An alternative P_{II} protein in the regulation of glutamine synthetase in *Escherichia coli*

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Summary

The P_{II} protein has been considered pivotal to the dual cascade regulating ammonia assimilation through glutamine synthetase activity. Here we show that P_{II}, encoded by the *glnB* gene, is not always essential; for instance upon ammonia deprivation of a *glnB* deletion strain, glutamine synthetase can be deadenylated as effectively as in the wild-type strain. We describe a new operon, *glnK amtB*, which encodes a homologue of P_{II} and a putative ammonia transporter. We cloned and overexpressed *glnK* and found that the expressed protein had almost the same molecular weight as P_{II}, reacted with polyclonal P_{II} antibody, and was 67% identical in terms of amino acid sequence with *Escherichia coli* P_{II}. Like P_{II}, purified GlnK can activate the adenylation of glutamine synthetase *in vitro*, and, *in vivo*, the GlnK protein is uridylylated in a *glnD*-dependent fashion. Unlike P_{II}, however, the expression of *glnK* depends on the presence of UTase, nitrogen regulator I (NRI), and absence of ammonia. Because of a NRI and a σ^N (σ^{54}) RNA polymerase-binding consensus sequence upstream from the *glnK* gene, this suggests that *glnK* is regulated through the NRI/NRII two-component regulatory

system. Indeed, in cells grown in the presence of ammonia, glutamine synthetase deadenylation upon ammonia depletion depended on P_{II}. Possible regulatory implications of this conditional redundancy of P_{II} are discussed.

Introduction

In enteric bacteria, glutamine synthetase (GS), one of the central enzymes in nitrogen anabolism, is strongly regulated by the nitrogen status of the cell (for a review, see Reitzer and Magasanik, 1987). Glutamine synthetase catalyses the incorporation of ammonia into glutamine. The intracellular concentrations of glutamine and 2-oxo-glutarate monitor the nitrogen status of the cell and are sensed by uridylyltransferase (UTase) (Engleman and Francis, 1978; Kamberov *et al.*, 1994), and P_{II} (Kamberov *et al.*, 1995), the products of the *glnD* gene and the *glnB* gene, respectively. UTase transduces the signal to the P_{II} protein by adjusting the degree of uridylylation of the latter. Native P_{II} signals a nitrogen-rich status, and P_{II}-UMP a nitrogen-poor cell status. From P_{II}, the signal is further transduced through two divergent cascades. One of these controls the expression of *glnA*, which encodes glutamine synthetase, via the NRI (NtrC)/NRII (NtrB) two-component regulatory system. The other affects the GS activity by adenylation of the enzyme (Reitzer and Magasanik, 1987). The trimeric P_{II} protein, of which the crystal structure has been determined (Cheah *et al.*, 1994; Carr *et al.*, 1996), can interact with at least three proteins: UTase, for which P_{II} is a substrate, NRII, and adenylyltransferase (ATase), for which P_{II} functions as a regulator. The native P_{II} protein regulates the NRI/NRII system by stimulating the NRI-phosphatase activity of NRII, which results in a decreased expression of the *glnA* gene. In nitrogen limitation, NRII phosphorylates NRI. NRI phosphate then stimulates the expression of *glnA* (Bueno *et al.*, 1985; Ninfa and Magasanik, 1986; Ninfa *et al.*, 1987; Keener and Kustu, 1988; Weiss *et al.*, 1991; 1992; Porter *et al.*, 1993; Atkinson *et al.*, 1994). In the GS adenylation cascade, P_{II} stimulates ATase to adenylylate GS to the inactive form of GS. Upon nitrogen limitation, P_{II}-UMP activates ATase to deadenylylate GS-AMP (Brown *et al.*, 1971; Engleman and Francis, 1978).

In agreement with the regulation of ammonia assimilation which is described above and generally accepted,

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Foor *et al.* (1980) showed that in a *Klebsiella aerogenes* *glnB* transposon-insertion mutant, the rate of GS-AMP deadenylation after removal of ammonia was strongly decreased when compared to the wild type. The same was found in a *Klebsiella pneumoniae* *glnB* insertion mutant (W. C. van Heeswijk, and D. Molenaar, unpublished). However, when we attempted to quantify the control exerted by P_{II} on the deadenylation of glutamine synthetase in the light of modular metabolic control analysis (Kahn and Westerhoff, 1991), we came upon an anomalous result: we observed no difference in the deadenylation rate between a *glnB* deletion mutant and the corresponding wild-type *Escherichia coli* (van Heeswijk *et al.*, 1995; W. C. van Heeswijk *et al.*, in preparation). Here we show that this paradox is explained by the existence of an alternative P_{II} protein in *E. coli*, encoded by a gene called *glnK*. GlnK and P_{II} are related in intriguing ways: similar to *glnB*-encoded P_{II} , the GlnK protein can activate the adenylation of glutamine synthetase *in vitro*. Second, the GlnK protein is uridylylated in response to nitrogen deficiency. Unlike *glnB*, however, the *glnK* gene is regulated by nitrogen status in *E. coli*.

Results

glnK, a gene encoding a P_{II} homologue

In preliminary work (see van Heeswijk *et al.*, 1995), we obtained indications that *E. coli* may contain a protein that is very similar to P_{II} . Accordingly, we set out to amplify DNA from the *glnB* deletion strain RB9060, using degenerated oligonucleotides corresponding to the most conserved amino-acid regions of the P_{II} protein. The derived amino acid sequence of one of the amplified fragments was 67% identical to that of *E. coli* P_{II} (van Heeswijk *et al.*, 1995). This DNA fragment was used as a probe to screen the Kohara phage library (Kohara *et al.*, 1987) for a phage containing the complete gene encoding the P_{II} homologue. The probe hybridized with the Kohara phages, number 149 and 150, but, under our hybridization and washing conditions, not with the phages containing the *glnB* gene (data not shown). DNA was isolated from phage number 150. After gel electrophoresis of phage DNA digested with *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I or *Pvu*II, a Southern blot was made using the same probe as described above. The Southern blot confirmed the restriction pattern of the chromosome at 485 kbp as described (Kohara *et al.*, 1987; Médigue

Fig. 1. Physico-genetical map of the *glnK* *amtB* region on the *E. coli* chromosome. Arrows indicate the orientation of transcription. The chromosomal physical map co-ordinate of the *Eco*RV restriction site in the *glnK* gene is indicated in kb (Médigue *et al.*, 1990). The regions cloned in various plasmids are indicated. Restriction sites: A, *Eae*I; B, *Bam*HI; E, *Eco*RI; F, *Eco*RV; P, *Pvu*II; R, *Rsr*II; S, *Ssp*I.

et al., 1990), and furnished the location of the gene encoding the P_{II} homologue (data not shown). The gene was cloned into pBluescript-II KS+ as a 3 kb *Rsr*II–*Bam*HI DNA fragment from phage number 150 (Fig. 1). The insert was sequenced. It contained two open reading frames (ORF), located clockwise, between the *mdl* gene (Allikmets *et al.*, 1993) and the *tesB* gene (Naggart *et al.*, 1991) (Figs 1 and 2) on the chromosome.

