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GLUTAMATE DEHYDROGENASE OF LUPIN NODULES: PURIFICATION AND PROPERTIES

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Key Word Index—*Lupinus luteus*; Leguminosae; lupin nodules; glutamate dehydrogenase; purification; ammonia assimilation.

Abstract—Glutamate dehydrogenase, GDH (L-glutamate:NAD^+ oxidoreductase (deaminating) EC 1.4.1.2) was purified from the plant fraction of lupin nodules and the purity of the preparation established by gel electrophoresis and electrofocusing. The purified enzyme existed as 4 charge isozymes with a MW of 270000. The subunit MW, as determined by dodecyl sulphate electrophoresis, was 45000. On the basis of the results of the MW determinations a hexameric structure is proposed for lupin-nodule GDH. The pH optima for the enzyme were pH 8.2 for the amination reaction and pH 8.8 for the deamination reaction. GDH from lupin nodules showed a marked preference for NADH over NADPH in the amination reaction and used only NAD^+ for the deamination reaction. Pyridoxal-5′-P and EDTA inhibited activity. The enzyme displayed Michaelis–Menten kinetics with respect to all substrates except NAD^+. When NAD^+ was the varied substrate, there was a deviation from Michaelis–Menten behaviour towards higher activity at high concentrations of NAD^+.

INTRODUCTION

In lupin nodules, ammonia formation takes place within the *Rhizobium lupini* bacteroids [1, 2]. These bacteroids contain insufficient glutamine synthetase (EC 6.3.1.2) and glutamate dehydrogenase, GDH (L-glutamate:NAD^+ oxidoreductase (deamination) EC 1.4.1.2) to assimilate more than a small part of the ammonia formed by nitrogenase [2–4] and ammonia is probably excreted during rapid nitrogen fixation into the plant cell cytoplasm. Excretion of ammonia in association with nitrogen fixation has been demonstrated with isolated bacteroids and free-living cells of *Rh. japonicum* [5–7].

Studies with $^{15}N_2$ fixation in serradella (*Ornithopus sativus*) nodules (*Rh. lupini* microsymbiont) showed that ammonia was the primary stable product of nitrogen fixation [8]. Pulse-chase labelling produced a rapid displacement of ^{15}N from the amide group of glutamine [8] consistent with the involvement of glutamate synthase (EC 1.4.1.13) in the assimilation of ammonia. However, as both nitrogen atoms of glutamine contained ^{15}N after 2 min exposure [9], no clear distinction can be made between glutamate synthesis by glutamine synthetase *plus* glutamate synthase, or directly by GDH.

GDHs from animal and microbial sources have been extensively studied and reviewed recently [10]. In contrast, relatively few detailed studies have been conducted on plant GDHs. The enzyme has been purified to apparent homogeneity from two plant sources: pea roots [11] and cowpea seedlings [12]. Both of these enzymes display a preference for NADH in the amination reaction and can use only NAD^+, and not $NADP^+$, in the deamination reaction. Other studies have been carried out on partially purified GDHs from a number of plant tissues [13, 14].

In this paper, a procedure for purification of the GDH from the plant fraction of *Lupinus luteus* nodules is described. An examination of properties which may be relevant in deciding the role of this enzyme in symbiotic nitrogen fixation by lupin nodules is included. This enzyme has not been previously purified from nitrogen-fixing plant tissue, although it has been located with significant activity in the plant fraction of a number of legume nodules [2, 3, 15–18]. While it is probable that

Table 1. Purification of glutamate dehydrogenase from lupin nodules

Fraction	Volume (ml)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification
Crude extract	2780	1550	0.04	100	—
1% Protamine sulphate	2940	1940	0.08	125	2.0
4–10% Polyethylene glycol 6000	260	1290	0.69	83	17.3
CM-Sepharose 6B-CL eluate	160	1360	14.4	87	360
NAD-agarose eluate	12.4	1590	317	102	7930

Lupin nodules (3 kg) were extracted as described. Glutamate dehydrogenase activity was measured as the reductive amination of 2-oxoglutarate.

ammonia assimilation involves the concerted action of glutamine synthetase and glutamate synthase in the plant fraction of lupin nodules [4], a significant role for nodule GDH in ammonia assimilation is also possible.

