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P_{II} Signal Transduction Proteins, Pivotal Players in Microbial Nitrogen Control

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INTRODUCTION

Today it is widely recognized that there is probably no ecological niche on earth where bacteria have not evolved to exploit whatever nutrients are available to support life. Despite this enormous versatility of bacterial metabolism, certain fundamental mechanisms have to exist to regulate and integrate enzyme synthesis and enzyme activity. At the center of this metabolic control is the need to coordinate the catabolism and assimilation of carbon and nitrogen sources so as to maximize potential growth rates under any particular nutritional regime. There appear to be very few, if any, absolutely conserved mechanisms for achieving these ends among the major families of the eubacteria and the archaebacteria. However, the subject of this review, a small signal transduction protein commonly known as P_{II}, is undoubtedly one of the most conserved signal proteins in the bacterial world. From its initial identification in 1969 as the second peak eluting from a gel filtration column, hence the designation P_{II} (206), this protein family has since been found to play a significant role in the coordination of nitrogen metabolism in a wide variety of bacteria. The recent identification of members of the P_{II} family in plants extends the biological role of these proteins even further. In this review, we have tried to bring together information, old and new, to give a broad perspective of our current knowledge of this intriguing molecule and to suggest new areas where it may also play a pivotal role in nitrogen metabolism.

HISTORICAL PERSPECTIVE

Nitrogen is one of the most important elements required for life, as it is necessary for the production of amino acids, nucleotides, amino sugars (required for the synthesis of lipopolysaccharides and peptidoglycan), NAD, and *p*-aminobenzoate (a precursor in folate biosynthesis). Consequently bacteria have developed a number of mechanisms by which nitrogen can be assimilated from a variety of sources, ranging from ammonium to atmospheric dinitrogen (N_2). Ammonium is almost always the preferred nitrogen source, as it can be assimilated directly into glutamine and glutamate, the key donors for biosynthetic reactions, and is therefore the least energetically expensive substrate to process. By contrast, organic sources such as amino acids must first be degraded to ammonium, and inorganic sources such as NO_3, NO_2, and N_2 must be reduced before assimilation (190).

There are two major assimilation pathways for ammonium, the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, which is ubiquitous in bacteria, and the glutamate dehydrogenase pathway, which is an alternative route of assimilation in many bacteria, including the enterobacteria. While glutamate dehydrogenase is energetically more efficient than GS/GOGAT, it has a low affinity for ammonium (K_m of about 1 mM) and is consequently rather ineffective in cells growing in nitrogen-limited conditions.

Glutamine synthetase (GS) is a highly regulated enzyme at both the transcriptional and posttranslational level (for a review, see references 146, 155, 190, and 194). In enteric bacteria, GS is reversibly covalently modified by the bifunctional enzyme adenylyltransferase (ATase) in response to nitrogen availability, and the adenylylation state of GS regulates its catalytic activity. The mechanism of this regulation was determined by a series of elegant biochemical experiments initiated in the late 1960s. Initial studies showed that two protein components, P_I and P_{II}, were concerned with the adenylylation and deadenylylation process in *Escherichia coli* (206). The P_I fraction contained an adenylytransferase whose ability to adenylylate or deadenylylate GS was specified by the P_{II} protein and by the concentrations of P_I, ATP, UTP, glutamine, and 2-ketoglutarate (5). It was subsequently determined that the P_I fraction could be resolved into an ATase and a uridylyltransferase (UTase/UR) activity, the latter being capable of both uridylylation/uridylyl-removing and deuridylylation of P_{II} (30, 148). P_{II} stimulated adenylylation of GS by ATase, and P_{II}-UMP stimulated the reverse reaction. P_{II} was subsequently purified and proposed to be a tetramer with a molecular mass of about 44 kDa (1). The site of uridylylation on P_{II} was identified as one of the two tyrosine residues in the protein, and the sequence of a tryptic peptide containing the covalently bound nucleotide was also determined (1, 193). The uridylylation and deuridylylation reactions were demonstrated to be catalyzed by a single bifunctional protein when the enzyme was finally purified to homogeneity as a single polypeptide of 95 kDa (78, 117). Hence, by 1984 the biochemical characterization of P_{II} and the reactions that it regulated was well advanced, and a detailed model of the process of GS adenylylation had been proposed (1, 218).

In the meantime, genetic analysis of the regulation of GS and histidase in *Klebsiella aerogenes* had begun to shed light on the genes involved in these processes. Mutations in the gene encoding P_{II} (*glnB*) were first isolated in *K. aerogenes* in 1973 by selection for glutamine-requiring mutants (184). Two classes of mutant were identified, those in which GS levels were undetectable, which were designated as *glnA* mutants, and a second class, represented by a single mutant, with very low but detectable levels of GS, which was designated the *glnB* mutant. It was then correctly postulated that *glnA* was the structural gene for GS and that *glnB* encoded an activator required for the synthesis of GS and for regulation of synthesis in response to nitrogen availability. Significantly, the *glnB* mutant was also unable to induce histidase expression when cells were nitrogen limited, giving the first indication that P_{II} might have a role beyond the regulation of GS activity.

The product of *glnB* was finally identified as P_{II} by Foor et al., who showed that in the original *K. aerogenes glnB* mutant, P_{II} was altered so that it could not be uridylylated in nitrogen-limited conditions and consequently could not stimulate dead-

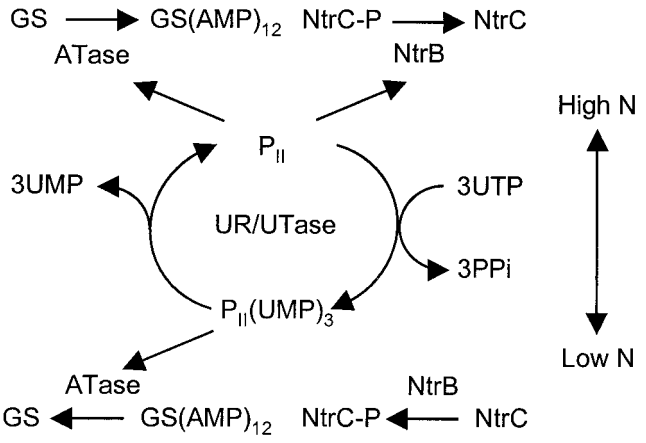

FIG. 1. Nitrogen regulation (Ntr) system of enteric bacteria. The activities of both GS and NtrC are regulated in response to the intracellular nitrogen status. UTase (*glnD* product) catalyzes the uridylylation and deuridylylation of P_{II} (*glnB* product). ATase catalyzes the adenylylation and deadenylylation of GS. NtrB catalyzes the phosphorylation and dephosphorylation of NtrC.

enylylation of GS by Atase (64, 65). A second class of *glnB* mutations resulted in loss of P_{II}, and these *glnB* null mutants were still able to adenylylate and deadenylylate GS, although the rates of these reactions were reduced. However, the absence of P_{II} also led to high levels of GS synthesis in the presence of ammonia, indicating a role of P_{II} in regulating GS expression as well as its activity. Further evidence for P_{II} having a role that is independent of its effect on ATase was provided by studies of *glnB* suppressor mutations which did not affect ATase activity but still affected levels of GS (191).

The *Escherichia coli glnB* gene was first sequenced by Son and Rhee (214). The deduced amino acid sequence agreed completely with that published earlier for the tryptic peptide containing the site of uridylylation (193) and showed that Tyr-51 was the modified residue. The isolation of further glutamine auxotrophs of both *K. aerogenes* and *E. coli* also led to identification of the structural genes for uridylytransferase (*glnD*) and adenylytransferase (*glnE*) that were finally cloned from *E. coli* and sequenced in 1993 (19, 25, 63, 64, 232).

Since the isolation of the first *glnB* mutant (184), there have been indications that P_{II} has a function beyond regulating ATase activity. Subsequent genetic studies with *E. coli, Salmonella enterica* serovar Typhimurium, *K. aerogenes*, and *Klebsiella pneumoniae* identified a two-component nitrogen regulatory (*ntr*) system encoded by the *ntrBC* genes that was responsible for the global transcriptional control of enzymes of nitrogen assimilation and catabolism (see reference 155 for a review). The link between NtrBC and P_{II} was demonstrated by further genetic studies in *E. coli* that led to the proposal that P_{II} modulated the activity of the histidine protein kinase sensor protein NtrB (31). This established P_{II} as the key link between changes in the intracellular nitrogen status and the activity of the transcriptional activator protein NtrC (Fig. 1).

Although by 1990 the molecular details of the enteric nitrogen regulation system seemed to be established, a number of inconsistencies were still apparent in the model. As the effects of UTase were predicted to be mediated through P_{II}, then in strains lacking P_{II} the *glnD* phenotype should be irrelevant, but this was not the case. *E. coli* cells lacking P_{II} are Ntr^+, as defined by their ability to utilize arginine as the sole nitrogen source, whereas both *glnD* mutants and *glnB glnD* double mutants cannot utilize arginine (13, 31). Hence, UTase has a role in Ntr regulation that is independent of P_{II}. Similarly, cells lacking P_{II} regulate GS adenylylation normally, whereas in vitro experiments showed that deadenylylation of GS by ATase absolutely requires P_{II}-UMP (219, 233). A *glnB glnD* double mutant does not deadenylylate GS correctly in N limitation, indicating that UTase is also required for GS adenylylation in the absence of P_{II}. Taken together, these results suggested the existence of at least one more protein that was involved in both Ntr regulation and GS adenylylation and that was also a substrate for UTase. Indications that the model was incomplete were not limited to *E. coli*, as studies on nitrogen control of nitrogen fixation (*nif*) genes in *K. pneumoniae* had prompted similar conclusions (55, 95).

The presence of a second P_{II} gene in *E. coli* was confirmed in 1995 with the identification of a *glnB* homologue that was designated *glnK* (231, 233): ironically, this gene had been sequenced in 1993 but had gone unnoticed by researchers in nitrogen regulation (3). The *E. coli* GlnK protein is 67% identical to GlnB at the primary sequence level, and the protein is encoded in an operon with a second downstream gene, *amtB*, that is believed to encode a high-affinity ammonium transporter (231). Tyrosine 51 is conserved in GlnK, and indeed in nitrogen-limiting conditions, the protein is subject to uridylylation at this residue in a similar manner to GlnB (89, 231).

The subsequent publication of the *E. coli* genome sequence confirmed that the organism has just two *glnB*-like genes, a situation that has since been found in many bacterial species. Indeed, as we discuss below, some organisms carry up to four copies of this gene family. This mutiplicity of P_{II}-like proteins raises complications with respect to terminology that we will discuss later, but from this point onwards we will use the term P_{II} as a generic term to describe any member of the P_{II} protein family.

OCCURRENCE OF P_{II}-LIKE PROTEINS

In recent years, the explosion in bacterial genomics together with recognition of the involvement of P_{II} proteins in a whole range of nitrogen regulation phenomena in bacteria led to the discovery that members of the P_{II} family are ubiquitous among prokaryotes. Genes encoding P_{II} proteins are found in the proteobacteria, the actinobacteria, the firmibacteria, the cyanobacteria, and the archaebacteria, and recently P_{II}-like proteins have also been found in higher plants. Within these different groups, P_{II} genes fall into a number of distinct classes with respect to both the primary amino acid sequences of their predicted products and their genetic linkage. Our current knowledge of the distribution of P_{II}-like proteins is summarized in Table 1. Information on expression of these genes varies considerably, with very detailed studies on some organisms and nothing at all to date on others.

γ Proteobacteria

The *E. coli glnB* gene is located at 50 min on the chomosome (139), between the *hmpA* gene (located at the 3′ end of *glnB*

TABLE 1. Organization of P_{II} genes in prokaryotes and plants

Organism[a]	Gene(s) present		
	glnB-like	*glnK*-like	*nif* linked
α Proteobacteria			
Azospirillum brasilense	*glnB, glnA*	*glnK, aat*	
Azorhizobium caulinodans	*glnB, glnA*	*glnK, amtB*	
Acetobacter diazotrophicus	*glnB, glnA*	$glnK_1$, *amtB;* $glnK_2$, $amtB_2$	
Bradyrhizobium japonicum	*glnB, glnA*		
Rhizobium etli	*glnB, glnA*	*glnK, amtB*	
*Rhodobacter capsulatus**	*glnB, glnA*	*glnK, amtB*	
Rhodobacter sphaeroides	*glnB, glnA*	*glnK, amtB*	
Rhodospirillum rubrum	*glnB, glnA*	*glnK*	
Sinorhizobium meliloti	*glnB, glnA*	*glnK, amtB*	
β Proteobacteria			
Azoarcus sp.	*nadE, glnB*	*glnK, amtB; gltB, glnY, amtY*	
Herbaspirillum seropedicae[b]	*nadE, glnB*	*glnK?*	
*Neiserria meningitidis**	*glnB*	*amtB*	
δ Proteobacteria			
Desulfovibrio gigas			$nifH$, $nifI_1$
γ Proteobacteria			
Azotobacter vinelandii		*glnK, amtB*	
*Escherichia coli**	*glnB*	*glnK, amtB*	
*Haemophilus influenzae**	*mog, glnB, ydgD*		
Klebsiella pneumoniae	*glnB*	*glnK, amtB*	
*Pseudomonas aeruginosa**		*glnK, amtB*	
*Vibrio cholerae**		*glnK, amtB*	
Xanthomonas citri		*glnA, glnK, amtB*	
Xylella fastidiosa		*glnA, glnK, amtB*	
Firmibacteria			
*Bacillus subtilis**		*amtB, glnK*	
*Clostridium acetobutylicum**		*amtB, glnK*	$nifH$, $nifI_1$, $nifI_2$, *nifD, nifK*
Clostridium cellobioparum			$nifH$, $nifI_1$
Clostridium longisporum		*glnK*	
*Lactococcus lactis**		*amtB, glnK*	
Actinobacteria			
Corynebacterium glutamicum		*amtB, glnK, glnD*	
*Mycobacterium tuberculosis**		*amtB, glnK, glnD*	
*Streptomyces coelicolor**		*amtB, glnK, glnD*	
Archaebacteria			
*Archaeoglobus fulgidus**		*amtB, glnK* (3x)	
Methanobacterium ivanovii			$nifH$, $nifI_1$, $nifI_2$, *nifD, nifK*
*Methanobacterium thermoautotrophicum**		*amtB, glnK* (2x)	$nifH$, $nifI_1$, $nifI_2$, *nifD, nifK*
*Methanococcus jannaschii**		*glnK, amtB*	
		amtB; glnK	
Methanococcus maripaludis			$nifH$, $nifI_1$, $nifI_2$, *nifD, nifK*
Methanococcus thermolithotrophicus			$nifH$, $nifI_1$, $nifI_2$, *nifD, nifK*
Methanosarcina barkeri			$nifH$, $nifI_1$, $nifI_2$, *nifD* (2x)
Deinococci			
*Deinococcus radiodurans**		*glnK, amtB*	
Thermatogales			
*Thermotoga maritima**		*amtB, glnK*	
Aquificaceae			
*Aquifex aeolicus**	*glnB, glnA, amtB*		$glnB_i$, *nasA, narB*
Cyanobacteria			
Anabaena PCC7120	*glnB*		
Freymella diplosiphon	*glnB*		
Nostoc punctiforme	*glnB*		
Prochlorococcus marinus	*glnB*		
*Synechocystis PCC6803**	*glnB*		
Synechococcus PCC7942	*glnB*		

Continued on following page

TABLE 1—*Continued*

Organism[a]	Gene(s) present		
	glnB-like	*glnK*-like	*nif* linked
Red algae[c]			
Cyanidium caldarium	*glnB*		
Porphyra purpurea	*glnB*		
Dicotyledenous plants			
Arabidopsis thaliana[d]	*GLB1*		
Glycine max	*GLB1*		
Lycopersicon esculentum	*GLB1*		
Ricinus communis	*GLB1*		

[a] Complete genome sequences are available for species marked with an asterisk. In these cases, all known *glnB*-like genes are shown; in other cases, the list is largely derived from independently cloned genes and should not therefore be considered comprehensive.
[b] *Herbaspirillum seropedicae* has two P_{II} genes but the second has not yet been characterised (21).
[c] In these cases the genes are located within the chloroplast genome.
[d] *A. thaliana GLB1* is a nuclear gene encoding a chloroplastic protein.

and transcribed in the opposite direction) and *yfhA* (also called ORF-2 or OrfXB), located directly upstream of *glnB* and transcribed in the same direction (139, 232). This genetic organization is conserved in *K. pneumoniae* (96).

