Partial purification and characterization of NADH-glutamate synthase from faba bean (Vicia faba) root nodules

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Abstract

The NADH-dependent glutamate synthase (EC 1.4.1.14) from the plant fraction of N 2-fixing faba bean (Vicia faba) nodules has been purified 74-fold to a specific activity of about 3 μmol min −1 mg protein −1 with a final yield of 32%. The NADH-GOGAT activity was associated with a single form of the enzyme that behaved as a monomeric protein with a subunit molecular weight of 195 kDa and a native molecular weight from 222 to 236 kDa estimated by gel filtration or PAGE, respectively. The NADH-GOGAT band on SDS-PAGE was cut out and used to produce antibodies. Western blots of SDS-PAGE of crude nodule proteins revealed a 195 kDa polypeptide in root extracts but not in those of leaves or bacteroids. The antiserum also cross-reacted with a polypeptide of comparable molecular weight (195 kDa) from both amide and ureide transporting species legume nodules, indicating that some antigenic epitopes have been conserved between nodule NADH-GOGAT of different species. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

In higher plants, ammonia is assimilated into organic form primarily via the glutamate synthase cycle, which is catalysed by GS and GOGAT [1]. Dinitrogen fixation by rhizobia in the root nodules of legumes generates ammonia which, after its transport to the plant cytosol, is also processed by the GS/GOGAT pathway [2]. Glutamine synthetase catalyses the incorporation of ammonia into the amide position of glutamate to yield glutamine, and GOGAT catalyzes the reductive transfer of the amide-nitrogen of glutamine to the α-keto position of 2-oxoglutarate, thereby forming two molecules of glutamate [3,4]. The glutamine and glutamate produced in the root nodules are then used to synthesize the nitrogen-transport compounds, asparagine (in temperate legumes) and ureides (in tropical legumes).

Three types of GOGAT activity have been detected in green and non-green plant tissues, one dependent on ferredoxin as the electron donor (Fd-GOGAT; E.C. 1.4.7.1), another using NADPH (NADPH-GOGAT) and yet another using NADH (NADH-GOGAT). Ferredoxin-GOGAT is involved in the assimilation of ammonia derived from the light-dependent reduction of nitrate and from photosynthesis [1,5,6]. The presence of Fd-GOGAT has also been demonstrated in soybean roots and nodules [5], where its physiological role is not well understood. Ferredoxin-GOGAT is restricted to higher plants,
algae and cyanobacteria, while NADPH- and NADH-dependent forms are found in plants, bacteria and fungi. There is strong physicochemical, immunological and genetic evidence that the NAD(P)H-dependent and Fd-dependent activities are catalysed by different proteins [7,8]. However, it is unclear whether the NADH- and NADPH-dependent GOGAT enzyme activities are a result of the presence of various proteins or a single protein that can utilize either of the pyridine nucleotide cofactors. Yelton and Yoch [9] suggest that the NADPH-dependent enzyme is found in bacteria, while the NADH-dependent would be found in plants. Like the NAD(P)H-dependent enzymes, Fd-GOGAT is a flavoprotein containing non-heme iron and acid-labile sulphur. In molecular structure, however, the enzymes differ markedly. While Fd-GOGAT is a monomeric enzyme of 130–180 kDa, NADH-GOGAT in Neurospora and in plants is a monomeric enzyme of around 230 kDa, and the bacterial NADPH-dependent form is an aggregate of four catalytically active dimers each composed of a heavy subunit (135–175 kDa) and a light subunit (51–55 kDa) [7,8].

NADH-GOGAT has been purified from soybean cell cultures [10], etiolated pea shoots [11], Chlamydomonas [12] and from root nodules of lupin [3], common bean [13,14], and alfalfa [15] but only Anderson et al. [15] obtained antibodies against this enzyme.

In view of the crucial role of GOGAT in nodule and plant nitrogen metabolism, this research was designed to characterize the NADH-dependent GOGAT from faba bean nodules. The aim of this work was to purify plant NADH-GOGAT from faba bean root nodules and to produce polyclonal antibodies using this enzyme. In addition, we examined the antigenic similarities, with respect to NADH-GOGAT, between different faba bean genotypes as well as between other legume species. Finally, we determined the molecular weight of NADH-GOGAT.

