Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB

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The Amt proteins are ammonium transporters that are conserved throughout all domains of life, being found in bacteria, archaea and eukarya. In bacteria and archaea, the Amt structural genes (amtB) are invariably linked to glnK, which encodes a member of the \(P_\text{II}\) signal transduction protein family, proteins that regulate enzyme activity and gene expression in response to the intracellular nitrogen status. We have now shown that in *Escherichia coli* and *Azotobacter vinelandii*, GlnK binds to the membrane in an AmtB-dependent manner and that GlnK acts as a negative regulator of the transport activity of AmtB. Membrane binding is dependent on the uridylylation state of GlnK and is modulated according to the cellular nitrogen status such that it is maximal in nitrogen-sufficient situations. The membrane sequestration of GlnK by AmtB represents a novel form of signal transduction in which an integral membrane transport protein functions to link the extracellular ammonium concentration to the intracellular responses to nitrogen status. The results also offer new insights into the evolution of \(P_\text{II}\) proteins and a rationale for their trigonal symmetry.

**Keywords:** ammonium transport/membrane sequestration/nitrogen regulation/\(P_\text{II}\) regulatory proteins/signal transduction

Introduction

The ammonium transport (Amt) family of proteins comprises a unique and ubiquitous group of integral membrane proteins found in all domains of life. They are present in bacteria, archaea, fungi, plants and animals, including humans, where they are represented by the Rhesus proteins (Saier *et al.*, 1999; Howitt and Udvardi, 2000; Marini *et al.*, 2000a; Thomas *et al.*, 2000b). The Amt proteins are high-affinity ammonium transporters that probably function to scavenge ammonium and to recapture ammonium lost from cells by diffusion across the cell membrane. In *Saccharomyces cerevisiae* and *Rhodobacter capsulatus*, Amt proteins have also been implicated in sensing ammonium in the external medium (Lorenz and Heitman, 1998; Yakunin and Hallenbeck, 2000).

Analysis of the predicted primary amino acid sequences of Amt proteins suggests that all members of the family adopt a similar topology within the cell membrane. A combination of *in silico* genetic and biochemical analyses suggests that most Amt proteins are likely to have 11 transmembrane (TM) helices, with the N-terminus being extracytosolic and the C-terminus being located in the cytoplasm (Marini and Andre, 2000; Thomas *et al.*, 2000b). Some bacteria, including *Escherichia coli*, appear to have an AmtB protein with a twelfth TM at the N-terminus such that in these organisms the N-terminus would also be cytosolic (Thomas *et al.*, 2000b). The C-terminal region of the Amt proteins is very variable in length, comprising a minimum of ~30 residues but in some cases extending to >150 residues. As such, this region is predicted to comprise by far the largest part of the protein exposed to the cytosol.

Almost all bacteria and archaea encode at least one Amt protein, and with very few exceptions the gene encoding the transporter (amtB) is found in an operon together with a second gene (glnK) encoding a small signal transduction protein (Thomas *et al.*, 2000a; Arcondéguy *et al.*, 2001). GlnK is a member of the \(P_\text{II}\) protein family, which act as sensors of the cellular nitrogen status in prokaryotes and which have also been identified in plants (Hsieh *et al.*, 1998; Arcondéguy *et al.*, 2001). The \(P_\text{II}\) proteins have been studied in most detail in the Proteobacteria, especially in *E. coli*, which encodes two \(P_\text{II}\) proteins, GlnK and GlnB. GlnK is homologous to GlnB at the primary sequence level and this is reflected in the very similar tertiary and quaternary structures adopted by both proteins (Carr *et al.*, 1996; Xu *et al.*, 1998). They are trimers and take the form of a squat barrel ~50 Å in diameter and 30 Å high, above the surface of which protrude three loops (the T-loops). In response to nitrogen deprivation, the proteins are covalently modified by uridylylation of residue Tyr51 at the apex of the T-loop, and this process is reversed in nitrogen sufficiency (Jaggi *et al.*, 1996; Atkinson and Ninfa, 1999). \(P_\text{II}\) proteins regulate the activities of other proteins by protein–protein interaction, as exemplified by the role of GlnK in modulating the activity of the transcriptional activator NtrC by interaction with the histidine protein kinase NtrB (Pioszak *et al.*, 2000). Whilst *E. coli* GlnK can substitute to some degree for GlnB, the primary function of GlnK has yet to be determined (van Heeswijk *et al.*, 1996; Atkinson and Ninfa, 1998).

