Minireview

Rhesus factors and ammonium: a function in efflux?

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Abstract

Completion of fungal, plant and human genomes paved the way to the identification of erythrocytic rhesus proteins and their kidney homologs as ammonium transporters.

Ammonium is the preferred nitrogen source of bacteria and fungi, and plants acquire nitrogen from the soil in the form of ammonium [1]. In animals and humans, assimilated forms of nitrogen - amino acids - are much preferred for nutrition, and, in the case of ammonotelic animals, ammonium is used for the excretion of nitrogen instead. In the human kidney, ammonium is produced, reabsorbed and excreted as a means to maintain pH balance and to get rid of surplus inorganic nitrogen. Whether ammonium transport also has a role in the pH regulation of other organs is not known and the molecular mechanisms were not, up to now, understood.

Properties and role of ammonium

In aqueous solution, ammonium (NH_4^+) is in equilibrium with ammonia (NH_3) with a pK_a of 9.25. Thus, at typical cytosolic pH, approximately 99% is present in the cationic form. Ammonia can pass through lipid bilayers along its concentration gradient, whereas ammonium is orders of magnitude less permeant. A transmembrane electrical and pH gradient will therefore affect NH_4^+/NH_3 equilibrium. Plants and some microorganisms acidify their external environment and utilize membrane-bound ammonium transport proteins, so internal ammonia concentration may be more than 100 times enriched compared to the external space. Besides pH, membrane potential and transporters, enzymatic reactions that generate or remove ammonium are also important in maintaining its concentration. For example, in microorganisms and plants, ammonium is rapidly and efficiently assimilated by enzymes such as glutamine synthetase and glutamate dehydrogenase. As high levels of ammonium can be cytotoxic, cytoplasmic ammonium has to be kept low. To maintain desired cytoplasmic concentrations and to promote nitrogen assimilation and growth, microorganisms and plants have evolved highly specific uptake and, because of passive leakage of ammonia out of the cells, re-uptake mechanisms. The mechanism of transport for import and export in different species - whether the transporters use ammonium or ammonia as a substrate and whether they couple the transport to protons or other ions - remains to be fully elucidated.

Transporters for ammonium

Ammonium transport has been studied for many decades in microorganisms, but the molecular structure of the transporters and the transport mechanism remained obscure. Genetic studies in *Escherichia coli* identified mutants defective in ammonium transport, but it was not possible to identify a *bona fide* gene encoding a polytopic membrane protein [2]. In the late 1970s, using a thorough screen for yeast mutants resistant to methylammonium (a toxic analog of ammonium carried by most ammonium transporters), a strain defective in ammonium uptake was identified [3]. Through complementation, ammonium transporter genes were identified, and the respective proteins from both yeast (Mep) and plants (AMT) were characterized [4,5]. The genes from the two organisms are closely related and encode plasma membrane proteins that have an external amino terminus, an internal carboxyl terminus and 11 transmembrane spans [6]. Yeast was then used as a tool to study the biochemical properties of both transporters.

Transport was saturable with high affinities and was selective for ammonium and methylammonium [7]. It was also energy-dependent and was able to concentrate ammonium several hundred-fold inside the cells.

Selectivity of ammonium transporters

How can transport systems be so specific for ammonium and methylammonium? Although the physicochemical properties of NH_4^+ in aqueous solution, such as mobility and ionic radii, are different from most other basic cations (for example, Na^+), they are highly similar to K^+. The similarity is reflected in the low selectivity shown by potassium transport systems for K^+ versus NH_4^+ [8]. K^+ does not seem to be recognized by Mep or AMT transporters, however [4,5], suggesting that chemical interactions between the transporter and substrate are important for recognition and transport of the substrate. The fact that transport is energy-dependent and can concentrate ammonium in the cytosol suggests that the transport mechanism involves ammonium taken up along the electrical and pH gradient established by proton ATPases. Although available experimental data for ammonium transporters can be explained by a uniport mechanism for NH_4^+ (Figure 1a), it has not yet been possible to distinguish a uniport mechanism from NH_3/H^+ co-transport experimentally (Figure 1b) because the net flux is electrochemically identical. In molecular terms, however, NH_3 and H^+ may bind to different specific sites on the transporter, and the affinity of each site for the respective substrate may vary depending on whether the other site is occupied.

