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THE INVOLVEMENT OF ASPARTATE AMINOTRANSFERASES IN AMMONIUM ASSIMILATION IN LUPIN NODULES

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Key Word Index—*Lupinus angustifolius*; Leguminosae; Uniwhite; *Rhizobium lupini*; nitrogen fixation; ammonium assimilation; aspartate aminotransferase; isoenzymes.

Abstract—Aspartate aminotransferase (AAT) activity has been detected in the plant and bacteroid fractions of lupin nodules, and in free-living *Rhizobium lupini*. Two electrophoretically distinct forms of AAT were detected in the plant fraction of the nodule and a third form in the bacteroid fraction. AAT activity increased in the plant fraction during nodule development and this increase may be due to an increase in the activity of one of the AAT forms in this fraction. The single form of AAT detected in the bacteroid fraction had the same electrophoretic mobility as that detected in free-living *R. lupini*. The nodulated roots of lupins, grown in a media supplemented with nitrate and ammonium, had a 3- and 4-fold lower activity of AAT and nitrogenase activity respectively, compared to the nodulated roots of plants grown in the absence of added nitrogen. A role for the plant AAT in ammonium assimilation in lupin nodules is proposed.

INTRODUCTION

Scott *et al.* [1] have proposed a pathway by which ammonium produced by nitrogen reduction in the bacteroids of lupin nodules is utilized by enzymes in the plant fraction of the nodule for the synthesis of asparagine. Evidence supporting the involvement of a plant glutamine synthetase [2], glutamate synthase [3] and asparagine synthetase [1] in this pathway has been previously published. In addition to these enzymes, the pathway requires an aspartate aminotransferase (L-aspartate: 2-oxo-glutarate aminotransferase, EC 2.6.1.1) to provide aspartate for the asparagine synthetase and 2-oxo-glutarate for the glutamate synthase reactions. The possible involvement of a plant or bacteroid aspartate aminotransferase (AAT) in ammonium assimilation was first suggested by Ryan *et al.* [4], from their studies of the enzyme in soybean nodules. AAT activity has also been detected in the plant fraction of white clover, soybean, pea and broad bean nodules [5]. The present paper reports the detection of AAT activity in the plant and bacteroid fractions of lupin nodules, and provides evidence supporting a role for the plant AAT in ammonium assimilation.

RESULTS

Aspartate aminotransferase activities in various fractions of lupin nodules and in free-living Rhizobium lupini

AAT activity was detected in the plant cytosol fraction of lupin nodules (Table 1). For convenience the enzyme was routinely assayed in the direction of aspartate utilization. When assayed in the direction of aspartate formation, the activity of the enzyme in the plant fraction was the same as that for the reverse direction; the ratio of the respective maximum velocities being 0.99. Electrophoresis on polyacrylamide gels showed that the plant fraction contained two electrophoretically distinct forms of the enzyme, AAT-P_1 and AAT-P_2 (Fig. 1).

Table 1. Aspartate aminotransferase activities in various fractions of 18-day-old lupin nodules, and in free-living *Rhizobium lupini*

Fraction	Aspartate aminotransferase activity (units/g fr. wt nodules)	(units/mg protein)
Plant cytosol	4.29	1.20
Crude bacteroid	0.561	0.399
R. lupini	—	0.042
Crude mitochondrial	0.015	0.546

A soluble protein fraction from the bacteroids of the nodules was prepared by a previously published differential centrifugation and washing procedure [3]. AAT activity was detected in both this extract and in a similar one prepared from the free-living form of *R. lupini* (Table 1). Examination of these extracts by electrophoresis on polyacrylamide gels revealed that the bacteroid fraction contained two forms of AAT, one corresponding to that found in the free-living *R. lupini* (AAT-R_1) and a second form with the same mobility as the AAT-P_2 found in the plant fraction (Fig. 1). The bacteroids prepared by this procedure were shown by electron microscopy to be still largely enclosed by the peribacteroid membranes. The possibility existed, therefore, that the AAT-P_2 detected in the crude bacteroid fraction (Fig. 1) was due to the association of this enzyme with the peribacteroid membrane of space. In addition, however, the electron

