The K_m values for glutamine, α-ketoglutarate, and NADH were 466, 33, and 4.2 micromolar, respectively. Antibodies were produced to NADH-GOGAT. Specificity of the antibodies was shown by immunotitration of GOGAT activity. Alfalfa nodule NADH-GOGAT antibodies cross-reacted with polypeptides of a similar molecular weight in a number of legume species. Western blots probed with anti-GOGAT showed that the high GOGAT activity of nodules as compared to roots was associated with increased levels of GOGAT polypeptides. Nodule NADH-GOGAT appeared to be highly expressed in effective nodules and little if any in other organs.

A substantial portion of alfalfa (*Medicago sativa* L.) nitrogen is derived through symbiotic N_2 fixation (26). In root nodules fixed N, in the form of NH_3, is exported from bacteroids into the host plant cytoplasm where further assimilation into amino acids occurs (17, 26). *In vitro* enzymic and $^{15}N_2$ labeling studies showed that the enzymes GS[2] (EC 6.3.1.2) and GOGAT (EC 1.4.1.14) catalyze the first two steps in NH_3 assimilation (9, 17, 26).

$$\text{Glu} + \text{NH}_3 + \text{ATP} \xrightarrow{\text{GS}} \text{Gln} + \text{ADP} + \text{Pi}$$

$$\text{Gln} + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GOGAT}} 2\ \text{Glu} + \text{NAD}$$

Similarly to GS, GOGAT activity increases several-fold during root nodule development (2, 9), and treatments that reduce N_2 fixation result in low GOGAT activity (9). In addition, nodule GOGAT activity of ineffectively nodulated plants is strikingly lower than that of effectively nodulated plants (27). While substantial effort has been expended on physiological, biochemical, and molecular regulation of root nodule GS (6, 28), comparable studies have not been undertaken for GOGAT.

GOGAT catalyzes the transfer of the amide N of Gln to the α-keto position of 2-oxoglutarate forming glutamate (1, 29). In higher plants GOGAT can occur as three forms that differ in mol wt, kinetics, and reductant specificity (20): (a) NADH-GOGAT, (b) NADPH-GOGAT (EC 1.4.1.13), and (c) ferredoxin (Fd)-GOGAT (EC 1.4.7.1). The Fd-dependent GOGATs range in mol wt from 125 to 180 kD, are usually localized in chloroplasts, and are involved in the assimilation of NH_3 derived from the light dependent reduction of NO_3^- and from photorespiration (20, 29). The Fd-GOGAT form has also been demonstrated in roots (20–22) and most recently in soybean (*Glycine max* L. Merr) nodules (23). The NAD(P)H-dependent GOGATs have been reported in roots (20), shoots (12, 22, 24), and nodules (2, 9) and ranged in mol wt from 200 to 240 kD. Antibodies have been produced to rice (*Oryza sativa* L.) (22) and maize (*Zea mays* L.) (21) leaf Fd-GOGAT. Monocot root Fd-GOGAT is antigenically quite different than the leaf enzyme, suggesting that they are distinct proteins (20–23). Antibodies to Fd-GOGAT generally do not recognize NAD(P)H-GOGATs, thus suggesting that these enzymes are also structurally distinct proteins. By contrast, antibodies to rice leaf Fd-GOGAT readily recognize soybean nodule Fd-GOGAT, implying that these two proteins are structurally related (23). Antibodies have not been produced to other GOGAT species.

In comparison to the Fd-GOGATs in photosynthetic tissue, the NAD(P)H-GOGATs in nongreen tissue have received less attention. The lack of reports on the characterization of GOGAT from roots or nodules may be due to the difficulty in maintaining *in vitro* activity in extracts of these organs.

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[2] Abbreviations: GS, glutamine synthetase; FPLC, fast protein liquid chromatography; GOGAT, glutamate synthase; HTP, hydroxylapatite; IEF, isoelectric focusing.

The only GOGAT to be purified and characterized from root nodules is the NADH-GOGAT of lupine (*Lupinus angustifolius* L.) (1, 3). The plant cytoplasmic nodule enzyme was purified 500-fold and was comprised of a single polypeptide with a mol wt of 235-kD. In view of the lack of information on nodule GOGAT, the unavailability of antibodies to the NADH form of the enzyme, and the crucial role of GOGAT in nodule and plant N metabolism, the objectives of this research were to: (a) purify plant NADH-dependent GOGAT from alfalfa root nodules, (b) produce polyclonal antibodies to the enzyme, and (c) characterize the mol wt and some basic kinetic parameters of the enzyme.

MATERIALS AND METHODS

Plant Materials

Alfalfa (*Medicago sativa* L. cv Saranac), birdsfoot trefoil (*Lotus corniculatus*, L. cv Norcen), adzuki bean (*Vigna angularis* Willd. Ohioi and Ohashi, cv Takara), soybean (*Glycine max* L. Merr. cv Hodson), pea (*Pisum sativum*, L. cv Alaska), Texas bluebonnet (*Lupinus subcarnosus* L. cv Hook), clover (*Trifolium pratense* L. cv Lakeland), common bean (*Phaseolus vulgaris* L.), broadbean (*Vicia faba* L. var. *major* (Alef.) cv Ipro), and horsebean (*V. faba* L. var *equina* (Pers.) cv Outlook) plants were grown in glasshouse in sand fertilized with macro- and micronutrients (except N) and watered daily. Plants were grown under natural light supplemented by fluorescent lamps with a 16/8 h day/night photoperiod and quantum flux of 400 μmol m^{-2} s^{-1} as previously described (9, 14, 27). Before flowering, plants were gently uprooted and washed free of sand. Nodules were manually removed from moistened roots, placed on ice, and stored at −80°C until needed.

