

## Ammonium Sensing in *Escherichia coli*

### ROLE OF THE AMMONIUM TRANSPORTER AmtB AND AmtB-GlnK COMPLEX FORMATION\*

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**The Amt proteins are high affinity ammonium transporters that are conserved in all domains of life. In bacteria and archaea the Amt structural genes (*amtB*) are invariably linked to *glnK*, which encodes a member of the P<sub>II</sub> signal transduction protein family, proteins that regulate many facets of nitrogen metabolism. We have now shown that *Escherichia coli* AmtB is inactivated by formation of a membrane-bound complex with GlnK. Complex formation is reversible and occurs within seconds in response to micromolar changes in the extracellular ammonium concentration. Regulation is mediated by the uridylylation/deuridylylation of GlnK in direct response to fluctuations in the intracellular glutamine pool. Furthermore under physiological conditions AmtB activity is required for GlnK deuridylylation. Hence the transporter is an integral part of the signal transduction cascade, and AmtB can be formally considered to act as an ammonium sensor. This system provides an exquisitely sensitive mechanism to control ammonium flux into the cell, and the conservation of *glnK* linkage to *amtB* suggests that this regulatory mechanism may occur throughout prokaryotes.**

The movement of ammonium across biological membranes is an important process in nearly all organisms from bacteria to man, and it is effected by a family of integral membrane proteins that is conserved throughout all domains of life (1, 2). The ammonium transporter (Amt)<sup>1</sup> protein family comprises the Amt/Mep proteins, found in bacteria, archaea, fungi, and plants, and the Rhesus proteins, which are found in animals from nematodes to man. The Amt/Mep proteins have a conserved core of 11 transmembrane helices with the C terminus always being intracellular (3, 4). They are typically 400–450 amino acids in length, although in some cases the C-terminal region is considerably extended, increasing their length to well over 500 residues, e.g. *Caenorhabditis elegans* Amt3 and *Synechocystis* Amt3. Purified *Escherichia coli* AmtB protein is a trimer, but the precise oligomeric status of Amt proteins from fungi and plants is not yet established (4–6). The Rhesus proteins have 12 transmembrane helices with both the N and C

termini being intracellular (7). They share considerable homology with the Amt proteins and have been demonstrated to function as ammonium transporters both by complementation of yeast *mep* mutants and by electrophysiology when expressed in *Xenopus* oocytes (2, 8, 9).

Almost all bacteria and archaea encode at least one Amt protein, and with very few exceptions, the structural gene (*amtB*) is linked genetically to a second gene (*glnK*) that encodes a small signal transduction protein (10, 11). GlnK is a member of the P<sub>II</sub> protein family, a group of trimeric proteins that act as sensors of the cellular nitrogen status in prokaryotes and that have also been identified in plants (11). They form a compact barrel around 50 Å in diameter and 30 Å high with a relatively unstructured loop (the T-loop) protruding from the upper surface (12). Residue Tyr-51, at the apex of the T-loop, is covalently modified by uridylylation in cells that are subject to nitrogen starvation, and the process is reversed in nitrogen sufficiency (13). The P<sub>II</sub> proteins have been shown to regulate the activities of a variety of other cytoplasmic proteins, both enzymes and transcription factors, by protein-protein interaction (11). *E. coli* encodes two P<sub>II</sub> proteins, GlnK and GlnB.

In those cases where it has been analyzed the *glnK* and *amtB* genes have been shown to form an operon. This transcriptional linkage led us to propose that the GlnK and AmtB proteins might physically interact to effect regulation of AmtB activity (10). We subsequently demonstrated that in *E. coli* and *Azotobacter vinelandii* GlnK is sequestered to the membrane in an AmtB-dependent fashion and that membrane binding of GlnK is modulated according to the cellular nitrogen status, being greatest in nitrogen-sufficient conditions (14). These data suggested that GlnK bound to AmtB when the cellular nitrogen status reached a certain level, so as to inhibit further ammonium transport. In this paper we now demonstrate that the two proteins do indeed form a complex. To be of physiological significance the model required that GlnK binding to AmtB should be sensitive, rapid, and reversible. We now report experiments that confirm all three of these properties. We also show that AmtB plays an active part in this process such that at physiologically relevant levels of ammonium sufficiency (around 50 μM) the transporter must be active to elicit GlnK deuridylylation and consequent sequestration. Hence the transporter is an integral part of the signal transduction cascade, and AmtB can be formally considered to act as an ammonium sensor.

The GlnK/AmtB system constitutes an exquisitely sensitive mechanism that allows the cell to regulate ammonium transport activity in precise accord with the metabolic need for ammonium. This report is the first demonstration of post-translational regulation of the activity of an Amt protein. We suggest that the need to regulate Amt protein activity is likely to apply in all organisms, and hence it is possible that eukary-

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<sup>1</sup> The abbreviations used are: Amt, ammonium transporter; GS, glutamine synthetase; GC/MS, gas chromatography/mass spectrometry; Ntr, nitrogen regulation.

TABLE I  
*E. coli* strains and plasmids used in this study

| Strain/plasmid         | Genotype or phenotype  | Ref./source |
|------------------------|--|-------------|
| <i>E. coli</i> strains |  |             |
| ET8000                 | <i>rbs lacZ::IS gyrA hutC<sub>k</sub></i>  | (42)        |
| FT8000                 | <i>rbs lacZ::IS gyrA hutC<sub>k</sub> ΔglnB1 ΔglnK1</i>                            | (43)        |
| GT1000                 | <i>rbs lacZ::IS gyrA hutC<sub>k</sub> ΔglnKamtB</i>                                | (14)        |
| GT1001                 | <i>rbs lacZ::IS gyrA hutC<sub>k</sub> ΔamtB</i>                                    | (3)         |
| ET8004                 | <i>rbs lacZ::IS gyrA hutC<sub>k</sub> glnA1885</i>                                 | (42)        |
| Plasmids <sup>a</sup>  |  |             |
| pAJ1001                | <i>glnK amtB1</i> in pUC9  | This work   |
| pAJ1002                | <i>glnK amtB1</i> in pBAD18  | This work   |
| pAJ1003                | <i>glnK11 (=glnKY51F) amtB1</i> in pUC9  | This work   |
| pAJ1004                | <i>glnK11 amtB1</i> in pBAD18  | This work   |
| pAJ2004                | <i>glnK amtB10 (=amtBD182E)</i> in pACYC184  | This work   |
| pAJ2006                | <i>glnK amtB11</i> derivative of pAJ2004 without the AmtB His <sub>6</sub> tag     | This work   |
| pAJ2008                | <i>glnK amtB12 (=amtBD182A)</i> derivative of pAJ2006                              | This work   |
| pBAD18                 | Medium copy number cloning vector  | (15)        |
| pESV0                  | Derivative of pACYC184 without EcoRI restriction site                              | This work   |
| pESV1                  | <i>glnK amtB8</i> (derivative of pESV0)  | This work   |
| pESV2                  | <i>amtB8</i> ( <i>ΔglnK</i> derivative of pESV1)                                   | This work   |
| pGC1                   | <i>glnK amtB</i> in pACYC184   | (14)        |
| pGC2                   | <i>glnK amtB1</i> in pACYC184  | (14)        |
| pGT104                 | <i>glnK amtB7</i> (derivative of <i>amtB3</i> with no internal NdeI site) in pUC19 | This work   |
| pJT1                   | <i>glnK</i> ( <i>ΔamtB</i> derivative of pGC1)                                     | This work   |
| pJT3                   | <i>glnK amtB7</i> ( <i>amtB7</i> derivative of pGC1)                               | This work   |
| pJT5                   | <i>glnK amtB8</i> (pJT3 derivative with some vector restriction sites deleted)     | This work   |
| pJT6                   | <i>glnK amtB9</i> (pJT5 derivative with PstI site near 5' of <i>amtB</i> )         | This work   |
| pMM285                 | <i>amtB3</i> in pT7-7  | (5)         |
| pTA43                  | <i>glnKY51F</i> in pWSK30  | (14)        |

<sup>a</sup> *amtB* alleles 3, 7, 8, 9, and 10 encode His<sub>6</sub>-tagged versions of the protein.

otic Amt or Rhesus proteins may also be regulated by interaction with intracellular signal proteins.