Four transcription starts involved in the transcription of *E. coli glnB* have been described, but none of these transcripts is regulated in response to nitrogen status (31, 87, 139, 232). Major promoters for *glnB* are located immediately upstream of the *glnB* gene (−33 and −95 from the ATG), but transcription of *glnB* can also be initiated at a promoter located upstream of *yfhA* (139). The *E. coli* genome sequence reveals a potential operon of three genes (*yfhKGA*) upstream of *glnB* and transcribed in the same direction. The product of *yfhA* has a high degree of homology to the nitrogen-regulatory protein NtrC (232), and YfhK and YfhA constitute a potential two-component regulatory pair, the function of which is unknown (161). It is therefore conceivable that under some conditions *glnB* is transcribed from a promoter upstream of *yfhK*.

A binding site for the repressor protein PurR, which is responsible for repression of enzymes required for purine nucleotide biosynthesis, is located between the two major *glnB* transcriptional start sites (from −68 to −83 nucleotides), and PurR downregulates the transcription of *glnB* twofold in conditions of purine excess (87). The physiological significance of this regulation during nitrogen excess may be to apply an additional subtle regulation of GS activity when the glutamine requirement for purine synthesis is low and little glutamine is required for protein synthesis (230).

Like *E. coli*, other members of the γ proteobacteria also have a second P_{II} gene, *glnK*, that is located upstream of the ammonium transporter gene *amtB* (101, 223, 231, 233). In both *E. coli* and *K. pneumoniae*, the region upstream of the *glnK* gene contains a σ^{54} binding site and a possible NtrC binding site, and expression of the operon is nitrogen regulated in an NtrC-dependent manner (13, 101, 231). Exceptions include *Azotobacter vinelandii*, which appears to have only one P_{II} gene that is linked to *amtB* and is expressed from a σ^{70}-like promoter at a low constitutive level (153), and *Haemophilus influenzae*, which has a single *glnB*-like gene flanked by an upstream gene with similarity to *E. coli mog* and a downstream gene encoding a protein with 28% identity to *E. coli* YdgG and YhhT, which are both presumptive integral membrane proteins. A single P_{II} gene is located between *glnA* and *amtB* in *Xylella fastidiosa* (accession no. AAF84648 to -50), and a similar linkage of *glnA* and *glnK* has been reported in *Xanthomonas citri* (accession no. AF182395 and -96).

α Proteobacteria

The majority of the α proteobacteria examined so far, including those in the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Acetobacter, Azospirillum, Rhodobacter*, and *Rhodospirillum*, have two P_{II} genes. One gene encodes a P_{II} protein that is very similar to *E. coli* GlnB and is located upstream of and cotranscribed with *glnA* (the structural gene for glutamine synthetase I) (7, 27, 41, 94, 113, 127, 151, 158, 262). The second gene encodes a protein similar to GlnK and is linked to an *amtB* homologue (125, 157, 182, 185, 222, 254) (Y. Zhang and G. Roberts, personal communication). One exception to this pattern is *Azospirillum brasilense*, in which the *glnK*-like gene (termed *glnZ*) is not linked to *amtB* (46, 49, 228), but instead an open reading frame encoding a protein with 26% identity to the aspartate aminotransferase of *Bacillus subtilis* is found just 300 bp downstream of *glnZ* (47). The second exception is *Acetobacter diazotrophicus*, which has both a *glnB-glnA* operon and two *glnK-amtB* operons (D. Meletzus, personal communication).

Expression of the *glnBA* and *glnK-amtB* operons has been studied in some detail in the α proteobacteria. Transcription of the *glnBA* operon varies somewhat from one organism to another but shows a number of common features. *Rhizobium leguminosarum* and *Sinorhizobium meliloti* have promoters upstream of both *glnB* and *glnA*. The *glnA* promoter does not contain a consensus sequence characteristic of other previously described promoters, but the *glnB* promoter region contains a −24, −12 motif characteristic of σ^{54}-dependent promoters. Expression of both *glnB* and *glnA* is partially NtrC dependent, but there are no clear NtrC binding sites upstream of *glnB* (6, 7, 36). In *R. leguminosarum*, the genes are cotranscribed, but neither the *glnBA* nor the *glnA* transcript is markedly nitrogen regulated (36, 195). However, expression of *glnB* is significantly downregulated during bacteroid differentiation (56).

Azospirillum brasilense also produces a *glnBA* and a *glnA* transcript, but in this case *glnB* is preceded by two promoters

(48). The gene is expressed from a σ^{70}-like promoter in nitrogen sufficiency, and transcription is elevated fivefold in nitrogen limitation as a second σ^{54}-dependent promoter is activated. However, expression of *glnB* is not NtrC dependent (48, 49), and it has been proposed that *glnB* is activated by an NtrC homologue. The *glnA* promoter does not bear any similarity to the known consensus sites for σ factors, and *glnA* transcription is downregulated when molecular nitrogen is the sole nitrogen source (48, 49).

In *Bradyrhizobium japonicum, glnB* and *glnA* expression is almost entirely separate, but *glnB* is transcribed from tandem promoters. The *glnA* gene is constitutively expressed from a σ^{70}-like promoter, and although *glnB* is expressed from the σ^{70}-like promoter in nitrogen sufficiency and from a σ^{54}-dependent promoter in nitrogen limitation, there is very little variation in the amount of transcript in response to the change in nitrogen status (151).

The *glnB* and *glnA* genes are also cotranscribed from tandem promoters in *Rhodobacter capsulatus*. The first promoter, *glnB*p1, is repressed by NtrC, while the second, *glnB*p2, is activated. In *R. capsulatus*, NtrC does not function with an RNA polymerase containing σ^{54}, and hence, not surprisingly, there are no σ^{54} binding sites upstream of *glnB* (43, 73). Borghese and Wall (26) studied *glnB* expression using a *glnB*::*lacZ* fusion and different growth conditions from those used by Foster-Hartnett and Kranz (73). In this case, *glnB* expression was found to occur at higher levels under nitrogen-rich conditions than under nitrogen-poor conditions even in an *ntrC* mutant background. Moreover, the data suggested a posttranscriptional processing event that resulted in unequal levels of expression of *glnB* and *glnA* (26). The *glnBA* promoter region of *Rhodobacter sphaeroides* is similar to that of *R. capsulatus*, suggesting similar regulation, including partial nitrogen control of a *glnB* expression (185, 262). Interestingly, in a ribulose bisphosphate carboxylase/oxygenase (RubisCO)-deficient mutant, *glnB* expression was repressed and GS activity was extremely low regardless of the nitrogen source, suggesting the presence of a weak constitutive *glnA* promoter (185).

In *Rhodospirillum rubrum, glnB* and *glnA* are cotranscribed from a weak σ^{70}-like promoter (*glnB*p1) and a strong σ^{54}-like promoter (*glnB*p2) in either nitrogen-rich or nitrogen-fixing conditions (34, 113). However, *glnB*p2 activity is enhanced by NtrC in nitrogen-fixing conditions. Although Northern blotting analysis revealed the presence of two transcripts, no *glnA* promoter was detected, suggesting that the *glnA* mRNA is produced by processing (34, 113). As in *Rhodospirillum rubrum* and *Rhodobacter capsulatus, Azorhizobium caulinaudans glnB* and *glnA* appear to be cotranscribed (158). In both nitrogen excess and nitrogen limitation, the *glnBA* operon is transcribed from two overlapping promoters having the same start site, one of them σ^{54} and NtrC dependent and the other uncharacterized. Again, no promoter was detected upstream of *glnA*, and an mRNA processing event was proposed (158).

Regulation of *glnK* expression is similar in all the α proteobacteria so far studied, *Azospirillum brasilense (glnZ), Azorhizobium caulinodans*, and *Rhizobium etli*. In each case, the primary transcript is expressed from a σ^{54}-dependent promoter that is active in nitrogen-limiting conditions and is NtrC dependent (47, 157, 222). In *Rhodobacter sphaeroides, glnK* is also expressed only under nitrogen-limiting conditions, and in a RubisCO-deficient mutant, *glnK* expression is partially derepressed in the presence of ammonium (185).

β Proteobacteria

Two representatives of the β proteobacteria, *Herbasprillum seropedicae* and an *Azoarcus* sp. have been studied in detail. *H. seropedicae* has two P_{II}-like genes, one of which is linked to a homologue of *E. coli nadE* (a gene encoding ammonia-dependent NAD synthetase) (21). The *Azoarcus* sp. has three P_{II}-like genes, one *glnB* homologue and two *glnK* homologues, each of which is linked to an *amtB* homologue (*glnK* to *amtB* and *glnY* to *amtY*) (119, 150, 188). Two of the *Azoarcus* P_{II} genes, *glnK* and *glnY*, are preferentially transcribed under conditions of nitrogen fixation (150).

δ Proteobacteria

Within the δ proteobacteria, there is evidence for one *glnB*-like gene in the form of an open reading frame downstream of the nitrogenase structural gene *nifH* of *Desulfovibrio gigas* (accession no. U68183). This situation is very similar to that seen in the diazotrophic methanogens (see below).

Firmibacteria

Among the gram-positive bacteria, there are relatively few reports of P_{II} genes to date. *Bacillus subtilis* has a single P_{II} gene that was originally designated *nrgB* and is located downstream of an *amtB* homologue (*nrgA*) (246). The upstream region contains a *B. subtilis* σ^{A}-dependent promoter, and characterization of two transcripts separated by a single nucleotide suggests a common promoter origin. The operon is markedly induced in nitrogen limitation, but regulation of an *nrgA* fusion is not altered in either a Δ*nrgB* background or a Δ*nrgAB* background, suggesting that these gene products are not required for their nitrogen regulation; rather, expression is activated by the *Bacillus* global nitrogen-regulatory protein TnrA (246–249).

Searches of the DNA sequence databases also reveal evidence for *glnB*-like genes in *Clostridium acetobutylicum* (http://www.genomecorp.com/genesequences/clostridium/clospage.html), *Clostridium cellobioparum* (accession no. U59414), *Clostridium longisporum* (29), and *Listeria monocytogenes* (accession no. AF104224).

Actinobacteria

P_{II} genes have been identified in a number of actinomycetes. *Mycobacterium tuberculosis* has a single gene, with an *amtB* homologue upstream and a *glnD* homologue downstream, forming a potential *amtB-glnK-glnD* operon (38). A similar organization is found in *Corynebacterium glutamicum* (104) and in *Streptomyces coelicolor*.

Cyanobacteria

The presence of a GlnB protein in cyanobacteria was first recognized with the N-terminal sequencing of a phosphorylated 13-kDa protein from *Synechococcus* sp. strain PCC6301 (85). Subsequent investigations of the occurrence of *glnB* genes in cyanobacteria included analysis of *Synechococcus* sp. strain

PCC7942, *Calothrix* sp. strain PCC7601, *Pseudanabena* sp. strain PCC6901, *Microcystis* sp. strain PCC7813, and *Nostoc* sp. strain PCC8009 and suggested that *glnB* is likely to be present in all cyanobacteria (225). Since then, the *glnB* genes from *Synechococcus* sp. strain PCC7942, *Synechocystis* sp. strain PCC6803, *Nostoc punctiforme*, and *Anabaena* sp. strain PCC7120 have been cloned and sequenced (76, 80, 84, 225). In all cases *glnB* appears to be monocistronic, and its transcription is enhanced by nitrogen limitation.

In both *Synechococcus* sp. strain PCC7942 and *Synechocystis* sp. strain PCC6803, *glnB* is expressed from two tandem promoters, a σ^{70} *E. coli*-type promoter, leading to constitutive expression of *glnB*, and a nitrogen-regulated promoter activated by the nitrogen-regulatory protein NtcA. Despite similarities, the physical organization of the promoters differs, and in *Synechocystis* sp. strain PCC6803 the regulated promoter is functional only in cells starved for nitrogen, whereas in *Synechococcus* sp. strain PCC7942 it is active both in the absence of nitrogen and in the presence of nitrate (76, 132). Moreover, in *Synechocystis* sp. strain PCC6803, *glnB* mRNA levels decrease when cells are either transferred to the dark or incubated in the presence of photosynthesis inhibitors, indicating that *glnB* transcription is under the control of the redox state of the cell (76).