Enzyme Purification

Frozen nodules (35 g) from approximately 700 plants were thawed and soluble protein was extracted by grinding nodules in a mortar and pestle using 175 mL of 20 mM K^+ phosphate buffer ($K_2HPO_4 \cdot KH_2PO_4$ (pH 7.6), containing 3 mM DTT. Particulate matter was removed by centrifugation at 19,000*g* for 30 min. The supernatant was applied to a 4 × 2 cm HTP column and GOGAT eluted with 100 mM K^+ phosphate buffer containing 3 mM DTT. Fractions showing GOGAT activity were pooled and treated with a saturated $(NH_4)_2SO_4$ solution. Protein precipitating between 35 and 50% saturation was pelleted by centrifugation and then resuspended in 8 mL of gel filtration buffer consisting of 200 mM K^+ phosphate buffer with 3 mM DTT. The protein was loaded onto a 2.6 × 90 cm Sephacryl S-300 gel filtration column and eluted at a flow rate of 30 mL/h. The most active fractions (80% of total activity) were pooled and dialyzed against 100 mM K^+ phosphate buffer with 3 mM DTT for 4 h. The dialysate (20 mL) was applied to a 2.0 × 25 cm Sephadex A-25 ion exchange column equilibrated in 100 mM K^+ phosphate buffer with 3 mM DTT and eluted with a 200 mL, 0 to 200 mM $(NH_4)_2SO_4$ linear gradient. The enzyme was further purified by loading the most active fractions after a twofold dilution onto a 5/5 HR FPLC Mono Q column (Pharmacia, Uppsala, Sweden) equilibrated in 30 mM Tris buffer at pH 8.0 and eluted with a 40 mL, 0 to 600 mM NaCl linear gradient at 1.0 mL/min. The enzyme was stored at −80°C after purification. All steps were carried out at 4°C except the separation with the mono Q column, which was performed at room temperature. Protein was estimated by the method of Smith *et al.* (19).

Enzyme Assays

Glutamate synthase activity was determined spectrophotometrically at room temperature by monitoring the oxidation of NADH at 340 nm (9). The assay buffer consisted of 100 mM K^+ phosphate buffer (pH 7.8), containing 0.1% 2-mercaptoethanol, 10% sucrose, 2.5 mM α-ketoglutarate, 1.0 mM NADH, 5 mM glutamine, and 1 mM aminooxyacetic acid.

Enzyme Kinetics

Kinetic measurements of nodule GOGAT were made on protein purified through gel filtration chromatography, stored in 50% glycerol, and frozen at −80°C. The K_ms for glutamine, α-ketoglutarate, and NADH were determined by assaying for GOGAT activity at various concentrations listed under the appropriate figure legend. One substrate concentration was varied while the others were kept at near saturation. Results were plotted in Lineweaver-Burke double reciprocal plots and K_ms were determined by linear regression.

Inhibitors were first screened at various concentrations and under near saturating substrate conditions (5 mM glutamine, 0.5 mM α-ketoglutarate, and 0.1 mM NADH). Further analysis on compounds showing greater than 20% inhibition ($P = 0.05$) was performed at the indicated inhibitor and substrate concentration. Data were plotted and analyzed using Lineweaver-Burke double reciprocal plots.

The pH optimum of GOGAT was established by assaying in buffers that could be titrated between pH 6 and 10: MES-NaOH (pKa 6.2), Mops (pKa 7.2), Tricine pKa 8.2), and glycine (pKa 9.8). Initial concentration of each buffer was 33 mM.

Electrophoresis

Purification progress and subunit mol wt were determined using Phast System SDS-PAGE 10 to 15% gradient gels (Pharmacia, Uppsala, Sweden). This system was useful due to its high sensitivity and small sample size requirement. The gradient gel was 0.45 mm thick with a 13 mm stacking zone and a 32 mm running zone. The stacking gel buffer at pH 6.4 contained 0.112 M acetate and 0.112 M Tris. The running buffer at pH 7.6 contained 0.20 M Tricine, 0.20M Tris, and 0.55% SDS. These gels were appropriate for SDS-treated proteins between 10 and 250 kD (SDS-PAGE Phast System Separation Techniques, File No. 110, 1986, Pharmacia, Uppsala, Sweden).

The isoelectric point of GOGAT was determined on Pharmacia Phast System IEF gels. The gels contained 5% polyacrylamide with 2% cross-linking and carrier ampholytes. Carrier ampholytes between pH 5 and 8 were prefocused before sample loading to establish a stable linear pH gradient.

Table I. *Typical Purification of Alfalfa Root Nodule NADH-GOGAT from 35 g of Nodules*

Data are representative of five separate purifications. All purifications were completed within 48 h.

Step	Total Protein	Total Activity	Specific Activity	Yield	Purification Factor[a]
	mg	*μmol·min⁻¹*	*μmol·min mg protein⁻¹*	%	*-fold increase*
Crude extract	331	21.5	0.063	100	1.0
Hydroxylapatite	118	26.8	0.227	124	3.6
$(NH_4)_2SO_4$ 35 to 50%	46	24.9	0.541	116	8.6
Gel filtration Sephacryl S-300	8.6	10.7	1.24	50	19.6
Ion exchange[b] Sephadex A-25	0.62	8.1	13.1	37	208.0

[a] The -fold increase in specific activity. [b] In some instances further purification of NADH-GOGAT was performed by FPLC Mono Q column. However, 75% of the remaining active GOGAT was lost during this step.