#### EXPERIMENTAL PROCEDURES

**Strains and Plasmids**—The bacterial strains and plasmids used are listed in Table I. *E. coli* strains were routinely grown in Luria medium, and for nitrogen-limited growth an M9 medium (M9Gln) was used in which ammonium was replaced by 200 μg/ml glutamine. The carbon source was 0.2% glucose or 0.4% glycerol plus 0.05% arabinose when working with pBAD plasmids (15). Ammonium shock was achieved by the addition of NH<sub>4</sub>Cl or [<sup>15</sup>N]NH<sub>4</sub>Cl (98 + % atom <sup>15</sup>N, Sigma-Aldrich) at the times and concentrations indicated. Antibiotics were used at the following concentrations: ampicillin 100 μg/ml, chloramphenicol 30 μg/ml.

Plasmid pAJ1002 was derived in two steps. The EcoRI-BamHI fragment encoding *glnKamtB1* from pGC2 was cloned into pUC9 to give pAJ1001, and the EcoRI-HindIII fragment from pAJ1001 was then cloned into EcoRI/HindIII-cut pBAD18 (15) to give pAJ1002. To construct pAJ1004 the EcoRV-BstEII fragment carrying *glnKY51F* (*glnK11*) from pTA43 was cloned into EcoRV/BstEII-cut pAJ1001 giving pAJ1003, and the SphI-BstXI fragment from pAJ1003 was cloned into SphI/BstXI-cut pAJ1002 to give pAJ1004. pGT104 contains the complete *glnKamtB7* operon cloned in pUC19. The *amtB7* allele is a derivative of *amtB3* from pMM285 in which the centrally encoded NdeI site has been removed by site-directed mutagenesis.

pJT1 contains only *glnK* and the *glnK-amtB* intergenic region with a new NdeI restriction site encompassing the initiation codon of *amtB*. PCR was performed with pGC1 as template and using two oligonucleotides: 5'-CTATGAAGCTGGTACCCTGAT-3' (BstEII site underlined) and 5'-GCGGATCCTATCTTCATATGTCGTTCC-3' (BamHI and NdeI sites underlined). The PCR fragment was cloned into BstEII-BamHI-cut pGC1 to give pJT1.

To construct pJT5, first the NdeI-BamHI from pJT1 was replaced by the NdeI-BamHI fragment from pGT104 to give pJT3. Second, the linker sequence (HindIII, EcoRV, EcoRI, PstI), which originates from pGT11 (5) and is located upstream of the *glnK* promoter in pJT3, was removed and replaced by a shorter linker (HindIII, SnaBI) to give pJT5. The linker was produced by annealing 5'-AGCTTTACGTAGTGCA-3' and 5'-CTACGTA-3'.

pJT6 was derived from pJT5 by adding a PstI site located over the codon for Ala25 of AmtB. This was performed by "overlap PCR," generating two overlapping mutated fragments and in a second PCR reaction the full-length mutated sequence. The two "cloning" oligonucleotides were 5'-CAGGAACGACATATGAAGATAG-3' and 5'-CGCGCGCC-

AGCGAGCTGGCGGCAAAAATCC-3' (NdeI and BstXI sites underlined). The mutagenic oligonucleotides were 5'-GTAATGGCTGCAC-CTGCAGTGGCCGATAAAG-3' and 5'-CTTTATCGGCCACTGCAGGTGCAGCCATTAC-3' (mutagenic bases in bold, PstI site underlined). The template was pJT5, and after digestion with NdeI and BstXI, the mutated fragment was cloned into NdeI-BstXI-cut pJT5 to give pJT6.

To construct pESV2, first the DraI fragment from pSU18 (16) replaced the equivalent fragment in pACYC184 to obtain an "EcoRI-free" version of the vector, designated pESV0. Second, the *glnKamtB8* HindIII-BamHI fragment from pJT5 was subcloned into pESV0 to give pESV1. Third, *glnK* was removed from pESV1 by replacing the EcoRI-NdeI fragment with a 16 bp linker containing the *amtB* Shine Dalgarno sequence. The linker was produced by annealing 5'-AATTCTGACCGGAGGGGACA-3' and 5'-TATGTCCCCTCCGGTCAG-3'.

Mutagenesis of AmtB was performed by overlap PCR using pJT6 as template. The cloning oligonucleotides were (forward) 5'-GCGCTGCA-GTGGCCGATAAAGCC GACAATGCGT-3' and reverse 5'-CGCGCGC-CAGCGAGCTGGCGGCAAAAATCC-3' (PstI and BstXI sites underlined). For D182E the mutagenic oligonucleotides were 5'-TCACAC-GGTGCGCTGGAATTCGCGGGTGTACC-3' (mutagenic bases in bold, DraIII site underlined) and 5'-GGTACCACCCGCAATTCAGCGCA-CCGTGTGA-3' (KpnI site underlined). The DraIII and KpnI sites allowed rapid preliminary screening for the new point mutation. The new restriction sites introduce silent mutations that do not alter the amino acid sequence. After digestion with PstI and BstXI, the mutated fragment was cloned into pJT6 to give pAJ2004 carrying *amtB10*. The His<sub>6</sub> tag was removed by replacing the PvuI-BamHI from pAJ2004 by the PvuI-BamHI fragment from pAJ1001 to give pAJ2006 carrying *amtB11*. The D182A mutant was constructed using the forward "cloning" and the mutagenic oligonucleotide 5'-CACGGTACCACCCGCGAAAGCCAGCGCACCGTGTGA-3' (KpnI site underlined). After digestion with PstI and KpnI, the mutated fragment was cloned into pAJ2006 to give pAJ2008 carrying *amtB12*.

**In vivo [<sup>14</sup>C]Methylamine Transport Assays**—These were performed as described previously using cells grown in M9Gln medium (3, 17). [<sup>14</sup>C]methylamine hydrochloride (2.15 Gbq/mmol) was obtained from Amersham Biosciences.

**Fractionation**—Cells were fractionated according to the procedures described previously (14), and membrane fractions were always subjected to two high salt (600 mM NaCl) washes prior to analysis.