Archaebacteria

In all the archaebacteria for which complete genome sequences are available, *glnB*-like genes are found linked to *amtB* homologues and are usually present in mutiple copies (223). However, in the diazotrophic methanogens, two additional *glnB*-like genes are located between *nifH* and *nifD* within the *nif* gene cluster (122, 123, 208, 210, 215). These genes have been anlayzed in most detail in *Methanococcus maripaludis*, in which they are apparently transcribed in a 7.6-kb *nif* mRNA transcript from a single promoter with a transcritional start site located 80 nucleotides upstream of the translational start site of *nifH* (122). Interestingly, a comparable gene organization is found in nitrogen-fixing microorganisms inhabiting the gut of the termite *Neotermes koshunensis*, in which two *glnB*-like genes are located between *anfH* and *anfD* (174).

Other Bacteria

The genome sequence of the extreme thermophile *Aquifex aeolicus* revealed two *glnB*-like genes (45). One of these is in a *glnB-glnA* operon similar to that found in the γ proteobacteria, and the other is the first gene in a cluster comprising $glnB_i$, *nasA*, and *narB*. This latter *glnB*-like gene apparently encodes a totally novel P_{II} polypeptide of 205 amino acids. The gene comprises a tandem duplication, so that the C terminus of the first "copy" is fused directly to the N terminus of the second copy. Both copies are predicted to have all the characteristic features known from structural studies of P_{II} (see later), with the exception that the N-terminal copy is truncated prior to the last two β-sheets that comprise the C-loop. This sequence would suggest that if the gene is expressed, it could encode a trimeric protein that comprises six P_{II}-like domains with three T-loops exposed on one face and another three similar but not identical T-loops on the opposite face. In alignments of P_{II} amino acid sequences, the predicted product of *Aguifex aeolicus* $glnB_i$ is most similar to the *glnB*-like proteins encoded downstream of *nifH* in the diazotrophic archaebacteria.

Organisms in Which P_{II} Is Absent

The P_{II} protein is not totally ubiquitous, and the completion of a significant number of bacterial genome sequences has identified a number of organisms that do not encode a P_{II}-like protein. These include *Helicobacter pylori, Mycoplasma genitalium, Mycoplasma pneumoniae, Campylobacter jejuni, Chlamydia muridarum, Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydophila pneumoniae, Treponema pallidum, Ureaplasma urealyticum, Rickettsia prowazekii, Aeropyrum pernix, Borrelia burgdorferi, Pyrococcus horikoshii*, and *Pyrococcus abyssi*. It is notable that a large number of these organisms are pathogens whose genomes have undergone reductive evolution to compete in specific niches within their respective hosts. As such, they frequently rely on their hosts for provision of nitrogen-containing compounds, and they probably do not face the homeostatic challenges that require constant monitoring and control of their intracellular nitrogen status.

P_{II} Genes in Plants

The first discovery of a P_{II} gene outside the bacteria was the identification of a *glnB*-like gene in the chloroplast genome of the red alga *Porphyra purpurea* (189), and a second such sequence has since been found in the chloroplast of *Cyanidium caldarium* (accession no. AF022186). However, more recent studies indicate that the P_{II} protein is probably quite widely distributed among higher plants. Genes encoding P_{II}-like proteins have been cloned from *Arabidopsis thaliana* and *Ricinus communis* (castor bean) (97), and similar genes have been sequenced in *Lycopersicon esculentum* (tomato) (accession no. AI773079) and *Glycine max* (soybean) (accession no. AW153272). In all cases, the predicted protein sequences show significant homology to the bacterial GlnB proteins but have an additional N-terminal domain of about 70 amino acids.

The P_{II} protein in *Arabidopsis thaliana* is encoded by the *GLB1* gene, mapped at 10.8 centimorgans from the top of chromosome IV (97). When extracted from leaves, this nucleus-encoded chloroplast protein exhibits a molecular mass of 18 kDa. However, its predicted molecular mass of 21.4 kDa (196 amino acids) suggests that P_{II} may be processed inside the plant cell. The transcription of *GLB1* mRNA is induced by light or sucrose and is repressed by the amino acids asparagine, glutamine, and glutamate (97).

P_{II} PROTEIN NOMENCLATURE

The recent rapid increase in the identification of genes encoding P_{II} homologues has led to an extremely complex and confusing situation with respect to nomenclature. It is clear that a number of genes that are currently designated *glnB* have very different functions and in some cases are predicted to encode proteins with significantly different structures. However, the number of sequences now available allows us to assess the similarities and differences between these proteins and to begin to make some predictions about their functions. In carrying out such an assessment, we have used two criteria: con-

servation of gene linkage and similarity at the level of primary amino acid sequence.

With the dramatic increase in availability of prokaryotic genome sequences, conservation of genetic linkage is increasingly being recognized as offering predictions of functional relationships between gene products (44, 177). Multiple sequence alignments can also provide information on sequence similarity, which, at least in some cases, may reflect similarity of function.

Multiple sequence alignments using Clustal W and analysis of the resultant dendrograms show, perhaps not surprisingly, that P_{II} sequences cluster into groups that reflect almost completely the taxonomic relationships of the originating organism, and furthermore, within certain groups, e.g., the archaebacteria, clear subgroups occur. We have combined this information with knowledge of gene linkage to define just three major groups of prokaryotic P_{II} proteins (Table 1). Based on this subdivision, we consider that it is now appropriate to review the current gene nomenclature, and we suggest a revised format that we hope will aid gene description in the future. This nomenclature is not meant to imply a strict link between gene designation and function, particularly given the recognized overlapping functions that can occur, e.g., with GlnB and GlnK in some organisms (see later). However, it is hoped that these suggestions will provide a rational framework for describing members of the P_{II} family.

glnB

The original *glnB* designation should be retained for genes encoding a large group of closely related proteins found predominantly in the proteobacteria and the cyanobacteria. With a few exceptions, these genes are either monocistronic operons or linked to *glnA* or *nadE*. The protein products of these genes are typified by lysine at residue 3 and glutamate or aspartate at residue 5.

glnK

The designation *glnK* was originally adopted for the *amtB*-linked gene of *E. coli* (231). It has since become apparent that the ammonium transporter gene *amtB* is almost invariably linked to a P_{II} gene, a phenomenon that may imply a functional relationship between GlnK and AmtB (223). We suggest that the *glnK* nomenclature be retained for all *amtB*-linked P_{II} genes. In some cases, e.g., *Methanobacterium thermoautotrophicum* and *Archaeglobus fulgidus*, organisms have multiple *amtB-glnK* operons, and in these cases the genes should be designated $amtB_1$-$glnK_1$, $amtB_2$-$glnK_2$, etc., to follow standard nomenclature for such situations. The GlnK proteins are often, but not invariably, distinguished from GlnB proteins in having a hydrophobic residue (leucine, isoleucine, methionine, or phenylalanine) at position 3 and isoleucine, threonine, or methionine at position 5. We suggest extending the *glnK* designation to include the exceptional *glnZ* gene of *A. brasilense*, which is not linked to *amtB* but encodes a P_{II} protein that is very similar to the GlnK proteins of *Rhizobium etli* and *Acetobacter diazotrophicus*. Likewise, *Azoarcus* sp. has two *glnK*-like genes, one of which has been designated *glnY* and is linked to an *amtB* homologue designated *amtY* (150). On the basis of amino acid sequence, GlnY clusters quite closely with other GlnK proteins and could therefore perhaps more appropriately be designated $GlnK_2$ rather than GlnY.

nifI

A quite distinct group of P_{II} proteins are encoded by the *nifH*-linked genes found in the diazotrophic methanogens. These genes always occur in pairs and fall into two discrete subgroups, the *nifH*-proximal gene, encoding a polypeptide of about 105 amino acids, and the *nifH*-distal gene, encoding a polypeptide of 120 to 130 amino acids. At present these genes have been designated *glnB*, with a variety of subdivisions. In *M. maripaludis*, the products of these genes have now been shown to function in ammonia switch-off of nitrogen fixation (123, 123a). For this reason, we propose that this pair of genes should be renamed $nifI_1$ and $nifI_2$, respectively, to reflect both their distinction from *glnB* and *glnK* and their specific biological role. The *nifI* genes are not restricted to the archaebacteria, as sequences of $nifI_1$ genes have also been identified downstream of *nifH* genes in *Desulfovibrio gigas, Clostridium acetobutylicum*, and *Clostridium cellobioparum*. The novel *glnB*-like gene in *Aguifex aeolicus* that is located upstream of *nasA* and *narB* and currently designated $glnB_i$ is also most similar to this group of P_{II} proteins.

BIOCHEMISTRY OF P_{II} PROTEINS

Initial characterization of *E coli* P_{II} by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and sedimentation centrifugation indicated that the *E. coli* protein formed a tetramer (1). However, subsequent crystallization studies and sedimentation equilibrium experiments have shown that it is in fact a trimer (33, 46, 236). The molecular mass of the P_{II} monomer was elucidated by matrix-assisted laser desorption mass spectrometry and shown to be 12,435 Da, which agrees very well with the prediction from the DNA sequence of 12,427 Da (235, 236).

Structure of GlnB

An X-ray crystal structure of *E. coli* GlnB was initially achieved at a resolution of 2.7 Å and subsequently refined to a resolution of 1.9 Å (32, 33, 46) (Fig. 2). Each monomer contains two α-helices and six β-strands arranged so that the two α-helices and β-strands 1 to 4 form a double βαβ motif connected by a large loop stretching from Gly-37 to Phe-55. At the apex of this loop is the site of uridylylation, Tyr-51, and consequently this is referred to as the T loop. A smaller loop (the B loop) stretching from Gln-82 to Asp-88 separates the second α-helix from the fourth β-sheet, and a third loop (the C loop) containing β-strands 5 and 6 is found at the C terminus. Analysis of the refined structure has revealed that hydrogen bonds are formed between Gly-27 and Val-64, stabilizing β-sheets 2 and 3, and between Glu-106 and Arg-101 and between Thr-104 and Gly-105, stabilizing the C loop.

The trimer is arranged so that the four major β-strands of each monomer are flanked on one side by the T loop from the second monomer and on the other side by the C-terminal loop of the third. The arrangement of the monomers relative to one another causes a cleft, which may have functional significance, to form between the T and B loops of one monomer and the C

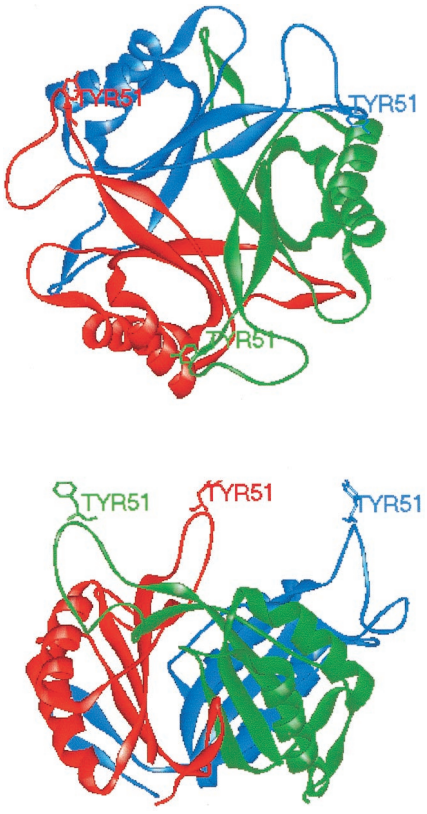

FIG. 2. Structure of the *E. coli* GlnB trimer viewed (top) from above and (bottom) from the side. Individual monomers are colored red, green, and blue.

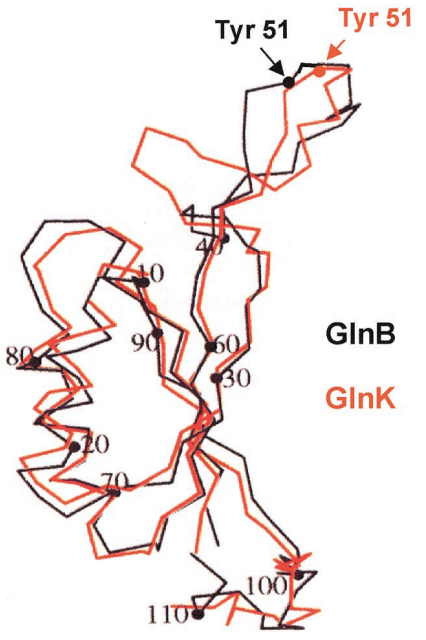

FIG. 3. Comparison of the structures of *E. coli* GlnB and GlnK. Superimposition of the C^{α} traces for GlnB (black) and GlnK (red) with the position of the Tyr-51 residues in each molecule is indicated. Adapted from reference 252.

loop of the next. Three six-stranded antiparallel β-sheets are arranged as a concave surface in the center of the trimer. The majority of the protein therefore packs into a squat barrel 30 Å high. The diameter of the cavity ranges from approximately 24 Å at the ends to 5.5 Å about 7.0 Å from either end, the diameter at the center of the cavity being about 12.0 Å. The cavity contains several ordered water molecules, and it has also been suggested that the cavity could contain metals (32). Each monomer is arranged so that an eight-residue stretch of the loop containing Tyr-51 at its apex is exposed at the same surface of the trimer about 13 Å above the surface of the barrel.

Structure of GlnK

The structure of *E. coli* GlnK has also been solved to a resolution of 2.0 Å (145, 252). GlnK also forms a trimer, with a core structure that is very similar to that of GlnB (Fig. 3). However, the GlnK protein was found to be present in two conformations in the crystals. They had core structures similar to each other's and to GlnB, but the structure of the T loop in each case was significantly different. It was only possible to solve the structure for one T loop which was stabilized by lattice contacts; the T loop of the other protein was disordered. The structure of the T loop differed from that of GlnB in that residues 47 to 49 of GlnK form a 3_{10} helix and the apex of the T loop was much closer to the top of the core of the molecule than seen with GlnB. It should be noted that the T loop of GlnK was only resolvable when it was constrained, and in the GlnB crystals the T loop was also latticed to an adjacent molecule. Nevertheless, the fact that the T loops form different conformations in the crystals might suggest that they behave differently in solution and that the T loop might be flexible rather than rigid. The T loop of *E. coli* GlnB is known to be essential for interaction with all three of its known targets, ATase, UTase, and NtrB (103, 112). These different targets may require different T-loop conformations to facilitate specific high-affinity interactions.