The first *orf* in our sequence encodes a protein of 112 amino acids (12.2 kDa), like P_{II} , and is 67% identical with *E. coli* P_{II} protein (Fig. 3). This gene will be called *glnK*. On the basis of preliminary single-strand DNA sequencing (M. Dean, personal communication), Allikmets *et al.* (1993) reported that an *orf* in between the *mdl* and *tesB* genes had 66% amino acid sequence identity with the *E. coli* P_{II} . They deposited a translated amino acid sequence, but not the DNA sequence, in the SWISSPROT database. This ORF was 128-amino-acids long, 16 amino acids longer than the ORF reported in this study. The amino acid sequence of the GlnK protein also revealed a very high homology with other P_{II} homologues (Fig. 3). The *glnK* gene was subcloned in pBluescript-II KS+, resulting in plasmid pWVH149 (Fig. 1). When transformed with plasmid pWVH149, the *glnB* deletion strain RB9060, grown in rich medium, expressed large amounts of a protein, with a molecular mass similar to that of P_{II} (i.e. 12 kDa) (Fig. 4A). A Western blot showed that the overexpressed protein reacted with the polyclonal P_{II} antibody (Fig. 4B), indicating that the product of the cloned gene is also immunochemically similar to P_{II} .

Fig. 2. Nucleotide sequence of the *glnK* *amtB* region and the deduced amino acid sequences. The NRI-binding consensus sequence and RNA polymerase σ^{N} -binding consensus sequence are boxed. Putative ribosome-binding sites are underlined. Inverted repeats are indicated by arrows. These sequence data appear in the GenBank Nucleotide Sequence Data Library under the Accession no. U40429. The first 105 amino acids of the GlnK protein is present in the SWISSPROT database (Accession no. P38504) (Allikmets *et al.*, 1993).

		UMP
		I
Ec-GlnK	MKLVTVIKPFKLEDDREALSSIGIQLTVTEVKGFGKQKH	-----AELYRGAEYS-----
Ec-GlnB	MKKIDAIKPFKLDVREALAEVGTGMTVTEVKGFGKQKH	-----TELYRGAEYM-----
Kp-GlnB	MKKIDAIKPFKLDVREALAEVGTGMTVTEVKGFGKQKH	-----TELYRGAEYM-----
Ab-GlnB	MKKIEAIKPFKLEDEKALHEVGIGITVTEAKGFGKQKH	-----TELYRGAEYV-----
Bj-GlnB	MKKIEAIKPFKLEDEVR-SLSGVGLQGITVTEAKGFGKQKH	-----TDLYRGAEYI-----
Rl-GlnB	MKKIEAIKPFKLEDEVR-SPSGVGLQGITVTEAKGFGKQKH	-----TELYRGAEYV-----
Rc-GlnB	MKKIEAIKPFKLEDEKALQAGVQGLSVTEVKGFGKQKH	-----TELYRGAEYV-----
Rs-GlnB	MKKIEAIKPFKLEDEKALQAGVQGLSVTEVKGFGKQKH	-----TELYRGAEYV-----
Rr-GlnB	MKKIEAIKPFKLEDEKALHEIGLQGITVTEAKGFGKQKH	-----TELYRGAEYV-----
Hi-GlnB	MKKIEAMKPFKLDVRESLSDIGISGMTITEVRGFGKQKH	-----TELYRGAEYM-----
Ss-GlnB	MKKIEAIKPFKLEDEKIALVNAGIVGMTVSEVRGFGKQKH	-----TERYRGEYIT-----
Bs-NrgB	MSGQMFKEIVIRTPANFEKLQELGKIGVTSLSFNSVHGCGLQKAH	-----TELYRGEVKIE-----
Ma-GlnBb	MKEVIAIIRPNKMKKTRDVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Mi-Orf122	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Mb1-Orf123	MKEVIAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Ml-Orf128	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Mb2-Orf125	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Ma-GlnBa	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Mi-Orf105	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Ml-Orf105	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Mb1-Orf105	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-
Mb2-Orf105	MKEIITAIIRPNKINRTKEVLDELGFSSFNATRVLRGKQRAIIDE	-----VTIPSPSPIDEV-RGT-

Fig. 3. Multiple alignment of GlnK and GlnB homologues. The uridylylatable tyrosine residue in the *E. coli* GlnB protein is indicated with UMP. Ec-GlnB, *E. coli* GlnB (Vasudevan *et al.*, 1991; Liu and Magasanik, 1993); Kp, *K. pneumoniae* (Holtelt and Merrick, 1988); Ab, *A. brasilense* (de Zamaroczy *et al.*, 1990); Bj, *B. japonicum* (Martin *et al.*, 1989); Rl, *R. leguminosarum* (Colonna-Romano *et al.*, 1987); Rc, *R. capsulatus* (Kranz *et al.*, 1990); Rs, *Rhodobacter sphaeroides* (Zinchenko *et al.*, 1994); Rr, *Rhodospirillum rubrum* (GenBank Accession no. X84158); Hi, *Haemophilus influenzae* (Fleischmann *et al.*, 1995); Ss, *Synechococcus* sp. PCC 7942 (Tsinoremas *et al.*, 1991); Bs, *B. subtilis* (Wray, *et al.*, 1994); Ma, *Methanobacterium thermoautotrophicus* (GenBank Accession no. X87971); Ml, *Methanococcus thermolithotrophicus* (Souillard and Sibold, 1989); Mb1 and Mb2, first and second *nifH* region of *Methanosarcina barkeri* 227 (Sibold *et al.*, 1991); Mi, *Methanobacterium ivanovii* (Sibold *et al.*, 1991). Dashes indicate gaps introduced to optimize the alignment. Amino acid identities are indicated in bold.

The GlnK protein is reversibly uridylylated

A *glnD glnB* double mutant (RB9065), transformed with plasmid pWVH149, growing on a plate with rich medium and ampicillin, formed much smaller colonies than did strain RB9065 containing pBluescript-II KS+ or strain RB9060 (Δ *glnB*) containing pWVH149. This growth defect could be supplemented by the addition of 14 mM L-glutamine. Thus overproduction of GlnK in a *glnD*⁻ strain but not in a *glnD*⁺ background results in a leaky glutamine auxotrophy. This suggested that GlnK, like P_{II}, can regulate GS synthesis or activity in a *glnD*-dependent manner, and that the GlnK protein can be uridylylated by the *glnD*-encoded UTase.