RESULTS

Purification of GDH

GDH was purified from the plant fraction of lupin nodules and details are given in Table 1. The crude extract contained no β-hydroxybutyrate dehydrogenase which is a marker enzyme for the bacteroids [2, 3]. This indicated that the plant extract was free of enzymes from the *Rhizobium lupini* bacteroids.

Polyethylene glycol (PEG) 6000 was chosen for the fractionation of the lupin-nodule extract because high concentrations of $(NH_4)_2SO_4$ caused loss of GDH activity. GDH was eluted from CM-Sepharose CL-6B as a single peak after the bulk of the bound protein had been eluted. The purification was completed using an affinity chromatography step with the GDH being eluted from the NAD-agarose column as a sharp peak at a concentration of 0.1 mM NADH. This procedure gave an excellent recovery of GDH from the lupin nodules with a typical overall purification of *ca* 8000-fold. The enzyme could be stored at −20° for up to a year without loss of activity.

Homogeneity

The homogeneity of the purified GDH was determined by polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) and electrofocusing. After PAGE, purified lupin-nodule GDH showed 4 bands of unequal intensity when stained for protein. The major band had the least mobility towards the anode. GDH activity was coincident with all 4 protein bands. The pattern of 4 protein bands coincident with GDH activity was obtained on 4, 5 and 6% (w/v) polyacrylamide gels. Similarly, electrofocusing of purified lupin-nodule GDH yielded 4 protein bands coincident with GDH activity. Only one polypeptide band was detected after SDS-PAGE. From these results, it can be concluded that the purification procedure yields 4 isozymes of lupin-nodule GDH which are free of other proteins.

Isozymes

Electrophoresis by the method of Hedrick and Smith [19] was used to determine the nature of the GDH isozymes from lupin nodules. A parallel pattern was obtained when the logarithm of relative mobility of the isozymes was plotted against the acrylamide concentration, which indicates that the isozymes differ in charge rather than in size. The 4 distinct bands which were obtained when the purified GDH was subjected to electrofocusing confirm that charge differences exist between the isozymes. The pI values of the GDH bands were 5.9, 6.2, 6.3 and 6.4.

MW

The MW of the native enzyme as determined on a standardized Sephadex G 200 column was 266000 ± 12000. This value is in good agreement with the value of 274000 ± 8000 obtained for the native enzyme by PAGE. The subunit MW of lupin-nodule GDH as determined by SDS-PAGE was 45000 ± 1000. The results of the MW determinations suggest that GDH from lupin nodules is a hexamer with subunits of equal MW.

Sedimentation coefficient

When subjected to sucrose density gradient centrifugation, lupin-nodule GDH activity sedimented in a single symmetrical peak. Using catalase ($s_{20^\circ,w} = 11.3$) as a standard, the $s_{20^\circ,w}$ value for the enzyme was 10.4. The same $s_{20^\circ,w}$ value was obtained when the initial concentration of enzyme was 0.1 and 1 mg/ml.

pH Optima

The pH optimum in constant ionic strength Tris–HCl buffer for the amination reaction was 8.2. In the same buffer, the pH optimum for the deamination reaction was 8.8. Enzymic activities of at least 90% of the maximum activity were obtained between pH 7.9 and 8.5 for the amination reaction and between pH 8.4 and 9.2 for the deamination reaction.

Substrate specificity

The activity of purified GDH from lupin nodules was assayed with a number of possible alternative substrates at pH 6 in 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, at pH 7 in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffer, at pH 8 in *N*-tris-(hydroxymethyl)-methyl-3-amino-propanesulphonic acid (TAPS) buffer, and at pH 9 and 10 in cyclohexyl-aminoethanesulphonic acid (CHES) buffer. For the amination reaction, NADPH was unable to substitute for NADH at any of the pH values tested other than pH 6. At this pH value, the activity with NADPH at a concentration of 0.125 mM was equal to the activity with NADH at the same concentration. This activity, however, represented less than 10% of the maximum activity of the enzyme with NADH as the coenzyme at pH 8.2. The keto-acids, oxaloacetate and pyruvate, at a concentration of 12.5 mM were unable to substitute for 2-oxoglutarate.

