



Original article

The *Escherichia coli* AmtB protein as a model system for understanding ammonium transport by Amt and Rh proteins

La protéine AmtB de *Escherichia coli*, un système modèle pour la compréhension du transport d'ammonium par AmtB et les protéines Rh

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Abstract

The *Escherichia coli* ammonium transport protein (AmtB) has become the model system of choice for analysis of the process of ammonium uptake by the ubiquitous Amt family of inner membrane proteins. Over the past 6 years we have developed a range of genetic and biochemical tools in this system. These have allowed structure/function analysis to develop rapidly, offering insight initially into the membrane topology of the protein and most recently leading to the solution of high-resolution 3D structures. Genetic analysis has revealed a novel regulatory mechanism that is apparently conserved in prokaryotic Amt proteins and genetic approaches are also now being used to dissect structure/function relationships in Amt proteins. The now well-recognised homology between the Amt proteins, found in archaea, eubacteria, fungi and plants, and the Rhesus proteins, found characteristically in animals, also means that studies on *E. coli* AmtB can potentially shed light on structure/function relationships in the clinically important Rh proteins.

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1. Introduction

The ammonium transport (Amt) protein family was recognised in 1994 following the cloning of genes encoding Amt proteins from both *Saccharomyces cerevisiae* [1] and *Arabi-*

dopsis thaliana [2]. These reports confirmed for the first time that the ability of cells to take up ammonium³ may not be a consequence of simple diffusion of NH₃ across the cell membrane but may, at least under some circumstances, be mediated by an integral membrane protein. This concept had been contentious for some time but, although the presence of such Amt proteins in bacteria had been proposed previously, convincing genetic evidence had been lacking [3]. Comparisons with the

³ The term ammonium is used to refer to both the protonated (NH₄⁺) and the unprotonated (NH₃) forms. Chemical symbols are used when the protonation state is important.

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predicted amino acid sequences of the *S. cerevisiae* and *A. thaliana* Amt proteins suggested that homologues may be present in a number of prokaryotes including *Bacillus subtilis*, *Rhodobacter capsulatus* and *Corynebacterium glutamicum* [1, 2] and in due course the *C. glutamicum amt* gene (now designated *amtA*) was cloned and shown to encode another Amt family member [4]. Since these early days, and with the explosion of genome data, it has become apparent that Amt proteins are present in organisms from all domains of life, including archaeobacteria, eubacteria, fungi, plants and nematodes, and that indeed there are very few examples to date of organisms that do not encode at least one member of the family [5]. The subsequent recognition in 1997 that the Amt proteins were related to the Rhesus (Rh) blood group polypeptides extended the distribution of Amt-related proteins throughout the animal kingdom [6]. Furthermore it raised the possibility that the Rh proteins, whose precise function at that time was unknown, might also act as ammonium transporters, an issue that remains controversial [7–9].

2. *Escherichia coli* AmtB—a model system

When Amt proteins were first described in 1994 very little was known about their likely structure or mode of action. In order to develop an understanding of their biology it was very desirable to identify a tractable model system that would offer the combination of a sophisticated genetic system and the potential to develop a variety of biochemical approaches for structure/function studies. These criteria were most likely to be satisfied by a bacterial system and in this regard *E. coli* was the organism of choice. We now know that many (possibly even most) organisms encode multiple Amt homologues, e.g. *S. cerevisiae* encodes three proteins Mep1, Mep2, Mep3 (Mep = methylammonium permease) [10], *A. thaliana* encodes five Amt proteins [11] and many prokaryotes have multiple *amt* genes [12]. Some of the attractions of *E. coli* were that it has just a single *amt* gene (*amtB*) and that regulation of nitrogen metabolism in this organism is well characterised. Likewise, in vivo assays for ammonium uptake, based on the use of a radioactively labelled analogue— ^{14}C methylammonium—were well established in *E. coli* [13] and methods for topology determination of polytopic membrane proteins were also well defined. In the last six years we have used these and other methods to develop *E. coli* AmtB to the point where it is now the system of choice to address many of the important structure and function questions that relate to Amt biology. Furthermore with the increasing interest in potential similarities between Amt proteins and their animal homologues, the Rh proteins, it is apparent that results coming from analysis of AmtB may also shed some light on Rh biology.

