

## Structural and mechanistic aspects of Amt/Rh proteins

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### Abstract

Amt/Rh proteins, which mediate movement of ammonium across cell membranes, are spread throughout the three kingdoms of life. Most functional studies on various members of the family have been performed using cellular assays in heterologous expression systems, which are, however, not very well suited for detailed mechanistic studies. Although now generally considered to be ammonia conducting channels, based on a number of experimental studies and structural insights, the possibility remains that some plant Amts facilitate net ammonium ion transport. The *Escherichia coli* channel AmtB has become the model system of choice for analysis of the mechanism of ammonia conductance, increasingly also through molecular dynamics simulations. Further progress in a more detailed mechanistic understanding of these proteins requires a reliable *in vitro* assay using purified protein, allowing quantitative kinetic measurements under a variety of experimental conditions for different Amt/Rh proteins, including mutants. Here, we critically review the existing functional data in the context of the most interesting and unresolved mechanistic questions and we present our results, obtained using an *in vitro* assay set up with the purified *E. coli* channel AmtB.

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### 1. Introduction

Ammonium is the preferred nitrogen source for many organisms and its transport across cellular membranes is a fundamental biological process. Ammonium transport is mediated by a family of ubiquitous membrane proteins (designated Amt), found in the three kingdoms of life (Huang and Peng, 2005), whose homologues in animals are the Rhesus (Rh) proteins (Marini et al., 1997b, 2000). The predominant view has long been that these proteins act as secondary active transporters for NH<sub>4</sub><sup>+</sup> but this has been challenged by Kustu and collaborators (Soupene et al., 1998, 2001, 2002) who have proposed that Amt and Rh proteins act as channels for NH<sub>3</sub> and CO<sub>2</sub>, respectively.

Strong support for this latter view came from the X-ray structure of the *Escherichia coli* channel AmtB (Khademi et al., 2004; Zheng et al., 2004) that revealed a predominantly hydrophobic channel, suggesting a high energy barrier for the conduction of the ammonium ion (Fig. 1). Based on this structural evidence, and confirmed by a number of subsequent functional studies with *E. coli* AmtB and human Rh proteins (Table 1), the predominant view has now become that the Amt/Rh proteins facilitate the transport of ammonia (Winkler, 2006). However, the possibility that Rh protein may also conduct CO<sub>2</sub> remains controversial (Van Kim et al., 2006). In this context the term gas channel is often used, apparently because the conducted substrates are small, relatively non-polar molecules occurring as gases in their pure form under ambient conditions. However, we consider this term incorrect from a physico-chemical point of view and will therefore not use it. It also

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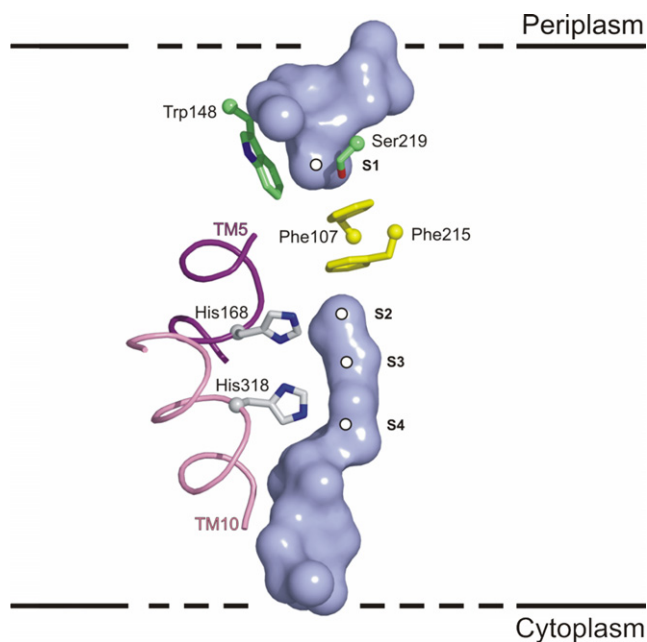


Fig. 1. Substrate translocation path of AmtB. The pore region of one monomer is shown with the water-accessible volume represented in space filling representation (light-blue). Four mechanistically distinct segments, (i) periplasmic ammonium binding site, (ii) phenylalanine gate, (iii) central pore with twin-His arrangement and (iv) cytoplasmic vestibule can be discriminated. Selected, highly conserved residues are shown in ball-and-stick representation for the ammonium binding site (green), phenylalanine gate (yellow) and central pore (grey), where parts of the two transmembrane helices TM5 (His168) and TM10 (His318) are also shown. S1-S4 designate the location of difference electron density peaks assigned to ammonia/water sites (Khademi et al., 2004; Zheng et al., 2004).

has to be noted that net ammonium ion conductance and thus electrogenic transport remains strongly indicated for some plant Amt homologues (Mayer et al., 2006a,b; Mayer and Ludewig, 2006). Here, we will critically review experimental data that relate to the nature of the species conducted through the Amt/Rh proteins and discuss important mechanistic questions which are largely unresolved.

## 2. Mechanistic questions

To define the most interesting mechanistic questions, we have considered the pore region located in the centre of each monomer of the *E. coli* AmtB trimer (Fig. 1) to consist of four distinct parts.