For the deamination reaction, no activity could be detected with $NADP^+$ (0.5 mM) as the coenzyme. The ability of lupin-nodule GDH to deaminate a wide range of amino acids was tested. No activity was detected with 25 mM concentrations of L-alanine, L-aspartate, L-asparagine, L-glutamine, L-isoleucine, L-leucine, L-norleucine, L-lysine, L-ornithine, or L-valine. The enzyme was able to deaminate L-norvaline: the activity with 25 mM L-norvaline was less than 5% of the activity of the enzyme with L-glutamate at the same concentration and pH.

Effect of pyridoxal-5′-P

The activity of lupin-nodule GDH was inhibited when the enzyme was assayed after it had been incubated with pyridoxal-5′-P. Fig. 1 shows the effect of incubation with pyridoxal-5′-P on the amination reaction. An inhibition of 50% was seen when the enzyme was incubated with 0.05 mM pyridoxal-5′-P in 0.2 M HEPES buffer (pH 7) for 15 min and then assayed (Fig. 1A). The extent of inhibition by pyridoxal-5′-P was dependent on incubation time (Fig. 1B) and also on the pH of the incubation mixture (Fig. 1C). The deamination activity was less

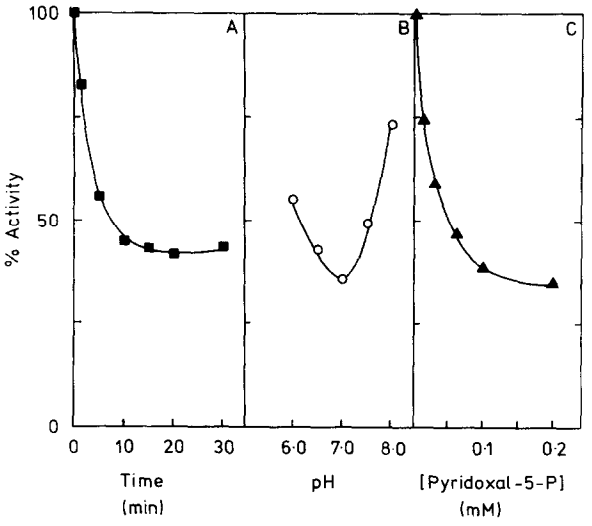

Fig. 1. Inhibition of glutamate dehydrogenase by incubation with pyridoxal-5'-P. Activities are compared with enzyme not treated with pyridoxal-5'-P. (A) The effect of incubation time on the inhibition by pyridoxal-5'-P. The enzyme was incubated with 0.1 mM pyridoxal-5'-P in 0.2 M HEPES buffer (pH 7) at 30°. At the times indicated samples were taken and assayed for amination activity under the standard conditions. (B) The effect of pH on pyridoxal-5'-P inhibition. Enzyme was incubated at 30° with 0.2 mM pyridoxal-5'-P in 0.2 M MES buffer (at pH 6.0 and 6.5) or in 0.2 M HEPES buffer (at pH 7.0, 7.5 and 8.0). After 15 min, the enzyme was assayed for amination activity under the standard conditions. (C) The effect of the concentration of pyridoxal-5'-P on glutamate dehydrogenase activity. Enzyme was incubated in 0.2 M HEPES buffer (pH 7) at 30° with various concentrations of pyridoxal-5'-P. After 15 min, the enzyme was assayed for amination activity under the standard conditions.

affected by pyridoxal-5'-P and 50% inhibition occurred following incubation with 0.2 mM pyridoxal-5'-P.

The inhibition of the amination activity by pyridoxal-5'-P was partially reversed when any one of the substrates of the amination reaction was added to the enzyme after the incubation (Table 2). The addition of NADH (0.1 mM) and 2-oxoglutarate (5 mM) in combination completely reversed the effects of pyridoxal-5'-P.

