INDUCTION OF GLUTAMATE SYNTHASE DURING NODULE DEVELOPMENT IN LUPIN

J. G. ROBERTSON and Marion P. WARBURTON
Applied Biochemistry Division, DSIR, Palmerston North, New Zealand

and

K. J. F. FARNDEN
Biochemistry Department, University of Otago, Dunedin, New Zealand

Received 13 March 1975

1. Introduction

The enzyme system comprising of glutamine synthetase (EC.6.3.1.2) and glutamine synthase (EC.2.6.1.53) has been suggested to play a role in ammonia assimilation in microorganisms [1] and in higher plants [2,3]. These enzymes have also been reported to occur in both the bacteroid and plant fractions of lupin nodules [4,5] although the physiological significance of the system in nodules is uncertain.

One approach to the problem of deciding which enzymes are important in the assimilation of the ammonia produced by the bacteroids as the first stable product of N-fixation [6], is to determine which enzymes increase in activity during nodule development. Recently it was shown that glutamine synthetase was induced in the plant fraction of lupin nodules over a time course which paralleled the induction of leghaemoglobin and nitrogenase [7]. Gel electrophoresis indicated that the induced glutamine synthetase was of plant, rather than bacteroid origin.

We now report that glutamate synthase is also induced in the plant fraction, but not in the bacteroid fraction, of lupin nodules during nodule development. The implication of this finding in relation to assimilation of ammonia, produced by the bacteroids during N-fixation, is discussed.

2. Experimental

Lupins (*Lupinus angustifolius* L-Uniwhite) inoculated with rhizobium (*Rhizobium lupini* NZP 2257) were grown in a controlled environment cabinet with a day length of 12 hr and a day/night temperature regime of 24/21°C [7]. Nodules were picked and crushed in 2 vol (w/v) of 0.5 M sucrose, 50 mM Tris–HCl (pH 8.0), 10 mM dithiothreitol, using a pestle and mortar at 4°C. The macerate was filtered through Miracloth (Chicopee Mills Inc. New York) and centrifuged at 300 *g* for 5 min. The supernatant was centrifuged at 6000 *g* for 5 min to yield the crude bacteroid pellet and a supernatant which was recentrifuged at 50 000 *g* for 30 min. The supernatant from this step was passed through Sephadex G25 [8] swollen in 10 mM Tris–HCl (pH 8.0), to yield the soluble protein fraction derived from the plant material of the nodule. The crude bacteroid pellet was resuspended in 0.5 M sucrose, 50 mM Tris–HCl (pH 8.0), 2 mM dithiothreitol, and washed twice by centrifuging at 6000 *g* for 5 min and resuspending. Care was taken not to resuspend the white starch-containing organelles which occurred underneath the bacteroids. The final suspension of morphologically intact bacteroids, which was demonstrated using electron microscopy to be free from other organelles, was sonicated with intermittent cooling and then centrifuged at 50 000 *g* for 30 min. The supernatant was passed through Sephadex G25

to give the soluble protein fraction derived from the bacteroid material of the nodule.

The nodule plant and nodule bacteroid soluble protein fractions, hereafter called the plant and bacteroid fractions, were assayed approximately 4 hr after crushing the nodules for glutamate synthase [9], glutamine synthetase [10] or glutamate dehydrogenase [11]. Assays containing no substrate were used as controls. Protein was determined by the Lowry method [12] following precipitation with trichloroacetic acid.

Xylem sap from 22 day old plants was collected using a pressure chamber [13] and stored under liquid nitrogen. The amino acids in the sap were separated two-dimensionally on paper by electrophoresis and chromatography and located by spraying with ninhydrin [14]. Amino acid analyses were carried out using a JEOL 6A-H analyser.

