The Amt/MEP/Rh Family: Structure of AmtB and the Mechanism of Ammonia Gas Conduction

The atomic structures of the first members of the Amt/MEP/Rh family show that they are 11-crossing membrane proteins that form trimers in the membrane. Each monomer supports a hydrophobic channel that conducts NH3 but not any water or ions. The reprotonation of NH3 on the receiving side raises the pH on that side in the absence of metabolism of NH3 and there is no transfer of protons through the protein.

Transport of ammonia across biological membranes is a key physiological process throughout all domains of life (7, 31, 32, 46, 60, 68), and most species have multiple different proteins of the family, underlining the importance of their roles in biology. Although NH4+/NH3+ (termed Am) is highly toxic to animals, it is the preferred source of nitrogen for most microorganisms. Once inside the cell, Am is directed to glutamine synthetase, which rapidly uses NH3 as substrate to synthesize the amino acid glutamine using ATP. Thus metabolism serves to maintain an inward gradient of the substrate. In this review, we will examine the current state of knowledge of the mechanism of conductance, beginning with the structure of Amts, the mechanistic implications of these structures, experimental tests of the mechanism, and implications for the other family members, including the Rh proteins of clinical importance.

Nomenclature and Diversity of NH3 Channels

Am exists in two forms, NH4+ and NH3, in an equilibrium controlled by a pKa of 9.25 in aqueous solution. However, a hydrophobic environment can reduce the pKa to favor the neutral form in the equilibrium. Neutral NH3 can diffuse through hydrophobic membranes and indeed can support growth of microorganisms, although when only low concentrations of Am are available, other regulated mechanisms of Am transport are used. Escherichia coli incorporates a single Amt (ammonium transporter) called AmtB carried on the glkK amtB operon. AmtB is barely expressed unless ammonium becomes growth limiting, whereupon the gene is induced. In bacteria, ammonia transporters in the family are termed Amts. Three are found in yeast and called methylammonium/ammonium permeases or MEPs. Ammonia is an important source of nitrogen for plants (21), and in plants they are termed Amts (51). Three Amts are found in tomato (33), three are found in Arabidopsis (15), others are found in rice (71). Amts are also found in invertebrates. The Caenorhabditis elegans genome, for example, contains four Amt homologs.

Members of the Rh branch of the family are found in animals, sometimes alongside Amts in invertebrates, but not so in mammals. The human erythrocyte carries the Rh proteins RhD and RhCcEe that express the antigens of the red blood cell and RhAg in complex, whereas RhBG and RhCG are found in kidney, brain, and many organs. In mammals, Am transport is vital to kidney physiology in maintaining pH and in renal ammonia secretion (30).

Marini and colleagues show, based on the partial functional complementation by Rh proteins of an S. cerevisiae Δ(mep1,2,3) mutant in which all three MEPs are deleted, that the Rh50, Rh-associated glycoproteins RhAG, RhBG, and RhCG can likewise conduct Am (43, 44). Since the structure determination of AmtB and alignment of the sequences of RhAG, RhBG, and RhCG especially show conservation of critical components with AmtB, which suggests they are structurally and functionally similar to AmtB (8, 29). Interestingly, invertebrates, and notably the green algae Chlamydomonas, have both Amts (of which there are four) and members of the Rhesus protein branch (Rh proteins, of which there are two) (32). Physiological considerations led Ouray et al. to propose that the function of Rh proteins might include transport of CO2 as diffusive channel (62). It has since been shown that RhAG and AmtB can indeed conduct both NH3 and CO2 (48).

