Ammonia Transport in Free-living and Symbiotic *Rhizobium* sp. ANU289

By SUSAN M. HOWITT,* MICHAEL K. UDVARDI, DAVID A. DAY AND
PETER M. GRESSHOFF

Botany Department, Australian National University, PO Box 4, Canberra 2601, Australia

(Received 20 April 1985; revised 12 September 1985)

[^{14}C]Methylamine uptake by free-living *Rhizobium* sp. ANU289 had Michaelis–Menten kinetics (apparent K_m 6.6 μM). Uptake was competitively inhibited by ammonia (K_i 0.4 μM) and was dependent on an energized membrane. Uptake by bacteria had an optimum at pH 7.0. Methylamine uptake by bacteroids from siratro root nodules was much slower than that by free-living bacteria at pH 7.0 but increased exponentially with the pH of the medium. Uptake by bacteroids did not show saturation kinetics and was insensitive to the presence of ammonia or uncouplers. These results suggest that free-living bacteria (grown under conditions where ammonia is limiting) have an active transport mechanism for the uptake of ammonium ions; this carrier is not operative in the symbiotic state, where passive diffusion of ammonia occurs. In the free-living state, the ammonium carrier is under genetic control, being repressed by growth on high concentrations of ammonia. Derepression occurs under conditions of nitrogen starvation.

INTRODUCTION

Rhizobium spp. fix N_2 in symbiotic association with legumes. The bacteria induce the formation of nodules on legume roots and differentiate inside the nodule to form bacteroids, which can convert atmospheric N_2 to ammonia. The ammonia produced in the bacteroids, however, is assimilated by plant enzymes in the plant cytosol. Thus, the mechanism by which ammonia passes from the bacteroid to the plant fraction of the nodule is of interest in understanding the *Rhizobium*/legume symbiosis. Free-living rhizobia induced to fix nitrogen *in vitro* also excrete ammonia into the environment (O’Gara & Shanmugam, 1976; Tubb, 1976; Bergersen & Turner, 1978).

Evidence for the existence of an ammonia carrier has been found in free-living *Rhizobium leguminosarum* (Glenn & Dilworth, 1984; O’Hara *et al.*, 1985) and in *Rhizobium* sp. 32H1 (Gober & Kashket, 1983). However, the characteristics and regulation of the carriers appear to differ between the two species.

The few published reports suggest that bacteroids lack an ammonia carrier. Laane *et al.* (1980) reported that bacteroids of *R. leguminosarum* were unable to accumulate ammonia and excreted ammonia in response to a ΔpH while Marsh *et al.* (1984) found reduced rates of ammonia uptake in bacteroids of *Rhizobium* sp. 32H1 compared to the free-living bacteria, and no uptake in *Rhizobium japonicum* bacteroids. Other free-living nitrogen-fixing bacteria, including *Klebsiella pneumoniae* (Kleiner, 1982), *Clostridium pasteurianum* (Kleiner & Fitzke, 1981), *Azotobacter vinelandii* (Jayakumar & Barnes, 1984) and *Anabaena cylindrica* (Rai *et al.*, 1984), do have ammonia carriers and it seems that the membrane potential may be the driving force for ammonia transport by these carriers (Kleiner, 1981).

The ammonia analogue methylamine has been used to study ammonia uptake in many bacteria (Gober & Kashket, 1983; Kleiner, 1982; Kleiner & Fitzke, 1981; Jayakumar & Barnes,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DES, diethylstilboestrol.

1984; Rai *et al.*, 1984); in these bacteria, methylamine and ammonia share a common carrier. We have found this to be true for the slow-growing *Rhizobium* sp. ANU289 and have used methylamine to investigate ammonia uptake in both free-living bacteria and bacteroids. The present report shows that a specific ammonia carrier, probably energized by the membrane potential, is present in free-living bacteria but not in bacteroids.