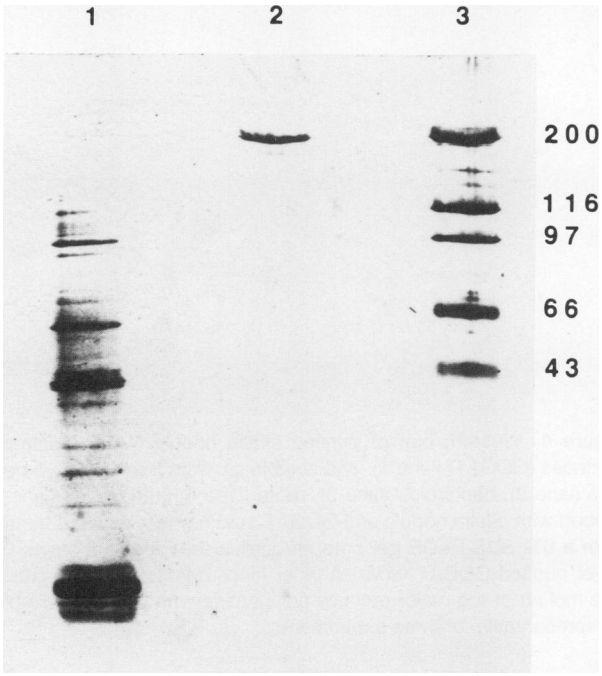

Figure 1. Purity and subunit mol wt of alfalfa nodule NADH-glutamate synthase (GOGAT) as determined on Pharmacia Phast System SDS-PAGE 10 to 15% gradient gels. Lane 1 contains 300 ng of nodule total soluble protein; lane 2 contains 34 ng of nodule NADH-GOGAT purified through FPLC; and lane 3 contains mol wt markers (myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase-b, 97 kD; bovine serum albumin, 66 kD; and ovalbumin, 43 kD). Protein was visualized by Ag staining. This gel is representative of three purifications.

Samples were loaded and run according to directions supplied by Pharmacia (Isoelectric Focusing and Electrophoresis Titration Curve Analysis, Phast System Techniques, File No. 100, 1986, Pharmacia, Uppsala, Sweden).

Activity Staining of Native PAGE

Native-PAGE was performed according to the procedure of Ornstein (15). Two 100 μL (6 μg) samples of purified active enzyme were loaded into separate lanes of a native-PAGE gel and run at 16 mA for 3 h at 4°C. After electrophoresis, one lane was stained for GOGAT activity according to Matoh *et al.* (11, 12), and the other lane was stained with Ag for protein according to Merrill *et al.* (13). The lane for activity stain was removed and equilibrated in 200 mM K^+ phosphate buffer (pH 7.5) for 30 min. Substrates were provided by incubating the gel strip in 100 mM K^+ phosphate buffer (pH 7.5), containing 30 mM NADH, 15 mM α-ketoglutarate, and 15 mM glutamine for 60 min at room temperature. The gel was rinsed three times in 100 mM Tris-HCl (pH 8.5), and incubated in a staining solution containing 50 mL of 50 mM Tris-HCl buffer (pH 8.5), 250 mg nitroblue tetrazolium (Bio Rad, Richmond, CA), and 30 mg phenazine methosulfate (Sigma, St. Louis, MO). A control without glutamine did not show activity, Glutamate synthase activity was indicated by negative staining (a clear band on a purple gel) due to the presence of NAD^+.

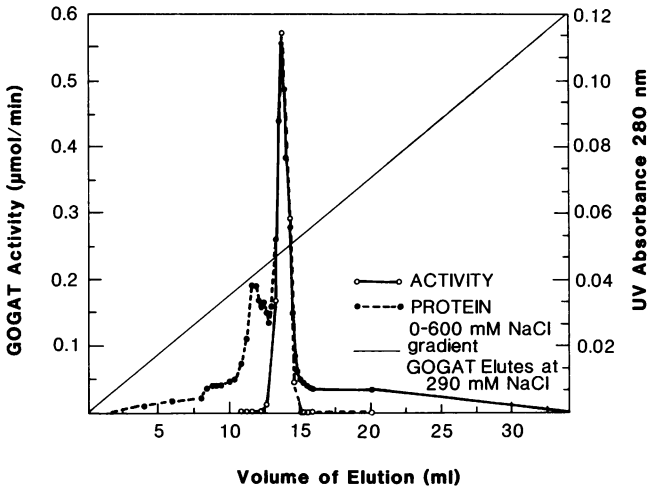

Figure 2. Elution profile of alfalfa nodule NADH-glutamate synthase (GOGAT) activity (○——○) and protein (●---●) from a FPLC 5/5 HR Mono Q column equilibrated in 30 mM TRIS-HCl buffer (pH 7.6), and eluted with a 0 to 600 mM NaCl (——) gradient. Glutamate synthase activity eluted at 290 mM NaCl. Protein was determined by μV absorption at 280 nm and NADH-GOGAT activity was monitored by the disappearance of NADH at 340 nm. Elution profiles are representative of three experiments.

