The ammonia channel protein AmtB from *Escherichia coli* is a polytopic membrane protein with a cleavable signal peptide

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Abstract

The *Escherichia coli* ammonia channel protein, AmtB, is a homotrimeric polytopic inner membrane protein in which each subunit has 11 transmembrane helices. We have shown that the structural gene *amtB* encodes a preprotein with a signal peptide that is cleaved off to produce a topology with the N-terminus in the periplasm and the C-terminus in the cytoplasm. Deletion of the signal peptide coding region results in significantly lower levels of AmtB accumulation in the membrane but modification of the signal peptidase cleavage site, leading to aberrant cleavage, does not prevent trimer formation and does not inactivate the protein. The presence of a signal peptide is apparently not a conserved feature of all prokaryotic Amt proteins. Comparison of predicted AmtB sequences suggests that while Amt proteins in Gram-negative organisms utilize a signal peptide, the homologous proteins in Gram-positive organisms do not.

Introduction

In bacteria, including *Escherichia coli*, cleavable signal peptides (SPs) are normally a feature of proteins that are to be secreted across the cytoplasmic membrane and they have not generally been implicated in the process of insertion of bacterial polytopic inner membrane proteins (IMPs) (Broome-Smith *et al*., 1994). However, there are a few known exceptions to this, namely *E. coli* FliP (Pradel *et al*., 2004), DsbD (Gordon *et al*., 2000), and CyoA (Celebi *et al*., 2006). The recognition of potential roles for SPs in IMP assembly has been hampered by the tendency of some topology prediction methods to predict an SP as a transmembrane helix (TMH) (Lao *et al*., 2002; Arai *et al*., 2003). The relative difficulty in purifying polytopic membrane proteins also means that in very few cases have such proteins been subjected to N-terminal sequence analysis that could reveal the removal of an SP during maturation. Nevertheless, comprehensive analyses of transmembrane topologies in prokaryotic genomes have predicted that 13–17% of membrane proteins have SPs (Arai *et al*., 2003; Käll *et al*., 2004).

The Amt proteins constitute a family of ammonia channel proteins found in all domains of life. They can be divided into the Amt/Mep proteins, found in eubacteria, archaebacteria, fungi and plants, and the related Rhesus proteins in animals, including humans (Westhoff *et al*., 2002; von Wirén & Merrick, 2004). Computer predictions of the topology of these proteins suggest that they are 11 or 12 TMH proteins with the C-terminus being intracytoplasmic and the N-terminus either intra- or extracytoplasmic. For the Amt/Mep proteins, the most detailed topology analysis to date was carried out with *E. coli* AmtB using protein fusions. This study concluded that the *E. coli* AmtB had 12 TMHs (Thomas *et al*., 2000) although it was subsequently suggested that the protein may have a signal sequence (van Dommelen *et al*., 2001). Furthermore, *in silico* analyses of other Amt proteins in a variety of organisms suggested that in many cases e.g. Amt proteins in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, the topology was likely to be 11 TMHs with an extracytoplasmic N-terminus (Thomas *et al*., 2000). For *S. cerevisiae* Mep2 this topology was also supported by mapping the extracytoplasmic location of an N-terminal glycosylation site (Marini & Andre, 2000).

Escherichia coli AmtB, which is the paradigm for the Amt protein family, is a stable trimer both in the native cell membrane and after purification (Blakey *et al*., 2002; Conroy *et al*., 2004). The X-ray crystal structure of *E. coli* AmtB shows a trimeric structure in which each monomer has 11 TMHs (Khademi *et al*., 2004; Zheng *et al*., 2004) consistent with the removal of a SP during AmtB assembly. We have used genetic and biochemical analyses to substantiate the

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presence of a SP in *E. coli* AmtB and to investigate its role. Furthermore, we predict from comparative sequence studies of bacterial Amt proteins that the SP is a feature of Amt proteins found in Gram-negative bacteria, but not of those from Gram-positive organisms.