First, on the periplasmic side, a putative  $\text{NH}_4^+$  binding site has been identified right at the entrance of the pore (Khademi et al., 2004; Zheng et al., 2004). The environment of this binding site is highly conserved within the Amt protein family. The apparent ammonium affinities reported for the Amt proteins from transport saturation and inhibition experiments (Howitt and Udvardi, 2000; Javelle et al., 2003, 1999, 2001; Marini et al., 1997a; Meier-Wagner et al., 2001) are in the micromolar range and are likely to relate to this binding site. In microorganisms and plants, the expression of Amt proteins is subject

to nitrogen repression, such that expression is essentially only induced at low external ammonium concentration (von Wirén and Merrick, 2004). Under this trophic condition, the high  $\text{NH}_4^+$  affinity of Amt proteins may be important for efficient substrate capture. Moreover, the specific features of the binding site may play an important role in discriminating against other cations and possibly water (Winkler, 2006). In the Rh family the apparent ammonium affinities are much lower, typically in the low millimolar range (Mayer et al., 2006b). Sequence alignments of Rh and Amt proteins (Callebaut et al., 2006; Conroy et al., 2005; Huang and Peng, 2005) show that the conserved Trp and Ser residues, implicated in direct interactions with  $\text{NH}_4^+$  in the Amt proteins (Fig. 1), are substituted by aliphatic residues in Rh proteins, which are expected to considerably lower the latter's affinity for  $\text{NH}_4^+$ .

Second, entry into the pore of AmtB appears to be sterically blocked by the partly stacked phenyl rings of residues F107 and F215. The high conservation of this 'dynamic gate' throughout the Amt/Rh family (Callebaut et al., 2006; Conroy et al., 2005; Huang and Peng, 2005) suggests an important mechanistic role, which is presently not understood. There is no indication that the protein can assume a distinct, stable structural state where this gate is open. It is believed, rather, that transient structural fluctuations are sufficient to permit entry (or exit) of small substrate molecules ( $\text{NH}_4^+/\text{NH}_3$ ,  $\text{H}_2\text{O}$ ,  $\text{CO}_2$ ) (Lin et al., 2006; Nygaard et al., 2006; Yang et al., 2007). However, the dynamics of these structural fluctuations may set an upper limit on the maximum conductance rate such that, in contrast to aquaporin channels (Agre, 2006), it can hardly be diffusion limited. Molecular dynamics simulations may help to clarify these questions and might also examine whether this gating mechanism confers some substrate specificity.

Third, the central part of the narrow pore is lined by hydrophobic side chains with two almost totally conserved histidine residues, the side chains of which are arranged such that a hydrogen bond forms between their  $\delta$  nitrogen atoms (Fig. 1). Their high conservation is suggestive of a distinct functional role, and a role in substrate deprotonation appears attractive at first sight. However, their location, deeply buried in the hydrophobic pore, raises questions with respect to the energetics of ammonium ion entry and subsequent proton release from the histidine residue (Winkler, 2006). Both possibilities, separate transfer of the proton through the pore (in the case of net  $\text{NH}_4^+$  flux) and transfer of the proton back to the periplasm from His168 (in the case of  $\text{NH}_3$  conductance), appear difficult. We have undertaken structural and functional studies on fourteen variant proteins with one or both of the histidines replaced by other polar or non-polar residues (Javelle et al., 2006). All these variants, except for H168E which has an activity of about 25% compared to wild-type AmtB, appear inactive in the conduction of methylamine. Crystallographic analysis of selected variants showed that their structures were essentially unaffected by the substitution. The results

Table 1  
Methods used and correspondingly deduced transport mechanisms of various member of the Amt/Rh protein family

Organisms	Proteins	Systems	Methods <sup>a</sup>	Mechanisms	References
<i>L. esculentum</i>	Amt1;1	Oocyte	EP	High affinity $\Delta\psi$ driven $\text{NH}_4^+$ uniporter	Ludewig et al. (2002, 2003)
	Amt1;2	Oocyte	EP	Low affinity $\Delta\psi$ driven $\text{NH}_4^+$ uniporter	Ludewig et al. (2003)
	Amt1;1	Oocyte/yeast	EP/TA/pH	$\text{NH}_4^+$ uniporter or $\text{NH}_3/\text{H}^+$ cotransporter	Mayer et al. (2006a)
	Amt1;2	Oocyte	EP/TA/pH	$\text{NH}_4^+$ uniporter or $\text{NH}_3/\text{H}^+$ cotransporter	Mayer et al. (2006b)
<i>A. thaliana</i>	Amt1;1	Oocyte	EP/TA/pH	$\text{NH}_4^+$ uniporter or $\text{NH}_3/\text{H}^+$ cotransporter	Mayer and Ludewig (2006), Ludewig (2006)
	Amt1;1	Oocyte/yeast	EP/TA	$\Delta\psi$ -driven transport of $\text{NH}_4^+$	Wood et al. (2006)
Mammals	RhAG	Oocyte	TA	Electroneutral $\text{NH}_4^+/\text{H}^+$ exchanger	Westhoff et al. (2002)
		Yeast	TA	Electroneutral $\text{NH}_4^+/\text{H}^+$ exchanger	Westhoff et al. (2004)
		Red Blood cells ghosts	pH	$\text{NH}_3$ channel	Ripoche et al. (2004)
		HeLa cells	pH	transport of $\text{NH}_4^+$ and $\text{NH}_3$	Benjelloun et al. (2005)
		Oocyte	EP/pH	Electroneutral $\text{NH}_3$ transport	Ludewig (2006)
		Red Blood cells	TA	$\text{NH}_4^+$ exporter	Hemker et al. (2003)
	RhBG	Oocyte	EP/TA/pH	Electroneutral $\text{NH}_4^+/\text{H}^+$ exchanger	Ludewig (2004)
		Oocyte	EP/pH	Electrogenic $\text{NH}_4^+$ transporter	Nakhoul et al. (2005)
		HEK293 and MDCK cells	pH	$\text{NH}_3$ channel	Zidi-Yahiaoui et al. (2005)
	RhCG	Oocyte	EP/TA	Electroneutral $\text{NH}_4^+/\text{H}^+$ exchanger	Mak et al. (2006)
		Oocyte	EP/pH	transport of $\text{NH}_4^+$ and $\text{NH}_3$	Bakouh et al. (2004)
		HEK293 and MDCK cells	pH	$\text{NH}_3$ channel	Zidi-Yahiaoui et al. (2005)
<i>S. cerevisiae</i>	Mep1,2,3	Oocyte	EP/TA/pH	Electroneutral $\text{NH}_3$ transport	Mayer et al. (2006b)
		Oocyte	EP/TA	Electroneutral $\text{NH}_4^+/\text{H}^+$ exchanger	Mak et al. (2006)
<i>S. typhimurium</i>	AmtB	<i>S. typhimurium</i> cells	TA	$\text{NH}_3$ channel	Soupene et al. (2001)
<i>E. coli</i>	AmtB	<i>E. coli</i> cells	GE	$\text{NH}_3$ channel	Soupene et al. (2002)
	AmtB	Proteoliposomes	TA/GE	$\text{NH}_3$ channel	Soupene et al. (1998)
	AmtB	<i>E. coli</i> cells	pH	$\text{NH}_3$ channel	Khademi et al. (2004)
	AmtB	<i>E. coli</i> cells	TA	$\text{NH}_3$ channel	Javelle et al. (2005)