Many homologs of the Mep and AMT ammonium transporters have been identified in yeast and plants. *Saccharomyces cerevisiae* has three paralogous Mep proteins with different affinities and capacities [9], and the higher plant *Arabidopsis thaliana* has six AMT paralogs [1,7]. Genetic evidence indicates there are regulatory interactions between the Mep proteins, which may point to an interaction as an oligomeric complex [10]. Furthermore, it is interesting to note that Mep2 is glycosylated and has been implicated in ammonium sensing during pseudohyphal growth [11].

The identification of yeast and plant transporters also led to the characterization of bacterial ammonium transporters because the genomes of archaebacteria and eubacteria contained homologous sequences. A bacterial homolog, like yeast Mep and plant AMTs, behaves strictly like an ammonium transporter [12,13]. The presence of homologs in all these species made it probable that related proteins might also be present in animals and humans. Indeed, they are, and the fascinating surprise was that the mammalian homologs, although distantly related, had previously been described but no transport function had been assigned: they were identified as the rhesus (Rh) blood group antigens [14]. Over stretches of approximately 200 amino acids, identity between Mep and AMT and Rh proteins can be as high as 25%.

The rhesus blood group antigens

Interestingly, Rh antigens had long been recognized for their role in transfusion-incompatible immune reactions and hemolytic disease of newborn babies. Although they have been biochemically characterized, a clear physiological role could not be assigned [15]. The Rh complex is expressed in erythrocytes and, perhaps similar to Mep proteins, is a heterotetramer consisting of two RhAG glycoproteins (also known as Rh50 for their electrophoretic mobility in SDS gels), which are glycosylated in topologically similar positions to Mep2, and two highly homologous non-glycosylated RhCE or RhD subunits (also referred to as Rh30). Each monomer contains 12 putative transmembrane domains, and both amino and carboxyl termini are cytosolic. Bacterial ammonium transporters have a similar structure [16]. The CE/D subunits of the complex present the epitopes of the Rh antigens D or E/e and C/c on their external loops. The RhAG subunits are essential for expression of the whole complex, whereas the combination with RhCE and RhD varies. Two different genetic defects can lead to the rare Rh_{null} disease, which results in changed erythrocyte morphology and physiology: either RhAG is mutated and thus does not translocate the Rh complex to the cell surface, or RhD is deleted and RhCE is simultaneously mutated [15].

The homology of Rh proteins with Mep and AMT proteins prompted Marini *et al.* [17] to investigate whether human RhAG expressed alone can mediate ammonium transport in ammonium-uptake deficient yeast. RhAG conferred growth on ammonium as sole nitrogen source. Surprisingly, and in contrast to the Mep proteins in yeast, RhAG also conferred resistance to very high concentrations of toxic methylammonium, indicating it has a role in export. RhAG promoted efflux of ammonium when cultured on an amino acid carbon source, the catabolism of which leads to ammonium production. These experiments suggest the efflux is mediated either by uniport of ammonia or by an antiport mechanism (Figure 1c,d). As the plasma membrane of erythrocytes seems to be permeable to NH_3 but not to NH_4^+ [18] and the pH of the cytosol is higher, facilitated diffusion of the uncharged molecule down its concentration gradient may be the actual mechanism. Such a mechanism would explain the coincidental uptake and efflux capabilities of RhAG.

Recently, two homologous glycoproteins were identified from mouse and human: RhBG, which is predominantly expressed in kidney, liver and skin [19] and RhCG (also known as RhGK), which is predominantly expressed in kidney and testis [17,20].

A role for RhBG and RhCG in kidney physiology

Ammonium excretion by the kidney is tightly coupled to the maintenance of systemic acid-base balance [21]. Ammonium is not primarily a constituent of the ultrafiltrate; it is generated by metabolism of glutamine in proximal epithelial cells