The conservation of genetic linkage in prokaryotes is characteristic of operons that encode either essential cellular components or proteins that physically interact (Dandekar *et al.*, 1998). Neither glnK nor amtB is essential for growth in *E. coli* (Atkinson and Ninfa, 1998; Soupe *et al.*, 1998), and this has led us to propose that their conserved association reflects a physical interaction and a related function between GlnK and AmtB (Thomas *et al.*, 2000b).
GlnK interacts with membrane-bound AmtB

**Fig. 1.** GlnK and GlnB associate with the membrane in an AmtB-dependent manner. Whole-cell extracts (W), cytoplasmic (C) and membrane (M) fractions were subjected to SDS–PAGE followed by western blotting using an anti-P$_H$ antibody. Extracts and fractions were prepared and loaded as described in Materials and methods. The strains used are indicated below each lane: wild type (ET8000), ΔglnB, ΔglnK (FT8000), ΔglnB (PT8000), ΔglnK (GT1002), ΔamtB (GT1001). The control lane contained 0.3 μg of purified E.coli GlnK.

2000a). We now report that in *E.coli* and *Azotobacter vinelandii* this is indeed the case. We show that GlnK is sequestered to the cell membrane in response to nitrogen shock in an AmtB-dependent manner and that this interaction is dependent on the C-terminal cytoplasmic domain of AmtB. We discuss the potential role of this interaction and the implications of its conservation throughout bacteria and archaea.

**Results**

**GlnK and GlnB associate with the membrane in an AmtB-dependent manner**

The highly conserved genetic linkage between the *glnK* and *amtB* genes in both bacteria and archaea led us to hypothesize that GlnK was functionally associated with AmtB and that the two proteins might physically interact (Thomas et al., 2000a). We therefore used a variety of wild-type and mutant strains of *E.coli* to assess whole-cell extracts, cytoplasmic fractions and membrane fractions for the presence of P$_H$ proteins (GlnK or GlnB) using SDS–PAGE and western blotting. The cells were grown in nitrogen-limiting media with glutamine as the nitrogen source (M9Gln medium), conditions under which the *glnK–amtB* operon is highly expressed (Atkinson and Ninfa, 1998). In initial experiments, the membrane fractions isolated by ultracentrifugation were subjected to repeated washes with 50 mM sodium phosphate buffer.

Cellular fractions prepared from wild-type cells (strain ET8000) indicated that P$_H$ protein was present in both the cytoplasmic and the membrane fractions, and control experiments with strain PT8000 (ΔglnB,ΔglnK) confirm that these signals are P$_H$ specific (Figure 1). The data confirm that some P$_H$ protein is membrane associated under these growth conditions but do not distinguish between GlnB and GlnK. To clarify the nature of P$_H$ association with the membrane and the role of AmtB in this association, three isogenic mutant strains were employed (ΔglnK, GT1002; ΔglnB, PT8000; and ΔamtB, GT1001). The ΔglnK mutation is an in-frame deletion within glnK that is not polar on *amtB* (Arcondéguy et al., 1999).

Analysis of the ΔglnB strain gave an identical pattern to that seen with the wild type, demonstrating that GlnK does indeed associate with the membrane (Figure 1). In the ΔglnK strain, the pattern was somewhat different and GlnB was only detected in the whole-cell lysate and the membrane fraction. Consequently, GlnB also appears to associate with the membrane, at least in the absence of GlnK; however, the signal was considerably weaker compared with that seen in the ΔglnB strain, suggesting that under these growth conditions there is considerably less GlnB than GlnK in the cell. Control experiments indicate that the anti-P$_H$ antibody used in these experiments is significantly less active against GlnK than GlnB (R.Little and T.Arcondéguy, personal communication), hence the GlnK:GlnB ratio is actually underestimated.

When the localization of the P$_H$ proteins was examined in extracts prepared from the ΔamtB strain GT1001, there was a striking absence of signal from the membrane fraction, while the proteins were just as abundant in the cytoplasmic fraction as in the wild type (Figure 1). Hence AmtB is essential for the association of the P$_H$ proteins with the membrane, most probably through a direct physical interaction between P$_H$ and AmtB.

**The membrane association of P$_H$ is affected by the cellular nitrogen status**

P$_H$ proteins typically function to regulate the activity of those proteins with which they interact in response to the nitrogen status of the cell (Arcondéguy et al., 2001). We therefore examined what effect, if any, a change in the cellular nitrogen status would have on the observed association between P$_H$ (in particular GlnK) and AmtB. Previous studies of this nature have investigated the role of GlnB in regulating adenylyltransferase, and thereby the adenylylation of glutamine synthetase, in response to a rapid addition of ammonium to a nitrogen-limited culture (termed ‘ammonia shock’) (Bender et al., 1977;
van Heeswijk et al., 1996). We therefore used the same ammonia shock conditions to investigate effects on GlnK–AmtB association.

