Ammonium ion transport by the AMT/Rh homologue LeAMT1;1

Maria MAYER¹, Marek DYNOWSKI¹ and Uwe LUDEWIG²

Zentrum für Molekularbiologie der Pflanzen (ZMBP), Pflanzenphysiologie, Universität Tübingen, Auf der Morgenstelle 1, 72076 Tübingen, Germany

AMT (ammonium transporter)/Rh (Rhesus) ammonium transporters/channels are identified in all domains of life and fulfil contrasting functions related either to ammonium acquisition or excretion. Based on functional and crystallographic high-resolution structural data, it was recently proposed that the bacterial AmtB (ammonium transporter B) is a gas channel for NH₃ [Khademi, O'Connell, III, Remis, Robles-Colmenares, Miercke and Stroud (2004) *Science* **305**, 1587–1594; Zheng, Kostrewa, Berneche, Winkler and Li (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 17090–17095]. Key residues, proposed to be crucial for NH₃ conduction, and the hydrophobic, but obstructed, pore were conserved in a homology model of LeAMT1;1 from tomato. Transport by LeAMT1;1 was affected by mutations of residues

that were predicted to constitute the aromatic recruitment site for NH₄⁺ at the external pore entrance. Despite the structural similarities, LeAMT1;1 was shown to transport only the ion; each transported ¹⁴C-methylammonium molecule carried a single positive elementary charge. Similarly, NH₄⁺ (or H⁺/NH₃) was transported, but NH₃ conduction was excluded. It is concluded that related proteins and a similar molecular architecture can apparently support contrasting transport mechanisms.

Key words: ammonium transport, ammonium transporter/Rhesus (AMT/Rh), homology modelling, ion channel, methylammonium, oocyte.

INTRODUCTION

There is an ongoing debate as to whether some or all AMT (ammonium transporter)/Rh (Rhesus) proteins function as NH₃ channels or as NH₄⁺ transporters [1]. Based on the recently obtained high-resolution structure of a bacterial homologue, AmtB (ammonium transporter B) from *Escherichia coli*, a channel-like transport of NH₃ in AMT/Rh proteins has been proposed [2,3]. Ammonium-mediated vesicular alkalization by reconstituted AmtB [2], and studies with bacterial strains compromised in ammonium assimilation and transport are in accordance with NH₃ transport [4]. However, mechanistic interpretations from a static structure should be taken with care. Though probably within the range of experimental error, some MeA (methylammonium) accumulation by AmtB was detected, which was only to be expected if AmtB transports NH₄⁺. MeA accumulation would also be expected if, hypothetically, the ion occasionally 'slipped' through the channel/transporter [4]. Additional *in vitro* and *in vivo* experiments, including flux measurements against ammonia gradients and/or current recordings, are required to establish the NH₃ transport mechanism of AmtB. According to its transcriptional and post-transcriptional regulation, the physiological role of AmtB appears to be the uptake of ammonium under low ammonium concentrations, but the NH₃ gradient is unfavourable for uptake under most conditions [4].

The transport mechanism proposed for AMT/Rh proteins from the study of the X-ray structures involves NH₄⁺ recruitment at the external pore mouth close to the aromatic residues Phe¹⁰³, Phe¹⁰⁷ and Trp¹⁴⁸, de-protonation and conduction as for NH₃ [2,3]. Hallmarks of the AMT/Rh family are two histidine residues that line the central hydrophobic pore. It has been proposed that NH₄⁺ cannot pass these histidines and thus no NH₄⁺ transport and no ionic currents would be expected in AmtB. An electroneutral

NH₃ transport by AMT/Rh homologues is compatible with our results on human RhBG (Rh B glycoprotein) and RhCG (Rh C glycoprotein). The studies suggest that ammonium transport by these proteins is electroneutral when heterologously expressed in oocytes and yeast [7,8], in accordance with results from other groups [9].

