The Ammonium Permease of *Rhizobium leguminosarum* MNF3841

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An ammonium permease was derepressed when *Rhizobium leguminosarum* was grown in chemostat culture under conditions of nitrogen limitation. The ammonium permease was characterized by direct measurements with an ion-specific ammonium electrode. Cells grown under ammonia, nitrate, glutamate or methylamine limitation had permease activity, while those grown with excess (10 mM) ammonium chloride or glutamate did not. On transfer from N-limited to N-excess conditions, the permease disappeared rapidly, and all activity was lost within 18 h. Uptake by the permease was sensitive to azide, carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol, nigericin and valinomycin. The apparent K_m for NH_4^+ was 0.015 mM; ammonium uptake had a narrow maximum around pH 7.5. The internal ammonia concentration of N-limited cells was 0.4 mM, with up to 60-fold gradients of NH_4^+ forming across the membrane within 30 min. Hydrazine and hydroxylamine strongly inhibited ammonium uptake, with methylamine, glutamine, aspartate and glycine less effective as inhibitors. Isolated pea bacteroids capable of transporting succinate did not possess the ammonium permease.

INTRODUCTION

An understanding of ammonia movement in nodule bacteria is of considerable importance in the study of symbiotic nitrogen fixation since ammonia is the first product of N_2 fixation (Bergersen, 1965; Kennedy, 1966) and appears to move from the site of fixation in the bacteroid (Bergersen & Turner, 1967) to the plant host for assimilation into amino acids and other compounds (Boland *et al.*, 1980). In this paper the term ammonia is used to refer to the compound without defining its state of protonation, while ammonium refers to the protonated species NH_4^+ and the uncharged species is specifically denoted by NH_3 .

It has been proposed that in free-living N_2 -fixing bacteria, ammonium transport systems have the dual function of (i) acquiring trace amounts of NH_4^+ from the environment, and (ii) cyclic retention of NH_4^+ produced by N_2 fixation and partially lost by outward diffusion of NH_3 (Kleiner, 1981). However, the situation is different for symbiotic rhizobia since most of the N_2 fixed as ammonia is liberated from the bacteroid and rapidly assimilated by the plant symbiont. It has been shown for *Rhizobium leguminosarum* that under conditions where ammonia is present in high concentrations (>1 mM), there is a rapid equilibration of ammonia across the cell membrane by a diffusive mechanism (Dilworth & Glenn, 1982). It has also been suggested that rhizobia may be unable to synthesize ammonium transport systems (Osburne, 1982; Wiegel & Kleiner, 1982). However, the presence of an ammonium transport mechanism would appear to be necessary for growth on low concentrations of NH_4^+ (Bergersen & Turner, 1976) similar to those found in the soil and rhizosphere.

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Active ammonium transport systems have been identified in some lower eukaryotes (Hackett *et al.*, 1970; Pateman *et al.*, 1974; Roon *et al.*, 1975; Breiman & Barash, 1980) and bacteria (Stevenson & Silver, 1977), including many N_2 -fixing bacteria (Kleiner, 1975, 1982; Barnes & Zimniak, 1981; Kleiner & Fitzke, 1979, 1981; Alef & Kleiner, 1982; Hartmann & Kleiner, 1982; Wiegel & Kleiner, 1982). An ammonium-sensitive methylamine transport system has also been reported in cowpea *Bradyrhizobium* 32H1 grown in batch culture under microaerobic conditions (Gober & Kashket, 1983).

An ammonium transport system has been demonstrated in *R. leguminosarum* MNF3841 grown in chemostat culture under nitrogen limitation (Glenn & Dilworth, 1984), where cells from ammonia-limited chemostats possessed a [^{14}C]methylamine uptake system which was sensitive to inhibition by ammonia. However, further study of this ammonium transport system using radioactive methylamine as an analogue of ammonium is complicated by the presence of a separate methylamine transport system unconnected with ammonium transport (Glenn & Dilworth, 1984). For this reason a direct approach using an ion-selective electrode rather than an analogue system has been used in the present paper to study ammonium transport in *R. leguminosarum*.

METHODS

Organism. *Rhizobium leguminosarum* MNF3841 is a *str^r* derivative of strain 300 (Johnston & Beringer, 1975).

Media. Batch cultures were grown at 28 °C in the minimal salts medium of Brown & Dilworth (1975) with sucrose (10 mM) as the carbon source in 20 mM-HEPES buffer, pH 7.2.