Indeed, the GlnK protein contains Tyr-51, which is known to be the site of uridylylation in P_{II} (Fig. 3; Adler *et al.*, 1975; Son and Rhee, 1987). To verify that GlnK can be uridylylated, cell-free extracts from the *glnB* deletion strain RB9060, labelled *in vitro* with [α -³²P]-UTP, showed a characteristic uridylylated protein which co-migrated with uridylylated P_{II} (Fig. 5). The covalent modification of GlnK was further confirmed on Western blots of high-resolution SDS-Tricine polyacrylamide gels using a

P_{II} antibody which cross-reacts with GlnK (see above) (Fig. 6). When grown on minimal medium without ammonia, the *glnB* deletion strain RB9060 showed two molecular species (Fig. 6, lane 6). Comparison with the mobilities

Fig. 4. Overexpression and immunochemical identification of GlnK. A. Coomassie brilliant blue-stained SDS-PAGE gel of whole-cell lysates. B. Western blot of a gel similar to that in (A); detection with polyclonal P_{II} antibody. Lanes 1 and 2, RB9060 cells; lanes 3–5, RB9065. Plasmids present in these strains are pBluescript-II KS+ (lanes 1 and 3), and pWVH149 (lanes 2, 4, and 5). Lanes 1–4 refer to cells grown in YT medium, and lane 5 to cells grown in YT medium supplemented with 14 mM L-glutamine.

Fig. 5. GlnK can be uridylylated. Cell extracts radiolabelled with [α - 32 P]-UTP *in vitro*. Lanes: 1, strain RB9010 (wild type); 2, strain RB9060 ($\Delta glnB$); 3, strain RB9040 ($glnD::Tn10$); and 4, strain RB9065 ($glnD::Tn10 \Delta glnB$). The four lanes are from the same gel and the same autoradiograph.

of P_{II} -UMP and P_{II} suggests that these species corresponded to the uridylylated and the native GlnK. As expected, the higher-molecular-weight species disappeared after an ammonia shock, corresponding with de-uridylylation of GlnK (Fig. 6, lane 8). This result indicates that GlnK uridylylation responds to the cellular nitrogen status, just as P_{II} uridylylation does.

Expression of the glnK gene is regulated by the nitrogen status

When RB9060 was grown in minimal medium with ammonia, expression of GlnK could no longer be detected (Fig. 6, lane 7), indicating that expression of the *glnK* gene is regulated by the nitrogen status of the cell. The upstream region of the *glnK* gene contains a perfect RNA polymerase σ^N (σ^{54})-binding site (Morett and Buck, 1989) 62 bp upstream of the translational start codon, as well as possibly half of a NRI-binding site and a full putative NRI-binding site (Reitzer and Magasanik, 1986; Ames and Nikaido, 1985) 159 bp and 130 bp upstream of the translational start codon, respectively (Fig. 2). This suggests that the transcription of the *glnK* gene may be regulated by the

two-component system NRII/NRI. Indeed, the NRI-deficient *glnG glnB* double mutant RB9067 expressed *glnK* neither with nor without ammonia (Fig. 6). Also, the *glnD glnB* double mutant RB9065 hardly contained any P_{II} -like protein in either medium.

In the promoter region of *glnK* an inverted repeat is located overlapping the full putative NRI-binding site. This structure may function as a ρ independent terminator of transcription of the upstream *mdl* gene. Two plasmids were constructed in attempts to overexpress the *glnK* gene under the control of the *lac* promoter; one plasmid without the *glnK* promoter and without the inverted repeat (pWVH148), and one with both the *glnK* promoter and the inverted repeat (pWVH147) (Fig. 1). The appearance of a Coomassie brilliant blue-stained SDS-PAGE gel (data not shown) suggested that DH5 α containing pWVH148 and growing in rich medium overproduced a 12 kDa protein, corresponding to GlnK, but DH5 α containing pWVH147 did not. This result is consistent with the possibility that the stem-loop structure located in the promoter region of the *glnK* gene and present in plasmid pWVH147 is indeed a terminator, and that *glnK* is not expressed by readthrough transcription from the *mdl* gene.

glnK, glnB and GS deadenylylation in vivo

The high amino acid sequence similarity between P_{II} and GlnK and the observation that both can be uridylylated suggested that the two proteins carry out similar functions. Indeed, in our attempt to quantify the control exerted by P_{II} on the deadenylylation of GS, we were surprised to find that the control by P_{II} was very small (W. C. van Heeswijk *et al.*, in preparation), suggesting a redundancy of P_{II} .

When the wild-type strain YMC10 and the *glnB* deletion strain RB9060 were grown in minimal medium without ammonia, and exposed to a 10 min ammonia pulse, the

Fig. 6. The expression of the *glnK* gene and the modification of the GlnK protein is regulated by nitrogen. Western blot of a Tricine-SDS gel of cell lysates; detection with polyclonal P_{II} antibody. Lanes 1 and 2, purified P_{II} -UMP and P_{II} , respectively; lanes 3–5, strain RB9010; lanes 6–8, strain RB9060; lanes 9 and 10, strain RB9065; lanes 11 and 12, strain RB9040; lanes 13 and 14, strain RB9066; lanes 15 and 16, strain RB9067. Lanes 3, 6, 9, 11, 13, and 15 refer to cells grown in medium without ammonia (–); lanes 4, 7, 10, 12, 14, and 16 refer to cells grown in medium with ammonia (+); and lanes 5 and 8 to cells grown in medium without ammonia and exposed to ammonia for 10 min (p).

Fig. 7. Deadenylation of GS-AMP upon removal of ammonia.

A. Cells grown in medium without ammonia and exposed to 30 mM NH_4Cl for 10 min. Cells were centrifuged at $3300 \times g$, washed twice in MOPS-glutamine medium (without glucose) and resuspended in MOPS-glucose medium (without nitrogen). Circles, YMC10 (wild type), $m=3$; squares, RB9060 ($\Delta glnB$), $m=2$; triangles, RB9040 ($glnD::Tn10$), $m=2$; diamonds, RB9065 ($glnD::Tn10, \Delta glnB$), $m=4$. B. Cells grown in medium with ammonia. Cells were resuspended in MOPS-glucose after washing the cells as in (A). Symbols are as described for (A). YMC10, $m=6$; RB9060, $m=4$; RB9065, $m=2$; and RB9040, $m=2$. m is the number of determinations. Error bars indicate the standard error of the mean; error bars that are not visible are smaller than the symbol.

rate of deadenylation of GS-AMP after removal of the ammonia was similar for both strains (Fig. 7A). Thus, unexpectedly, the *glnB* gene product seemed irrelevant for GS-AMP deadenylation in cells grown in nitrogen-poor medium. Contingent upon the discovery of the P_{II} homologue, GlnK, this can now be rationalized as *glnK* being expressed under these conditions, suggesting that GlnK complements the *glnB* deletion. Indeed conditions reducing expression of *glnK*, such as high ammonium, restored the control of P_{II} upon GS deadenylation (Fig. 7B). In the wild-type cells the deadenylation rate was greatly increased by growth in the presence of ammonia. In the *glnB* mutant the growth history hardly effected GS deadenylation.

GlnK can activate the adenylation of GS in vitro

To directly prove that the cloned *glnK* gene product can activate ATase, adenylation of GS was performed with purified components. To exclude contamination with P_{II} , P_{II} -UMP or GlnK-UMP, GlnK was purified from the *glnB glnD* strain RB9065 transformed with plasmid pWVH149. GS containing one adenylylated subunit was incubated with ATase and either GlnK or P_{II} . GS adenylation was monitored by measuring the decrease in γ -glutamyl transferase activity in the presence of Mg^{2+} ions (Stadtman *et al.*, 1979). To reduce P_{II} - or GlnK-independent adenylation of GS, we applied a high phosphate concentration (D. Molenaar, unpublished). As shown in Fig. 8, purified GlnK was able to activate the adenylation reaction in a concentration-dependent manner, as was P_{II} . This shows that the *glnK* gene product is indeed a functional P_{II} homologue.

