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Review article

Regulation of Nitrogen Fixation by Multiple P_{II} Homologs in the Photosynthetic Bacterium *Rhodospirillum rubrum*

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Abstract

In the photosynthetic bacterium *Rhodospirillum rubrum* the regulation of nitrogen fixation occurs at both transcriptional and posttranslational levels. Recently, three P_{II} homologs, named GlnB, GlnK, and GlnJ, have been identified in *R. rubrum*, and they play very important roles in the regulation of nitrogen fixation. Similar to that seen in *Azospirillum brasilense* and other N_2-fixation bacteria, the expression of the *nif* operon is controlled by posttranslational regulation of NifA activity in *R. rubrum*. Only the uridylylated form of GlnB is required for the activation of NifA activity, and GlnK and GlnJ do not appear to be involved in that process. The posttranslational regulation of nitrogenase activity involves the DRAT/DRAG regulatory system, which responds to fixed nitrogen and to energy limitations. Both GlnB and GlnJ, but not GlnK, are involved in the regulation of DRAT/DRAG activities. All three P_{II} homologs can support proper regulation of glutamine

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synthetase activity. The discovery through genome analysis of both DRAT/DRAG and P_{II} homologs in many organisms, including those incapable of nitrogen fixation, suggests that these proteins might interact to serve a variety of biological roles.

Keywords: P_{II} homologs, nitrogen fixation, ADP-ribosylation, transcriptional regulation, posttranslational regulation

1. Introduction

Biological nitrogen fixation is catalyzed by the nitrogenase complex, which consists of two proteins: dinitrogenase (also referred to as MoFe protein) and dinitrogenase reductase (also referred to as Fe protein) (Burris, 1991). Dinitrogenase is an $\alpha_2\beta_2$ tetramer of the *nifKD* gene products and contains the active site (FeMo-co) for reduction of N_2, C_2H_2 and other substrates. Dinitrogenase reductase is an α_2 dimer of the *nifH* gene product, and passes electrons to dinitrogenase. Nitrogen fixation is a very energy-demanding process and is therefore rigorously regulated at both transcriptional and posttranslational levels in many N_2-fixing bacteria.

Regulation of *nif* expression is best characterized in *Klebsiella pneumoniae* where it responds to both the fixed nitrogen and oxygen through both global *ntr* system and *nif*-specific systems (Merrick and Edwards, 1995). The P_{II} homologs, GlnB and GlnK, play a very important role in these regulatory systems in response to carbon and nitrogen status.

Recent work has characterized the function of the P_{II} family of proteins in detail in *Escherichia coli* and other bacteria (Arcondéguy et al., 2001; Ninfa and Atkinson, 2000). GlnB functions as a trimer and can directly sense the α-ketoglutarate level as an indicator of carbon status or carbon/nitrogen balance (Jiang and Ninfa, 1999; Kamberov et al., 1995). At a low α-KG concentration, GlnB trimers bind only one molecule of α-KG and can interact with NtrB, thus inhibiting its kinase activity and activating its phosphatase activity to dephosphorylate NtrC. However, at higher α-KG concentrations, GlnB binds additional molecules of α-KG and thereby is unable to interact with NtrB, so that NtrB acts as a kinase to phosphorylate NtrC. In enteric bacteria, the phosphorylated form of NtrC acts as a transcriptional activator of *nifLA*, *glnA ntrBC*, *glnK amtB*, *nac* and other operons involved in nitrogen fixation and assimilation.

It appears that GlnB does not directly sense the nitrogen status. Instead, a bifunctional, uridylyltransferase/uridylyl-removing enzyme (UTase/UR, gene product of *glnD*) is believed to be a primary sensor of the intracellular nitrogen status (as the glutamine level) in the cell and reversibly controls the activity

of GlnB by uridylylation or deuridylylation (Jiang et al., 1998). Under N-limiting conditions, the uridylylation of GlnB prevents its interaction with NtrB, so that NtrC is accumulated in the phosphorylated form.