In contrast, specific, saturable, voltage-dependent, pH_o (extracellular pH)-independent NH₄⁺ and MeA⁺ currents have been recorded from two tomato AMT protein homologues expressed in oocytes [5,6]. There is little doubt that ammonium uptake in plants occurs at least partially as NH₄⁺, since electrogenic ammonium transport in plants is well established [1]. Ionic currents are in conflict with the proposed NH₃ channel mechanism in AMT/Rh proteins, but NH₄⁺ currents might represent an ion 'slippage', while the major transport form is NH₃. It has not been tested if NH₃ is transported through LeAMT1;1.

The surprising similarity of an LeAMT1;1 homology model to AmtB triggered a further analysis of NH₄⁺ and MeA⁺ currents mediated by LeAMT1;1. Although NH₄⁺ and MeA⁺ currents were recorded, no 'slippery' gas (NH₃) transport was detected. It was additionally found that an aromatic residue at the outer pore mouth is involved in high-affinity recruitment of NH₄⁺. LeAMT1;1 may thus function either as an NH₄⁺ channel (or 'uniporter') or, physiologically equivalent, as an NH₃/H⁺ co-transporter.

EXPERIMENTAL

Plasmid constructs, preparation and injection of oocytes

The plasmid pO02-*LeAMT1;1* and oocyte handling have been described in previous studies [5,7]. Mutations were introduced into LeAMT1;1 by using the QuikChange[®] method from Stratagene and the complete coding region was verified by sequencing.

Abbreviations used: AMT, ammonium transporter; AmtB, ammonium transporter B; AQP1, aquaporin 1; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; CLC, chloride channel; GFP, green fluorescent protein; MeA, methylammonium; pH_i, intracellular pH; pH_o, extracellular pH; Rh, Rhesus; RhCG, Rh C glycoprotein; rmsd, root mean square deviation.

¹ These authors have contributed equally to this work.

² To whom correspondence should be addressed (email uwe.ludewig@zmbp.uni-tuebingen.de).

Electrophysiological measurements, radiotracer uptake and pH_i (intracellular pH) changes

Recordings were performed as described in [7]. Means $\pm$ S.E.M. are given. There was no special selection applied, but studies of ammonium transport in oocytes may be hampered by endogenous ion channels that are activated by high ammonium concentrations such as 10 mM [10,11]. Currents in the oocytes used in the present study were insensitive to ammonium and MeA at concentrations of 1 or 10 mM respectively [5,12], and care was taken to verify that these endogenous ammonium-inducible currents were not present in the oocyte batches used. Oocytes used in our laboratory had a background conductance of $\leq 0.9 \mu\text{S}$ (between 0 and -100 mV) and background currents as low as approx. -80 nA at -140 mV . This background current is approximately half that of the endogenous current in oocytes used in the laboratories that have recorded and investigated NH_4^+ -induced currents [11,13]. Interestingly, seasonal variations were reported for the endogenous amine-inducible current [11]. Although activation of such endogenous oocyte currents at lower ammonium concentrations has been reported [13], we and others have observed that ionic currents from oocytes are insensitive to ammonium at $\leq 1 \text{ mM}$ at physiological pH ([5–7,12,14]; results not shown). Although the nature of this discrepancy and the reason for natural variance in oocyte currents is unclear, *Xenopus* oocytes with reduced or absent endogenous MeA^+ and NH_4^+ currents have the great advantage that measurement of ammonium transport can be performed with minimal contamination from the endogenous ammonium-induced currents. The absence of endogenous MeA^+ and NH_4^+ currents in oocytes used in the present study suggests that internal pH_i will respond differently to ammonium concentration than in previous studies on oocytes with large NH_4^+ currents (see Figure 5) [10,11]. Oocytes expressing large endogenous ammonium-activated currents had been reported to acidify slowly when measured with pH-sensitive electrodes [11,13]. As NH_4^+ conductance was absent from the oocytes used in our study, native oocytes were alkalized upon addition of 10 mM ammonium (see Figure 5).