Table 2. Ability of the substrates of glutamate dehydrogenase to reverse the inhibition caused by pyridoxal-5'-P

Addition	Activity Units/mg	% Control
None	319	100
Pyridoxal-5'-P	109	34
Pyridoxal-5'-P + NAD^+ (0.5 mM)	105	33
Pyridoxal-5'-P + L-glutamate (20 mM)	133	42
Pyridoxal-5'-P + NADH (0.1 mM)	220	69
Pyridoxal-5'-P + 2-oxoglutarate (5 mM)	200	63
Pyridoxal-5'-P + NH_4Cl (80 mM)	192	60
Pyridoxal-5'-P + NADH (0.1 mM) + 2-oxoglutarate (5 mM)	332	104

The enzyme was incubated at 30° in HEPES buffer (pH 7.0) with 0.2 mM pyridoxal-5'-P for 15 min, after which the substrates indicated were added to the enzyme solution. The enzyme was incubated for a further 15 min and then assayed.

Effect of metabolites

AMP (final concentration 2 mM) and ADP (2 mM) inhibited the amination activity of lupin-nodule GDH by 32 and 27%, respectively. ATP (2 mM) did not affect the amination activity. AMP (2 mM), ADP (2 mM) and ATP (2 mM) inhibited the deamination reaction by 18, 30 and 16%, respectively. Citrate (5 mM) inhibited the amination reaction by 23% but had no effect on the deamination reaction. The following compounds had no significant effect on either the amination or deamination reaction: pyruvate (final concentration 5 mM), oxaloacetate (5 mM), succinate (5 mM), fumarate (5 mM), L-malate (5 mM), L-alanine (10 mM), 4-aminobutyrate (10 mM), L-aspartate (10 mM), L-asparagine (10 mM) and L-glutamine (10 mM).

Effect of inhibitors

When lupin-nodule GDH was incubated at 30° for 10 min with 0.01 mM EDTA and the amination reaction subsequently measured, the activity was inhibited by 50%. If the incubation concentration of EDTA was increased to 0.1 mM, no additional inhibition was observed. The deamination activity was inhibited by 5 and 36% when the enzyme was incubated with 0.01 and 0.1 mM EDTA, respectively before assaying. Incubation with 1 mM KCN at 30° for 10 min caused 12% inhibition of the amination activity and 60% inhibition of the deamination activity. Neither the amination nor the deamination activity was altered when the enzyme was incubated at 30° for 10 min with 0.1 mM *p*-chloromercuribenzoate or 1 mM NaF before the assays.

Preliminary kinetic studies

With L-glutamate as the varied substrate, typical Michaelis–Menten kinetics were observed in initial velocity studies of the deamination reaction; an intersecting pattern of double reciprocal plots was obtained. However, with NAD^+ as the varied substrate, there was a deviation from Michaelis–Menten behaviour towards higher activity with high concentrations of NAD^+. The double reciprocal plots with NAD^+ as the varied substrate (Fig. 2) showed two linear regions of different slope separated by a discontinuity.

To determine whether the biphasic plots in Fig. 2 resulted from the isozymic nature of the lupin-nodule GDH, the isozymes were separated by electrophoresis on 5% (w/v) polyacrylamide gels. The gels were stained for activity and then segments of the gels containing: (a) the major isozyme alone and (b) all 4 isozymes, were pulverized and the proteins extracted with 0.2 M TAPS buffer (pH 8.4).

In separate kinetic studies, both the preparation containing one isozyme and the preparation with all 4 isozymes gave biphasic double reciprocal plots with the same two apparent K_m values when NAD^+ was the variable substrate. The major isozyme band was subjected to PAGE again after extraction and only one band of GDH activity was seen. From these results it is concluded that the biphasic double reciprocal plots with NAD^+ as the varied substrate are not a result of the isozymic nature of lupin-nodule GDH. Kinetic constants for the deamination reaction at pH 8.8 are listed in Table 3. The K_m value for L-glutamate is not affected by the discontinuity in the double reciprocal plot in Fig. 2.

