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Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules

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Abstract Ammonia permeability of the peribacteroid membrane (PBM) from N_2-fixing soybean nodules was measured (8×10^{-5} m/s) using isolated PBM in a stopped-flow spectrofluorimeter. Ammonia (NH_3) uptake into PBM vesicles was inhibited by up to 42% by $HgCl_2$ ($EC_{50}=2.9$ μM, mercaptoethanol-reversible) and reduced by ATP pre-incubation. The activation energy of NH_3 uptake (52 kJ/mol) increased (118 kJ/mol) with $HgCl_2$. Water transport was also $HgCl_2$-sensitive ($EC_{50}=52.6$ μM), but increased by ATP pre-incubation. NH_3 and H_2O may permeate via different pathways through Nodulin 26 or there is another protein on the PBM that is permeable to NH_3.

Key words: Peribacteroid membrane; NH_3 permeability; Aquaporin; Activation energy; Mercury inhibition; Nodulin 26

1. Introduction

Reduced nitrogen released by symbiotic N_2-fixing bacteria is an important nitrogen source in legumes [1]. In soybean root nodules, nitrogen fixed by *Rhizobium* bacteria has to pass the peribacteroid membrane (PBM) to be assimilated in the plant cytoplasm. Whereas ammonium, as a charged molecule (NH_4^+), is supposed to enter the cytoplasm through specialised Ca^{2+}- and Mg^{2+}-regulated channels [2,3], ammonia (NH_3), as a small uncharged molecule, has been assumed to simply diffuse through the lipid phase of the membrane. However, this idea of dissolved gases simply permeating membranes by diffusion through the lipid phase has recently been challenged. It has been demonstrated that AQP1, a membrane intrinsic protein (MIP) in the red cell membrane, can facilitate CO_2 permeation [4,5]. AQP1 is an aquaporin that belongs to a large family of MIPs that are widespread in animals and plants [6–8]. Interestingly, Nodulin 26 (NOD26), a prevalent MIP in the PBM that is only expressed when the bacteria infect a root cell, is also a member of the aquaporin family [9]. NOD26 confers increased water permeability [10], but is also permeable to small molecules like formamide and glycerol [10,11]. It has been suggested that the main function of NOD26 in the symbiosome membrane is that of osmoregulation [11], the feature of its glycerol permeability being less understood.

Given the obvious importance of reduced nitrogen exchange in the *Bradyrhizobium*-soybean symbiosis, we tested the NH_3 permeability of native vesicles obtained from the PBM from soybean nodules with respect to known features of aquaporins and ion channels in the symbiosome membrane.

2. Materials and methods

2.1. Plant material

Soybeans, *Glycine max* (L. cv. Stephens), germinated on moist filter paper were planted in 5 l pots (12 plants to a pot) of a mixture of vermiculite and perlite and inoculated with *Bradyrhizobium japonicum* USDA 110. Plants were supplied with a nutrient solution [12] in a naturally illuminated greenhouse with supplementary lights to provide a 16 h light/8 h dark cycle. Nodules were collected from 5–6 week old plants.

2.2. Vesicle isolation

PBM vesicles were obtained as described by Christiansen and coworkers [13], but for addition of 5 μg/ml leupeptin, and for replacing the hypoosmotic step in the preparation by vortexing the symbiosomes in the resuspension medium for phase partitioning. 100 μM carboxyfluorescein (CF, a pH-sensitive, fluorescent dye) was added to this medium to load the dye into the vesicles during their formation. 40 g of nodules was processed and a 36 g phase system was used. The final pellet of CF-loaded vesicles in resuspension medium (330 mM sucrose, 5 mM potassium phosphate, pH 7.8) was stored at −80°C.

2.3. Enzyme assays

Enzyme assays were performed as described by Niemietz and Tyerman [14]. According to Christiansen et al. [13], glucansynthase II was considered a marker enzyme of the PBM, whereas cytochrome *c* oxidase activity served as a tracer of bacterial contamination.