Structure of Amts: The Paradigm for the Family

The X-ray structure of AmtB from E. coli was initially determined to 1.3 Å resolution at pH 6.8, with and without Am or MA (29). The structure shows that the protein is an 11-crossing membrane protein that homotrimers in a threefold symmetric fashion. Each monomer incorporates a hydrophobic channel in between relatively polar cytoplasmic and periplasmic vestibules. This, and “difference mapping” between pairs of the structures in presence of MA or Am, and the crystals of AmtB without any Am suggested that it recruits ammonium ions, the most available Am species...
FIGURE 1. Sequences of AmtB/MEP/Rh homologs from *E. coli*, *A. aeolicus*, *Neurospora*, *Saccharomyces cerevisiae*, *Lycopersicon esculentum*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, and human Rh proteins. Trans-membrane helices M1–M11 are indicated. The numbering is that of *E. coli* AmtB. Conserved amino acids are in white in red-filled rectangles. Similar residues are in red surrounded by blue lines. The signal sequences in *E. coli* and *A. aeolicus* are underlined. Red stars indicate residues that line the lumen of the channel.
at pH of <9, but conducts only neutral NH$_3^+$ leaving a proton behind, a mechanism supported by assays of AmtB reconstituted in proteoliposomes (29). The structure was independently determined to 1.8 Å at pH 4.6 and compared with our first-reported structure (76). A third structure of Amt-1, one of three Amts from the hyperthermophilic archaeon *Archaeoglobus fulgidus*, was later determined at 1.54 Å (3).

NH$_2$-terminal amino acid sequencing of AmtB shows that 20 amino acids are excised from the NH$_2$-terminus of *Aquifex aeolicus* AmtB (AmtB$_{AQFX}$), whereas 22 residues are cleaved from the *Escherichia coli* AmtB (AmtB$_{Ecoli}$) in the mature proteins (29). These are signal sequences that were appropriately cleaved during expression in *E. coli*. They are strongly predicted to be signal sequences using neural network approaches (50) (FIGURE 1). Thus the AmtB monomer (FIGURE 2A) is comprised of 11 transmembrane helices with the NH$_2$-terminus on the periplasmic side and the COOH-terminus on the cytoplasmic side (29). The trimer (FIGURE 2B) is also quite stable, even running as a trimer in SDS-PAGE, and the trimer is the physiological form of the protein (6, 9).

Consistent with the “positive inside” trend in membrane proteins, the trimer of AmtB$_{Ecoli}$ has net negative charge of –7.5 (13.5 positive + 21 negative) on the periplasmic surface and net positive charge of +9 (42 positive + 33 negative) on the cytoplasmic side. Several side chains of tyrosine and tryptophan with their partially polar character lie in positions to interact with the lipid head groups around the trimer.

To date, the 11-crossing structure of AmtB (and we presume of the associated MEP/Rh proteins) is the first of this fold among membrane proteins. It reveals a quasi-twofold symmetric structure within the monomer itself, in which membrane-spanning segments M1–M5 are related to M6–M10 by a quasi-twofold axis that lies in the mid-membrane plane. The implied gene duplication presumably occurred before the transport function arose since the two related domains are oppositely polarized with respect to the sidedness of the membrane. Alternatively, the primordial gene product before the duplication may have been of the type that could adopt either polarity in the membrane to form a functional anti-parallel dimer. This kind of alternate topology has recently been proposed to occur in membrane proteins based on fusion protein labels for determination of factors that control sidedness of membrane proteins (56). The final helix M11 is long and highly inclined to the membrane and lies on the outer surface of the trimer, in contact with the lipids. Together M1–M10 diverge outward from the central plane in a right-handed helical bundle to generate a vestibule on each side of the cell membrane (FIGURE 2, A AND B).

Such structural duplications with opposite polarity are increasingly seen in other membrane protein structures, including GlpF and the aquaporins (14, 18, 61, 66), the SecY protein of the translocon (69), the CIC H$^+$/Cl$^-$ exchange transporter (2, 11, 12), the bacterial homolog of the Na$^+$/Cl$^-$-dependent neurotransmitter transporters for biogenic amines (75).
and lactose permease LacY (1), GlpT (22), and their “major facilitator super-family” (MFS) homologs. The vestiges of structural duplication were recognized in the gene sequences of the aquaporins (53) and in the MFS (52) before structure determination, although not in the Amt/MEP/Rh superfamily. In hindsight, one could imagine algorithms that could detect such gene duplication based on the increasingly good ability to predict the topography of membrane-spanning segments (16) and the expectation that the duplicated segments would retain some structural similarity.