3. The topology of Amt proteins

When the first two *amt* genes were cloned, bioinformatic approaches were used to compute the likely topology of these novel integral membrane proteins. These analyses suggested that Amt proteins might have anything from nine to twelve

transmembrane helices (TMH) and consequently clarification of the topology of Amt proteins by empirical methods was one of the first tasks undertaken with *E. coli* AmtB. By combining in silico analysis, using a number of different algorithms, with topology mapping using protein fusions, we deduced that *E. coli* AmtB comprised twelve potential TMH with both the N- and C-termini of the polypeptide in the cytoplasm [14]. However, using this model to predict the topologies of other Amt and Rh proteins it was apparent that Amt proteins from eukaryotes, such as *S. cerevisiae* and *A. thaliana*, were most likely to have 11 TMH with the N-terminus being extracytoplasmic. For *S. cerevisiae* Mep2 this was supported by the localisation of an extracytosolic glycosylation site at residue Asn4 [15]. We therefore designated the core TMHs as TMH 1-11 and the N-terminal TMH of *E. coli* AmtB as TMH0 [14]. By comparison, earlier work had already indicated that Rh proteins were likely to have a 12 TMH topology [17] (discussed in Section 7).

Subsequent analyses showed that not all prokaryotic Amt proteins were predicted to have the atypical TMH0 and indeed it appears to be a characteristic of Gram-negative bacteria because sequence alignments suggest that Gram-positive bacteria, such as *B. subtilis*, encode an eleven TMH Amt protein. The apparent disparity of *E. coli* AmtB was subsequently rationalised when we recognised that the first 22 residues of AmtB (previously designated TMH0) encode a cleavable signal peptide so that the mature polypeptide has the typical 11 TMH topology with an extracytoplasmic N-terminus [5,16]. This maturation was subsequently confirmed by the solution of the X-ray crystal structure of *E. coli* AmtB [18,19]. The presence of signal peptides in integral membrane proteins has presently been reported in very few cases other than AmtB, namely the phage M13 procoat protein and the *E. coli* FliP and DsbD proteins [20–22]. The precise role of the signal peptide in AmtB remains unclear.

4. The 3D structure of Amt proteins

One of the original requirements of *E. coli* AmtB as a model system was to be able to apply biochemical as well as genetic analytical tools and in this context purification of the protein was a primary goal. Overexpression of AmtB impairs cell growth but this can be overcome by use of an *E. coli* B mutant strain that tolerates overproduction of at least some inner membrane proteins [23]. Our topological analysis also suggested that AmtB might be tagged at the C-terminus without significant impairment of function and this is indeed the case. Purification of AmtB revealed that the protein is a stable trimer that retains its quaternary structure on SDS-PAGE [24] and this trimeric state is very clearly seen using cryoelectron microscopy (cryoEM) and atomic force microscopy (AFM) of two-dimensional crystals of AmtB reconstituted in the presence of lipids [25]. Furthermore, probing of *E. coli* whole cell extracts and/or membrane fractions reveals the same trimeric state indicating that it is likely that this structure represents the in vivo state of the protein [24,25]. Analysis of Fourier phases from cryoEM images of 2D crystals combined with analysis of

AFM surface topographs of the crystals also revealed a clear pseudo-two-fold symmetry suggesting a possible internal homology between the N- and C-terminal halves of the protein, although no clear homology was apparent at the level of the primary amino acid sequence [25].

Our understanding of the structure of Amt proteins advanced dramatically in 2004 with the publication, by two groups, of very high resolution X-ray crystal structures for *E. coli* AmtB [18,19], complemented more recently by a structure for the homologous Amt-1 protein of *Archaeoglobus fulgidus* [26]. These structures confirm the predicted 11 TMH topology of Amt proteins and the pseudo-two-fold symmetry seen in the 2D crystals. Indeed the proteins are characterised by two contiguous five-helix bundles, TMH1–5 and TMH6–10, and a long C-terminal helix TM11, with the two five helix bundles being related by an approximately two fold axis in the plane of the membrane. The structure of the cytoplasmic C-terminal tail is unresolved in the AmtB structures but is resolved in the highly homologous Amt-1 structure where it forms two short helices that make up a significant part of the cytoplasmic face [26]. Given the high level of homology observed within the Amt family it seems highly likely that at least the tertiary structure, and predictably the quaternary structure, of all Amt proteins will be comparable to that of *E. coli* AmtB and *A. fulgidus* Amt-1.