For these experiments, we used strain GT1000 (pMM280), which has chromosomal deletions of both glnK and amtB and contains glnK and amtB in trans with a modified version of amtB that encodes a C-terminally His-tagged version of the protein (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation). The modified AmtB protein retains methylammonium transport activity at a level comparable to the wild-type protein (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation). The cells were grown in M9Gln medium and then subjected to ammonia shock with 30 mM NH4Cl. Samples were taken immediately prior to addition of ammonium and 15 min later, and then fractionated as before. We monitored the membrane fractions for the presence of both P11 and AmtB, the latter being detected using an anti-His tag antibody (Qiagen) to detect the C-terminal His tag on the modified version of AmtB. The levels of AmtB in the membrane fraction remained constant both pre- and post-ammonia shock (Figure 2). In contrast, there was a very significant increase in the amount of P11 protein associated with the membrane after ammonia shock, indicating that membrane association is modulated by the nitrogen status (Figure 2). The levels of P11 (GlnB + GlnK) in the cytoplasmic fractions showed a significant reduction after ammonia shock (data not shown).

Membrane association of GlnK reflects the uridylylation state of the protein

Given that GlnK is a trimer, the protein can exist in four states in the cell depending upon the number of subunits that are modified by uridylylation. Ammonia shock is known to cause rapid deuridylylation of both GlnB and GlnK (van Heeswijk et al., 1996; Jiang et al., 1998) and the uridylylation states of the P11 trimers can be assessed by separating the different forms on native polyacrylamide gels (Forchhammer et al., 1999; van Heeswijk et al., 2000). Under these conditions, the non-uridylylated form of the protein has the lowest mobility and the fully uridylylated form the highest (Jaggi et al., 1996). For these experiments, we used a ΔglkB strain (PT8000) and a ΔglkB,ΔamtB strain (AT8000) in order to assess specifically the membrane association of GlnK. We also subjected the membrane fractions to a more stringent wash using 50 mM sodium phosphate supplemented with 600 mM NaCl to ensure that only those proteins that were tightly associated with the membrane were detected.

In whole-cell lysates from ΔglkB cells grown in M9Gln medium, four bands were detected corresponding to the uridylylated forms of GlnK (GlnK-UMP1, GlnK-UMP2 and GlnK-UMP3) and the unmodified protein (Figure 3). Following ammonia shock, this profile changes to reveal only two bands, the major one being the non-uridylylated form of GlnK and the minor species being GlnK-UMP1. The cytoplasmic fraction showed a very similar pattern, although GlnK-UMP1 was apparently less abundant than GlnK-UMP2 and GlnK-UMP3 pre-ammonia shock. Most strikingly, analysis of the membrane fractions gave a relatively weak signal pre-ammonia shock, with only GlnK-UMP3 being revealed, and a very much stronger signal post-ammonia shock, which was almost exclusively the non-uridylylated form of GlnK (Figure 3A). Identical amounts of membrane material were loaded in each lane and, as shown earlier, the amount of AmtB present pre- and post-ammonia shock did not vary significantly. Parallel analyses of the ΔglkB,ΔamtB strain produced very similar profiles for the whole-cell lysate and for the cytoplasmic fraction, but there was no membrane-associated GlnK either pre- or post-ammonia shock (Figure 3B). In similar experiments using the less stringent salt wash, we detected membrane association of other uridylylated species of GlnK in the pre-ammonia shock samples, suggesting that whilst there is association of GlnK in nitrogen-limited conditions, most of this protein is only weakly bound (data not shown).

We carried out experiments comparable to those described above using the Δglnk strain (GT1002) to examine the behaviour of GlnB in the absence of GlnK. Our initial experiments on nitrogen-limited cells had indicated that in the absence of GlnK essentially all GlnB was membrane associated (Figure 1). Nevertheless, we saw a very marked apparent increase in the amount of GlnB in the membrane fraction after ammonia shock (data not shown). We interpret this to reflect an increased affinity of GlnB for AmtB in response to ammonia shock so that membrane-associated GlnB that was lost from pre-ammonia shock samples during the salt wash was retained in post-ammonia shock samples.

Finally, we repeated the experiment with wild-type cells, but in this case interpretation of the results is complicated due to the potential presence of heterotrimers between GlnB and GlnK whereby at least 16 different species of P11 protein can occur in principle (van Heeswijk et al., 2000). Using native gel electrophoresis, we did indeed observe more bands in western blots of whole-cell extracts, cytoplasmic and membrane fractions both pre- and post-ammonia shock than we detected in either the ΔglkB or Δglnk strains (data not shown). Assignment of these bands was assisted by the previously reported differential migration of the homotrimeric GlnK and GlnB proteins (van Heeswijk et al., 2000). The membrane fractions from post-ammonia shock samples contained
three major species of which the dominant form had a mobility consistent with that of the non-uridylylated GlnK homotrimer, the other species most probably being heterotrimers containing predominantly GlnK subunits. Hence it would appear that although GlnB can associate with AmtB in the absence of GlnK, it does not compete significantly with GlnK in wild-type cells.