Continuous culture. Cells were grown in continuous culture in the liquid medium of Glenn & Dilworth (1984) with sucrose (10 mM) as carbon source. Chemostats had working volumes of either 75 ml or 500 ml and were operated at 28 °C. Nitrogen-limited cells were cultured ($D = 0.1 \text{ h}^{-1}$) with ammonia, nitrate, methylamine or glutamate (0.5 mM) as nitrogen source. Cultures grown under phosphate limitation (0.08 mM; $D = 0.1 \text{ h}^{-1}$) with either NH_4Cl or glutamate (10 mM) as nitrogen source were used to provide nitrogen-excess cells.

Nodulation and preparation of bacteroids. Pea plants (*Pisum sativum* L. cv Greenfeast) grown in sterile sand were nodulated by *R. leguminosarum* MNF3841, and bacteroids were prepared and assayed for [^{14}C]succinate uptake as described by Glenn *et al.* (1980). Bacteroids were used immediately after isolation.

Determination of ammonium transport. Cells from batch or continuous culture were prepared for ammonium uptake experiments by centrifugation at 10000 *g* at 22 °C and washing (once for N-limited cells, three times for N-excess cells) with sterile salts medium minus HEPES, phosphate and nitrogen source, but with sucrose added at 0.5 mM and buffered with 5 mM-sodium phosphate, pH 7.2. Ammonium uptake was determined using cell suspensions (20 ml) stirred in a water-jacketed vessel maintained at 28 ± 0.5 °C. The ammonia concentration of the bacterial suspension was monitored continuously using an ammonium ion-selective electrode (Model IS 561- NH_4^+ , Philips) that had been calibrated with an ammonia solution of the same pH. Output potentials were collected from the electrode through a Philips model PW 9414 digital ion activity meter, and an analogue to digital convertor connected to a CBM microcomputer. Under the conditions used, ammonia concentrations of 0.001 mM could be measured. The usual time course for an experimental run was 15–30 min. The ammonia concentration in the cell suspension was recorded at specified time intervals (usually 15 s) by the CBM microcomputer and stored on a sequential disc file. Output from the stored data was graphed with a multipen Digi-Plot flat bed plotter (Watanabe Instrument Corp., Tokyo, Japan). In most experiments the initial ammonia concentration in the cell suspension was in the range 0.01–0.05 mM.

Derepression of ammonium permease activity. The appearance of the ammonium uptake system was followed using cells grown in a chemostat under ammonia-excess conditions to a cell density of approximately 0.23 mg dry wt ml^{-1} . The cells were centrifuged and washed three times in minimal salts medium, then resuspended to a cell density of 0.14 mg dry wt ml^{-1} in minimal salts medium minus nitrogen source and grown in batch culture at 28 °C. Samples of the cell suspension were taken at regular intervals, centrifuged and washed, and the activity of the ammonium permease measured.

Determination of intracellular ammonia. A suspension (100 ml) of N-limited *R. leguminosarum* (0.48 mg dry wt ml^{-1}) was incubated in the standard assay using the ammonium-selective electrode to monitor changes in ammonia concentration in the cell supernatant. The ammonia concentration in the supernatant was measured chemically at the start and completion of the experiment. After incubation, duplicate samples (40 ml) were centrifuged (11800 *g*, 15 min, 4 °C), and the pellets were weighed and resuspended in 1 ml of sterile minimal salts medium. To measure the volume of entrained liquid (Stock *et al.*, 1977), [3H]inulin (0.46 MBq) was added to the suspension, duplicate samples (0.1 ml) were removed and radioactivity was determined by liquid scintillation counting. The remaining suspension was centrifuged (11800 *g*, 15 min, 4 °C) and the pellet was resuspended in minimal salts medium (1.5 ml) containing toluene (0.025 ml) to release intracellular ammonia (Dilworth & Glenn, 1982). After mixing and incubation at 4 °C for 15 min, the suspension was centrifuged as above and samples of the supernatant were taken for determination of radioactivity and ammonia concentration.

Cell water was also measured on a set of suspensions using the combined [^3H]water and [^{14}C]inulin technique of Stock *et al.* (1977) to derive a value relating cell water to dry weight [$1.45\text{ ml (g dry wt)}^{-1}$; Glenn *et al.*, 1984].

Radioisotopes. [^3H]Inulin ($72.5\text{ GBq mmol}^{-1}$), [^{14}C]inulin (185 MBq mmol^{-1}) and [$1,4\text{-}^{14}\text{C}$]succinate ($4.36\text{ GBq mmol}^{-1}$) were from Amersham, and $^3\text{H}_2\text{O}$ ($0.67\text{ MBq mmol}^{-1}$) was from New England Nuclear.