In light of the structure of AmtB, the highly conserved residues Asp 160 (7 residues) His168 at the NH$_2$-terminal end of M5 and Asp 310 (7 residues) His 318 located 150 residues downstream at the NH$_2$-terminal end of M10 are some of the most notable vestiges of duplication in the amino acid sequences.

**FIGURE 3.** The ammonia-conducting channel in AmtB

*A:* the lumen of the ammonia channel is colored according to electrostatic potential. Surfaces of helices M9 and parts of M8 and M10 are removed and replaced by helical backbones (yellow). The positions of two conserved histidines near the three NH$_3$ sites (blue spheres) are shown in green and blue stick representation and were not included in the surface electrostatic calculation. *B:* stereo views of the CH$_3$NH$_3^+$/NH$_4^+$ seen at the base of the vestibule. Red dashed lines show hydrogen bonds between the NH of CH$_3$NH$_3^+$ and Oy of Ser$^{219}$, and between Ne1H of Trp$^{148}$ and O of Asp$^{160}$, with bond distance in yellow.

**The Mechanism of NH$_3$ Conduction Deduced from the Structure**

To probe the mechanism of conductance, 25 mM AmSO$_4$ at pH 6.5 or 100 mM MASO$_4$ at pH 6.5 was added to crystals of AmtB to see whether there were any discreet binding sites for Am/MA (29). No significant conformational changes are induced, suggesting that AmtB acts as a channel that serves to conduct Am in the direction of an Am gradient. Subsequently, we developed biochemical assays with AmtB reconstituted into liposomes (proteoliposomes) to test and validate our deduced mechanism.

The conducting pathway within each monomer of AmtB lies between two vestibules, one on the outer surface and the other on the cytoplasmic surface (FIGURE 3A). Each vestibule contains approximately 30 sites occupied by water molecules that are hydrogen bonded to one another and to the carbonyls and...
polar groups in the vestibules. Between the vestibules, the lumen is narrow and mostly formed by aliphatic nonpolar side chains throughout its approximately 20-Å length. This would only seem consistent with conduction of a neutral molecule such as uncharged NH₄⁺. To test the hydrophobic nature of the pore, a hydrophobic gas, xenon, was diffused into the crystals of Amt-1. Two xenon atoms were found in the lumen of the pore, emphasizing the hydrophobicity of the substrate channel (3).

There is no discreet water visible in the lumen, even though the structure is at 1.35-Å resolution, which is almost sufficient to resolve the positions of hydrogen atoms (29). This could be because any water is in dynamic motion or it could be because the lumen is too hydrophobic to retain or transport water. We surmise that it is probably the latter, since no water is conducted by the channel (29). The single exception to the hydrophobic walls lies on either side of the mid-membrane center of the pathway where there are two in-line, almost coplanar imidazole side chains of His168 and His318. The two are hydrogen bonded to one another, and they are highly conserved throughout the AmtB/MEP/Rh family (although not in erythroid RhCcEe and RhD).

The entrance to the lumen has a narrow 1.2-Å diameter hydrophobic constriction on each side that must move apart dynamically during any conduction event. This necessity for a dynamic opening to allow entry to and exit from the hydrophobic portion of the pathway does not change the inference that the protein acts as a diffusive channel. Likewise, the relatively low flux for ammonia for a channel, estimated from various free-energy calculations to be <10⁴/s (76), may reflect these dynamic structural and electrostatic barriers to NH₄⁺.