<sup>a</sup> EP, electrophysiological measurements; TA, transport assays using  $^{14}\text{C}$ -methylamine; pH, pH measurement; GE, growth experiments.

establish that an optimally conducting protein has to fulfill some very precise requirements in terms of pore shape and in offering hydrogen bonding capacity, but they do not reveal us whether transient protonation of the histidines is required. It also remains open whether only one or both of the possible tautomeric states of the twin His arrangement is/are functionally important.

Fourth, in contrast to the periplasmic side, the structural elements shaping the cytoplasmic vestibule appear to be structurally flexible and can exist in different conformations (Zheng et al., 2004) whose functional significance is not known. It is unclear whether reprotonation of  $\text{NH}_3$  occurs at a defined site involving specific residues or whether it is simply the result of pH dependent equilibration in the water filled cytoplasmic vestibule. Interestingly, recent studies suggest that the C-terminal tail, disordered in the AmtB structures, might mediate cooperativity between the three subunits of AmtB (Severi et al., in press).

Resolving these and similar questions will not only require more functional and structural studies including engineered mutant proteins, but also computational studies taking into account the possible importance of different structural states.

### 3. Functional data

There is a lack of quantitative kinetic data characterizing the activity of Amt/Rh proteins at the single channel

level. Up to now, essentially all functional studies for Amt and Rh proteins were carried out using intact cells or cell-derived vesicles (Table 1) and no channel densities have been reported, with one exception (Ripoche et al., 2004). In all these systems, the parameters measured to analyze the substrate conduction (pH change, electric current, uptake of labelled analogue) are likely to be affected by other physiological phenomenon, requiring careful controls. An *in vitro* assay set up with purified protein would not suffer from such problems and would also make it easier to vary experimental parameters like substrate concentration, pH, and presence of potential inhibitors. In particular, it would allow assessment of the activity of variant proteins designed to test mechanistic hypotheses under controlled conditions. Such an assay has been reported for the *E. coli* channel AmtB (Khademi et al., 2004), but, in view of our results presented below, its reliability appears questionable.

#### 3.1. *In vitro* assays with purified protein

Recording protein-mediated transport of small neutral molecules like water, carbon dioxide, or ammonia is complicated by the fact that these molecules are highly lipid permeable (Lande et al., 1995). Liposome alkalisation, due to  $\text{NH}_3$  influx following an ammonium pulse, occurs in fractions of a second and requires stopped-flow analysis in order to follow the kinetics (Lande et al., 1995; Ripoche

et al., 2004; Zidi-Yahiaoui et al., 2005). Protein-mediated conduction of  $\text{NH}_3$  can then only be observed if it significantly exceeds diffusion through the lipid bilayer.

We have carried out a large number of stopped-flow experiments with AmtB reconstituted into liposomes (proteoliposomes) and with control liposomes. The pH variation inside the vesicles was monitored via the fluorescence change of a pH-sensitive dye. Disappointingly, in none of the many different conditions tested and briefly described below, has a significant AmtB-dependent increase of the alkalisation rate been observed.

We have used the standard detergent-mediated reconstitution method (Rigaud and Levy, 2003) to incorporate *E. coli* AmtB into liposomes. Three different techniques, namely dialysis, dilution and adsorption of detergent by polystyrene beads (Bio-beads) have been used to remove the detergent (Rigaud and Levy, 2003). As apparent from electron micrographs of freeze fractured samples (Fig. 2), the most homogenous protein distribution was obtained using Bio-beads. Furthermore, the size distribution of these liposomes/proteoliposomes, assessed by static light scattering, was also more homogenous, with the majority having diameters around 250 nm (data not shown). Different types