In the cases where currents 'induced by ammonium' are given; these were obtained by subtracting the current in the absence of ammonium (background) from the current in the presence of ammonium at each voltage. NH_3 and NH_4^+ concentrations were determined from total ammonium concentration using the Henderson–Hasselbach equation: $\log_{10} [\text{NH}_3]/[\text{NH}_4^+] = \text{pH} - 9.25$. A pK_a of 10.66 was used for calculations involving MeA. The concentration dependence of current/uptake was fitted using the following equation: $I = I_{\text{max}}/(1 + K_m/c)$, where I_{max} is the maximal current at saturating concentration, K_m is the substrate concentration permitting half-maximal currents, and c is the experimentally used concentration. [^{14}C]MeA uptake was determined as in [7].

pH_i changes

pH_i changes were monitored using the pH-sensitive dye BCECF [2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein] and were calibrated as described in [7]. Briefly, oocytes were incubated in uptake buffer (ND96, containing $5 \mu\text{M}$ BCECF acetoxymethyl ester, from a 50 mM stock in DMSO) for 30 min protected from light. Fluorescence of BCECF-loaded oocytes placed in a recording chamber and superfused with the standard solution ($\pm \text{NH}_4\text{Cl}$) was monitored using an inverted Leica fluorescence microscope at maximal rate (5 s) using a spinning wheel system with excitation at 410 ± 30 and $470 \pm 20 \text{ nm}$ and emission at $535 \pm 20 \text{ nm}$. In some experiments, oocytes were voltage-clamped during fluorescence recording.

Homology modelling

The primary sequence of LeAMT1;1 was aligned with AmtB using Clustal W, followed by manual inspection of functionally important residues. The alignment was identical with the alignment in Khademi et al. [2] for the first transmembrane helix (see Supplementary Figure at <http://www.BiochemJ.org/bj/396/bj3960431add.htm>). Structural fitting was done using MODELLER 7v7 (available at <http://salilab.org/modeller/>), a well-established structure prediction program [15]. The structures were evaluated using the WHATIF program (<http://biotech.ebi.ac.uk:8400/>) and PROCHECK (<http://www.biochem.ucl.ac.uk/~roman/procheck/procheck.html>). The LeAMT1;1 model used had 90.5% residues in the most favoured regions in a Ramachandran plot (compared with 92.7% in the AmtB structure). Only nine amino acids were identified that had 'bad' stereochemistry. All of these were located outside of the core structure of the transmembrane helices. Further evaluation was carried out by energy minimization of the monomers of AmtB (PDB accession code 1U7G; [2]) and the LeAMT1;1 model for 10 ps using NAMD2.5 (<http://www.ks.uiuc.edu/Research/namd/>), using the Amber7 force field. The stability of the structure was used as a measure for the quality and was assessed by measuring the rmsd (root mean square deviation) between backbone residues of the structures. Minor attention was given to reconstruction of loops, as these are expected to minimally influence the core structure and transport mechanism. The pore diameter was accessed using the program HOLE2 [16]. Monomeric bovine AQP1 (aquaporin 1; PDB accession code 1J4N) was analysed in parallel.

LeAMT1;1 expression in yeast

The open reading frames of LeAMT1;1 and a translational LeAMT1;1–GFP (green fluorescent protein) fusion were cloned into plasmid pDR196 and heat-shock-transfected in the *ura⁻ Saccharomyces cerevisiae* wild-type strain (23344c) or the ammonium transporter-defective yeast strain (31019b; $\Delta\Delta\Delta\text{mep1};2;3$) [17]. The nitrogen-deficient growth medium for strain 31019b was YNB (1.7 g/l yeast nitrogen base and 5 g/l glucose; Difco), supplemented with 3% (w/v) glucose and appropriate NH_4Cl concentrations. The medium was adjusted to pH 5.5 using 50 mM Mes buffer. The MeA toxicity medium contained YNB, 3% glucose, 0.1% arginine and 125 mM MeA. Growth of yeast was not affected by expression of the different constructs under non-selective conditions.