The C-terminal domain of AmtB is required for GlnK association

The C-terminal 32 residues of E.coli AmtB are predicted to be located on the cytoplasmic side of the membrane and to constitute the most extensive part of the protein exposed to the cytoplasm (Thomas et al., 2000b). The region contains a number of residues that are highly conserved amongst members of the Amt family and is therefore likely to be either functionally and/or structurally important. We reasoned that this region constitutes a potential interaction domain for GlnK and we therefore examined the consequences of deleting the C-terminus of the transporter.

The amtB gene was truncated (amtB4) so that 25 residues were removed from the C-terminus and the encoded protein terminated at residue 403. The activity of the truncated protein was compared with that of wild-type AmtB by introducing the wild-type glnK–amtB operon (on pGC2) and the derivative operon glnK–amtB4 (on pGC4) into the ΔglnK,amtB strain GT1000. The truncated transporter had 28% of the wild-type AmtB activity as measured by [14C]methylamine transport. We used a polyclonal antibody raised against purified E.coli AmtB (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation) to assess the presence of the truncated AmtB in the cell membrane and found levels comparable to the wild-type protein both pre- and post-ammonia shock (data not shown). Hence the reduced [14C]methylamine transport activity of the truncated protein apparently is not due to reduced levels of the protein in the membrane, but rather to decreased activity of the protein.

Using strain GT1000 (pGC4), which synthesizes only the C-terminally truncated form of AmtB, we analysed the location of P1H in cell fractions both pre- and post-ammonia shock using western blots after denaturing SDS–PAGE. The protein was detected in whole-cell lysates and cytoplasmic fractions at levels comparable to those observed previously, but there was almost no signal in the membrane fractions (Figure 4). Although this strain synthesizes both GlnK and GlnB, a combination of the differential expression of glnB and glnK, together with the multicopy effect of the plasmid-borne glnK gene, means that nearly all of the P1H in these cells is expected to be GlnK. These data strongly suggest that the C-terminal domain of AmtB either constitutes the site of interaction between GlnK and AmtB or is required to allow AmtB to adopt a conformation required for GlnK association.

GlnK–AmtB association is found in other bacteria

We have proposed previously that the conservation of linkage between the glnK and amtB genes in both bacteria and archaea reflects a highly conserved interaction between the two proteins (Thomas et al., 2000). Having demonstrated evidence for such an association in E.coli, we sought to support the concept that this is a general phenomenon by carrying out a similar analysis in another species, namely the γ-proteobacterium A.vinelandii. Azotobacter vinelandii contains a single glnK–amtB operon and, unlike E.coli, is proposed to encode only a single P1H protein, namely GlnK (Meletzus et al., 1998). We used a wild-type strain (UW136) and an amtB mutant (MV560) that has been shown to lack [14C]methylamine transport activity (Meletzus et al., 1998). Cells were grown in a nitrogen-limited medium and then subjected to ammonia shock in a similar manner to that used for E.coli. Membrane fractions were isolated pre- and post-ammonia shock, subjected to stringent washing and then run on native polyacrylamide gels prior to western blotting with antibodies against E.coli GlnB and AmtB. As in E.coli, GlnK was found to be associated with the membrane in an AmtB-dependent manner and the level of membrane-associated GlnK increased post-ammonia shock whilst the level in the cytoplasm was reduced (Figure 5). The AmtB antibody confirmed that the levels of AmtB present in the membrane do not vary pre- and post-ammonia shock and that membranes from the amtB mutant

Fig. 4. The C-terminal domain of AmtB is required for GlnK association. Whole-cell extracts (W), and cytoplasmic (C) and membrane (M) fractions were prepared from strain GT1000 (pGC4) [ΔglnK amtB (glnK amtb4)] both before (–N) and after (AS) ammonia shock. Extracts and fractions were prepared as described in Materials and methods and were subjected to SDS–PAGE followed by western blotting using an anti-P1H antibody.

Fig. 5. GlnK–AmtB association is found in other bacteria. Whole-cell extracts (W), and cytoplasmic (C) and membrane (M) fractions were prepared from A.vinelandii strains (A) UW136 (wild type) and (B) MV560 (amtB1::KIXX) both before (–N) and after (AS) ammonia shock. Extracts and fractions were prepared as described in Materials and methods and subjected to native PAGE followed by western blotting using an anti-P1H antibody. Under these conditions, all four possible forms of GlnK (UMP 0–3) can be identified.
showed no cross-reacting material (data not shown). Hence the GlnK and AmtB proteins of *A. vinelandii* show very similar behaviour to that seen in *E. coli* in response to a rapid change in the cellular nitrogen status.

**The GlnK protein negatively regulates the transport activity of AmtB**

Having found evidence for an interaction between P$_{II}$ proteins and the ammonium transporter AmtB, we considered what functions such an interaction might serve. One possibility is that the interaction serves to regulate the activity of the transporter and, with this in mind, we carried out a series of experiments comparing the transport activity of AmtB in the wild type with that in strains either lacking or overexpressing *glnK*. The substrate in these assays is [*14C]*ammonium, an analogue of ammonium, and as transport of methylammonium by AmtB is competitively inhibited by micromolar concentrations of ammonium it is not possible to assess the effect of GlnK under those conditions where its interaction with AmtB is maximal, i.e. after ammonia shock. Therefore, all these experiments were carried out using cells grown in nitrogen limitation, a condition where any effects of GlnK on AmtB activity were expected to be relatively small.