2. Materials and methods

2.1. Plant material and inoculation

Five inbred lines (VF51, VF61, VF52, VF60, and VF184) of V. faba L. selected by Dr. A. Martin (CSIC, Córdoba, Spain) and the commercial cv. Alborea (Semillas Pacifico S.A., Sevilla, Spain) were used in this study. Other legumes used included Vicia sativa L., Phaseolus vulgaris L., Medicago sativa L., Pisum sativum L. and Glycine max L. Merr. Plants were grown in controlled environmental chambers under a 16/8 h light/dark cycle, 23/16°C day/night temperature, and a relative humidity of 55–75%. The photosynthetic photon flux density (400–700 nm) was 450 μmol m⁻² s⁻¹, supplied by Sylvania Cool-White Life-line fluorescent lamps (F96T12-CW-VHO, Sylvania, Que., Canada) and incandescent lamps (30% fluorescent wattage).

Seedlings were planted in sterile 1 l Leonard jars with vermiculite and a nutrient solution [16] containing 2 mM KNO₃ [17]. Nodules were formed by inoculating seedlings with a culture of the appropriate rhizobium (10⁹ cells/seedling) grown in solid medium [18]. Inoculations were as follows: V. faba; V. sativa and P. sativum seedlings were inoculated with Rhizobium leguminosarum biovar viciae GRA19; M. sativa was inoculated with R. meliloti GR4B; P. vulgaris with R. etli GR12; G. max with Bradyrhizobium japonicum USDA 110. Nodules, young portions of roots and leaves were harvested before flowering, frozen in liquid nitrogen and stored at −80°C.

2.2. Partial purification of NADH-GOGAT

All purification steps were performed at 4°C.

1. Crude Extract. Frozen root nodules (50 g) of faba bean cv. Alborea plants were homogenized in a Sorval Omni Mixer using six pulses of 30 s at maximum speed in the presence of 250 ml of extraction buffer (20 mM potassium phosphate buffer at pH 7.5 containing 0.5% (v/v) 2-mercaptoethanol and 1 mM PMSF). The brei was filtered through two layers of muslin, and centrifuged twice at 20000 × g for 30 min, producing a clear solution which was used for stepwise ammonium sulphate precipitation. The pellet, precipitated by 30–60% saturation with ammonium sulphate, was redissolved in 10 ml of running buffer [50 mM potassium phosphate at pH 7.5, containing 0.5% (v/v) 2-mercaptoethanol], and dialysed in the same buffer.

2. Anion-exchange chromatography. The extract was loaded onto a 2.5 × 22 cm DEAE-Sephacel column previously equilibrated with run-
ning buffer. After washing overnight with 500 ml of the same buffer containing 0.1 M KCl, was eluted at a flow rate of 40 ml h⁻¹ with 800 ml of a linear gradient of 0.1–0.4 M KCl in running buffer. Fractions of 4.35 ml were collected, placed on ice, and then assayed for NADH-GOGAT. Fractions (54–65) containing NADH-GOGAT activity were pooled and concentrated by ultrafiltration in Centricon-30 vials (Amicon).

3. Gel filtration on Sephacryl S-300. The partially purified extract was then loaded onto a 1.5 × 38 cm Sephacryl S-300 column pre-equilibrated with running buffer. Proteins were eluted with the same buffer at a flow of 15 ml h⁻¹ and fractions of 3 ml were collected. Fractions containing NADH-GOGAT activity were pooled, concentrated as described above, and stored at −80°C in 50% glycerol.

2.3. Chromatography on Q-Sepharose column

After chromatography of crude extract on DEAE-Sepharose (2.6 × 14.5 cm) as described for DEAE-Sepahel, fractions with the highest NADH-GOGAT activity were pooled, concentrated and dialysed. The dialysed sample was loaded on a Q-Sepharose ‘Fast-Flow’ column (1.5 × 16 cm) equilibrated with running buffer. After washing with the same buffer containing 0.1 M KCl, NADH-GOGAT activity was eluted at a flow rate of 40 ml h⁻¹ with 100 ml of a linear gradient of 0.1–1.0 M KCl in running buffer.