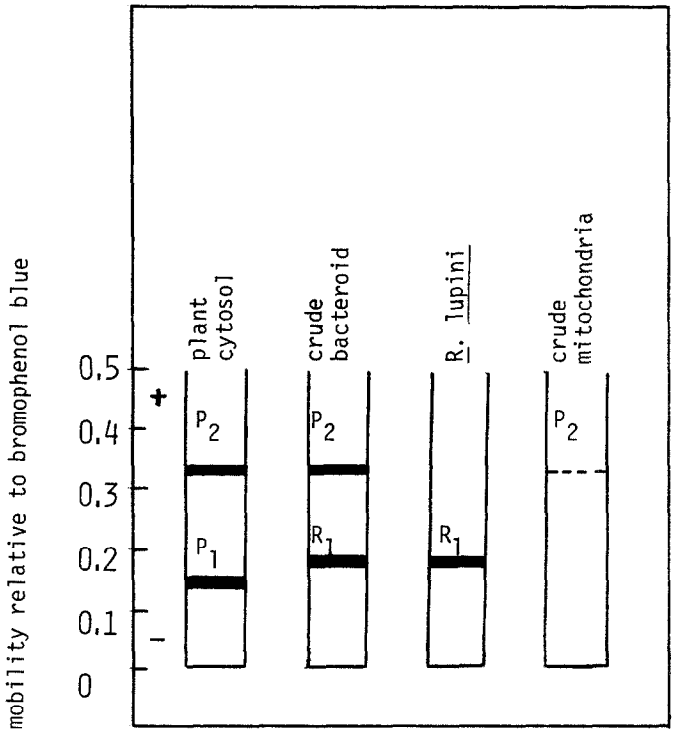

Fig. 1. Localization of aspartate aminotransferase activity on polyacrylamide gels following electrophoresis of the soluble protein fractions (20 μg protein/gel) from 18-day-old lupin nodules and *R. lupini*. The relative intensities of the aminotransferase bands are indicated by shading.

micrographs revealed that these bacteroid preparations were substantially, but not completely, free of cellular debris, mitochondria or plastids. Peribacteroid membrane-enclosed bacteroids, free of all other plant membranes and organelles, were then prepared by the sucrose step-gradient technique of Robertson *et al.* [7]. A cell-free extract of these membrane-enclosed bacteroids was found by polyacrylamide gel electrophoresis to contain both AAT-P_2 and AAT-R_1. Removal of the peribacteroid membrane by osmotic shock and the separation of this shock fraction (peribacteroid membranes and peribacteroid space material) from the intact bacteroids on a further sucrose step-gradient [7], gave a purified bacteroid preparation containing only AAT-R_1, whereas the shock fraction contained AAT-P_2 and trace amounts of AAT-R_1. Of the total AAT activity originally found in the peribacteroid membrane-enclosed bacteroids, 85% was recovered in this purified bacteroid fraction (AAT-R_1) with the remaining 15% being found in the shock fraction (AAT-P_2).

Table 2. NAD^+- and $NADP^+$-dependent isocitrate dehydrogenase activities in various lupin nodule fractions

Fraction	NAD^+-dependent isocitrate dehydrogenase (units/g fr. wt nodules)	$NADP^+$-dependent isocitrate dehydrogenase (units/g fr. wt nodules)
Plant cytosol	<0.004	0.46
Crude bacteroid	<0.003	0.49
Crude mitochondrial	0.023	<0.001

When expressed in terms of fr. wt, a crude mitochondrial fraction of the nodule had a low AAT activity compared to that of the plant cytosol fraction (Table 1). One band of AAT activity of the same mobility as the plant fraction AAT-P_2 was detected in this extract on polyacrylamide gels (Fig. 1). It was subsequently found that if the crude mitochondrial pellet used to prepare this fraction was washed a further 4 times, 80% of this AAT-P_2 activity was released into the wash solutions with only a 14% loss in NAD^+-dependent isocitrate dehydrogenase activity (a mitochondrial marker enzyme). We concluded, therefore, that most of the AAT-P_2 activity found in the crude mitochondrial extract was probably not of mitochondrial origin, and the problem of whether or not the mitochondria did contain AAT activity was not pursued.