3. Results and discussion

Glutamate synthase activity was detected in the plant and the bacteroid fractions from lupin nodules (table 1). Activity was lost rapidly when fractions were held at room temperature. The loss of activity on storage at 0°C was approx. 2%/hr for the plant enzyme and 27%/hr for the bacteroid enzyme. The glutamate synthase activity in both plant and bacteroid fractions was specific for NADH (table 1). The different NADH, NADPH requirements of the glutamate synthase and the glutamate dehydrogenase activities (table 1) showed that the observed glutamate synthase activity was not due to combined glutaminase–glutamate dehydrogenase activity [4] since NADH-linked glutamate dehydrogenase activity was not detected. It seemed unlikely that the plant glutamate synthase could have arisen by leakage from the bacteroids since previous studies [15], carried out under similar conditions, had shown inverse levels of fumarase and invertase in the plant and bacteroid fractions, suggesting that significant intermixing of these fractions had not occurred. Evidence confirming that the plant and bacteroid enzymes were different was obtained from a study of the effects of various compounds on enzymic activity (table 2). It was of interest, though expected [9], that both glutamic acid and glycine inhibited enzyme activity. Both these amino acids are present in the plant fraction from nodules unless removed by dialysis or gel filtration. This fact, together with the observed instability of the enzymes, possibly explains why glutamate synthase activity has not previously been detected in the plant fraction from nodules [16–18].

The level of glutamate synthase activity increased in the plant fraction over the same time course as glutamine synthetase (fig.1). There was little change in the activity of the bacteroid glutamate synthase over the period of the most rapid increase in the plant enzyme. The initial fall in bacteroid glutamate synthase activity and the subsequent rise (fig.1)

Table 1
Activities and co-factor requirements of glutamate synthase and glutamate dehydrogenase in plant and bacteroid soluble protein fractions from nodules from 18 day old plants

Nodule fraction	Cofactor	Glutamate synthase (nmol glutamate/min/mg protein)	Glutamate dehydrogenase (nmol glutamate/min/mg protein)
Plant	NADH	60.6	0
	NADPH	0	7.0
Bacteroid	NADH	43.2	0
	NADPH	0	5.0

Table 2
Effects of various compounds on glutamate synthase activity in plant and bacteroid soluble protein fractions from lupin nodules from 18 to 22 day old plants

Compound	Inhibition (−) or stimulation	
	Plant	Bacteroid
	%	%
L-glutamate	−81 ± 1	−93 ± 2
glycine	−14 ± 2	−30 ± 2
L-cysteine	27 ± 9	−29 ± 6
NH_4Cl	40 ± 6	4 ± 8
$(NH_4)_2SO_4$	82 ± 6	19 ± 11
Na_2SO_4	85 ± 2	12 ± 7

Assay mixtures (1 ml) contained 0.16 mM NADH, 0.25 mM α-ketoglutarate, 0.5 mM L-glutamine, 1 mM EDTA, 50 mM HEPES, pH 7.5 and 50 mM indicated compound. Assays were carried out in triplicate and standard errors are presented.

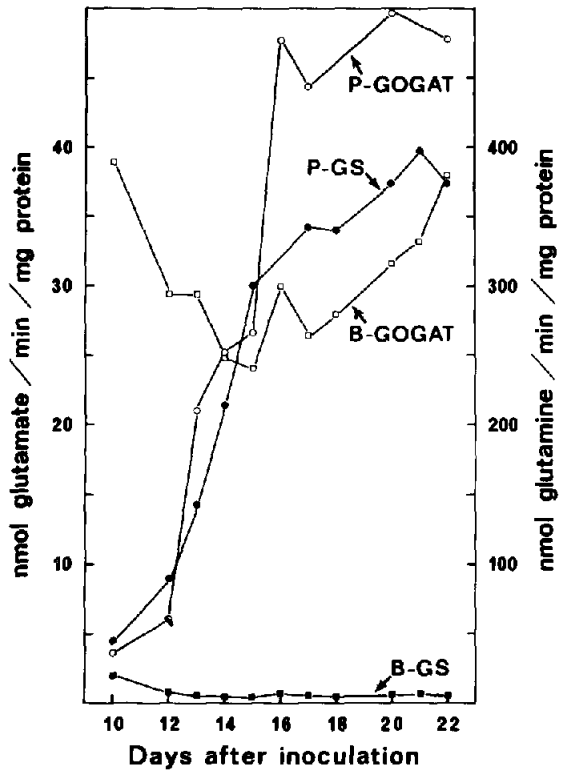

Fig.1. Induction of glutamine synthetase and glutamate synthase during nodule development in lupin. Nodules were collected from plants harvested at intervals following inoculation and the plant and bacteroid soluble protein fractions were prepared and assayed. (■) glutamine synthetase activity in the bacteroid soluble protein fraction (B-GS). (□) glutamate synthase activity in the bacteroid soluble protein fraction (B-GOGAT). (●) glutamine synthetase activity in the plant soluble protein fraction (P-GS). (○) glutamate synthase activity in the plant soluble protein fraction (P-GOGAT).