RESULTS

Homology model of LeAMT1;1

To identify the molecular determinants for differences in the transport mechanism between AMT/Rh homologues, a homology model of LeAMT1;1 with AmtB (PDB code 1U7G) as template was constructed. Both proteins are 25% identical and 44% similar within the protein core (excluding the N- and C-termini). The most stable model (see the Experimental section) was obtained by using a slightly modified alignment from that published by Khademi et al. [2], which is given as a Supplementary Figure (<http://www.BiochemJ.org/bj/396/bj3960431.add.htm>) and only differs in the first transmembrane region. A ribbon representation of the structural backbone of AmtB and the model of LeAMT1;1 is shown in Figures 1(A) and 1(B). Major deviations are seen in the extrahelical loops, consistent with the low similarity between these regions. The model was less stable than AmtB after a minimization for 10 ps, which reduces 'bad' contacts within the

Figure 1 Analysis of the LeAMT1;1 homology model

(A) Ribbon structure of an AmtB monomer backbone (PDB code 1UG7). (B) Structure of the homology model of LeAMT1;1. (C) Upper panel: rmsd from a minimization of the AmtB structure and comparison with the homology model. Lower panel: pore diameter of AmtB (red) and AQP1 (blue) calculated with the program HOLE2. (D) Pore diameter of AQP1 calculated with HOLE2. (E) Top view of the trimeric assembly of the LeAMT1;1 model. (F) Pore diameter in AmtB. Residues Phe¹⁰³, Phe¹⁰⁷ and Trp¹⁴⁸ participate in the recruitment (or binding) site for the NH₄⁺ ion and Phe¹⁰⁷ and Phe²¹⁵ obstruct the pore. The conserved residues His¹⁶⁸ and His³¹⁸ line the hydrophobic pore. Helices M7 and M8 were removed for better visualization. The pore diameters below 0.8 Å are shown in red, between 0.8 and 2.3 Å in green, and diameters above 2.3 Å are shown in blue. (G) Position of similar residues in the model of LeAMT1;1.

protein (Figure 1C). Three monomers preferably assembled in a trimeric form (Figure 1E).

AmtB does not form an 'open' channel-like structure as e.g. AQP channels. Several AQPs transport ammonia in addition to water [18,19], which justifies the comparison between these protein classes. The substrate pore of AQP monomers is illustrated for bovine AQP1 (PDB code 1J4N) [20] using the program HOLE2, a program that identifies putative ionic and solute pores in proteins based on the van der Waals radii of amino acid residues close to the pore [16]. The AQP1 pore constriction does not go below 0.8 Å (1 Å = 0.1 nm) (Figure 1D) [20],

but the solute pathway in AmtB is blocked by residues Phe¹⁰⁷ and Phe²¹⁵ and partially by Phe³¹ (Figures 1C and 1F). In accordance with this finding, preliminary comparative steered molecular dynamics studies did reveal solute flux in AQPs, but not in AmtB (M. Dynowski and U. Ludewig, unpublished work). The fundamental difference appears to be important, since the proposed channel-like mechanism was recently illustrated by a misleading 'open-pore' cartoon-like model [21]. Minimal conformational changes at least are necessary to obtain a channel-like open pore structure. Another possibility is that transport-cycle-dependent conformational changes are involved in ammonium transport.

Figure 2 Analysis of LeAMT1;1 wild-type and NH_4^+ recruitment site mutants (W178L and Y133I) in yeast

Spotted 100-fold dilutions and growth of the $\Delta\Delta\Delta\text{mep1;2;3}$ strain expressing the respective constructs on limiting (A) 0.2 mM, (B) 1 mM and (C) 3 mM ammonium. (D) Dilution series (10 $\times$) of wild-type yeast expressing the constructs on toxic MeA (125 mM). (E) Expression and subcellular localization of GFP-tagged wild-type, Y133I and W178L mutants in yeast; left panel: bright field; right panel: GFP fluorescence. Scale bars, 5 μm .