**In Vivo Interaction between AmtB and GlnK**—A 1-ml aliquot of membrane extract was prepared from 250 ml of culture grown in M9Gln and subjected to an ammonium shock by the addition of NH<sub>4</sub>Cl to a final concentration of 30 mM for 15 min. AmtB6H and GlnK were co-purified

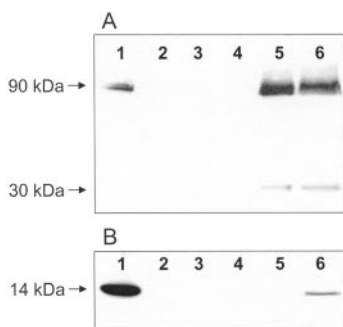


FIG. 1. *In vivo* interaction between GlnK and AmtB. Strains used were: lane 2, wild type (ET8000); lane 3, *glnK*<sup>+</sup>  $\Delta$ *amtB* (GT1001); lane 4,  $\Delta$ *glnK*  $\Delta$ *amtB* (GT1000); lane 5,  $\Delta$ *glnK* *amtB8* (GT1000 pESV2); lane 6, *glnK*<sup>+</sup> *amtB9* (GT1000 pJT6). Both *amtB8* and *amtB9* encode AmtB6H. Extracts were incubated with His Mag<sup>TM</sup> agarose beads and washed extensively with buffer containing 15 mM imidazole, and proteins bound to the beads were eluted with 500 mM imidazole, and protein samples were then analyzed by Western blotting using anti-His (A) or anti-GlnK (B) antibody. Both the trimeric (90 kDa) and monomeric (30 kDa) forms of AmtB are detected in extracts. Lane 1 contained 0.3 μg of purified *E. coli* AmtB (A) or GlnK (B).

from the membrane extract using His Mag<sup>TM</sup> Agarose Beads (Novagen). The binding buffer used was 60 mM CaCl<sub>2</sub>, 40 mM K<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 5 mM imidazole, pH 8. The imidazole concentration was 15 mM in the wash buffer and 500 mM in the elution buffer.

**Protein Purification**—GlnK from *E. coli* was purified as described previously (18) and stored in 50 mM Tris-HCl, 50% v/v glycerol, pH 7.6. *E. coli* AmtB was purified as described previously (5) and used to raise polyclonal anti-rabbit antibodies.

**Western Blotting**—Western blotting was performed as described previously (14). Proteins were reacted with an appropriate antibody: anti-P<sub>II</sub> antibody, raised against purified *E. coli* GlnB protein but also cross-reactive against *E. coli* GlnK (18); anti-*E. coli* GlnK and anti-*E. coli* GlnB antibodies (19, 20); anti-*E. coli* glutamine synthetase (GS) antibody (from W. van Heeswijk); anti-His antibody (Qiagen); anti-AmtB (this work).

**Amino Acid Extraction and Analysis**—Amino acids were extracted twice from 2 ml of overnight culture using cold methanol, which prevents metabolic depletion of the glutamine pools (21). The GC/MS analysis was performed as described previously (22). The data quoted for each extraction are the means of four independent analyses.

## RESULTS

**Evidence for *in Vivo* Complex Formation between GlnK and AmtB**—The highly conserved genetic linkage between the *glnK* and *amtB* genes in both eubacteria and archaea (10) and the demonstration of AmtB-dependent membrane sequestration of GlnK after an “ammonium shock” led us to propose that GlnK interacts physically with AmtB to regulate the activity of the transporter (14). To investigate the proposed interaction, membrane fractions were prepared from cells grown under nitrogen-limiting conditions and then subjected to an ammonium shock with 30 mM NH<sub>4</sub>Cl for 15 min (subsequently referred to as “high” ammonium shock). The strains used were ET8000 (wild type), GT1001 (*glnK*<sup>+</sup>  $\Delta$ *amtB*), GT1000 ( $\Delta$ *glnK*  $\Delta$ *amtB*), and GT1000 carrying pESV2 (*amtB8*) or pJT6 (*glnK* *amtB9*). Both *amtB8* and *amtB9* encode C-terminally His<sub>6</sub>-tagged AmtB (AmtB6H). The extracts were incubated with His-Mag<sup>TM</sup> agarose beads and washed extensively, and then the bound proteins were eluted with 500 mM imidazole buffer. The protein samples were analyzed by Western blotting using specific anti-GlnK and anti-His antibodies. No signals were detected in the 15 mM imidazole wash using either antibody (data not shown).

As expected AmtB6H was detected only in extracts from strains carrying *amtB8* or *amtB9* (Fig. 1A, lanes 5 and 6). As previously shown (5) AmtB is predominantly detected as a trimer with an apparent molecular mass of 90 kDa; the monomer (30 kDa) is a minor species. GlnK was detected only in the eluate when AmtB6H was co-expressed (Fig. 1B, lane 6) and no

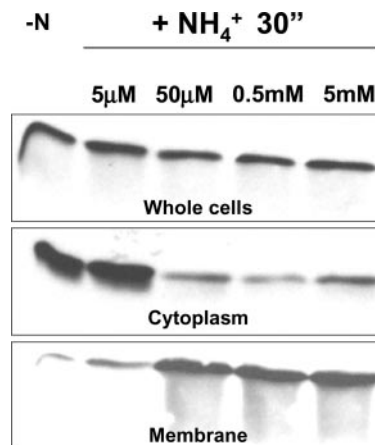


FIG. 2. Sequestration of GlnK by AmtB is sensitive and rapid. Whole cell extracts and cytoplasmic and membrane fractions were subjected to SDS-PAGE followed by Western blotting using anti-P<sub>II</sub> antibody. Extracts were prepared from wild type cells (ET8000) grown in M9Gln. Lanes show extracts before shock (–N) and 30 s after ammonium addition to various final concentrations.

GlnK was found in eluates from wild type or *glnK*<sup>+</sup>  $\Delta$ *amtB* cells (Fig. 1B, lanes 2 and 3) indicating that GlnK itself has no ability to interact with the beads. This result was also confirmed using purified GlnK (data not shown). Co-purification of AmtB6H and GlnK from GT1000(pJT6) extracts was also demonstrated using Ni<sup>2+</sup> affinity chromatography (data not shown). These results confirm that AmtB interacts with GlnK *in vivo*.

Western blot analysis of the  $\Delta$ *glnK* strain GT1000(pESV2) using anti-P<sub>II</sub> instead of anti-GlnK antibodies showed that GlnB can interact with AmtB when GlnK is absent. However, using protein-specific antibodies to estimate the relative amounts of GlnK and GlnB, we have shown that in nitrogen-limited cells the intracellular levels of GlnK are at least 500-fold greater than those of GlnB.<sup>2</sup> Consequently we consider that in wild type cells GlnB will show little, if any, interaction with AmtB.

**Sequestration of GlnK by AmtB Is Rapid and Responds to μM Ammonium Concentrations**—We have demonstrated that GlnK interacts with AmtB in response to elevation of the extracellular ammonium concentration to 30 mM for a period of 15 min. However, given that the *K<sub>m</sub>* of AmtB for ammonium is around 10 μM (23), we hypothesized that the role of GlnK is to regulate AmtB such that it is only active when the extracellular ammonium concentration is in the low μM range. In this case we would expect that GlnK sequestration by AmtB should occur in response to a relatively “low” ammonium shock. To provide the necessary fine-tuning of AmtB activity the response should also be rapid.