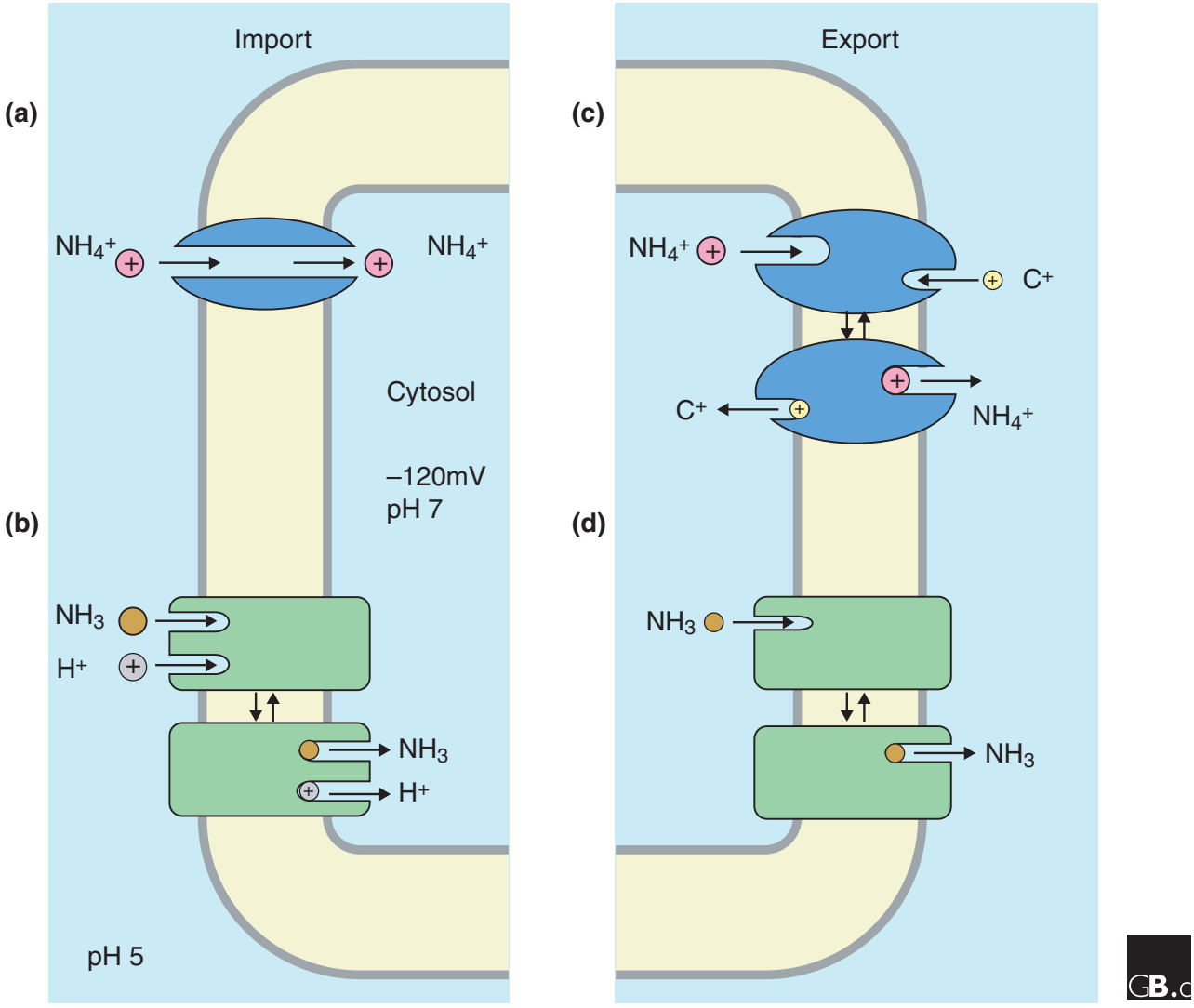

Figure 1
Models for NH_3 and NH_4^+ transport. **(a)** Channel- or carrier-mediated uniport for NH_4^+. Both concentration gradient and transmembrane voltage influence the direction of transport. A firmly established example of such NH_4^+ transport is through potassium channels. **(b)** NH_3/H^+ co-transporter. Driven by the pH gradient and transmembrane voltage, ammonia may be taken up against a concentration gradient for NH_3. The electrochemical situation is similar as for NH_4^+ uniport in (a). **(c)** Carrier-mediated antiport for NH_4^+ with unknown secondary substrate(s), for example, cation C^+. If both substrates are univalent only the concentration gradients determine the direction of transport. In the kidney, C^+ could be sodium. **(d)** Channel- or carrier-mediated uniport for NH_3. Independent of the membrane potential, ammonia will equilibrate along its concentration gradient (depending on the external and internal pH). A net inflow of ammonium into the cytoplasm will depend on ammonium metabolism, which acts as a sink for NH_3/NH_4^+.