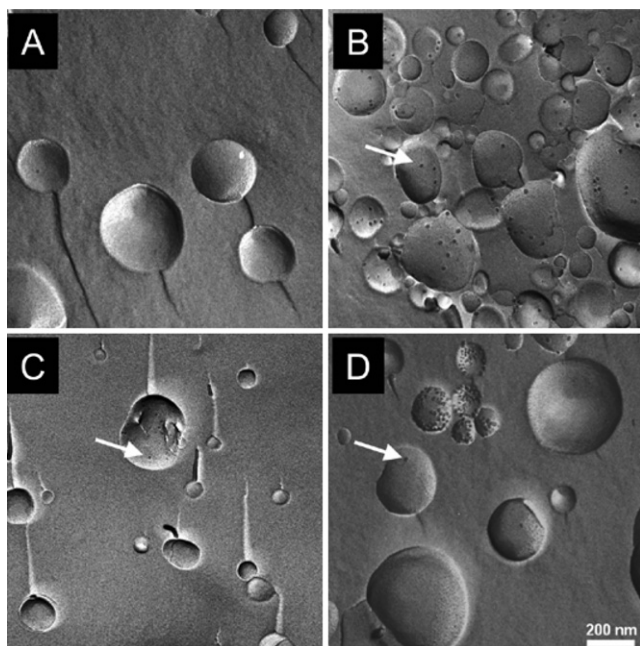


Fig. 2. Freeze fracture electron microscopy. Liposomes (A) and proteoliposomes reconstituted into *E. coli* lipids solubilised in  $\beta$ -OG using polystyrene beads (B), dilution (C) and dialysis. (D). White arrows indicate the AmtB particles (ratio protein to lipids of 1:100 w/w) revealed by platinum deposit. Suspensions were harvested by centrifugation at 100,000g. The pellet was placed between a thin copper holder and a thin copper plate before quenching in liquid propane, as previously described (Aggerbeck and Gulik-Krzywicki, 1986). The frozen sample was fractured at  $-125^\circ\text{C}$  in a vacuum of about  $10^{-7}$  torr in a Balzers BAF300 freeze-etching unit by removing the upper plate with a knife that had been cooled by liquid nitrogen. The fractured sample was replicated with a 1-nm thick platinum-carbon deposit (rotary shadowing). The replica was washed with distilled water and observed with a Hitachi 7000 electron microscope operating at 100 kV.

Table 2  
Alkalinisation rate constants of liposomes<sup>a</sup>

Lipids	k (s <sup>-1</sup> )	
	$\beta$ -OG	C <sub>12</sub> E <sub>8</sub>
<i>E. coli</i> <sup>b</sup>	5.1	3.3
PA:PC <sup>c</sup>	21.5	23.5
<i>E. coli</i> <sup>b,d</sup>	5.0	nd

<sup>a</sup> Different types of lipids in combination with different detergents were tested for the liposome preparations. The pH<sub>i</sub> variation, generated by an inwardly directed ammonium gradient of 10 mM, was measured for each condition by stopped-flow analysis as described in Fig. 3.

<sup>b</sup> *E. coli* total lipids from AVANTI (Avanti polar lipids, INC) were used.

<sup>c</sup> A ratio of 1:9 for PA:PC w/w was used.

<sup>d</sup> Liposomes were loaded with 2 mM carboxyfluorescein. nd, not determined.

of lipids in combination with different detergents were tested, and the combination of *E. coli* lipids with *n*-octyl- $\beta$ -D-glucopyranoside ( $\beta$ -OG) or octaethyleneglycol-*n*-dodecyl monoether (C<sub>12</sub>E<sub>8</sub>) yielded the most homogeneous liposome preparations. The internal pH change due to an ammonium pulse of 10 mM was monitored for each condition by stopped-flow analysis. Two pH-sensitive dyes, carboxyfluorescein and pyranine, were used and gave very similar results (Table 2). It is well known that the presence of residual detergent in liposomes can alter their permeability (Rigaud and Levy, 2003). In order to test the integrity of our liposome preparations, the pH change due to an ammonium pulse was followed for 50 s (Fig. 3A). After a rapid alkalisation, the pH remains essentially constant, indicating no significant proton leakage. The most precise method to analyse liposome integrity consists of measuring the proton flux generated by an external acid/base pulse (Rigaud and Levy, 2003). Following such a procedure, the apparent proton permeability of our liposomes was determined to be  $2 \cdot 10^{-6}$  cm/s at  $6^\circ\text{C}$  (Fig. 3B), indicating that the detergent was efficiently removed by the Bio-beads during liposome formation.

The alkalisation rate constants measured for liposomes and proteoliposomes were not significantly different (Fig. 4), which led us to investigate possible reasons. We have shown elsewhere (Javelle et al., 2005) that the C-terminally-(His)<sub>6</sub>-tagged version of Amt reconstituted into our proteoliposomes is fully active in an *in vivo* transport assay. To test possible orientation effects of AmtB into proteoliposomes, we followed the internal acidification upon applying an outwardly directed ammonium gradient. Similar equilibration rate constants were observed and, again, no significant difference between proteoliposomes and liposomes was measured (data not shown).