2.4. Electrophoresis

Purification progress and subunit molecular mass were determined using a 7% polyacrylamide SDS gel [19]. The samples were treated (4:1, v/v) with sample buffer [0.36 M Tris, 30% (v/v) glycerol, 45% (v/v) 2-mercaptoethanol, 18% (w/v) SDS, 0.015% (w/v) bromophenol blue], heated to 100°C for 2 min and loaded onto SDS gel. After electrophoresis, the gels were stained for proteins with Coomassie brilliant blue.

Native-PAGE was performed at 4°C using a 4.5 – 10 polyacrylamide linear gradient. Samples (100 µg protein) of partially purified active enzyme were loaded. After electrophoresis the gel was stained for NADH-GOGAT activity according to Anderson et al. [15].

2.5. Assay of NADH-GOGAT activity

NADH-glutamate synthase (EC 1.4.1.14) activity was determined spectrophotometrically at 30°C by monitoring the oxidation of NADH at 340 nm essentially as indicated by Groat and Vance [20] and Singh and Srivastava [21]. Two controls (without α-ketoglutarate and without glutamine) were used to correct for endogenous NADH oxidation. The decrease in absorbance (linear for at least 10 min) was recorded for 10 min in a Beckman DU-70 spectrophotometer.

2.6. Molecular mass determination

Native molecular mass of NADH-GOGAT was determined on a 1.5 × 66 cm Sephacryl S-300 ‘Fast-Flow’ gel filtration column equilibrated with running buffer containing 0.1 M KCl and eluted at a flow rate of 50 ml h⁻¹. The column was calibrated with molecular weight standards (thyroglobin, 669 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa).

Native molecular mass was also estimated by native-PAGE as described above. The molecular weights used were thyroglobin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine albumin (67 kDa).

The molecular mass of NADH-GOGAT subunit was determined by SDS-PAGE [22] using samples of partial purified enzyme (9.3 µg). The molecular weight markers were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

The molecular weight for NADH-GOGAT was determined by linear regression.

2.7. Antibody production and specificity

Polyclonal antibodies against V. faba nodule NADH-GOGAT were produced by inoculating New Zealand-California rabbits with SDS-PAGE purified enzyme in 50 mM Tris–HCl pH 6.8. Four intradermal injections of the purified enzyme (100 µg) were given at 15 days intervals. Ten days after the last injection, blood was taken from ears using heparinized vacutainers (Becton–Dickinson). The blood was centrifuged at 3000 × g and comple-
ment inactivated by heating for 10 min at 56°C. Plasma was stored at −20°C until use.

Antibody specificity was determined by Western blotting of crude extracts subjected to SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose blots according to the procedure of Towbin et al. [23]. Protein blots were reacted with anti-GOGAT antibodies and visualized with peroxidase-conjugated anti-rabbit antibodies.

The immunoprecipitation curve of nodule NADH-GOGAT activity was performed using aliquots of crude extract (100 μl) incubated with increasing amounts of anti-GOGAT (from 5 to 60 μl), PBS (50 mM potassium phosphate at pH 7.2 containing 150 mM NaCl) up to 0.4 ml and 100 μl of Protein A Sepharose 10% (w/v) (100 μl) at 4°C for 12 h. The samples were centrifuged at 2500 × g for 10 min and NADH-GOGAT activity was assayed in the supernatant. Pre-immune serum was incubated under identical conditions as a control.

2.8. Protein extraction

Nodule, root and leaf protein for Western blots and enzyme assays were ground in a mortar and pestle with an extraction medium containing 100 mM maleic acid-KOH, pH 6.8, 100 mM sucrose, 2% (v/v) 2-mercaptoethanol, and 15% (v/v) ethylene glycol. The homogenate was centrifuged at 3500 × g at 2°C for 8 min and afterwards at 30 000 × g for 20 min. The bacteroid protein was extracted as described by Delgado et al. [24].