We examined two isogenic sets of strains, one derived from ET8000 and one from YMC10, for the effects of *ΔglnK* and *ΔglnB* mutations on uptake (Figure 6A and B). In each case, the *ΔglnB* strain was indistinguishable from the wild type, whereas the activity of the *ΔglnK* strain was considerably elevated (50% in the ET8000 background, Figure 6A; and 100% in the YMC10 background, Figure 6B). In principle, this effect of the *ΔglnK* mutation could arise in a number of ways. It could be due to a direct effect on the activity of AmtB or to an indirect effect (through NtrB and NtrC) leading to elevated *glnK–amtB*
transcription. Alternatively, as methylammonium is metabolized by glutamine synthetase to methylglutamine and the transport assay effectively measures metabolically trapped methylammonium (Soupene et al., 1998), there is also the potential for effects on the activity of glutamine synthetase. The effects of glnK mutants of E. coli on both glnK–amtB transcription and glutamine synthetase activity have been assessed previously and, in each case, the mutant was shown to have little if any effect (Atkinson and Ninfa, 1998). Indeed, the most marked effect is on glnK–amtB transcription where the glnK mutant decreases, rather than increases, expression. To confirm independently that the levels of AmtB are unaffected by the glnK mutation, we used western blots to assess the levels of AmtB present in whole-cell extracts of wild type, and ΔglnK and ΔglnB mutants. We found no significant differences in the levels of AmtB in each of the strains (data not shown). We therefore conclude that the elevated transport activity of the glnK mutant is attributable to an increase in the activity of AmtB.

The deduced negative effect of GlnK on AmtB was confirmed by examining the activities of strains in which GlnK levels were elevated. We introduced into a wild-type strain low-copy-number plasmids on which either glnK (pTA40) or a mutant glnK allele (glnKY51F, carried on pTA43) is expressed from the lacZ promoter. AmtB activity was unchanged in the presence of wild-type GlnK, but a non-uridylylatable form of the protein, GlnKY51F, which is expected to associate strongly with AmtB, significantly depressed activity (Figure 6C).

The negative effect of GlnK on AmtB activity was also apparent when we compared plasmids expressing either amtB alone (pGT14) or the complete glnK–amtB operon (pGT11) into strain GT1000, which carries a chromosomal glnK–amtB deletion (Figure 6D). The presence of multicopy glnK–amtB gave an activity 4-fold greater than wild type, but when glnK was deleted from the plasmid and amtB was expressed alone from the same native promoter, the activity was elevated 9-fold. Hence AmtB is significantly more active in the absence of GlnK.

Discussion

In almost all members of the bacteria and archaea, genes encoding the high-affinity ammonium transporter AmtB are linked to and potentially co-transcribed with glnK, which codes for a member of the P1f signal transduction protein family (Thomas et al., 2000a; Arcendéguy et al., 2001). We have now shown that in E. coli and in A. vinelandii this genetic linkage almost certainly reflects a physical interaction between the two proteins such that GlnK can be sequestered specifically to the cell membrane by AmtB. The affinity of non-uridylylated GlnK for AmtB appears to be considerably greater than that of the uridylylated protein, such that the interaction of GlnK with AmtB is promoted by an increase in the intracellular ammonium status. AmtB activity increases in the absence of GlnK, indicating that GlnK acts as a negative regulator of the transporter.

We propose that the physiological role of this interaction is to modulate the activity of the transporter so as to optimize ammonium uptake under all growth conditions, and a model for this regulation is outlined in Figure 7. The transcriptional control of amtB by the nitrogen regulation (NtrB/NtrC) system ensures that amtB is only expressed in nitrogen-limited growth conditions. However, during growth in nitrogen limitation, GlnK can fine-tune the activity of AmtB in response to minor or transient fluctuations in ammonium availability. In nitrogen-limited conditions, GlnK is predominantly in its fully uridylylated state and is not strongly membrane associated (non-shaded GlnK indicates the minority species in each situation). AmtB is active and will effectively scavenge ammonium from the surrounding medium. When the cellular nitrogen status changes, e.g. as a consequence of a sudden rise in the availability of extracellular ammonium, GlnK is deuridylylated rapidly and the unmodified form of the protein associates tightly with AmtB in the inner membrane. This association reduces the activity of AmtB and ammonium is no longer transported actively into the cell. This process is rapidly reversible, but in the event that the nitrogen status remains high, transcription of the glnK–amtB operon will cease due to dephosphorylation of the activator protein NtrC.