The pore analysis using HOLE2 did not reveal a continuous solute pathway in the LeAMT1;1 homology model. Most residues, if not all, that have been suggested to be crucial for NH_4^+ recruitment and NH_3 conduction in the occluded pore of AmtB are identical or similar in LeAMT1;1. The residues Phe¹⁰³, Phe¹⁰⁷, Trp¹⁴⁸ and Ser²¹⁹ in AmtB have been predicted to participate in the recruitment and co-ordination of the NH_4^+ ion; the equivalent residues in LeAMT1;1 are at positions Tyr¹³³, Phe¹³⁷, Trp¹⁷⁸ and Ser²⁶³ respectively. The aromatic residues Phe¹⁰⁷ and Phe²¹⁵ block the pore in AmtB; these residues are at positions Phe¹³⁷ and Phe²⁵⁹ in LeAMT1;1. The model has a similar hydrophobic pore to AmtB, with two conserved residues at positions His²⁰⁷ and His³⁷⁴ that line the putative pore lumen (Figure 1G). Although the model cannot be a substitute for a crystal structure, its similarity to the NH_3 -transporting AmtB is striking.

The aromatic nature of two residues forming the ion-binding cavity (Tyr¹³³ and Trp¹⁷⁸ in LeAMT1;1) is conserved between bacterial and plant homologues, but not in Rh glycoproteins. In Rh proteins, non-polar small neutral residues occupy these positions (isoleucine and leucine in RhCG). These residues might thus be responsible for the different substrate affinity that is observed in Rh glycoproteins [7,8]. The overall quality of the LeAMT1;1 model was tested by mutational analysis of the residues Tyr¹³³ and Trp¹⁷⁸ that are putatively involved in substrate affinity.

Ammonium transport by LeAMT1;1 pore mutants

Initial attempts to functionally express EcAmtB (AmtB from *E. coli*) in oocytes failed (results not shown), so we concentrated on the function of LeAMT1;1. Residues Tyr¹³³ and Trp¹⁷⁸ were exchanged with the respective residues found in RhCG (Y133I and W178L). LeAMT1;1 and, to a lesser extent, the two mutants functionally complemented the ammonium transporter-deficient yeast strain $\Delta\Delta\Delta\text{mep1;2;3}$ with ammonium as the

sole nitrogen source (Figure 2). The mutations did not largely alter the expression pattern as assessed by confocal microscopy of translational C-terminal GFP fusions in yeast. A cell-to-cell variability in fluorescence levels was observed in homogeneous yeast cultures when analysed with the identical microscopy settings at low magnification (Figure 2E). LeAMT1;1 and the mutants showed the same subcellular localization when analysed at higher magnification (Figure 2E).

At 125 mM, MeA is toxic to wild-type yeast and expression of Rh glycoproteins has been shown to rescue growth on toxic MeA. Rh glycoproteins probably export uncharged MeA along its chemical gradient, even though Rh glycoproteins are saturated well below these high MeA concentrations [8]. Both mutants and LeAMT1;1 showed increased sensitivity to MeA, which is most simply explained by MeA^+ import by LeAMT1;1 and the mutants (Figure 2D). In all assays, the W178L mutant displayed less transport activity than the Y133I mutant.

Altered saturation of NH_4^+ transport in an LeAMT1;1 mutant

For a more detailed analysis, the ionic currents elicited by the mutant transporters were assayed using the two-electrode voltage clamp in oocytes. No significant [^{14}C]MeA uptakes and currents were detected with W178L, precluding further analysis. In contrast, the mutant Y133I displayed small, but reliable currents on treatment with ammonium. Currents were more than 10-fold smaller than in the wild-type (Figure 3), in accordance with its reduced overall transport rate or reduced functionality compared with wild-type LeAMT1;1 in the yeast-growth experiments. Importantly, the half-maximal current, which is a measure that is independent of the expression level, was saturated at very low concentrations. In contrast with wild-type ($K_m = 12 \mu\text{M}$), the K_m for NH_4^+ in the Y133I mutant was only $1 \mu\text{M}$ at -120 mV . Similarly, MeA^+ currents were saturated at a half-maximal

Figure 3 Functional analysis of the Y133I mutant in oocytes and comparison with wild-type LeAMT1;1

