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# PURIFICATION AND PROPERTIES OF LUPIN NODULE GLUTAMINE SYNTHETASE

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**Key Word Index**—*Lupinus luteus*; Leguminosae; lupin nodules; symbiotic nitrogen fixation; ammonia assimilation; glutamine synthetase purification; ADP-Sepharose affinity chromatography.

**Abstract**—Glutamine synthetase (GS) from the cytoplasm of *Lupinus luteus* nodules was purified to apparent homogeneity using a final step of ADP-Sepharose affinity chromatography. Mercaptoethanol and divalent metals were essential to maintain the enzyme activity and keto compounds enhanced the stability during purification. From gel filtration a $M_r$ for the native enzyme of 347 000 was determined with subunits of 41 500 indicated by SDS-PAGE. The pH optima for the biosynthetic and transferase activities were 7.9 and 6.5 respectively. $Mg^{2+}$-activated GS was strongly inhibited by $Mn^{2+}$ and $Ca^{2+}$; $Co^{2+}$, while also inhibitory, allowed an alternate, more active form of GS after addition of glutamate. Activity was also inhibited by possible feedback inhibitors. The apparent $K_m$ values for glutamate, $NH_4^+$, ATP, glutamine, $NH_2OH$ and ADP were 8.58 mM, 12.5 μM, 0.22 mM, 48.6 mM, 3.37 mM and 59.7 nM respectively.

## INTRODUCTION

In legume nodules, dinitrogen is fixed in the *Rhizobium* bacteroids. Most of the initial product, ammonia, is excreted into the cytosol of nodule tissue where it is considered to be assimilated into glutamine by plant glutamine synthetase (GS) (EC 6.3.1.2.) [1].

Assimilation by plant GS is consistent with the results of a kinetic analysis of $^{15}N_2$ fixation indicating ammonia was assimilated in a compartment different to that where it was fixed [2]. By $^{15}N_2$-pulse labelling with serradella (*Ornithopus sativus*) nodules [3] it was further shown that both the amide and the α-amino nitrogens of glutamine acted as precursor nitrogen for other amino compounds. In soybean [4] and lupin [5] nodules, most of the GS activity was found in the nodule cytosol, consistent with the idea that most of the ammonia is taken up by plant enzymes. In return for ammonia, the plant supplies carbon sources and energy and helps provide oxygen at a suitable activity for the nitrogen fixation to take place—forming a highly beneficial symbiotic system [6].

It is well established that GS plays a very important role in ammonia assimilation and nitrogen metabolism of organisms. Extensive studies of GS have been conducted in higher plants [7–16], animals [17, 18] and bacteria [19, 20]. These have used both biosynthetic assays and an artificial assay—an exchange catalysed by GS to form γ-glutamylhydroxamate from glutamine and hydroxylamine in the presence of arsenate, ADP and $Mn^{2+}$.

In an earlier paper from this laboratory [21], the purification to homogeneity and properties of lupin (*Lupinus luteus*) nodule glutamate dehydrogenase (GDH) (EC 1.4.1.2), another plant enzyme possibly involved in nitrogen assimilation, was described. Here we report the purification of nodule GS from the same species and describe some of its properties.

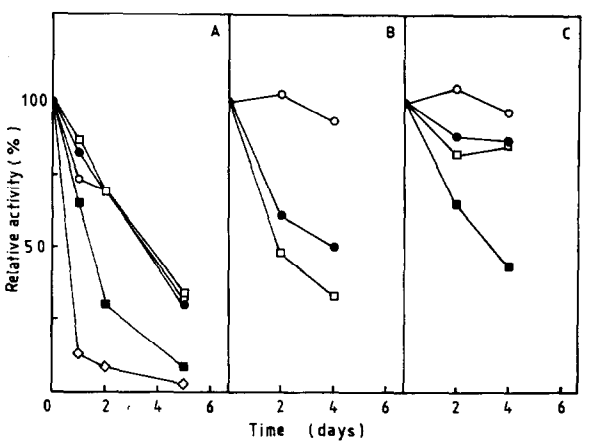


Fig. 1. Effect of various agents on the stability of GS. (A), (□) 10 mM Na-pyruvate; (●) 10 mM 2-oxoglutarate; (○) 10 mM oxaloacetate; (■) 2°; (◇) room temperature. (B), 5 mM 2-oxoglutarate and 2 mM $MnSO_4$ plus (○) 10 mM mercaptoethanol; (●) nil; (□) 4 mM DTT. (C), (○) 10 mM pyruvate, 2 mM $MnSO_4$ and 11 mM mercaptoethanol; (●) 2 mM $MnSO_4$ and 11 mM mercaptoethanol; (□) 20 mM mercaptoethanol; (■) no stabilizer. GS transferase activity was assayed.