Fig. 7. Model for regulation of the interaction between GlnK and AmtB. In conditions of nitrogen limitation (−N), GlnK is predominantly in its fully uridylylated state and is not strongly membrane associated (non-shaded GlnK indicates the minority species in each situation). AmtB is active and will effectively scavenge ammonium from the surrounding medium. When the cellular nitrogen status changes, e.g. as a consequence of a sudden rise in the availability of extracellular ammonium, GlnK is deuridylylated rapidly and the unmodified form of the protein associates tightly with AmtB in the inner membrane. This association reduces the activity of AmtB and ammonium is no longer transported actively into the cell. This process is rapidly reversible, but in the event that the nitrogen status remains high, transcription of the glnK–amtB operon will cease due to dephosphorylation of the activator protein NtrC.
cytoplasmic targets. To date, only one specific cytoplasmic target of GlnK is known, the nitrogen fixation regulatory protein complex NitLA (Jack et al., 1999). In Klebsiella pneumoniae, GlnK is required to relieve the inhibitory effects of NitL on the activator protein NitA and, somewhat surprisingly, uridylylation of GlnK is not required to regulate this process (He et al., 1998). This led to the question of how NifL-mediated inhibition could be restored when ammonium is added back to a nitrogen-limited medium and to the suggestion that GlnK might be proteolyzed or covalently modified by a mechanism other than uridylylation upon replenishment of ammonium (He et al., 1998). Our present observations offer an alternative explanation in that the sequestration of deuridylylated GlnK by AmtB would rapidly lower the cytoplasmic GlnK pool and thereby release NitL to inhibit NitA activity. A further implication of this mechanism is that the pivotal role of AmtB in such a process would give the transporter the appearance of acting as a sensor of extracellular ammonium, a role that had been suggested previously for Amt proteins in R. capsulatus (Yakunin and Hallenbeck, 2000) and in S. cerevisiae (Lorenz and Heitman, 1998).

The C-terminal region of E. coli AmtB is required for the membrane sequestration of GlnK and potentially could constitute the specific site of interaction. In S. cerevisiae Mep proteins, a point mutation in this region can have a transdominant phenotype, suggesting that C-terminal residues are involved in interactions between subunits of the transporter (Marini et al., 2000b). In E. coli, deletion of this region does not abolish transport, though it does significantly reduce it. Hence, one function of this region of the transporter could be regulatory, and it is notable that whilst the C-terminal region in E. coli AmtB is conserved in Amt proteins, many members of the family have considerably extended C-terminal domains of up to 150 residues. These domains could potentially constitute interaction sites for regulatory proteins.

We suggest that the interaction of GlnK and Amt proteins is a general phenomenon that is fundamental to the biology of bacterial and archaeal ammonium transporters. In those organisms in which multiple copies of the *amtB* gene are present, e.g. *Archaeoglobus fulgidus* and *Azoarcus*, a linked *glnK* homologue is also duplicated, suggesting that the two proteins co-evolved. The major exception to this situation is in the cyanobacteria, which have a single *P*II gene (*glnB*) that is not *amt* linked and which can have multiple homologues of *amtB* (Montesinos et al., 1998). The conservation of the *glnK*-*amtB* operon leads us to suggest that *P*II proteins originally evolved together with the Amt proteins and that the primary role of the *P*II protein was to regulate the activity of the ammonium transporter. Based on this model, the *P*II paralogues that are now found in the bacteria and the archaea, namely the GlnB proteins of the Proteobacteria and the NifL proteins in the nitrogen fixation gene clusters of some archaea (Arcondeguy et al., 2001), are likely to be the result of subsequent gene duplication and specialization. Of particular interest in this context is the fact that the *P*II proteins are trimers and to date the reason for this quaternary structure has not been apparent, particularly as none of their known targets has trigonal symmetry. However, we have recently shown that the purified AmtB protein from *E. coli* is also trimeric (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation) and hence, if GlnK did evolve in concert with AmtB, its trimeric structure may reflect a symmetry required for optimal interaction between the two proteins.

In *E. coli*, the interaction of GlnK with AmtB apparently is modulated by uridylylation, and a generalization of our model would imply that covalent modification of the *P*II protein is required to regulate interaction with the transporter. In the cyanobacteria, *P*II is modified by phosphorylation of Ser49 rather than uridylylation of
Tyr51 (Forchhammer and Tandeau de Marsac, 1994) and this could serve the same purpose. However, it is not known whether the Ptu proteins of archaea are subject to covalent modification. A uridyl/transferase (GlnD homologue) is not present in the archaea and their Ptu proteins do not contain a potential phosphorylation site, i.e. a conserved serine residue, in the T-loop. It is also possible that the transporter plays an active role in the interaction with GlnK and that their association is also modulated by conformational changes in the transporter in response to increased levels of extracellular ammonium.