Since X-rays are scattered by electrons, it is difficult to “visualize” NH₃ vs. NH₄⁺, H₂O, Na⁺, Mg⁺², or even F⁻ since all of these species have the same electron content of 10 electrons. Thus any possible binding site for Am could only be defined using the alternate substrate, methyl ammonium. In the presence of MA, there is one partially occupied MA site seen in a “difference electron density map” (29). However, even though the MA concentration (100 mM) is 2,000-fold higher than levels that would reach 50% maximal conduction rate [K₉₅ (MA) ~50 μM], the site is only 60% ordered, reflecting the fact that it is not rigidly held in place but rather is an electrostatically favorable location for the Am-related cations. That is to say that there is no real binding site as such, although this site, located at the very bottom of the vestibule as it enters the hydrophobic portal to the lumen, may be on the pathway and help catalyze the removal of a proton from ammonium ion, to yield the neutral NH₃ for transport.

Interestingly, this “recruitment” site is located against three aromatic rings of F103, F107, and W148 and allows a hydrogen bond to Ser219. The π-electron orbitals within such aromatic rings provide for a so-called “π-cation site,” favorable for cations such as NH₄⁺ and CH₃NH₃⁺ (FIGURE 3B). The K₉₅ (Am) for transport of Am is approximately 10 μM, and, in presence of 25 mM Am rather than MA, a 67% occupied peak lies in the π-cation site. There is a similar well-defined H₂O peak at this site in the absence of Am. Overall, the site may help catalyze the deprotonation of the ammonium ion as it proceeds on to the hydrophobic lumen that then acts as a channel for NH₄⁺. Each vestibule offers approximately eight carbonyl oxygens (each with 0.4e charge) to act as an attractive funnel for water and for positively charged NH₄⁺. The idea that this “recruitment” site may act as a transient site in the mechanism is consistent with the finding that there is competition between Am and MA (26) for transport, which can also be competed by nontransported dimethylamine or ethylamine (45). Interestingly, K⁺ or alkali cations do not compete with NH₃ transport.

In AmSO₄ vs. no Am present, three weakly occupied sites (Am2, Am3, and Am4) lie adjacent to imidazole rings of conserved quasi twofold related His168 and His318 and hint at transient intermediate sites along the pathway (FIGURE 4). On average, these peaks indicate only approximately 20% occupancy at each site (approximately 1.4 electrons each), so they provide a lower limit to the occupancy by NH₃. There are suggestions that these weak peaks may be water and seem to be observed at pH 4.6 with or without NH₃ (3, 76). However, the peaks have such low occupancy and are most sensitively quantitated in the difference between protein with Am and protein without Am. In addition, we show that water is not conducted by AmtB (29); thus it is unlikely that there is any even disordered water in the channel. There are at best only very weak hydrogen bonds to Am2, Am3, and Am4 from the Ce1-H of the imidazole rings of His168 to Am2 (3.2 Å) and from His318 to Am3 (3.4 Å). We presume that these histidine side chains are neutral since they lie in the hydrophobic lumen with no evidence of ordered water for solvation nearby. Since they are hydrogen bonded together between Nδ of H168 and Nδ of H318, sharing one hydrogen atom, they could not both be protonated without moving apart quite significantly. It is most likely that their conjoint pKa is significantly lowered by the environment to preserve their neutrality within the hydrophobic lumen. Indeed, the structure at pH 4.6 shows no difference in this low dielectric environment, suggesting that the pKa may be below pH 4.

In general, for example, in enzyme active sites, imidazoles of histidine often facilitate acid-base mechanisms with their pKa when solvated near 6.8. The conservation of these residues leads one to query the possibility of a role in proton removal from NH₄⁺ by the two-imidazole pair. The pair of imidazoles might somehow facilitate removal or conceivably rotate to pass such a proton on through the lumen and eventually reprotonate the NH₃ at the end of its travel. This
fluorescein-loaded vesicles in buffer with ammonium chloride (0.5 mM or 5 mM) was initiated at pH 6.8 in a stopped-flow spectrometer. NH₄⁺ is in equilibrium with NH₃ with a pKa of 9.25 in aqueous solution. Any influx of NH₃ would reacquire a proton inside forming NH₄⁺ and OH⁻ and so raise intravesicular pH. The addition of 5 mM NH₄Cl outside led to a rise in pH with a rate of 115.6 ±13.2 s⁻¹ (n = 6) that was >10-fold higher when AmtB was incorporated vs. 12.8 ±0.7 s⁻¹ (n = 6) for protein-free liposomes. This clearly implies that, indeed, NH₃ is transported to equilibrium, down the concentration gradient. It gains a proton inside the proteoliposomes to raise the pH inside the proteoliposomes.