(A) Saturation of ammonium-induced currents at -120 mV for wild-type LeAMT1;1 (open circles, $K_m = 12 \mu\text{M}$, $n = 5$) and the Y133I mutant (closed triangles, $K_m = 1 \mu\text{M}$, $n = 9$). (B) Same data, but normalized to the same maximal current with a focus on low NH_4^+ concentrations. (C) Saturation of MeA-induced currents at -120 mV for wild-type LeAMT1;1 (open circles, $K_m = 584 \mu\text{M}$, $n = 5$) and the Y133I mutant (closed triangles, $K_m = 4.5 \mu\text{M}$, $n = 5$). (D) Same data, but normalized to the same maximal current with a focus on low MeA⁺ concentrations.

concentration of $584 \mu\text{M}$ at -120 mV in LeAMT1;1, while the small currents of the mutant were half-maximal at $4.5 \mu\text{M}$ (Figure 3).

Charge coupling of MeA⁺ transport in LeAMT1;1

The ratio of charged versus uncharged solute transport was assayed using the analogue MeA labelled at its carbon ($[^{14}\text{C}]\text{MeA}$). Transport and charge translocation were monitored simultaneously in native and LeAMT1;1-expressing oocytes from the same batch. LeAMT1;1-expressing oocytes incubated in $1 \text{ mM } [^{14}\text{C}]\text{MeA}$ accumulated $9.0 \pm 1.5 \text{ pmol of } [^{14}\text{C}]\text{MeA}$ ($\text{H}_3^{14}\text{C-NH}_3^+/\text{H}_3^{14}\text{C-NH}_2$) $\cdot \text{min}^{-1}$. During incubation, oocytes had a membrane potential of $+4 \pm 1$ mV. The accumulation rate can be converted using Avogadro's constant into molecules of $\text{MeA} \cdot \text{min}^{-1}$. If each molecule transported is charged ($\text{H}_3^{14}\text{C-NH}_3^+$ carries one elementary charge), this corresponds to an ionic current of -14.4 ± 2.4 nA. At the measured resting potential of $+4$ mV, MeA induced -12.5 nA inward current (Figure 4), suggesting that roughly 100% of MeA is taken up as charged $\text{H}_3^{14}\text{C-NH}_3^+$. The MeA⁺/MeA-induced current was specific to LeAMT1;1-expressing oocytes, was time-independent over several minutes and was strongly inwardly rectifying. Taking the error bar into account, the maximal slippage of uncharged $\text{H}_3^{14}\text{C-NH}_2$ must be below 10%. Identical results were obtained with three independent batches of oocytes; one of these experiments is shown in Figure 4. The results show that $\text{H}_3^{14}\text{C-NH}_3^+$ is preferentially transported but, at this resolution, the possible transport of $\text{H}_3^{14}\text{C-NH}_2$ cannot be excluded (Figure 4).

Ionic currents and acidification by LeAMT1;1

To determine if a residual flux of NH_3 is maintained by LeAMT1;1, current and pH_i recordings were combined. Current–

Figure 4 MeA⁺ transport in LeAMT1;1

(A) Induced ionic currents by MeA⁺ in LeAMT1;1-expressing oocytes (background currents have been subtracted). The inset magnifies a part of the Figure. (B) Uptake of $[^{14}\text{C}]\text{MeA}$ in LeAMT1;1-expressing oocytes (black bar), not injected (n.i., grey bar) and LeAMT1;1-mediated (LeAMT1;1 - n.i., dark grey bar). The uptake of $7.8 \text{ pmol} \cdot \text{min}^{-1}$ corresponds to 12.5 nA currents measured from the same oocytes (white bar). Constants used for current–charge conversion were: $1 \text{ pmol} = 6.02 \times 10^{23}$ particles; elementary charge $e^0 = 1.6 \times 10^{-19} \text{ C}$.