Both the B and C loops of GlnK also differ from those of GlnB. These loops form part of the cleft thought to be the important in the binding of effector molecules and proteins. The apex of the B loop of GlnK differs from that of GlnB by 4.3 Å, and the C loop contains a single rather than two β-strands and a 3_{10} helix which causes the end of the peptide to turn back into the bottom of the cleft. The structure of ATP-bound GlnK was also elucidated and showed that ATP makes contacts along the length of the cleft and bonds with residues from both subunits. In particular, the nitrogen and carbonyl oxygen of Ala-64 (which is not a conserved residue and is valine in GlnB) are thought to form hydrogen bonds with the adenine group. The B loop has a consensus sequence of Thr-Gly-X-X-Gly-Asp-Gly-Lys-Ile-Phe, which is very similar to that of mononucleotide-binding proteins such as adenylate kinase. The architecture of the phosphate-binding loop is different from that of other nucleotide-binding proteins, and there is no evidence for Mg^{2+} binding as seen in adenylate kinase and $p21^{ras}$. The motif is one of the most highly conserved regions in all the P_{II} proteins from eubacteria, archaebacteria, and plants. This suggests that ATP binding is probably a common property of P_{II}, and indeed it has also been demonstrated for the GlnB protein

of *Synechococcus* sp. (67). The precise role of ATP is presently unknown, although biochemical data suggest that high-affinity binding of 2-ketoglutarate requires ATP (67, 118). The binding of ATP may also cause alterations in the structure and mobility of the T loop and in the structure of the B loop, both of which could affect molecular recognition.

Heterotrimer Formation

E. coli GlnB and GlnK can form heterotrimers in vivo when *glnB* and *glnK* are coexpressed from a plasmid or when the wild-type strain is grown in nitrogen-limiting conditions (68, 234). GS adenylylation experiments in vitro demonstrate that the fully uridylylated heterotrimer is still capable of stimulating the deadenylylation activity of ATase, albeit to a lower extent than homotrimeric P_{II}-UMP. It has been proposed that heterotrimers could facilitate fine-tuning of the signal transduction cascade (234). While the physiological consequences of heterotrimer formation have yet to be fully established, heterotrimer formation is possibly not restricted to *E. coli* because it is also observed between *Synechococcus* GlnB and *E. coli* GlnB or GlnK when the cyanobacterial protein is expressed in *E. coli* (68). Given this observation, one might expect heterotrimer formation to occur in other organisms which express more than one form of P_{II} concurrently. The factors controlling such events could be of considerable interest and certainly complicate the interpretation of mutant phenotypes.

Analysis of sequence comparisons revealed that residues that are either involved in the formation of the trimer (residues 30, 32, 34, 93, 95, and 98) or interact in the interior of the central cavity (residues 3, 5, 60, and 62) are not always conserved in P_{II} proteins (252). In particular, residues 3 and 5 vary between the different subgroups of P_{II} proteins (101). In *E. coli* GlnB, Lys-3 and Asp-5 form a ring of alternating charge in the central cavity of the trimer, but in GlnK these residues are replaced by neutral Leu-3 and Thr-5 residues (252). The distinction between the nature of residues 3 and 5 in GlnB and GlnK extends to other members of the P_{II} family, e.g., $NifI_1$ polypeptides are characterized by Met-3 and Arg-5 or Lys-5, while $NifI_2$ is characterized by Glu-3 and Ile-5. The significance of this signature is not clear.

Structure-Function Analysis

A considerable number of mutations have been studied in *E. coli glnB*, and with the available crystal structure, these can help to shed some light on structure-function relationships in the protein. Uridylylation of GlnB is prevented by mutation of Tyr-51 to Phe, Asn, or Ser (14, 89, 103, 111). It is also severely affected by other alterations in the exposed part of the T loop, including E50Q, the same alteration as that produced by the *K. pneumoniae glnB502* mutation (96, 111). Residue Tyr-46 is highly conserved in P_{II} proteins, and a Y46F variant is uridylylated at a much lower rate than the wild type (103). The mutation does not alter the structure of the protein or binding of ATP or 2-ketoglutarate, suggesting that the residue may play an important role in recognition and binding by UTase (103, 111). Removal of the apex of the T loop (Δ47–53) also prevents uridylylation. Indeed, it alters interaction with all three known receptors but not the binding of small effector molecules, implying that the T loop is necessary for interaction with GlnB targets (111).

Mutations of different residues within the T loop of GlnB affect inteaction with all targets to some degree, but two of them, G41A and A49P, are notable exceptions dramatically eliminating interaction with UTase or NtrB, respectively, without having any dramatic effect on their other targets (111). A number of other alterations have also been made to residues found in the conserved region comprising the B loop and the fourth β-sheet of *E. coli* GlnB. The effects of these changes on the interaction of GlnB with 2-ketoglutarate, ATP, and the signal-transducing enzymes were then studied. Mutations around the B loop and the base of the T loop tend to affect the binding of the small-molecule ligands and some or all of the three protein receptors UTase/UR, ATase, and NtrB (111). It may be that the effect on enzyme interactions is a result of poor ligand binding. This second group of mutations cluster around the cleft formed by the T and B loops of one monomer and the C loop of the neighboring monomer, adding weight to the theory that this structure plays an important role in the interaction with receptor proteins and ligands.

Finally, two other alterations with marked effects on GlnB function fall outside these regions. A Thr29Met mutation was identified by the mutant's inability to activate *glnA* transcription, but further investigation revealed that the protein was affected in all its interactions. As this mutation destroys the hydrogen bond link with Glu-62 (found at the base of the third β-sheet), it may result in an overall change in protein conformation. A Gly89Ala mutation causes an almost total loss of function, probably linked to the inability to bind small-molecule effectors. Gly-89 is at the base of the B loop at a sharp turn, which allows the Lys-90 of β-4 to line the cleft. It is possible that this mutation causes a conformational change that prevents this turn.

Experiments investigating the functionality of heterotrimers comprising Δ47–53 GlnB and wild-type GlnB showed that the T loops can act independently of one another and a single loop is sufficient to allow interaction with the receptor proteins. Heterotrimers of Gly89Ala and wild-type monomers had different effects on interaction with different protein receptors, indicating that changes at the base of the B loop probably affect intersubunit interactions and thereby the activity of the wild-type subunit. Heterotrimers of Gly89Ala (containing a normal T loop but unable to bind small-molecule effectors) and Δ47–53 (missing the T loop) remained inactive, suggesting that there is no *trans* complementation between subunits (112).

Modification of P_{II} in Response to Nitrogen

A principal characteristic of the P_{II} proteins is their ability to be switched between two forms by covalent modification of a residue in the T loop. This modification was initially recognized as uridylylation of Tyr-51 in the T loop of *E. coli* GlnB. This residue is highly conserved, being present in the GlnB and GlnK proteins of the proteobacteria and the actinobacteria. Uridylylation of P_{II} proteins has been demonstrated in *Rhizobium leguminosarum* (40), *Klebsiella pneumoniae* (55), *Azospirillum brasilense* (47, 50), *Azotobacter vinelandii* (196), *Herbaspirillum seropedicae* (20), and *Azoarcus* sp. strain BH72 (150) and suggested in *Sinorhizobium meliloti* (8), *Rhodospiril-*

lum rubrum (114), and *Corynebacterium glutamicum* (104). While this process is widespread, it is not universal; P_{II} proteins in the cyanobacteria are phosphorylated on a serine residue, Ser-49, in the T loop, and in other organisms, e.g., the archaebacteria and higher plants, modification of P_{II} proteins has yet to be investigated.

Uridylylation of P_{II} proteins. Uridylylation of *E. coli* GlnB in vitro is a noncooperative reaction (15). The transferase reaction shows linear kinetics, with GlnB binding before ATP and pyrophosphate being released before GlnB-UMP. By contrast, the uridylyl-removing reaction proceeds with rapid equilibrium binding of substrate and random release of products. Both reactions are activated by ATP and 2-ketoglutarate, while glutamine inhibits the transferase reaction. The kinetic data are consistent with a single binding site on GlnD for GlnB and GlnB-UMP and with a single catalytic center on the enzyme, which could be located at the conserved nucleotidyl transferase site (108). In vitro, GlnK is uridylylated as effectively as GlnB, but deuridylylation of GlnK-UMP is slower than that of GlnB-UMP (14).

Under physiological conditions, the uridylylation state of P_{II} appears to be regulated mainly by the glutamine concentration, suggesting that the UTase/UR enzyme serves as an intracellular nitrogen sensor (108). This hypothesis is consistent with the constitutive expression of *glnD*, so that UTase/UR is always present in the cell and its activity is modulated in response to nitrogen availability, with the result that the uridylylation level of P_{II} reflects the intracellular nitrogen status of the cell.

The UTase/UR structural gene *glnD* has been cloned and sequenced from *E. coli* (232), *K. pneumoniae* (55), *Azotobacter vinelandii* (42), *Rhizobium tropici* (176), *Vibrio fischeri* (accession no. AF152563), *Rhizobium leguminosarum* biovar *viciae* (199), and *Sinorhizobium meliloti* (accession no. AF227730). Genome sequence analysis shows that *glnD* homologues are present in most proteobacteria and also in the actinobacteria, e.g., *Streptomyces coelicolor* (accession no. AL023797), *Mycobacterium tuberculosis* (38), and *Corynebacterium glutamicum* (104), but *glnD* is not found in the gram-positive firmibacteria (e.g., *Bacillus subtilis*), the cyanobacteria (e.g., *Synechocystis*), or the archaebacteria (*Methanobacterium thermoautotrophicum* and *Methanococcus jannaschii*). Relatively little is known about *glnD* expression, but the *E. coli glnD* gene is monocistronic and expressed at a very low level (117, 124, 232), and this is also the case for *Rhizobium tropici glnD* (176).

GlnD proteins have predicted molecular masses of about 100 kDa and contain a conserved nucleotidyltransferase superfamily motif (AVGGYGRXXLXPXSDIDLL) located in the amino-terminal region (93). This conserved motif is thought to be located in the active site of the enzyme, suggesting that the two catalytic activities of UTase reside at the same site unless one of the two catalytic sites does not contain the conserved nucleotidyltransferase sequence. The activities of GlnD proteins are indeed conserved as *Azotobacter vinelandii glnD* can complement *E. coli* and *K. aerogenes glnD* mutants, and likewise, *E. coli glnD* complements an *Azotobacter vinelandii glnD* mutant (42).

In *E. coli*, only one *glnD* mutation (*glnD*99::Tn*10*) has been physically mapped within the *glnD* gene (229), and the Tn*10* is inserted 567 bp upstream of the *glnD* stop codon (T. Arcondéguy and M. Merrick, unpublished results). The leaky nature of this mutation (31, 214) led Atkinson and Ninfa (16) and Son and Rhee (214) to propose that the Tn*10* does not completely inactivate UTase, which is consistent with initial data reporting a GlnB-UMP activity equivalent to 8% of the total purified GlnB from a *glnD99*::Tn*10* mutant strain (214). The predominance of mutations at the 3′ end of the gene is a notable feature in many organisms; a Tn*5* insertion in *Azotobacter vinelandii glnD* was located about 80 bp upstream of the stop codon (42), and in *Rhizobium tropici* a Tn*5* insertion was located at the 3′ end of the gene (176).

In *Azotobacter vinelandii*, a stable *glnD* null mutation could only be maintained when GS adenylylation was prevented by a *glnA* Y407F mutation or a suppressor presumptively located in the ATase gene *glnE* (39, 196). An extensive *glnD* deletion was constructed in *K. pneumoniae* (55), but the possibility that this mutation was maintained as a consequence of secondary suppressor mutations cannot be excluded. A *glnD* null mutant of *E. coli* exhibits a severe growth defect in minimal medium with ammonium as a nitrogen source, but the occurrence of suppressors in this strain has also not been investigated (T. Arcondéguy and M. Merrick, unpublished results). Finally, a *glnD* insertion mutant of *Corynebacterium glutamicum* was unstable (104). It therefore appears that *glnD* may be essential in some (or all) organisms and that maintenance of a null mutation may require the presence of secondary mutations that can occur spontaneously at high frequencies. This may be consistent with the important role of UTase as an intracellular nitrogen sensor but could also suggest that the protein has other, as yet unidentified, roles in the cell.

Phosphorylation of P_{II} proteins. Although Tyr-51 is present in GlnB from the cyanobacterium *Synechococcus* sp. strain PCC6803, this protein is not uridylylated but is phosphorylated at the nearby Ser-49 residue (69, 71). This is consistent with the absence of a *glnD* homologue. Despite this difference, heterologous expression of *Synechococcus* sp. strain PCC7942 GlnB in *E. coli* results in uridylylation of this protein, albeit with low efficiency (67, 68). P_{II} was originally identified in cyanobacteria as a 13-kDa protein in *Synechococcus* sp. strain PCC7942 that exhibited a posttranslational modification dependent on the nitrogen source and the spectral light quality (225). However, the spectral light quality dependency is only seen in the presence of nitrate, and hence, as in enterics, the primary role of GlnB appears to be in sensing nitrogen status (69). In the presence of ammonium, GlnB is unmodified, and in nitrate GlnB is predominantly modified. Moreover, in cells grown in ammonium but in the presence of L-methionine sulfoximine (a GS inhibitor), GlnB exhibits the modified pattern, indicating that ammonium assimilation is required to elicit dephosphorylation.

The modification state of GlnB in cyanobacteria is not controlled by a bifunctional enzyme but by a kinase and a phosphatase activity that can be biochemically separated, suggesting that these two activities reside on different proteins (100). In vivo studies of the phosphorylation state of GlnB under different growth conditions (70) and studies of the in vitro kinase activity (using a *glnB* null mutant extract) show that the kinase activity is stimulated by 2-ketoglutarate (71). However, unlike GlnD, the kinase does not respond to glutamine. Because dephosphorylation of GlnB is regulated by synergistic inhibition by ATP and 2-ketoglutarate and phosphorylation

depends on the same metabolites, it seems that ATP and 2-ketoglutarate binding of P_{II} (67) inversely affects its recognition by the kinase and the phosphatase (71, 100).

Modification of GlnB in cyanobacteria has also been reported in *Synechococcus* sp. strain 6301 (85) in *Synechocystis* sp. strain PCC6803 (92) and in the two filamentous nitrogen-fixing cyanobacteria *Calothrix* sp. strain PCC7504 (137) and *Anabaena* sp. strain PCC7120 (257). When *Synechocystis* sp. strain PCC6803 cells are exposed to conditions impairing the photosynthetic electron flow, GlnB remains unphosphorylated, suggesting that GlnB modification is also regulated by the redox state of the cells (92).