To further clarify, in this carefully controlled experiment, the vesicles are first loaded with the carboxyfluorescein, then washed and spun down twice. The rise in fluorescence with the increase in pH is small, and thus that change is normalized to 1.0 to plot the change in “relative fluorescence” (29) vs. time, and the entire reaction is complete within fractions of a second. The pH of the vast volume on the outside of the vesicles does not change. Thus any fluorescence change reflects only what takes place inside the liposomes. Any dye that might have remained on the outside of the vesicles does not change on addition of AmSO₄. The diffusion of NH₃ is much faster than for the bulky carboxyfluorescein molecule and is complete within 0.1–0.4 s. Likewise, any leakage of dye out of the proteoliposomes would change only the magnitude of the small change, the “signal” but not its time dependence. The lack of any drift in this value over the relevant time scale shows that leakage is in any case not a factor. This was verified by replacing AmSO₄ by NaCl as a control. Using NaCl did not cause any change in relative fluorescence (29), and this also shows that the fluorescence change is not because of leakage of dye out of liposome.

To see whether water passed through AmtB, we again used the fluorescence of carboxyfluorescein. This time, the concentration of dye was monitored by its alteration in self-quenching vs. time. Indeed, the AmtB proteoliposomes show no difference from liposomes in their rate of water conductance, as reflected in response to an osmotic challenge using sucrose, showing that essentially no water passes through the hydrophobic channel (29).

The Mechanism Deduced from Structure

The mechanism therefore involves the following elements: a site that can recruit and possibly assist in deprotonation of NH₄⁺ and a hydrophobic channel for NH₃ that must lower the pKa of Am, probably to a very low level ensuring that it is always in its gas-like neutral form inside the hydrophobic portion. The energy to accomplish lowering of pKa, perhaps by as much as 8 pH units, perhaps approximately 12 kcal/M is
already much less than the cost of dehydrating an NH₄⁺ ion. Thus the hydrophobic channel, and its affinity for hydrophobic NH₄⁺ coupled with the lack of any water of hydration within, easily suffices. Put another way, the dehydration of NH₃ is much more easily accomplished than the dehydration of the NH₄⁺ ion. This difference in energy easily reaches that required to accommodate the necessary change in pKa. From our observations that were made at pH 6.8, solely to exploit the most sensitive region of the fluorescent dye, the pKa of the NH₃ when it is in the channel must be pKa < 6. It is probably much lower. Once it leaves the channel, the pKa reverts to its aqueous value and takes up a proton to yield NH₄⁺ + OH⁻, thus raising the pH inside the proteoliposomes.

All the observations noted so far are reconciled by the recruitment of NH₄⁺, reduction of its pKa, and conductance of NH₃ as the primary mechanism. In some ways, the proton-stripping mechanism we propose makes it seem as if a proton is transferred from inside to outside since NH₄⁺ leaves a proton starting on the outside, to pick one up on the other side. Unlike an antiporter, however, there would be no net transfer of a proton.

One problem that has been raised with this mechanism is that it would tend to alkalize the cell, effectively removing protons from inside to outside against the normally 0.2-V proton motive force across the plasma membrane in E. coli. If so, the process would cease to work. This would only work either if the NH₃ were metabolized as NH₂, rather than NH₄⁺, which it is by GS, or if there was an energy source that helped to drive the process. GS is the enzyme that provides the source of energy by ATP hydrolysis that serves to fix the NH₃ into glutamine and maintain the inward gradient of NH₃.