Materials and methods

Bacterial strains and plasmids

The host strain used throughout this work was *Escherichia coli* strain GT1000 (*rbs lacZ*::IS*1 gyrA hutC*$_K$ Δ*glnKamtB*) (Coutts *et al.*, 2002). The plasmids used are listed in Table 1. Plasmid pMM291 was constructed by ligating an *Eae*I–*Bam*HI fragment carrying most of *amtB* from pGT104 together with a short cassette (made by annealing two oligonucleotides 5′-GGGAATTCCATATGGCACCTGCGGT -3′ and 5′-GGCCACCGCAGGTGCCATATGGAATTCCC-3′, followed by restriction with *Nde*I and cloning into *Nde*I–*Bam*HI cut pT7-7. The *Nde*I–*Bam*HI fragment from pMM291 was subsequently recloned into pJT1 to give pJT4. Plasmids pJT8–pJT21 were all constructed in the same manner. A region extending from the *Bst*EII site in *glnK* to the *Pst*I site introduced around the Pro24 codon of *amtB* in pJT6 (Javelle *et al.*, 2004) was PCR amplified using a wild-type *glnK* primer and a mutagenic *amtB* primer. The PCR fragment was digested with *Bst*EII and *Pst*I and cloned into a derivative of pJT6 called pJT7 where the natural *Bst*EII–*Pst*I region had been replaced with a 960 bp 'stuffer' *Bst*EII–*Pst*I fragment from pACYC177. In this way cloning of the mutant fragment was ensured. All mutations were confirmed by sequencing.

Table 1. Plasmids

Plasmid number	Genotype	AmtB phenotype	Reference or source
pGT104	*glnKamtB7*	Wild type	Javelle *et al*. (2004)
pJT1	*glnK*Δ*amtB*	ΔAmtB	Javelle *et al*. (2004)
pJT4	*glnKamtB17*	Δ (M1-A22)	This work
pJT6	*glnKamtB9*	Wild type	Javelle *et al*. (2004)
pJT8	*glnKamtB19*	A22L	This work
pJT9	*glnKamtB20*	A22F	This work
pJT10	*glnKamtB21*	A22I	This work
pJT12	*glnKamtB22*	V20I, A22I	This work
pJT13	*glnKamtB23*	V20M, A22I	This work
pJT17	*glnKamtB18*	Δ (I6-A22)	This work
pJT18	*glnKamtB24*	V20E	This work
pJT19	*glnKamtB25*	V20K	This work
pJT20	*glnKamtB26*	A22E	This work
pJT21	*glnKamtB27*	A22K	This work
pMM285	*glnKamtB3*	Wild type	Blakey *et al*. (2002)
pMM291	*glnKamtB17*	Δ (M1-A22)	This work

Western blotting

Cell fractionation and western blotting were performed as described previously (Coutts *et al.*, 2002) and proteins were detected with an anti-His antibody (Qiagen, Crawley, UK).

[^{14}C]-methylammonium transport assays

Assays were carried out as described previously for 'washed' (Thomas *et al.*, 2000) and 'unwashed' assays (Javelle *et al.*, 2005), respectively.

Purification of *Escherichia coli* AmtB

This was carried out as described previously (Blakey *et al.*, 2002).

N-terminal sequence determination

Purified AmtB (in 0.03% dodecylmaltoside, 50 mM Tris-HCl, 100 mM NaCl, 10% glycerol) was transferred to a polyvinylidene fluoride membrane and analysed using an Applied Biosystems (Foster City, CA) Procise 494 Protein Sequencer.