2.4. Vesicle size determination

Electron micrographs were used to determine vesicle diameters. PBM vesicles had an average diameter of 202 nm, while liposomes were smaller, with an average diameter of 125 nm (results not shown).

2.5. Ammonia uptake experiments

In a stop-flow fluorimeter (Applied Photophysics, Leatherhead, UK), vesicles loaded with the pH-sensitive dye CF (100 μM, excitation 492 nm; emission >515 nm, GG 515 cut-on filter, Schott, supplied by Applied Photophysics, Leatherhead, UK) and suspended in dye-free fluoro-buffer (250 mM sorbitol, 30 mM KCl, 10 mM HEPES-KOH, pH 6.8) were mixed with identical solutions containing NH_4Cl. NH_3 crossing the membrane was protonated according to its pH-dependent equilibrium with NH_4^+ and thus caused alkalinisation of the weakly buffered vesicle interior and reduced CF fluorescence. Preliminary experiments, where Anti-CF antibody (Molecular Probes) was added to quench extra-vesicular fluorescence [15,16], revealed only a negligible amount of external fluorescence. The antibody was hence omitted. The time-course of fluorescence change was fitted with either a single or a double exponential equation.

2.6. Determination of NH_3 permeability

Theoretical curves for the kinetics of intravesicular pH change were generated using the differential equations of Boron and DeWeer [17] solved using Mathcad V8.0 (MathSoft) for the conditions used in the

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experiments (internal vesicle buffer capacity, surface area to volume ratio, pH and external NH_4^+ concentration) and for a range of NH_3 and NH_4^+ permeabilities. The vesicle membrane potential was assumed to be close to zero since the PBM is very permeable to K^+ [3] and there was no significant K^+ concentration gradient across the membrane. Also inclusion of 5 μM valinomycin did not affect the kinetics of the experimental curves (results not shown). The theoretical curves were fitted to a single exponential (case of very low NH_4^+ permeability) or the sum of two exponentials (case of significant NH_4^+ permeability) and the rate constant of the fast phase plotted against the NH_3 permeability coefficient. This gave a calibration curve from which the experimental measurements of rate constant could be used to estimate NH_3 permeability (P_{NH3}).

2.7. Water permeability experiments

Light scattering experiments to determine the osmotic water permeability (P_{OS}) of PBM vesicles were principally conducted as described by Niemietz and Tyerman [14]. PBM vesicles suspended in Iso370 (330 mM sucrose, 5 mM HEPES-KOH, pH 7, 100 μM $CaCl_2$, at 370 mOsm) were simultaneously injected with a hyperosmotic solution (0.2 M extra sucrose in Iso370 to create an inwardly directed 100 mOsm osmotic gradient). The time-course of vesicle shrinking was followed as an increase in light scattering at 500 nm.

2.8. ATP pre-incubation

Vesicles in resuspension medium pH 7 were incubated for 1 h at room temperature with additional 6 mM $MgSO_4$, 1 mM $CaCl_2$ and 4 mM K-ATP. Vesicles were then pelleted at 100 000 × *g* and resuspended respectively in Iso370 for H_2O flux measurements (light scattering), or in fluoro-buffer for NH_3 uptake (CF fluorescence).

3. Results and discussion

Following a procedure by Christiansen et al. [13], PBM vesicles were isolated from the nodules of 6 week old soybean plants. Monitoring protein content and glucansynthase II and cytochrome *c* oxidase as markers of respectively PBM and bacteroids (Table 1), we obtained a PBM fraction virtually devoid of bacterial contamination.

Ammonia uptake into PBM vesicles leads to alkalinisation of the vesicle interior. In CF-loaded PBM vesicles, this pH shift resulted in a decrease of CF fluorescence (Fig. 1A). The time-course of decrease was well fitted by a single exponential. No alkalinisation was observed when NH_4Cl was substituted by KCl (results not shown). It was established that the observed shift in CF fluorescence was directly proportional to the concentration of protons (Fig. 1B).