**Chemistry of NH₃ Transport**

Based on the growth behavior of both enteric bacteria and S. cerevisiae, the Kustu group proposed that AmtB/MEP proteins and their homologs increase the rate of diffusion of the uncharged species NH₃ across the cytoplasmic membrane and deduced that there was no evidence for concentrative uptake of NH₃ against a gradient (63, 65). This is now borne out by the flux assay of purified AmtB in reconstituted liposomes (29). This shows that the channel is indeed a passive channel that conducts NH₃ bidirectionally in the absence of any energy source. Seeking a direct measure of conduction rates in vivo, and since the radioactive nitrogen isotope is very short lived, transduction rates of most Amt/MEP proteins has been monitored by transport of radioactive ¹⁴C-labeled CH₃NH₂/CH₃NH₃⁺ (termed MA) (17). When carried out in vivo, this ¹⁴C/MA assay has led to misunderstandings in the past, since at least in E.coli ¹⁴C/MA is converted into ¹⁴C/methylglutamine by glutamine synthetase (26, 63); thus, before this realization, accumulation of radioactivity erroneously appeared to be a concentrative MA uptake against a gradient, which sometimes led incorrectly to suggestions of active, energy-coupled, or electrogenic transport through the transporter. Consequently, experiments that rely on conductance of ¹⁴C/MA need to be reevaluated.

A modification of the method (27) was generated by the Merrick group to yield the activity of AmtB alone and shows AmtB to act as a slow diffusive channel (26). It was also shown that transport is not dependent either on membrane potential or on ATP, which thereby supercedes other attempts to monitor ¹⁴C/MA uptake using protonophores like CCCP or adjustments of membrane potential.

The dependence of MA uptake by AmtB on external pH was shown to increase by a factor of 2.7, between pH 6.25 and pH 7.25, and by a factor of 3.8, between pH 7.25 and pH 8.25 (26). This factor of approximately 10 between pH 6.25 and 8.25 does not correspond to the approximately 100-fold change in concentration of NH₃ in the bulk solution, as had been implied by Soupene (63). It therefore invalidates the notion that NH₃ from the bulk solution is conducted and supports the mechanism deduced from the X-ray crystal structure of AmtB in which NH₃⁺ is recruited into the periplasmic vestibule and deprotonated to allow NH₃ to pass through the channel (29).

Intriguingly, the Merrick group took advantage of this observation to show that the apparent Kₘ of the substrate MA for GS is much lower in vivo (Kₘ = 380 μM) than it is for the enzyme in isolation (Kₘ = 79 mM). They infer that AmtB and multiple allosterically regulated GS may be metabolically coupled together in some way (24). This makes sense since the GS reaction uses NH₃ as substrate and thus bypasses any tendency of the NH₃ transport to change the pH inside the cell. Physical proximity would help channel the substrate more directly to the enzyme and maintain the inward gradient of NH₃. Arrhenius plots for GS indicate activation energy of 14.1 kcal/M. The energy of activation (Eₕ) for AmtB was 1.6 kcal/M, consistent with its action as a slow channel for NH₃.

**Simulation of Conductance by AmtB**

Molecular dynamic simulations of AM transport through AmtB support the role of the recruitment site in the vestibule (36), which is followed by passage of neutral NH₃ through the hydrophobic pathway. Specifically, these calculations show a quite small Eₕ of 3.1 kcal/M for NH₃⁺ ion as it enters the vestibule, which compares well with the measured 1.6 kcal/M at 37°C (26). “Molecular mechanics” are an important guide as to whether processes deduced from static or “time-averaged” crystal structures can take place, and in what time scale. They bridge the time-averaged crystal structure and biochemical assays to test mechanism
by means of simulation in a computational environment. The simulations of AmtB show how a hydrogen bond to \( \text{NH}_3^+ \), first with the backbone carbonyl oxygen of Phe161 then with the hydroxyl group of Ser219, compensates for the loss of a hydrogen bond to water and how the aromatic rings of Trp148 and Phe107 assist in the \( \pi \)-cation interactions. At the end of the vestibule, the phenyl ring of Phe107 dynamically switches to an open state accompanied by a slight rotation and shifting of the indole ring of Trp148. This allows access of the initially buried and highly conserved carboxylate group of Asp160 to the vestibule, where it may become the base that ultimately helps catalyze the deprotonation of \( \text{NH}_3^+ \) via water molecules (36). This might be a second reason for the high conservation of Asp160 and its twofold counterpart Asp 310 in Amt proteins and could be a factor in why the D160A mutant (25) or D160N in MEPs, RhAG, and RhCG are inactive as transporters (42). Once \( \text{NH}_3^+ \) deprotonates, the phenyl ring of Phe215 rotates to open, and the subsequent passage of \( \text{NH}_3 \) through the channel is straightforward.