5. The mode of action of Amt proteins

The nature of the substrate for Amt proteins and their mode of action has been a contentious issue. The most widely accepted view has been that they are secondary transporters mediating the uptake of the ammonium cation NH_4^+ [3,10,11] but it has alternatively been proposed that Amt proteins are channels that increase the rate of equilibration of NH_3 across the cell membrane [27,28]. The 3D structures of AmtB and Amt-1 revealed that each subunit of the trimer contains a narrow, mainly hydrophobic, pore that was predicted to be the substrate conduction channel [18,19,26]. The size and properties of this channel are consistent with the conduction through the channel of uncharged NH_3 rather than charged NH_4^+ . Furthermore, based on observation of the structure and on the location of novel density in crystals derived in the presence of methylammonium (MA), a potential binding site for ammonium within a vestibule on the periplasmic face of the protein was proposed. It is suggested that prior to transit through the pore NH_4^+ is deprotonated and subsequently re-protonated on the cytoplasmic side of the membrane by an undefined mechanism [18,19]. Within both the conduction channel and the periplasmic vestibule a number of highly conserved residues have been identified that potentially play critical roles in NH_4^+ binding and NH_3 translocation [18,19,29]. These concepts are now being tested by analysis of appropriate mutants of AmtB (Javelle and Merrick, unpublished, and paper by Li et al., in this volume).

It has been suggested that no major conformational change in AmtB occurs upon NH_4^+ binding [18] but the present models do require at least transient movement of particular residues

to facilitate substrate conduction. Indeed two slightly different conformations of the cytoplasmic end of the substrate channel have been observed [19] and studies with xenon, to locate potential cavities within the protein, have led to the suggestion that Amt proteins may be much more flexible than typical passive channels, such as those that mediate potassium or chloride movement [26].

In vivo studies of ammonium uptake have traditionally been based on a washed cell assay of [^{14}C]MA accumulation, but we have recently unambiguously demonstrated that, at least in *E. coli*, this assay actually measures accumulation of intracellular [^{14}C]glutamine and that the kinetics derived from such assays are those of the ammonium assimilation enzyme glutamine synthetase (GS) [30]. However, a previously described variation of this assay that dispenses with the washing step (in which accumulated [^{14}C]MA is lost from the cells) allows specific assessment of the activity of AmtB [13]. In vivo data derived in this way are consistent with the channel model for *E. coli* AmtB activity [30]. This assay methodology incidentally allowed the first in vivo determination of the kinetics of *E. coli* GS and surprisingly these differ markedly from in vitro derived kinetics. They reveal a significantly higher substrate affinity in vivo (K_m for MA = 380 μM) than when calculated in vitro with the purified enzyme (K_m for MA = 79 mM), suggesting that the activities of AmtB and GS may be coupled, in an as yet unidentified manner [30]. It would be of considerable interest to determine whether this is a common feature of Amt proteins.

6. Regulation of Amt proteins

In most systems that have been studied to date the expression of Amt structural genes is induced in response to nitrogen limitation [5]. However in bacteria we have shown that Amt activity is also regulated post-translationally. The ubiquitous nature of Amt proteins in prokaryotes is paralleled by the almost invariant linkage of the Amt structural gene to a second gene called *glnK* [12]. Such conservation frequently reflects related functions of the encoded proteins, in this case suggesting that the function of GlnK, which encodes a member of the P_{II} family of signal transduction proteins, is linked to that of AmtB. In a series of studies we have shown that this is indeed the case. Like other P_{II} proteins in proteobacteria, GlnK is subject to covalent modification such that in nitrogen-limited growth conditions the protein is uridylylated on each of its three identical 12 kDa subunits and in nitrogen-sufficient conditions, e.g. when ammonium is added to the growth medium, these modifying groups are rapidly removed. In *E. coli*, deuridylylation of GlnK, as a consequence of ammonium shock, leads to its membrane sequestration by AmtB [31]. This process occurs rapidly (within seconds), in response to relatively low concentrations of ammonium (50 μM), and is completely reversible [32]. Binding of GlnK to AmtB apparently inhibits ammonium uptake suggesting that the role of GlnK may be to regulate AmtB activity in response to the intracellular nitrogen status. This status is perceived via a combination of the glutamine pool that controls activity of the uridylyltrans-

ferase/uridylyl-removing enzyme GlnD, and the 2-oxoglutarate pool which mediates its effect by direct binding of 2-oxoglutarate to GlnK. The presence of the C-terminal cytoplasmic tail of AmtB is necessary for complex formation [31] (Severi and Merrick, unpublished) and indeed in a hypothetical model of the complex, based on the structure of *A. fulgidus* Amt-1 and a model of *A. fulgidus* GlnB-1, it has been suggested that the Amt C-terminus is necessarily involved in interaction with GlnB-1 [26]. Analysis of the purified *E. coli* GlnK-AmtB complex indicates that the stoichiometry of the complex is 1:1 and that all molecules of GlnK within the complex are fully deuridylylated (Durand and Merrick, unpublished). The stability of the complex is also very sensitive to salt, suggesting that electrostatic interactions could play an important part in stabilising the complex.