METHODS

Bacterial strain. *Rhizobium* sp. ANU289 is a streptomycin resistant, non-mucoid derivative of strain CP283 (Mohapatra *et al.*, 1983).

Media and growth conditions. For all experiments strain ANU289 was grown in medium N⁻FGM supplemented with 10 mM-glutamate as described previously (Howitt & Gresshoff, 1985).

Nodulation and isolation of bacteroids. Bacteroids were isolated from nodules induced by strain ANU289 on siratro (*Macroptilium atropurpureum*). The growth of plants and bacteroid isolation procedure was described by Sandeman & Gresshoff (1985).

Uptake experiments. Free-living strain ANU289 cells were harvested in mid-exponential phase, washed twice, and resuspended in N⁻FGM before being used for uptake experiments. Bacteroids were finally resuspended in a buffer containing 50 mM-sucrose, 50 mM-MOPS, 2 mM-MgCl₂, 0.1 mM-dithiothreitol and 4% (w/v) polyvinylpyrrolidone. For experiments in which the pH dependence of methylamine uptake was investigated, 50 mM-Tris was added to the buffers used for both bacteria and bacteroids to buffer at the higher pH range. Cells (bacteria or bacteroids) were pre-incubated at 30 °C. [¹⁴C]Methylamine (10 µM unless otherwise stated; 56 mCi mmol⁻¹, 2.07 GBq mmol⁻¹) was added and, after 1 min, a sample was taken and the cells were collected by filtration. Cells were then washed three times in N⁻FGM containing 50 µM-methylamine. Radioactivity in the filters was measured by liquid scintillation counting after placing them in a scintillation vial with 10 ml scintillation fluid (Permafluor/Triton X-100/toluene; 2:25:50, by vol.).

Protein determinations These were made using a modified Lowry procedure (Schacterle & Pollack, 1973).

RESULTS AND DISCUSSION

The uptake of [¹⁴C]methylamine was linear for at least 10 min in both bacteria and bacteroids (Fig. 1). Free-living bacteria had nearly 10-fold higher transport activity than bacteroids. In free-living bacteria, methylamine uptake showed a distinct optimum at pH 7.0 while in bacteroids, methylamine uptake increased exponentially with increasing pH (Fig. 2). The dependence of methylamine uptake on pH in bacteroids suggests that diffusion of uncharged methylamine was occurring. The pK_a of methylamine is 10.6 and at the lower pH values shown in Fig. 2 very little methylamine will exist in the uncharged form. Consequently the uptake by bacteroids was much less than that by bacteria. However, as the pH increases more methylamine becomes uncharged and uptake by bacteroids becomes proportionally greater. The pH optimum observed in free-living bacteria indicates that a methylamine carrier is likely to be present.

Methylamine uptake in free-living bacteria showed Michaelis-Menten kinetics (apparent K_m 6.6 µM, Fig. 3). Uptake was competitively inhibited (K_i 0.4 µM) by 5 µM-ammonia. In bacteroids, methylamine uptake did not show saturation kinetics but increased linearly with increasing methylamine concentration up to 1 mM (Fig. 3). Uptake of methylamine by bacteroids was not affected by the presence of ammonia (results not shown).

Uptake in bacteria was inhibited by about 90% by 5 mM-azide (an electron transport inhibitor), and by 80% by 5 µg valinomycin ml⁻¹ (a potassium ionophore) suggesting that transport required energy. However, neither the ATPase inhibitor DES (100 µM), nor the protonophore CCCP (2 µM) inhibited uptake. Similar results were obtained in *Klebsiella pneumoniae* by Kleiner (1982), who argued that this would occur if methylamine uptake was energized by a membrane potential which was largely due to cations other than protons. CCCP would not dissipate this potential but, if K⁺ contributed a large part of the membrane potential, valinomycin would. *Rhizobium* sp. ANU289 therefore seems to be similar to *K. pneumoniae* in this respect.