The gene downstream of the *glnK* gene may encode an ammonia transporter

Conceptual translation of the *orf* downstream of the *glnK*

Fig. 8. GlnK-dependent adenylation of GS *in vitro*.

A. The formation of γ -glutamylhydroxamate (γ -gh) by the non-adenylylated GS ($n=1$) was measured. Open triangles, no P_{II} or GlnK added; open circles, 6.7 nM P_{II} ; open squares, 26.9 nM P_{II} ; closed circles, 27.3 nM GlnK; closed squares, 137 nM GlnK. B. Coomassie brilliant blue-stained SDS-PAGE gel (15%) of purified P_{II} and GlnK. Lane 1, P_{II} (1 μg); lane 2, GlnK (0.8 μg).

gene revealed several methionines, one of which is preceded by a Shine–Dalgarno sequence as predicted by Stormo *et al.* (1982). We suggest that this methionine is the start of a protein of 401 amino acids (42 kDa). Analysis of the hydrophilicity of the translated *orf* with the algorithm of Kyte and Doolittle (1982) revealed 12 putative membrane-spanning domains, suggesting that this *orf* encodes an integral transmembrane protein (data not shown). Comparison of the amino-acid sequence of this ORF with proteins in the databases (GENPEPT, SWISS-PROT, and PIR) revealed that it is homologous to the ammonia transporters MEP1 (29% identical) and MEP2 (30% identical) of *Saccharomyces cerevisiae* (Marini *et al.*, 1994; GenBank Accession no. X83608, respectively), to AMT1 of *Arabidopsis* (26% identical) (Ninnemann *et al.*, 1994), and to the hydrophobic protein NrgA of *Bacillus subtilis* (42% identical) (Wray *et al.*, 1994). Interestingly, the latter gene product forms an operon together with a P_{II} -like protein NrgB. The *B. subtilis* *nrgB* gene is located downstream from the *nrgA* gene. In *E. coli*, a gene (*amtA*) involved in ammonium transport has been cloned and sequenced (Fabiny *et al.*, 1991; Jayakumar *et al.*, 1989). The AmtA protein has been predicted to be a cytoplasmic component of an ammonium-transport system (Fabiny *et al.*, 1991) and is not homologous to the ORF cloned in this study. To highlight the high homology of the ORF with the ammonium transporters we call the gene encoding this protein *amtB*. The AmtB protein might be an integral membrane subunit of an ammonium-transport system, the cytoplasmic AmtA protein constituting another subunit. The Trk K^+ transport system of *E. coli* is organized in this manner (Bossemeyer *et al.*, 1989).

No clear termination sequence or promoter sequence was found between the *glnK* gene and the *amtB* gene. This suggests that the *glnK* gene and the *amtB* gene are co-transcribed. Growth of strain DH5 α carrying a high-copy-number plasmid (pWVH142; Fig. 1), in which this putative *glnK amtB* operon resided downstream from a *lac* promoter, resulted in very small colonies on plates with rich medium supplemented with ampicillin. This phenotype may be explained by the overproduction of an integral transmembrane protein in *E. coli* affecting membrane permeability. The suggestion that the two genes are co-transcribed implies that the *amtB* gene is also regulated by the nitrogen status of the cell.

An inverted repeat resides in between the *amtB* gene and the *tesB* gene. Perhaps both transcription of the *amtB* gene and transcription, in the opposite direction, of the *tesB* gene terminate at this stem-loop structure.

Discussion

We were surprised to find that the P_{II} protein was not necessary for the deadenylation of GS-AMP. This result

was obtained with cells that had been grown in the absence of ammonia (Fig. 7A and van Heeswijk *et al.*, 1995). In a previous study (van Heeswijk *et al.*, 1995) we had already found that 45 s after ammonia deprivation, there was little difference in GS-AMP deadenylation between the wild type and the *glnB* mutant. The kinetic study in this report showed that neither $t_{1/2}$ nor the final extent of GS-AMP deadenylation are strongly affected by *glnB* deletion. A Western blot showed that cells grown in this medium contained a protein cross-reacting with the P_{II} antibody, with a size similar to that of P_{II} (van Heeswijk *et al.*, 1995). This suggested the presence of a P_{II} -like protein in this medium. Indeed, we have now cloned and sequenced a *glnK amtB* operon encoding a P_{II} homologue and a putative ammonia transporter (Fig. 2).

We conclude that the GlnK protein is structurally similar to P_{II} on the basis of its size similarity with P_{II} , its high amino acid sequence identity with all known P_{II} proteins (Fig. 3), its cross-reactivity with a polyclonal P_{II} antibody, and its nitrogen-regulated uridylylation (Figs 5 and 6).

P_{II} deletion did not greatly reduce the rate of deadenylation of GS-AMP induced by ammonia downshift (Fig. 7A) after pregrowth in the absence of ammonia. Combined with the observations of Bueno *et al.* (1985) and Ninfa *et al.* (1995) that *glnD* has a nitrogen-regulated phenotype in a *glnB*-negative background in terms of GS expression and adenylation level, this shows that P_{II} can be functionally redundant. Our finding of the P_{II} homologue, GlnK, makes us propose that GlnK can take over the function of P_{II} . The functional similarity of GlnK and P_{II} is evident from the *in vitro* adenylation of GS. Both GlnK and P_{II} can activate the adenylation reaction, although GlnK is less active than P_{II} .

In vivo, however, it is less clear as to what extent GlnK activates GS-AMP deadenylation. This is because, when growing the *glnB* deletion strain in the presence of ammonia, when cells hardly express *glnK*, the rate of deadenylation of GS-AMP was hardly reduced compared with the same cells grown in the absence of ammonia. Residual GlnK-UMP, escaping detection by Western blotting (Fig. 6), may be responsible for this remaining activity. This is consistent with the observation that, independent of whether the cells were grown in the absence or presence of ammonia, GS-AMP of the *glnD glnB* double mutant did not deadenylate at all during this time interval (Fig. 7). Both expression and uridylylation of GlnK are strongly though perhaps not completely *glnD* dependent and it is presumably GlnK-UMP that stimulates deadenylation.

For cells that lacked P_{II} we were able to show that the amount of GlnK was greatly reduced when cells were grown in the presence of ammonia. Because of the great similarity of P_{II} and GlnK we could not prove that also in the *glnB*-positive cells, GlnK was repressed by ammonia.

If, however, we presume this to be the case (this is supported by the more intense P_{II} plus GlnK band in cells grown in the absence of ammonia (Fig. 6)), comparison of the GS-AMP deadenylylation of the wild-type cells in Fig. 7B with those in Fig. 7A suggests that GlnK inhibits rather than stimulates GS-AMP deadenylylation when P_{II} is present. One possible explanation is that GlnK-UMP competes effectively with P_{II} -UMP in terms of its binding to ATase, and the maximum activation of ATase by GlnK-UMP is lower than that by P_{II} -UMP.