Dynamic changes in the subcellular localization of proteins are emerging as significant features in bacteria with respect to both cellular physiology and cell differentiation (Shapiro and Losick, 2000). A number of examples of membrane sequestration of regulatory proteins have been described. The transcriptional repressor of the Salmonella typhimurium proline utilization (put) operon, PutA, shuttles between the membrane and the cytoplasm depending on the intracellular concentration of proline (Muro-Pastor et al., 1997). Similarly, in the presence of glucose, the E. coli repressor protein Mlc binds to the activated glucose transporter PtsG, thereby becoming sequestered to the membrane and allowing derepression of Mlc-regulated promoters (Lee et al., 2000; Tanaka et al., 2000). In the case of GlnK and AmtB, the cytoplasmic component is a signal transduction protein rather than a transcriptional regulator but, as with Mlc, the system potentially provides a means not only of responding to the presence of an extracellular substrate but also of sensing how much is entering the cell. One can imagine that such mechanisms may be much more widespread and indeed the potential role of transport proteins as part of signal transduction pathways in bacteria and archaea is perhaps considerably greater than has been recognized so far. One possible example, which also involves a Ptu protein, is in the cyanobacterium Synechococcus, where GlnB has been shown to regulate nitrate uptake and might interact with the C-terminus of one of the ATP-binding subunits of the nitrate transporter NrtC (Kobayashi et al., 1997; Hisbergues et al., 1999).

Materials and methods

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table I. The E. coli strains were grown routinely in Luria medium (Luria broth or Luria agar LA). For growth under nitrogen limitation, an altered M9 medium was used in which the ammonia was replaced by 200 μg/ml glutamine (M9Gln medium). Ammonia shock was achieved by addition of NH4Cl to a final concentration of 30 mM. Acetobacter vinelandii was grown in Burk’s succrose medium supplemented with 25 mM ammonium acetate for +N conditions and 200 μg/ml glutamine for –N conditions. Antibiotics were used at the following concentrations: chloramphenicol 30 μg/ml and kanamycin 1 μg/ml.

Plasmid pGT11, which contains the complete glnK–AmtB operon, was created by subcloning an Spc–BamHI fragment from pWVH141 (van Heeswijk et al., 1996) into Spc–BamHI-cut pWSK29 (Wang and Kusner, 1991). pG2C was derived from pGT11 in two steps: first, the HindIII–BamHI 1.8 kb fragment of pGT11 was cloned into pACYC184 (Chang and Cohen, 1978) to give pGCl. Secondly, using the primers AMT1 (GGGCAATCATGTTGCTG) and AMT2 (CCGATCCATTAC- AGAATCTGTTAATAGCATTGCGC), BamHI and BglII sites underlined and termination codon italicized, the 3’ end of amtb was amplified on a PvuI–BamHI fragment. This fragment then replaced the wild-type PvuI–BamHI fragment, giving pG2C in which the BamHI site was moved closer to the termination codon of amtb and a unique BglII site encoding two additional residues (aspartate and leucine) was introduced 5’ to the stop codon. The modified amtb gene in pG2C was designated amtb1.

A short linking amino acid-coding sequence was added to the C-terminus of Amtb by the ligation of a 3’-adenylated cassette into pG2C cut with BglII and BamHI. The cassette was produced by annealing AMT3 (GATCAAGCCACACCGCCTACACGAGTCTCGATAAG) and AMT4 (GATCCTCTAGCTGAGATCGTCTGCTGA-GCGGGCTGTTGGCGCTTTCAT) to produce pGCS. A His6 tag [made by annealing oligos AMT5 (GATCATACCCAACCATCTACAT- TC-AG) and AMT6 (GATCCCTAGTCATGATCATGGTCCTGAT)] was added to the C-terminus of the protein by the ligation of the oligonucleotide cassette between the BglII and BamHI sites in pGCS to give a further allele of amtb-amtb3, encoded on pMM280.

The plasmid pGC4 was created by using two primers Amt1 (GGGCAATCATGTTGCTG) and Amnt5 (CCGATCCATTACGTA- AGAATCTGTTAATAGCATTGCGC, BamHI and XhoI sites underlined and termination codon italicized) to amplify part of the end of amtb. This fragment was then ligated into pG2C (glnK-amtb1) on a PvuII–BamHI fragment to give pGC4 (glnK-amtb4), which encodes GlnK and a truncated form of Amtb comprising the wild-type sequence to residue Thr402 with a newly introduced XhoI site resulting in one extra codon (Ser403) just prior to the stop codon.

Plasmid pTA40 was constructed by cloning an EcoRI–SacI fragment carrying glnK from pWVH149 (van Heeswijk et al., 1996) into pWSK30 (Wang and Kusner, 1991) so that glnK is expressed from plac-Z. The derivative plasmid pTA43 is identical to pTA40 but contains a site-directed mutation that converts the TAC codon for Y51 to a TTC codon, giving E51.