The above results have shown that the plant cytosol fraction of the nodule contains AAT-P_1 and AAT-P_2 activity and that low levels of AAT-P_2 activity were found in the mitochondrial and peribacteroid membrane-enclosed bacteroid fractions. The plant fraction itself was not significantly contaminated with mitochondrial or bacteroid proteins since no NAD^+-dependent isocitrate dehydrogenase or AAT-R_1 was found in this fraction (Table 2 and Fig. 1). In addition, the absence of NAD^+-dependent isocitrate dehydrogenase activity in the bacteroid fraction (Table 2) supports the electron microscopic evidence above that this fraction

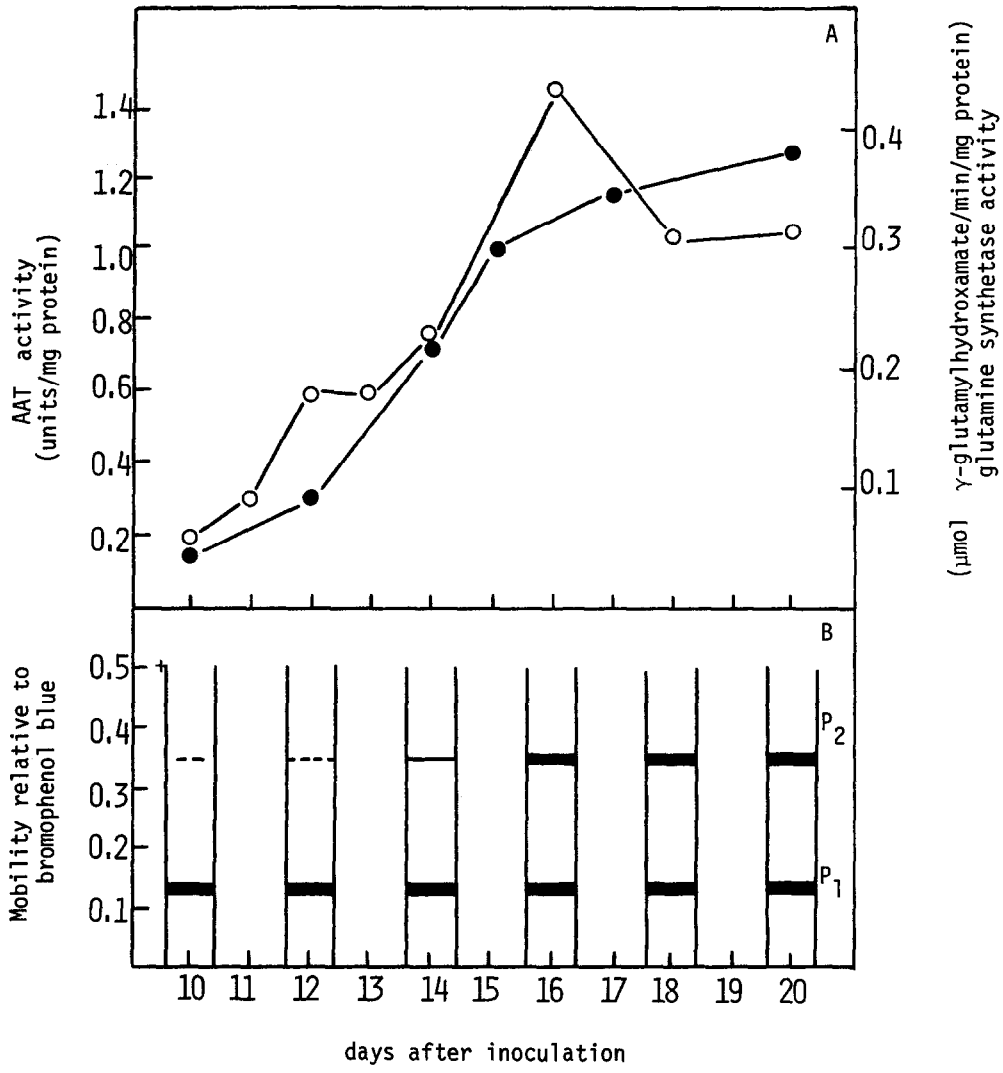

Fig. 2. (A) Aspartate aminotransferase (○) and glutamine synthetase (●) (replotted from ref. [3]) activities in the plant cytosol fraction of developing lupin nodules. (B) Aspartate aminotransferase from the plant cytosol fraction (20 μg protein/gel) of developing lupin nodules separated by polyacrylamide gel electrophoresis. The relative intensities of the aminotransferase bands are indicated by shading.

was substantially free of mitochondrial contamination. Conversely, the mitochondrial fraction was free of bacteroid contamination (no $NADP^+$-dependent isocitrate dehydrogenase and no AAT-R_1, Table 2 and Fig. 1).

Aspartate aminotransferase activity in lupin nodules during nodule development

AAT activity in the plant cytosol fraction of lupin nodules increased 5-fold in the period 10 to 18 days after rhizobial inoculation (Fig. 2A). The increase in activity seen after day 10 is similar to that already reported for the plant glutamine synthetase (ref. [3] and replotted here, Fig. 2A). Examination of the fractions by polyacrylamide gel electrophoresis revealed that the intensity of AAT-P_2 increased during nodule development, whereas the intensity of AAT-P_1 remained constant (Fig. 2B). The intensity of the bacteroid AAT-R_1 band (not shown here) did not change significantly during the same developmental period.

The effect of supplementary nitrogen on aspartate aminotransferase activity in nodulated and non-nodulated lupin roots

Lupin plants inoculated with rhizobia and supplied with a 'plus-nitrogen' nutrient solution exhibited poor nodule development and had a lower nitrogenase activity than that of nodulated plants supplied with a nitrogen-free nutrient solution (24 and 95 nmol ethylene produced/plant/min, respectively). Therefore, rather than attempt to prepare extracts of the few small nodules on the nitrogen supplemented plants, extracts were prepared from the nodulated tap roots of these plants. A similar