Since our initial demonstration of GlnK–AmtB complex formation in *E. coli* and in *Azotobacter vinelandii* [31], similar processes have been reported in *C. glutamicum* [33], *B. subtilis* [34] and *Azospirillum brasilense* [35]. Hence this mechanism appears to be widespread in prokaryotes and may reflect the fact that AmtB and GlnK constitute an ancient prototypical nitrogen regulation system in prokaryotes [36]. Although P_{II} proteins are also found in plants (where they are apparently exclusively located in the plastid), they are not present in fungi or other eukaryotes and there is presently no indication of a complex analogous to that of GlnK–AmtB outside the prokaryotes. Even so, the *E. coli* system has introduced the concept that the activity of Amt proteins might be regulated by interaction with cytoplasmic factors and it is certainly conceivable that analogous regulatory systems might exist in higher organisms including animals.

7. Comparison of Amt and Rh proteins

The Rh proteins in animals are now recognised to be related to Amt proteins and consequently to be potential ammonium

transporters [7,9]. Consequently this raises questions as to whether we might predict certain aspects of Rh structure/function by using *E. coli* AmtB as a model. Phylogenetic analysis of Rh proteins identifies four major groups in vertebrates, from fish to mammals, designated Rh30, RhAG, RhBG and RhCG, and two primitive clusters (Rhp1 and Rhp2) in eukaryotic microbes, invertebrates and non-mammalian vertebrates [37]. Of all these, by far the best studied are the human proteins, the non-glycosylated Rh30s (RhCE and RhD) and the glycosylated Rh50s, (RhAG, BG and CG), all of which are predicted to have 12 TMH [17,38]. The erythrocyte membrane is considered to contain an Rh complex that comprises RhAG and two very similar Rh30 proteins, RhCE and RhD. Furthermore, it has been suggested that this complex could be an RhAG₂ Rh30₂ tetramer [17] and this model has been widely quoted [39]. This contrasts with the homotrimeric structure of *E. coli* AmtB, although when expressed in *S. cerevisiae*, RhAG can function independently of Rh30 proteins to mediate ammonium uptake [7]. Likewise RhBG and RhCG, which are found in kidney, liver or skin, function independently of Rh30 proteins [40,41].

Using the structure of *E. coli* AmtB as a template, together with secondary structure predictions for Rh polypeptides and the extensive available biochemical data on Rh proteins, we have constructed homology models for human RhAG and RhD [42]. The core of the Rh polypeptide (TMH1 to TMH11) has the potential to adopt a very similar fold to that of the Amt proteins, and our models suggest that RhAG, and the homologous RhBG and RhCG, have a very similar channel architecture to AmtB (Fig. 1). By comparison, Rh30 proteins have a notably different arrangement of residues indicating that they are unlikely to function as ammonia channels. The Rh50 proteins retain the two highly conserved His residues that characterise the conduction channel of Amt proteins and are predicted to facilitate NH₃ conduction (Fig. 1B). Likewise Rh50s have analogues of AmtB Phe107 and Phe215 that lie at the