Alternatively, GlnK uridylylation proceeds more slowly than P_{II} uridylylation and the native GlnK competes P_{II} -UMP away from ATase. The formation of heterotrimers offers additional possible explanations.

Unlike in *E. coli*, in *K. aerogenes* grown in minimal medium without ammonia and briefly exposed to ammonia, the rate of deadenylylation depends on P_{II} (Foor *et al.*, 1980). The fact that this dependence was incomplete (Foor *et al.*, 1980) suggests the existence of a second P_{II} protein in this closely related organism, albeit with a lower activity than the GlnK protein described in this study.

Recently, a second P_{II} -like protein has also been found in other bacteria. In *Azospirillum brasilense* this protein was nitrogen regulated and detected on the basis of its reactivity with *A. brasilense* P_{II} antibody (de Zamaroczy *et al.*, 1995). In several methanobacteria, two copies of a *glnB*-like gene have been identified in between *nifH* and *nifD* on the basis of their amino acid sequence similarity with other P_{II} proteins (Fig. 3) (Souillard and Sibold, 1989; Sibold *et al.*, 1991; Kessler *et al.*, 1995). The functions of these P_{II} -like proteins are still not known, but their nitrogen regulability in *A. brasilense* and the location of the *glnB*-like genes within a *nif*-gene cluster in the methanobacteria, invites the speculation that they are involved in nitrogen regulation.

Expression of *glnK* is regulated by nitrogen in a NRI-dependent manner (Fig. 6). This is consistent with the presence of a characteristic RNA polymerase σ^N -dependent promoter sequence and putative NRI-binding sites upstream of *glnK*. Moreover, expression of *glnK* depends on the presence of the *glnD*-encoded UTase in the absence of P_{II} . This may indicate that non-uridylylated GlnK stimulates the dephosphorylation of NRI, thereby affecting transcription from the *glnK* promoter. The greatly reduced expression of the *glnK* gene in a *glnD*⁻ background may explain why Bueno *et al.* (1985) did not find a *glnD glnK* double mutant as suppressor for the *glnD* mutation.

Although expression of the *E. coli glnB* gene is constitutive (van Heeswijk *et al.*, 1993; Liu and Magasanik, 1993), it is not unusual for the expression of a *glnB*-like gene to be regulated by nitrogen. In *A. brasilense*, transcription of *glnB* is elevated five- to sixfold by nitrogen

limitation, although this expression is not NRI dependent (de Zamaroczy *et al.*, 1993). As in *A. brasilense*, the *glnB* gene in both *Bradyrhizobium japonicum* (Martin *et al.*, 1989) and in *Rhodobacter capsulatus* (Foster-Hartnett and Kranz, 1994) is transcribed from tandem promoters. Although NRI is involved in the expression of the *glnB* gene in the latter two bacteria, their *glnB* transcription is hardly regulated by the nitrogen status. *R. capsulatus glnB* promoters lack an RNA polymerase σ^N -binding site (Foster-Hartnett and Kranz, 1994). In *Rhizobium leguminosarum* the *glnB* gene is transcribed from a single promoter, which does contain an RNA polymerase σ^N -binding site but no NRI-binding site. Its expression is elevated threefold by nitrogen limitation (Chiurazzi and Iaccarino, 1990). In *E. coli* (van Heeswijk *et al.*, 1993; Liu and Magasanik, 1993) and the four organisms described here, expression of the *glnB* gene is differently regulated. The transcriptional regulation of the *E. coli glnK* gene adds additional regulatory options, i.e. strong regulation of transcription by nitrogen status in an NRI-dependent manner.

The *glnK* gene is closely linked to a gene, *amtB*, homologous to various ammonia transporters (Fig. 9). Such a situation is also found in the *nrgAB* operon of *B. subtilis*. However, the *glnB*-like *nrgB* gene is located downstream from the *nrgA* gene (Wray *et al.*, 1994). Recently, an operon similar to the *E. coli glnK amtB* operon has been found in *Azotobacter vinelandii* (Meletzus *et al.*, 1995). The *E. coli glnK amtB* sequence suggests that the two genes are co-transcribed and thus that expression of *amtB* is nitrogen regulated. This suggestion is consistent with the observation that (methyl)ammonium uptake into *E. coli* is repressed by ammonium and depends on *rpoN* and *glnG* (Servín-González and Bastarrachea, 1984; Jayakumar *et al.*, 1986).

Why does *E. coli* have two genes encoding a P_{II} -like protein, one of which is regulated by nitrogen? If the products of the two genes, *glnK* and *glnB*, are completely homologous functionally, then the question can be reduced to: why does *E. coli* have a nitrogen-regulated P_{II} protein? Subtlety in regulation could be a reason. The $t_{1/2}$ of deadenylylation in the wild type, upon removal of ammonia, is lower when the expression of *glnK* is reduced (Fig. 7B) than when both GlnK and P_{II} are present (Fig. 7A). Accordingly, the apparent redundancy of GlnK and P_{II} gives rise to both a fast and a slow signal-transduction system. Recently, the functional significance of the inducibility or repressibility of signal-transducing proteins has been emphasized (Hellingwerf *et al.*, 1995). If a signal-transducing protein is induced by its own signal (or response) then the response time and response efficiency itself can depend on the growth history. The Ntr system and GlnK may constitute an example. The signal that the P_{II} -like proteins transmit depends on their