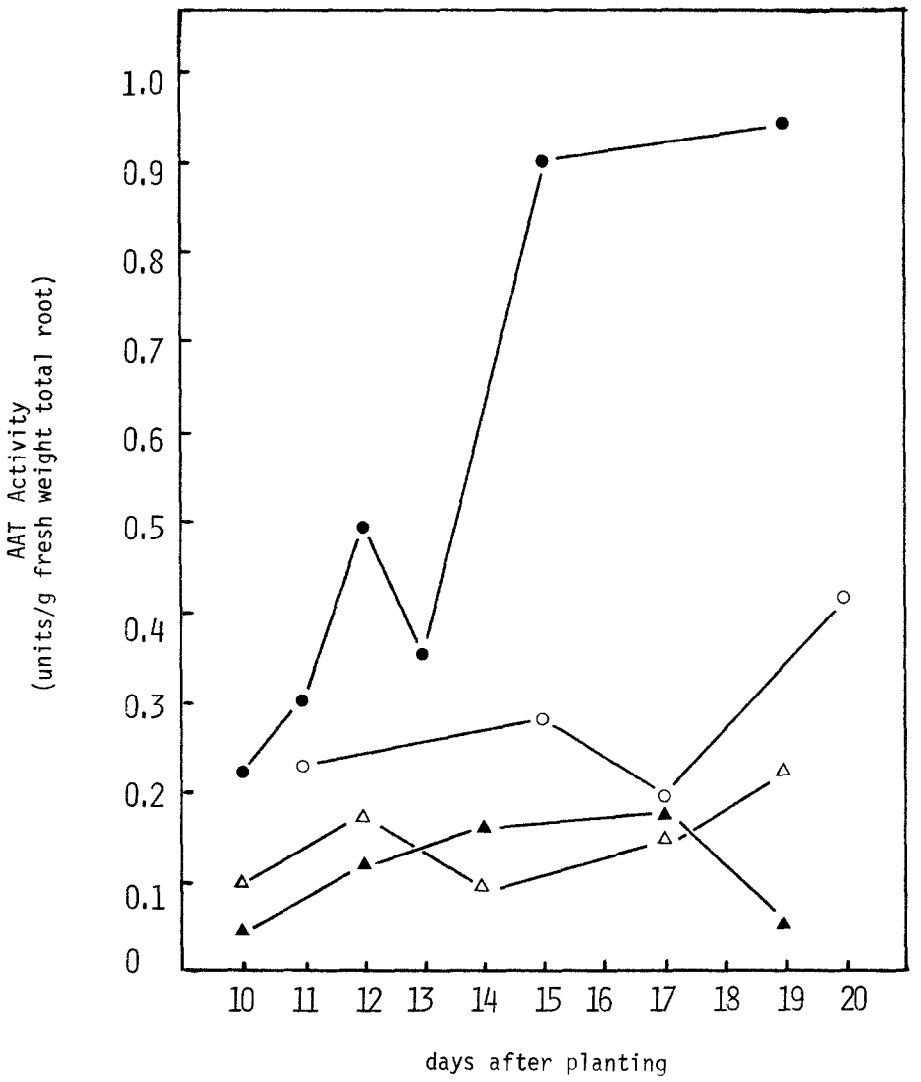

Fig. 3. Aspartate aminotransferase activities in the plant-soluble protein fraction of the roots of (i) nodulated plants grown in the absence (●) or presence (○) of added nitrogen and (ii) non-nodulated plants grown in the absence (▲) or presence (△) of added nitrogen. Fractions were prepared from plants at various times after planting.

root plus nodule extract was prepared from nodulated plants supplied with the 'nitrogen-free' nutrient solution. AAT activity was determined in these extracts at various times after rhizobial inoculation and the results are shown in Fig. 3. AAT activity in the extracts from the nodulated plants grown in the absence of added nitrogen increased in the time period 11 to 15 days after inoculation (Fig. 3). This increase was not seen in extracts from nodulated plants grown in the presence of supplementary nitrogen (Fig. 3). Root extracts from non-nodulated plants grown in the presence or absence of added nitrogen had low levels of AAT activity (Fig. 3). Electrophoresis on polyacrylamide gels showed that the root extracts from the nodulated plants supplied with either the plus-nitrogen or nitrogen-free nutrient solutions had similar levels of AAT-P_1 (Figs. 4A, B). However, the levels of AAT-P_2 in these same extracts differed markedly in that lower levels of AAT-P_2 were found in the root extracts from the nodulated plants supplied with added nitrogen (Figs. 4A, B). It was these plants that also had lower nitrogenase activity (see above).

DISCUSSION

Two electrophoretically distinct forms of aspartate aminotransferase (AAT-P_1 and AAT-P_2) were detected in the plant cytosol fraction of lupin nodules. A third form (AAT-R_1), was found in the bacteroids of the nodule and in broth cultured *R. lupini*. A further electrophoretically distinct form of AAT has been reported by Ryan *et al.* [4] in the mitochondria of soybean nodules. A separate mitochondrial AAT was not detected here in lupin nodules, but low levels of AAT-P_2 activity were found in the crude mitochondrial fraction. However, most of this activity was removed from this fraction by increasing the number of washes. This suggests that the