In many N_2-fixing bacteria, NifA activity is also regulated in response to NH_4^+. In *K. pneumoniae* and *Azotobacter vinelandii*, NifA activity is inhibited by another *nif* protein, NifL, in response to NH_4^+ and oxygen, through protein-protein interactions (Dixon, 1998; Govantes et al., 1996; Money et al., 1999; Reyes-Ramirez et al., 2001). Another P_{II} homolog, GlnK, has been found to be involved in the relief of NifL inhibition of NifA in *K. pneumoniae* under N_2-fixing conditions (He et al., 1998; Jack et al., 1999). However, a different type of posttranslational regulation of NifA activity has been found in other N_2-fixing bacteria and is discussed below.

Posttranslational regulation, which has also been termed "short-term inhibition" or "switch-off", causes a rapid control of nitrogenase activity. This regulatory approach has been found in diverse nitrogen-fixing bacteria, and has been well characterized in photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodobacter capsulatus*, and associative N_2-fixing bacteria *Azospirillum brasilense* and *Azospirillum lipoferum* (Ludden and Roberts, 1989; Masepohl et al., 1993; Nordlund, 2000; Zhang et al., 1997). This regulation involves the reversible mono-ADP ribosylation of dinitrogenase reductase and is catalyzed by two enzymes (Fig. 1). Dinitrogenase reductase ADP-ribosyl transferase (referred to as DRAT, the gene product of *draT*) carries out the transfer of the ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase homodimer, resulting in the inactivation of that enzyme. The ADP-ribose group attached to dinitrogenase reductase can be removed by another enzyme, dinitrogenase reductase activating glycohydrolase (referred to as DRAG, the gene product of *draG*), thus restoring nitrogenase activity. Recent work showed that P_{II} homologs play a significant role in the regulation of DRAT/DRAG activity in *R. rubrum*.

2. Transcriptional Regulation of Nitrogen Fixation in *R. rubrum*: The Uridylylated Form of GlnB is required for the Activation of NifA Activity

Unlike the case in *K. pneumoniae*, analysis of *ntrB*, *ntrC*, and *glnB* mutants of *R. rubrum* has shown that *glnB* is essential for nitrogen fixation, but *ntrBC* mutants have a Nif^+ phenotype (Ludden and Roberts, 1989; Masepohl et al., 1993; Nordlund, 2000; Zhang et al., 1995b; Zhang et al., 2000). *nifA* in *R. rubrum* is actually expressed under conditions that do not favor nitrogen fixation, such as in the presence of fixed nitrogen or air. The regulation of the expression of *nif* operons is controlled by the posttranslational regulation of NifA activity. The