2.9. Protein determination

The soluble proteins were determined by Bradford [25] assay, using bovine serum albumin (Merck, fraction V) as the standard.

### Table 1

<table>
<thead>
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<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (μmol min⁻¹)</th>
<th>Specific activity (μmol min⁻¹ mg protein⁻¹)</th>
<th>Purification factor (-fold)</th>
<th>Recovery (%)</th>
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<tr>
<td>Crude extract</td>
<td>695</td>
<td>25.0</td>
<td>0.036</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
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<td>23.0</td>
<td>0.092</td>
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<td>92</td>
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<tr>
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<td>15.7</td>
<td>2.00</td>
<td>55</td>
<td>63</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>2.97</td>
<td>7.9</td>
<td>2.66</td>
<td>74</td>
<td>32</td>
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</table>

*Data are representative of six separate purifications, all purifications were completed within 48 h.*
Fig. 1. Elution profile of *V. faba* nodule NADH-GOGAT activity from a DEAE-Sepharose column (A) or a Q-Sepharose column (B).

Fig. 2. A. SDS gel electrophoresis pattern of partially purified NADH-GOGAT from *V. faba* nodules. Lane 1, purified by DEAE-sephacel column (25 µg protein); lane 2, purified by Sephacryl S-300 column (9.3 µg protein); lane 3, molecular weight markers; lane 4, crude extract (23 µg protein); lane 5, concentrated by ammonium sulfate precipitation (30–60% saturation) (25 µg protein). B. Non-denaturing linear gradient (4.5–10%) PAGE of the NADH-GOGAT partially purified (100 µg protein/lane) and stained by activity. The position, and size in molecular weight of protein markers are indicated. The native molecular weight (236 kDa) was determined by regression analysis ($y = 1.482x + 6.047; r = 0.999$) against native-PAGE separated protein markers.

corresponded in weight (195 kDa) to purified GOGAT. Western blot also revealed a comparable 195 kDa polypeptide in root extracts (Fig. 4B, lane 1) but not in those of leaves or bacteroids (Fig. 4B, lanes 3 and 4).

Antibodies to faba bean nodule NADH-GOGAT recognize a 195 kDa polypeptide from nodules of five inbred lines (VF51, VF61, VF52,
Fig. 4. A. Western blot of SDS-PAGE of partially purified *V. faba* nodule NADH-GOGAT. Lane 1, 5 µg protein; lane 2, 9 µg protein. B. Western blot of SDS-PAGE of soluble proteins (25 µg) from root (lane 1), effective nodules (lane 2), bacteroids (lane 3), and leaves (lane 4). C. Western blot of SDS-PAGE of nodule soluble proteins of different genotypes of *V. faba*. Lanes: (1) Alborea; (2) VF51; (3) VF61; (4) VF52; (5) VF60 and VF184 (0.083, 0.009, 0.075, 0.023, 0.065 and 0.069 µmol NADHox min⁻¹ mg⁻¹ protein, respectively). D. Western blot of SDS-PAGE of nodule soluble proteins of various legume species. Lanes: (1) *V. faba*; (2) *V. sativa*; (3) *M. sativa*; (4) *G. max* and (5) *P. sativum* (0.065, 0, 0.123, 0.070 and 0.096 µmol NADHox min⁻¹ mg⁻¹ protein, respectively). For each lane, 35 µg protein.

VF60, and VF184) of *V. faba* (Fig. 4C) and for a number of legumes (Fig. 4D). The lack of a cross-reactive band in *V. sativa* reflects the lack of activity in the crude extract from nodules (Fig. 4).

4. Discussion

The partial purification of NADH-GOGAT from *V. faba* nodules included anion-exchange and gel filtration chromatography (Fig. 2A). All purifications were carried out within 48 h because of the instability showed by the enzyme during the purification process as has been described for the nodule NADH-GOGAT of common bean [13] and alfalfa [15]. In contrast the lupin nodule NADH-GOGAT showed more stability [3].

