Uncoupling of Ionic Currents from Substrate Transport in the Plant Ammonium Transporter AtAMT1;2*

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Background: The substrate transported by AMT/Rh proteins (NH₄⁺/NH₃⁻/NH₃ H⁺) is strongly disputed.

Results: In the net NH₄⁺ transporter AtAMT1;2 mutants led to a change in coupling of NH₄⁺ and H⁺ transport.

Conclusion: AMT/Rh proteins might share a general mechanism of NH₃ transport with different H⁺ coupling ratios.

Significance: Different NH₃/H⁺ coupling ratios in AMT/Rh proteins can explain the contrasting results for their transported substrate (NH₃⁻/NH₃⁺/NH₃ H⁺).

Ammonium (this term designates the sum of NH₄⁺ and NH₃⁻) is an important nutrient and ubiquitous intermediate in nitrogen metabolism. Some passive leakage of NH₃ across lipid membranes may occur, but most cells regulate the ammonium permeability by the expression of AMT/Rh² transporters (1, 2). In microorganisms and plants, the AMT/Rh-mediated high affinity ammonium transport is critical at low concentrations to provide sufficient nitrogen for growth (3, 4). At acidic pH, the NH₄⁺ ion is by orders of magnitude more abundant than NH₃⁻, with an equilibrium constant of pKₐ = 9.25.

The high-resolution crystal structures of AmtB form Escherichia coli, AMT-1 from Archaeoglobus fulgidus, and Rh-1 from Nitrosomonas europaea show that these proteins share a highly similar overall (homo-)trimeric structure (5–8). Each subunit forms a hydrophobic pore in its center that is aligned by two pore-facing conserved histidines, which are essential for excluding other cations, such as K⁺ (9). The pore is occluded to different levels by a “gate” that is formed by two conserved phenylalanines facing into the pore. Although functional assays with liposomes initially supported a facilitated diffusion mechanism for NH₃ in EcAmtB (5), these results could not be confirmed by others (10). Heterologous expression of EcAmtB in oocytes is in agreement with a net NH₃ transport mechanism (11), but NH₄⁺ transport was proposed based on the capacity of an EcAmtB mutant to accumulate ammonium in cells (12). The structure of EcAmtB strongly suggests that an aromatic NH₄⁺ recruitment site selects against water and other cations at the external pore entrance of each subunit. Structural, computational, and mutational analysis revealed that the ion is likely deprotonated before NH₃ passes the pore (5, 6, 13). Simulations may suggest that NH₄⁺ in the pore lumen is deprotonated close to the more externally situated pore histidine and that NH₃ diffuses some distance in the pore lumen (14). In EcAmtB, the proton likely associates with water molecules that fill up the pore and might then be transported in a stoichiometric manner. However, the NH₄⁺ recruitment site, the transient deprotonation acceptor, the NH₃ conduction mechanism, and the H⁺ acceptor are disputed (15–19). After the pore transfer and exit into a cytosolic compartment with nearly neutral pH, NH₃ is finally re protonated at the cytoplasmic pore exit.

By contrast to the uncertain functional mechanism in EcAmtB, direct electrogenic transport and membrane potential-driven accumulation of ionic NH₄⁺ and methylammonium (MeA⁺) have been measured for several plant AMTs (20, 21). The substrate/charge coupling of 1:1 was determined for AMTs from tomato and wheat, respectively. These data are compatible with an NH₄⁺ uniport or an NH₄⁺/H⁺ co-transport mechanism, but cannot distinguish between these mechanisms (22, 23). By contrast, an NH₄⁺/H⁺ co-transport mechanism has been suggested for a bean homolog, based on reversal potential measurements (24). On the other hand, two plant AMTs from the AMT2 sub-branch (25, 26) did not show electrogenic transport in oocytes and were concluded to transport only the uncharged substrate. Structural models of these functionally

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The abbreviations used are: AMT, ammonium transporter; Rh, Rhesus; MeA, methylammonium.
different plant AMTs were not helpful to understand different transport mechanisms (25).

In this study, plant ammonium transporter mutants with reduced transport capacity were identified. AtAMT1;2 from the plant Arabidopsis was chosen as the model because this protein generated very large ammonium uptake rates and NH4\(^+\)-dependent currents in oocytes (20). These large currents allowed a clear distinction from putative endogenous background currents in oocytes. In an attempt to characterize these mutants further, it was identified that these mutants, despite retaining residual transport capacity, lacked (or had minimal) electrogenic ammonium transport currents. The positions of the mutations are surprisingly not found in the pore lumen, but rather on subunit contact sites within the trimer. The data are discussed in light of the proposed transport mechanisms for AMTs.