Results

The effects of modifying the signal peptide of *Escherichia coli* AmtB

The N-terminal sequence of purified wild-type AmtB encoded on pMM285 was determined by automated Edman degradation. A single sequence was obtained giving Ala, Pro, Ala, Val, Ala for the first five residues which unambiguously identified the N-terminus as starting at residue 23 of the predicted polypeptide sequence. These data indicate that mature AmtB is processed from a preprotein by cleavage between residues Ala22 and Ala23 and that the first 22 residues of AmtB encode a SP. Analysis of the amino-acid sequence using the programme Signal P (Dyrlov Bendtsen *et al.*, 2004) is in complete agreement with the empirical data giving a SP (*S*) score of 0.967 and a cleavage site (*C*) score of 0.917.

To examine the role of the SP in AmtB maturation we initially constructed a derivative in which we deleted the entire coding region for the SP giving a variant *amtB* gene (*amtB17*) in which the initiating methionine was in the position of Ala22 in the wild-type protein. The mutant gene was carried on a pACYC184-derived plasmid (pJT4) in which *amtB17* replaced the wild-type allele within the *glnKamtB* operon such that the transcription and translation signals were unaltered. To guard against the possibility that translation of the initial codons of the variant *amt* gene was less efficient than that of the wild-type gene we also constructed a variant (pJT17) in which we retained the first

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Table 2. AmtB variants with modified signal peptides

Plasmid	Mutation	Signal peptide sequence*	AmtB activity†	GS activity†
pJT6	Wild type	MKIATIKTGLASLAMLPGLVMAAPAVAD	100	100
pJT4	ΔM1-A22	---------------------MAPAVAD	2	0
pJT17	ΔI6-A22	MKIAT----------------AAPAVAD	5	0
pJT8	A22L	MKIATIKTGLASLAMLPGLVM**L**APAVAD	42	41
pJT9	A22F	MKIATIKTGLASLAMLPGLVM**F**APAVAD	49	40
pJT10	A22I	MKIATIKTGLASLAMLPGLVM**I**APAVAD	49	50
pJT12	V20I, A221	MKIATIKTGLASLAMLPGL**I**M**I**APAVAD	41	39
pJT13	V20M, A22I	MKIATIKTGLASLAMLPGL**M**M**I**APAVAD	59	44
pJT18	V20E	MKIATIKTGLASLAMLPGL**E**MAAPAVAD	76	96
pJT19	V20K	MKIATIKTGLASLAMLPGL**K**MAAPAVAD	69	79
pJT20	A22E	MKIATIKTGLASLAMLPGLVM**E**APAVAD	86	100
pJT21	A22K	MKIATIKTGLASLAMLPGLVM**K**APAVAD	78	105

*Residues altered by mutation are shown in bold.
†AmtB activity was determined in an "unwashed" [^{14}C] methylammonium uptake assay and GS activity in a "washed" assay. Activities are computed as a percentage of the wild-type (pJT6) and are the means of two or more replicate experiments. GS activity of the wild-type strain GT1000(pJT6) was 0.293 nmol methylglutamine mg^{-1} dry wt min^{-1}. AmtB activity of the wild-type strain was 0.104 nmol methylammonium mg^{-1} dry wt s^{-1}. The host strain GT1000 shows no AmtB activity in this assay. Errors were typically ±5%.
GS, glutamine synthetase.

five codons of *amtB* and deleted codons for residues Ile6 to Met21 (Table 2). Expression and location of the altered AmtB proteins was compared with wild-type AmtB in the Δ(*glnKamtB*) host strain GT1000, and assessed by western blotting of whole cell lysates together with cytoplasmic and membrane fractions. In whole cell extracts, the levels of AmtB from pJT4 or pJT17 were very significantly reduced compared with the wild type but all of this protein was correctly located to the membrane and ran on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels with a mobility characteristic of a trimer (Fig. 1).