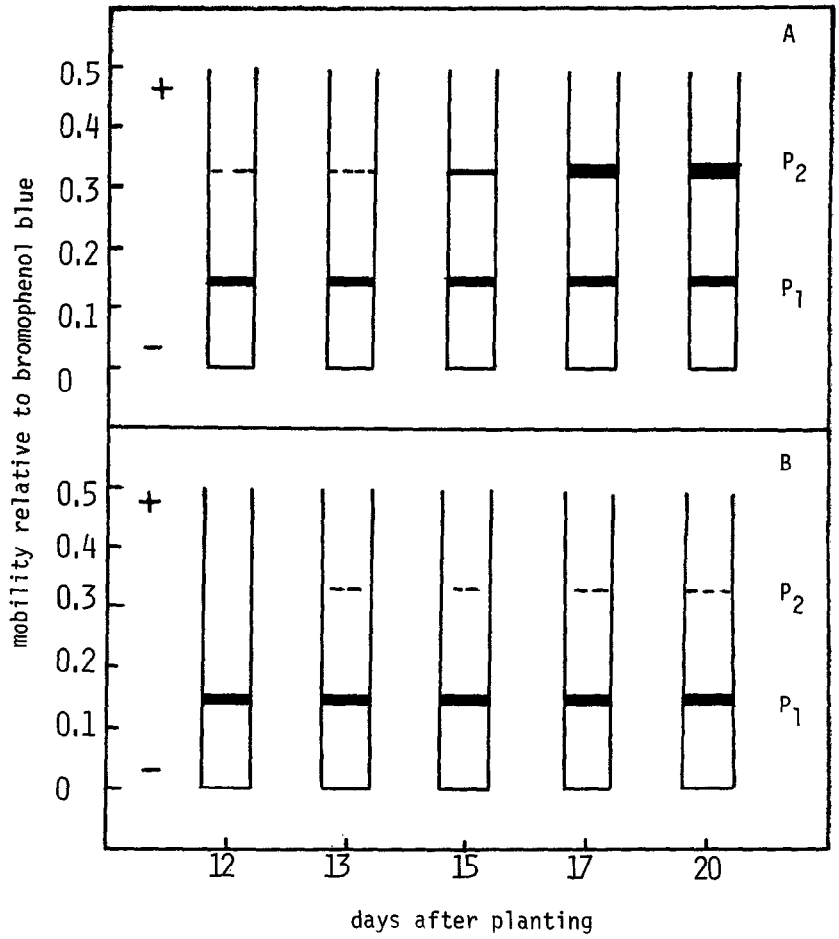

Fig. 4. Localization of aspartate aminotransferase activity on polyacrylamide gels following electrophoresis of the plant soluble protein fractions (20 μg protein/gel) from the nodulated roots of plants grown in the absence (A) or presence (B) of added nitrogen. Fractions were prepared from plants at various times after inoculation with *R. lupini* and planting. The relative intensities of the aminotransferase bands are indicated by shading.

AAT-P_2 activity was not of mitochondrial origin and may have come from the non-specific binding of the enzyme to the mitochondrial membranes; but the problem was not pursued here. The crude bacteroid fraction also contained AAT-P_2 activity. These bacteroids were then purified on sucrose step-gradients and were found to still contain AAT-P_2 as well as the rhizobial AAT-R_1. The bacteroids prepared by the techniques employed here are still enclosed by the peribacteroid membranes. Removal of these membranes by osmotic shock gave a bacteroid fraction containing only AAT-R_1. AAT-P_2 was found in the shock fraction which contained the peribacteroid membranes and peribacteroid space material. This result does not necessarily mean that AAT-P_2 is associated with the peribacteroid membrane or space *in vivo*. Non-specific binding of AAT-P_2 to the peribacteroid membrane during the fractionation procedure has not been discounted. Indeed, although the work reported here has demonstrated the presence of two forms of AAT in the plant fraction of the nodule, the subcellular localization of these enzymes within the nodule cell is a problem for future study.