**Regulation of Ammonia Transport**

In most bacteria, Amt genes are induced by nitrogen limitation, which is controlled by an activator, NtrC. However, PII signal transduction proteins that can also block transport also tightly regulate nitrogen metabolism posttranslationally. In almost all bacteria, the **amtB** gene is linked to **glnK** (67). GlnK inhibits \( \text{NH}_3 \) transport by direct association with AmtB. Inhibition by GlnK is controlled by the uridylylation/deuridylylation of GlnK. GlnK is also a trimer but soluble protein that binds to the AmtB trimer and blocks conduction directly (10, 46). Each of three PII-like encoding genes, paralogs **glnB**, **glnK** (**GlnK1** and **GlnK2**), and **glnY** are found in close association with Amts. In each case, the unmodified protein is inhibitory. Models of this assembly have been made (3) and, along with structure determinations, show how association prevents \( \text{NH}_3 \) progressing through the cytoplasmic side of the channel. The COOH terminal 20 amino acids of **E. coli** AmtB are extremely polar, highly charged, and, not surprisingly, not ordered in the structure. They are likely to be involved in the regulatory interface with the PII-proteins (3).

**Function and Structure of the Rh Proteins and Rh-Associated Glycoproteins**

Rh found in animalia fall into a distinct branch of the Amt/MEP/Rh family. RhAG can specifically mediate ammonium transport when expressed in ammonium-uptake-deficient yeast by testing the growth of triple-**mep**\( \Delta \) strains on media containing <5 mM ammonium (43). Expression of RhAG enhances resistance to a toxic concentration of methylammonium (250 mM) in such triple-**mep**\( \Delta \) yeast, consistent with RhAG promoting export of the ammonia analog. Although bacteria, plants, and fungi can acquire nitrogen from their environment in the preferred form of ammonia (72), mammals prefer assimilated forms of nitrogen, amino acids, for nutrition, and they excrete nitrogen as Am mediated by Rh proteins (43).

Multiple Rh-associated glycoproteins are identified in various organs (73, 74). RhAG is expressed in erythrocytes and erythroid precursors along with the Rh proteins RhCcEe and RhD (5, 40), where it facilitates export of Am (19). Nonerythroid RhBG and RhCG are expressed in important sites for ammonia metabolism, including kidney, liver, skin, testis, the central nervous system, and the intestinal tract (4, 30, 39, 41, 49, 55, 73).

Based on the structure of AmtB and sequence conservation, the Rh-associated glycoproteins and the Rh proteins most probably have the same structural framework and trimeric state as AmtB, despite earlier suggestions that they had 12 crossings (13): the first helix M1 lies in the center of the trimer. Thus any portion of the protein that was coded for by sequences ahead of the mature \( \text{NH}_3 \) terminus of AmtB, or its homologous position in other proteins of the family, would be difficult to accommodate within the trimeric structure (Figure 2). Thus we propose that all of the Rh-associated glycoproteins, RhAG, RhBG, and RhCG, will also follow the same pattern with cleaved signal sequences and that they will form trimeric assemblies.

It is therefore worth reexamining the data again that suggested 12 crossings and a cytoplasmic \( \text{NH}_3 \) terminus. The conclusion was based on antibody binding of anti-peptide polyclonal Rh30A antibodies to intact red cells vs. to leaky red cells that opened access to the inside. The \( \text{NH}_3 \) terminus of a full-length protein was seen in Western blots to be on the cytoplasmic side. This remains a conundrum that is apparently confusing in the field. However, the signal sequence could have been only partially cleaved from Rh or it could have been cleaved from the functional protein and been left with the exposed epitope on the signal peptide, with its \( \text{NH}_3 \) terminus detectable only from the inside.