Ec-AmtB	MKIATIKTGL	ASLAMLPGLV	MAAPAVADKA	DNAFMMIC-T	ALVLFMTIPG	49
Bs-NrgA	MQM-----	-----	-----G	DTVFMFFC-A	LLVWLMT-PG	22
Sc-Mep1	M-----	-ESRTTGPLT	TE---TYDGP	TVAFMILG-A	ALVFFMV-PG	35
Sc-Mep2	MSYNFTGTPT	GEGTGGNSLT	TDLNTQFDLA	NMGWIGVA-S	AGVWIMV-PG	48
At-Amt1	MSCSATDLAV	LLGPNATAAA	NYICGQLGDV	NNKFIDTAF	IDNTYLLFSA	50
Ec-AmtB	IALFYGGILR	GKNVLSMLTQ	VTVTFAVLCI	LWVVYGYSLA	F---GEGNNE	96
Bs-NrgA	LALFYGGMVK	SKNVLSSTAMH	SFSSIAIVSI	VWVLFGYTLA	F---APGNSI	69
Sc-Mep1	LGFLYSGLAR	RKSALALIIV	VLMTLVGIL	QWYFWGYSLA	FSKSAPNNKF	85
Sc-Mep2	IGLLYSGLSR	KKHALSLLWA	SMMASAVCIF	QWFFWGYSLA	FSHNTRNGGF	98
At-Amt1	YLVFSMQLGF	AMLCAGSVRA	KNTNMIMLTN	VLEPAAGGLF	YYLFGYAFAF	100
Ec-AmtB	FGNINWMLK	NIELTAV--M	GSIQYIHVA	FQGSFACITV	GLIVGALAER	144
Bs-NrgA	IGGLEWAGLK	GVGFDPGDYS	DTIPHSLEFMM	FQMTFAVLTT	AIISGAFER	119
Sc-Mep1	IGNLDSFGFR	NV-YGKKFDE	DAYELAYAT	FQMMFSCVNL	SIIAGATAER	134
Sc-Mep2	IGTLEFFGFR	NV-LGAPSSV	SSLPDILFAV	YQGMFAAVTG	ALMLGGACER	147
At-Amt1	GSPSNGFIGK	HY-FGLKDIP	TASADYSNFL	YQWAFATAAA	GITSGSIAER	149
Ec-AmtB	IRFSAVLIFV	VVWLTLSYIP	IAHMVWGGG-	L-----	-LASHGALDF	183
Bs-NrgA	MRFGAFLIFS	VLWASLVYTP	VAHWVWGGG-	W-----	-IGQLGALDF	158
Sc-Mep1	GRLLPHMVFL	FILATIGYCP	VTYIWLSPGG	W-----	-AYQWGVLDW	174
Sc-Mep2	ARLFPMVFL	FLWMTIVYCP	IACVWVNAEG	W-----	-LVKLGSLDY	187
At-Amt1	TQFVAYLIYS	SFLTGFVYPV	VSHWFWSVDG	WASPFRTDGD	LLESTGAIDF	199
Ec-AmtB	AGGTVVHINA	AIAGLVGAYL	IGKRVG----	FGKE-AFKPH	NLEPMVFTGTA	228
Bs-NrgA	AGGNVVIHSS	GVAGLVLAIV	LGKRKD----	-GTA-S-SPH	NLIYTFLGGA	201
Sc-Mep1	AGGGNIEILS	AVSGEFVYSWF	LGKRNEKL--	--LI-NFRPH	NVSLVTLGTS	219
Sc-Mep2	AGGLCVHLTS	GHGGLVYALI	LGKRNDPVTR	KGMP-KYKPH	SVTSVVLGTV	236
At-Amt1	AGSGVVMVG	GIAGLWGALI	EGPRLGRFDN	GGRAIALRGH	SASLVVLGTF	249
Ec-AmtB	ILYIGWFEGFN	AGS---AGTA	NEIAAL----	-AFVNTVVAT	A---AAILGW	267
Bs-NrgA	LIWFGWEGFN	VGS---ALTL	DGVAMY----	-AFINTNTAA	A---AGIAGW	240
Sc-Mep1	ILWFGWLLFN	SAS---SLSP	NLRSVY----	-AFMNTCLSA	I---TGGMTW	258
Sc-Mep2	FLWFGWMFFN	GGG---AGNA	TIRAWY----	-SIMSTNLAA	A---CGGLTW	275
At-Amt1	LLWFGWYGFN	PGSFNKILVT	YETGTYNQW	SAVGRTAVTT	TLAGCTAALT	299
Ec-AmtB	IFGEW-ALRG	KPSLLGACSG	AIAGLVGVTP	ACGYIGVGGA	LIIGVVAGLA	316
Bs-NrgA	ILVEW-IINK	KPTMLGAVSG	AIAGLVAITP	AAGFVTFPAS	LIIGIIGGAV	289
Sc-Mep1	CLLDY-RSEK	KWSTVGLCSG	IISGLVAATP	SSGCITLYGS	LIQGIVAGVV	307
Sc-Mep2	MVIDYFRGCR	KWTTVGLCSG	IIAGLVGITP	AAGFVPIWSA	VVIGVVTGAG	325
At-Amt1	TLFGKRLLSG	HWNVTDVCNG	LLGGEFAITG	GCSVVEPWAA	IICGFVAALV	349
Ec-AmtB	GLWGVTMLKR	LLRVDDPCDV	FGVHGVCGIV	GCIMTGIFAA	S-SLGGVG--	363
Bs-NrgA	CFWGVFSLKK	KFGYDDALDA	FGLHGIGGTW	GGIATGLFAT	T-SVNSAG--	336
Sc-Mep1	CNFAT-KLKY	YAKVDDAMDI	LAEHGVAGVI	GLIFNALFGA	D-WVIGMDGT	355
Sc-Mep2	CNLAV-DLKS	LLRIDDGLDC	YSIHGVGGCI	GSVLTGIFAA	D-YVNATAGS	373
At-Amt1	-LLGCNKLAE	KLKYDDPLEA	AQLHGGCGAW	GLIFTALFAQ	EKYLNQIYGN	398
Ec-AmtB	----FAEG-V	TMGHQLL-VQ	LESIAITIVW	-SGVVAFIGY	KLADL-TVGL	405
Bs-NrgA	----ADGL-F	YGDASLIWKQ	IVAIAATYVF	-VFIVTFVII	KIVSL-FLPL	379
Sc-Mep1	--TEHEGGWV	THNYKQMYKQ	IAYIAASIGY	TAAVTAIICF	VLGYIPGMRL	403
Sc-Mep2	YISPIDGGWI	NHHYKQVGYQ	LAGICAALAW	TVTITSILLL	TMNAIPFLKL	423
At-Amt1	KPGRPHGLFM	GGGKLLGAQ	LIQIIVITGW	VSATMGTLFF	ILKKM-KL-L	446
Ec-AmtB	RVPEEQEREG	LDVNSHGENA	YNA-----	-----	-----	428
Bs-NrgA	RATEEEESLG	LDLTMHGEGA	YQ-----	-----	--DSM----	404
Sc-Mep1	RISEEAEEAG	MDEDQIGEFA	YDYVEVRRDY	YLWGVDEDSQ	RSDVNHRVNN	453
Sc-Mep2	RLSADEEELG	TDAAQIGEFT	YE-----ESTA	YI-----PEPI	RSK-----TS	460
At-Amt1	RISEDEEMAG	MDMTRHGGEFA	YM-----	YF-----	-DD-----DE	474
Ec-AmtB	-----	-----	-----	-----	428	
Bs-NrgA	-----	-----	-----	-----	404	
Sc-Mep1	AHLAAERSSS	GTNSSSDGNG	EMIQSEKILP	IHQEDPANR	492	
Sc-Mep2	AQMPPPHENI	DDKIVGNTDA	EKNSTPSDAS	STKNTDHIV	499	
At-Amt1	SHKAIQLRRV	EPRSPSPSGA	NTPTTPV---	-----	501	

Fig. 9. Multiple alignment of AmtB and ammonia transporter homologues. MEP1 and MEP2 of *S. cerevisiae* (Marini *et al.*, 1994, and GenBank Accession no. X83608), AMT1 of *Arabidopsis* (Ninnemann *et al.*, 1994), and NrgA of *B. subtilis* (Wray *et al.*, 1994). Dashes indicate gaps introduced to optimize the alignment. Amino acid identities are indicated in bold.

concentration in the cell, hence on the growth condition of the cell (growth history) (Fig. 7). When the cell has adapted to a nitrogen-poor condition, the concentrations of NRI, NRII (Reitzer and Magasanik, 1983) and the P_{II}-like proteins (Fig. 6) are high. A single short exposure to ammonia should not lead to a strong reduction in these concentrations. After a prolonged ammonia exposure, the concentration of the signalling proteins will decrease. In the former condition the cells' machinery has been adapted to growth in the absence of ammonia. Then rapid adaptation upon disappearance of a short ammonia pulse is not useful. In the latter condition, the cell has grown accustomed to the luxury of high ammonia concentration and has lost much of its ability to do without. In this condition, a quick adaptation may well be important. The concentration of these proteins may therefore be the way in which the cell remembers the condition to which it has been adapted. NRI, NRII, and GlnK may function as 'memory' proteins (Hellingwerf *et al.*, 1995).