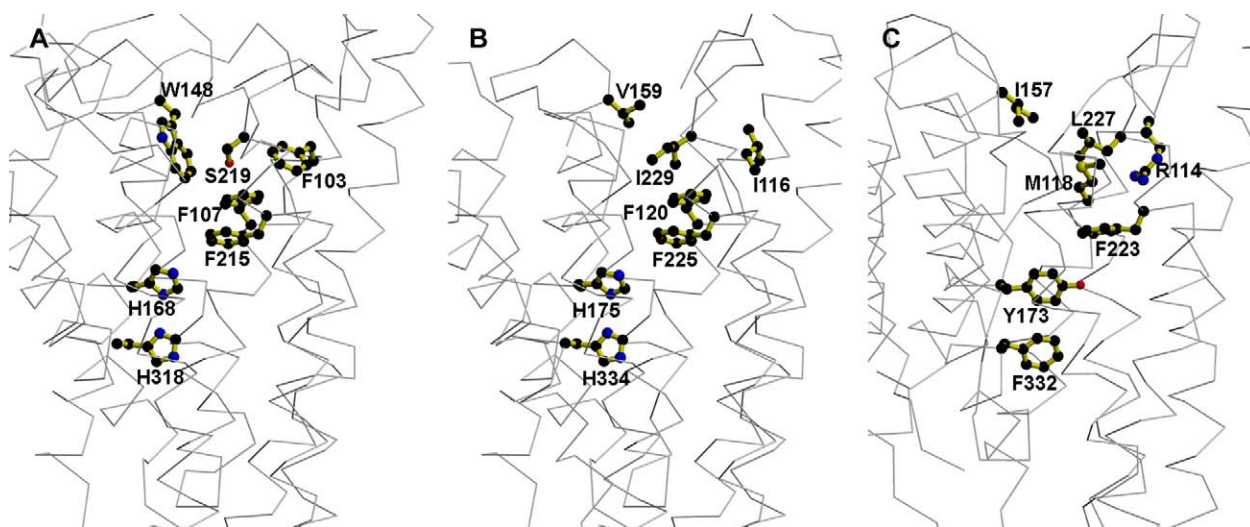


Fig. 1. Comparison of the predicted structures of the conduction channels in Rh proteins compared with that in *E. coli* AmtB. (A) Important vestibule and channel residues in the *E. coli* AmtB structure (PDB code 1u7g). Corresponding residues in human RhAG (B) and human RhD (C) as predicted by homology modelling using *E. coli* AmtB as the template [41].

entrance to the channel and are also highly conserved. However, the conserved vestibule residues of Amt proteins that are proposed to contribute to the NH_4^+ binding site, Phe103, Trp148 and Ser219, are all replaced by Ile, Leu or Val in Rh50s [42]. By comparison with RhAG the Rh30 proteins lack conservation of the pseudo-symmetrical histidines in the conductance channel, the equivalent residues in Rh30s being Tyr and Phe (Fig. 1C), and hence if Rh30 proteins were to conduct ammonia they must do so by a different mechanism. These modelling predictions are consistent with present studies on human Rh proteins that indicate that RhAG can mediate ammonium uptake [7,9] but RhD or RhCE cannot [9,43].

As mentioned earlier, the erythrocyte Rh complex has been suggested to be an RhAG₂Rh30₂ heterotetramer, based largely on size determination of the complex in detergent-solubilised membranes [44]. However, in the light of the homotrimeric structure of Amt proteins and our modelling of Rh polypeptides, these ideas merit reassessment. The major differences between Amt and Rh proteins concern the presence of an extra N-terminal helix (TMH0) in Rh proteins, which effectively replaces the SP found in some Amt proteins, and notable differences in the sequences of the C-terminal cytoplasmic tails. It is not possible by modelling to predict with accuracy what effect the presence of the extra TMH might have on the tertiary and quaternary structures of Rh proteins. However we predict from bioinformatic analysis that Rh proteins contain a long extra-cytoplasmic loop of 12–23 residues between TMH0 and TMH1 and consequently TMH0 is not constrained to lie close to TMH1. In this case then it would be possible for the Rh proteins to adopt a trimeric structure analogous to that of Amt proteins and for the additional TMH0 to be accommodated on the outer face of the trimer near the interface between adjacent subunits [42]. Alternatively if TMH0 were to remain adjacent to TMH1 then this would significantly alter the subunit interface and the quaternary structure might be altered.

Whilst it seems likely that the Rh family evolved from the Amt proteins and that the original event may have occurred in the bacteria, phylogenetic analysis does not yet suggest a clear evolutionary path [37]. Nevertheless it is interesting to consider that the change from the 11 TMH that characterise Amt proteins, to the 12 TMH that are characteristic of Rh proteins, could have arisen by the simple loss of a signal peptidase cleavage site, in a protein like *E. coli* AmtB, and the subsequent adoption of the signal peptide as a new TMH.

With regard to the potential composition of the erythrocyte-Rh complex, data on the relative amounts of RhAG and Rh30 in the complex vary, such that 1:2 or 2:1 ratios could as well be accommodated as the presently accepted 1:1 [42]. Furthermore, although RhAG, RhD and RhCE have been implicated as members of a single Rh antigen complex [39] there is no empirical evidence that the RhCE and RhD polypeptides are ever present in the same complex [42,45]. In summary, when the present biochemical data are taken together with considerations from modelling, then RhAGRh30₂ or RhAG₂Rh30 complexes are at least as likely as the RhAG₂ Rh30₂ tetramer model. Resolution of these alternative models by the isolation and char-

acterisation of one or more Rh proteins is undoubtedly a major challenge for the future.

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