Phytochemistry, Vol. 21, No. 3, pp. 505–507, 1982.
Printed in Great Britain.

0031-9422/82/030505-03$03.00/0

ENZYMES OF AMMONIA ASSIMILATION IN ROOT NODULES OF *TRIGONELLA FOENUM-GRAECUM*

C. S. NAUTIYAL and V. V. MODI

Department of Microbiology, Faculty of Science, M. S. University of Baroda, Baroda 390 002, India

(*Revised received* 15 *June* 1981)

Key Word Index—*Trigonella foenum-graecum*; Leguminosae; nitrogen fixation; root nodules; nodule cytosol; bacteroids; ammonia assimilation.

Abstract—The main pathway of ammonia assimilation in the root nodules of *Trigonella foenum-graecum* is via nodule cytosol glutamine synthetase–glutamate synthase.

INTRODUCTION

Ammonia, the primary end-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 for the assimilation of ammonia[5]. The assimilation of ammonia in legume nodules may proceed via either glutamate dehydrogenase (GDH; EC 1.4.1.3) or the glutamine synthetase–glutamate synthase (GS; EC 6.3 1.2/GOGAT; EC 2.6 1.53) pathway[6–8]. However, the presence of GDH and the GS/GOGAT pathway in symbiotic nitrogen-fixing associations have been the subject of several contradictory reports[9–15]. In the present communication, therefore, an attempt has been made to ascertain the probable pathway for ammonia assimilation in the nodules of *Trigonella foenum-graceum* L.

RESULTS

The nitrogenase activity of the intact root nodules of *T. foenum-graecum* was maximal 5 weeks after planting (Fig. 1A). The activities of the nodule cytosol GS (Fig. 1B) and GOGAT (Fig. 1C) increased simultaneously with the increase in the nitrogenase activity up to the fifth week of plant growth after which they declined. The decrease in the ammonia assimilatory enzyme activities in the older tissues was not due to the presence of inhibitors in the older tissues, since mixing crude cell-free extracts from older tissues (10-week-old root nodules) with crude cell-free extracts of 5-week-old tissues (where the levels of all the enzymes were maximal), did not inhibit the enzyme activities of the 5-week-old tissues (data not presented). There was no significant change in the activities of bacteriodal GS (Fig. 1B) and GOGAT (Fig. 1C). Unlike GS/GOGAT, the levels of

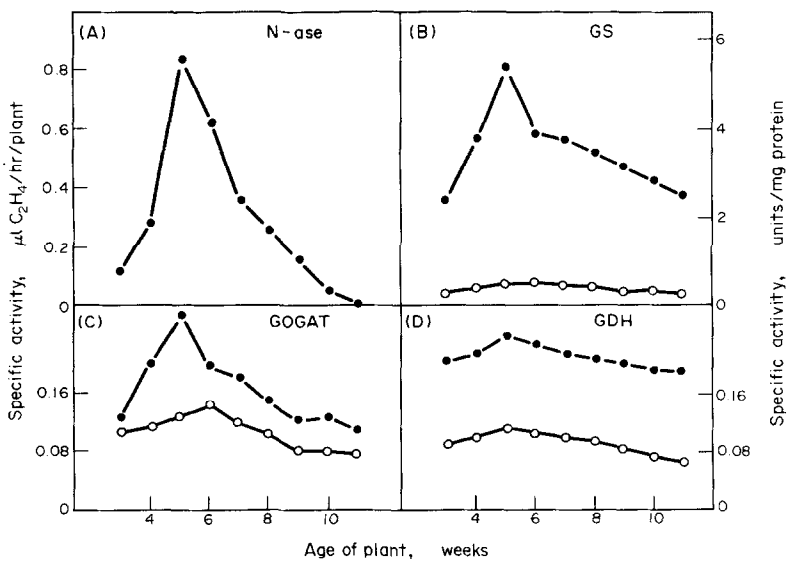

Fig. 1. Activities of different enzymes in developing root nodules of *T. foenum-graecum*. (A) Nitrogenase activity in intact root nodules (●–●); (B) GS activity in nodule cytosol (●–●) and bacteroid (○–○); (C) GOGAT activity in nodule cytosol (●–●) and bacteroid (○–○); (D) GDH activity in nodule cytosol (●–●) and bacteroid (○–○).

GDH (Fig. 1D) in both the nodule cytosol and bacteroidal fractions did not change significantly throughout plant growth.