Insufficient loading with protein was considered another possible reason for the apparent lack of AmtB activity. Possibilities to overcome this problem are: (i) increasing the protein to lipid ratio, (ii) reduction of the substrate gradient; indeed the millimolar concentrations of ammonium used are far above the apparent ammonium saturation concentration for AmtB of about 10  $\mu\text{M}$ , (iii) use of substrate

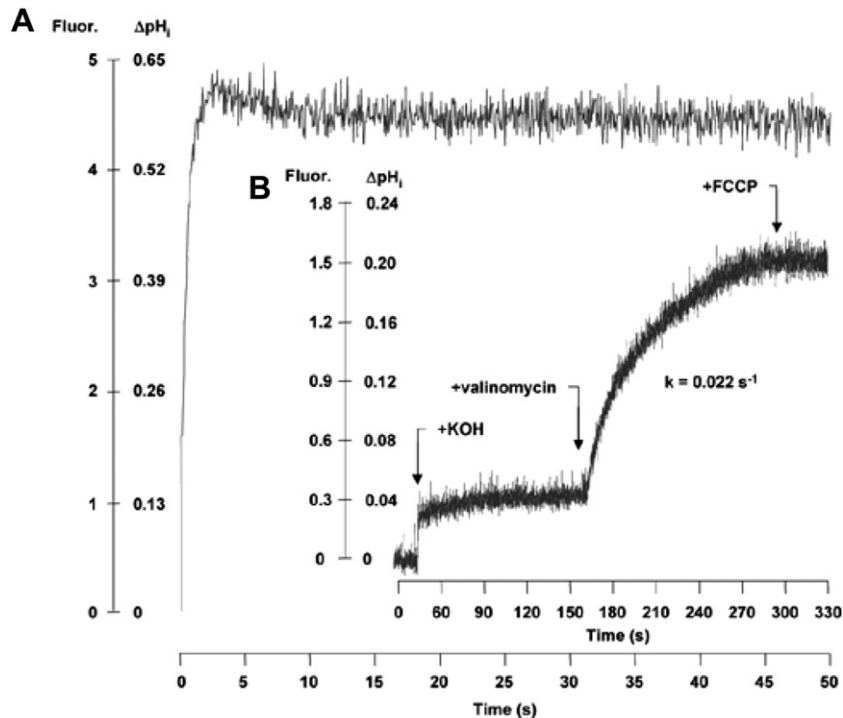


Fig. 3. Time course of fluorescence (fluor) and  $\text{pH}_i$  changes ( $\Delta\text{pH}_i$ ) in liposomes. (A) Liposomes prepared by the step-by-step methods (Rigaud and Levy, 2003) using polar *E. coli* lipids solubilised in  $\beta$ -OG were submitted iso-osmotically to a 10 mM inwardly directed ammonium gradient (pH 6.8) at 6 °C. The  $\text{pH}_i$  variation was followed by the fluorescence change of pyranine (200  $\mu\text{M}$ ) in a solution containing 10 mM Hepes (pH 6.8) and 50 mM  $\text{K}_2\text{SO}_4$  using a stopped-flow spectrofluorometer (SFM3, Biologic, Grenoble, France) as previously described (Ripoche et al., 2004). The excitation wavelength was 460 nm, and the emitted light was filtered with a 520 nm cut-on filter. Using a previously described titration method (Ripoche et al., 2004), the relative pyranine fluorescence was determined to be proportional to pH over the range used (6.8–7.6). The trace shown is an average of 6 runs. (B) Two ml of liposomes from the same preparation were submitted to a pH pulse of 0.2 units by adding 1 mM KOH (final concentration). Subsequently, 0.25  $\mu\text{M}$  valinomycin (final concentration) was added, followed by the addition of carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP) to a final concentration of 0.25  $\mu\text{M}$ .  $\text{pH}_i$  was recorded using the titration device (Biologic) as previously described (Ripoche et al., 2004). The alkalisation phase was fitted to a single exponential function by using the Simplex procedure of the BIOKINE software (Biologic) to calculate the kinetic rate constants  $k$  ( $\text{s}^{-1}$ ). The apparent permeabilities ( $P'$ ) were determined as  $P' = k V/S$  where  $V/S$  is the volume-to-surface-area ratio of liposomes.

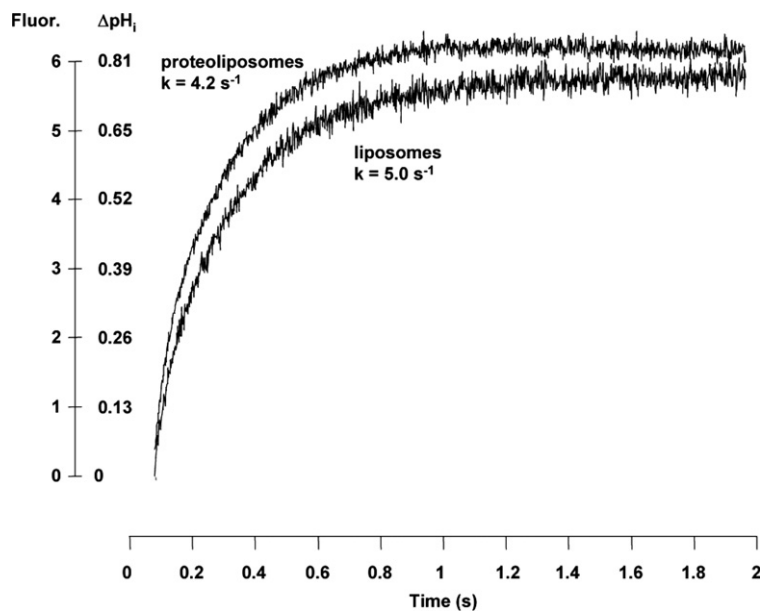


Fig. 4.  $\text{NH}_3$  transport in liposomes and proteoliposomes. Liposomes and proteoliposomes prepared by the step-by-step methods, using the same conditions as described in Fig. 3, were submitted iso-osmotically to a 10 mM inwardly directed ammonium gradient. A lipid to protein ratio of 10:1 (w/w) was used for the reconstitution of AmtB prepared as previously described (Rigaud et al., 1997; Rigaud and Levy, 2003). The trace is an average of 6 runs. Analysis of the kinetics was performed as described in Fig. 3.

analogues with lower lipid permeability but similar protein-mediated conductance, or (iv) use of lipids with lower membrane fluidity and thus decreased  $\text{NH}_3$  permeability (Lande et al., 1995; van Blitterswijk et al., 1987), but not affecting the efficient membrane insertion of active protein. We have explored the first three of these possibilities, thus far without success. The last one is currently under investigation.