voltage relationships from LeAMT1;1-expressing oocytes are shown in Figure 5. The open circles represent a current–voltage relationship from LeAMT1;1-expressing oocytes in the absence of MeA⁺ and the closed circles show the same after addition of $200 \mu\text{M}$ ammonium (Figure 5A). The ammonium-induced current was inward and the reversal potential of the total currents shifted to more positive membrane potentials. No pH_i changes upon $500 \mu\text{M}$ ammonium treatment were observed in BCECF-loaded, LeAMT1;1-expressing oocytes and in water-injected controls (Figure 5B). Although NH_4^+ transport is almost saturated at $500 \mu\text{M}$ in LeAMT1;1 (Figure 3) [5], 10 mM ammonium was also used to compare the results with older studies on native oocytes that used these excess ammonium concentrations. Only negligible NH_4^+ influx is expected from non-voltage-clamped, LeAMT1;1-expressing oocytes at depolarized potentials (compare the current–voltage relationship in Figure 5A), but significant NH_4^+ influx was observed in voltage-clamped oocytes at a negative membrane potential. The negative membrane potential mimics the *in vivo* situation of LeAMT1;1 in tomato root epidermal cells [22].

The pH_i of water-injected and LeAMT1;1-expressing oocytes clamped to -80 mV is shown in Figures 5(C) and 5(D). Ammonium at $500 \mu\text{M}$ induced -150 nA current in LeAMT1;1 and a persistent acidification, in accordance with NH_4^+ influx, but in opposition to significant NH_3 flux. This acidification was not observed in water-injected control oocytes. It is necessary to recall that at the cytosolic pH_i of 7.4 , the pH_i is highly sensitive to NH_3 influx, as almost every incoming NH_3 will bind H^+ to form NH_4^+ . This situation is distinct from NH_4^+ transport: most of the influxed NH_4^+ will remain as NH_4^+ and only a minor fraction ($\sim 1.5\%$) will dissociate into NH_3 and H^+ , and thus acidify the cytosol.

The pH_i changes were not due to possible oocyte to oocyte variability, as shown in a continuous recording from a single LeAMT1;1-expressing oocyte in Figure 5(D). Low ammonium concentration induced acidification and a -170 nA current during voltage clamping, while no acidification was seen when the membrane potential was then allowed to change freely. Interestingly, the voltage-clamped LeAMT1;1 oocytes that acidified with NH_4^+ uptake did not recover the pH_i to the baseline level after the withdrawal of ammonium. This suggests that ammonium that passively exits the oocyte as NH_3 is reabsorbed as NH_4^+ and thus the cytosolic ammonium concentration and pH_i are maintained.

Figure 5 NH_4^+ transport by LeAMT1;1

(A) Ionic currents in oocytes expressing LeAMT1;1 without ammonium (open circles) and with $200 \mu\text{M}$ NH_4Cl (closed circles). (B) pH_i in water-injected oocytes (upper trace) and LeAMT1;1-expressing oocytes (lower trace) induced by 1 mM MeA, 500 μM ammonium and alkalization by 10 mM ammonium. Note the pH_i overshoot induced by 10 mM ammonium in LeAMT1;1 oocytes. (C) As in (B), but cytosolic pH_i measured in voltage-clamped oocytes (to -80 mV). (D) Representative pH_i trace from an LeAMT1;1-expressing oocyte at -80 mV, then not voltage-clamped, and again clamped to -80 mV. Alkalinization is shown as upward, and acidification as downward deflection throughout the Figure.

DISCUSSION

Prediction of the function of proteins based on their crystal structure is an exceptionally elegant method, but we cannot yet distinguish a channel from a transporter that couples conformational changes with transport on the basis of the crystal structure. The structure of the bacterial AMT/Rh homologue AmtB does not show a clearly open transmembrane pore (Figure 1), which would be required for a channel but is unlikely in any well-coupled exchanger. This is also true for the model of LeAMT1;1, which shows that key residues are highly conserved between these proteins. Thus, even in AmtB, conformational changes or side chain movements are required to allow solute flow. If, however, AmtB undergoes a conformational change that is not detected by the crystallographic approach, then hydrogen bond acceptors may become available for interaction with ammonium.