and is released into the tubular fluid. Interestingly, RhBG localizes to proximal tubules [19], and ammonium is concentrated several-fold at the end of the proximal tubule. A significant proportion of ammonium is reabsorbed in the thick ascending limb of the loop of Henle, the apical membrane of which is highly permeable to NH_4^+ but not to NH_3 [22]. Lumen-positive voltage drives paracellular absorption of NH_4^+. The final nephron segment, the collecting duct, seems impermeable to NH_4^+, but permeable to NH_3. Protons are secreted into the lumen and ammonia released from cortical cells will be protonated to form ammonium, trapped in the lumen and excreted. RhCG, which is also able to efflux ammonium [17], localizes to the collecting duct [20].

Functional and evolutionary relationships

Taken together, the results suggest that different transporters from the same superfamily do transport the same substances, but use different transport mechanisms. This may not be too surprising, since different functional relationships are found

in diverse classes of membrane transporters, such as glucose transporters of the major facilitator superfamily (for example, the yeast protein ScHXT1 compared with the *Arabidopsis* protein AtSTP1) and amino acid transporters of the amino acid/polyamine/choline (APC) family (for example, the human protein HsCAT1 compared with the *Arabidopsis* protein AtCAT1). In both cases, the plant protein probably mediates proton co-transport, whereas the other mediates uniport.

One might speculate that Rh-like proteins are NH_3 uniporters or NH_4^+/cation antiporters (Figure 1c,d). In contrast, Mep and AMT ammonium transporters may either function as NH_3/H^+ co-transporters or as NH_4^+ uniporters (Figure 1a,b). A chemical interaction of ammonium or ammonia with the proteins might explain why ammonium transporters are so selective for ammonium (compared with K^+, for example). It might be possible that members of different clades of the superfamily recognize either uncharged or charged forms of the substrate. Does this fit the evolutionary pattern? Phylogenetic analyses suggest that Rh proteins and Mep/AMT have an ancestral relationship (Figure 2). Interestingly, the nematode worm and fruitfly contain both Mep/AMT and Rh homologs in their genomes. One higher plant sequence from *Arabidopsis*, that of AtAMT2, groups closely to bacterial sequences, which are also closely related to yeast sequences [23]. The archaebacterial sequences are closely related to some plant and animal sequences. Within a single species, co-assembly of different subunits into a heteromeric complex may lead to fine tuning of ammonium transport by Mep, AMT and Rh proteins.