**EXPERIMENTAL PROCEDURES**

**Yeast Transformation and Growth**—The constructs involving AtAMT1;2 (At1g64780) were based on earlier constructs (20). Mutations were verified by full-length sequencing. The plasmids were heat shock-transformed in the wild type (23344c) and ura\(^-\) ammonium transporter-defective yeast strains (31019b; *triple-Δmep*) (3). Selection for transformed yeast was done on solid arginine medium (2% agar, 0.17% yeast nitrogen base without amino acids and ammonium sulfate (YNB) without amino acids and ammonium sulfate (Difco), supplemented with 3% glucose and 0.1% Arg as nitrogen source, buffered with 20 mM MES/Tris, pH 6.1). Yeast was grown in liquid Arg medium until As995 reaching 0.6–0.8. Cells were harvested, washed, and resuspended in water to a final concentration of 2.11 Gbq/mmol, Amersham Biosciences. 50-ml samples were prepared using the software PyMOL. Homology modeling was performed using the EXPRESSO module of the T-Coffee suite. Structures of the templates. Multiple sequence alignments were performed using the EXPRESSO module of the T-Coffee suite. Modeling was carried out using MODELLER 9v7. Several homology models were generated for the target sequence, and the most stable model was chosen. Graphical representations were prepared using the software PyMOL.

**RESULTS**

Identification of AtAMT1;2 Mutants That Improved MeA Resistance of Yeast—The expression of plant AMT1s, such as AtAMT1;2, increases the yeast susceptibility to MeA due to...
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AtAMT1;2 on nonselective conditions (2 mM arginine, 1 mM ammonium). The sequencing of a number of variable size even on nonpermissive MeA concentrations supported yeast growth on 1 mM ammonium. The sequencing of a number of variable size even on nonpermissive MeA concentrations supported yeast growth on 1 mM ammonium.

The translated protein sequence, namely Q67K, M72I, W145S, V179L, and G291S. Two other mutants (G357V and L399R, isolated in duplicate) improved the growth of the wild type yeast on MeA above the level of the empty plasmid transformed controls. However, these plasmids failed to support the growth of the triple-$\Delta mep$ strain on low ammonium (Fig. 1A). These latter two mutations and the two mutations V179L and G291S occurred in residues that were conserved among most AMT/Rh proteins, but the three other mutations (Q67K, M72I, and W145S) were confined to stretches that were exclusively conserved among plant AMT1 sequences, but not in AmtB, AtAMT2, and Rh sequences. These three mutants were chosen for further analysis. Their reduced ammonium transport capacity as compared with AtAMT1;2 wild type was further supported by their differential effects on yeast growth at high ammonium concentrations in yeast (data not shown).

Position of Mutant Residues at Subunit Contact Sites—The reduced transport activity in the mutants may be a consequence of altered pore properties or altered protein stability in the membrane or rigidity. Structural modeling clearly indicated that the altered residues were likely not in direct contact with the hydrophobic pore lumen (and thus likely not in direct contact with the substrate) (Fig. 1B). By contrast, these residues clustered at opposite surfaces between adjacent subunits and may therefore be important for subunit interactions within the functional trimers. Subunit interactions indeed appear to be crucial for the transport activity (29).

MeA and Ammonium Substrate Transport without Ionic Currents in AtAMT1;2 Mutants—AtAMT1;2 and the mutants were expressed in parallel as GFP-tagged proteins in oocytes. The fluorescence pattern at the rim of the oocytes was similar for all constructs and distinct from that of free GFP; this may indicate their plasma membrane localization, but identified different protein expression levels. The Q67K and W145S mutants expressed to high levels in oocytes, whereas a reduced protein level was detected for the M72I mutant (Fig. 2A). As all mutants increased $[^{14}\text{C}]$methylamine uptake into oocytes, the mutant proteins must be inserted into the plasma membranes. However, the uptake activity of all mutants was significantly decreased by about ~70% of the wild type (Fig. 2B), which was consistent with the data from yeast (data not shown). This 30% of residual transport activity was expected to elicit about 30% of the ionic currents that were recorded from the AtAMT1;2 wild type upon the addition of MeA$^+$. Surprisingly, no measurable residual MeA-associated currents above background were observed (Fig. 2, C and D), but it should be noted that the expected MeA$^+$-induced ionic currents were relatively small, even in the AMT1;2-expressing oocytes.

In the absence of MeA or ammonium, AtAMT1;2-expressing oocytes had similar small, almost linear background currents, which were not distinguishable from water-injected control oocytes. This small background leak was also recorded from mutant-expressing oocytes. Thus, in the absence of the substrate, the electrical resistance of expressing and nonexpressing oocytes was highly similar. In the presence of ammonium, however, large time-independent ionic inward currents were recorded in the wild type AtAMT1;2-expressing oocytes (Fig. 2E). Such a large NH$_3^+$-induced current was absent in water-injected controls and in mutant-expressing oocytes. When the relative ammonium uptake into AtAMT1;2- and mutant-expressing oocytes was quantified with $^{15}$N isotope label, it was

