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SUBCELLULAR LOCALIZATION OF ENZYMES INVOLVED IN THE ASSIMILATION OF AMMONIA BY SOYBEAN ROOT NODULES

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Key Word Index—*Glycine max*, Leguminosae, soya bean, nitrogen fixation, *Rhizobium japonicum*, root nodules, ammonia assimilation, enzyme localization

Abstract—Ammonia, the primary product of nitrogen fixation is rapidly incorporated into a number of amino acids such as glutamate and aspartate. A novel enzyme system glutamine 2-oxoglutarate aminotransferase oxidoreductase, which probably has an important role in ammonia assimilation has been detected, in the present studies, in the rhizobial fraction of soybean root nodules and in *Rhizobium japonicum* grown in culture. The role of this latter enzyme and other enzymes such as glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase in ammonia assimilation by soybean nodules is discussed.

INTRODUCTION

AMMONIA, the primary product of nitrogen fixation, causes repression of nitrogenase synthesis.[1–3] Therefore, to maintain a high rate of nitrogen fixation over extended periods of time, as in nodulated legumes,[4] it is obvious that an efficient system must exist in legume nodules for the assimilation of ammonia.

Detached serradella (*Ornithopus sativus*) nodules very rapidly incorporate newly-fixed nitrogen into glutamic acid, glutamine, aspartic acid, asparagine and alanine.[5] However, the metabolic pathways involved in the synthesis of these amino acids in nodules and the relative roles of the plant and bacterial components of the nodule in these syntheses have not been elucidated. It has been concluded[6] that the initial reaction in ammonia assimilation is catalysed by glutamate dehydrogenase (GDH), although Kennedy[5] has calculated that the activity of this enzyme in serradella nodules was not sufficient to account for the observed rate of ammonia assimilation. Furthermore, others have been unable to detect any GDH activity in nitrogen-fixing *Bacilli*[7] or in *Clostridium pasteurianum*.[8] Recently however, a novel enzyme system has been demonstrated in several nitrogen-fixing bacteria

Abbreviations glutamate dehydrogenase (GDH), aspartate aminotransferase (AAT), alanine aminotransferase (GPT), glutamine 2-oxoglutarate aminotransferase oxido-reductase (GOGAT), pyridoxal-5′-phosphate (PLP)

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[1] DALTON, H., POSTGATE, J. R. (1966) *J. Gen. Microbiol.* **56**, 307
[2] SMITH, R. V. and EVANS, M. C. W. (1971) *Nature* **225**, 1253
[3] OPPENHEIM, J. and MARCUS, L. (1970) *Bacteriol. Proc.* p. 148
[4] HARDY, R. F. W., HOLSTEN, R. D., JACKSON, E. K. and BURNS, R. C. (1968) *Plant Physiol.* **43**, 1185
[5] KENNEDY, I. R. (1966) *Biochim. Biophys. Acta* **130**, 285
[6] BERGERSEN, F. J. (1971) *Ann. Rev. Plant Physiol.* **22**, 121
[7] HONG, M. M., SHEN, S. C. and BRAUNSTEIN, A. E. (1959) *Biochim. Biophys. Acta* **36**, 288
[8] DAINTY, R. H. and PEEL, J. L. (1970) *Biochem. J.* **117**, 573

and in *Rhizobium*;[9–12] the enzyme, termed glutamine (amide) 2-oxoglutarate aminotransferase oxido-reductase (GOGAT) (or glutamate synthase) catalyses the irreversible reaction

$$\text{Glutamine} + \text{2-oxoglutarate} + \text{NAD(P)H}^{+} \rightarrow 2\,(\text{glutamate}) + \text{NAD(P)}^{+}$$

In conjunction with glutamine synthase, this GOGAT enzyme system will produce glutamate from 2-oxoglutarate and ammonia i.e. the same reactants and products involved in the GDH reaction.

In the present paper the specific activities and relative distribution of the enzymes GDH, GOGAT, aspartate aminotransferase (AAT) and alanine aminotransferase (GPT) in the cytosol, rhizobial and mitochondrial fractions of soybean root nodules are examined and possible roles are assigned to each enzyme in the assimilation of ammonia by root nodules.

RESULTS

Enzyme levels in nodule fractions

The specific activities and relative distribution of the enzymes GDH, GOGAT, AAT and GPT in the cytosol, rhizobial and mitochondrial fractions of soybean root nodules are given in Table 1. Details of the fractionation procedure and enzyme assays are given in the Experimental section. The degree of cross-contamination between fractions, as determined by starch gel zymograms specifically stained for AAT and GPT isoenzymes[13] was negligible. Previous studies[13] have shown that specific isoenzymes of AAT and GPT are present in cytosol and rhizobial fractions of soybean nodules and serve as useful markers for these fractions.