## RESULTS

### *Stability of crude lupin nodule GS*

An initial difficulty in purifying GS was the pronounced instability of the enzyme when prepared in crude extracts. In Fig. 1, it is shown that the half-life of crude GS activity was only several hr. It was necessary to perform a large number of tests to obtain stabilizing conditions before purification of GS could be attempted. The results obtained with various agents are also shown in Fig. 1. It was found that mercaptoethanol (but not DTT) was obligatory to obtain reasonably stable enzyme. $MnSO_4$

and keto acids (2-oxoglutarate, oxoloacetate, pyruvate) also stabilized the enzyme. A 10 mM imidazole buffer containing 10 mM K-pyruvate, 1 mM $MnSO_4$ and 5 mM mercaptoethanol was used in the purification procedure, providing optimum stabilization.

*Purification of GS*

GS was purified as indicated in Experimental and details of the purification are given in Table 1.

Four purification stages were employed with a final step using ADP-Sepharose affinity chromatography (Fig. 4). Overall, GS was purified about 39-fold to a specific activity of 60–70 $U_t$/mg (transferase) with a purification yield of about 20%. About 3% of the total soluble nodule protein was thus GS. The total amount of biosynthetic activity ($U_{bs}$), measured by estimating ADP formation under optimum conditions, was 3.5 μmol $NH_4^+$ assimilation (= $NAD^+$ formation)/min/g (fresh nodule weight). This is considerably higher than the rate of ammonia production by lupin bacteroids *in vivo*, which is 0.15 μmol $NH_4^+$/min/g as calculated from an acetylene reduction datum [22]. This clearly indicates that adequate GS activity is present in nodules to assimilate ammonia produced during the nitrogen fixation.

In order for binding of GS to ADP-Sepharose to occur, it was necessary to include $Mn^{2+}$ in the buffer. Binding did not occur with $Mg^{2+}$.

*Purity and* $M_r$

GS was purified to near homogeneity as determined by PAGE (Fig. 2). In DEAE-Sephacel chromatography (Fig. 3), most of the GS eluted with 145 mM KCl, with a minor shoulder of activity (less than 10%) eluting at about 290 mM KCl. The main peak of activity was purified separately from this minor fraction. Both fractions, when purified, were found to have the same $M_r$ (within experimental error) on Sephacryl S-300, the same $M_r$/charge ratio by PAGE, the same subunit size on SDS-PAGE and the same $K_m$ values for ATP, glutamate, hydroxylamine and glutamine, as well as a similar specific activity. The two fractions from DEAE-Sephacel also gave similar transferase/synthetase ratios.

Cullimore *et al.* [16] have described the occurrence of three forms of plant GS in *Phaseolus* root nodules, with the two major peaks of activity eluting from DEAE-Sephacel at 25 mM and 65 mM KCl respectively. These