Here it may be important that the expression of *glnB* in *E. coli* does not depend on the nitrogen status (van Heeswijk *et al.*, 1993; Liu and Magasanik, 1993). Consequently, cells have a basal response activity towards changes in nitrogen status plus a response activity that depends on the cells' nitrogen history. The dependence of *glnK* expression on nitrogen status might make the constitutive P_{II} necessary.

Experimental procedures

Bacterial strains and media

The bacterial strains used are listed in Table 1. Cells were grown overnight at 37°C to an OD₆₀₀ of 0.3, in 40 mM MOPS medium with 22 mM glucose (Neidhardt *et al.*, 1974) plus 14 mM L-glutamine ('nitrogen poor') or 14 mM L-glutamine and 14 mM NH₄Cl ('nitrogen rich') as nitrogen source. Rich medium (YT) contains 8 g l⁻¹ tryptone (Difco), 5 g l⁻¹ yeast extract (Difco) and 5 g l⁻¹ NaCl. A solution of L-glutamine (Sigma) was freshly prepared before use.

Table 1. *E. coli* K-12 strains and genotypes.

Strain	Genotype	Reference
YMC10	<i>endA1 thi-1 hsdR17 supE44</i> <i>ΔlacU169 hutC_{klebs}</i>	Chen <i>et al.</i> (1982)
RB9010	As YMC10, but Mu lysogen	Bueno <i>et al.</i> (1985)
RB9040	As RB9010, but <i>glnD99::Tn10</i>	Bueno <i>et al.</i> (1985)
RB9060	As RB9010, but <i>ΔglnB2306</i>	Bueno <i>et al.</i> (1985)
RB9065	As RB9010, but <i>ΔglnB2306</i> , <i>glnD99::Tn10</i>	Bueno <i>et al.</i> (1985)
RB9066	As RB9010, but <i>glnG::Tn5</i>	Bueno <i>et al.</i> (1985)
RB9067	As RB9010, but <i>glnG::Tn5</i> , <i>ΔglnB2306</i>	Bueno <i>et al.</i> (1985)
DH5α	F ⁻ <i>supE44 hsdR17 recA1 endA1</i> <i>gyrA thi-1 relA1 Δ(lacZYA-argF)</i> _{U169} (m80/lacZΔM15)	Raleigh <i>et al.</i> (1989)

Phage DNA manipulations

The Kohara phage library (Kohara *et al.*, 1987) was used to screen for a phage containing the gene encoding the P_{II} homologue. From each phage sample from a Kohara phage library 1 μl was spotted onto a filter and probed with the digoxigenin-labelled amplified DNA fragment from strain RB9060 (van Heeswijk *et al.*, 1995). Hybridization was performed in a 5× SSC (1× SSC: 150 mM NaCl, 15 mM Na₃-citrate, pH 7.0) incubation buffer (non-radioactive detection kit from Boehringer Mannheim) at 65°C. Filters were washed three times with 2× SSC and 0.1% SDS at 65°C. DNA from phage number 150 was isolated using LambdaSorb phage adsorbent (Promega). A Southern blot was performed by digesting the phage DNA with *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I or *Pvu*II and probed with the DNA fragment described above.

Plasmid constructions

The gene encoding the P_{II} homologue was cloned as a 3 kb *Rsr*II (blunted with T4 DNA polymerase) –*Bam*HI DNA fragment from phage number 150 into pBluescript-II KS+ (Stratagene) and digested with *Sma*I and *Bam*HI, resulting in pWVH141 (Fig. 1). Plasmid pWVH142 was constructed by digesting pWVH141 with *Eco*RI and then religating the plasmid. Plasmid pWVH146 was constructed by digestion of pWVH141 with *Ssp*I and *Bam*HI and ligation of the 2.2 kb fragment into pBluescript-II KS+ digested with *Sma*I and *Bam*HI. Constructs pWVH147 and pWVH148 were made by cloning a 1 kb or 0.83 kb *Hind*III (polylinker)–*Pvu*II fragment from pWVH146 or pWVH142, respectively, into pBluescript-II KS+ digested with *Hind*III and *Sma*I. A 0.47 kb *Eco*RI–*Eae*I fragment of pWVH141 was cloned into pBluescript-II KS+ digested with *Eco*RI and *Not*I, resulting in pWVH149.

DNA sequencing

To sequence the cloned phage fragment, several subclones from pWVH141 were made in M13 and pBluescript-II SK+ (Stratagene) and sequenced using the Applied Biosystems 373A DNA sequencer. Oligonucleotides were synthesized to sequence the gaps. Nucleotides 1823 to 1890 (Fig. 2) were copied from Naggart *et al.* (1991) after partial sequencing of the overlap. DNA sequence and deduced amino acid sequences were analysed using the computer programs SEQUENCHER (Gene codes corporation), BLAST (Altschul *et al.*, 1990), GENWORKS (IntelliGenetics), Multalin (Corpet), and GENEMARK (Borodovsky *et al.*, 1994).

Gel electrophoresis and Western blot analysis

From the cells grown in minimal medium with or without ammonia, 10 μg aliquots of protein were loaded onto a 20 cm SDS–Tricine gel (16.5% acrylamide, 6% bisacrylamide) (Schägger and von Jagow, 1987). For purified P_{II} and P_{II}-UMP (D. Molenaar *et al.*, in preparation) 1 ng of protein in 0.75 mg ml⁻¹ BSA was loaded. As a visible marker, 50 μg of cytochrome *c* (12.5 kDa) (Boehringer Mannheim)

was loaded. After the run, the gel was cut just above cytochrome *c* and the lower part was blotted onto nitrocellulose and probed with polyclonal P_{II} antibody. The bands were visualized using the ECL system of Amersham. The same cell suspensions were loaded onto a 10% acrylamide mini-gel, blotted onto nitrocellulose, and probed with polyclonal GS antibody. DH5 α , RB9060 or RB9065 cells carrying plasmids that contain the *glnK* gene or the *glnK amtB* operon were grown overnight in YT medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin at 37°C. Cell suspensions, equivalent to 15 μg of protein, were loaded onto an SDS-PAGE (15% acrylamide) Mini-gel (Bio-Rad). After electrophoresis the gel was stained with Coomassie brilliant blue. For the Western blot analysis cell suspensions, equivalent to 1.5 μg of protein, were used. Polyclonal P_{II} antibody was used as probe. Protein concentration of the cell suspensions was measured according to the modified Lowry procedure with bovin serum albumin (BSA) (Sigma) as the standard (Markwell *et al.*, 1978).