Mol Wt and Isoelectric Point Determination

Native mol wt of GOGAT was determined on a 2.6 by 90 cm Sephacryl S-300 gel filtration column equilibrated in 50

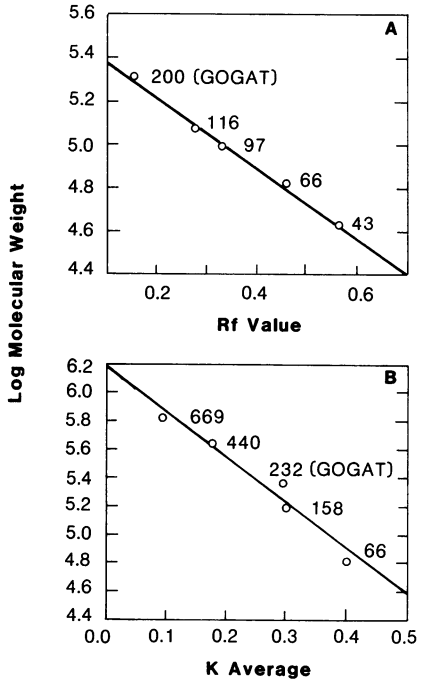

Figure 3. Subunit and native mol wt of alfalfa nodule NADH-glutamate synthase (GOGAT). A, A subunit mol wt of 200 ± 7 kD was determined by regression analysis (R^2 = 0.99) against SDS-PAGE-separated protein standards (myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase-B, 97 kD; bovine serum albumin, 66 kD; ovalbumin, 43 kD). B, A native mol wt of 235 ± 16 kD was determined by regression analysis (R^2 = 0.96) against the elution profiles of standard proteins from a Sephacryl S-300 gel filtration column (standards = thyroglobin, 669 kD; ferritin, 440 kD; catalase, 232 kD; aldolase, 158 kD; bovine serum albumin, 66 kD). Arrows point to location of alfalfa nodule NADH-GOGAT. Results are the average of five experiments.

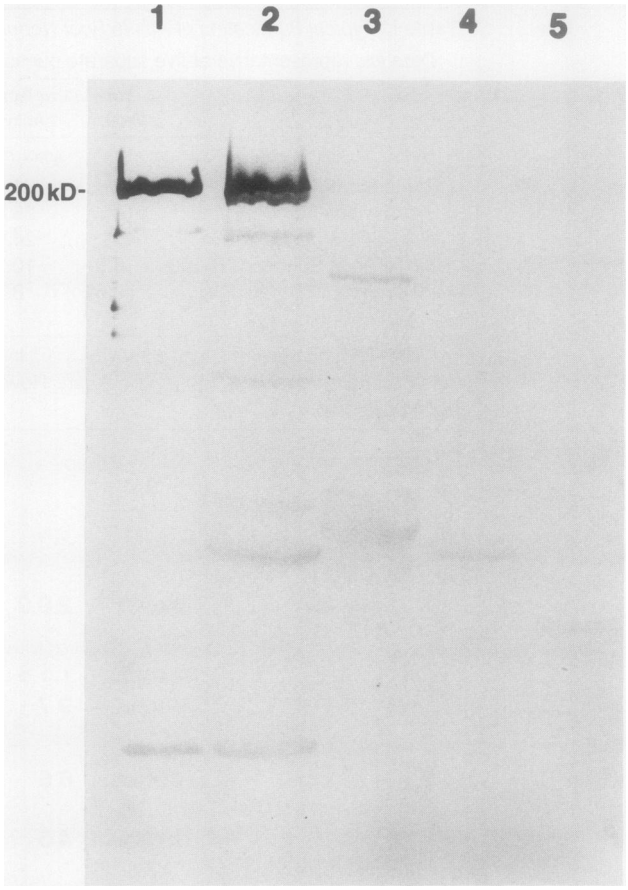

Figure 4. Western blot of purified alfalfa nodule NADH-glutamate synthase (GOGAT) (lane 1), and soluble proteins from effective nodules (lane 2), bacteroids (lane 3), roots (lane 4), and leaves (lane 5) probed with alfalfa nodule anti-GOGAT serum. Proteins were blotted from a 6% SDS-PAGE gel onto nitrocellulose. Lane 1 contains 0.5 μg of purified GOGAT while all other lanes contain 200 μg protein. The mol wt of the major reactive polypeptide was 200 kD. This blot is representative of three experiments.

mM K^+ phosphate buffer, 150 mM NaCl, 3 mM DTT and eluted at a flow rate of 20 mL/h. The column was calibrated with mol wt standards (Pharmacia) (thyroglobin, 669 kD; ferritin, 440 kD; catalase, 232 kD; aldolase, 158 kD; bovine serum albumin, 66 kD). The K_{ave} method with linear regression was used to determine mol wt (7, 18).

Subunit mol wt was determined by SDS-PAGE (10). Purified GOGAT (1 μL, 34 ng), crude nodule protein (1 μL, 300 ng), and mol wt markers (myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase-b, 97 kD; bovine serum albumin, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 31 kD; trypsin inhibitor, 21 kD; and hen lysozyme, 14 kD) were treated (1:1, v:v) with Pharmacia Phast System sample buffer at pH 8.0 (20 mM Tris, 2 mM EDTA, 5.0% SDS, 10% 2-mercaptoethanol), heated at 100°C for 2 min and loaded onto a 10 to 15% gradient SDS gel. The gel was processed and Ag stained (13).