For all mutants, two assays of [^{14}C] methylammonium uptake were performed. The 'unwashed' assay is a direct measure of the activity of the AmtB protein, whereas the 'washed' assay measures methylglutamine synthesis and is effectively a measure of *in vivo* glutamine synthetase activity (Javelle *et al*., 2005). If coupling of ammonium uptake and assimilation is unperturbed by the alterations to AmtB then these two assays are expected to give similar results in the parental strain and the mutants. Both deletion mutants had levels of activity no greater than that in the control *amtB* deletion mutant GT1000 (Table 2). However, it is conceivable that these data could reflect proteins with significant activity but present at a relatively low level in the membrane. Hence a protein with 50% of the wild-type activity but present in the membrane at only 10% of the normal level would effectively have 5% activity in our assays.

Previous studies on the role of the amino-acid sequence in the SP cleavage region were carried out using *Escherichia coli* alkaline phosphatase and demonstrated that alterations of the −1 and −3 positions in the SP had marked effects on processing (Karamyshev *et al*., 1998; Kajava *et al*., 2002).

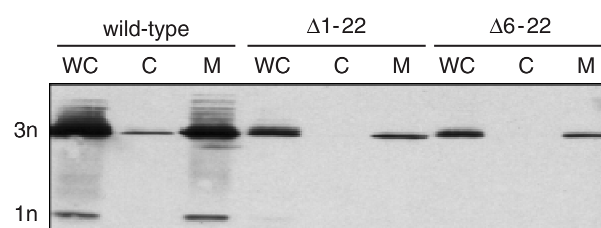

Fig. 1. Western blots of AmtB signal peptide deletion mutants. Whole cell extracts (WC), cytoplasmic (C) and membrane (M) fractions separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis gels and revealed by western blotting with anti-His antibody. 3n and 1n identify the trimer and monomer forms of AmtB, respectively.

We therefore constructed a variety of mutants with changes at positions −1, −3 or both (Table 2) to examine the effect of inhibiting SP cleavage in AmtB. Again all of the mutants were analysed in activity assays and by western blotting. All were correctly targeted to the membrane with no significant amount of AmtB in the cytoplasmic fraction (Fig. 2). Furthermore, unlike the ΔSP mutants, the level of protein detected in whole cell extracts of all of these variants was similar to that seen in the wild-type (Fig. 2). However, based on activity assays and western blots the variants fell into two groups.

One group included the five mutants with either single changes introducing a large neutral residue at −1 (A22L, A22F, A22I) or two changes introducing large neutral residues at −1 and −3 (V20I/A22I, V20M/A22I). These all had activities in the range of 41–59% of the wild-type (Table 2) and in contrast to wild-type, when extracts were analysed on SDS-PAGE, they showed a very significant proportion of the protein with a mobility equivalent to that

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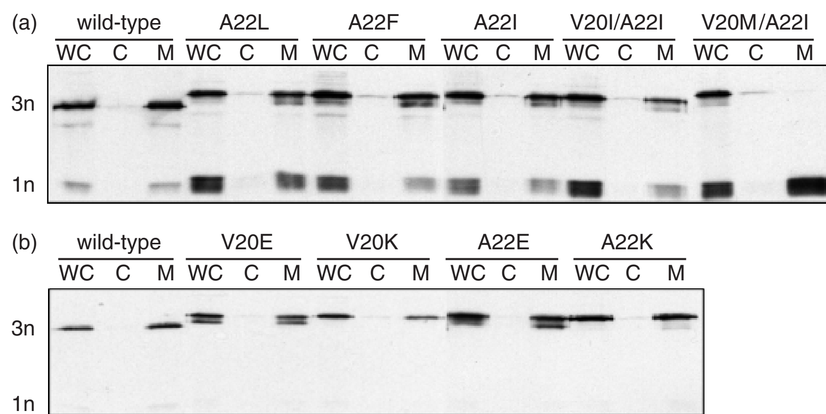

Fig. 2. Western blots of AmtB signal peptide variants. Whole cell extracts (WC), cytoplasmic (C) and membrane (M) fractions separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis gels and revealed by western blotting with anti-His antibody. 3n and 1n identify the trimer and monomer forms of AmtB respectively.