As in *Synechococcus* sp. strain PCC7942, *Anabaena* GlnB is phosphorylated when filaments are grown in the presence of nitrate and dephosphorylated in the presence of ammonium, although the kinetics of *Anabaena* GlnB phosphorylation-dephosphorylation is slower than in the *Synechococcus* sp. (80, 257). Interestingly, mutation of *pknD*, a eukaryote-type protein kinase, enhances phosphorylation of GlnB under nitrogen-fixing conditions. It has been proposed that PknD is probably not involved in the phosphorylation process itself but that the effect on GlnB phosphorylation is rather the consequence of impaired nitrogen metabolism or trafficking (257).

Other possible modifications. P_{II} modification has not yet been studied in the archaebacteria. A Tyr-51 residue (or its equivalent) is present in the P_{II} proteins encoded by the *amtB*-linked genes of *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*, but there is no *glnD* homologue in these organisms, suggesting that in these cases modification, if it occurs at all, is not by uridylylation (35). The archebacterial *nif* cluster GlnB homologues fall into two classes, $NifI_1$, with a predicted T loop of only 15 residues, and $NifI_2$, with a predicted T loop of 27 or more residues. Both contain conserved tyrosine residues, but they are not predicted to be at the apex of the T loop. Tyr-51 is also absent in *Bacillus subtilis* GlnK (246) and *Clostridium longisporum* GlnK (29), and consistent with this, there is no *glnD* homologue in the *Bacillus subtilis* genome. Both organisms have a serine residue in the T loop which could constitute a potential modification site.

In all the P_{II} sequences so far identified in plants, i.e., *Arabidopsis thaliana GLB1*, and similar sequences in *Glycine max, Lycopersicon esculentum*, and *Ricinus communis*, the position equivalent to Tyr-51 is occupied by phenylalanine. However, all these proteins contain serine residues in the T loop at the equivalent of positions 49 and 52 that could potentially be phosphorylated. Hence, nitrogen and/or carbon status could be sensed through the activity of a serine-threonine kinase and its associated phosphatase, which are common regulatory components of signal transduction pathways in eukaryotes (221). Transgenic plants overexpressing *A. thaliana* GlnB exhibit a phenotype consistent with the involvement of GlnB in perceiving the status of carbon and nitrogen (97).

Role of small effector molecules. The interaction of GlnB with three proteins, UTase/UR, GS, and NtrB, has been studied in depth, and the mechanisms that control these interactions are now beginning to be elucidated. In all cases the interactions depend on the nitrogen and carbon/energy status of the cell, which is signaled primarily by three small effector molecules, glutamine, 2-ketoglutarate, and ATP.

The ratio of glutamine to 2-ketoglutarate in the cell rises in conditions of nitrogen sufficiency and drops in conditions of nitrogen starvation (129, 205). Consequently, it was originally thought that the ratio between 2-ketoglutarate and glutamine was the critical factor influencing the uridylylation state of GlnB. However, detailed analysis in vitro showed that the concentration of 2-ketoglutarate in the cell is always sufficient for uridylylation to occur (117, 118). It has since been established that while uridylylation of GlnB requires 2-ketoglutarate and ATP and is repressed by glutamine, deuridylylation also requires ATP and 2-ketoglutarate but is stimulated by glutamine (117, 118). A low concentration of ATP or GlnB limits the uridylylation reaction, but the addition of excess GlnB does not inhibit it. This suggests that the binding of ATP to GlnB is required for GlnB to bind to UTase (108). ATP and 2-ketoglutarate are also required for the deuridylylation of GlnB-UMP, and there is evidence to suggest that this also involves their binding to GlnB (108).

Hence, glutamine is the nitrogen signal, and at physiological levels it regulates UTase/UR independently of 2-ketoglutarate (109). Glutamine inhibits uridylylation of GlnB by affecting the rate of the transfer reaction. Glutamine stimulates deuridylylation of GlnB-UMP fourfold in the presence of Mg^{2+}, and there is evidence that glutamine interacts with UTase/UR at a single site. Both UTase and UR activities can also be stimulated by a second metal ion cofactor, Mn^{2+}, but this is not thought to be physiologically significant for either reaction (108).

The main carbon signal appears to be 2-ketoglutarate, for which the trimeric GlnB molecule has three binding sites. The physiological significance of 2-ketoglutarate becomes clear when the interaction of GlnB with NtrB is investigated. The dephosphorylating activity of NtrB (and therefore the inactivation of NtrC) is stimulated by interaction with GlnB. This interaction is inhibited in two ways, first, by the uridylylation of GlnB, which, as discussed above, is dependent on the glutamine concentration of the cell, and second, by the binding of 2-ketoglutarate directly to GlnB. In contrast to the effect of 2-ketoglutarate on the uridylylation state of GlnB, the concentration of 2-ketoglutarate that affects the GlnB-NtrB interaction is significant within a physiologically relevant range, 0.1 to 0.9 mM (109), and very low levels of 2-ketoglutarate were found to stimulate the interaction of GlnB with NtrB in vitro (118). In vitro experiments reexamining the regulation of NtrB by GlnB in the presence of a low 2-ketoglutarate concentration showed that GlnB is an inhibitor of the kinase activity (107). Under these conditions, GlnB actively binds NtrB and activates its phosphatase activity, whereas at high 2-ketoglutarate concentrations, GlnB does not bind efficiently to NtrB, does not activate the phosphatase activity, and releases inhibition of NtrB kinase activity. As for other transmitter proteins (98, 99, 142, 197), it has been proposed that the kinase and phosphatase activities of NtrB are coordinately and reciprocally regulated (107).

GlnB also controls the adenylylation state of GS in response to glutamine and 2-ketoglutarate concentrations. Here again the glutamine concentration determines the uridylylation state of GlnB (GlnB is required for adenylylation and GlnB-UMP is required for deadenylylation of GS), but in addition to this control, 2-ketoglutarate binding prevents the stimulation of ATase activity by binding directly to GlnB and preventing the

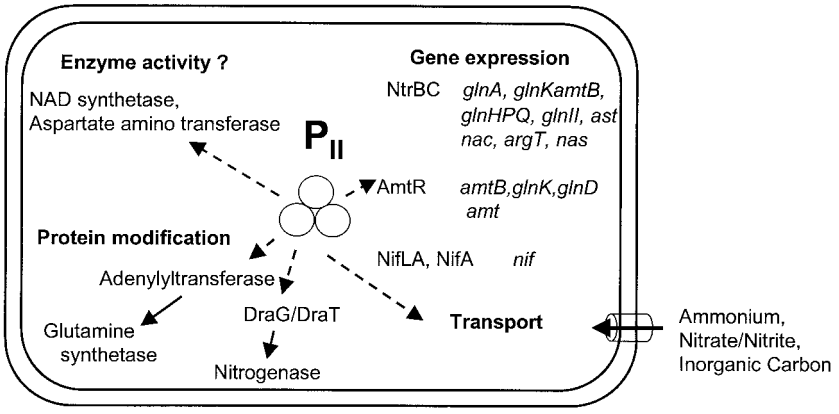

FIG. 4. Schematic representation of the potential mutiplicity of roles for P_{II} proteins. This cartoon summarizes information (both definitive and predicted) obtained from many different organisms.

GlnB-ATase interaction (110). Moreover, glutamine alone stimulates the adenylylation of GS even at low glutamine concentrations, and GlnB is required to prevent GS adenylylation under these conditions (110).

In summary, the antagonistic effect between 2-ketoglutarate and glutamine in the regulation of either the GS adenylylation state or NtrC-dependent promoter expression is due to the effects of glutamine on the UTase and ATase enzymes and the effect of 2-ketoglutarate on the conformation and activity of GlnB. A high concentration of 2-ketoglutarate is required for the binding of GlnB to UTase/UR, for stimulation of the adenylyl-removing activity of ATase, and for inhibition of the interaction with NtrB. Conversely, lower 2-ketoglutarate levels promote interaction of GlnB-UMP with UTase/UR, activating deuridylylation, stimulation of ATase, and enhanced binding of GlnB to NtrB. This is explained by the fact that 2-ketoglutarate binding results in a strong negative cooperativity for the binding of additional 2-ketoglutarate molecules, so that the protein only becomes saturated with 2-ketoglutarate at the high end of the physiological range. Consequently, GlnB may exist in different conformations depending on the number of 2-ketoglutarate molecules bound (107). Mutagenesis experiments suggest a mutual influence of 2-ketoglutarate binding and T-loop conformation (111), but further structures with different combinations of effectors bound to the protein are likely to shed more light on how effector binding influences P_{II} structure. The potential for a regulatory function of ATP binding to P_{II} proteins has also been remarked upon by Xu et al. (252). The ATP-binding site is highly conserved in P_{II} proteins, and binding of ATP and 2-ketoglutarate to GlnB is synergistic; hence, there is the potential to integrate signals that reflect both the carbon and the energy status of the cell.

Like GlnB, GlnK appears to bind one molecule of 2-ketoglutarate with a high affinity and is allosterically regulated by 2-ketoglutarate. Moreover, it seems that the binding of additional effector molecules at high 2-ketoglutarate concentrations results in another conformation of GlnK that is unable to interact with NtrB or ATase. However, unlike GlnB, low concentrations of 2-ketoglutarate do not stimulate GS adenylylation by GlnK (14).

Apart from the *E. coli* P_{II} proteins, the only other in vitro studies have been carried out with *Synechoccocus* GlnB. This protein also binds 2-ketoglutarate and ATP, and as with *E. coli* GlnB, high-affinity binding of 2-ketoglutarate requires ATP (66, 67, 118). Studies of ATP-binding characteristics show that in the absence of 2-ketoglutarate, *Synechococcus* GlnB binds ATP with a lower affinity than *E. coli* GlnB. However, higher concentrations of 2-ketoglutarate reduce this difference, and by using an ATP competitor, 2-ketoglutarate can be shown to increase the specificity of the ATP-binding site. Under physiological conditions the 2-ketoglutarate concentration might determine the ligand status of GlnB, and ATP could function as a cofactor. Hence, as in *E. coli*, the primary function of GlnB in this *Synechococcus* sp. could be to sense 2-ketoglutarate (67).

When growing with CO_2 as a carbon source, cyanobacteria use the reductive tricarboxylic acid (TCA) cycle to form 2-ketoglutarate directly. The only 2-ketoglutarate-consuming reaction is conversion to glutamate by GOGAT, and therefore changes in carbon and nitrogen assimilating reactions will immediately influence the pool size of 2-ketoglutarate. The central position of this metabolite may explain why cyanobacteria rely on 2-ketoglutarate as a sensor of carbon and nitrogen status (66). In contrast, in organisms using the oxidative TCA cycle, e.g., most proteobacteria, the 2-ketoglutarate pool depends on the flux through the TCA cycle, which might explain the need to use a nitrogen-containing metabolite, namely glutamine, as an indicator of nitrogen status.

ROLE OF P_{II} PROTEINS IN GENETIC AND METABOLIC CONTROL

Although P_{II} was originally discovered as a consequence of studies on factors controlling the activity of GS in *E. coli*, P_{II} proteins are now known to have many complex roles in the regulation of microbial nitrogen metabolism. The mutiplicity of possible roles for P_{II} proteins is presented schematically in Fig. 4. The recognition that many organisms have multiple P_{II} genes has added a further layer of complexity, and it is extremely likely that there are still new roles for P_{II} waiting to be discovered. In the following sections we discuss those functions

of P_{II} that have already been recognized and consider the evidence for other possible targets.

Regulation of GS Activity

The most common form of GS in bacteria is termed GSI. It is encoded by *glnA* and consists of 12 identical subunits of 55 kDa, which form two superimposed hexagonal rings (256; for a review, see reference 155). In a number of species, including *E. coli*, GSI is posttranslationally modified by the adenylylation of a tyrosine residue (Tyr-397) on each subunit by the ATase enzyme encoded by *glnE* (64, 207). This allows the progressive inactivation of GSI as each subunit is independently inactivated in response to increasing levels of intracellular nitrogen. It has been proposed that the physiological significance of the posttranslational regulation of GS is to prevent a sustained decrease in the ATP concentration and to protect the cellular glutamate pool, allowing rapid growth (129, 204).

ATase is one of the best characterized of the known P_{II} targets, but there is presently no information on the precise site of interaction of GlnB with ATase. The gene for ATase (*glnE*) has been cloned from *E. coli* and sequenced, and the protein has been shown to be a monomer of 115 kDa (232). Like UTase, ATase is a bifunctional enzyme catalyzing either the addition of AMP to Tyr-397 of GS using ATP as substrate or the removal of the adenylyl group, which results in the release of ADP. Separation of the two antagonistic activities of the protein, adenylylation and deadenylylation, has been demonstrated by the formation of two truncated proteins. The N-terminal domain of ATase deadenylylates GS, and this activity is regulated by GlnB-UMP and 2-ketoglutarate but is not affected by glutamine, whereas the C-terminal domain adenylylates GS in a glutamine-dependent manner. GlnB is required for the latter activity to occur in the complete enzyme but not when only the C-terminal region is present, giving rise to the hypothesis that the N-terminal region masks the adenylylation site and that GlnB binding causes a conformational change which exposes this site (102). *glnE* genes have also been found and sequenced in *Streptomyces coelicolor* (58), *Haemophilus influenzae* (62), *Mycobacterium tuberculosis* (38), *Neisseria meningitidis* (accession no. AE002380), *Pseudomonas aeruginosa* (accession no. U63816), and *Aquifex aeolicus* (accession no. AE000755).

A second form of posttranslational modification of GS in response to nitrogen status, ADP-ribosylation, has been identified in *Rhodospirillum rubrum, Streptomyces griseus*, and *Synechocystis* sp. strain. PCC6803 (181, 209, 244), although the physiological significance of this has not yet been determined. In *Synechocystis* sp. strain PCC6803, reversible inactivation of GSI is also controlled by interaction with two inhibitory polypeptides, GifA and GifB (77, 78). P_{II} has not been implicated in either of these regulatory processes.

Other forms of GS in addition to GSI have been found in a number of organisms. GSII is an octamer of 36-kDa subunits encoded by *glnII*; GSIII, encoded by *glnN*, is a hexamer with a subunit molecular mass of 75 kDa; and the fourth form of the enzyme is an octamer of 47 kDa encoded by *glnT*. None of these enzymes has been reported to be adenylylated, and to date P_{II} has not been implicated in regulation of their activities.