Preliminary kinetic studies were performed on the

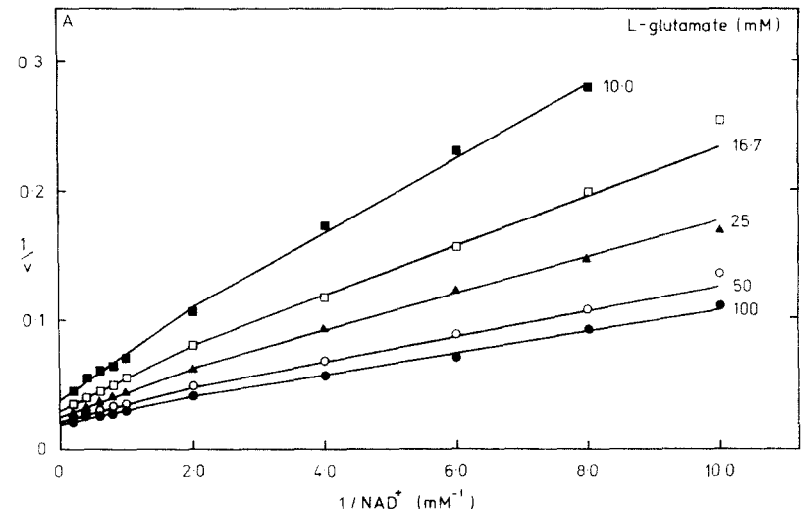

Fig. 2. The effect of varying NAD^+ concentration on the initial velocity of the deamination reaction. Velocities are expressed as μmol of NADH formed/min per mg.

amination reaction by varying the concentration of two reactants while holding the third reactant at a fixed concentration. The enzyme displayed typical Michaelis–Menten kinetics with each of the substrates and an intersecting pattern of double reciprocal plots was observed in all cases. The values for the apparent kinetic constants at pH 8.2 (Table 4) were obtained graphically by the method of Dalziel [20]. These constants do not represent the maximum velocity or the true K_m values, as saturating concentrations of the fixed substrate could not be obtained in the assays.

DISCUSSION

The MW of lupin-nodule GDH was 270000 and this is similar to values reported for GDHs from several plants [11, 21]. The lupin-nodule enzyme is suggested to exist as a hexamer; a similar subunit structure has been proposed for GDHs from a number of animal and microbial sources [22, 23]. The subunit structure of GDHs from other plants has not been investigated in detail.

Lupin-nodule GDH exists as 4 isozymes which differ in charge but not in molecular size. The major isozyme was not converted to the other enzyme forms as shown by electrophoresis. Charge isozymes have been shown to occur in the GDH from *Pisum sativum* [13] and *Medicago sativa* [14].

Lupin-nodule GDH showed a marked preference for NADH over NADPH in the reductive amination of 2-oxoglutarate. The enzyme was active only with NAD^+ in the oxidative deamination of L-glutamate. This coenzyme specificity is similar to that of other plant GDHs

Table 3. Kinetic constants for the deamination reaction of glutamate dehydrogenase from lupin nodules

NAD^+ concentration (mM)	V (units/mg enzyme)	K_m (mM) NAD^+	K_m (mM) L-Glutamate
0.05–0.5	46.5 ± 1.1	0.26 ± 0.02	14.7 ± 0.2
1.0 –5.0	64.1 ± 0.4	0.52 ± 0.03	14.4 ± 0.2

Values for the kinetic constants at pH 8.8 were obtained by replotting the intercepts and slopes of the double reciprocal plots against the reciprocal of the fixed substrate concentration. Two values for each kinetic constant were obtained, one for low NAD^+ concentrations (0.05–0.5 mM) and one for high NAD^+ concentrations (1.0–5.0 mM). These ranges correspond to the two linear regions of the plots in Fig. 2.