We therefore used the wild type strain ET8000 to examine the speed and sensitivity of the process. Cells were grown in M9Gln and then subjected to ammonium shock at a range of concentrations. Samples were taken prior to the addition of ammonium and after just 30 s of exposure to ammonium; all were frozen immediately with liquid nitrogen. We monitored whole cells, cytoplasm, and membrane fractions for the presence of GlnK by Western blots. There was a very significant increase in the amount of GlnK associated with the membrane after a 50 μM NH<sub>4</sub><sup>+</sup> shock, in contrast to a 5 μM NH<sub>4</sub><sup>+</sup> shock (Fig. 2). These experiments confirm that GlnK sequestration by AmtB is extremely rapid and is sensitive to μM concentrations of ammonium.

<sup>2</sup> A. Javelle and M. Merrick, unpublished data.

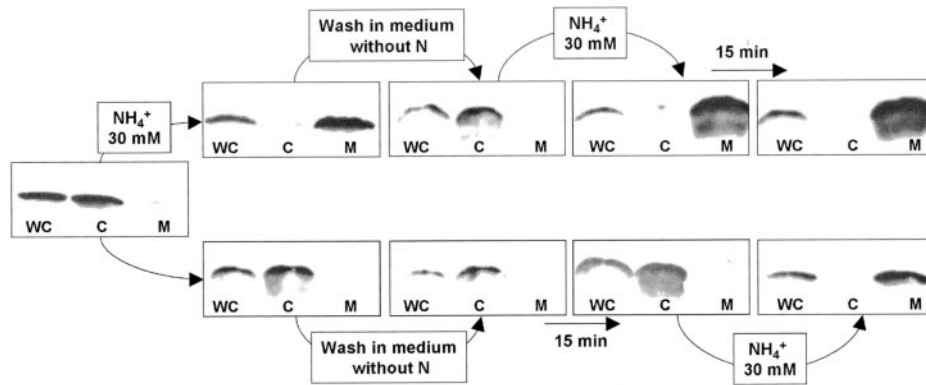


FIG. 3. **Sequestration of GlnK by AmtB is reversible.** Wild type cells (ET8000) grown in M9Gln. *Upper panels*, cells subject to high ammonium shock (30 mM for 15 min) were washed, resuspended in medium without nitrogen, and sampled after 5 min. They were then subjected to a repeat high ammonium shock, sampled after 15 min and again 15 min later. *Lower panels*, control. Cells were not shocked but were still washed and resuspended in medium without nitrogen to ensure that washing has no effect on GlnK-AmtB association. These cells then received a high ammonium shock. At each step, samples were frozen immediately with liquid nitrogen, and GlnK was identified by Western blotting SDS-polyacrylamide gels using anti-P<sub>II</sub> antibody.

*Sequestration of GlnK by AmtB Is Reversible*—The P<sub>II</sub> family of signal transduction proteins are characterized by their ability to regulate the activities of other proteins by protein-protein interaction in a reversible manner (11). Consequently we wished to determine whether binding of GlnK to AmtB was also reversible. As previously described, wild type (ET8000) cells were grown in M9Gln and then subjected to a high ammonium shock; they were washed and then resuspended in a medium without any nitrogen source. In parallel, to confirm that the washing step itself had no effect on GlnK-AmtB association, a control was used in which the cells were washed and resuspended in a medium without any nitrogen source before the high ammonium shock. After each treatment cells were frozen with liquid nitrogen, and the sequestration of GlnK by AmtB was again examined by Western blotting. When the ammonium shock was followed by a washing step to remove the nitrogen source rapidly, there was a very significant decrease in the amount of GlnK associated with the membrane, and GlnK was relocated to the cytoplasm (Fig. 3). Controls showed that the wash itself had no effect and that after a second ammonium shock GlnK was relocated to the membrane (Fig. 3). Consequently it is clear that the process is rapidly reversible and responds to the nitrogen source.

*Binding of GlnK to AmtB Is Dependent on Its Uridylylation State*—As GlnK is a trimer, the protein can exist in four states depending upon the number of subunits that are uridylylated. Ammonium shock causes deuridylylation of GlnK, and the uridylylation states of GlnK trimers can be assessed on native polyacrylamide gels (14). We previously reported that membrane association of GlnK reflects the uridylylation state of the protein (14), and we reasoned that the uridylylation state of GlnK should govern its binding to AmtB. We therefore examined localization of GlnK in cells expressing a variant of the protein, which cannot be uridylylated. GlnK is uridylylated on residue Tyr-51 at the apex of the T-loop (24, 25), and we therefore constructed a Y51F variant of GlnK (encoded by *glnK11*) and expressed both a *glnK11amtB* (on pAJ1004) and a *glnKamtB* (on pAJ1002) operon in the  $\Delta$ *glnK* $\Delta$ *amtB* strain (GT1000) under control of the P<sub>BAD</sub> promoter. We used this inducible promoter to avoid any potential effects of GlnKY51F on *glnKamtB* expression.

The uridylylation state of GlnK in whole cell lysates was investigated by native PAGE (Fig. 4A). In GT1000(pAJ1002) grown under nitrogen limitation, one major band was detected corresponding to the fully uridylylated form of GlnK (GlnK-UMP<sub>3</sub>); following ammonium shock four bands were revealed, the major one being the non-uridylylated form of GlnK (Fig.

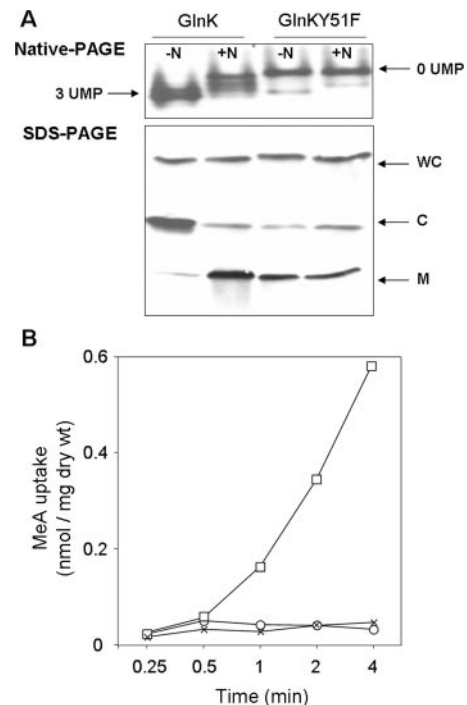


FIG. 4. **Binding of GlnK to AmtB regulates the activity of the transporter.** A, whole cell extracts (WC) and cytoplasmic (C) and membrane (M) fractions were prepared from GT1000(pAJ1002) and GT1000(pAJ1004) expressing GlnK and GlnKY51F, respectively, both before (–N) and after (+N) high ammonium shock. Extracts and fractions were subjected to native and SDS-PAGE, respectively, followed by Western blotting with anti-P<sub>II</sub> antibody. Native-PAGE reveals all four forms of GlnK (UMP<sub>0</sub>–UMP<sub>3</sub>). B, [<sup>14</sup>C]methylammonium (MeA) transport activities for strains GT1000 ( $\Delta$ *glnK* $\Delta$ *amtB*) (○), GT1000(pAJ1004) ( $\Delta$ *glnK* $\Delta$ *amtB*) (*glnK11amtB*<sup>+</sup>) (×), and GT1000(pAJ1002) ( $\Delta$ *glnK* $\Delta$ *amtB*) (*glnK*<sup>+</sup> *amtB*<sup>+</sup>) (□).