Global Nitrogen Regulation (Ntr) System

As discussed in detail above, there were early indications from the analysis of *glnB* mutants that P_{II} played a role in addition to the modulation of ATase activity. A combination of genetic and biochemical experiments subsequently led to the recognition that in enteric bacteria (and indeed in most, if not all, of the proteobacteria), expression of nitrogen assimilation and catabolism genes is coordinated by a central regulatory system (147, 155, 190). This global nitrogen regulatory (Ntr) system was originally considered to consist of four proteins, UTase/UR, GlnB, and the proteins of the two-component histidine protein kinase system, NtrB and NtrC, which in enterics are cotranscribed in the *glnA-ntrBC* operon. The later discovery of GlnK in *E. coli* and many other bacteria increased the complexity of the system, although it also explained some previous anomalies. For clarity, the role of GlnB in the Ntr system (Fig. 1) will be described first, and the role of GlnK and its importance in both genetic and metabolic control will then be considered.

Enteric Ntr model. Activation and repression of nitrogen-regulated genes is coordinated by the action of the central regulatory protein NtrC, a typical σ^{54}-dependent transcriptional activator protein (166). The protein comprises a DNA-binding carboxy-terminal domain, a highly conserved central domain required for the activation of transcription, and an N-terminal domain which is characteristic of two-component response regulator proteins (178, 220). These proteins have a highly conserved tertiary structure in the N-terminal region, containing an aspartic acid residue, Asp-54, which, in the case of NtrC, is phosphorylated in response to low nitrogen, giving NtrC-P, the activated form of the protein (121, 239, 242). NtrC exists as a dimer in its nonphosphorylated form, but for the activation of transcription to take place, oligomerization to a tetramer or higher-order oligomer must occur. Phosphorylation induces both DNA binding and the oligomerization of NtrC, and many NtrC-dependent promoters contain more than one binding site, facilitating oligomerization (18, 156, 240, 241, 251).

Control of NtrC activity in response to nitrogen status is mediated by NtrB, which acts as a phosphate donor to NtrC. NtrB is a 36-kDa protein comprising two distinct domains, the N-terminal sensor domain and the C-terminal kinase domain, typical of histidine protein kinases (168, 178). NtrB exists as a dimer which is autophosphorylated on the conserved histidine (His-139) at the amino end of the C-terminal domain (169, 171). ATP binds to one subunit, and phosphorylation occurs on the conserved histidine residue of the second subunit (172).

NtrB is stimulated to dephosphorylate NtrC in the presence of GlnB and ATP. This is termed regulated phosphatase activity (117, 118, 120, 140, 170). Mutations in *ntrB* have shown that the kinase and phosphatase activities of NtrB are separate. Mutation of His-139 to arginine or mutation of the ATP-binding site prevents and significantly slows kinase activity, respectively, but does not affect the phosphatase activity of the protein. However, in these mutants GlnB is not required to stimulate phosphatase activity (17, 116). Other mutations in the N-terminal domain of NtrB affect the interaction of GlnB without affecting kinase activity (117). These data suggested that P_{II} interacts with the N-terminal domain of NtrB, but

subsequent cross-linking experiments indicated that P_{II} regulates both the kinase and phosphatase activities of NtrB by binding to the kinase domain of the NtrB transmitter module (183). Functional dissection of the activities of NtrB confirmed this model, showing that NtrB consists of three domains: an N-terminal domain involved in signal transduction, a central phosphotransferase/phosphatase/dimerization domain, and a C-terminal kinase domain. Binding of P_{II} to the kinase domain appears to result in an altered conformation that is transmitted to the other two domains and causes the central domain to assume a conformation with potent phosphatase activity (106).

The modification of GlnB in response to the cellular nitrogen status provides the intracellular switch that in turn regulates the phosphatase and kinase activities of NtrB and hence the transcriptional activity of NtrC. In nitrogen excess, unmodified GlnB stimulates dephosphorylation of NtrC by NtrB, and conversely, in nitrogen starvation, when GlnB is modified, GlnB-UMP no longer interacts with NtrB and the kinase activity predominates, so that NtrC is phosphorylated and transcriptionally active. In enterics, a number of genes are transcriptionally regulated by NtrBC; these include the *glnK-amtB* operon (13, 101, 231), the glutamine transport *glnHPQ* operon (37), the arginine catabolism *astCADBE* operon (202), the *nac* regulatory gene (167), *S. enterica* serovar Typhimurium *argT* (200), the nitrogen fixation regulatory genes *nifLA*, and the *nasR* and *nasFEDCBA* genes of *K. pneumoniae* (52, 136, 250).

As discussed earlier, the discovery of GlnK in *E. coli* helped to explain a number of anomalies in the Ntr model and rationalized certain aspects of regulation that had been observed in the absence of GlnB. Nevertheless, the problem of determining the primary physiological role of GlnK remains. Studies of single (*glnB* or *glnK*) and double (*glnB glnK*) mutants give some information, though by definition they do not relate to the normal physiological situation. Likewise, in vitro data can shed light on the potential activities of the proteins but do not necessarily identify their primary function. These observations are even further complicated by the recognition that GlnB and GlnK can from heterotrimers in vivo under normal physiological conditions, so that given the potential for uridylylation of both GlnB and GlnK, a large number of different P_{II} molecules may exist in the cell in different situations.

In vivo, GlnK can complement the *glnB* defect in deadenylylating GS (13, 231). However, in vitro experiments show that GlnK is less efficient than GlnB in activating GS adenylylation, suggesting that in vivo, when GlnB is present, GlnK may not play a significant role in regulating ATase (14, 231). Interestingly, under some physiological conditions, neither GlnB nor GlnK is necessary for GS adenylylation (68). In vitro, GS adenylylation can be triggered by glutamine alone (110), and the adenylyl transfer process of the carboxy-terminal domain of ATase is activated by glutamine (102). Moreover, adenylylation activity of the C-terminal domain is independent of GlnB (or GlnB-UMP), whereas in the intact enzyme, GlnB is required for this activity (102). Hence, depending on the physiological conditions of the cells, the adenylylation of GS may be P_{II} dependent or P_{II} independent (68).

GlnK, like GlnB, can act through NtrB to regulate NtrC-P concentration, but GlnK appears to be less potent than GlnB in controlling the level of NtrC-P. Hence, GlnK may play some role in the regulation of Ntr promoters requiring a high concentration of NtrC-P (e.g., *E. coli* p*glnK*), although in *K. pneumoniae* p*glnK* expression is constitutive in the absence of GlnB (101). By contrast, GlnK has little effect on expression from the *glnA*p2 promoter, which is extremely sensitive to NtrC-P, which is consistent with *glnA* expression in the absence of GlnB (13, 14, 31).

The precise physiological role of GlnK may well not be determined until the full complement of potential P_{II} targets is known. However, the conserved linkage of *glnK* to the ammonium transporter gene *amtB* may suggest one possible target (see later).

Regulation of the Ntr system in other organisms. Outside the enterics, the role of the Ntr system has been studied almost exclusively in diazotrophic members of the proteobacteria. In all of these organisms except *Azotobacter vinelandii*, both GlnB and GlnK are present, although the phenotypes of both single and double mutants have not always been reported. Nevertheless, in many cases the roles of GlnB and GlnK are similar to those reported in *E. coli*.

An investigation of GlnB function in *Sinorhizobium meliloti* using both an in-frame *glnB* null mutation and a Y51F point mutation showed that in free-living conditions, GlnB is required for the expression of the NtrC-dependent GSII gene (*glnII*) and for regulation of the adenylylation state of GSI. As in enterics, GSI adenylylation is stimulated by native GlnB, whereas GlnB-UMP induces GSI deadenylylation. Furthermore, nonuridylylated GlnB prevents expression of NtrC-dependent genes such as *glnII*, presumably by stimulating NtrB phosphatase activity. However, unlike the enteric bacteria, a GlnB-deficient strain shows no *glnII* expression, suggesting that GlnB-UMP is required either for NtrC phosphorylation or for expression of *ntrC* itself (8). Unlike *Sinorhizobium meliloti*, a *Rhizobium leguminosarum* Δ*glnB* mutant exhibits constitutive expression of NtrC-dependent promoters, suggesting that in this organism, as in *E. coli*, the absence of GlnB results in NtrC activation independent of the N status (4).

In *Azospirillum brasilense*, neither GlnB nor GlnK is essential for GSI adenylylation or deadenylylation (47, 50). Moreover, a *glnB* mutation results in constitutive expression of NtrC-dependent promoters, such as that for *glnK* (47).

In *Azorhizobium caulinodans*, GlnB and GlnK are not necessary for GSI adenylylation, whereas both are required for complete GS deadenylylation. In the free-living state and under nitrogen-fixing conditions, the high level of GS adenylylation in a Δ*glnBK* background results in ammonium excretion. These results suggest that in this organism, GlnB and GlnK are interchangeable in regulating deadenylylation of GS (159).

In *Rhodospirillum rubrum*, regulation of GS adenylylation and deadenylylation was initially studied with purified GlnB and GlnB-UMP and extracts from *R. rubrum* grown in different conditions. Unlike the situation in *Azorhizobium caulinodans*, native GlnB and glutamine stimulate adenylylation of GS and 2-ketoglutarate totally inhibits the process, whereas none of the effectors tested induce the deadenylylation process (115). Mutations in *glnB* have no effect on *glnA* expression or GS modification, but the effects of *glnK* and *glnB, glnK* mutations have not yet been studied (261).

In *Azotobacter vinelandii*, GS is the only route for ammonium assimilation, and the organism cannot take up glutamine; therefore, *glnA* null mutations are lethal (224). Several at-

tempts to construct a *glnK* null mutant of *A. vinelandii* also failed (153), and as a stable *glnD*-deficient strain can only be maintained in the absence of GS adenylylation, it seems that in *A. vinelandii*, GlnK-UMP may be absolutely required for deadenylylation of GS (39, 196).

Nitrogen Control in Other Bacteria

P_{II} proteins are of course not restricted to the proteobacteria, but their roles in other bacteria are much less well understood. Nitrogen control systems have been characterized in members of the actinobacteria, the cyanobacteria, and the firmibacteria, but only in the actinobacteria (e.g., *Corynebacterium*) does it appear that P_{II} may have a central regulatory role.

***Corynebacterium glutamicum*.** In *Corynebacterium glutamicum*, nitrogen-regulatory protein AmtR regulates transcription of the *amtB-glnK-glnD* operon and the *amt* gene. AmtR represses transcription of these genes in the presence of ammonium, and repression is relieved upon nitrogen starvation (105). AmtR does not respond to nitrogen limitation in a *glnB* deletion strain, indicating that GlnB acts directly or indirectly to signal the nitrogen status to AmtR (A. Burkovski, M. Jacoby, and R. Krämer, personal communication).

Cyanobacteria. As in enteric bacteria, ammonium is a good nitrogen source for cyanobacteria, and in its presence, these organisms are unable to assimilate alternative inorganic nitrogen sources such as nitrate, nitrite, or dinitrogen. However, no homologues of the *ntrBC* genes are found in cyanobacteria, and nitrogen control is mediated by the NtcA protein, which belongs to the Crp family of transcriptional regulators (90b, 144, 237, 238). Moreover, GS activity is not regulated by reversible adenylylation but by protein interaction with two inhibitory peptides (77, 78). Despite the fact that no GlnB targets have been identified in cyanobacteria, the phenotype of a *glnB* mutant of *Synechococcus* sp. strain PCC7942 suggests that GlnB does not modulate the activity of NtcA (70). Indeed, the phosphorylation state of GlnB depends on NtcA (132, 198) as well as 2-ketoglutarate (66, 71, 100).

***Bacillus subtilis*.** There is no evidence for an NtrBC system in the firmibacteria, and despite some minor similarities with the enteric system, namely the presence of a σ^{54}-like sigma factor (σ^{L}) and a GlnK homologue (NrgB), *B. subtilis* controls nitrogen assimilation in a quite different manner (60). The GlnK homologue NrgB does not seems to regulate the activity or expression of any σ^{L}-dependent transcriptional activators, and the only phenotype reported for a Δ*nrgB* mutant is one of slow growth on nitrate (79, 246).

In *B. subtilis*, nitrogen metabolism is not governed by a single global nitrogen-regulatory system but by three regulatory proteins, GlnR, TnrA, and CodY, which are active under different nutritional conditions. These multiple systems allow noncoordinate gene expression under different growth conditions. GlnR represses gene expression during growth in nitrogen excess, whereas TnrA activates or represses gene transcription during nitrogen-limited conditions, and finally, CodY represses transcription in cells growing rapidly with amino acids. CodY-dependent regulation appears to reflect the total nutritional state of the cell (61), whereas GlnR and TnrA respond to nitrogen availability. None of the signals regulating the activity of these three proteins has been identified, but GlnK (NrgB) has not been implicated, and the only factor so far known to be involved in the regulation of GlnR and TnrA activity is GS (249).

Regulation of Nitrogen Fixation

For those bacteria that are capable of diazotrophic growth, the fixation of atmospheric nitrogen can be a major route of nitrogen assimilation, and in nearly all cases P_{II} proteins appear to play a significant role in the regulation of this process. Due to the sensitivity of the nitrogenase enzyme to oxygen and the high energy requirement for the process of nitrogen fixation, the nitrogen fixation activity of diazotrophs is regulated in response to both the redox and nitrogen status of the cell, so that nitrogen fixation occurs only when it is both favorable and necessary. Nitrogen regulation of nitrogen fixation has been studied most extensively in the proteobacteria, and it is becoming increasingly apparent that P_{II} proteins play a pivotal role in this process. The primary mode of regulation is by control of the transcription of the nitrogen fixation (*nif*) genes, and this can be achieved by regulating both the expression and the activity of the transcriptional regulator. Some organisms also regulate the activity of the nitrogenase enzyme at a posttranslational level in response to ammonia and other fixed nitrogen sources, and here again P_{II} proteins play their part.

***nif*-specific regulator NifA.** In diazotrophs of the α and γ proteobacteria, *nif* gene transcription is initiated at σ^{54}-dependent promoters in conjunction with a specific transcriptional activator, NifA (51, 59, 154). Control of *nif* transcription is mediated by regulating either the expression or the activity of NifA in response to oxygen and fixed nitrogen. Nearly all NifA proteins have a three-domain structure typical of σ^{54}-dependent transcriptional activators (53, 166). The three domains are functionally different, and experiments have shown that isolated domains are structurally and functionally independent (22, 23, 54). The carboxy-terminal domain contains a conserved helix-turn-helix motif which is involved in DNA binding (160, 165). The central domain contains the site for ATP binding and for interaction with σ^{54} (81, 166). The N-terminal domain of σ^{54}-dependent activators is highly variable and is typically the target for regulatory signals.