Table 4. Apparent kinetic constants for the amination reaction of glutamate dehydrogenase of lupin nodules

Fixed substrate	Varied substrates	Apparent V (units/mg enzyme)	Apparent K_m (mM) NADH	Apparent K_m (mM) 2-Oxoglutarate	Apparent K_m (mM) NH_4Cl
NADH (0.25 mM)	2-Oxoglutarate NH_4Cl	534 ± 8		12.1 ± 0.1	86.4 ± 0.2
2-Oxoglutarate (25 mM)	NADH NH_4Cl	801 ± 32	0.274 ± 0.002		59.0 ± 6.0
NH_4Cl (200 mM)	NADH 2-Oxoglutarate	513 ± 13	0.133 ± 0.005	9.1 ± 0.2	

Values of the apparent kinetic constants at pH 8.2 were obtained graphically from initial velocity studies as described in ref. [20].

[11, 12]. The lupin-nodule enzyme is highly specific for the substrates 2-oxoglutarate and L-glutamate.

The biphasic double reciprocal plots, which were obtained when NAD^+ was the varied substrate in the deamination reaction, indicate that lupin-nodule GDH binds more than one molecule of NAD^+ and that the NAD^+ binding sites are either not equivalent or display negative cooperativity. Similar biphasic double reciprocal plots have been reported for GDHs from other sources [10, 13].

From the intersecting initial velocity patterns obtained for both the amination and deamination, it can be concluded that GDH from lupin nodules follows a sequential mechanism in both directions. Detailed qualitative and quantitative kinetic studies on both the amination and deamination reactions are consistent with a fully ordered sequential reaction mechanism for lupin-nodule GDH [24, 25].

The inhibition of GDH by pyridoxal-5'-P has been observed with the enzyme from a number of sources [10]. For the GDH from pea mitochondria, the effect has been suggested as a possible control which acts under conditions of nitrogen limitation [26]. The inhibition by EDTA and KCN are suggestive of metal involvement in the activity of the enzyme.

The main pathway for the assimilation of newly fixed ammonia into glutamate in nitrogen-fixing plant tissue is proposed to be via the glutamine synthetase and glutamate synthase reactions [4]. The average concentration of ammonia in root nodule tissue fixing nitrogen is *ca* 5 mM [8, 27]. In this concentration range glutamine synthetase would be effectively saturated with ammonia; a K_m value for NH_4^+ of 0.02 mM has been reported for glutamine synthetase in peas [28]. The amination activity of GDH at a concentration of ammonia of 5 mM would be low relative to the maximum velocity, but the enzyme would be responsive to small changes in the level of ammonia. This could be an advantage during active nitrogen fixation and may indicate a role for GDH under such conditions. Comparison of the apparent maximum velocities for both reactions indicates a greater potential for amination than deamination. The value of K_{eq}, which favours the amination reaction, the tight binding of 2-oxoglutarate in an enzyme-NAD-2-oxoglutarate complex, and the other kinetic parameters reported for the enzyme [24, 25] are consistent with such a role.

EXPERIMENTAL

Plant material. Lupins (*Lupinus luteus* L. cv Weiko III) were inoculated with *Rhizobium lupini* (WU 425) and grown in the field. At flowering entire plants were harvested and the nodules frozen in liquid N_2.

Enzyme purification. All steps were carried out at 4° unless stated otherwise. Frozen nodules were macerated in an equal vol. of 0.05 M K-Pi buffer (pH 7) containing 0.2 M sucrose, 5 mM β-mercaptoethanol and 2% (w/v) insoluble PVP. The macerated tissue was squeezed through cheesecloth and centrifuged at 5000 *g* for 10 min. The supernatant was termed the crude extract.

Protamine sulphate soln (1% w/v) was added slowly to the crude extract at 20° to a final concn of 1 g protamine sulphate per kg nodules extracted. The pH was adjusted to 5.7 with N HOAc and the extract allowed to stand at 20° for 30 min. The resulting ppt. was removed by centrifugation at 15000 *g* for 20 min and discarded. The supernatant was brought to 4% (w/v) with respect to PEG 6000 and stirred for 30 min. Further PEG 6000 was added to the 4% PEG supernatant until the concn was 10% (w/v). The soln was stirred for 30 min, centrifuged and the supernatant discarded. The ppt. was resuspended in 200 ml of 20 mM MES buffer (pH 5.7) containing 5 mM β-mercaptoethanol (buffer I) and the suspension dialysed overnight against 100 vol. of the same buffer. Protein which had not dissolved after dialysis was removed by centrifugation at 40000 *g* for 20 min and discarded.