In theory, fast initial NH_3 uptake should be followed by a slower phase of NH_4^+ uptake through previously characterised NH_4^+-permeable channels [2], leading to fluorescence reversal. In most experiments, this second phase of NH_4^+ uptake was virtually undetectable over a period of 10 s, indicating the vastly higher permeability of the membrane for NH_3 over NH_4^+. In some experiments, however, there was clearly a second phase of fluorescence reversal that necessitated careful mathematical evaluation of the curves to ascertain that this did not affect the estimation of NH_3 permeability from the initial rapid alkalinisation phase. A system of differential equations developed by Boron and DeWeer [17] allows an estimate of the effect of the second phase of fluorescence reversal (acidification) on the permeability coefficient for NH_3 as measured from the rate constant for fast alkalinisation. The theoretical curves demonstrated that the sum of two exponentials could be well fitted to the time-course and that the rate constant for the fast phase could be used to estimate the NH_3 permeability for a wide range of NH_4^+ permeabilities.

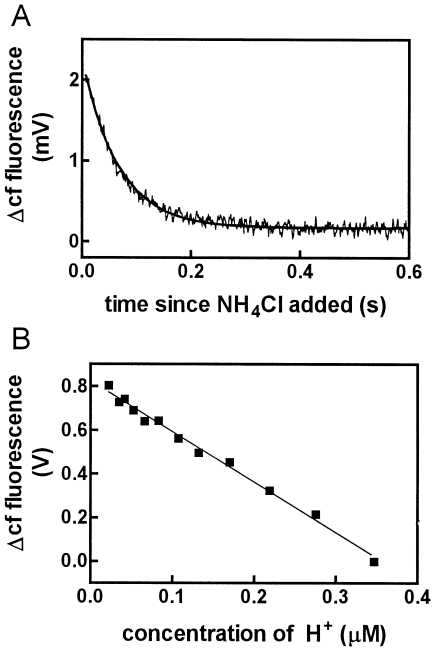

Fig. 1. Time-course of CF fluorescence in PBM vesicles representing decrease in internal H^+ concentration. (A) pH-dependent fluorescence decrease in CF-loaded PBM vesicles in fluoro-buffer (250 mM sorbitol, 30 mM KCl, 10 mM HEPES-KOH, pH 6.8) injected vs. fluoro-buffer plus 5 mM NH_4Cl (final NH_4Cl concentration 2.5 mM). A single exponential was fitted to the time-course giving a rate constant of 14.9 s^{-1}. (B) Linear relationship between CF fluorescence and H^+ concentration when 100 μM CF in fluoro-buffer was titrated with successive additions of HCl.

When CF-loaded PBM vesicles were incubated with increasing concentrations of NH_4Cl (Fig. 2A), the rate of uptake was directly proportional to the NH_3 concentration (Fig. 2B). A shift in pH to a more alkaline value causing a higher proportion of NH_3/NH_4^+ resulted in a corresponding change in the slope of the concentration-dependence (Fig. 2). Such non-saturable uptake of NH_3 would be expected for permeation through channels or via the lipid pathway. Unstirred layer effects can be excluded using the same arguments given for H_2O permeation in [14].

If NH_3 uses the same pathway as other small solutes (H_2O,

Table 1
Distribution of protein- and marker enzyme activity for PBM (glucansynthase II) and bacteroid activity (cytochrome *c* oxidase) in the microsomal fraction and in the membranes recovered after three partitioning steps

	Protein (μg/ml fraction)	Glucansynthase II (nmol UDPG/h/μg (protein))	Cytochrome *c* oxidase (μmol/min/μg (protein))
Microsomes	390 ± 69.5	0.922 ± 0.122 (100%)	1.49 ± 0.23 (100%)
UIII	245 ± 23.4	1.031 ± 0.306 (112%)	0.11 ± 0.0072 (7%)
LIII	242 ± 30.15	1.74 ± 0.231 (188%)	9.047 ± 0.772 (607%)

Values are mean (S.E.M.), $n = 4$.