AAT and GOGAT were almost entirely confined to the nodule rhizobia, while GPT and GDH were almost exclusively located in the cytosol fraction. No significant amounts of any of the four enzymes were associated with the mitochondrial fraction.

Enzyme levels in Rhizobium japonicum *grown in free culture*

Rhizobia grown in free culture are not capable of fixing nitrogen but can assimilate ammonia from the growth medium.[14] These free-grown bacteria may thus serve as a useful substitute for nodule rhizobia in ammonia assimilation studies. In the present experiment, *Rhizobium japonicum* (strain 392) was grown in a defined medium with 14 mM NH_4Cl + 2·5 mM 2-oxoglutarate as nitrogen source.[14] The specific activities of the enzymes GDH, GOGAT, AAT and GPT from these bacteria and from rhizobia isolated from soybean root nodules are compared in Table 2. The total protein in both extracts was similar but all four enzymes were present in higher concentration in the free grown bacteria. This is probably attributable to the induction of these enzymes by ammonium chloride and 2-oxoglutarate in free grown bacteria.[14]

An interesting feature of these experiments was the affect of PLP on the activity of AAT from *Rhizobium japonicum* grown in free culture, where over 50% stimulation of enzyme

[9] MEERS, J. L., TEMPEST, D. W. and BROWN, C. M. (1970) *J. Gen. Microbiol.* **64**, 187
[10] ELMERICH, C. and AUBERT, J. B. (1971) *Biochem. Biophys. Res. Commun.* **42**, 371
[11] NAGATANI, H., SHIMIZU, M. and VALENTINE, R. C. (1971) *Arch. Microbiol.* **79**, 164
[12] DAINTY, R. H. (1972) *Biochem. J.* **126**, 1055
[13] RYAN, E., BODLEY, F. and FOTTRELL, P. F. (1972) *Phytochemistry* **11**, 957
[14] FOTTRELL, P. F. and MOONEY, P. (1969) *J. Gen. Microbiol.* **59**, 211

activity was obtained on addition of co-enzyme to the assay mixture (Table 2). No stimulation of AAT activity was obtained on addition of excess PLP to the nodule rhizobial fraction (Table 2) or to the nodule cytosol fraction (Table 1). This stimulation, in the case of rhizobia grown in free culture, is explained by the fact that over 50% of the AAT is present as the apo form.[17]

TABLE 1 SPECIFIC ACTIVITY AND RELATIVE DISTRIBUTION OF AAT, GPT, GDH AND GOGAT IN THE CYTOSOL, RHIZOBIAL AND MITOCHONDRIAL FRACTIONS OF SOYBEAN ROOT NODULES

Nodule fraction*	Enzyme† AAT	GPT	GDH	GOGAT
Cytosol				
Specific activity	0 005	0 007	0·007	0 001
% of Total	11	81	97	4
Rhizobial				
Specific activity	0 21	0 007	0 013	0 13
% of Total	87	15	3	96
Mitochondrial				
Specific activity	0 001	0 002	0 000	0·000
% of Total	2	4	0	0

* Details of fractionation procedure and enzyme assays are given in Experimental section
† Specific activity μmoles substrate min/mg protein % of Total activity· calculated with respect to total protein in each fraction

It has previously been shown[15–17] that the physico-chemical properties of AAT from *Rhizobium japonicum* were identical with those of the same strain of rhizobia isolated from root nodules, whereas AAT from the nodule cytosol was different. The coenzyme PLP was very loosely bound to the rhizobial enzyme but was tightly bound to nodule cytosal AAT. It may be that rhizobia grown under conditions where AAT synthesis is induced are not capable of synthesizing sufficient PLP to activate all the newly formed apo enzyme, whereas in the root nodule the plant tissue supplies coenzyme, thus making the full potential activity of rhizobial AAT available

TABLE 2 SPECIFIC ACTIVITY OF AAT, GPT, GDH AND GOGAT IN THE RHIZOBIAL FRACTION OF SOYBEAN ROOT NODULES IN *Rhizobium japonicum* GROWN IN DEFINED MEDIUM

Preparation from*	Specific activity† AAT	GPT	GDH	GOGAT
Nodule rhizobia	0 21 (0 21)	0 007 (0 003)	0 017	0 13
Rhizobium japonicum	0 77 (0 30)	0 033 (0 030)	0 09	0 27