of the monomer. Indeed for V20M/A22I the membrane fraction contained almost entirely the monomeric form (Fig. 2a). In whole cell extracts and membrane fractions most of these variants showed two bands of varying intensity and with a mobility equivalent to or less than that of the wild-type trimer (Fig. 2a). The second group included the four mutants with negative or positive charged residues at -1 or at -3 (V20E, V20K, A22E, A22K). They had activities in the range of 69–86% of the wild-type (Table 2). In western blots they did not show any significant levels of monomer, and whereas both glutamate mutants (V20E and A22E) showed two approximately equivalent high molecular weight bands, both lysine mutants (V20K and A22K) showed only one of these species (Fig. 2b).

To try and determine exactly what effect the SP mutations had on cleavage of the SP we purified a number of the proteins (variants A22F, A22L, A22E, A22K, V20E, V20K). N-terminal sequence analysis showed that only the A22F variant showed the wild-type cleavage pattern (Fig. 3a). The A22L variant gave a predominant species that resulted from cleavage between Gly18 and Leu19, with at least four other minor species. The V20E andV20K variants were very similar to each other with major cleavage between Ser12 and Leu13, and the A22E and A22K variants were also very similar to each other with a major cleavage site between Ala14 and Met15 (Fig. 3a). On SDS-PAGE all of the proteins showed a reduced mobility compared with the wild-type and this was most apparent using 7.5% gels (Fig. 3b). [Note that whereas on 10% SDS-PAGE the AmtB trimer runs with an apparent molecular mass of $\sim$90 kDa (Blakey *et al.*, 2002), on 7.5% gels this changes to an apparent molecular mass of $\sim$60 kDa.]

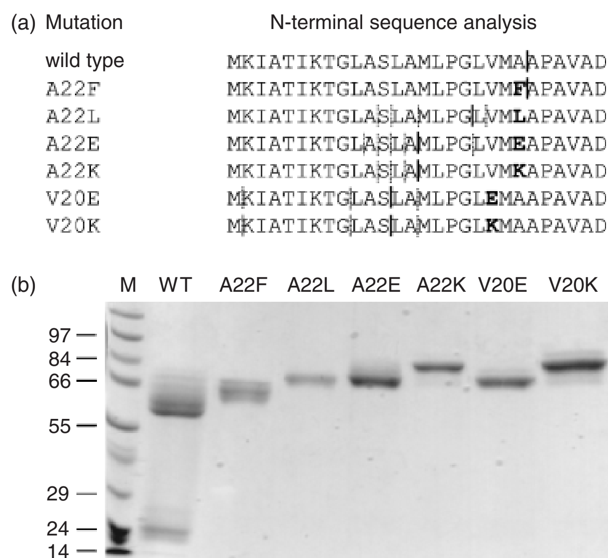

Fig. 3. (a) Results of N-terminal sequence analysis. Vertical lines show N-termini; solid lines, major product; dashed lines, minor products. (b) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified variant AmtB proteins. Proteins were purified as described in Materials and methods and analysed on 7.5% SDS-PAGE gels with 5 g of protein loaded in each lane. M, molecular weight markers.

Prediction of signal peptides in other Amt proteins

The presence of an SP in *E. coli* AmtB raises the question as to whether this is widespread amongst bacterial Amt proteins. The relatively high level of sequence similarity between members of the Amt family means that alignment of primary amino-acid sequences using Clustal W (Thompson *et al.*, 1994) provides a clear indication of the regions that constitute the TMHs. It is then apparent that the discrepancies between sequences that have previously been predicted to have either 11 TMHs or 12 TMHs are accounted for in most cases by differences at the N-terminus, and in the predicted sequences of AmtB from a variety of Gram-negative organisms, TMH1 is preceded by between 28 and 46 residues (Fig. 4). These regions are often identified by topology algorithms as a TMH (Lao *et al.*, 2002) but when analysed with Signal P they invariably showed a very high