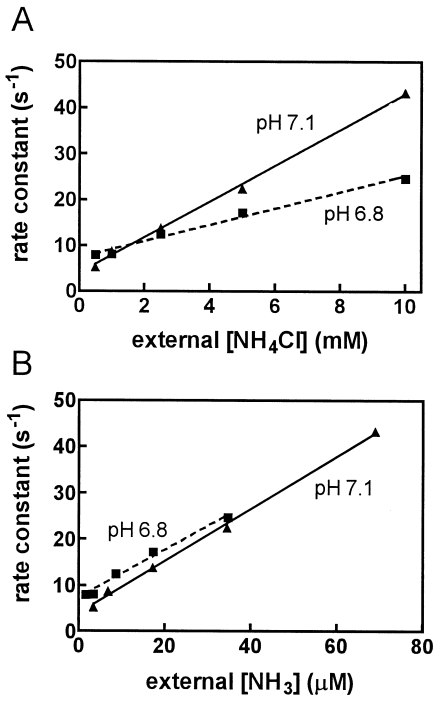

Fig. 2. Concentration-dependence of NH_4Cl uptake into PBM vesicles at two pH values. PBM vesicles in fluoro-buffer, pH 6.8 or pH 7.1, were injected vs. 1, 2, 5, 10, 20 mM NH_4Cl. Rates of fluorescence decrease were plotted against the final NH_4Cl concentration (A). Between pH 6.8 and pH 7.1, the proportion of NH_3/NH_4^+ increases 2.4 times ($pK_{NH3} = 4.74$). (B) Rate of NH_4Cl uptake at two pH values normalised for NH_3 concentration.

glycerol) then it could potentially permeate via NOD26 and display features of those transports. Rivers et al. [18] demonstrated that NOD26 when expressed in *Xenopus* oocytes induced water channel activity that was mercury-sensitive and which lowered the activation energy for water uptake. Such mercury-sensitivity and low activation energy for water permeation was also observed in isolated PBM vesicles. These vesicles also showed mercury-sensitive glycerol and formamide permeability which was taken as an indicator that these solutes also permeate NOD26. The purified protein, when incorporated into liposomes also increased glycerol permeability while retaining mercury-sensitivity and conveying a low activation energy for glycerol uptake [19].

To investigate protein involvement in NH_3 uptake, we tested the effect of $HgCl_2$ (Fig. 3) known to interact with membrane proteins, and specifically NOD26. Pre-incubation of PBM vesicles with $HgCl_2$ reduced the rate of NH_3 uptake in a concentration-dependent manner (Fig. 3A), with half-maximal inhibition being reached at 2.9 μM $HgCl_2$ and a maximal inhibition of 42%. When $HgCl_2$-treated vesicles were pelleted and resuspended in a mercury-free medium containing excess mercaptoethanol, inhibition of NH_3 uptake was fully reversible (results not shown). For comparison, we also tested the effect of $HgCl_2$ on P_{OS} in PBM vesicles (Fig. 3B), and found that, similar to the situation in *Xenopus* oocytes expressing NOD26 [18], and in liposomes reconstituted with NOD26 [19], water flow in native PBM vesicles was inhibited by $HgCl_2$. Half-maximal inhibition occurred at 52.6 μM $HgCl_2$ and maximal inhibition was in excess of 75% (Fig. 3B). When liposomes, prepared by sonication of a crude soybean lecithin preparation in CF-containing resuspension medium, were incubated with $HgCl_2$, no inhibition of NH_3 uptake was observed (Fig. 3C). This pattern suggests that both NH_3 and H_2O fluxes through the native PBM are at least partially protein-mediated.