Purified protein (1 μL, 34 ng) and a mixture of protein standards (Bio Rad, Richmond, CA) (equine myoglobin, pI 7.0; human carbonic anhydrase, pI 6.5; bovine carbonic anhydrase, pI 6.0) were loaded into separate lanes and run according to directions supplied by Pharmacia. The pI for GOGAT was determined by linear regression.

Antibody Production and Specificity

Polyclonal antibodies to nodule NADH-dependent GOGAT were produced by injecting 500 μg of SDS-PAGE purified enzyme in PBS at pH 7.2 emulsified in Freund's complete adjuvant into a New Zealand White Rabbit. Subsequently, boosts of 200 μg of purified GOGAT with Freund's incomplete adjuvant were injected into the rabbit every 2 weeks for 6 weeks. Serum was obtained from four bleedings 6 to 12 weeks after the initial injection and the IgG fraction was purified by double $(NH_4)_2SO_4$ precipitation between 0 and 40% saturation. Antibody IgGs were obtained by Protein A Sepharose chromatography.

Antibody specificity was determined by Western blotting against SDS treated GOGAT. Crude nodule extracts in SDS-PAGE sample buffer were loaded onto the 0.75 mm 6% polyacrylamide slab gel and electrophoresed at 16 mA for 4 h with constant cooling according to the method of Maizel (10). After electrophoresis, proteins were transferred to nitrocellulose blots according to the procedure of Towbin *et al.* (25). Protein on blots was reacted with anti-GOGAT and visualized with goat anti-rabbit conjugated peroxidase. Immunotitration of GOGAT activity was performed as described by Miller *et al.* (14).

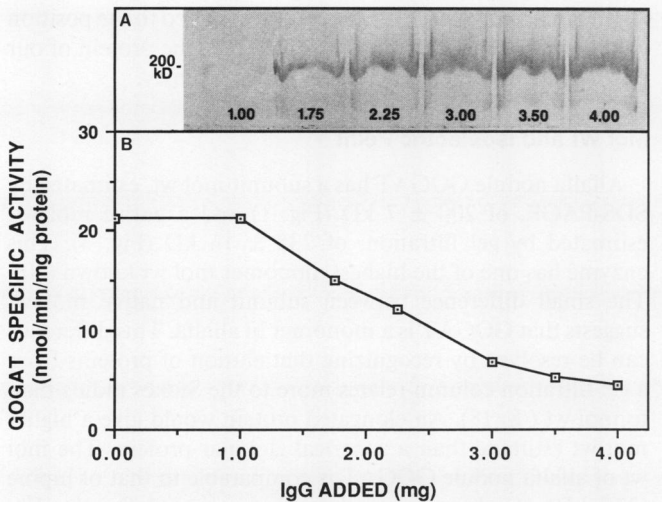

Figure 5. Immunotitration of alfalfa nodule NADH-GOGAT activity using anti-alfalfa GOGAT IgGs: A, Western blot of immune complexes; B, immunotitration curve of nodule NADH-GOGAT activity. A constant quantity of protein (485 μg) was incubated with Protein A purified anti-GOGAT IgGs (1000–4000 μg). Goat anti-rabbit IgGs (120 μg) were added to each reaction to precipitate the immune complexes and the GOGAT activity remaining in the supernatant after centrifugation was assayed spectrophotometrically. Precipitated immune complexes were dissolved in SDS sample buffer, electrophoresed in an SDS-PAGE system (10), then blotted to nitrocellulose. The Western blot was probed with anti-GOGAT serum. The mol wt of the major reactive immunoprecipitated polypeptide was 200 kD. The number beneath the lanes refers to quantity of immune IgG(mg) added. Purified preimmune IgGs were incubated with nodule protein as a control. Preimmune IgGs did not reduce GOGAT activity. The figure is representative of three experiments.