Enzyme assays. Cell-free extracts were prepared from chemostat-grown ammonia-limited cells by centrifuging at 22°C , washing the cells in 5 mM -sodium phosphate buffer, ($\text{pH } 7.2$) and resuspending in the same phosphate buffer. Cells were then broken at 4°C in a French pressure cell (Paton Industries, Adelaide, S. Australia) at 69000 kPa and the supernatant, after centrifugation at 14000 g for 20 min at 4°C , was used in enzyme assays.

Biosynthetic glutamine synthetase (EC 6.3.1.2), and glutamate synthase (EC 1.4.1.13) were measured as described by Shapiro & Stadtman (1970) and Tempest *et al.* (1973), respectively.

Analytical methods. Ammonia was measured spectrophotometrically over the range $0\text{--}50\text{ nmol}$ using the phenol-hypochlorite method of Fawcett & Scott (1960), as modified by Dilworth & Thorneley (1981).

In experiments on methylamine inhibition of ammonium uptake it was necessary to separate methylammonium and ammonium, since the former reacts in the phenol-hypochlorite assay. Samples (3 ml) were loaded onto $12.5 \times 0.55\text{ cm}$ diam. columns of Dowex 50W-X8 or X12 (Na^+ form; $100\text{--}200\text{ mesh}$) and eluted with 60 mM -sodium pyrophosphate ($\text{pH } 8.7$). Ammonia eluted first and was then estimated chemically as above.

Bacterial protein was measured by the Lowry method with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Validation of ammonium ion specific electrode assay

Since *R. leguminosarum* MNF3841 grown in a chemostat under N-limitation produces an active transport system for methylamine which is extremely sensitive to ammonium (Glenn & Dilworth, 1984), we attempted to measure ammonia disappearance from the extracellular medium directly, using an ammonium electrode. Nitrogen-limited cells removed ammonia (Fig. 1), while cells from N-excess (P-limited) chemostats did not. There was no electrode response when NaCl replaced NH_4Cl , but the same changes were noted when $(\text{NH}_4)_2\text{SO}_4$ replaced NH_4Cl on an equimolar NH_4^+ basis. To validate the electrode method samples were taken from the vessel at intervals (5 min) and the ammonia in the culture supernatants measured chemically using the phenol-hypochlorite method. Experiments using both N-limited and N-excess cells showed that the results from the electrode assay exactly paralleled those from the chemical assay (Fig. 1). Thus, the electrode system successfully monitored changes in ammonia concentration in the cell supernatant. All subsequent experiments used the electrode system which allowed direct calculation and plotting of rates of ammonia disappearance.

Chemostat culture studies

R. leguminosarum MNF3841 grown in continuous culture under ammonia, nitrate, glutamate or methylamine limitation contained ammonium permease activity [4.8 , 4.3 , 4.9 and $5.1\text{ nmol min}^{-1} (\text{mg protein})^{-1}$, respectively], whereas cells grown with either excess ammonia or glutamate did not. Cells grown on agar media with no added nitrogen and in low-N (0.5 mM-NH_4^+) batch cultures contained the transport system, but high-N (10 mM-NH_4^+) cells from agar plates and batch cultures did not show any evidence of an ammonium uptake system.

Regulation of the ammonium permease

Cells grown in a chemostat under ammonia-excess, washed and resuspended in nitrogen-free medium, showed limited growth for 4 h , after which growth ceased (Fig. 2). An ammonium transport system was detectable 2 h after transfer of the cells to nitrogen-free conditions. This developed slowly until maximum activity was achieved 12 h following transfer to batch culture (Fig. 2). The addition of chloramphenicol (0.2 mg ml^{-1}) prevented the formation of the permease. This derepression of the ammonium permease in *R. leguminosarum* was slow compared to the derepression of ammonium transport systems reported for other free-living N_2 -fixing bacteria (Hartmann & Kleiner, 1982; Kleiner, 1982). However, similar times have been reported for the induction of histidase or the *p*-hydroxybenzoate catabolic enzymes in *R. leguminosarum* MNF3841 grown in minimal media (Dilworth *et al.*, 1983).

The repression of the ammonium uptake system in *R. leguminosarum* by ammonia was examined using chemostat cultures. Cells were grown under ammonia-limitation to steady state,

Fig. 1. Uptake of ammonium by *R. leguminosarum* MNF3841 grown in nitrogen-limited (○, ●) or nitrogen-excess (□, ■) chemostat cultures. The cells were harvested, washed and resuspended in minimal salts medium containing 5 mM-sodium phosphate (pH 7.2) and 0.5 mM-sucrose. The ammonia concentration was measured using either the ion specific ammonium electrode (open symbols) or the phenol-hypochlorite method (closed symbols).

