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Nitrous Oxide Reduction in Nodules: Denitrification or N_2 Fixation?

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Detached cowpea nodules that contained a nitrous oxide reductase-positive (Nor^+) rhizobium strain (8A55) and a nitrous oxide reductase-negative (Nor^-) rhizobium strain (32H1) were incubated with 1% $^{15}N_2O$ (95 atom% ^{15}N) in the following three atmospheres: (i) aerobic with C_2H_2 (10%), (ii) aerobic without C_2H_2, and (iii) anaerobic (argon atmosphere) without C_2H_2. The greatest production of $^{15}N_2$ occurred anaerobically with 8A55, yet very little was formed with 32H1. Although acetylene reduction activity was slightly higher with 32H1, about 10 times more $^{15}N_2$ was produced aerobically by 8A55 than by 32H1 in the absence of acetylene. The major reductive pathway of N_2O reduction by denitrifying rhizobium strain 8A55 is by nitrous oxide reductase rather than nitrogenase.

Nitrogen fixation and denitrification are dramatically opposed processes usually regarded as independent and separated by space, if not also by time. Nevertheless, over the last decade, denitrification has been demonstrated in a few genera of N_2-fixing bacteria (6, 18, 19, 25, 29). Comparatively more is known about N_2O as an intermediate of denitrification and a substrate of N_2O reductase (20) than as a substrate of nitrogenase. N_2O is a competitive inhibitor of nitrogen fixation in free-living nitrogen fixers (15, 22), and both N_2O and N_2 compete for the same enzyme site (24). Mozen and Burris (16) found equal amounts of $^{15}N_2O$ and $^{15}N_2$ incorporated as $^{15}NH_3$ in sliced nodules. In contrast, very little N_2O was incorporated as ammonia, and most was released as N_2 with cell extracts of free-living N_2 fixers (8, 13).

Hoch et al. (10) also observed the evolution of $^{15}N_2$ from $^{15}N_2O$ with detached soybean nodules. Because all of the N_2 fixation studies with $^{15}N_2O$ described above were conducted before the discovery by Murphy and Elkan (17) that some strains of *R. japonicum* were denitrifiers, it was never considered that $^{15}N_2$ production could arise from anything other than nitrogenase.

Nitrate reduction to nitrite has been readily observed in nodules (3, 23) and bacteroids (26) of *R. japonicum*. By using a denitrifying cowpea rhizobium, Zablotowicz and Focht (30) showed that $^{15}NO_3^-$ was reduced to $^{15}N_2O$ during active reduction of acetylene by bacteroid preparations incubated anaerobically. These studies clearly establish that bacterial respiratory nitrate reduction can supply ATP for nitrogenase activity of nodules under anaerobic conditions. However, nitrogen fixation coupled to respiratory N_2O reduction has not been demonstrated because measurements of nitrogen fixation invariably involve the use of C_2H_2 reduction, and C_2H_2 completely inhibits N_2O reductase (1, 28) at concentrations (1%, vol/vol) lower than those used for acetylene reduction activity (ARA; 10%, vol/vol).

Since N_2O reductase can be induced solely by low oxygen tension without previous exposure to nitrate or nitrite (14, 20, 21), it should be expressed in nodules as soon as the oxygen tension is low enough for nitrogenase to function. Which of the two processes, N_2 fixation or denitrification, accounts for the primary evolution of $^{15}N_2$ from $^{15}N_2O$ in nodules has not been established. Thus, the study presented here was undertaken to compare the relative importance of the two nitrous oxide-reducing reactions between two denitrifying cowpea rhizobia which differed only in their ability to reduce N_2O to N_2 under denitrifying conditions.

Cowpea rhizobial strains 8A55 and 32H1 were obtained from the Nitragin Co., Milwaukee, Wis., courtesy of J. C. Burton. 32H1 is a truncated denitrifer which lacks dissimilatory N_2O reductase (Nor^-) and accumulates N_2O as the terminal product of denitrification, while 8A55 possesses a dissimilatory N_2O reductase (Nor^+) and produces N_2 as the final product of denitrification (M. S. Coyne, M.S. thesis, University of California, Riverside, Calif. 1984). Both strains were maintained at 4°C on yeast extract-mannitol slants (4). Inocula were prepared by growing rhizobia in sidearm flasks (275 ml) containing 25 ml of glucose-yeast extract medium (4) on a rotary platform shaker (100 rpm) at 28°C to an optical density (525 nm) of 0.70.