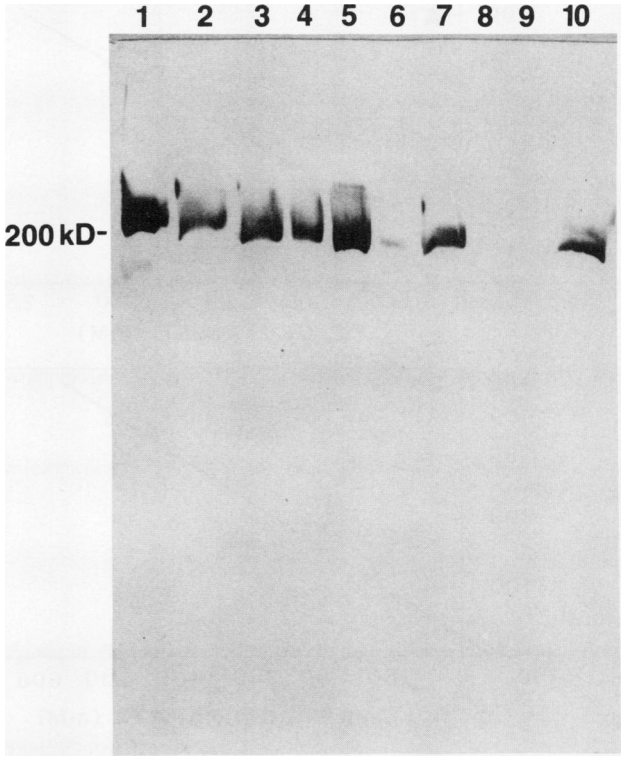

Figure 6. Cross-reactivity of alfalfa nodule anti-GOGAT serum with effective nodule polypeptides of various legume species. Nodule soluble proteins were electrophoresed in SDS-PAGE and denatured polypeptides were transferred to nitrocellulose, then the blot was probed with alfalfa nodule anti-GOGAT serum. Each lane contained 100 μg of protein. Lane designations are: (1) *Medicago*, alfalfa; (2) *Pisum*, pea; (3) *Trifolium*, clover; (4) *Lotus*, trefoil; (5) *Lupinus*, Texas bluebonnet; (6) *Glycine*, soybean; (7) *Phaseolus*, field bean; (8) *Vigna*, adzuki bean; (9) *Vicia*, broadbean; (10) *Vicia*, horsebean. Mol wt is indicated in kD. Enzyme activity for GOGAT is given in text. This blot is representative of three experiments.

Protein Extraction

Nodule protein for Western blots and enzyme assays was extracted as previously described (9). Leaf protein was obtained by grinding leaves (1:4 w/v) in 30 mM Tris-NaOH and Mes (pH 7.5) containing 0.5 M sucrose, 5 mM EDTA, and 5 mM DTT. Particulate matter was removed by centrifugation for 30 min at 15,000*g*. Bacteroid protein was extracted according to Robertson *et al.* (16). Protein was estimated by the bicinchoninic acid method after TCA precipitation of protein to remove interfering substances (19).

RESULTS AND DISCUSSION

Purification of GOGAT

GOGAT was purified 208-fold after ion exchange chromatography to a specific activity of 13.1 μmol · min^{-1} · mg protein^{-1} with a final yield of 37% (Table I). The HTP column removed leghemoglobin and other proteins resulting in a 3.6-fold purification, while also concentrating the crude preparation of enzyme. Small HTP columns were used for concentrating GOGAT whenever necessary. Ammonium sulfate precipitation of proteins and gel filtration chromatography resulted in a further 5-fold increase in specific activity. Ion exchange chromatography gave the highest purification, with a further 11-fold increase in specific activity. The 208-fold increase in specific activity suggests that GOGAT must constitute no more than 0.5% of the nodule total soluble protein.

Purification to near homogeneity was achieved with FPLC Mono Q column chromatography (Fig. 1). The preparation was estimated to be 95% pure given that the detection limit of the Phast System Ag stain is 0.5 ng and 34 ng of GOGAT was loaded onto the gel. Further increases in enzyme specific activity were not obtained due to substantial loss of activity on the FPLC Mono Q column. The activity loss may have been due to the use of Tris buffer since K^+ phosphate buffer is incompatible with the Mono Q column at pH 7.6.

In contrast to the stability of lupine nodule GOGAT activity in simple K^+ phosphate buffer (1), alfalfa nodule GOGAT activity declined 3% · h^{-1} in the crude extract and 8% · h^{-1} after gel filtration despite attempts to stabilize the enzyme with reducing compounds (3 mM DTT, 1% 2-mercaptoethanol), substrates, protease inhibitors (PMSF, antipain), and stabilizers (20% ethylene glycol and 10% sucrose). Partial stability, for up to 1 month, was eventually achieved by storage at −80°C in 50% glycerol, 100 mM K^+ phosphate, and 3 mM DTT. All purifications were effected within 48 h because of enzyme instability.