Like AmtB, the erythroid Rh proteins are usually oligomers of RhAG with Rh proteins RhCcEe and RhD (13, 57, 70). We would expect them to be trimers, but heterotrimers rather than the homotrimer of AmtB. By the principles of assembly, one would expect that a heterotrimer would contain three different proteins in a uniform arrangement. The erythroid Rh proteins that carry the antigenic sites RhCcEe and RhD are less similar to AmtB in that they do not conserve the second Asp 310 His 318 pair. The first pair is present but separated by only six amino acids vs. seven. Nevertheless, these proteins make a complex in the red cell with RhAG. The quaternary structure of this complex has been thought of as a tetramer; however, considerations of assembly would suggest that the
A single Rh complex unit has been estimated to have a conductance of about $1 \times 10^4$ molecules NH$_3$/s consistent with action as a channel (58, 70). It has been suggested that Rh, proteins, prominent in mammals that do not contain more Amt-like subfamily members, may function physiologically as the much sought-after channels responsible for CO$_2$ transport in the red cell (64). Recently, Boron and colleagues (48) showed that the red cell RhAG, and indeed AmtB, can conduct both NH$_3$ and CO$_2$. They also found that the red cell RhAG, and indeed AmtB, can conduct NH$_3$. AtTIP2;1 from Arabidopsis roots, an AQP homolog in plants, is unregulated in response to nitrogen starvation (38), suggesting that TIP2 functions in remobilization of ammonium from vacuoles by transporting ammonium across the membrane (23). Using functional complementation of a yeast mutant deficient in all three ammonium transporters (Dmepl-3) by wheat (Triticum aestivum) TIP2 AQPs and also using expression in Xenopus oocytes, TaTIP2;1 and mammalian AQP3, AQP8, and AQP9 have been shown to conduct NH$_3$ (20, 23, 37). Such AQPs are called aquaammoniaporins. AQP3 is found in erythrocytes, and AQP8 and AQP9 are primarily found in nitrogen-handling organs such as the liver and the kidney.

Six high-resolution crystal structures of AQP homologs cover the various AQP subclasses (34), the aquaglyceroporin GlpF (14), AQP1 (66), AQPZ (61), AQP0 (18), and the probable H2S channel AQPM (35). In GlpF, the channel is slightly wider, and key hydrophobic residues allow the passage of the carbon backbone of glycerol. In pure water channels, the selectivity filter is surrounded by R197/F58/H182/C191. In the aquaammoniaporin TIPs, isoleucine or valine replace H182, and glycine or alanine substitute at C191. F58 of bovine AQP1 is substituted by histidine, tryptophan, leucine, and alanine in aquaammoniaporins. These substitutions make the selectivity filter in aquaammoniaporins wider and more hydrophobic. Both the dipole moment (1.49 for NH$_3$ vs. 1.85 for H$_2$O) and the dielectric constant of the liquid (22 for NH$_3$ vs. 80 for H$_2$O) are considerably lower than that of H$_2$O. A more hydrophobic selectivity region favors NH$_3$ transport in the AQP5s. Likewise, mutation of the selectivity filter residues in TIP2;1 to the corresponding residues from AQP1 resulted in the loss of NH$_3$ conduction in TIP2;1 (23).

Since all K$^+$ channels also conduct the ionic form of NH$_3$ with similar ionic radius (0.148 vs. 0.149) and hydration shells, it is crucial that the mechanism of Amt/MEPs and Rh absolutely exclude K$^+$ ion leakage. Although the K$^+$ channels must conduct charged cations and mimic the hydration shell with oxygen (28, 47), ammonia channels utilize the titratable character of ammonia to stabilize and then allow passage of the unhydrated NH$_3$, and thereby ensure that there is no leakage of any hydrated ions.

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References