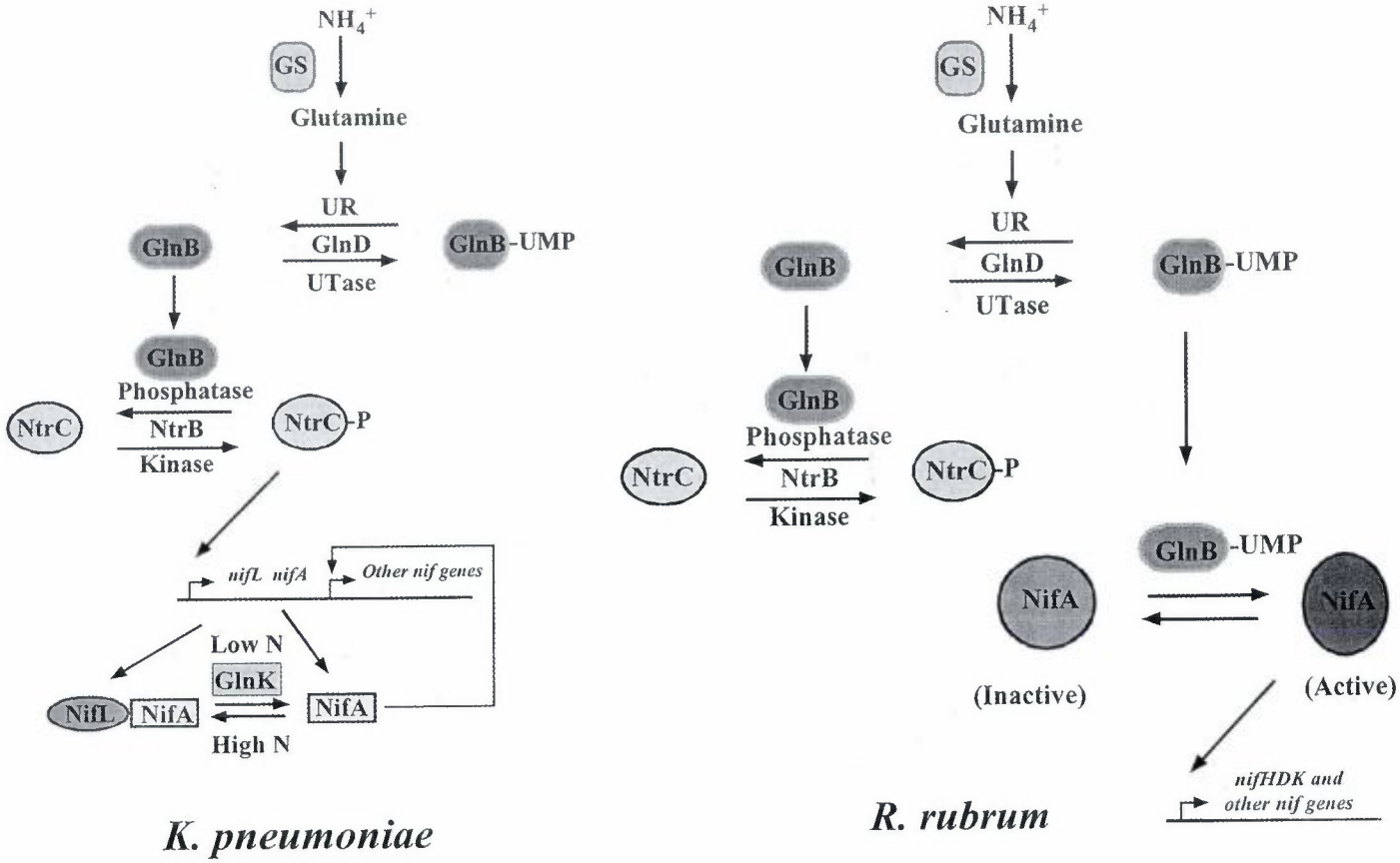

Figure 1. Model for the regulation of nitrogenase activity by ADP-ribosylation of dinitrogenase reductase in *R. rubrum*. This regulation is catalyzed by the DRAT/DRAG regulatory system.