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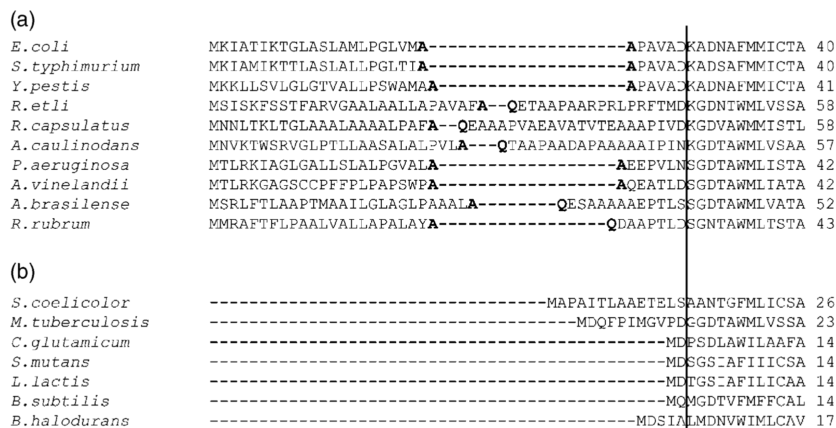

Fig. 4. Alignment (using Clustal W) of the N-terminal sequences of a number of predicted AmtB sequences from Gram-negative (a) and Gram-positive (b) bacterial species. The vertical line designates the predicted start of TMH1 derived from the known position of TMH1 in the *Escherichia coli* AmtB X-ray crystal structure. Residues flanking the signal peptide cleavage sites as predicted by SignalP are shown in bold type. The cleavage site in *E. coli* AmtB was determined empirically.

probability of constituting an SP with a predicted cleavage site, of either Ala-Ala or Ala-Gln, 22–28 residues from the N-terminus (Fig. 4). The resultant mature AmtB proteins would have N-terminal periplasmic regions varying in length from six (e.g. *E. coli*) to 23 (e.g. *Rhodobacter capsulatus*) residues. By contrast, in Gram-positive AmtB proteins there is little or no predicted N-terminal sequence preceding the start of TMH1 indicating that none of these proteins is expected to have an SP (Fig. 4).

Discussion

We have shown that the *Escherichia coli* ammonia channel protein AmtB is encoded by a preprotein with a SP of 22 amino acids. Deletion of the AmtB SP resulted in a very significant reduction in AmtB activity that appears to be primarily a consequence of the reduced level of AmtB accumulated in the membrane, indicating that in the absence of the SP AmtB is very inefficiently translocated to the membrane. The protein is not observed to accumulate in the cytoplasm, suggesting that in the absence of correct membrane insertion it is rapidly degraded.

To our knowledge this is only the fourth characterized example of a polytopic IMP with an SP, and the precise role of the SP in each of those proteins is also unclear. In the case of FliP, a protein that is essential for flagellum assembly and for bacterial motility, deletion of the SP partially impairs function, but whether this reflects reduced levels of protein accumulated in the membrane has not been determined (Pradel *et al.*, 2004). Studies on CyaO, subunit II of cytochrome *bo* oxidase, while not directly addressing the role of the SP, suggest that insertion of the N-terminal part precedes and is a prerequisite for the insertion of the C-terminal region of the protein (Celebi *et al.*, 2006). No studies on the role of the SP in DsbD, which is a disulphide oxidoreductase, have been reported (Gordon *et al.*, 2000).

We introduced a number of modifications into the AmtB SP, based on changes that completely inhibit SP cleavage in alkaline phosphatase (Karamyshev *et al.*, 1998). By contrast in the AmtB variants SP cleavage was not completely inhibited but rather they were mostly cleaved aberrantly and as a consequence the mature AmtB monomer had an unusually extended N-terminus. All of the AmtB variants were still targeted to the membrane, to a greater or lesser degree, and all showed AmtB activity.