In the γ proteobacteria, the product of an additional gene, *nifL*, is required to regulate NifA activity in response to both oxygen and fixed nitrogen. NifL proteins have some similarity to the sensor components of the two-component sensor regulator systems, such as NtrB, having N-terminal sensor and C-terminal transmitter domains tethered by a flexible Q linker. However, there is no evidence that NifL is a histidine protein kinase; indeed, the appropriate histidine residue is absent in *K. pneumoniae* NifL, and in *Azotobacter vinelandii*, NifL, where it is present, it is not required for regulation of *nif* gene expression (245). Both *A. vinelandii* and *K. pneumoniae* NifL are flavoproteins with flavin adenine dinucleotide as the prosthetic group (51, 91, 201). The NifL and NifA proteins form a complex in vivo and in vitro, and stoichiometric levels of the two proteins are required for effective modulation of NifA activity (82, 90, 162).

Regulation of *nifA* and *nifLA* transcription. The factors that regulate *nifA* transcription reflect the lifestyle of the organism,

so that in those that can fix nitrogen in the free-living state, the availability of fixed nitrogen is a major factor, whereas in symbiotic diazotrophs, the critical factor controlling *nifA* expression is the availability of oxygen.

Among the free-living diazotrophs, regulation of *nifA* expression varies considerably. In *K. pneumoniae, nifLA* transcription is nitrogen regulated by NtrC and is therefore dependent on GlnB (52, 95), while in *Azotobacter vinelandii, nifLA* transcription is independent of nitrogen sources (24, 186). In both *Herbaspirillum seropedicae* and *Rhodobacter capsulatus, nifA* expression is regulated with respect to nitrogen by NtrC, and hence GlnB may be involved (57, 72, 215). By contrast, *nifA* expression in *Azospirillum brasilense* is independent of NtrC and only partially repressed by either ammonium or oxygen, maximal repression being achieved by the synergistic effect of both effectors (57). In *Rhodospirillum rubrum, nifA* transcription appears to be independent of nitrogen source, and nitrogen control of *nif* gene expression is only exerted through NifA activity (261).

In the symbiotic diazotrophs of the genera *Rhizobium, Bradyrhizobium*, and *Azorhizobium, nifA* expression is predominantly regulated in response to oxygen availability (for a review, see reference 59). However, in *Azorhizobium caulinodans, nifA* expression is also regulated in response to nitrogen (187). This may be due to the fact that *A. caulinodans* can fix nitrogen not only symbiotically within root or stem nodules of *Sesbania rostrata* but also in a free-living state where the source of nitrogen is less reliable. Nitrogen control of *nifA* expression in *A. caulinodans* is mediated by two nitrogen-responsive systems, NtrB/C and NtrY/X (179, 180). In *glnB* or *glnK* mutants, *nifA* is expressed only during nitrogen-fixing conditions, whereas in a Δ*glnBK* mutant, *nifA* is expressed under nitrogenase-derepressing conditions and in the presence of ammonia. Consequently, it has been suggested that GlnB and GlnK are involved in the repression of NifA synthesis (159).

Nitrogen control of NifA activity. Whereas the role of P_{II} proteins is somewhat variable with respect to regulation of *nifA* expression, a much more consistent picture is emerging regarding the function of these proteins in regulating NifA activity. In this context, information comes from studies with *Azospirillum brasilense, Herbaspirillum seropedicae, Rhodospirillum rubrum*, and *Azorhizobium caulinodans*.

The first indication that a P_{II}-like protein might be involved in the nitrogen regulation of NifA activity came from the observation that in *A. brasilense*, a *glnB* mutant was Nif^- (135), while a *glnK* mutation had no effect on nitrogen fixation (47). As the *glnB* mutation did not affect *nifA* expression, it appeared that GlnB is required to maintain the active form of NifA. Deletions within the N-terminal domain of NifA restore *nif* gene expression, suggesting that GlnB is required to activate NifA by preventing the inhibitory affect of its N-terminal domain (11). Mutation of residue Tyr-18 to Phe in the N-terminal domain of NifA results in an active NifA that does not require GlnB; however, whether GlnB interacts directly with NifA or modulates the activity of another protein that in turn regulates NifA activity remains unsolved (12). A *glnB* Y51F mutant and a *glnD* mutant both exhibit a Nif^- phenotype (12, 227), which is consistent with the fact that during nitrogen fixation, *A. brasilense* GlnB is uridylylated (47) and suggests that it is GlnB-UMP which is required for NifA activation.

A similar situation occurs in *H. seropedicae*: a *glnB* mutant is Nif^-, while *nifA* expression, which is NtrC dependent, would be expected to be constitutive in this background (21). Studies of the *H. seropedicae* NifA protein in vivo show that the full-length protein expressed in *A. brasilense* is active only under low oxygen and in the absence of ammonium, but NifA is not active when expressed in *E. coli* or *K. pneumoniae* (217). By contrast, the amino-terminally truncated form is still active in the presence of ammonium in *A. brasilense, E. coli*, and *K. pneumoniae*, again indicating that the N-terminal domain is involved in nitrogen control. Furthermore, when expressed in *trans*, this domain can inhibit the activity of the truncated NifA (163, 164, 217). If GlnB were to interact with the N-terminal domain, then the inactivity of *H. seropedicae* NifA in *E. coli* could be due to the absence of the cognate P_{II}.

In *R. rubrum*, a Δ*glnB* mutant has no nitrogenase activity, whereas a *glnB* Y51F mutant shows about 10% of wild-type nitrogenase activity. Expression of *R. rubrum nifA* from a multicopy plasmid does not restore nitrogenase activity in a Δ*glnB* mutant, whereas a *glnB* Y51F mutant is complemented. These results suggest that GlnB is essential for NifA activity, but it is not clear whether the effect of GlnB on NifA is direct or indirect (261). An attractive model is that modified GlnB leads to activation of NifA, while unmodified GlnB inhibits NifA. However, the role, if any, of GlnK in this system has still to be investigated.

The situation is somewhat different in *A. caulinodans*. Single *glnB* and *glnK* mutants express NifA constitutively but behave like the wild type and show ammonium repression of *nif* expression. However, the *glnBK* double mutant shows derepressed *nif* expression in ammonium, indicating that in this case NifA activity can be controlled by either GlnB or GlnK (159) During nitrogen fixation conditions, *glnB* and *glnK* are dispensable (even if a *glnBK* mutant is less able to activate *nif* expression), and unlike *H. seropedicae* or *A. brasilense* NifA, an amino-terminally truncated NifA is inactive (159).

The theme of P_{II} proteins being involved in modulating the activity of NifA in response to nitrogen availability has now been extended to the γ proteobacteria. Initial studies in *K. pneumoniae* indicated that neither GlnB nor GlnD played a direct role in NifL-mediated regulation of NifA activity in response to fixed nitrogen (55, 95). However, in *Azotobacter vinelandii*, GlnD has been shown to be necessary for the sensing of nitrogen status by the *A. vinelandii* NifLA system, and the Nif^- phenotype of a *glnD* mutant was suppressed by a second mutation in *nifL*, indicating that UTase is involved in NifL-dependent regulation of NifA activity (42).

In their studies on the role of GlnB in *nif* regulation, Holtel and Merrick (95) concluded that at least one other nitrogen-sensing system is present in *K. pneumoniae* in addition to GlnB, and Edwards and Merrick subsequently proposed that this system might involve an alternative P_{II}-like protein (55). The discovery of a GlnK in *E. coli* (231) offered just such a candidate, and the demonstration by He et al. (88) that derepression of NifA activity is dependent on NtrC was then entirely consistent with a role for GlnK. Genetic studies in both *E. coli* and *K. pneumoniae* indicated that the *K. pneumoniae* NifL-NifA interaction is indeed modulated by the presence of GlnK in such a way that in N-limiting conditions, GlnK is required to relieve the inhibitory effect of NifL on NifA (89, 101). In an *E.*

coli glnBK double mutant, the modulation of NifA activity by NifL is dependent on the concentration of GlnK, and when overproduced to nonphysiological levels, GlnB can substitute for GlnK (10). The effect of GlnK is independent of its uridylylation state, which raises the question of how NifL inhibition is rapidly reestablished in response to elevated N status. Studies of the NifL-NifA interaction in *E. coli* suggest that in normal physiological conditions, GlnB can counteract the positive action of GlnK, and it is possible that this negative effect might be mediated by GlnB-GlnK heterotrimer formation (10). Site-directed mutagenesis of GlnB indicates that residue 54 in the T loop is the single most important amino acid in determining the discrimination between GlnK and GlnB proteins in the context of the modulation of NifA activity. A single alteration of GlnB Asp-54 to Asn, found at this position in GlnK, makes GlnB quite effective in regulating the NifLA interaction, and if combined with a second T43A alteration, the resultant GlnB D54N T43A protein mimics the GlnK protein almost perfectly in relieving NifL inhibition. It has been proposed that the D54N T43A changes modify the characteristics of the T loop in such a way that the altered GlnB protein is now fully competent to interact with the target, be that NifL or NifA or the complex of both (9).

While P_{II} is also implicated in modulating NifLA control in *Azotobacter vinelandii*, the situation differs from that in *K. pneumoniae*. *A. vinelandii* NifL responds to the availability of fixed nitrogen when expressed in *E. coli* (217), but studies in vivo and in vitro indicate that neither GlnB nor GlnK is required for derepression. However, in N excess conditions, the inhibitory function of NifL is dependent on either of the nonmodified forms of *E. coli* GlnB or *A. vinelandii* GlnK (192). In vitro analysis indicates that the inhibitory activity of *A. vinelandii* NifL is stimulated by interaction with the nonuridylylated form of GlnK and that the NifL-NifA system is also directly responsive to 2-ketoglutarate (138). Interestingly, sequence comparison of T-loop sequences suggests that the *A. vinelandii* T loop is similar to the *E. coli* GlnB T loop, which is consistent with the fact that it is *E. coli* GlnB rather than *E. coli* GlnK that is effective in regulating *A. vinelandii* NifLA function.

Hence, while a common theme is emerging of regulation of NifA activity by a P_{II} protein, the precise details, e.g., whether GlnB or GlnK or both are involved, whether they act in concert with NifL or independently, and whether they activate or inactivate NifA, appear to differ from one organism to another. It is attractive to imagine that a common mechanism could underlie this variety, but at present it is difficult to suggest a unified model, and the challenge now is to elaborate the mechanism of regulation, preferably in more than one system.

Regulation of nitrogenase activity. Just as GS and NifA can be regulated at the level of transcription and activity, so at least some organisms can regulate both the expression and the activity of the nitrogenase enzyme, here again P_{II} is now recognized as a key regulator. A number of diazotrophic α proteobacteria, including *Rhodospirillum rubrum, Rhodobacter capsulatus, Azospirillum brasilense*, and *Azospirillum lipoferum* can regulate the activity of nitrogenase by ADP-ribosylation in response to ammonia, energy limitation, or anaerobiosis shifts (86, 133, 143, 152, 258, 260). This is a reversible reaction driven by the enzymes dinitrogenase reductase ADP-ribosyl transferase (DRAT), which ribosylates a specific arginine residue (Arg-101) of one subunit of the dinitrogenase reductase homodimer, inactivating the enzyme, and dinitrogenase reductase activating glucohydrolase (DRAG), which removes the ribosyl group, reactivating the enzyme (143). The *R. rubrum* DRAT and DRAG system has been shown to function in *K. pneumoniae*, in which the target residue Arg-101 of nitrogenase is conserved, and as no *draG* or *draT* genes have been identified in *K. pneumoniae*, it would appear that the sensing system that modulates DRAG and DRAT activity is also present in this organism (74).

In *Rhodospirillum rubrum*, addition of 10 mM ammonia during nitrogen fixation causes a slow decrease in nitrogenase activity, finally reaching 20 to 30% of the initial activity. In contrast, a *glnB* Y51F mutant shows a much more rapid loss of nitrogenase activity. Moreover, during nitrogen fixation conditions, a *draG* mutant exhibits normal nitogenase activity, whereas a *glnB* Y51F *draG* double mutant shows no nitrogenase activity and complete modification of the nitrogenase (261). Using a heterologous system in which DRAG and DRAT are expressed in *K. pneumoniae*, it has been shown that DRAT activity is unaffected in a *glnK* mutant, whereas regulation of DRAG activity is completely abolished, and DRAG stays active even after ammonia addition (261a). These results suggest that both GlnB and GlnK play an important role in regulating the DRAG-DRAT system. In *Rhodobacter capsulatus*, a *glnB* mutation does not affect nitrogenase switch-off, but a double *glnB glnK* mutant is impaired in switch-off (83, 125).

The phenomenon of switch-off has also been reported in *Azorhizobium caulinodans*, but it is not known whether this occurs by ADP-ribosylation (128). In *Azospirillum brasilense* and *Rhodobacter capsulatus*, a second mechanism for nitrogenase switch-off in addition to ADP-ribosylation has been shown to occur, and this could therefore also be true in other organisms (253, 255, 259). Nevertheless, in a *glnBK* double mutant of *A. caulinodans*, nitrogenase is active in the presence of 10 mM NH_4^+, whereas it is inactive in a wild-type strain. Hence, both GlnB and GlnK may be involved in the regulation of *A. caulinaudans* nitrogenase activity (159). As discussed earlier, *A. caulinodans glnB* and *glnBA* mutants induce Fix^- nodules on *Sesbania rostrata* roots despite expressing a *pnifH-lacZ* fusion at a similar level to the wild type, a situation which again suggests control of nitrogenase activity by GlnB (158).