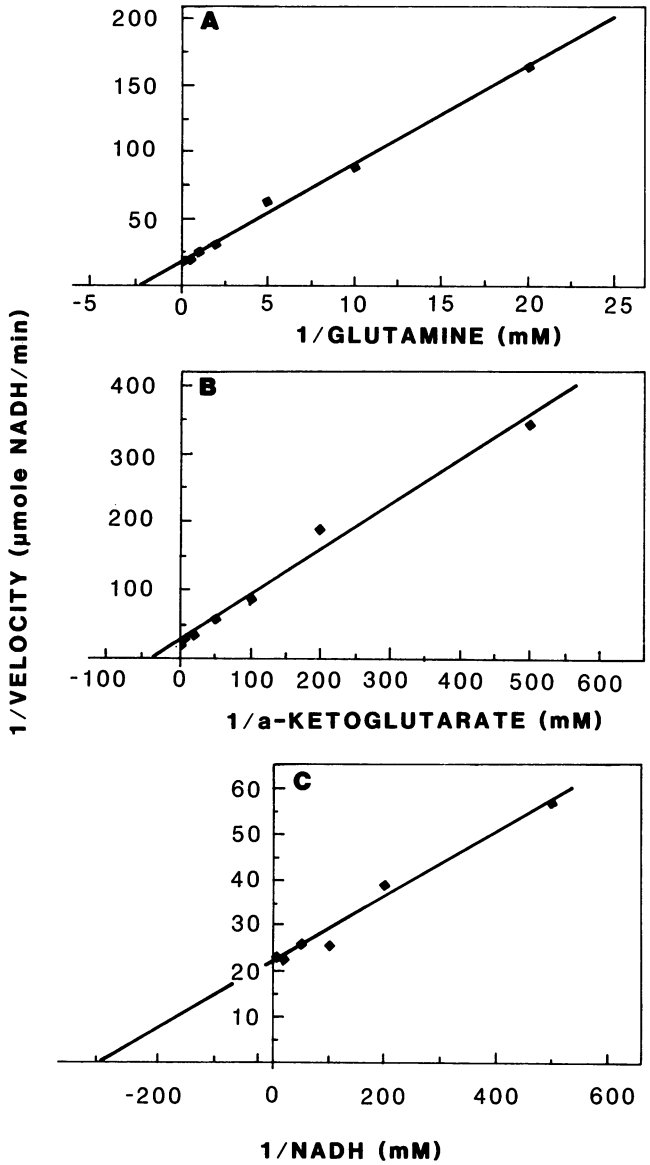

Figure 7. Lineweaver-Burke plots for apparent K_m determination of alfalfa nodule NADH-glutamate synthase (GOGAT): A, glutamine, 466 ± 91 μM; B, α-ketoglutarate, 33 ± 8 μM; and C, NADH, 4.2 ± 0.49 μM. Glutamate synthase was assayed as described in "Materials and Methods." Concentrations of substrates measured are noted in figures. All other substrates were kept at saturating concentrations (2.5 mM α-ketoglutarate, 5 mM glutamine, 1 mM NADH). Regression analysis was used to determine K_m and V_{max} (R^2 = 0.96). Plots are the average of four experiments.

GOGAT Identification

GOGAT preparations passed through ion exchange chromatography and FPLC showed a single Ag-stained protein band after SDS-PAGE on 10 to 15% gradient gels (Fig. 1). The FPLC elution profile (Fig. 2) showed one protein peak coincident with the peak for GOGAT activity, indicating that the major component of the fraction was GOGAT. Moreover, a native gel loaded with active GOGAT purified through ion exchange chromatography showed a major protein band when Ag stained, and GOGAT activity corresponded to the position of the major band (data not shown). Thus, the protein of our purification scheme was GOGAT of high purity.

Mol Wt and Isoelectric Point

Alfalfa nodule GOGAT has a subunit mol wt, estimated by SDS-PAGE, of 200 ± 7 kD (Fig. 1) and a native mol wt, estimated by gel filtration, of 235 ± 16 kD (Fig. 3). This enzyme has one of the highest monomer mol wt known (20). The small difference between subunit and native mol wt suggests that GOGAT is a monomer in alfalfa. This difference can be resolved by recognizing that elution of proteins from a gel filtration column relates more to the Stokes radius than to mol wt (8, 18). An elongated protein would give a higher mol wt estimate than a spherical globular protein. The mol wt of alfalfa nodule GOGAT is comparable to that of lupine (225 kD) (1), but higher than that reported for the Fd-dependent GOGATs from maize and spinach leaves and soybean nodules (145–180 kD) (11, 23, 24).

Alfalfa nodule GOGAT has an isoelectric point of 6.6 substantially higher than the 4.2 reported for maize leaf Fd-dependent GOGAT (11). Our mol wt and isoelectric point determinations for alfalfa nodule NADH-GOGAT support previous suggestions (20) that Fd-GOGATs and NADH-GOGATs are distinct proteins.

Antibody Production

Specific high titer polyclonal antibodies were produced against purified alfalfa nodule NADH-GOGAT (Fig. 4). A major reactive band was seen on Western blots of SDS-PAGE separations of crude nodule protein that corresponded in mol wt (200 kD) to purified GOGAT. A comparable 200 kD polypeptide was not seen in crude soluble protein fractions from roots, leaves, or bacteroids even when 200 μg of protein was loaded on the blot. In addition, the staining intensity of polypeptides on Western blots directly reflected the NADH-GOGAT activity of 0.820 ± 0.06 and 0.008 ± 0.002 μmol · min g fresh weight^{-1} for nodules and roots, respectively. The data indicate that alfalfa nodule NADH-GOGAT may be nodule-specific and that a comparable protein is either not expressed or occurs at much reduced levels in roots and leaves. Thus, alfalfa nodule GOGAT represents the third enzyme of N assimilation in nodules that may be nodule-specific. Previously, *Phaseolus* nodule GS and soybean nodule uricase were shown to be nodule specific (6, 28).

Alfalfa nodule GOGAT antibodies showed only faint nonspecific reaction with bacteroid proteins, further confirming that the alfalfa nodule NADH-GOGAT that we purified was of plant origin. These data also provide additional support for bacteroid- and nodule-GOGAT being two distinct enzymes.

The lack of comparable polypeptides in leaves and roots may indicate that Fd-GOGAT is the active GOGAT species in these organs. The major form of GOGAT in leaves of spinach, maize, and rice is Fd-dependent (20). In addition, antibodies to Fd-GOGAT generally do not recognize NADH-GOGAT (21, 23). Lack of recognition of a leaf polypeptide by our antibodies further supports the immunological distinctiveness of NADH- and Fd-GOGATs.

Table II. *Inhibition of Alfalfa Nodule NADH-GOGAT by Azaserine, Malate, Citrate, Glutamate, and NAD+*

Inhibitor	Inhibitor Concentration	Substrate	Substrate Concentration	Inhibition range
	mM		μM	%
Azaserine	2–10	All[a]	Saturating[b]	80 to 100[c]
Malate	40	α-Ketoglutarate	20–40	44 to 51
Citrate	40	α-Ketoglutarate	20–40	31 to 43
Glutamate	1–12	α-Ketoglutarate	13–100	−9 to 66
NAD+	3–12	α-Ketoglutarate	13–100	14 to 34
NAD+	3–12	Glutamine	500–2000	−6 to 42
NAD+	3–12	NADH	2.5–10	69 to 81

[a] Assays run in the presence of saturation conditions for all substrates. [b] Saturating substrate concentrations (1 mM α-ketoglutarate; 10 mM glutamine; 1 mM NADH) were utilized unless otherwise stated. [c] All inhibition range values are the average of three determinations.