4A). On the other hand, GT1000(pAJ1004) cells showed only non-uridylylated GlnK both pre- and post-ammonium shock (Fig. 4A). The ability of GlnKY51F to bind to AmtB was examined by running cellular fractions on SDS-PAGE, and contrary to wild type GlnK, GlnKY51F was found to be bound to AmtB both pre- and post-ammonium shock (Fig. 4A). Taken together these results show that the uridylylation state of GlnK does indeed govern its binding to AmtB.

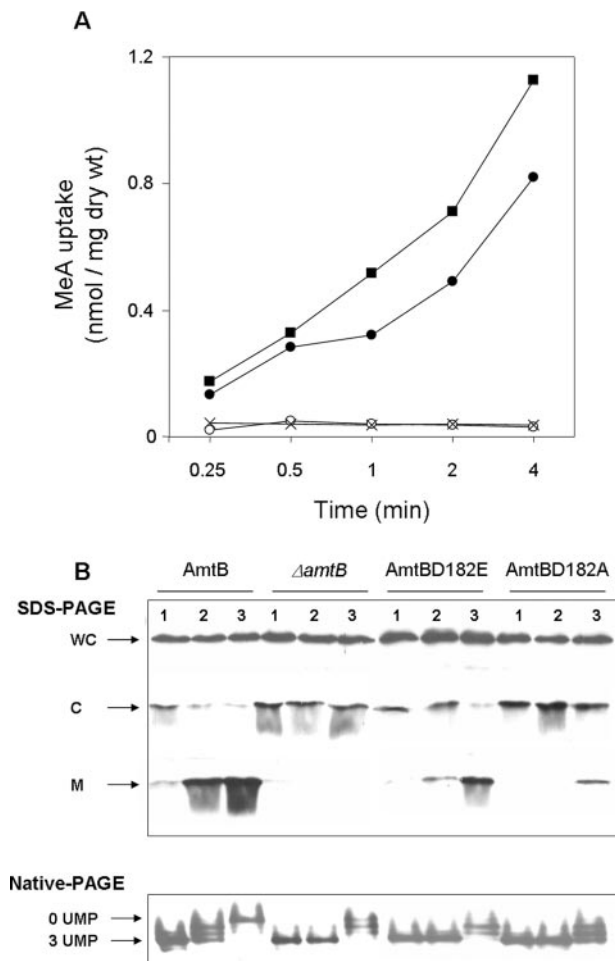
*Binding of GlnK to AmtB Regulates the Activity of the Transporter*—It has been suggested previously that the interaction between GlnK and AmtB negatively regulates the transport

activity of AmtB (14). However in those experiments, the transported substrate was [ $^{14}$ C]methylammonium, an analogue of ammonium. The transport of methylammonium by AmtB is competitively inhibited by  $\mu$ M concentrations of ammonium. Therefore it was not previously possible to assess the effects of GlnK on methylammonium transport under conditions in which its interaction with AmtB is maximal, *i.e.* after ammonium shock. Having shown that the GlnKY51F protein behaves as if it is permanently locked in the AmtB-bound state, it was possible to measure [ $^{14}$ C]methylammonium transport in cells that simulate ammonium shock conditions. We carried out a series of experiments comparing the transport activity of AmtB in the  $\Delta$ *glnK* $\Delta$ *amtB* background (GT1000) expressing either GlnK and AmtB or GlnKY51F and AmtB. We used Western blots to compare the levels of AmtB in whole cell extracts of GT1000 carrying pAJ1002 (*glnK*<sup>+</sup>) and pAJ1004 (*glnK11*) and found no significant differences (data not shown). However, when compared with wild type GlnK the presence of GlnKY51F completely inhibited AmtB activity, which was reduced to the background level observed in the  $\Delta$ *glnK* $\Delta$ *amtB* strain (GT1000) (Fig. 4B). Hence, AmtB is inactive in the presence of GlnKY51F, demonstrating that deuridylylated GlnK inhibits the ammonium flux through the transporter by protein-protein interaction.

**Ammonium Transport through AmtB Is Required for GlnK Deuridylylation**—The demonstration that the uridylylation state of GlnK governs its binding to AmtB raises the question of whether ammonium binding to and/or transport through AmtB is necessary to propagate the signal that leads to uridylylation of GlnK. Residue Asp-182 (between TM IV and V) is highly conserved in the Amt proteins of bacteria, fungi, and plants (3, 22, 26, 27). An aspartic acid residue (Asp-50) is found at the ammonium binding site of *E. coli* glutamine synthetase (28), and by analogy Thomas *et al.* (3) suggested that residue Asp-182 of AmtB is a potential candidate for an initial ammonium binding site within the transporter.

We therefore constructed two mutant versions of *amtB* encoding conservative (D182E, *amtB11*) and nonconservative (D182A, *amtB12*) substitutions of this residue and introduced them into the *glnKamtB* operon giving pAJ2006 and pAJ2008, respectively. We then compared the transport activities of the different versions of AmtB when the plasmids were introduced into the  $\Delta$ *glnK* $\Delta$ *amtB* strain GT1000. To confirm independently that the levels of AmtB are unaffected by the mutations, we used Western blots to assess the levels of AmtB present in whole cell extracts of each strain and found no significant differences (data not shown). When compared with wild type the activities of AmtBD182E and AmtBD182A were 71 and 0%, respectively (Fig. 5A). These results support the hypothesis that residue Asp-182 plays a crucial role in the binding of ammonium and/or its transport through AmtB.

We then asked whether transport activity of AmtB was required to induce the GlnK-AmtB interaction by examining sequestration of GlnK by each variant of AmtB. We reasoned that the most physiologically relevant treatment would be to use a 50  $\mu$ M shock for 30 s. The wild type strain ET8000 showed the expected deuridylylation of GlnK and consequent binding to AmtB, but in strains lacking AmtB (GT1001) or expressing AmtBD182A (GT1000 with pAJ2008) GlnK remained fully uridylylated and localized to the cytoplasm (Fig. 5B). In GT1000 with pAJ2006, which expresses AmtBD182E, the partial activity of AmtB apparently allowed some complex formation, and a significant amount of GlnK was found in the membrane fraction. These data indicate that when *E. coli* experiences a transient increase in extracellular ammonium to a level at which the metabolic demand for ammonium is exceeded, the conse-