increased uptake of this cytotoxic compound (28). We noted that occasionally AtAMT1;2-transformed yeast formed colonies of variable size even on nonpermissive MeA concentrations. This resistance was likely due to spontaneous mutations in the high copy number plasmid carrying AtAMT1;2, potentially leading to an abolished or reduced MeA$^+$ import, improved compartmentation, or even efflux of MeA. The plasmids from initially more than 80 isolated colonies were isolated and reintroduced into the triple-$\Delta mep$ yeast strain that lacks endogenous ammonium transporters. Most plasmids did not support yeast growth on 1 mM ammonium. The sequencing of a few of these revealed that mutations in the coding sequence of AtAMT1;2 introduced premature STOP codons, in accordance with loss-of-function mutations. Thus, in the original colonies, the lack of functional AMT led to a similar MeA sensitivity as had wild type yeast transfected with an empty plasmid. However, the uptake activity of all mutants was significantly decreased by about ~70% of the wild type (Fig. 2B), which was consistent with the data from yeast (data not shown).

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found that the uptake activity by AtAMT1;2 mutants was significantly reduced, but readily detectable. These combined data from MeA and ammonium transport studies and parallel electrical recording indicated that the substrate transport in the mutants was not associated with significant electric charge transport, indicating that neither the ammonium ion nor H\(^+\) is transported at larger amounts in the mutants.

**DISCUSSION**

In a screen involving yeast survival on toxic MeA, functional AtAMT1;2 mutants that lowered the susceptibility of yeast to MeA and that were capable of rescuing growth on ammonium were identified. The mutations in the residues Gln-67, Met-72, and Trp-145 were characterized in more detail. The function of these mutants in yeast was consistent with the idea that ammonium and methylamine were transported at a reduced rate or that less AMT transporter protein was expressed at the plasma membrane of yeast.

The more detailed electrophysiological characterization of the mutants in oocytes revealed that the mutants not only had reduced transport activity, but failed to elicit the expected NH\(_4^+\)-dependent ionic currents that were associated with the transport in the AtAMT1;2 wild type. The fluorescence of oocytes expressing GFP-tagged wild type and mutants was similar, potentially indicating the same subcellular localization at the plasma membrane of oocytes, analogous to other diverse plant proteins expressed in oocytes (30, 31). However, the resolution of the confocal pictures of oocyte plasma membrane microvilli may not allow us to clearly distinguish plasma membrane localization from near plasma membrane subcompartments (32). Nevertheless, the fluorescence clearly indicated that at least the Q67K and W145S mutants were expressed at high levels and their functional activity proved their plasma membrane localization. Importantly, ammonium or MeA did not evoke any detectable ionic currents above background in oocytes expressing these three mutants, despite a residual substrate transport activity of 10–30%. These electrically silent transporters were mutated at positions that were in the membrane core of subunits, but not directly lining the pore lumen. The residues Gln-67, Met-72, and Trp-145 were located at the protein surface between monomers, where they are in contact with adjacent subunits. These residues may affect cooperative interactions in the functional trimer, which also involve the C terminus in plant AMTs (20, 29, 34, 35). The structure of the related EcAmtB was unaffected by point mutations (13, 33), which may argue that the overall architecture was not drastically changed by the mutations. Importantly, the residual electroneutral substrate transport in the mutants cannot be explained by simple NH\(_4^+\) diffusion through the mutant pores, as at the used low external pH 6, less than 0.1% of the ammonium is available as NH\(_3\), which is not sufficient to account for
the observed transport rates. As a consequence, an NH₄⁺ deprotonation step is likely to occur in the mutants.

Structural constraints, simulation studies, and electrophysiological data clearly indicate that NH₃⁺ is recognized in the external pore vestibule of AMTs and that a charged substrate is transported, but it remains unclear whether this is NH₄⁺, or H⁺ together with NH₃. The fact that K⁺ is transported in EcAmtB mutants, in which the essential pore twin histidine motif was removed (9), is seemingly more compatible with an NH₄⁺ conduction mechanism in the wild type AMT. However, the functional twin histidine motif was shown to be essential for ammonium transport, and the deletion of this motif must alter mutant EcAmtB pore properties, which now also pass other ions, such as an alkali cation. Alternatively, a H⁺/(uncharged substrate) mechanism may explain the excellent selectivity of AMTs against the transport of alkali cations of similar size and may explain why deprotonable substrates with very different size (NH₄⁺ and MeA⁺) are transported at various levels. Such an NH₄⁺ deprotonation step is likely occurring in AtAMT1;2 mutants with intact twin histidine motifs. Furthermore, the transport mechanism in a bean AMT homolog is mechanistically equivalent to a 2H⁺/NH₃ co-transport mechanism (24). Larger electric currents elicited in oocytes by the AtAMT1;1 Q57H mutant may be explained by a higher H⁺/substrate coupling ratio (35). This mutation was in the homologous residue to Gln-67 of AtAMT1;2. This highlights the importance of the respective glutamine in AMTs and opens the possibility that different residues at this position may result in different transport/charge coupling ratios.

In conclusion, further experiments seem to be required to unequivocally answer whether NH₄⁺ or H⁺/NH₃ are transported by individual AMTs.

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