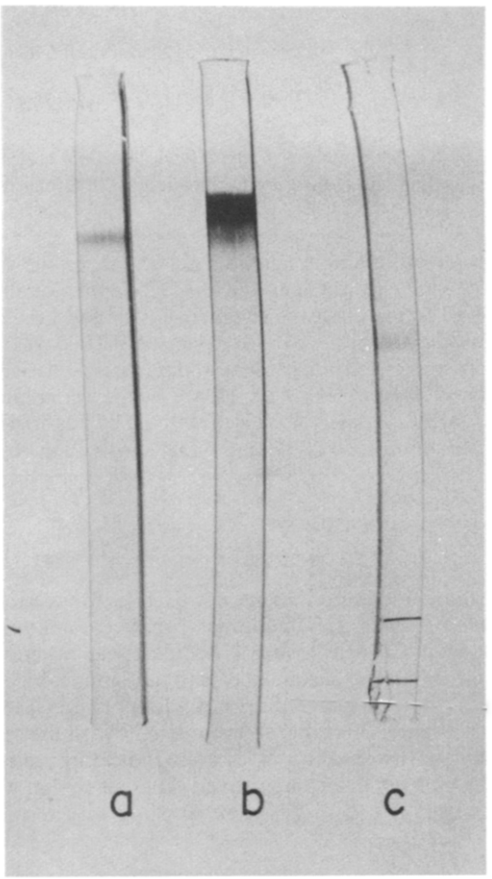


Fig. 2. Purified GS on PAGE and SDS-PAGE gels. (a) *ca* 5 μg; (b) *ca* 50 μg of native GS. (c) *ca* 10 μg GS subunit on SDS-PAGE gel.

Table 1. Purification of GS from lupin nodules

| Fraction | Total activity ($U_t$)* | Total protein (mg) | Specific activity ($U_t$/mg) | Yield | Purification factor |
|---|---|---|---|---|---|
| Crude extract | 4136 | 2538 | 1.6 | 100 | — |
| 0–33% $(NH_4)_2SO_4$ Supernatant | 3143 | 1562 | 2.0 | 76 | 1.3 |
| 33–50% $(NH_4)_2SO_4$ precipitation | 2721 | 573 | 4.7 | 66 | 3.0 |
| DEAE-Sephacel | 1140 | 130 | 8.8 | 28 | 5.5 |
| Sephacryl S-300† | 890 | 42.6 | 20.9 | 22 | 13 |
| ADP-Sepharose‡ | 709 | 11.4 | 62.0 | 18 | 38.8 |

*Enzyme Unit ($U_t$) = 1 μmol γ-glutamylhydroxamate/min.

†15–20% of the total main GS fraction from DEAE-Sephacel step was employed for this procedure.

‡5–10% of the total activity from Sephacryl S-300 was used for this step.

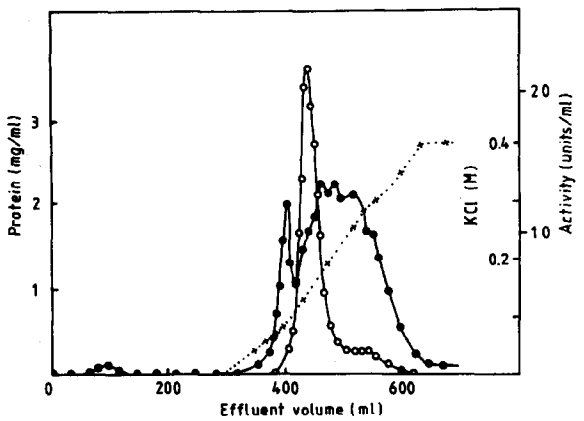


Fig. 3. Elution profile of nodule GS from DEAE-Sephacel. (●) protein; (○) enzyme activity; (×) KCl concentration.

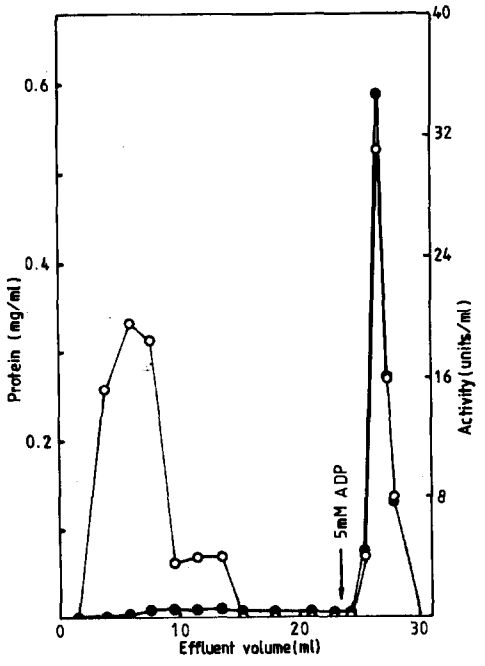


Fig. 4. A typical purification on ADP-Sepharose. 5.9 ml (75 $U_t$) of GS activity from Sephacryl S-300 was absorbed onto the affinity column. The enzyme was eluted with 5 mM ADP in buffer II. (●) enzyme activity; (○) protein.

were distinguished by their slight separation on PAGE and the ratio of transferase/synthetase activity, although in most other respects they appeared almost identical. We have not been able to clearly distinguish the two GS fractions by any tests other than by their position of elution. On PAGE gels, their relative positions did not vary more than experimental error.