Cowpea seeds (*Vigna unguiculata* (L.) Walp.) were surface sterilized with 0.1% (wt/vol) $HgCl_2$, rinsed, and added aseptically to sterilized masonry sand contained in clay pots, which were continually moistened with a sterile nutrient solution (27) from below in accordance with procedures previously described (12). The emerged seedlings were inoculated with 2.0 ml of inoculum (optical density at 525 nm, 0.70) applied to the base of the stem 1 week after planting and then thinned to three per pot after 2 weeks. Five weeks after planting, plants were removed, roots were washed free of sand, and the nodules from each inoculum strain were pooled separately.

Detached nodules were incubated statically at 25°C in 50-ml Erlenmeyer flasks containing gas-tight serum stoppers. Each flask contained 218 ± 52 mg (dry mass; 65°C) of nodules. Before injection of 0.5 ml of $^{15}N^{15}NO$ (95 atom% ^{15}N; U.S. Services, Inc., Summit, N.J.) into all flasks, appropriate atmospheric conditions were established with a gasing manifold to give the following treatments: (i) aerobic, (ii) aerobic plus 10% (vol/vol) acetylene, and (iii) anaerobic

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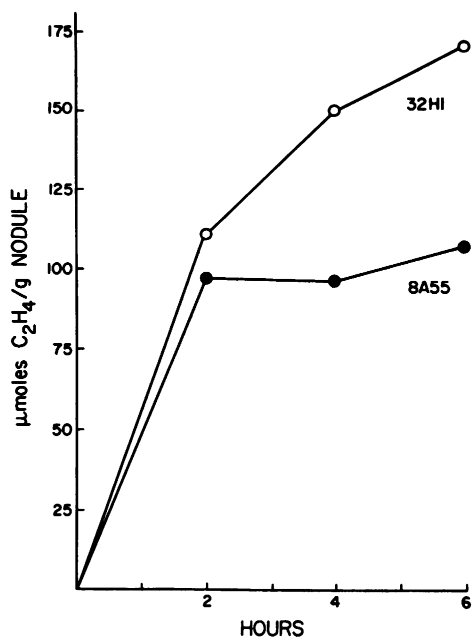

FIG. 1. Nodule-specific ARA by 8A55 (Nor^+) and 32H1 (Nor^-) incubated aerobically with 10% (vol/vol) acetylene.

(argon). Each treatment consisted of eight flasks, two of which contained inactivated nodules (before immersion in boiling water for 1 min) for sampling at zero time. Duplicates of the remaining six flasks for each treatment were likewise removed at 2, 4, and 6 h, analyzed for headspace $^{15}N_2$ and $^{15}N_2O$, and analyzed later for $^{15}NH_4^+$ incorporation into nodules. Approximately 90 min elapsed from the time at which plants were harvested until $^{15}N_2O$ was injected.

Headspace gas was analyzed for $^{15}N_2$ by removing a 5.0-ml sample and storing it in a 3.0-ml VACUTAINER for later analyses by gas chromatography-mass spectrometry on a Finnigan 4000 mass spectrometer (7). Nitrous oxide and ethylene were analyzed immediately with a Varian 3700 gas

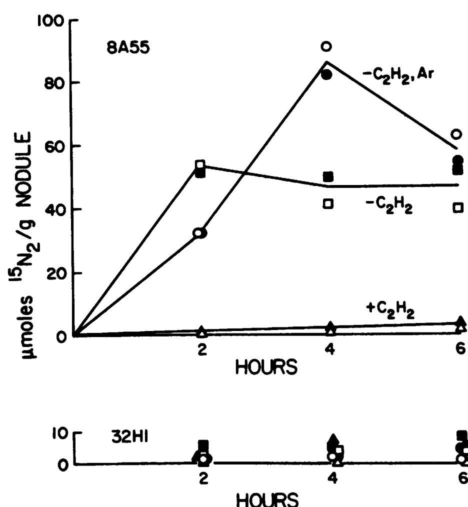

FIG. 2. Cumulative $^{15}N_2O$ consumption (closed symbols) and $^{15}N_2$ production (open symbols) in nodules inoculated with strains 8A55 and 32H1: ambient atmosphere (□, ■), ambient atmosphere with 10% (vol/vol) acetylene (▲, △), argon atmosphere (●, ○).

chromatograph with a fixed 0.5-ml injection loop. A Porapak Q (2.0 m by 1.6 mm inside diameter) column with an electron capture detector (340°C) was used for N_2O, and a Porapak T column (2.0 m by 1.6 mm inside diameter) with a flame ionization detector (340°C) was used for ethylene. An N_2 carrier gas flow rate of 25 ml/min and oven temperature of 65°C were used for both analyses. After gas sampling of the headspace, nodules were removed, dried at 65°C overnight, weighed, and ground with a mortar and pestle. Total N was determined by Kjeldahl digestion and NH_3 distillation (2), N_2 was converted from NH_3 by the Rittenberg method (9), and $^{15}N_2$ was determined by direct inlet to the Finnigan 4000 mass spectrometer (11).