Another criterion for protein-mediated membrane permeation of NH_3 is the activation energy for NH_3 uptake as reflected in the temperature-dependence of the process. Temperature-dependence of uptake was measured between 13°C and 22°C (Fig. 4). Ammonia uptake showed strong temperature-dependence with an activation energy of 55 kJ/mol. This value is rather higher than the activation energies reported for water and glycerol permeation through NOD26 [10,19] and makes it unlikely that NH_3 shares a common pathway with these substrates. The value is more in line with those reported for water

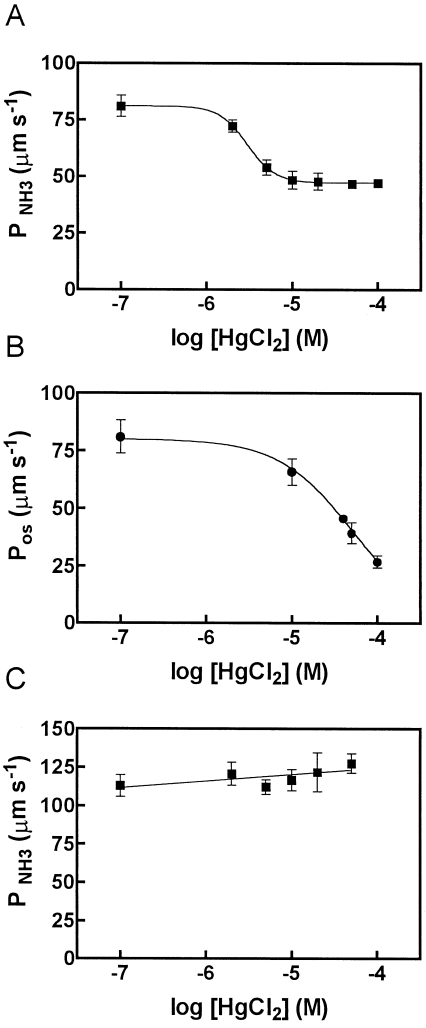

Fig. 3. Effect of mercury on NH_3 and H_2O permeability of PBM vesicles and on NH_3 permeability of soybean lecithin liposomes. (A) PBM vesicles in fluoro-buffer were pre-incubated for 5 min with increasing concentrations of $HgCl_2$ (0, 10, 20, 50, 100 μM) before being injected vs. 20 mM NH_4Cl; (B) PBM vesicles in Iso370 were pre-incubated for 5 min with increasing concentrations of $HgCl_2$ (0, 10, 50, 100, 200 μM) and injected vs. extra 0.2 M sucrose in Iso370; (C) Soybean lecithin liposomes in fluoro-buffer were pre-incubated for 5 min with increasing concentrations of $HgCl_2$ (0, 10, 20, 50, 100 μM) before being injected vs. 20 mM NH_4Cl. The permeabilities for A and B were fitted to dose-response curves to determine half-maximal inhibition.

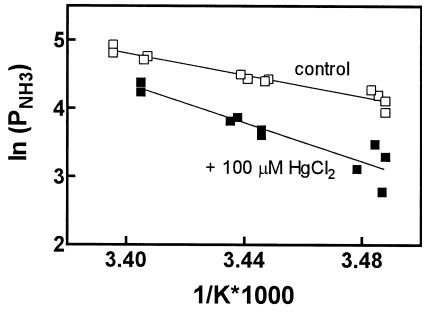

Fig. 4. Temperature-dependence of P_{NH3} for PBM vesicles with or without $HgCl_2$ pre-incubation. The experiment was performed four times each on different batches of PBM vesicles. The fitted linear regressions were used to calculate the activation energies reported in the text.

and glycerol permeation through the lipid phase of the membrane [20]. However, other, carrier-mediated transport processes show similar, high, activation energies [21–23]. When $HgCl_2$ was present, the activation energy for NH_3 uptake more than doubled to 118 kJ/mol (Fig. 4).