The $M_r$ of the native enzyme as determined by gel filtration on Sephacryl S-300 was 347 000 ± 20 000. The subunit $M_r$ of GS as determined by SDS-PAGE was 41 500 ± 1000. Trace amounts of an additional band on SDS-PAGE of about 34 000 $M_r$ is derived from a minor contaminant, visible when very heavy loadings of purified GS (*ca* 50 μg) are run on PAGE as shown in Fig. 2. It is suggested that lupin nodule GS is an octamer with subunits of equal $M_r$, as reported for other plant GS enzymes [4, 23].

### *pH optima*

pH optima were established as 6.5 for the transferase, and 7.9 for the biosynthetic assay using coupling enzymes to estimate ADP formation. pHs of 7.0 (90% of the activity at pH 6.5) and 7.9 were used routinely in the transferase and biosynthetic assay respectively.

### *Kinetic properties*

The apparent $K_m$ values for GS, determined by calculation from initial rates of enzyme activity with a non-linear regression program [24] on an Apple IIe minicomputer are given in Table 2.

In the transferase assay, the $K_m$ values obtained for glutamine and $NH_2OH$ were comparable to those of soybean hypocotyl enzyme [14]. The $K_m$ value for ADP was so low that it was impossible to demonstrate an ADP requirement without very thorough dialysis ($10^6$ excess of dialysis buffer) to remove ADP used in elution of GS from ADP-Sepharose.

The $K_m$ for ammonium was also determined using direct analysis of glutamate-dependent phosphate formation from ATP (Table 2).

### *Inhibition of GS by selected amino acids and other compounds*

A number of amino acids and compounds related to nitrogen metabolism were tested for effects on the enzyme activity. At 10 mM concentrations, aspartic acid, glycine, alanine and serine inhibited 43%, 33%, 22% and 20% respectively. Only small or no effects were observed with histidine, DL-tryptophan, proline, L-glutamine, arginine, asparagine, L-ornithine and 2-oxoglutarate. Activation by 10 mM cysteine varied from 2–23% on different occasions. γ-Amino-*n*-butyric acid, a prominent compound in nodules [2] and urea had no effect on the enzyme activity. At 5 mM glutamate, rather than 50 mM of the standard assay, the inhibitions were similar, suggesting they were not competing for the same binding site. Combined inhibitions by glycine, alanine and serine were cumulative,

Table 2. $K_m$ values for transferase and biosynthetic reactions

| Assay | Substrate | $K_m$ values |
|---|---|---|
| γ-Glutamyltransferase assay | $NH_2OH$ | 3.37 ± 0.23 mM |
| | Glutamine | 48.6 ± 1.6 mM |
| | ADP | 59.7 ± 4.2 nM |
| Biosynthetic assay | $NH_4^+$ | 12.5 ± 0.6 μM |
| | Glutamate | 8.58 ± 0.14 mM |
| | ATP | 0.22 ± 0.01 mM |
| Phosphate assay* | $NH_4^+$ | 13.7 ± 1.4 μM |

*Determined using GS without affinity chromatography step of purification.

as calculated by the method of Woolfolk and Stadtman [20], suggesting that each inhibitory amino acid is bound at a different site. The three amino acids were uncompetitive with respect to glutamate as reported for pea leaf GS [9].

Inorganic phosphate inhibited both transferase and biosynthetic activities, but with a stronger inhibition in the transferase assay ($I_{50}$ = 7.8 mM) than in the biosynthetic assay ($I_{50}$ = 20 mM). Phosphate inhibition was found to be uncompetitive with respect to ATP. This is in contrast to GS from pea leaf [9].