ARA was lower in 8A55 (Nor^+) nodules than in 32H1 (Nor^-) nodules (Fig. 1). Reduction of $^{15}N_2O$ to $^{15}N_2$ was not observed in this treatment since 10% acetylene blocks reduction of N_2O by either nitrogenase or nitrous oxide reductase. Despite the higher ARA, in the absence of acetylene the nodules inoculated with Nor^- bacteria reduced much less $^{15}N_2O$ than the nodules inoculated with Nor^+ bacteria (Fig. 2).

$^{15}N_2O$ consumption was attributable to ^{15}N production (Fig. 2), and in no instance could we detect significant enrichment in nodule ^{15}N content. Virtually all (97 ± 5%) of the ^{15}N could be accounted for as $^{15}N_2$ and $^{15}N_2O$ in all samples. This is not unexpected if N_2O reduction by nitrogenase proceeds through N_2, as suggested by Lockshin and Burris (13), rather than directly to $^{15}NH_4$. The dilution effect of $^{15}N_2$ (produced from $^{15}N_2O$) with ambient N_2 would be most pronounced at the beginning, when both ARA and ambient N_2 concentrations were highest and $^{15}N_2$ was lowest.

If ARA is a valid measurement of N_2 fixation, then it follows that the proportional quantity of $^{15}N_2$ attributable to nitrogenase activity in the nodules inoculated with Nor^+ bacteria at 6 h (Fig. 2) can be calculated from the product of $^{15}N_2$ in the nodules inoculated with Nor^- bacteria (4.3 μmol/g) multiplied by the ratio of ARA of both strains (108/170; Fig. 1), which gives 2.7 μmol of $^{15}N_2$ per g. Since the total $^{15}N_2$ production was 39 μmol/g (Fig. 2), only 7% of the total $^{15}N_2$ production in the aerobic incubation could be attributable to nitrogenase. The amount and proportion of $^{15}N_2$ produced anaerobically is higher yet. Moreover, the comparative kinetics of both reductive processes greatly favor denitrification versus N_2 fixation since the $^{15}N_2O$ concentration was well above half-saturation for nitrous oxide reductase (5), but not for nitrogenase (8). We therefore conclude that the major reductive pathway of N_2O in strain 8A55 is by nitrous oxide reductase and not by nitrogenase.

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LITERATURE CITED

1. **Balderston, W. L., B. Sherr, and W. J. Payne.** 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. **31:**504–508.
2. **Bremner, J. M., and C. S. Mulvaney.** 1982. Nitrogen—total, p. 595–624. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed), Methods of soil analysis, part 2, 2nd ed. Chemical and microbiological properties. American Society of Agronomy, Madison, Wis.
3. **Cheniae, G. M., and H. J. Evans.** 1957. On the relation between nitrogen fixation and nodule nitrate reductase of soybean root nodules. Biochim. Biophys. Acta **26:**654–655.
4. **El Hassan, G. A., B. S. Hernandez, and D. D. Focht.** 1986. Comparison of the Hup trait and intrinsic antibiotic resistance

for assessing rhizobial competitiveness axenically and in soil. Appl. Environ. Microbiol. **51**:546–551.