DISCUSSION

Studies in which $^{15}N_2$ was fed to detached nodules have indicated that ammonia is the first stable product of dinitrogen fixation by nitrogenase[16]. The bacteroid fraction of nodules is without any doubt, the site of nitrogen fixation[17]. Many of the early experiments carried out on the mechanism of ammonia assimilation were based on the fundamental assumption (which has recently been challenged) that ammonia is assimilated in the bacteroid and then excreted into the nodule cytosol as amino acids. This theory is supported by the demonstration of the presence of both nitrogenase and ammonia assimilatory enzymes in the bacteroid[18–20]. However, it has now been suggested that although some of the ammonia produced by bacteroid nitrogenase may be assimilated by the bacteroids for cell growth and maintenance, a large proportion of it is excreted from the bacteroids and is assimilated in the nodule cytosol[21–24].

The observation that the levels of ammonia assimilatory enzymes are less in bacteroids as compared to the nodule cytosol, further suggests the significant role played by the ammonia assimilatory enzymes of nodule cytosol in the fixation of ammonia excreted by the bacteroids. Higher levels of GS in the nodule cytosol could be of importance in the nitrogen-fixing symbiotic relationship, since it would generate a steep concentration gradient of ammonia away from the bacteroids and allow continued fixation to occur.

The activity of GS/GOGAT in the nodule cytosol is considerably higher than that of GDH during nodule development. On the contrary there is no significant change in the activity of nodule cytosol GDH as compared with the nitrogenase and GS/GOGAT, throughout plant growth. These results suggest that the nodule cytosol GS/GOGAT is the major ammonia assimilatory pathway in the root nodules of *T. foenum-graecum*. These observations are in accord with those of Wolk's group, who by using $^{13}N_2$ has provided further evidence that in the *Anabaena cylindrica*[25–28], tobacco cells[29] and soyabean root nodules[30], GS/GOGAT is the primary route of ammonia assimilation.

EXPERIMENTAL

Growth of plants. *T. foenum-graecum* plants were grown and maintained as described earlier[31].

Enzyme preparation. All the isolation steps were performed at 0–4°. Nodules were ground with extraction buffer (30% W/V, 0.05 M Tris–HCl, pH 7.5) in a precooled mortar and pestle. The extract was filtered through four layers of cheesecloth and centrifuged for 10 min at 400 *g*. The supernatant was centrifuged at 6000 *g* for 10 min to yield the crude bacteriod pellet and a supernatant, which was recentrifuged at 32000 *g* for 20 min. The resultant supernatant was used for assaying nodule cytosol enzymes. The bacteroid pellet was resuspended in extraction buffer, subjected to ultrasound and centrifuged at 32000 *g* for 20 min, the supernatant obtained was used for assaying bacteroidal enzymes. The crude enzyme extracts were applied to a column of Sephadex G-15 preequilibrated and eluted with extraction buffer as described earlier[32], before assaying for enzyme activities. No measureable β-hydroxybutyrate dehydrogenase (a bacteroid marker enzyme) activity[33] was detected in the nodule cytosol extracts, whereas sonicated bacteroids had high activity.

Enzyme assays. The nitrogenase activity of the intact root nodule was assayed as described in ref. [34]. The activity was expressed as μl C_2H_4 produced/plant hr. GS, GDH and GOGAT were assayed by the methods of refs. [35], [36] and [37], respectively. The unit of GS was defined as the amount of enzyme that brings about the formation of 1.0 μmol product (hydroxamic acid)/min at 30°. Hydroxamic acid formed in the reaction was estimated by the method of ref. [38]. The unit for both GDH and GOGAT was defined as the amount of enzyme which brings about an oxidation of 1.0 μmol NADH measured at 340 nm/min at 30°. The activities of all the enzymes are expressed in terms of sp. act. (units/mg protein) and are the average values of five determinations. The protein was estimated by the method of ref. [39] using bovine serum albumin as standard.

Acknowledgements—We thank Dr. H. S. Chhatpar and Dr. N. N. Rao for helpful discussion during the preparation of the manuscript. This investigation was supported by grants from the Indian Council of Argicultural Research, New Delhi, India.