Strain GT1000 containing an unmarked deletion that removes the majority of the glnK–AmtB operon was constructed in a manner comparable with that used previously to construct the unmarked amtb deletion strain GT1001 (Thomas et al., 2000b). PCR primers GlnKno (5’-CGCGATCCCGTGGTCGAGACGCGCGAAGA-3’ and GlnKn (5’-CGCGATCCCGTGGTCGAGACGCGCGAAGA-3’) were used, with plasmid pWVH141 as template DNA, to amplify a 500 bp region upstream of glnK, which ends with the first six codons of glnK. A similar pair of primers, Amtbci (5’-TGTTTAATGCTAGGATGGGATGTCAACCGCAAGGGCGAG-3’) and AmtbCo (5’-CGCGATCCCGTGGTCGAGACGCGCGAAGA-3’), was used to amplify a 500 bp region extending from the last seven codons of amtb into the downstream isxb gene. The two PCR products were purified, mixed and used as templates for a second PCR using primers GlnKno and AmtbCo. The resulting PCR product was digested with BamHI and cloned into BamHI-cut pKO3 (Link et al., 1997). This unmarked deletion of glnK–amtB was then crossed onto the chromosome of strain ET8000. Resolution of the recombination was checked extracellularly by PCR. The unmarked deletion of glnK (ΔglnK) that is not polar on amtb was transduced from strain WCH30 into ET8000 selecting for gentamicin-resistant mutants. Candidate transductants were checked using PCR and the resultant strain was designated GT1002.

In vivo [3H]methylamine transport assays

These assays were performed as described previously using cells grown in M9Gln medium (Jack et al., 1999; Thomas et al., 2000b). [3H]Methylamine hydrochloride (2.15 Gbq/nmol) was obtained from Amersham Pharmacia, UK.

Western blotting

Protein concentrations of various cell fractions were determined using the Sigma Bradford reagent/Bio-Rad protein assay system using bovine serum albumin as a standard. In all cases, 5 μg of total protein was separated by either SDS–PAGE (15% polyacrylamide) or native PAGE (7.5% polyacrylamide). After transfer to a nitrocellulose membrane (Hybond ECL nitrocellulose membrane; Amersham), the proteins were reacted with either anti-Pu antibody (initially raised against purified E. coli GlnB protein, but also cross-reactive against E. coli and A. vinelandii GlnK), anti-AmtB antibody or anti-(tetra-His) antibody (Qiagen). Antibody against E. coli AmtB was a polyclonal antibody raised in rats against purified protein prepared in this laboratory (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K-findlay and M.Merrick, in preparation). A 100 μl aliquot of whole-cell extract from strain FT000 (AmpR, AmpS) was added routinely to the anti-Pu primary incubations to reduce non-specific interactions. Signals were then detected using the ECL system (Amersham).
Fractionation

A pre-culture grown in Luria broth was used to inoculate 500 ml of modified M9 medium, M9Gln (or Burk’s sucrose medium –N for Azotobacter) using a 1:50 dilution. The cells were grown to an OD660 of 1.3–1.4 when they were harvested by centrifugation at 5000 r.p.m. at 4°C for 10 min in an SLA-1500 rotor and resuspended in 0.75 ml of 50 mM sodium phosphate buffer pH 7.0. For ammonia shock experiments, an initial 11 culture was split into two cultures of 500 ml just prior to shock and one was treated by addition of NH4Cl to a final concentration of 30 mM and harvested 15 min later. Breakage was achieved by five 15 s sonications on ice in an MSE Soniprep 150 sonicator at an amplitude of 10 μm with 15 s intervals between bursts. The extract was clarified by centrifugation in a microfuge at 16,000 g for 4 min. A 1 ml aliquot of the supernatant was then transferred to an 11 x 34 mm polycarbonate ultracentrifuge tube (Beckman) and centrifuged at 250,000 g at 4°C for 30 min using a Beckman TL-120.2 rotor in a Beckman TL-100 ultracentrifuge; the remaining low-speed supernatant was used as the whole-cell fraction. After ultracentrifugation, the top 0.1 ml was taken from the tube and used as the cytoplasmic fraction; the rest of the supernatant was disposed of. The membrane pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer and centrifuged again at 250,000 g. The supernatant was discarded, and the membrane pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer and centrifuged again at 250,000 g. The pellet from this third centrifugation was resuspended in 50 μl of 50 mM sodium phosphate buffer and used as the membrane fraction. The purity of the membrane fractions was confirmed by western blotting with an antibody against a known cytoplasmic protein, namely pyruvate formate lyase.

To wash the membranes stringently, the cells were treated as above but after the third centrifugation the membrane pellet was resuspended in 50 μl of 10 mM Tris pH 8.0, centrifuged 3 times at 100,000 x g for 1 h, and centrifuged again at 250,000 g using the same high-salt buffer, after which the pellet was resuspended in 16 μl (or other suitable volume) of 50 mM sodium phosphate buffer.

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References


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