Similar to the case of AMT/Rh proteins, the X-ray structures of a prokaryotic homologue of CLC (chloride channel) anion channels did not reveal its mechanism, which was later identified as rapid $2\text{Cl}^-/\text{H}^+$ antiport [23]. Although the pathway for anions is clearly apparent from the atomic CLC structures, it has not yet been resolved how H^+ is transported via CLCs. Similarly, although residues involved in sugar/ H^+ co-transport via LacY transporters had long been known, the mechanism of H^+ co-transport was not readily apparent from the structure [24]. Crystal structures provide a static view, and even if structures look alike in the presence or absence of substrate, this does not exclude the existence of other conformations, since the protein may preferably crystallize in one state. Solute transport in proteins needs to be viewed as dynamic, but crystallographic structures are, by nature, static. A full understanding of the transport mechanism will only result from a resourceful combination of structural and functional biochemical approaches.

The three-dimensional model of LeAMT1;1 was successfully used to predict residues involved in NH_4^+ recruitment (or binding). Although no attempt is made to derive mechanistic conclusions from the homology model and the structure of the hydrophobic pore of LeAMT1;1, the molecular modelling may be useful in guiding future experimental work on this transporter/channel class. A very similar AMT/Rh structure has recently been reported for a thermophilic archaeon [25], which emphasizes that AMT/Rh proteins adopt a similar structure. The functional data for LeAMT1;1 in Figures 4 and 5 show that LeAMT1;1 is a net NH_4^+ transporter (or 'channel'). Based on the AmtB structure and the hydrophobic pore in the LeAMT1;1 model, one may speculate that the molecular mechanism is NH_3/H^+ co-transport. If NH_4^+ dissociates into NH_3 and H^+ during passage, an unresolved pathway would exist for H^+ . This had already been discussed [1] and explains the superb selectivity of NH_4^+ transporters against alkali cations [5]. NH_4^+ transport is equal at external pH_o 5.5 and 8.5 [5] and exceeds that of NH_3 , which suggests that LeAMT1;1 can transport against NH_3 gradients.

Despite similar key residues in its structure, AmtB apparently uses a different transport mechanism to LeAMT1;1, as it transports at least some NH_3 , shown by the rapid alkalization of AmtB vesicles in ammonium solution [2]. It is currently impossible to exclude the possibility that AmtB is sloppier and passes more NH_3 together with NH_4^+ . It remains to be shown that AmtB dissipates NH_3 gradients and thus acts as a channel. Even if AmtB does transport 95 % in the form of NH_4^+ and only 5 % in the form of NH_3 , alkalization would be expected due to a net inward NH_3 flux (at physiological pH_i total ammonium is $\sim 98.5\%$ NH_4^+ and $\sim 1.5\%$ NH_3).

The data on LeAMT1;1 presented here are in accordance with previous data [5] and argue against the speculation that LeAMT1;1 currents may be endogenous to oocytes [2]. The

saturation of currents was altered by more than 10-fold (NH₄⁺) and 100-fold (MeA⁺) in LeAMT1;1 mutants and differed from endogenous ammonium-induced oocyte currents [8]. Transport of NH₄⁺ is not restricted to LeAMT1;1, since other plant homologues have now been identified in our laboratory that also induce saturable NH₄⁺ and MeA⁺ currents when expressed in oocytes [26]. Similar to the plant transporters, the *Saccharomyces* homologues (MEP1–MEP3; where MEP1 is methylammonium permease 1 from *S. cerevisiae*) are involved in re-uptake and accumulation of lost ammonium [17]. Micro-organisms and plants maintain a near neutral cytoplasmic pH_i, but the acidic pH_o often requires the acquisition of NH₄⁺/NH₃ against an NH₃ concentration gradient.

Small conformational changes that may represent ‘gating’ of AmtB have already been documented in the different crystal structures [2,3], but more structures showing different conformations of AMT/Rh proteins are required to distinguish between channel and transporter-like mechanisms. In any case, the functional results suggest that the classical concept of clear discrimination between channels and transporters is diminishing on a molecular level: highly similar proteins from the AMT/Rh family may function as equilibrative ‘gas’ or ion channels/transporters.

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