The glutamine synthetase/glutamate synthase pathway is the sole route of ammonia assimilation in strain ANU289 (Howitt & Gresshoff, 1985). In an attempt to distinguish

Fig. 1

Fig. 2

Fig. 1. Time course of $[^{14}\text{C}]$ methylamine uptake by free-living bacteria (O) and bacteroids (●). Results for bacteria are the mean of three experiments (SE generally less than 10%); results for bacteroids are from one typical experiment.

Fig. 2. pH dependence of $[^{14}\text{C}]$ methylamine uptake by free-living bacteria (O) and bacteroids (●). Results for bacteria are the average of three experiments (SE generally less than 10%); results for bacteroids are from one typical experiment.

Fig. 3. (a) Concentration dependence of $[^{14}\text{C}]$ methylamine uptake by free-living bacteria (O) and bacteroids (●) up to 30 μM -methylamine. (b) Concentration dependence of $[^{14}\text{C}]$ methylamine uptake by bacteroids up to 1 mM-methylamine. Results for bacteria are the average of three experiments (SE generally less than 10%); results for bacteroids are from one typical experiment.

between uptake of methylamine and its metabolism, we examined the effect of the glutamine synthetase inhibitor methionine sulfoximine on methylamine uptake. Methionine sulfoximine (5 mM), which completely inhibited both the glutamine synthetases that are present in strain ANU289, inhibited methylamine uptake by about 30% (results not shown). This suggests that some methylamine is converted to γ -*N*-methylglutamine, as it is in *Rhizobium* sp. 32H1 (Gober & Kashket, 1983). However, most of the uptake observed was independent of metabolism. The fact that methylamine uptake was inhibited by valinomycin and azide confirms that methylamine uptake, and not metabolism was occurring.

When 10 mM- NH_4Cl was added to a nitrogen starved strain ANU289 culture, 90% of the methylamine uptake activity was lost after 24 h (Fig. 4). Resuspension of these cells in a medium

Fig. 4. Recovery of methylamine uptake after repression by ammonia. NH_4Cl (10 mM) was added to a mid-exponential phase culture, which was then allowed to grow for a further 24 h. The cells were then washed and resuspended in N-FGM plus 10 mM-glutamate with (○) and without (●) 0.2 mg chloramphenicol ml^{-1} . Samples were taken and [^{14}C]methylamine uptake activity was determined at the times indicated.

containing 10 mM-glutamate, but no NH_4Cl , resulted in the recovery of two-thirds of the initial activity within 5 h (Fig. 4). The recovery of methylamine uptake activity was completely inhibited by chloramphenicol (Fig. 4). The carrier thus appears to be regulated at a genetic level, with ammonia repressing the synthesis of the carrier and nitrogen starvation derepressing it. Regulation of the ammonia carrier of *R. leguminosarum* (O'Hara *et al.*, 1985) and of *K. pneumoniae* (Kleiner, 1982) occurs in a similar manner and appears to be part of the general nitrogen control system in the latter species. This contrasts with the case of *A. vinelandii* where, in the presence of excess ammonia, a build up of metabolites inhibitory to ammonia uptake occurs (Jayakumar & Barnes, 1984).

The ammonia carrier in bacteroids of strain ANU289 and other *Rhizobium* spp. (Laane *et al.*, 1980; Marsh *et al.*, 1984; O'Hara *et al.*, 1985) may be absent because the ammonia produced by N_2 fixation represses the carrier, as it does in the free-living state. O_2 has also been implicated in the regulation of the ammonia carriers of other *Rhizobium* spp. but different reports are contradictory. Marsh *et al.* (1984) found that low O_2 tension repressed the ammonia carrier of both the cowpea strain 32H1 and *R. japonicum*. However, Gober & Kashket (1983), also working with 32H1, suggested that the carrier would be induced in the bacteroid by low O_2 and would function to actively export ammonia to the plant cytosol. Our results show that this latter explanation definitely does not hold for strain ANU289 bacteroids.

S. M. H. is the recipient of an Australian Government Postgraduate Research Award. Agrigenetics Research Associates supported parts of this study.