REFERENCES

1. Dalton, H. and 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. (1976) *Bacteriol. Proc.* 148.
4. Hardy, I. R. W. F., Holsten, R. D., Jackson, E. K. and Burns, R. C. (1968) *Plant Physiol.* **43**, 1185.
5. Ryan, E. and Fottrell, P. F. (1974) *Phytochemistry* **13**, 2647.
6. Dougall, D. K. (1974) *Biochem. Biophys. Res. Commun.* **58**, 639.
7. Fowler, M. V., Jessup, W. and Sarkissian, G. S. (1974) *FEBS Letters* **46**, 340.
8. Lea, P. J. and Miflin, B. J. (1974) *Nature* **251**, 614.
9. Nagatani, H., Shimizu, M. and Valentine, R. C. (1971) *Arch. Mikrobiol.* **79**, 164.
10. Kennedy, I. R., Parker, C. A. and Kidby, D. K. (1966) *Biochim. Biophys. Acta* **130**, 517.
11. Kennedy, I. R. (1966) *Biochim. Biophys. Acta* **130**, 293.
12. Mooney, P. and Fottrell, P. F. (1968) *Biochem. J.* **110**, 17.
13. Planque, K., Kennedy, I. R., Devaries, G. E., Quispel, A. and Van Brussel, A. A. N. (1977) *J. Gen. Microbiol.* **102**, 95.
14. Scott, D. D. (1977) in *Limitation and Potentials for Biological Nitrogen Fixation in the Tropics* (Dobereiner, J., Burris, R. H. and Hollander, A., eds.) p. 223. Plenum Press, New York.
15. Boland, M. J., Fordyce, A. M. and Greenwood, R. M. (1978) *Aust. J. Plant Physiol.* **5**, 553.
16. Kennedy, I. R. (1966) *Biochim. Biophys. Acta* **130**, 285.
17. Bergersen, P. J. and Turner, G. L. (1967) *Biochim. Biophys. Acta* **141**, 507.
18. Kennedy, I. R. (1973) *Proc. Aust. Biochem. Soc.* **6**, 33.

19. Dunn, S. D. and Klucas, R. V. (1973) *Can. J. Microbiol.* **19**, 1493.
20. Sloger, C. (1973) *Plant Physiol.* **51** (Suppl.), 354.
21. Miflin, B. J. and Lea, P. J. (1976) *Phytochemistry* **15**, 873.
22. Yates, M. G. and Eady, R. R. (1979) in *Recent Advances in Biological Nitrogen Fixation* (Subba Rao, N. S., ed.) p. 88. Oxford and IBH Publishing Co., New Delhi.
23. Givan, C. V. (1979) *Phytochemistry* **18**, 375.
24. Rawsthorne, S., Minchin, F. R., Summerfield, R. J., Cookson, C. and Coombs, C. (1980) *Phytochemistry* **19**, 341.
25. Wolk, C. P., Austin, S. M., Bartins, J. and Galousky, A. (1974) *J. Cell. Biol.* **61**, 440.
26. Thomas, J., Wolk, C. P., Shaffer, P. W., Austin, S. M. and Galonsky, A. (1975) *Biochem. Biophys. Res. Commun.* **67**, 501.
27. Wolk, C. P., Thomas, J., Shaffer, P. W., Austin, S. M. and Galonsky, A. (1976) *J. Biol. Chem.* **251**, 5027.
28. Thomas, J., Meeks, J. C., Wolk, C. P., Shaffer, P. W., Austin, S. M. and Chien, W. S. (1977) *J. Bacteriol.* **129**, 1545.
29. Skokut, T. A., Wolk, C. P., Thomas, J., Meeks, J. C., Shaffer, P. W. and Chien, W. S. (1978) *Plant Physiol.* **62**, 299.
30. Meeks, J. C., Wolk, C. P., Schilling, N., Shaffer, P. W., Avissar, Y. and Chien, W. S. (1978) *Plant Physiol.* **61**, 980.
31. Nautiyal, C. S., Chhatpar, H. S. and Modi, V. V. (1980) *Indian J. Exp. Biol.* **18**, 362.
32. Nautiyal, C. S. and Modi, V. V. (1981) *Experientia* **37**, 121.
33. McParland, C. H., Guevara, J. G., Becker, R. R. and Evans, H. J. (1978) *Biochem. J.* **153**, 597.
34. Kurz, W. G. W., Rokosh, D. A. and LaRue, T. A. (1973) *Can. J. Microbiol.* **21**, 1009.
35. Nautiyal, C. S. and Modi, V. V. (1981) *Curr. Microbiol.* **5**, 329.
36. Thomulka, K. W. and Moat, A. G. (1972) *J. Bacteriol.* **109**, 25.
37. Roon, R. J., Even, H. L. and Larimase, I. (1974) *J. Bacteriol.* **118**, 89.
38. Lipmann, F. and Tuttle, L. C. (1945) *J. Biol. Chem.* **159**, 21.
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.