In contrast to our negative results, Stroud and colleagues (Khademi et al., 2004) reported a significantly enhanced alkalisation rate constant for AmtB proteoliposomes, which confirmed their structure-based conclusion that AmtB is an  $\text{NH}_3$  channel rather than an  $\text{NH}_4^+$  transporter. For reconstitution they used a relatively high lipid to protein ratio of 200:1 (w/w) which would correspond to a maximum channel density of about 300 per  $\mu\text{m}^2$ , assuming that all protein becomes incorporated in a functional state. From this, we estimate a minimum single channel conductance rate of about  $3 \times 10^4$  molecules/s (Table 3) at an initial ammonium concentration gradient of 5 mM (at 12 °C). With ammonium as the sole nitrogen source, we estimate that an *E. coli* cell needs an uptake of  $10^4$ – $10^5$  ammonium molecules ( $\text{s } \mu\text{m}^2$ )<sup>-1</sup> to support the observed growth rates (Zheng et al., 2004). Assuming that AmtB is saturated at concentrations above 10–50  $\mu\text{M}$  and that all ammonium uptake is protein-mediated, less than 10 channels/ $\mu\text{m}^2$  would be needed with the above (minimum) single channel conductance rate of  $3 \times 10^4 \text{ s}^{-1}$ . Based on these considerations and our failure to observe AmtB-mediated transport, we consider it possible that the rate is 10–100 fold less. However, convincing experimental evidence will be needed to clarify this issue. It could also be important to note that while we have used His-tagged wild-type version of AmtB, Stroud and colleagues (Khademi et al., 2004) have used an AmtB variant carrying three substitutions (F68S, S126P, and K255L).

### 3.2. *In vitro* assays with cell-derived vesicles

We have carried out stopped-flow analysis on *E. coli*-derived right-side-out vesicles prepared using the lysozyme method as previously described (Kaback, 1974) and loaded with 200  $\mu\text{M}$  pyranine. In this system, AmtB becomes inserted into the membrane via the cellular machinery, which should avoid any potential problem of protein orientation or protein inactivation during purification or *in vitro* reconstitution. We have measured the internal alkalisation rate generated by an inwardly directed ammonium gradient pulse of 25 mM in vesicles prepared from strain GT1000 (*amtB*<sup>-</sup>) (Coutts et al., 2002), overexpressing (i) wild-type AmtB, (ii) the C-terminally-(His)<sub>6</sub>-tagged version used in the *in vitro* reconstitution, or (iii) an H318A AmtB variant shown to be inactive in methylamine transport (Javelle et al., 2006). No significant differences in the alkalisation rate constants were measured between these conditions (Fig. 5), nor when vesicles prepared from the

wild-type strain ET8000 (Jayakumar et al., 1986) or the *amtB*<sup>-</sup> strain GT1000 were used (data not shown).

Ripoche and colleagues (Ripoche et al., 2004) studied the alkalisation of human red blood cell ghosts after an ammonium pulse and concluded that the human erythroid Rhesus protein RhAG acts as an ammonia channel. For this system the channel density is known (~85,000 RhAG molecules/red blood cell) such that approximate single channel conduction rates of  $4 \times 10^4$  at an initial gradient of 32.5 mM can be derived (Table 3). Reported ammonium inhibition constants of methylamine uptake by Rh proteins are in the millimolar range (Ludewig, 2004; Mayer et al., 2006b) and it would appear that the channel conducts at its maximum capacity under these conditions. However, this may be questioned and experimental verification is needed.

### 3.3. Cellular assays

*In vivo* transport assays measuring the uptake of the ammonium analogue <sup>14</sup>[C]-methylamine (<sup>14</sup>C-MA) by bacterial or fungal cells have been a useful tool to study the physiological role of Amt proteins and to assess the activity of variant proteins (Javelle et al., 2005, 2004, 2006; Marini et al., 2006; Severi et al., in press). However, for studying the detailed mechanism of substrate conductance these assays have limitations.

A large number of studies, based on heterologous expression in oocytes, have been used to measure the transport of ammonium and/or methylammonium by Amt and Rh proteins. This system has been widely used for measuring electrogenic transport and appeared ideally suited to clarify whether Amt/Rh protein conduct charged  $\text{NH}_4^+$  or neutral  $\text{NH}_3$ . However, measurement of the current or intracellular pH variations induced by an ammonium pulse in *Xenopus* oocytes expressing Amt or Rhesus proteins has led different groups to draw quite different conclusions (Table 1). The discrepancies suggest that heterologous expression in oocytes, which contain various endogenous channels and transporters, is problematic for the functional analysis of Amt/Rh proteins. It is well known, for example, that  $\text{NH}_4^+$  enters oocytes via non-specific endogenous channels activated by local alkalisation (Boldt et al., 2003). If we only consider studies using ammonium pulses of less than 1 mM, which apparently avoids this problem, it seems that Rh proteins act as  $\text{NH}_3$  channel, whereas the plant Amt proteins studied to date (Ludewig et al., 2002; Ludewig et al., 2003; Mayer et al., 2006a,b; Mayer and Ludewig, 2006) appear to facilitate a net transport of  $\text{NH}_4^+$ . Measuring local pH by microelectrodes is possible within the large oocytes, but the non-uniform buffer capacity (al-Baldawi and Abercrombie, 1992; Swietach et al., 2003) and the presence of microdomains especially near the negatively charged membrane complicate a simple interpretation (for review see (DeCoursey, 2003)).