REFERENCES

- BERGERSEN, F. J. & TURNER, G. L. (1978). Activity of nitrogenase and glutamine synthetase in relation to availability of oxygen in continuous cultures of cowpea *Rhizobium* sp. supplied with excess ammonium. *Biochimica et biophysica acta* **538**, 406–416.
- DILWORTH, M. J. & GLENN, A. R. (1982). Movements of ammonia in *Rhizobium leguminosarum*. *Journal of General Microbiology* **128**, 29–37.
- GLENN, A. R. & DILWORTH, M. J. (1984). Methylamine and ammonium transport systems in *Rhizobium leguminosarum* MNF3841. *Journal of General Microbiology* **130**, 1961–1968.
- GOBER, J. W. & KASHKET, E. R. (1983). Methylammonium uptake by *Rhizobium* sp. 32H1. *Journal of Bacteriology* **153**, 1196–1201.
- HOWITT, S. M. & GRESSHOFF, P. M. (1985). Ammonia

- regulation of glutamine synthetase in *Rhizobium* sp. ANU289. *Journal of General Microbiology* **131**, 1433–1440.
- JAYAKUMAR, A. & BARNES, E. M. (1984). The role of glutamine in regulation of ammonium transport in *Azotobacter vinelandii*. *Archives of Biochemistry and Biophysics* **231**, 95–101.
- KLEINER, D. (1981). The transport of NH_3 and NH_4^+ across biological membranes. *Biochimica et biophysica acta* **639**, 41–52.
- KLEINER, D. (1982). Ammonium (methylammonium) transport by *Klebsiella pneumoniae*. *Biochimica et biophysica acta* **688**, 702–708.
- KLEINER, D. & FITZKE, E. (1981). Some properties of a new electrogenic transport system: the ammonium (methylammonium) carrier from *Clostridium pasteurianum*. *Biochimica et biophysica acta* **641**, 138–147.
- LAANE, C., KRONE, W., KONINGS, W. & VEEGER, C. (1980). Short term effect of ammonium chloride on nitrogen fixation by *Azotobacter vinelandii* and by bacteroids of *Rhizobium leguminosarum*. *European Journal of Biochemistry* **103**, 39–46.
- MARSH, S. D., WYZA, R. E. & EVANS, W. R. (1984). Uptake of ammonia and methylamine by free-living and symbiotic *Rhizobium*. *Plant Physiology* **75S**, abst. 155, p. 28.
- MOHAPATRA, S. S., BENDER, G. L., SHINE, J., ROLFE, B. G. & GRESSHOFF, P. M. (1983). *In vitro* expression of nitrogenase activity in *Parasponia Rhizobium* strain ANU289. *Archives of Microbiology* **134**, 12–16.
- O'GARA, F. & SHANMUGAM, K. T. (1976). Regulation of nitrogen fixation by rhizobia. *Biochimica et biophysica acta* **437**, 313–321.
- O'HARA, G. W., RILEY, I. T., GLENN, A. R. & DILWORTH, M. J. (1985). The ammonium permease of *Rhizobium leguminosarum* MN3841. *Journal of General Microbiology* **131**, 757–764.
- RAI, A. N., ROWELL, P. & STEWART, W. D. P. (1984). Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria. *Archives of Microbiology* **137**, 241–246.
- SANDEMAN, R. A. & GRESSHOFF, P. M. (1985). Nitrogenase activity and inactivation in isolated bacteroids from the legume siratro and the non-legume *Parasponia rigida*. *Plant Science Letters* **37**, 199–204.
- SCHACTERLE, G. R. & POLLACK, R. L. (1973). Simplified method for quantitative assay of small amounts of protein in biological material. *Analytical Biochemistry* **51**, 654–655.
- TUBB, R. S. (1976). Regulation of nitrogen fixation in *Rhizobium* spp. *Applied and Environmental Microbiology* **32**, 483–488.