Both the mercury effect per se and the strong effect of $HgCl_2$ on the activation energy of NH_3 uptake indicate protein mediation of NH_3 penetration into PBM vesicles. The intrinsically high activation energy for NH_3 permeation might be a feature of a highly specialised membrane, where a control of gas exchange is more important than an overall high permeability to gases.

This aspect of controlled permeation is emphasised by the finding that the PBM contains a Ca^{2+}-dependent protein kinase [24], which could modulate activity of membrane proteins by phosphorylation. NOD26 is phosphorylated by this Ca^{2+}-dependent protein kinase and its transport activity is altered as a result [25]. Pre-incubation of PBM vesicles with ATP significantly reduced P_{NH3} from 79.00 (± 4.76)$\times 10^{-6}$ m/s to 55.27 (± 6.18)$\times 10^{-6}$ m/s (S.E.M., $n=5$, t-test $P<0.05$) but increased P_{OS} from 59.96 (± 4.145)$\times 10^{-6}$ m/s to 70.10 (± 2.937)$\times 10^{-6}$ m/s (S.E.M., $n=4$, t-test $P<0.05$).

The NH_3 permeability of the PBM is quite low compared to the erythrocyte membrane (2.1×10^{-3} m/s) [26]. The high permeability of the erythrocyte membrane is not due to the CHIP28 (AQP1) water channel [16]. However, the PBM has a higher permeability than for bacteria and cyanobacteria (2–6×10^{-6} m/s) [27,28] and the apical membrane of gastric parietal cells (4.4×10^{-5} m/s) [15].

To compare the contribution of NH_3 flux as described in this paper to the previously described NH_4^+ release through a specialised cation channel in the PBM [2], a number of parameters need to be considered: flux through both pathways depends on pH and NH_4^+ concentration in the cytoplasm and symbiosome space, as well as the proton pump rate across the PBM, and PBM membrane potential. Using an estimate of 12 mM for the symbiosome space NH_4^+ concentration [29] and a value of 0.2 mM for the cytoplasmic concentration (near the K_m for glutamine synthases) [26], it can be calculated that the NH_3 flux from symbiosomes is critically dependent on symbiosome space pH. For example, at symbiosome space pH of less than 5.5, the flux of NH_3 could actually be in the reversed direction (into the symbiosome space from the cytoplasm). Under these circumstances, which would be when the proton pump was very active, the nitrogen flux from symbiosomes would be via the NH_4^+ channel that can easily accommodate the calculated flux [30] from symbiosomes [2]. With the pump active, and with a low pH in the symbiosome space, closing the NH_3-permeable channel would reduce the reverse flux. In this respect, it is interesting that ATP pre-incubation reduced NH_3 permeation. If the pH of the symbiosome space was to rise to above 6, signifying decreased proton pump rate, there could be a large efflux of NH_3 to the cytoplasm via both the membrane lipid and channel pathways (flux of 4×10^{-7} mol/m^2/s at pH 6) that could be equivalent to the flux through the NH_4^+ channel and approximately 100 times that necessary to account for the estimated symbiosome nitrogen efflux (4×10^{-9} mol/m^2/s) [30]. Thus the activity of the proton pump and the pH of the symbiosome space are critical in determining the relative contribution of the NH_4^+ flux compared to the NH_3 flux. It would be interesting to see if pH also affects the channel-mediated NH_3 flux particularly in view of the recent discovery that an animal aquaporin is regulated by pH [31].

In conclusion, NH_3 permeation is partially protein-mediated in native PBM vesicles. The H_2O (osmotic) and NH_3 permeabilities were both inhibited by mercury and modulated by ATP pre-incubation. However, P_{NH3} was 10 times more sensitive to mercury inhibition than P_{OS}, and P_{NH3} was reduced by ATP pre-incubation, whereas P_{OS} increased after ATP pre-incubation. We propose that either NH_3 and H_2O permeate via different pathways through the NOD26 tetramer aggregates or that there is another protein in the PBM that allows permeation of NH_3.

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