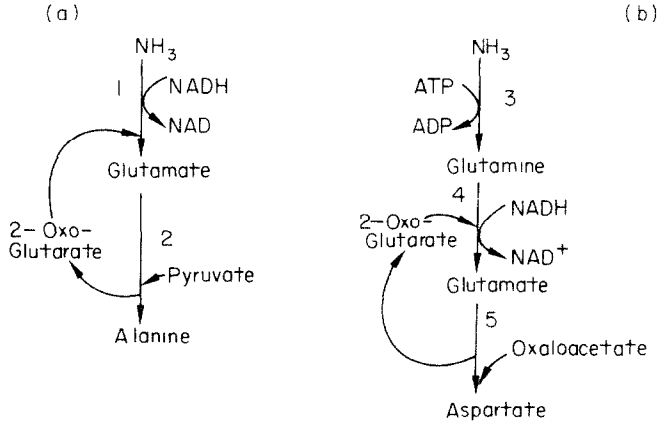
* Experimental details are given in the text Specific activity is expressed as μmoles substrate min/mg protein.
† Bracketed values represent the enzyme activity obtained when PLP was omitted from the assay system The activities of GDH and GOGAT were not affected by PLP

[15] RYAN, E., BODLEY, F and FOTTRELL, P F (1971) *Plant and Soil* (Special Vol) Proc Technical Meetings on Biological Nitrogen Fixation of the International Biological Programme, Prague and Wageningen, Lie, T. A and Mulder, E G, eds, p 545
[16] RYAN, E and FOTTRELL, P F (1972) *J Gen Microbiol* **70,** 395
[17] RYAN, E and FOTTRELL, P F (1972) *FEBS Letters* **23,** 73

DISCUSSION

The present studies have shown that the enzyme GOGAT was found almost exclusively in the rhizobial component of soybean nodules and that the specific activity of rhizobial GOGAT was ten times higher than GDH.

Jordan[18] found high glutamine synthetase activity in both rhizobial and cytosol fractions of soybean root nodules. It is therefore proposed that the glutamine synthetase-GOGAT couple catalyses the first step of ammonia assimilation in soybean rhizobia. Glutamate production is dependent on the supply of ammonia, ATP, 2-oxoglutarate and $NADH^+$ and this glutamate in turn serves as substrate for AAT and GPT, yielding aspartate and alanine respectively. The kinetic constants of rhizobial AAT are consistent with this enzyme operating in the direction of aspartate formation.[19] A proposed pathway for ammonia assimilation (Scheme 1) requires a constant supply of 2-oxoglutarate, oxaloacetate and pyruvate and the relative amounts of the nitrogenous products synthesized will be determined by the supply of these keto acids. Since glutamic acid and glutamine are, in fact, the principal products formed,[5] 2-oxoglutarate is probably the principal keto acid substrate available for amination. Keele *et al.*,[20] have shown that in *Rhizobium japonicum*, glucose was converted to pyruvate solely by the Entner–Doudoroff pathway[21] and that these bacteria also contained an active TCA cycle. The 2-oxoglutarate required for amination is presumably diverted from the TCA cycle, and the cycle is replenished by oxaloacetate formed from pyruvate by the enzyme pyruvate carboxylase.[22]



Scheme 1. PROPOSED PATHWAYS OF AMMONIA ASSIMILATION IN CYTOSOL (A) AND RHIZOBIAL (B) COMPONENTS OF SOYBEAN ROOT NODULES.
Enzymes: (1) GDH, (2) GPT, (3) glutamine synthetase, (4) GOGAT, (5) AAT. For further details see text.

It is of note that one molecule of ATP is required per molecule of ammonia assimilated. In this connection, Minchin and Pate[23] calculated that 62% of the carbohydrate translocated from the photosynthesizing shoot to the nodulated roots of *Pisum sativum* was lost

[18] JORDON, D. C. (1962) *Bacteriol. Rev.* **26**, 119.
[19] RYAN, F. and FOTTRELL, P. F. In preparation.
[20] KEELE, B. B., HAMILTON, P. B. and ELKAN, G. H. (1969) *J. Bacteriol.* **97**, 1184.
[21] MELOCHE, H. P. and WOOD, W. A. (1964) *J. Biol. Chem.* **239**, 3505.
[22] LILLICH, T. T. and ELKAN, G. H. (1971) *Can. J. Microbiol.* **17**, 683.
[23] MINCHIN, F. and PATE, J. F. (1971) personal communication.

in respiration while 23% was returned back to the shoot in the form of nitrogenous compounds. The remaining 15% was consumed in growth (dry matter accumulated) of the roots and nodules.