Fig. 2

Fig. 3

Fig. 2. Derepression of ammonium transport after transfer of cells of *R. leguminosarum* MNF3841 grown with 10 mM- NH_4^+ to a minimal salts medium minus nitrogen source. The cells were harvested, washed three times and resuspended in the new medium. Ammonium uptake (□, ■) and cell growth (○, ●) were measured in the presence (open symbols) and absence (closed symbols) of 0.2 mg chloramphenicol ml^{-1} .

Fig. 3. Repression of ammonium transport in chemostat-grown *R. leguminosarum* MNF3841. An ammonia-limited chemostat culture of *R. leguminosarum* growing at a concentration of 0.5 mM- NH_4^+ in the inflowing medium was changed to a medium containing 10 mM- NH_4^+ . Ammonium uptake (●) and cell growth (○) were measured.

then NH_4^+ (10 mM) was introduced to the culture in the inflowing medium. A decrease in the activity of the ammonium uptake system was evident 8 h after the addition of NH_4^+ (Fig. 3) and the system was totally repressed within 18 h of the introduction of a non-limiting ammonium concentration.

In the later stages, between 12 and 18 h, the rate of loss of activity is greater than that predicted by simple dilution, implying either a degradation of the permease or its inhibition.

Similar results have been reported for the repression by NH_4^+ of ammonia-sensitive methylamine uptake systems in free-living N_2 -fixers, with 10 mM- NH_4^+ repressing uptake systems in *Azospirillum* spp. (Hartman & Kleiner, 1982) and *Klebsiella pneumoniae* (Kleiner, 1982), and 15 mM- NH_4^+ repressing the uptake system in *Rhodospirillum rubrum* (Alef & Kleiner, 1982). The repression of the ammonium permease in *R. leguminosarum* by its substrate, NH_4^+ , indicates a similar genetic control to that reported in *K. pneumoniae* (Kleiner, 1982).

Fig. 4. Effect of medium pH on ammonium uptake by *R. leguminosarum* MNF3841. Cells from an ammonia-limited chemostat were incubated for 30 min as described in Fig. 2, except the buffer was 5 mM-sodium phosphate for pH 5.8–7.8 and 10 mM-Tris/HCl for pH 7.1–8.5.

Characteristics of the ammonium permease

Ammonium uptake by cells from ammonia-limited chemostats was inhibited by more than 95% by carbonyl cyanide *m*-chlorophenylhydrazon (0.02 mM), valinomycin (0.01 mM), nigericin (0.01 mM), azide (1 mM) or 2,4-dinitrophenol (0.1 mM), indicating that this was an active system dependent on an energized membrane.

The effect of pH on ammonium uptake was examined using 5 mM-sodium phosphate buffer (pH 5.8–7.8) and 10 mM-Tris/HCl buffer (pH 7.1–8.5). Ammonium uptake showed a narrow maximum around pH 7.5 (Fig. 4). No ammonia was removed when cells were incubated below pH 6.5 or above pH 8.3. This result indicates that ammonium uptake by the permease characterized here would not occur when *R. leguminosarum* MNF3841 was in an environment with a pH of 6.5 or less, which would limit its usefulness to the organism in the soil. Whether other ammonium transporting systems operate at lower pH values remains to be determined.

Similar effects of external pH on ammonium uptake systems in other N_2 -fixing organisms have been measured using [^{14}C]methylamine as an analogue for ammonia, with pH profiles being reported for *Clostridium pasteurianum* (pH 6.0–7.5, optimum pH 6.4; Kleiner & Fitzke, 1981), and *R. rubrum* (pH 5.5–8.0, optimum pH 7.2; Alef & Kleiner, 1982). pH optima of 7.5 for *Azospirillum brasilense* (Hartman & Kleiner, 1982) and 6.8 for *K. pneumoniae* (Kleiner, 1982) have also been reported, but there is no mention in these studies of the buffers used or the pH range over which uptake was measured.

Kinetics of the ammonium permease

The effect of substrate concentration on ammonium uptake in N-limited cells was investigated in detail with the apparent K_m values quoted being the means of five separate determinations. The K_m for ammonium uptake in ammonia-limited cells was determined over the range 0.004–0.05 mM- NH_4^+ using the non-linear regression method of Duggleby (1981). The K_m was 0.015 mM with a V_{max} of 7.2 nmol min $^{-1}$ (mg protein) $^{-1}$. The K_m for ammonium uptake by nitrate-limited cells was 0.012 mM with a V_{max} of 6.8 nmol min $^{-1}$ (mg protein) $^{-1}$.