The data in Table 3 show that HEK-293 cells and oocytes have membrane permeabilities for ammonia and

Table 3  
Quantitative data on ammonium (A) and methylammonium (MA) transport of Amt/Rh proteins<sup>a</sup>

Study <sup>b</sup>	Assay	rad ( $\mu\text{m}$ )	$V/S$ ( $\mu\text{m}$ )	pH/T( $^{\circ}\text{C}$ )	$\Delta c_0 B_{\text{tot}}$ (mM) <sup>c, d</sup>	$P'(B_{\text{tot}})^e$ $\mu\text{m/s}$	$k$ ( $\text{s}^{-1}$ )	$J_0^f$ ( $10^{-6} \cdot N_B/\text{s} \cdot \mu\text{m}^2$ ) <sup>e</sup>	L/P ratio (w/w) (channel density)	Single channel conductance
Khademi	Liposomes	0.09 <sup>a</sup>	0.03	<b>6.8/12<sup>o</sup></b>	<b>5 (A)</b>	0.39	<b>12.8</b>	1.2 L		
	AmtB liposomes	0.09 <sup>a</sup>	0.03	<b>6.8/12<sup>o</sup></b>	<b>5 (A)</b>	3.5	<b>115.6</b>	10 P + L	<b>200:1</b> (300/ $\mu\text{m}^2$ )	$3.3 \times 10^4$
This study	Liposomes	<b>0.12</b>	0.04	<b>6.8/10<sup>o</sup></b>	<b>10 (A)</b>	0.2	<b>5.0</b>	1.2 L	<b>200:1</b> (300/ $\mu\text{m}^2$ )	$2.1 \times 10^4$
	AmtB liposomes					0.17	<b>4.2</b>			
Ripoche	RBC ghosts (-) <sup>g</sup>	<b>2.5</b>		<b>7.0/15<sup>o</sup></b>	<b>32.5 (A)</b>	<b>2.54</b>	<b>2.48</b>	50 L		
	RBC ghosts (+)			<b>7.0/15<sup>o</sup></b>	<b>32.5 (A)</b>	<b>4.46</b>	<b>4.95</b>	87 P + L	(~1000/ $\mu\text{m}^2$ )	$3.7 \times 10^4$
	RBC ghosts (-)	<b>2.5</b>		<b>7.0/15<sup>o</sup></b>	<b>32.5 (MA)</b>	<b>0.12</b>	<b>0.14</b>	2.3 L		
	RBC ghosts (+)			<b>7.0/15<sup>o</sup></b>	<b>32.5 (MA)</b>	<b>0.88</b>	<b>0.95</b>	17 P + L	(~1000/ $\mu\text{m}^2$ )	$1.5 \times 10^4$
Zidi-Yahiaoui	HEK293, WT	<b>8.6</b>	<b>0.81</b>	<b>7.0/15<sup>o</sup></b>	<b>32.5 (A)</b>	<b>0.24</b>	<b>0.18</b>	4.7 L		
	+RhBG					<b>1.5</b>	<b>1.0</b>	29 P + L		
	+RhCG					<b>1.31</b>	<b>0.88</b>	26 P + L		
	HEK293, WT	<b>8.6</b>	<b>0.81</b>	<b>7.0/15<sup>o</sup></b>	<b>32.5 (MA)</b>	<b>0.02</b>	<b>0.029</b>	0.4 L		
	+RhBG					<b>0.65</b>	<b>0.53</b>	13 P + L		
Mayer	+RhCG					<b>0.10</b>	<b>0.11</b>	2 P + L		
	Oocyte uninjected	<b>500</b>		<b>7.5/RT</b>	<b>10 (-MA)</b>	0.017		0.1 L		
	+RhCG			<b>7.5/RT</b>	<b>10 (MA)</b>	0.05		0.33 P + L		
	+LeAMT1;2			<b>7.5/RT</b>	<b>0.5 (MA)</b>	0.05		0.017 P + L		

<sup>a</sup> Numbers in bold are directly taken from the cited work, other numbers are derived from these or are assumed in the case of the mean liposome radius (Khademi).

<sup>b</sup> References are: Khademi, (Khademi et al., 2004); Ripoche, (Ripoche et al., 2004); Zidi-Yahiaoui, (Zidi-Yahiaoui et al., 2005); Mayer, (Mayer et al., 2006b).

<sup>c</sup> Initial concentration gradient across membrane of total base ( $B_{\text{tot}}$ ).

<sup>d</sup> A, ammonium; MA, methylamine.

<sup>e</sup> Apparent permeability of  $B_{\text{tot}}$ , calculated as  $P' = k \cdot V/S$  for stopped-flow equilibration experiments ( $k$  = exponential rate constant) or from the uptake rate ( $k'$  in moles/s per oocyte) using the equation  $P' = k' \cdot \Delta c_0 \cdot A_{\text{oocyte}}$  ( $A$  = surface area).

<sup>f</sup>  $J_0$  initial flux (number of molecules ( $N$ ) per second per  $\mu\text{m}^2$ ), L and P stand for lipid- and protein-mediated transport, respectively.