The dialysed 4–10% PEG 6000 fraction was applied to a CM-Sepharose CL-6B column (2.6 × 40 cm). The column was washed with 2 bed-vol. of buffer I and GDH was eluted with a gradient obtained by introducing 500 ml 0.5 M KCl in buffer I into 500 ml buffer I. Fractions containing GDH activity were dialysed against 0.1 M HEPES buffer (pH 8.3) containing 5 mM β-mercaptoethanol (buffer II) and concd to 20 ml using an Amicon pressure cell (XM-50 membrane under 300 kPa/m^2 N_2 pressure). The prepn was applied to a column of 8-(6-aminohexyl)-amino-NAD-agarose (0.8 × 7 cm) which was then washed with buffer II until *A* at 280 nm was zero. GDH was eluted with a gradient obtained by introducing 10 ml of 0.5 mM NADH in buffer II into 10 ml of buffer II and the enzyme dialysed to remove NADH.

Measurement of enzyme activity. The amination reaction was measured by the rate of decrease of *A* at 340 nm during the NH_4^+ and 2-oxoglutarate dependent oxidation of NADH. The standard assay medium for this reaction contained in a final vol. of 1 ml: 200 μmol Tris–HCl buffer (pH 8.2), 0.125 μmol NADH, 12.5 μmol 2-oxoglutarate, 200 μmol NH_4Cl and enzyme equivalent to 0.01–0.02 μg of purified glutamate dehydrogenase. The pH of the mixtures was adjusted to 8.2 before addition to the reaction cuvette. The enzyme reactions were initiated by the addition of NH_4Cl. Correction for the oxidation of NADH in the absence of NH_4Cl was necessary for GDH assays in the early stages of the purification procedure. Constant ionic strength was maintained by adding an appropriate vol. of 2 M KCl soln. One unit of GDH activity is defined as the amount of enzyme which catalyses the conversion of 1 μmol of substrate per min.

The deamination activity of GDH was measured by the rate of increase of *A* at 340 nm during the L-glutamate dependent reduction of NAD^+. The assay medium contained in a final vol. of 1 ml: 200 μmol Tris–HCl buffer (pH 8.8) and levels of L-glutamate and NAD^+ indicated. Reactions were initiated by the addition of 0.1 μg of enzyme.

Gel electrophoresis. PAGE was performed at 4° according to the method in ref. [29]. Gels were stained for protein with Coomassie Brilliant Blue R [30]. GDH activity was detected in the gels by a modification of the method in ref. [31]. The reaction mixture contained in a final vol. of 10 ml: 1 mmol Tris–HCl buffer (pH 8.5), 1 mmol L-glutamate, 50 mol NAD^+, 2.5 mg nitroblue tetrazolium and 0.25 mg phenazine methosulphate. The method in ref. [19] was used to estimate the MW of lupin-nodule GDH and to characterize the nature of the isozymes. SDS-PAGE was performed according to the method in ref. [30].

Electrofocusing. Electrofocusing was performed with LKB Ampholine 10 cm PAG plates at 15°. 50 μg of purified GDH was electrofocused for 2.5 hr at 300 V/50 mA; the gel plate was cut into strips which were stained for protein or GDH activity as described above.

MW determination by gel filtration. The method in ref. [32] was used to determine the MW of lupin-nodule GDH on a Sephadex G 200 column (1.5 × 55 cm).

Sucrose density gradient centrifugation. A sedimentation coefficient for lupin-nodule GDH was determined by the

method in ref. [33]. Centrifugation was carried out at 64000 rpm in a Beckman SW65L rotor for 210 min at 4°.

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