Accumulation of intracellular ammonia in ammonia-limited cells

The internal ammonia concentration of ammonia-limited cells (0.09 mg protein ml $^{-1}$) was determined after a known amount of ammonium had been removed from solution by the cells. The initial ammonia concentration of the supernatant was 0.079 mM and at the conclusion of the experiment (32 min) the final ammonia concentration was 0.0075 mM. Determination of the internal ammonia concentration yielded values of 0.42 mM (entrained liquid assay) or 0.45 mM (cell water from dry weight method). Either result represents a significant concentration increase over the final ammonia concentration in the medium (0.0075 mM).

These results, taken together with the pH profile for ammonium uptake and the inhibitor studies, suggest that in *R. leguminosarum* MNF3841 grown under nitrogen limitation there is an active accumulation and retention of ammonium in the cells against a concentration gradient.

Assimilation of ammonia in nitrogen-limited *R. leguminosarum* cells proceeds via the glutamine synthetase/glutamate synthase system (Brown & Dilworth, 1975), and the activities of these enzymes in the cells used in this study were 52.4 and 22.8 nmol min⁻¹ (mg protein)⁻¹, respectively. These levels of enzyme activity are in agreement with previously published results for another strain of *R. leguminosarum* (Brown & Dilworth, 1975). In the uptake experiment described above, each culture sample (40 ml) took up 2860 nmol of ammonium; recovery as intracellular ammonia accounted for 12 nmol. The activities of the ammonia assimilatory enzymes were consistent with this rate of incorporation.

The measured intracellular ammonia concentration of 0.4 mM in N-limited *R. leguminosarum* is similar to values reported previously for *Azotobacter vinelandii* (0.6 mM, Kleiner, 1975) and *K. pneumoniae* (0.3–0.4 mM; Kleiner, 1976). Both of these organisms are free-living nitrogen-fixing bacteria which have been shown to possess active ammonium uptake systems (Kleiner, 1982; Wiegel & Kleiner, 1982).

Effect of amino acids and other N-containing compounds on ammonium permease activity

The addition of ethylamine, glutamate or nitrate to ammonia-limited cell suspensions had no effect on the removal of ammonium by cells, even when present in a 100-fold molar excess. Hydrazine and hydroxylamine were both potent inhibitors of ammonium uptake in ammonia-limited cells, with K_i values of 0.02 mM and 0.09 mM, respectively. Glutamine, aspartate and glycine were less effective inhibitors with K_i values of 0.12, 0.25 and 2.44 mM, respectively. Previously, Barnes & Zimniak (1981) found hydrazine and hydroxylamine to be strong inhibitors of methylammonium uptake in *A. vinelandii*, with K_i values of 0.0023 mM and 0.0063 mM, respectively, but amides and amino acids were not inhibitory to methylammonium accumulation in *A. vinelandii*.

Since ammonia is a strong inhibitor of [¹⁴C]methylamine uptake in N-limited cells of MNF3841 (Glenn & Dilworth, 1984), methylamine would be expected to inhibit ammonium uptake. We were unable to measure directly the effect of methylamine on the ammonium permease since methylamine interfered with the electrode response at the low concentrations of ammonia used. However, after separation of ammonium and methylammonium by ion-exchange chromatography, we were able to use the chemical assay for ammonia to show that methylamine inhibited ammonium uptake in ammonia-limited MNF3841 cells, with 1 mM-methylamine inhibiting ammonium uptake by 62%.

Bacteroid assays

Isolated pea bacteroids did not show ammonium permease activity though they were able to accumulate [¹⁴C]succinate, as described previously (Glenn *et al.*, 1980; Glenn *et al.*, 1984). This absence of the ammonium permease in bacteroids of *R. leguminosarum* MNF3841 confirms previous work by Glenn & Dilworth (1984), and shows that there is no specific active ammonium uptake system in N₂-fixing pea bacteroids. This contrasts with the situation in N₂-fixing cells of the free-living bacteria *A. vinelandii* (Laane *et al.*, 1980) and *K. pneumoniae* (Kleiner, 1982), the free-living heterocystous cyanobacterium *Anabaena variabilis*, and the symbiotic form of *Anabaena azollae* (Rai *et al.*, 1984) which all possess an active NH₄⁺ uptake system.

If this lack of an ammonium permease in the bacteroids reflects the true situation in the nodule, our results suggest that this permease is unlikely to be of importance in the symbiotic state. We suggest that the neutral NH₃ molecule diffuses from the bacteroid to the plant cytosol where it is assimilated by plant enzymes.

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