5. **El Hassan, G. A., R. M. Zablotowicz, and D. D. Focht.** 1985. Kinetics of denitrifying growth by fast-growing cowpea rhizobia. Appl. Environ. Microbiol. **49**:517–521.
6. **Eskew, D. L., D. D. Focht, and I. P. Ting.** 1977. Nitrogen fixation, denitrification, and pleomorphic growth in a highly pigmented *Spirillum lipoferum*. Appl. Environ. Microbiol. **34**:582–585.
7. **Focht, D. D., N. Valoras, and J. Letey.** 1980. Use of interfaced gas chromatography-mass spectrometry for detection of concurrent mineralization and denitrification in soil. J. Environ. Qual. **9**:218–223.
8. **Hardy, R. W. F., and E. Knight.** 1986. Reduction of N_2O by biological N_2-fixing systems. Biochem. Biophys. Res. Commun. **23**:409–414.
9. **Hauck, R. D.** 1982. Nitrogen-isotope ratio analysis, p. 735–780. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2, 2nd ed. Chemical and microbiological properties. American Society of Agronomy, Madison, Wis.
10. **Hoch, G. E., K. C. Schneider, and R. H. Burris.** 1960. Hydrogen evolution and exchange, and conversion of N_2O to N_2 by soybean root nodules. Biochim. Biophys. Acta **37**:273–279.
11. **La Favre, J. S., and D. D. Focht.** 1983. Comparison of N_2 fixation and yields in *Cajanus cajan* between hydrogenase-positive and hydrogenase-negative rhizobia by *in situ* acetylene reduction assays and direct ^{15}N partitioning. Plant Physiol. **72**:971–977.
12. **La Favre, J. S., and D. D. Focht.** 1985. Nitrogen fixation and hydrogen evolution by the pigeon pea-*Rhizobium* symbioses. Trop. Agric. **62**:285–288.
13. **Lockshin, A., and R. H. Burris.** 1965. Inhibitors of nitrogen fixation in extracts from *Clostridium pasteurianum*. Biochim. Biophys. Acta. **111**:1–10.
14. **Matsubara, T.** 1971. Studies of denitrification. XIII. Some properties of the N_2O-aerobically grown cell. J. Biochem. **59**: 991–1001.
15. **Molnar, D. M., R. H. Burris, and P. W. Burris.** 1948. The effect of various gases of nitrogen fixation by *Azotobacter*. J. Am. Chem. Soc. **70**:1713–1716.
16. **Mozen, M. M., and R. H. Burris.** 1954. The incorporation of ^{15}N-labeled nitrous oxide by nitrogen fixing agents. Biochim. Biophys. Acta **14**:577–578.
17. **Murphy, S. G., and G. H. Elkan.** 1965. Nitrogen metabolism of some strains of *R. japonicum* having different nodulating capacities. Can. J. Microbiol. **11**:1039–1041.
18. **Neal, J. L., G. C. Allent, R. D. Morse, and D. D. Wolf.** 1983. Anaerobic nitrate-dependent chemolithotrophic growth by *Rhizobium japonicum*. Can. J. Microbiol. **29**:316–320.
19. **Neyra, C. A., J. Dobereiner, R. Lalande, and R. Knowles.** 1977. Denitrification by *Spirillum lipoferum*. Can. J. Microbiol. **23**: 300–305.
20. **Payne, W. J.** 1981. Denitrification. John Wiley & Sons, Inc., New York.
21. **Payne, W. J., P. S. Riley, and C. D. Cox, Jr.** 1971. Separate nitrite, nitric oxide, and nitrous oxide reducing fractions from *Pseudomonas perfectomarinus*. J. Bacteriol. **106**:356–361.
22. **Repaske, R., and P. W. Wilson.** 1952. Nitrous oxide inhibition of nitrogen fixation by *Azotobacter*. J. Am. Chem. Soc. **74**:3101–3103.
23. **Rigaud, J. F., F. J. Bergersen, G. L. Turner, and R. M. Daniel.** 1973. Nitrate dependent anaerobic acetylene reduction by soybean bacteroids. J. Gen. Microbiol. **77**:137–144.
24. **Rivera-Ortiz, J. M., and R. H. Burris.** 1975. Interactions among substrates and inhibitors of nitrogenase. J. Bacteriol. **123**:537–545.
25. **Satch, T., S. S. Hom, and K. T. Shanmugan.** 1981. Production of nitrous oxide as a product of nitrite metabolism of enteric bacteria, p. 473–480. *In* J. H. Lyons, R. C. Valentine, D. A. Phillips, D. W. Rains, and R. C. Huffaker (ed.), Genetic engineering of symbiotic nitrogen fixation and conversation of fixed nitrogen. Plenum Publishing Corp., New York.
26. **Stephens, B. D., and C. A. Neyra.** 1983. Nitrate and nitrite reduction in relation to nitrogenase activity in soybean nodules and *Rhizobium japonicum* bacteroids. Plant Physiol. **71**: 731–735.
27. **Weaver, R. W., and L. R. Frederick.** 1982. *Rhizobium*, p. 1043–1070. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2, 2nd ed. Chemical and microbiological processes. American Society of Agronomy, Madison, Wis.
28. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. **69**:705–710.
29. **Zablotowicz, R. M., D. L. Eskew, and D. D. Focht.** 1978. Denitrification in *Rhizobium*. Can. J. Microbiol. **24**:757–760.
30. **Zablotowicz, R. M., and D. D. Focht.** 1979. Denitrification and anaerobic nitrate-dependent acetylene reduction in cowpea rhizobium. J. Gen. Microbiol. **111**:445–448.