The possible presence of distinct NADH-GOGAT isoforms in the plant fraction of root nodules of faba bean was not detectable by ion-ex-
change chromatography (Fig. 1). Chen and Cullimore [13], using a weak anion-exchanger (DEAE) separated two NADH-GOGAT isoforms (I and II) in common bean nodule; primarily isoenzyme II increases during the development of nodules. In nodules of V. faba only one peak of NADH-GOGAT activity was clearly distinguishable in the eluate from a weak anion-exchanger (DEAE-Sephacel) (Fig. 1A) or from a strong anion-exchanger (Q-Sepharose) (Fig. 1B), and thus NADH-GOGAT activity in nodule of faba bean is associated with a single form of the enzyme. In alfalfa and lupin nodules, NADH-GOGAT activity is also associated with a single form of the enzyme [3,15]. In relation to the above results, Gregerson et al. [26], reported a single NADH-GOGAT gene or a small gene family in alfalfa. The small difference between subunit (195 kDa) and native molecular weight (about 229 kDa) suggests that NADH-GOGAT is a monomer in faba bean, as was described for Neurospora [7] and for other legumes [3,5,13,15]. The molecular mass of the NADH-GOGAT from the faba bean were similar to those described for alfalfa (235 kDa), lupin (225 kDa) and Neurospora (230 kDa) [3,7,15], but higher than that reported for the enzyme of common bean (about 200 kDa) [13] and for the Fd-GOGAT (130–180 kDa [5,27].

The immunoprecipitation pattern of NADH-GOGAT from nodules of faba bean (Fig. 3) shows relatively high reactivity. The 100% of the nodule NADH-GOGAT was immunopreciptated. Anderson et al. [15] obtained antibodies against alfalfa nodule NADH-GOGAT using the native form of the enzyme and could immunoprecipitate only 80% of the total activity NADH-GOGAT of nodule crude extracts.

A reactive band was detected in Western blots of SDS-PAGE separations of crude root protein from faba bean that corresponded in molecular weight to NADH-GOGAT (195 kDa). The staining intensity of polypeptides on Western blots were fainter than that obtained from faba bean nodules (Fig. 4B). A comparable 195 kDa polypeptide was not found in crude soluble protein fractions from leaves or bacteroids (Fig. 4B), in agreement with Anderson et al. [15] studying alfalfa. The lack of comparable polypeptides in leaves may indicate that Fd-GOGAT is the active GOGAT in this organ. This finding agrees with the results reported for greening barley, pea leaves [4] and green leaves of tomato [27], where NAD(P)H-GOGAT remains steady during the greening process and while Fd-GOGAT increases. Leaf Fd-GOGAT antibodies do not recognize NADH-GOGAT protein [28,29], suggesting that these enzymes are structurally distinct proteins and are encoded by separate genes [26,30]. The lack of recognition of bacteroid polypeptide by anti-GOGAT serum also confirm that bacteroid and nodule GOGAT are two different enzymes [15,29], but can use either pyridine nucleotide co-factor (NADH and NADPH) [9,13].

Anti-GOGAT serum from faba bean nodule cross-reacted with a 195 kDa polypeptide from nodules of other genotypes of the same specie (VF51, VF61, VF52, VF60, and VF184) (Fig. 4C). The antiserum also cross-reacted with a polypeptide of comparable molecular weight (195 kDa) from nodules of different legume species (Fig. 4D), indicating that some antigenic epitopes have been conserved between nodule NADH-GOGAT of these species.

In summary, NADH-GOGAT of V. faba nodules was partially purified and its molecular weight was characterized. Using the protein from SDS-polyacrylamide gels high titre polyclonal antibodies were produced. The NADH-GOGAT of V. faba showed antigenic similarity with a similar-sized protein of other legumes. Further studies are now in progess for the isolation of the NADH-GOGAT gene and for intracellular localization of the enzyme using the antibodies. Moreover, the antibodies may prove useful in studying the expression and regulation of NADH-GOGAT throughout the development of the symbiosis.

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