Uridylylation of the P_{II} homologue

Cell extracts were labelled *in vitro* as described by Colonna-Romano *et al.* (1993), as modified by Edwards and Merrick (1995). Cells were grown in minimal medium without ammonia. After sonication, 40 μl of cell extract equivalent to 25 μg of protein was incubated in the presence of 1 μl [α - ^{32}P]-UTP (800 Ci mmol $^{-1}$) (Amersham) and 7 μl of the incubation mix was separated by SDS-PAGE. A total of 10 μg of protein was used for the Western blot analysis. Protein concentration of the cell extracts was measured with the bicinchoninic acid (BCA) protein assay reagent (Pierce) according to the manufacturer, and using BSA as the standard.

Glutamine synthetase adenylylation assay

Growth of cells, harvesting, and preparation of CTAB (hexadecyltrimethylammonium bromide)-treated cells was carried out as described (van Heeswijk *et al.*, 1995). Essentially, cells grown in medium without ammonia were exposed to 30 mM NH_4Cl for 10 min at 37°C. Cells that had been pulsed with ammonia and cells grown in medium in the presence of ammonia were chilled on ice for 10 min, harvested by centrifugation at 3300 $\times g$, and washed twice in ice-cold MOPS-glutamine medium (without glucose). The pellet was resuspended in 100 μl MOPS-glutamine. At time zero, 5 μl of this suspension was analysed for the adenylylation state. The remainder of the cell suspension was added to 30 ml of prewarmed (37°C) MOPS-22 mM glucose medium (without nitrogen) at time zero. Aliquots of 0.8 ml were pipetted into liquid nitrogen at the indicated time points. After CTAB treatment cells were resuspended in 50 μl of imidazole buffer (van Heeswijk *et al.*, 1995). The adenylylation state (*n*) of glutamine synthetase was determined by the γ -glutamyl transferase assay (Stadtman *et al.*, 1979) performed in microtitre plates. For this assay, 5 μl of CTAB-treated cell suspension was incubated with 100 μl of any of the four imidazole buffers (with Mg^{2+} , pH 7.20 or without Mg^{2+} , pH 7.38; with or without ADP and AsO_4^{3-}) and incubated for 30 min at 37°C followed by the addition of 200 μl of the acidic FeCl_3 stop mixture. The imidazole buffers were freshly prepared before use.

The microtitre plates were centrifuged for 5 min at 335 $\times g$ and 200 μl of every incubation mixture was transferred to a well of another microtitre plate. The absorbance was measured at 490 nm.

Protein purifications

Purification of glutamine synthetase (*n* = 1), adenylyl-transferase, and P_{II} will be described in a future study (D. Molenaar *et al.*, in preparation). Each protein was more than 95% pure, as judged by its appearance on Coomassie brilliant blue-stained SDS-PAGE gels. GlnK has been purified from strain RB9065 transformed with pWVH149.

Cells were grown for several hours in 10 ml of YT medium supplemented with 14 mM L-glutamine and 50 $\mu\text{g ml}^{-1}$ ampicillin at 37°C. This culture was used to inoculate 2 l of the same medium and then grown overnight to saturation. Cells were collected by centrifugation at 7000 $\times g$, resuspended in 50 ml of 20 mM potassium phosphate buffer, pH 7.2, and stored at -80°C. The cell suspension was passed two times through a French Pressure Cell and clarified by centrifugation at 20 000 $\times g$. Streptomycin sulphate was added to the supernatant to a final concentration of 1%. After 15 min of stirring on ice the suspension was centrifuged at 20 000 $\times g$. β -mercaptoethanol was added to the supernatant to a final concentration of 26% and stirred at room temperature for 20 min. After centrifugation at 3600 $\times g$, the supernatant was dialysed twice (2 h at room temperature and 16 h at 4°C) against 5 l of 50 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA. The filtered supernatant was loaded onto a Mono Q HR 10/10 column (Pharmacia), and equilibrated with 50 mM Tris-HCl, pH 7.6, at a flow rate of 4 ml min $^{-1}$. The column was eluted with a 160 ml linear gradient of 50 mM Tris-HCl, pH 7.6, 0 mM NaCl to 50 mM Tris-HCl, pH 7.6, 1 M NaCl. GlnK eluted at approx. 300 mM NaCl. The pooled fractions containing GlnK were dialysed against 50 mM Tris-HCl, pH 7.6. The total fraction was loaded again onto a Mono Q column (HR 5/5) at a flow rate of 1 ml min $^{-1}$. The column was eluted with 30 ml of the same gradient as described above. The pooled fractions containing GlnK were concentrated with Aquacide (Calbiochem), filtered, and loaded onto a Superose 12 column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.6. The column was eluted with 50 mM Tris-HCl, pH 7.6, at a flow rate of 0.5 ml min $^{-1}$. The fractions containing GlnK were pooled and concentrated with Aquacide. Glycerol was added to the GlnK solution to a final concentration of 55%, and the solution then stored at -20°C. GlnK was more than 95% pure, as judged by its appearance on Coomassie brilliant blue-stained SDS-PAGE gels (Fig. 8B).

In vitro adenylylation reactions

Quoted molarities refer to the GS dodecamer, ATase monomer, and P_{II} /GlnK trimer (Vasudevan *et al.*, 1994; Cheah *et al.*, 1994; de Mel *et al.*, 1994; Carr *et al.*, 1996). The adenylylation reaction was monitored by the formation of γ -glutamyl-hydroxamate determined by the γ -glutamyl transferase assay (Stadtman *et al.*, 1979), performed in microtitre plates. All incubations were performed at 30°C. ATase, P_{II} , and GlnK were diluted in 1 mg ml $^{-1}$ BSA. Reaction mixture and buffer B were freshly prepared before use. The reaction started

after addition of 100 µl of reaction mixture to 100 µl ATase diluted in BSA, both pre-warmed at 30°C for 5 min. Final concentration of the components were 50 mM Hepes-HCl, pH 7.6, 25 mM potassium phosphate, 5 mM MgCl₂, 1 mM ATP, 1 mM L-glutamine, 1.5 mg ml⁻¹ BSA, 80.4 nM GS (n = 1), 23.1 nM ATase, and P_{II} or GlnK as indicated in the appropriate figure legend. At the indicated time points, 10 µl of the incubation mixture was added to 100 µl buffer B, pre-warmed at 30°C. Buffer B contained 0.16 M imidazole-HCl, 0.16 M L-glutamine, 42 mM NH₂OH, 0.42 mM ADP, 21 mM Na₂HASO₄, 0.42 mM MnCl₂, and 62.5 mM MgCl₂, pH 7.2. After 30 min the reaction was stopped by adding 200 µl of the acidic FeCl₃ stop mixture. The absorbance was measured at 490 nm.

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