Transmembrane helix-1 of AmtB is located at the centre of the trimer (Khademi *et al.*, 2004; Zheng *et al.*, 2004) such that an aberrantly extended N-terminus might well interfere with oligomerization as a consequence of being at the trimer interface. Consistent with this concept we observed that many of the mutants showed an increased level of the monomeric AmtB species when cell extracts were analysed by SDS-PAGE (Fig. 2). However, we cannot distinguish whether this reflects an increased level of monomer in the native *E. coli* membrane, or a reduced stability of the trimer on SDS-PAGE. Nevertheless there is some correlation between the *in vivo* AmtB activity and the amount of monomer we observe on gels. This could indicate that, although each AmtB monomer contains an ammonia channel, only the trimer is active and that the reduced activity is a direct reflection of the fact that in these variants only a proportion of the AmtB is in a trimeric form *in vivo*.

The A22F variant of AmtB was anomalous, in that N-terminal sequencing of the purified protein showed it to be the only variant that was correctly cleaved (Fig. 3a). Despite this the protein showed anomalous migration on SDS-PAGE, both by western blotting of cell extracts (Fig. 2) and by protein purification (Fig. 3b) and furthermore it had a reduced activity (Table 2). Hence it would appear that some event prior to SP cleavage influences the final conformation of the mature trimer, suggesting that SP cleavage is closely coupled to assembly of the trimer.

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The AmtB variants carrying a charged residue near the normal cleavage site (A22E, A22K, V20E, V20K) did not dissociate readily or show significantly impaired activity. However, SP cleavage was aberrant such that they had an unnaturally extended N-terminus. As the proteins were purified in the presence of dodecylmaltoside and they retain their oligomeric state in the presence of SDS, their mobility on SDS-PAGE gels does not relate directly to their molecular mass. Consequently, the differences in mobility shown by the different variants on SDS-PAGE, both in cell extracts and after purification, suggests that they may adopt slightly different conformations from the wild-type (Figs 2 and 3). One possible explanation for the relative stability of the variants with a novel charged residue at the N-terminus is that the charge prevents insertion of the incorrectly cleaved N-terminal region into the membrane at the trimer interface, thereby ensuring formation of a stable active trimer. In this regard the predicted amino-acid sequences of some Amt proteins, e.g. *Saccharomyces cervisiae* Mep2, suggest that they may naturally have relatively large N-terminal extracytoplasmic regions; that of Mep2 would be 28 residues in length and would include two negatively charged residues.

Bioinformatic analysis strongly suggests that an SP is a conserved feature of Amt proteins from Gram-negative organisms, but it would appear that the otherwise highly conserved Amt proteins from Gram-positive bacteria such as *Bacillus subtilis* (Detsch & Stulke, 2003) or from some archaea, e.g. *Archaeoglobus fulgidus* Amt1 and Amt3 (Andrade *et al*., 2005), do not have an SP but are presumably still efficiently targeted to the membrane. *Archaeoglobus fulgidus* Amt1 has been shown to be trimeric (Andrade *et al*., 2005) but the oligomeric nature of *B. subtilis* AmtB has not been examined (Detsch and Stulke, 2003). It therefore remains to be determined why AmtB should depend on an SP in *E. coli* but not in *B. subtilis* or *A. fulgidus*. It is interesting to note that AmtB is not unique in this respect because the *B. subtilis* FliP protein is likewise homologous to *E. coli* FliP throughout its length with the exception that it also lacks a SP (Bischoff *et al*., 1992).

Although it has been suggested that a significant percentage of bacterial IMPs may be encoded as preproteins with a signal peptide (Arai *et al*., 2003; Käll *et al*., 2004) there is presently little empirical data to support this. The use of a SP in such a context ensures that the N-terminus of the mature protein is located extracytoplasmically. However, not all polytopic IMPs with extracellular N-termini utilize a SP. Further experiments are currently in progress to dissect the pathway of *E. coli* AmtB assembly into the inner membrane.

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