<sup>g</sup> RBC ghosts (-): Rhnull regulator of P1-P4 cells not expressing RhAG; RBC ghosts (+): RhD-positive cells expressing about 85.000 RhAG molecules per red blood cell.

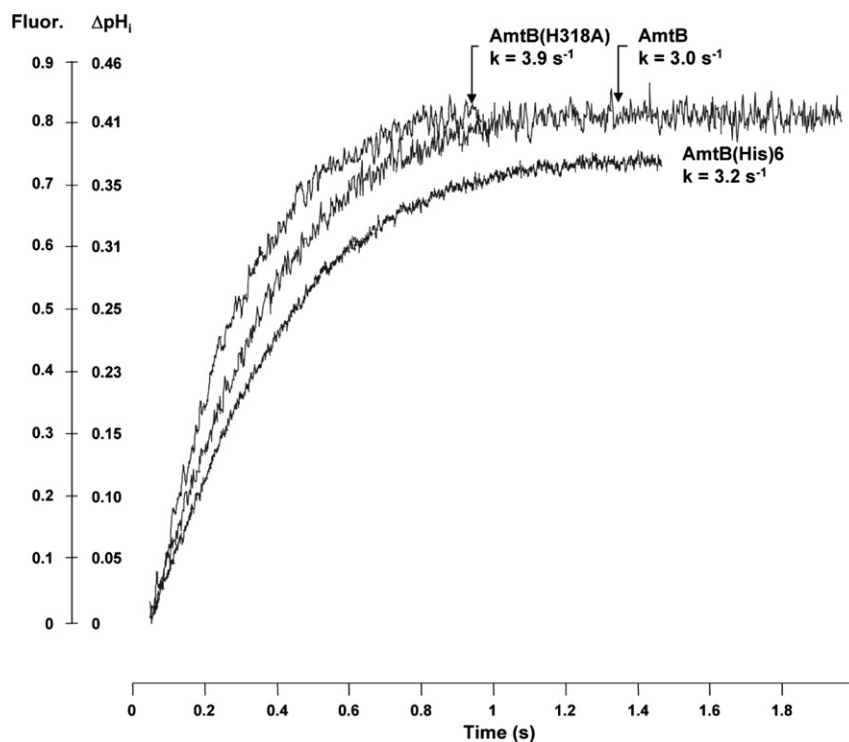


Fig. 5. Time course of fluorescence and  $\text{pH}_i$  changes in *E. coli* vesicles. RSO membrane vesicles in a solution containing 10 mM Hepes (pH 6.8), 50 mM  $\text{K}_2\text{SO}_4$  and 200  $\mu\text{M}$  pyranine, were prepared by osmotic lysis as described previously (Kaback, 1974), and diluted to a total protein concentration of about 10 mg/ml, frozen in liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$  until use. The trace is an average of 6 runs. The  $\text{pH}_i$  variation of vesicles submitted iso-osmotically to an inwardly directed pulse of 25 mM ammonium gradient (pH 6.8) at  $10^\circ\text{C}$  was followed as described in Fig. 3. Vesicles were prepared from strain GT1000 (*amtB*<sup>-</sup>) (Coutts et al., 2002) overexpressing AmtB from (i) plasmid pJT6E (Javelle et al., 2006) carrying a wild-type version of AmtB (trace AmtB in the figure), or (ii) plasmid pAJ2011 (Javelle et al., 2006) carrying the inactive H318A mutated version of AmtB (trace AmtBH318A in the figure) or (iii) plasmid pJT6 (Javelle et al., 2004) carrying a C-terminally-His<sub>6</sub>-tagged version of AmtB (trace AmtB(His)<sub>6</sub> in the figure).

methylamine that are 10- and 6-fold smaller, respectively, than those of red blood cell ghosts. The apparent protein-mediated methylamine uptake rate observed with the tomato protein LeAmt1;2 expressed in oocytes is rather small ( $\sim 10^4$  molecules/s  $\mu\text{m}^2$ ) but appears to be consistent with the small increase in the measured current (Mayer et al., 2006b). Whether this small value is due to low protein expression or to a low intrinsic transport rate is unknown.

#### 4. Conclusions

The structural insights and an increasing number of functional studies have greatly increased our knowledge of the Amt/Rh protein family. The detailed information gained from the structure analysis of Amt proteins (Andrade et al., 2005; Javelle et al., 2006; Khademi et al., 2004; Zheng et al., 2004) has generated a number of specific hypotheses concerning the mechanism of substrate conduction. Some of these are now being addressed by molecular dynamics simulations (Lin et al., 2006; Liu and Hu, 2006; Luzhkov et al., 2006; Nygaard et al., 2006; Yang et al., 2007). Thus far, these studies have led to substantially different conclusions with respect to the mechanistic role of different conserved residues along the pore although they all infer transport of  $\text{NH}_3$ . Clearly, we do not yet under-

stand the mechanism at a detailed level, but the possibility that some Amt proteins conduct the ammonium ion remains a challenge for our mechanistic understanding in general, given the high conservation of the pore-lining residues.

A reliable assay using purified protein will be crucial for further progress on the experimental side. Unfortunately, we have not been able to reproduce the result reported by Stroud and co-workers (Khademi et al., 2004), despite extensive efforts. We consider it possible that the maximum single channel conduction rate of AmtB is considerably lower than the rate derived from that study (Table 3). If indeed the case, our failure would be explained by the fact that, at the millimolar ammonium gradients needed to measure a signal, protein-mediated transport is too low to be visible against lipid-mediated diffusion. We hope that future studies by us and others will resolve this issue.

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