represented less than a 2-fold change in activity in comparison with a 10-fold change in activity in the plant fraction. The physiological significance of the change in activity of bacteroid glutamate synthase should be interpreted with caution, especially in view of the instability of the enzyme. The activity of glutamine synthetase in the bacteroid fraction showed an initial fall. This decrease was consistent with previous analyses [7] which showed that the level of glutamine synthetase in cultured rhizobia was 10- to 20-fold higher than in bacteroids from 21 day old plants.

The low levels of glutamate dehydrogenase activity in both the plant and bacteroid fractions compared to the activities of glutamine synthetase, and glutamate synthase (table 1 and fig.1) support the view [4] that these latter enzymes are of greater importance than glutamate dehydrogenase for the assimilation of ammonia. The observation that the enzymic activity of glutamine synthetase and of glutamate synthase increased dramatically in the plant fraction and not in the in the bacteroids at the onset of nitrogen reduction strongly suggests that this enzyme system plays a major role in ammonia assimilation. It follows that ammonia is very probably the major product of N-fixation excreted from the bacteroids in vivo as it is in vitro [19,20]. Furthermore, if the in vitro activities of the bacteroid and plant glutamine synthetases do reflect their activity in vivo, then calculations based on the rate of ammonia production during N-fixation by 19 day old plants [7] show that only 3% of this ammonia could be assimilated by the bacteroid glutamine synthetase whereas the plant enzyme could assimilate more than 2-fold the amount of ammonia actually produced. The presence of the glutamine synthetase-glutamate synthase system in the plant fraction avoids the requirement for carbon compounds to be transported into the bacteroids simply to provide a carrier for ammonia excreted from the bacteroids in the form of amino aicds. The system would be consistent with ^{15}N studies in which the highest enrichment of ^{15}N in amino compounds from root nodules of *Myrica* was found in the amide nitrogen of glutamine [21].

It is of interest that the bacteroid glutamine synthetase was less active than the bacteroid glutamate synthase, whereas the reverse was true for the plant enzymes (fig.1). It is tempting to speculate that the decreasing level of glutamine synthetase found in the bacteroid during nodule development resulted from ammonia repression, a situation known to occur in free living rhizobia [4]. However, if the bacteroid glutamine synthetase is a regulator of nitrogenase synthesis as has been found in *Klebsiella* [22,23], then the level of ammonia in the bacteroid is probably critical since complete ammonia repression of the glutamine synthetase would ultimately result in the cessation of nitrogenase systhesis. The lack of any regulation of the bacteroid glutamate synthase by ammonia, as suggested by Dilworth [4], could account for the higher levels of this enzyme maintained

Table 3
Amino acid analyses of xylem sap from 22-day old plants

Amino acid[a]	μmol amino acid/ml sap
Asparagine[b]	3.30
Glutamine[b]	0.60
Aspartate	0.25
Glutamate	0.20
Serine	0.16
Glycine	0.09
Alanine	0.04
Valine	0.08

[a] The level of all other acidic and neutral amino acids was less than 0.01 μmol/ml sap.

[b] Determined as the increase in aspartate or glutamate on hydrolysis for 4 hr at 105°C in 1 N HCl.

in the bacteroid. Conversely, the increasing level of glutamine synthetase found in the plant fraction could be due to induction by ammonia excreted from the bacteroid. The levels of glutamine synthetase in lupins and peas have in fact been shown to increase following $(NH_4)_2SO_4$ uptake [7,24].