*Effect of divalent metals*

$Mg^{2+}$-activated biosynthetic GS activity was strongly inhibited by $Mn^{2+}$ and $Ca^{2+}$, with respective $I_{50}$ of 0.025 mM and 0.189 mM. At higher concentrations $Mn^{2+}$ and $Ca^{2+}$ inhibited 91% (0.75 mM) and 89% (3.16 mM) of the activity respectively. Part of the inhibition might have been due to a shift of pH optimum with different metals, as suggested by O'Neal and Joy [8], but the lupin nodule GS is much more sensitive to these metals and the $I_{50}$ for $Mn^{2+}$ was only about one-fifteenth of that required for the pea leaf GS.

The inhibitory effect of $CoCl_2$ was complicated in that a glutamate-dependent re-activation of $Co^{2+}$-inhibited GS occurred after 4–5 min, restoring about one-third of the uninhibited GS activity (Fig. 5).

*$Mg^{2+}$/ATP ratio*

With ATP concentration constant and $Mg^{2+}$ varied, a sigmoid response curve was observed, with a maximum activity at $Mg^{2+}$/ATP of 3:1. The enzyme activity decreased sharply with $Mg^{2+}$/ATP less than 1. This result is similar to others reported [8, 11]. Since $MgATP^{2-}$ is probably the true substrate [8, 12], the optimum ratio of 3:1 indicates a requirement for free $Mg^{2+}$ binding to GS, as suggested by Pushkin *et al.* [12].

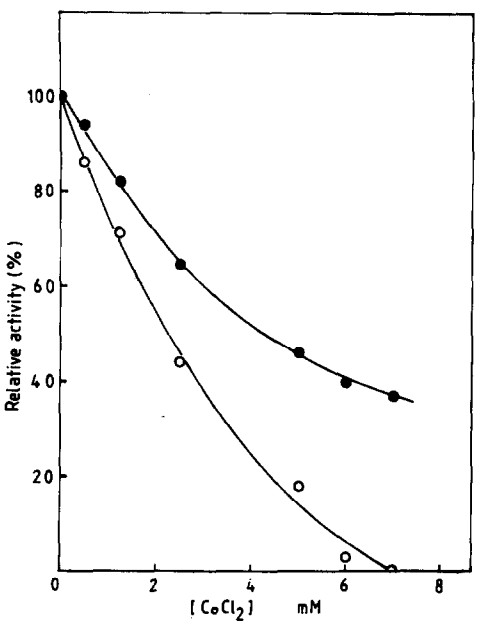


Fig. 5. Inhibition of GS biosynthetic activity by $CoCl_2$. A standard assay substrate without EDTA was used. GS was added to start the reaction and a time curve was followed; (●) final activity; (○) initial activity.

## DISCUSSION

The lupin nodule GS is very unstable both in crude extracts and when purified. Keto acids enhanced the stability of GS in crude extracts, but had little effect on the enzyme activity, at least in the case of 2-oxoglutarate. The purified enzyme from the ADP-Sepharose chromatography was also unstable when in high concentration and precipitated noticeably, beginning during dialysis. Considerable denaturation of GS was also observed during storage in frozen buffer at −20°. Similar spontaneous denaturation of GS was noted by O'Neal and Joy [7] with pea leaf GS. However, purified GS in 30% glycerol retained 70% of the initial activity after two months' storage at −20°, while the GS without glycerol lost 90% activity.

We initially attempted purification using glutamate and ATP-linked affinity media under various conditions without success. The extremely high affinity of GS for ADP noted for the transferase assay was consistent with our success using 2′,5′-ADP-Sepharose, even though the structures are not identical. This contrasts with the failure of 5′-ADP-Agarose in purification of pea seed GS [25]; possibly, the use of $Mg^{2+}$ rather than $Mn^{2+}$ ions in the buffer may explain their result. Dowton and Kennedy (unpublished) have successfully employed 2′,5′-ADP-Sepharose chromatography to purify an insect GS and this affinity procedure may prove of general use in purification of GS enzymes.