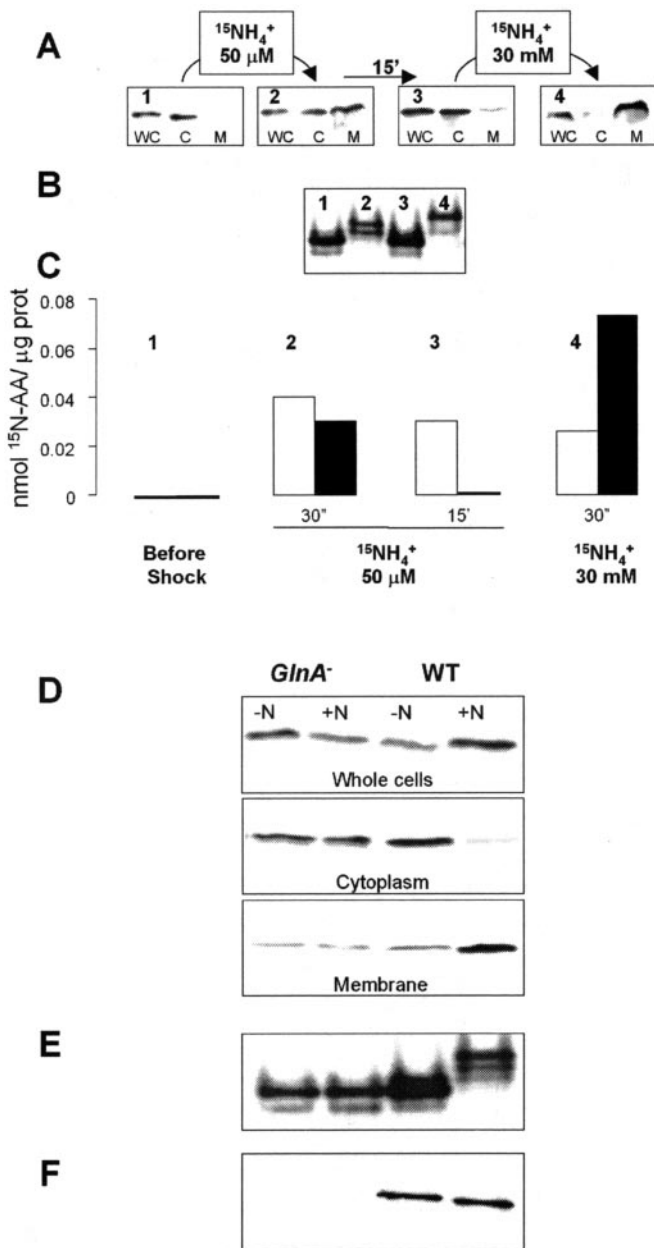


**FIG. 5. Ammonium transport through AmtB is required for GlnK sequestration.** A, [ $^{14}$ C]methylammonium transport activities for GT1000(pJT6) (*glnK*<sup>+</sup> *amtB*<sup>+</sup>) (■), GT1000 ( $\Delta$ *glnK* $\Delta$ *amtB*) (○), GT1000(pAJ2006) ( $\Delta$ *glnK* $\Delta$ *amtB*) (*glnK*<sup>+</sup> *amtB11*) (●), and, GT1000(pAJ2008) ( $\Delta$ *glnK* $\Delta$ *amtB*) (*glnK*<sup>+</sup> *amtB12*) (×). B, whole cell extracts (WC) and cytoplasmic (C) and membrane (M) fractions were prepared from strains ET8000 (*amtB*<sup>+</sup>), GT1001 ( $\Delta$ *amtB*), GT1000(pAJ2006) expressing AmtBD182E, and GT1000(pAJ2008) expressing AmtBD182A before (1) and after ammonium shock at 50  $\mu$ M for 30 s (2) or 30 mM for 15 min (3). Extracts and fractions were subjected to native and SDS-PAGE, respectively, followed by Western blotting with anti-P<sub>II</sub> antibody.

quent deuridylylation of GlnK and inactivation of AmtB is dependent on the activity of the transporter.

In previous studies in which we used a high ammonium shock, we observed deuridylylation of GlnK protein in an *amtB* deletion (14). We therefore repeated and extended those studies by examining the results of a high ammonium shock on each of the AmtB variant strains. We observed GlnK-AmtB complex formation with both variants, but AmtBD182A showed significantly less GlnK sequestration than AmtBD182E and wild type (Fig. 5B).

**The Level of Intracellular Glutamine Is the Signal That Controls GlnK-AmtB Interaction**—P<sub>II</sub> proteins typically function to regulate the activity of those proteins with which they interact in response to the nitrogen status of the cell. Previous studies have concluded that the intracellular glutamine concentration is the main effector of this response in that it regulates the activity of the uridylyltransferase protein and, thereby, the uridylylation state of GlnB or GlnK (for review see Ref. 11). An *in vitro* analysis of the factors that regulate the activity of uridylyltransferase concluded that the binding of glutamine to a single site on the enzyme is responsible for both the inhibition



**FIG. 6. The level of intracellular glutamine is the signal that controls GlnK sequestration.** Wild type strain ET8000 grown in M9Gln (panel 1) was subjected to ammonium shock with 50  $\mu\text{M}$   $^{15}\text{NH}_4^+$  and sampled after 30 s (panel 2) and again after 15 min (panel 3) and then finally subjected to a second shock with 30 mM  $^{15}\text{NH}_4^+$  and sampled after 15 min (panel 4). Whole cell extracts (WC) and cytoplasmic (C) and membrane (M) fractions were subjected to SDS-PAGE (A) and native PAGE (B) followed by Western blotting with anti- $\text{P}_{\text{II}}$  antibody. Concentrations of  $^{15}\text{N}$ glutamate (white bars) and  $^{15}\text{N}$ glutamine (black bars) within the cells were measured on the same samples (C). Extracts from strains ET8000 (wild type) and ET8004 (*glnA*<sup>-</sup>) were subjected to SDS-PAGE (D) and native PAGE (E) before (-N) and after (+N) high ammonium shock and analyzed by Western blotting with anti- $\text{P}_{\text{II}}$ . Whole cell extracts were also subjected to SDS-PAGE and analyzed by Western blotting with anti-GS antibody (F).

of the uridylyltransferase activity and the stimulation of uridylyl-removing activity (13). However we considered it important to provide further supporting evidence that fluctuations in the glutamine pool *in vivo* were the signal for regulation of AmtB activity. We wished to determine whether the kinetics of *in vivo* changes in the intracellular glutamine pool were consistent with the kinetics of AmtB-GlnK association and to confirm that ammonium assimilation is required to

generate the signal. These experiments should exclude the possibility that ammonium plays any direct role in the deuridylylation of GlnK and its sequestration by AmtB.

For this experiment, the effects of a 50  $\mu\text{M}$  followed by a 30 mM  $^{15}\text{N}$ ammonium shock on the sequestration of GlnK by AmtB in the wild type strain (ET8000) were examined. We monitored GlnK by running cellular fractions with both SDS-PAGE (Fig. 6A) and native PAGE (Fig. 6B), and we also measured the concentrations of  $^{15}\text{N}$ glutamate and  $^{15}\text{N}$ glutamine within the cells using the same samples (Fig. 6C). In response to a 50  $\mu\text{M}$  ammonium shock, the  $^{15}\text{N}$ glutamine pool increased dramatically by 30-fold after 30 s, and as found previously, GlnK was rapidly deuridylylated and sequestered to the membrane (Fig. 6, A–C). Analysis of GC/MS data showed that 100% of the glutamine pool was labeled with  $^{15}\text{N}$  after the shock. After 15 min the newly synthesized glutamine was completely metabolized, the  $^{15}\text{N}$  label appeared in other amino acids, the  $^{15}\text{N}$ glutamine pool returned to its basal level (0.0001 nmol/ $\mu\text{g}$  protein), and GlnK was uridylylated again. A second high ammonium shock generated the same response as the first one, namely a rapid rise in the glutamine pool and a concomitant membrane sequestration of GlnK. The  $^{15}\text{N}$ glutamate level was much less responsive and stayed essentially stable between the two shocks (Fig. 6C).