A role for AAT in ammonium assimilation in lupin nodules has been proposed by Scott *et al.* [1]. These authors outlined a pathway for the synthesis of asparagine from oxaloacetate and ammonium involving the plant enzymes glutamine synthetase, glutamate synthase, asparagine synthetase and AAT. The results reported here support the inclusion of a plant AAT in this pathway. Firstly, the *in vitro* activity of AAT-P_1 plus AAT-P_2 is several-fold higher than that of both the rate of ammonium production by the bacteroid nitrogenase and the rate of asparagine transportation from the nodules. For example, in 18-day-old plants, the AAT activity in the plant fraction of the nodules expressed on a per plant basis, is 38 μmol aspartate produced/hr/plant. The nitrogenase activity of these same plants can be estimated as 7.7 μmol ammonium produced/hr/plant (using previously published data [2] and an ethylene/N_2 conversion factor of 3.9 [13]). Asparagine, which accounts for 70% of the nitrogen leaving the nodules as amino acids, is transported at a rate of 2 μmol/hr/plant in these 18-day-old plants [1]).Secondly, a role for a plant AAT in ammonium assimilation is supported by the increase in AAT activity found in the plant fraction of the nodule 11 days after inoculation with rhizobia (Fig. 2). The timing

of this increase in activity is similar to that observed previously for nitrogenase activity in the bacteroids [2] and leghaemoglobin [2], glutamine synthetase [2, 3], glutamate synthase [3] and asparagine synthetase [1] levels in the plant fraction of lupin nodules. Polyacrylamide gel electrophoresis studies revealed that this increase in plant AAT was due to the increase in specific activity of the AAT-P_2 form of the enzyme. No increases in AAT-P_1 or AAT-R_1 were detected during nodule development. A role for AAT-P_2 in ammonium assimilation in the nodule was further supported by the observation that a decrease in nitrogenase activity (produced by supplying the plants with nitrate and ammonium) resulted in a decrease in the level of AAT-P_2 in the nodulated roots.

Presently we are attempting to purify the two forms of the plant AAT to determine the kinetic characteristics of each form. It will be of interest to see if the kinetic properties of AAT-P_2 correlate with the proposed role of this enzyme in ammonium assimilation.

EXPERIMENTAL

Plant material and bacterial cultures. Lupins (*Lupinus angustifolius* var. Uniwhite) inoculated with *Rhizobium lupini* NZP2257 were grown in a controlled environment cabinet in stainless steel troughs containing sterile pumice [2]. Plants were supplied with either a N-free nutrient soln or one supplemented with 4.5 mM KNO_3, 4.5 mM $Ca(NO_3)_2$ and 0.9 mM NH_4NO_3. *R. lupini* was maintained on yeast extract–mannitol–agar slopes [6]. Liquid cultures were grown at 26° in a broth medium [2].

Preparation of enzyme extracts. Plant cytosol and crude bacteroid extracts of the nodules were prepared as described in ref. [3]. Peribacteroid membrane-enclosed bacteroids, free from all other plant membranes and organelles, were prepared by the sucrose step-gradient fractionation procedure of ref. [7]. The peribacteroid membranes were released from the bacteroids by osmotic shock and the shock fraction and bacteroids were separated on a further sucrose step-gradient [7]. A crude mitochondrial fraction was prepared by centrifuging the post-bacteroid supernatant [3] at 14500 *g* for 5 min. The pellet obtained was washed by resuspending in the homogenizing buffer and centrifuging at 6000 *g* for 5 min to remove any remaining bacteroids. The supernatant was centrifuged for a further 5 min at 14500 *g* to pellet the mitochondria. The final suspension of mitochondria was sonicated with intermittent cooling to give the crude mitochondrial fraction. A cell-free extract of the cultured *R. lupini* was prepared by the method of ref. [2].

Enzyme assays. AAT activity was assayed routinely in the direction of aspartate utilization by the method of ref. [8]. Assays in the reverse direction were carried out by the method of ref. [9]. NAD^+- and $NADP^+$-isocitrate dehydrogenase activities were estimated in extracts prepared in the presence of 5 M glycerol by the method of ref. [10]. Lowry protein determinations, nitrogenase and glutamine synthetase assays were carried out as described in ref. [2].

Localization of AAT on acrylamide gels. Tube gels, 7.5% acrylamide [11] were prepared with 0.1 M Tris–0.1 M glycine buffer, pH 8.9, which was also used in the electrophoresis reservoirs. Bromophenol blue was used as a marker dye. Following electrophoresis, the gels were immersed in the AAT staining solution of ref. [12].

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