The antiserum showed a slight reaction with a few bands other than GOGAT in highly loaded (200 μg crude protein) Western blots of all samples tested. Those bands probably reflect nonspecific staining since they were not apparent when lower amounts of protein were probed and blots were washed more extensively.

Further support for the specificity of our antibodies was demonstrated by immunotitration of 80 ± 5% of the *in vitro* GOGAT activity of nodule crude extracts through addition of anti-GOGAT IgGs (Fig. 5). Electrophoresis (SDS-PAGE) of the immunoprecipitate followed by Western blotting using anti-GOGAT serum as a probe showed that as GOGAT activity was immunoprecipitated, increasing quantities of a 200 kD polypeptide were precipitated out of the nodule extract. The lack of complete or proportional inhibition by our antiserum was unexpected and may reflect either one or both of the following: (a) the presence of another form of NADH-GOGAT that is antigenically different than the GOGAT we purified and (b) that our antibodies were raised against the SDS-denatured form of the enzyme thus resulting in incomplete recognition of the large monomeric native form of the enzyme.

Antibodies to alfalfa nodule NADH-GOGAT cross-reacted with a 200 kD polypeptide in a number of legumes (Fig. 6). The intensity of the cross-reactive band in general reflected the specific activity (μmol · min g fresh weight^{-1} of nodule NADH-GOGAT: lane 1, *Medicago*, 0.900; lane 2, *Pisum*, 0.668; lane 3, *Trifolium*, 0.934; lane 4, *Lotus*, 0.137; lane 5, *Lupinus*, 1.376; lane 6, *Glycine*, 0.290; lane 7, *Phaseolus*, 0.217; lane 8, *Vigna*, 0.113; lane 9, *Vicia faba*—broadbean, 0; lane 10, *Vicia faba*—horsebean, 0.242. Cross-reaction occurred with both amide and ureide transporting species. The lack of a cross-reactive band in *Glycine* and *Vigna* may reflect instability of the protein during the extraction process and electrophoresis and/or too little protein loaded on the gel. The lack of a cross-reactive band in these species could also reflect an alternate form of GOGAT. Soybean nodules have a high Fd-GOGAT as compared to NADH-GOGAT (23). Alfalfa nodules are also reported to have Fd-GOGAT activity (A Suzuki, personal communication). Cross-reaction of alfalfa nodule NADH-GOGAT antibodies with a polypeptide of comparable mol wt from other legume nodules is consistent with the cross-reaction of *Phaseolus* nodule GS (5) and alfalfa nodule phosphoenol-pyruvate carboxylase (PEPC) antibodies (14) with other legume GS and PEPC polypeptides.

Enzyme Characteristics

Alfalfa nodule GOGAT has an apparent K_m of 466 ± 91 M for glutamine, 33 ± 8 μM for α-ketoglutarate, and 4.2 ± 0.5 μM for NADH (Fig. 7). These values agree quite closely with those found for lupine nodule GOGAT (1). Low cofactor K_ms have been demonstrated in Fd-GOGATs from soybean nodules and rice leaves (K_ms 1.3–7.0 μM) (20–23).

Alfalfa GOGAT was inhibited by malate, citrate, glutamate, NAD^+, and azaserine (Table II). Azaserine (a glutamine antagonist) completely inhibited GOGAT activity at 10 mM. Inhibition by high concentrations (40 mM) of citrate and malate supports the involvement of both terminal carboxylic acids for binding of α-ketoglutarate, similar to lupine (4). The enzyme was not inhibited by 2-2′ dipyridil and *N*-ethylmaleimide suggesting that neither Fe, sulfhydryl, nor lysine residues are involved in catalysis (7). Alfalfa nodule GOGAT showed a pH optimum between 7.5 and 8.5. Below pH 7 and above pH 9 activity declined rapidly (data not shown).

Recently, two NADH-GOGATs were demonstrated in root nodules of *Phaseolus vulgaris* (6; and FL Chen, JV Cullimore, personal communication). The *Phaseolus* GOGATs were purified approximately 100-fold and had physical and kinetic characteristics similar to those reported here for alfalfa and those previously reported for lupine (1, 3). Traces of activity for both forms were found in roots. No antibodies were produced to the *Phaseolus* GOGATs. Our antibodies cross-reacted with a 200 kD *Phaseolus* band (Fig. 7), indicating antigenic similarity between alfalfa nodule GOGAT and a *Phaseolus* polypeptide. In support of our results, FL Chen and JV Cullimore (personal communication) also were unable to detect a second form of NADH-GOGAT in alfalfa nodules. The inability to detect two NADH-GOGATs in alfalfa and the presence of high Fd-GOGAT activity in soybean nodules (23) indicate that the expression of root nodule GOGAT, similar to GS, varies between and within species.

In summary, NADH-dependent GOGAT was purified to near homogeneity, high titer polyclonal antibodies were produced, and GOGAT was characterized with respect to mol

wt, isoelectric point and K_ms for three substrates. The antibodies may be useful for further characterization of nodule GOGAT with respect to intracellular localization, and polypeptide expression throughout plant and nodule development. Moreover, the antibodies may prove useful in isolation of the GOGAT gene and in studying molecular regulation of GOGAT throughout development of the symbiosis.

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