The phenomenon of ammonia switch-off is also found in the diazotrophic methanogens *Methanosarcina barkeri* and *Methanococcus maripaludis* (123, 141). However, as *draG* and *draT* homologues are not found in the genome of the related *Methanobacterium thermoautotrophicum*, the mechanism of this inactivation may well be novel. Despite this, P_{II} proteins are again involved in the phenomenon. As discussed earlier, two *glnB*-like genes, $nifI_1$ and $nifI_2$, are located between the nitrogenase structural genes *nifH* and *nifDK* in a number of diazotrophic methanogens. In *M. maripaludis*, the involvement of these proteins in regulation of *nif* expression has been ruled out, but a mutant carrying an in-frame deletion of both $nifI_1$ and $nifI_2$ does not show the ammonia switch-off phenomenon; nitrogenase remains active after addition of ammonia, whereas in a wild-type strain nitrogenase activity is rapidly lost. Hence, at least one of the genes is involved in the switch-off process (123, 123a). A similar posttranslational control of nitrogenase

may also be present in *Clostridium acetobutylicum, Clostridium cellobioparum*, and *Desulfovibrio gigas*, as *glnB*-like genes are also linked to *nifH* in these organisms.

Regulation of Transport Systems

The transport of nitrogenous compounds into the cell is another important process in nitrogen metabolism, and here again evidence is emerging for control by P_{II} proteins.

Nitrate transport. P_{II}-like proteins play a role in nitrate utilization or uptake in many organisms, including *Bacillus subtilis, Rhizobium leguminosarum, Azospirillum brasilense, Synechococcus* sp. strain PCC7942, and *Synechocystis* sp. strain PCC6803. *glnB* mutants of *B. subtilis* and *R. leguminosarum*, fail to utilize nitrate as a sole nitrogen source, suggesting that GlnB might participate in nitrate utilization (4, 246). A *glnB* mutant of *A. brasilense* excretes ammonium when cells are grown in the presence of nitrate. It has been proposed that this effect could be linked to deregulation of the nitrate assimilation pathway (which is regulated by the *ntr* system), with the consequent accumulation of intracellular ammonium leading to ammonia excretion (134). This phenotype is restored by complementation with *glnB* or elevated expression of GS (47).

In the cyanobacterium *Synechococcus* sp. strain PCC7942, the nitrate/nitrite ABC transporter and nitrate reductase are rapidly inhibited upon addition of ammonium (126). In a *glnB* mutant, nitrate uptake activity remains high after addition of ammonium and a *glnB* S49A mutant (which mimics the unphosphorylated form of GlnB) does not exhibit any nitrate uptake regardless of nitrogen source. This suggests that GlnB is involved in control of the short-term inhibition of either nitrate uptake or nitrate reductase activity (131). Both *glnB* S49D and S49E mutants (which mimic phosphorylated GlnB) behave like the wild-type strain, indicating that both GlnB forms have a negative effect on nitrate uptake or assimilation. Because the "phosphorylated-like" forms of GlnB allow nitrate uptake only in the absence of ammonia, it has been suggested that an additional effector signaling N deficiency (such as 2-ketoglutarate) is required to establish the noninhibitory form of phosphorylated GlnB (130, 131). As in *Synechococcus* sp. strain PCC7942, in *Synechocystis* sp. strain PCC6803, nitrate uptake is correlated with the phosphorylation level of GlnB. However, when cells are grown in the presence of low concentrations of inorganic carbon, nitrate uptake is not completely abolished even though GlnB is unphosphorylated. Moreover, a *glnB* mutant exhibits constitutive nitrate uptake, which is consistent with a role for GlnB in regulating nitrate uptake or assimilation (92). Deletion of the C terminus of one of the ATP-binding subunits (NrtC) of the transporter allows nitrate accumulation in the presence of ammonium (126), suggesting that this domain may interact with an effector protein, perhaps GlnB.

Another organism in which P_{II} may control nitrate metabolism is *Aquifex aeolicus*. One of the two *glnB*-like genes in this organism is located immediately upstream of *nasA* (encoding a nitrate transporter) and *narB* (encoding a nitrate reductase). The gene organization suggests that all three genes are contranscribed, and as they are all potentially translationally coupled, it is quite likely that all three gene products are functionally related.

Methylammonium and ammonium transport. The ubiquitous linkage of genes encoding the proposed high-affinity ammonium transporter AmtB and the P_{II} protein GlnK has led to the proposal that these proteins are functionally related (223), and some evidence to support this comes from *Azospirillum brasilense* and *Synechococcus* sp. In *A. brasilense*, GlnK (Glnz) negatively regulates methylammonium transport (47). In this case, although *amtB* expression is regulated by the *ntr* system, GlnK is not required for NtrC-dependent regulation (228), and therefore it has been suggested that GlnK modulates the activity of AmtB (47). A *glnB* mutant of *Synechococcus* sp. strain PCC7942 exhibits a peculiar phenotype; no methylammonium transport is observed when cells are grown in the presence of nitrate, whereas in the absence of combined nitrogen, transport activity increases rapidly to reach a higher level than in a wild-type strain (70).

Other transporters. The *glnB* gene of *Haemophilus influenzae* is located immediately upstream (3 bp) of an open reading frame (HI0338) that encodes a protein of unknown function. However, BLAST searches reveal that this protein is a member of a large class of integral membrane proteins represented in *E. coli* by YdgG and YhhT. The tight linkage between *glnB* and HI0338 and their presumptive cotranscription is highly suggestive of a functional relationship and might imply that *H. influenzae* YhhT is involved in the transport of some nitrogenous compound.

Inorganic carbon uptake. In *Synechocystis* sp. strain PCC6803, inorganic carbon (C_i) uptake involves a constitutively expressed low-affinity transporter and a high-affinity transporter expressed under a low concentration of C_i. In a *glnB* mutant, the activity of the C_i high-affinity transporter (as measured by bicarbonate uptake) is constitutive, and the regulation appears to be independent of GlnB phosphorylation. It has been proposed that in the *glnB* mutant, the absence of a 2-ketoglutarate–GlnB complex could activate or repress the high-affinity transporter even when the C_i concentration is high (92). Because GlnB also controls nitrate uptake in *Synechocystis* sp. strain PCC6803, the protein appears to be involved in the coordination of carbon and nitrogen metabolism, as was previously suggested for *Synechochococcus* sp. strain PCC7942 (69).

A similar link between carbon and nitrogen metabolism has been observed in *Rhodobacter sphaeroides*, in which a RubisCO-deficient mutant fails to express *glnB* under photoheterotrophic growth with either ammonium or glutamate as the nitrogen source (185). While *glnK* expression is strictly under nitrogen control in a wild-type strain, in the RubisCO mutant *glnK* is expressed in the presence of ammonium.

Nodule development. Given the pivotal role of P_{II} proteins in regulating nitrogen metabolism in many bacteria, the symbiotic interaction between *Rhizobium* spp. and legumes is a potentially important biological process in which these proteins might well be expected to play a significant part. The development of a legume nodule and the concomitant differentiation of the free-living bacterium into a nitrogen-fixing bacteroid involve a major change in the nitrogen assimilation pathways of the *Rhizobium* cell.

In *Rhizobium leguminosarum*, the *glnB* promoter is downregulated during bacteroid differentiation at a time coincident with the arrest of bacterial division (56). However, in *Sinorhizobium meliloti*, construction of both an in-frame deletion of

glnB and a *glnB* Y51F mutant implicated GlnB in nodule development. Both mutants exhibit similar symbiotic phenotypes, albeit somewhat stronger for *glnB* Y51F, with defective infection on alfalfa and heterogenous nodules on 3-week-old plants. The plants are chlorotic despite being able to reduce dinitrogen to appreciable levels. On addition of ammonium, chlorosis is lost, confirming that the plants are suffering nitrogen starvation despite a high level of nitrogenase activity. This paradoxical Nif^+ Fix^- phenotype may be explained by the involvement of GlnB in regulation of a bacteroid ammonium transporter (8), and the critical role of the AmtB protein in nodule development has been demonstrated independently in *Rhizobium etli* (222). In this context, the phenotype of a *glnK* mutant in one or more of the *Rhizobiaceae* would be of considerable interest.

Regulation of Other Nitrogen-Related Systems

Mutations in the *nadE* genes of *Salmonella enterica* serovar Typhimurium (formerly *nit*) and *Rhodobacter capsulatus* (formerly *adgA*) prevent growth with low concentrations (<1 mM) of ammonia (2, 28, 203, 243). The *nadE* gene encodes an ammonia-dependent NAD synthetase with a K_m for ammonia of 65 μM, lower than that for any known amidotransferase. It has therefore been proposed that the phenotype of the *nadE* mutants is due to a low residual NAD synthetase activity that is insufficient when ammonia becomes limiting. Such an ammonia-dependent enzyme is a good candidate for posttranslational regulation by P_{II}, so that activity is coordinated with ammonia availability. It is therefore perhaps no coincidence that in *Herbaspirillum seropedicae* and *Azoarcus, nadE* is genetically linked to a *glnB*-like gene, and it would certainly be of interest to study the NAD synthetase activity in *glnB* and *glnK* mutants.

A further extrapolation of this observation is related to the DRAG-DRAT system that is responsible for switch-off of nitrogenase activity in *Rhodospirillum rubrum* and other diazotrophs (see above). The donor of the ADP-ribose moiety for the DRAT-mediated inactivation of dinitrogenase is NAD^+ (213), and it has been proposed that an increase in NAD^+ concentration is the signal for DRAT activation in *R. rubrum* (175). Given the ammonia dependence of NadE and a potential regulation of NadE activity by P_{II}, this could suggest one step in the pathway at which P_{II} could influence the response of the DRAG-DRAT system to ammonia shock.

Another interesting relationship is the close linkage in *Azospirillum brasilense* between the GlnK structural gene (termed *glnZ*) and a gene (*aat*) encoding a protein with significant similarity to an aspartate amino transferase. In *E. coli* this enzyme (AspC) is involved in the utilization of glutamate as a carbon source; it catalyzes the reaction between L-glutamate and oxaloacetate, producing L-aspartate and 2-ketoglutarate (149), and a similar pathway exists in *K. aerogenes* (226). Given that the reaction generates 2-ketoglutarate, it is clearly a candidate for regulation by a P_{II} protein, and the gene linkage in *A. brasilense* may be indicative of just such an interaction.

The above considerations suggest that there could still be many other undiscovered targets for P_{II}. Indeed, the activity of any enzyme or the transcription of any gene that is involved in nitrogen assimilation or metabolism is a potential candidate. Recruitment of any such candidate into the P_{II} "network" would facilitate the optimal coordination of cellular nitrogen metabolism.

Role of P_{II} Proteins in Plants

Nitrogen is largely absorbed by plants as nitrate, which in *Arabidopsis thaliana* is reduced primarily in the cytosol of leaf cells to nitrite by nitrate reductase. After its import into the chloroplast, nitrite is converted by nitrite reductase to ammonium, which is then assimilated into glutamine by the plant GSII. In addition, in green leaves of C3 plants, ammonium resulting from photorespiratory metabolism is imported to the chloroplast and reassimilated by GSII. Interestingly, a new member of the plant ammonium transporter (AMT) family was recently described in *Arabidopsis thaliana*. This protein (AtAMT2) is more highly expressed in shoots than in roots (212), and it was suggested that this protein may play a different role to the AtAMT1 proteins (173). One possibility is that AtAMT2 could be responsible for the transport of ammonium produced during photorespiration into the chloroplast. The localization of the P_{II}-like protein GLB1 to the chloroplast (97), together with known functions of P_{II} proteins in bacteria, suggests many possible targets for GLB1. The protein could regulate the activity of the nitrite or ammonium transporter or the metabolic enzymes nitrite reductase and GSII, all of which will affect the nitrogen status of the chloroplast. It should also be noted that regulation of the N-C balance in photosynthetic bacteria seems to be integrated by GlnB (92, 185), in which case the chloroplastic P_{II}-like protein GLB1 could similarly be involved in coordinating nitrogen assimilation and photosynthesis.

Is P_{II} an Essential Gene Product in Some Organisms?

An *E. coli* null mutant in which both P_{II} genes are deleted is viable, albeit slow-growing. Likewise, double mutants of *Azorhizobium caulinodans* and *Rhodobacter capsulatus*, in which there are probably only two P_{II} genes, are viable. Hence, P_{II} is not by definition an essential protein. However, in *Azotobacter vinelandii* (153) and in *Nostoc punctiforme* (84), several attempts to create a P_{II} mutant failed, which led the authors to suggest that in these organisms, P_{II} may be essential. In *A. vinelandii*, the requirement for a certain level of GS activity has been considered the likely reason for the lethality of both *glnA* and *glnD* mutations (39). However, the apparent lethality of *glnK* mutations cannot be attributed to a possible effect on the adenylylation of GS, because a *glnK* mutation was still unstable in a mutant carrying a nonadenylylatable GS (*glnA* Y407F) (153).

CONCLUDING REMARKS

In summarizing the state of knowledge in the field of bacterial nitrogen control 5 years ago, we noted that one of the few apparently unifying concepts that appeared to be emerging at that time was the ubiquity of the P_{II} proteins and their pivotal role in nitrogen metabolism (155). The very significant advances in our understanding of this protein family since then have undoubtedly served only to confirm this view. In this review, we have attempted not only to summarize the detailed

information that we now have on the structures and functions of bacterial P_{II} proteins but also to draw attention to indicators of the much wider roles that these proteins might play in coordinating a great many facets of nitrogen metabolism, including acquisition of nitrogen compounds and their catabolism and assimilation.

The explosion in genome sequence information in the last 5 years has served to reveal the ubiquitous nature of the P_{II} protein family, and the developments in the concept of functional coupling of genes (177) are already suggesting new targets for P_{II} proteins. However, in the near future the application of transcriptome and proteome analysis will rapidly allow us to move to a much more comprehensive analysis of the potential targets for P_{II}, in terms of regulating both gene expression and protein activity. We would not be surprised if these analyses reveal, particularly in the proteobacteria, that we have only begun to scratch the surface.

One particularly notable feature of the P_{II} proteins is their degree of conservation, as demonstrated by the GlnB and GlnK proteins. However, there are some interesting challenges ahead in understanding the more unusual members of the family, such as the NifI proteins, the novel $GlnB_I$ protein of *Aquifex aeolicus*, and the newly emerging P_{II} proteins of higher plants with their additional N-terminal domains. While the availability of X-ray structures for both GlnB and GlnK offers insights into the mode of action of P_{II}, further structures of other forms, e.g., with 2-ketoglutarate bound or with P_{II} complexed to a "target" protein, would undoubtedly help to illuminate exactly how the protein functions.

There is still much to learn about this fascinating signal transduction protein that appears to have proved so versatile as a regulatory molecule. Our understanding of the biology of these proteins will undoubtedly be served by the continued endeavors of microbiologists to study as wide a spectrum of organisms as possible, and given the enormous diversity of prokaryotic organisms, there are likely many novel stories still waiting to be told.

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