The $K_m$ values given in Table 2 are quite similar to those for GS from other sources [26] except for hydroxylamine. To obtain the $K_m$ for ammonia, a method reported by Orr and Haselkorn [27], relying on the form of the time course as ammonia is consumed, was used, giving a $K_m$ value of 12.5 $\mu$M. This was confirmed by the $K_m$ value of 13.7 $\mu$M obtained using a phosphate assay.

Lupin nodule GS shows an absolute requirement for divalent metal cations for activity, as noted for all GS enzymes studied. Extensive dialysis to remove metal ions led to rapid loss of activity, indicating the need for free $Mn^{2+}$ or $Mg^{2+}$ to maintain an active conformation. The effect of $Co^{2+}$ ions was not clear-cut, producing marked inhibition of $Mg^{2+}$-activated biosynthetic activity immediately after addition of glutamate to commence reaction, but allowing a degree of reactivation several min later at pH 7.9. This suggests a partial reversion to an active form in the presence of glutamate.

The maximum specific activity observed for purified GS was 21 $U_{bs}$/mg. This provides a turnover number of 7290 moles of catalytic activity/mole of enzyme/min, a value similar to that observed with some other GS enzymes [27]. The turnover number observed with lupin nodule GDH was 32 times greater, but the GDH was only about 0.02% of nodule cytoplasmic protein compared with the 2–3% of cytoplasmic protein represented by GS. The apparent $K_m$ value for $NH_4^+$ of 12.5 $\mu$M also contrasts with the $NH_4^+$ $K_m$ for GDH of about 60 mM [21]. An average concentration of $NH_4^+$ observed in nitrogen-fixing serradella nodules was about 3–5 mM [2], intermediate between these $K_m$ values.

Obviously, lupin nodule GS could assimilate $NH_4^+$ at all

likely concentrations effectively, given adequate ATP and glutamate. GDH activity in $NH_4^+$ assimilation would be low relative to the maximum activity possible. The contrast in kinetic parameters between GS and GDH points to the main role of GS in symbiotic nitrogen fixation (with glutamate synthase) as suggested earlier [1]. It is of interest, however, that $Ca^{2+}$ and $Mn^{2+}$ which inhibited GS, have also both been implicated in the activation of NADH-dependent GDH [28], indicating that the role of GDH in $NH_4^+$ assimilation *vis-a-vis* GS may remain undecided until the significance of other factors such as these is understood. Certainly, crucial experiments excluding GDH from any role in symbiotic nitrogen assimilation have not so far been performed, but the primacy of GS is clearly indicated.

## EXPERIMENTAL

*γ-Glutamyltransferase assay.* GS transferase activity was assayed by a method modified from that of ref. [29]. The concns of reactants in 1 ml assay were: Hepes buffer, pH 7, 50 mM; L-glutamine, 25 mM; $NH_2OH$, 20 mM; $MnSO_4$ 1 mM; Na or K-ADP 0.5 mM; K-arsenate 20 mM and the enzyme. Reaction was initiated with the addition of GS. After 10 min incubation at 30°, the reaction was terminated by adding 1.5 ml of a mixture containing a 4-fold dilution of 10% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.2 M HCl, 24% (w/v) TCA and 50% HCl. The *A* at 540 nm was measured and the enzyme unit ($U_t$) was defined as 1 μmol glutamylhydroxamate formed/min using commercial γ-glutamylhydroxamate (Sigma) as a standard.

*Assay of GS by phosphate formation.* Reaction was carried out in a 0.2 ml mixture containing imidazole buffer (pH 7), 50 mM; $MgSO_4$, 30 mM; Na-glutamate, 24 mM and ATP, 10 mM. GS (1.3 m$U_{bs}$) was added and the reaction was terminated after 30 min with 1.8 ml of 0.8% $FeSO_4 \cdot 7H_2O$ in 7.5 mM $H_2SO_4$ and 0.15 ml of the ammonium mólybdate reagent of ref. [30] added and *A* measured at 660 nm.