A two dimensional separation on paper of the amino acids in the xylem sap of nodulated lupins followed by ninhydrin spraying revealed that asparagine was the predominant amino acid. Glutamine, and to a lesser extent aspartate, glutamate, and glycine were also detected on the chromatogram. Amino acid analysis (table 3) of an aliquot of the sap confirmed that asparagine was the predominant amino compound present, amounting to 70% of the total. Glutamine levels represented a further 13% of the total. Low levels of aspartate, glutamate, serine, glycine, alanine and valine were also found (table 3). If the ammonia produced by N-fixation is assimilated into glutamine and glutamate in the plant fraction, then it seems likely that the asparagine transported from the nodule to the aerial parts of the plant would be synthesised by the plant glutamine dependent asparagine synthetase [25,26]. Results from ^{15}N studies support such a sequence of reactions for ammonia assimilation [27].

In conclusion we have shown that glutamine synthetase and glutamate synthase are induced in the plant, but not in the bacteroid fraction of lupin nodules and that this ammonia assimilating system is more active than glutamate dehydrogenase. It is suggested that this is strong evidence for a pathway in which the bacteroids excrete ammonia which is then assimilated through the plant glutamine synthetase-glutamate synthase enzyme system.

Acknowledgements

We wish to thank Mrs Sally Roughan for growing the plants, Dr M. F. Beardsell and Professor M. J. Dilworth for helpful advice and Dr G. S. Bailey for the amino acid analyses.

References

[1] Tempest, D. W., Meers, J. L. and Brown, C. M. (1970) Biochem. J. 117, 405–407.
[2] Lea, P. J. and Miflin, B. J. (1974) Nature 251, 614–616.
[3] Fowler, M. W., Jessup, W. and Sarkissian, G. S. (1974) FEBS Lett. 46, 340–342.
[4] Dilworth, M. J. (1974) Ann. Rev. Plant Physiol. 25, 81–114.
[5] Sloger, C. (1973) Plant Physiol. 57, 34s.
[6] Bergersen, F. J. (1965) Aust. J. Biol. Sci. 18, 1–9.
[7] Robertson, J. G., Farnden, K. J. F., Warburton, M. P. and Banks, J. M., Aust. J. Plant. Physiol. in press.
[8] Neal, M. W. and Florini, J. R. (1973) Anal. Biochem. 55, 328–330.
[9] Miller, R. E. and Stadtman, E. R. (1972) J. Biol. Chem. 247, 7407–7419.
[10] Elliott, W. H. (1955) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 2 pp. 337–342, Acad. Press, London and New York.
[11] Schmidt, E. (1963) in: Methods of Enzymatic Analysis (Bergmeyer, H. U., ed). pp. 752–756. Acad. Press, New York and London.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Boyer, J. S. (1967) Plant Physiol. 42, 133–137.
[14] Lees, E. M., Farnden, K. J. F. and Elliot, W. H. (1968) Archives Biochem. Biophys. 126, 539–546.
[15] Robertson, J. G. and Taylor, M. P. (1973) Planta 112, 1–6.
[16] Dunn, S. D. and Klucas, R. V. (1973) Canad. J. Microbiol. 19, 1493–1499.
[17] Ryan, E. and Fottrell, P. F. (1974) Phytochem. 13, 2647–2652.
[18] Brown, C. M. and Dilworth, M. J. (1975) J. Gen. Microbiol. 86, 39–48.
[19] Bergersen, F. J. and Turner, G. L. (1967) Biochim. Biophys. Acta 141, 507–15.
[20] Koch, B., Evans, H. J. and Russell, S. A. (1966) Plant Physiol. 41, 1748–50.

[21] Leaf, G., Gardner, I. C. and Bond, G. (1959) Biochem. J. 72, 662–667.
[22] Streicher, S. L., Shanmugam, K. T., Ausubel, F., Morandi, C. and Goldberg, R. B. (1974) J. Bact. 120, 815–821.
[23] Tubb, R. S. (1974) Nature 251, 481–485.
[24] Evstigneeva, Z. G., Aseeva, K. B., Motchalkina, N. A. and Kretovich, W. L. (1971) Biokhimiya 36, 388–392.
[25] Rognes, S. E. (1970) FEBS Lett. 10, 62–66.
[26] Kanamori, T. and Matsumoto, H. (1975) Z. Pflanzenphysiol. Bd. 74, s. 264–266.
[27] Kennedy, I. R. (1966) Biochim. Biophys. Acta 130, 295–303.