It is also possible that some NH_3 is secreted from the nodule rhizobia into the nodule cytosol and is assimilated by enzymes originating from the legume. In this case GDH may catalyse glutamate formation, which could then serve as substrate for cytosol AAT and GPT. The relative quantity of glutamate metabolized by these transaminases is not known but since the cytosol fraction contains 81% of the total GPT activity of the nodule but only 11% of the total AAT activity, it is probable that the more favoured pathway is in the direction of alanine formation. Note, that the transamination reaction will regenerate 2-oxoglutarate and thus, assuming that the main flux through the pathway is in the direction of alanine formation, stoichiometric quantities of pyruvate only are required. In postulating separate sites of ammonia assimilation within the nodule, it may be noted that the ^{15}N labelling experiments of Kennedy[5] determined only the sum of assimilation by both rhizobial and legume components since no attempt was made to fractionate the nodules prior to extraction of the labelled nitrogenous compounds.

The physiological significance of loosely bound pyridoxal coenzyme to rhizobial AAT[16] is not known at present. An interesting possibility might be that free PLP is directly aminated by rhizobial nitrogenase to form PMP which would then serve as an intermediate carrier of ammonia to the apotransaminase. This could explain the observation made by Kennedy[5] "that (free) ammonia is not the only vehicle of nitrogen flow (from nitrogenase) to amino acids and other organic compounds" While such coenzyme transfer between nitrogenase and AAT has not been demonstrated, there is very good evidence for transfer of PLP between rat liver cystathionase and pig heart AAT.[24]

EXPERIMENTAL

Plants Soybeans (*Glycine max* var Vent, seeds obtained from U S Department of Agriculture, Beltsville Md) were grown in nitrogen free medium and inoculated as described previously[25] with *Rhizobium japonicum* strain 392

Nodule fractionation Nodules were removed after 6 weeks growth, crushed and extracted with 0 5 vols of 0 4 M sucrose in 50 mM Tris–4 cl buffer pH 7 5 The brei was filtered through cheesecloth and centrifuged at 34000 *g* for 20 min The supernatant was the cytosol fraction The residue was washed several times with buffered sucrose and fractionated by differential centrifugation to give rhizobial (10000 *g* for 15 min) and mitochondrial (30000 *g* for 15 min) fractions The entire fractionation procedure was performed at 4°

Bacteria Rhizobium japonicum and rhizobia isolated from soybean root nodules were disrupted by violent agitation with Ballatoni beads in a Braun (Melsungen, Germany) disintegrator The following components were mixed to a thick paste and cooled to 2° before disintegration 10 g (wet wt) bacteria + 40 g Ballatoni beads (0 17–0 18 mm) + 10 ml 50 mM Tris–HCl pH 7 5 The disintegrator was operated for 40 sec during which time the temperature within the extraction vessel was maintained below 10° with solid CO_2 Cell debris and glass beads were removed by centrifugation at 34000 *g* for 30 min

Enzyme assays Aspartate aminotransferase was assayed by the method of Bergmeyer and Bernt[26] in which the product oxaloacetate was converted to malate by malate dehydrogenase and the concomitant oxidation of NADH was monitored at 340 nm Glutamate pyruvate transaminase was assayed by a similar method in which the product pyruvate was converted to lactate by lactate dehydrogenase Glutamate dehydrogenase and glutamate synthase were assayed as outlined by Meers *et al* [9] The final concentration of each component (in a total volume of 3 0 ml) was as follows 5 mM 2-oxoglutarate, 0 1 mM $NADH_2$, 50 mM Tris–HCl pH 7 5, diluted

[24] CHURCHICH, J E (1970) *Biochim Biophys Res Commun* **40,** 1374
[25] FOTTRELL, P F (1966) *Nature* **210,** 198
[26] BERGMEYER and BERNT (1963) in *Methods of Enzymatic Analysis* (BERGMEYER, H U , ed) p 837 Academic Press, London

enzyme extract, 40 mM NH_4Cl (glutamate dehydrogenase assay), or 5 mM glutamine (glutamate synthase assay) Protein was estimated by a modified Biuret method [27]

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[27] GORNAL, A G, BARDAWILL, C J and DAVID M M (1949) *J Biol Chem* **177**, 751