*Biosynthetic GS assay.* A coupled enzyme system was used routinely to assay the ADP formed in the reaction, according to ref. [31]. The modified reaction mixture in 1 ml was: Hepes buffer, pH 7.9, 50 mM; KCl, 20 mM; $MgCl_2$, 5 mM; K-glutamate 50 mM; ATP, 2 mM; EDTA, 0.5 mM; phosphoenolpyruvate, 1 mM, lactate dehydrogenase, 17 U (37°); pyruvate kinase, 4 U (37°) and ammonia *ca* 26 mM, added in the coupling enzymes. The reaction mixture was incubated at 30° for 15–30 min to obtain a satisfactory blank before initiation with GS or glutamate.

*Protein* was determined by the method of ref. [32], using BSA as standard.

*Stabilization studies.* A 25–45% satd $[NH_4]_2SO_4$ fraction was dialysed against 0.05 M imidazole buffer, pH 7.3. One of a large number of possible stabilizers was added to a 1 ml aliquot of GS (5–10 $U_t$) and the mixture stored at 2–4°. Aliquots of enzyme containing about 0.3 $U_t$ of initial activity were assayed at intervals over several days.

*Purification of GS.* All steps were performed at 0–4° and centrifugations were at 13000 *g* for 15 min. Nodules were harvested when the yellow lupins (*Lupinus luteus* L. cv Weiko III) were in full bloom, frozen in liquid $N_2$ and stored at −20°. A crude extract was prepared from 400 g nodules by homogenization under a continuous stream of highly purified $N_2$ in the following buffer (buffer I): imidazole buffer, 50 mM (pH 7.3); sucrose, 0.4 M; 2% (w/v) soluble PVP; K-pyruvate, 10 mM; $MnSO_4$, 1 mM; and mercaptoethanol, 5 mM. The macerated tissue was squeezed through 4 layers of cheesecloth and centrifuged to remove debris and bacteroids. The total vol of extract was 380 ml.

Solid $(NH_4)_2SO_4$ was added slowly to the crude extract to 33% satn with continuous stirring and the pH was adjusted to 7.3. The soln was allowed to stand for 20 min and the resulting ppt. was centrifuged and discarded. The $(NH_4)_2SO_4$ concn was increased to 50% satn and the soln was recentrifuged after 20 min. The pellet was suspended in the 10 mM imidazole buffer (pH 7.3) with 10 mM K-pyruvate, 1 mM $MnSO_4$ and 5 mM mercaptoethanol (buffer II) and dialysed in the same buffer with two changes overnight. Undissolved proteins were discarded by centrifugation.

The 33–50% $(NH_4)_2SO_4$ fraction was applied to a DEAE-Sephacel (Pharmacia) column (2.6 cm × 21 cm) that had been equilibrated with the buffer II used above. The column was washed with 170 ml buffer and GS was eluted with a gradient of 0–0.4 M KCl in 300 ml buffer II followed by 0.4 M KCl. KCl concn was determined from conductivity measurements. The fractions (5.5 ml) of the main peak of GS activity (Fig. 3) were pooled and concd in an Amicon pressure cell (PM-10 membrane). The cell was flushed with $N_2$ for 15 min with stirring and each group of enzyme was concd to 12 ml at 207 kPa (30 lb/in$^2$). The concd enzyme was distributed into 4 ml fractions and frozen at −20°.

The enzyme was thawed and 4 ml was applied to a Sephacryl S-300 column equilibrated with buffer II. Fractions of 3.6 ml were collected and those of highest sp. act. were combined. This GS was absorbed to an 2′,5′-ADP-Sepharose (Pharmacia) column (0.8 cm × 7 cm) in buffer II. After extensive washing with buffer II, the enzyme was eluted with 5 mM K-ADP in buffer II and the high GS activity fractions were pooled and dialysed against the same buffer to remove ADP. The purified enzyme was kept frozen in 30–50% glycerol at −20°.

*Polyacrylamide gel electrophoresis* was performed at 4° according to the method of ref. [33] but using a single concn of gel (5%).

*Subunit $M_r$ determination.* GS subunit $M_r$ was determined by the modified method of ref. [34] using a 9% gel. Phosphatase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin were used as standards.

*$M_r$ determination.* GS $M_r$ was determined by gel filtration on a Sephacryl S-300 column (2.6 cm × 57.5 cm). Aldolase (158000), catalase (232000), ferritin (400000) and thyroglobulin (669000) were used as standards.

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