These results are in complete accord with the hypothesis that the intracellular glutamine level is the main effector of the process, but they do not rule out a role of ammonium. To address this possibility we examined sequestration of GlnK as well as its uridylylation state in response to high ammonium shock using a *glnA* mutant strain (ET8004) in which no signal is detectable by Western blotting with a glutamine synthetase-specific antibody (Fig. 6F). In this strain no  $^{15}\text{N}$ glutamine was detected after the shock, whereas the  $^{15}\text{N}$ glutamate level increased 3-fold (from 0.003 nmol/ $\mu\text{g}$  protein to 0.01 nmol/ $\mu\text{g}$  protein; not shown in Fig. 6). The ammonium shock did not cause deuridylylation of GlnK, and there was no GlnK association to AmtB (Fig. 6, D and E). Taken together these results demonstrate that the deuridylylation of GlnK and the consequent GlnK-AmtB association is due solely to the increase in the intracellular glutamine level and not to an increase in intracellular ammonium.

#### DISCUSSION

The remarkable conservation of genetic linkage between the structural genes encoding the ammonium transporter AmtB and the signal transduction protein GlnK originally led us to propose that these two proteins were functionally related in both the eubacteria and the archaea (10). Subsequent demonstration that in both *E. coli* and *A. vinelandii* GlnK could be sequestered to the membrane in an AmtB-dependent fashion was consistent with the hypothesis that the two proteins could form a complex; but the underlying physiological parameters and the metabolic consequences remained to be elaborated (14).

We have now proved that GlnK does indeed form a complex with AmtB and that the complex forms rapidly (within 30 s) when cells growing under nitrogen-limiting conditions are presented with as little as 50  $\mu\text{M}$   $\text{NH}_4^+$  in the external medium. The observation of such coordinated rapid *in vivo* responses of bacteria to sudden small changes in the cellular nitrogen status has not previously been reported. Earlier studies in *E. coli* and in *Salmonella typhimurium* demonstrated that nitrogen-limited cells respond to an ammonium up-shift by a rapid increase in the size of the intracellular glutamine pool. However those studies utilized additions of 10 mM  $\text{NH}_4^+$  and assessed the pool size up to 10 min after the addition (29, 30). We have now shown that the glutamine pool responds rapidly to much smaller fluctuations in external ammonium concentration.

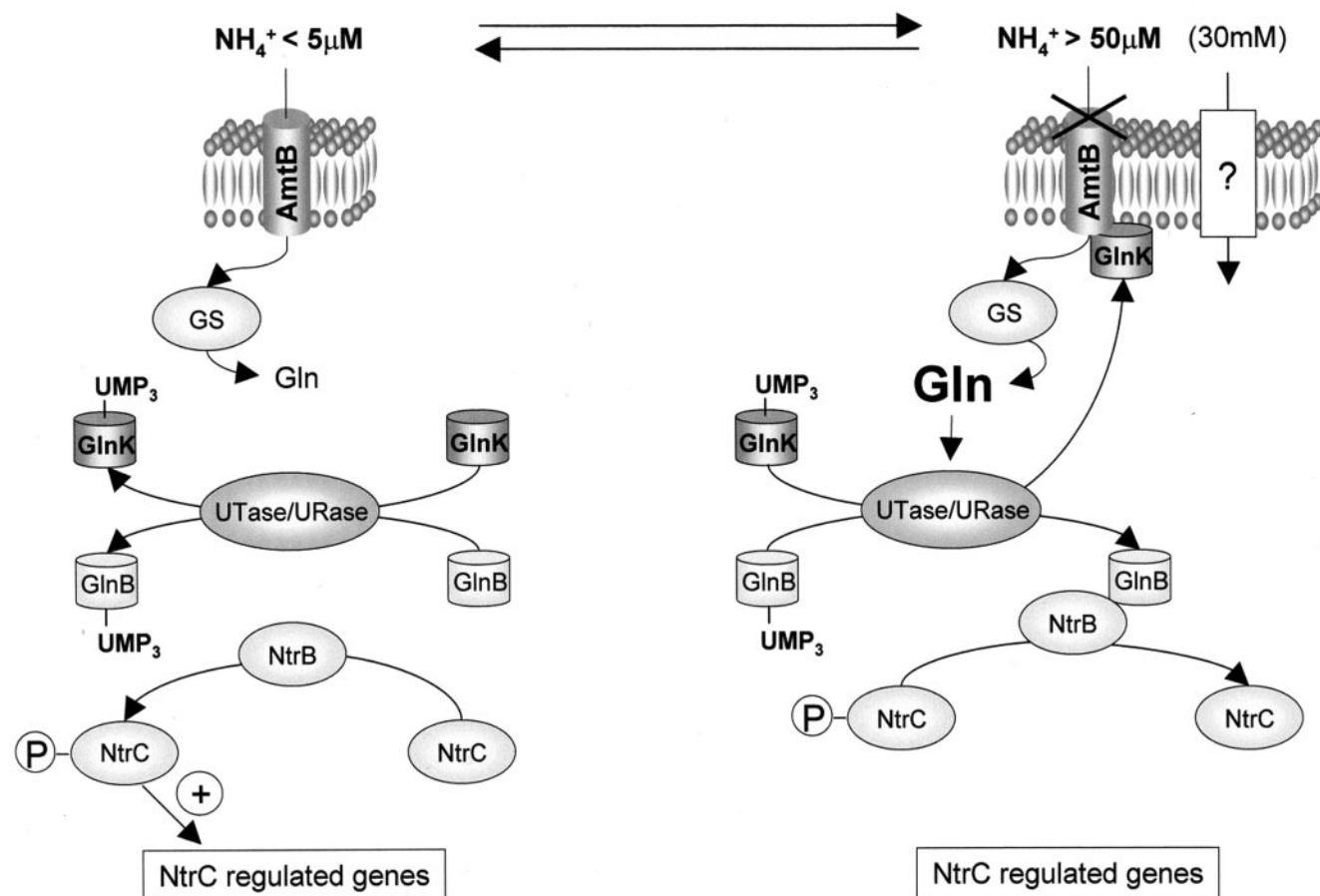


FIG. 7. Model for functions of AmtB and GlnK in ammonium transport and their integration into the Ntr system of *E. coli*. When extracellular  $\text{NH}_4^+$  concentrations are around  $5 \mu\text{M}$  or less, ammonium enters the cell via AmtB and is converted by glutamine synthetase (GS) to glutamine, which is utilized in metabolism. The intracellular glutamine pool is low, and uridylyltransferase (UTase) uridylylates both GlnK and GlnB. NtrB phosphorylates NtrC, and NtrC-dependent gene expression is activated. When extracellular  $\text{NH}_4^+$  concentrations rise above  $\sim 50 \mu\text{M}$ , the metabolic demand for glutamine is exceeded, the intracellular glutamine pool rises, and uridylyltransferase deuridylylates GlnK and GlnB. GlnK complexes with AmtB, thereby inhibiting the transporter. GlnB interacts with NtrB and activates its phosphatase activity leading to dephosphorylation of NtrC, and NtrC-dependent gene expression ceases. At high extracellular  $\text{NH}_4^+$  concentrations (e.g. 30 mM), ammonium enters the cell either by an unidentified low affinity transport system or by free diffusion.