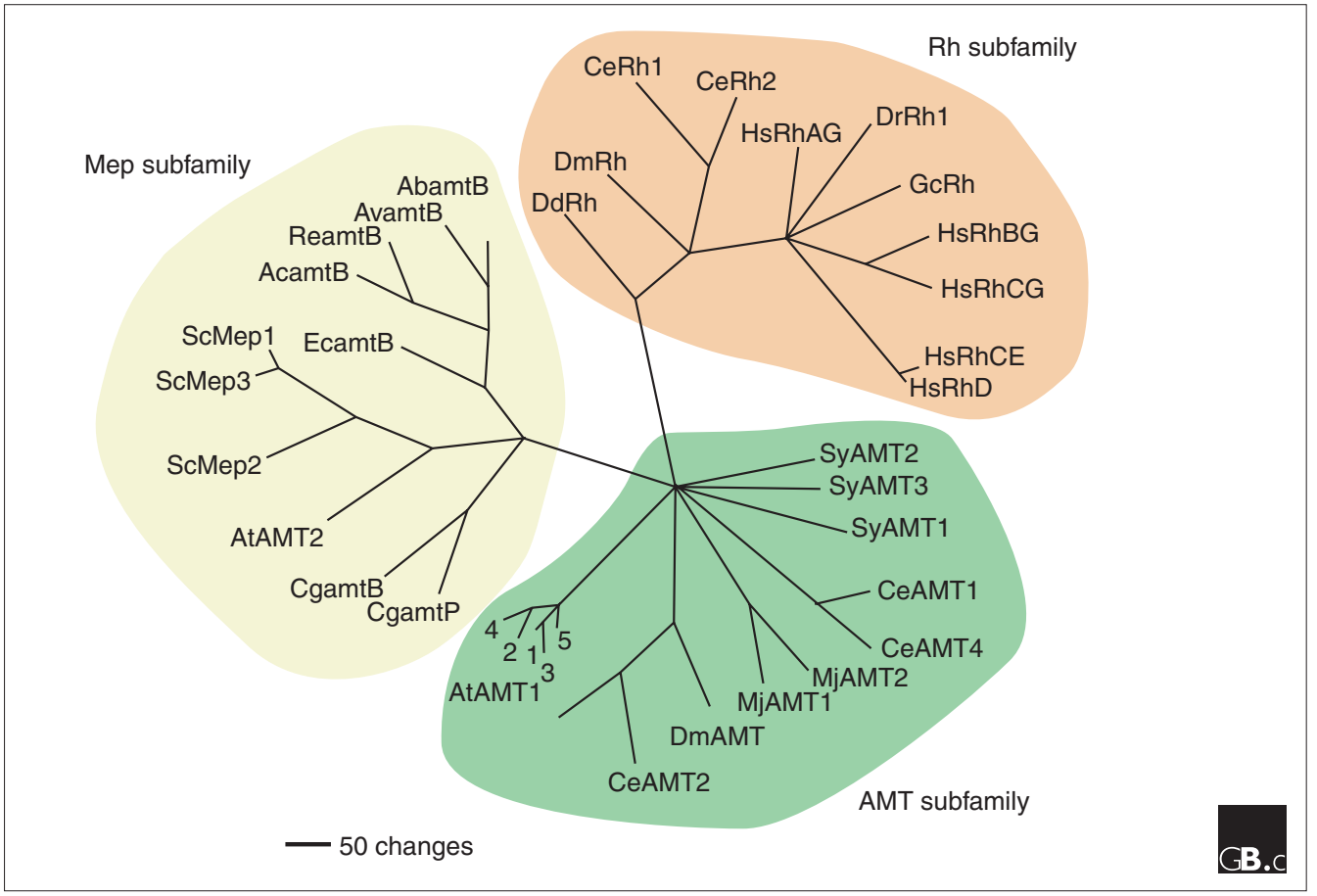

Figure 2
A phylogenetic tree of Mep, AMT and Rh-like proteins. Rh and AMT/Mep proteins are found in bacteria, archaebacteria and eukaryotes. The amino acid sequence alignment was created using the program ClustalW, and phylogenetic analysis was done using the program PAUP 4.0b4a. Highly variable amino- and carboxy-terminal sequences were removed before analysis. Only one archaebacterial sequence (*Mj, Methanococchus janaschii*) and those prokaryotic sequences that have been analyzed for ammonia transport have been included: *Ec, Escherichia coli; Sy, Synechocystis sp.; Ac, Azorhizobium caulinodans; Av, Azotobacter vinelandii; Ab, Azospirillum brasilense; Re, Rhizobium etli; Cg, Corynebacterium glutamicum.* With the exception of human (*Hs, Homo sapiens*), slime mold (*Dd, Dictyostelium discoideum*), marine sponge (*Gc, Geodia cydonium*), and zebrafish *(Danio rerio*), only eukaryotic sequences from fully sequenced organisms (*Dm, Drosophila melanogaster* (fruit fly); *Ce, Caenorhabditis elegans* (nematode); *At, Arabidopsis thaliana* (plant); *Sc, Saccharomyces cerevisiae* (yeast)) have been included.

The absence of Rh-like proteins in higher plants suggests that either some of the AMT proteins also use a different coupling mechanism or that other, sequence-unrelated proteins are responsible for ammonium/ammonia export. In contrast to urea-excreting mammals, fish excrete mainly ammonium, through the gills. We would therefore predict that Rh-like proteins (such as AAF63256, also known as DrRh1) may be highly expressed in the gills and may be responsible for ammonium/ammonia excretion in fish. Knock-out mutants in plants, mice and fish will be excellent tools for dissecting the individual roles of AMT, Mep and Rh-like proteins in the different genomes and their relative contribution to net ammonium flux compared with other transporters that can mediate ammonium transport (such as potassium transporters, Na^+/H^+ antiporters or $Na^+/K^+/Cl^-$ cotransporters). Genome-wide analysis will also help to unravel the evolution of the mechanism of ammonium/ammonia transport.

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