These experiments are limited by the technical feasibility of obtaining accurate data at shorter time points, but we consider it likely that the metabolic response is even more rapid than reported here.

We observed deuridylylation of GlnK and the concomitant formation of a complex between AmtB and GlnK within 30 s of ammonium addition. This is consistent with the predicted inhibition of the uridylyltransferase activity of GlnD and activation of its uridylyl-removing activity in response to an elevated glutamine pool (13). These changes were completely dependent on ammonium assimilation, and by studying a variant of GlnK that is locked in the deuridylylated state we showed that the consequence of GlnK-AmtB complex formation is the inactivation of AmtB. We conclude that this system constitutes an exquisitely rapid and sensitive post-translational control of ammonium uptake.

Other researchers (24, 25) have reported conflicting data on the rate of deuridylylation of *E. coli* GlnK. Experiments on GS adenylation led to the conclusion that the reversible modification of GlnK *in vivo* was very rapid (24). However subsequent *in vitro* studies concluded that deuridylylation of GlnK-UMP is very slow and that rapid uridylylation/deuridylylation of GlnK is not necessary for the function of the protein (25). We cannot reconcile those data with our observations, but we suggest that *in vivo* the relative concentrations and cellular locations of GlnK and GlnB may have significant effects on their covalent

modification. The rapid reversible modification of GlnK that we report here is certainly consistent with both the rapid fluctuations in the intracellular glutamine pool and the speed of AmtB-GlnK complex formation.

The high ammonium shock that involves transition from nitrogen starvation to 30 mM  $\text{NH}_4^+$  is probably unlikely to be experienced by *E. coli* in its natural habitat. The *glnKamtB* operon is not expressed in high ammonium medium because of transcriptional control by the nitrogen regulation (Ntr) system (24). Hence control of AmtB activity is almost certainly of most physiological significance when *E. coli* cells are subjected to transient small elevations in the external ammonium concentration, e.g.  $5\text{--}50 \mu\text{M}$   $\text{NH}_4^+$ .

Strikingly, when we used a  $50 \mu\text{M}$   $\text{NH}_4^+$  shock it was clear that GlnK deuridylylation was completely dependent on AmtB activity, indicating that in this situation ammonium enters the cell solely through the action of AmtB. Elevation of the intracellular glutamine pool is therefore intimately coupled to AmtB activity. These experiments confirm that AmtB acts as a sensor of the extracellular ammonium concentration and lead us to propose that AmtB and GlnK should be considered as integral components of the Ntr system, which until now has been characterized by four proteins: uridylyltransferase GlnD; the signal transduction protein GlnB; and the two-component regulatory system, NtrB/NtrC. The Ntr system has previously been considered only as a monitor of the intracellular nitrogen status,

but the inclusion of AmtB and GlnK rationally integrates the sensing of the extracellular ammonium status into the system (Fig. 7).

Amt proteins have been suggested to act as ammonium sensors in other systems, namely ADP-ribosylation of nitrogenase in *Rhodobacter capsulatus* (31) and pseudohyphal growth, which occurs in a number of fungi in response to nitrogen limitation (32–34). Formal linkage of the transporter to a signal transduction system has yet to be demonstrated in those systems, although P<sub>II</sub> proteins have been implicated in regulating ADP-ribosylation (31, 35).

Deuridylylation of GlnK after a 30 mM NH<sub>4</sub><sup>+</sup> shock, in either an AmtB (D182A) strain or an *amtB* deletion, suggests that in this case ammonium enters the cell either by free diffusion of NH<sub>3</sub> or by NH<sub>4</sub><sup>+</sup> uptake through an as yet unidentified low affinity ammonium transporter (Fig. 7). Based on physiological data, low affinity transport proteins have been proposed in a number of systems, particularly in plants (36), but whether such a low affinity system is present in *E. coli* is not known.

The data presented in this paper reinforce our earlier proposal that the regulation of AmtB activity is the primary cellular role of GlnK in *E. coli*, and they lend credence to the concept that this is the primary function of all GlnK proteins encoded in a *glnKamtB* or *amtBglnK* operon (14). Other authors (24, 37, 38) have suggested a variety of alternative functions for GlnK, at least in *E. coli*. These possible functions of GlnK include regulation of the expression level of NtrC-P during severe nitrogen starvation (24, 37, 38), and action as a so called “memory protein” that serves to prevent inappropriate termination of the stress response by a transient improvement in the environment (18). Most recently it has been suggested that one function of GlnK is to prevent AmtB-mediated antagonism of GlnB signaling after nitrogen starvation (39). However these suggestions are in general only applicable in those organisms like *E. coli* that also have an Ntr system. Furthermore, these hypotheses are primarily derived from studies of *glnK* mutants (18, 25, 38) in which the absence of GlnK causes GlnB to be unnaturally sequestered to the membrane by AmtB. We propose that in wild type *E. coli* GlnK and GlnB normally have quite distinct functions, GlnK being concerned with AmtB regulation and GlnB with control of NtrB (Fig. 7).

A direct consequence of GlnK sequestration by AmtB is a rapid reduction in the intracellular GlnK pool that will have direct effects on intracellular processes controlled by GlnK. GlnK has been reported to be a potent activator of NtrB phosphatase activity *in vitro* and has therefore been suggested to play a role in “fine-tuning” the Ntr system (25). However, as the intracellular concentration of GlnK in nitrogen-limited cells is some 500-fold greater than that of GlnB, deuridylylation of GlnK in response to an ammonium shock could lead to rapid activation of NtrB phosphatase. The consequent dephosphorylation of NtrC would rapidly impair transcription of promoters that require high NtrC-P concentrations, including the promoter of the *glnK amtB* operon. We consider that this is unlikely to occur because of the sequestration of deuridylylated GlnK by AmtB, which will modulate the otherwise rapid inactivation of NtrC-P and thereby buffer the Ntr system against extreme fluctuations in response to small and transient changes in extracellular ammonium concentrations. Similar systems in which membrane sequestration is used to control the intracellular pool of a bacterial regulatory protein have been described recently, one example being the sequestration of the *E. coli* transcriptional regulator Mlc by the glucose transporter PtsG (40).

The GlnK protein is known to have some well defined intracellular targets in other organisms, and GlnK binding to AmtB

may also constitute a component of the regulatory process in these systems. They include the nitrogen fixation regulatory proteins (NifLA), which are controlled by GlnK in *Klebsiella pneumoniae* and *A. vinelandii* (17, 41), and ADP-ribosylation of nitrogenase in *Rhodospirillum rubrum*, which is regulated by the GlnK homologue GlnJ (35).

The role of GlnK in regulating the transport activity of AmtB may also have general implications for ammonium transport biology. Although the GlnK-AmtB system is restricted to prokaryotes, the activity of Amt proteins in eukaryotes (including the human Rhesus proteins) could conceivably also be controlled by interaction with one or more other intracellular signal proteins. For example the proposed ammonium-sensing role for certain Amt proteins in fungi would require that they communicate with an intracellular signal transduction system, most probably by direct interaction with other proteins (32–34). It is notable that the cytoplasmic C-terminal domain is by far the most variable region of Amt proteins, a variation that could reflect the capacity of Amt proteins to interact with different regulatory proteins in different organisms.

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