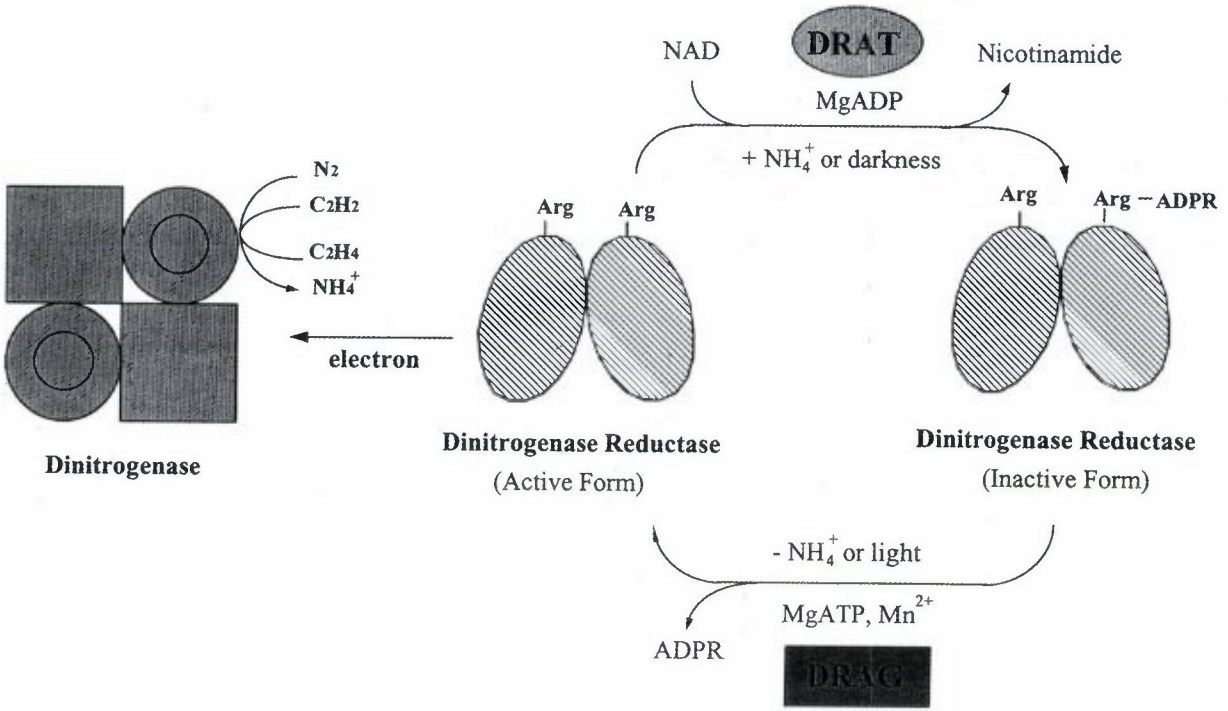

Figure 2. Transcriptional regulation of *nif* expression in *K. pneumoniae* and *R. rubrum*.

presence of *nifA* from *K. pneumoniae* can restore the Nif^+ phenotype of a *glnB* mutant of *R. rubrum*, whereas *nifA* from *R. rubrum* cannot (Zhang et al., 2000). This indicates that NifA of *R. rubrum* is synthesized in an inactive form and that GlnB is required for its activation. Furthermore, the activation of NifA requires the uridylylated form of GlnB, since a *glnB* mutant with a Y51F substitution (the site of uridylylation) has low nitrogenase activity (Zhang et al., 2000). Although the sequences of two other P_{II} homologs, GlnK and GlnJ, are very similar to that of GlnB, neither is involved in the activation of NifA activity in *R. rubrum* (Zhang et al., 2001b). The comparison of the regulation of *nif* expression between *K. pneumoniae* and *R. rubrum* is shown in Fig. 2.

A similar mechanism for NifA regulation has been reported in some other N_2-fixing bacteria, such as *A. brasilense* and *Herbaspirillum seropedicae*. Deletion analysis of *nifA* revealed that the N-terminal domain is involved in the regulation of NifA activity (Arsene et al., 1996; Monteiro et al., 1999; Souza et al., 1999). It has been suggested that the N-terminal domain inhibits NifA-dependent transcriptional activation by interacting with the catalytic C-terminal domain of NifA. Consistent with this model, the N-terminal domain of *H. seropedicae* NifA interacts in vitro with the C-terminal domain and inhibits its ATPase and DNA-binding activities (Monteiro et al., 2001). GlnB-UMP might bind the N-terminal domain of NifA, relieving or preventing its inhibitory interaction with the catalytic domain.

3. Posttranslational Regulation of Nitrogenase Activity in *R. rubrum*: Either GlnB or GlnJ is Sufficient for the Proper Regulation of the DRAT/DRAG System

As mentioned above, the nitrogenase activity in *R. rubrum* is regulated by the DRAT/DRAG regulatory system in response to negative stimuli such as NH_4^+ and darkness (Fig. 1). Furthermore, the regulation of the ADP-ribosylation of dinitrogenase reductase is effected through the posttranslational regulation of both DRAT and DRAG activities (Zhang et al., 1997). Under nitrogen-fixing conditions, DRAT is inactive, so that dinitrogenase reductase is in its active form. Following a negative stimulus, DRAT is activated, resulting in the loss of nitrogenase activity and the modification of dinitrogenase reductase. However, DRAT activation is only transient and it becomes inactive again even in the presence of continued pressure of the negative stimulus. In contrast, DRAG is active under nitrogen-fixing conditions, and it is inactivated by a negative stimulus. Unlike DRAT, however, the regulation of DRAG is not transient, but reflects the current physiological status. After removal of the negative stimulus, DRAG becomes active again, and it then reactivates dinitrogenase reductase by cleavage of the ADP-ribose group.

Part of this regulation is through the differential abilities of DRAT and DRAG to regulate oxidized and reduced forms of dinitrogenase reductase. In other words, the electron flux through the protein substrate of DRAT and DRAG regulates their ability to posttranslationally modify it (Halbleib et al., 2000a; Halbleib et al., 2000b). However, this regulation cannot explain all of the regulation of the DRAT/DRAG system. The observation that both DRAT and DRAG always are active in *in vitro* assays, either in extracts or when purified, has suggested the hypothesis that they might loosely bind negative effector(s), although the nature of these has been unclear (Ludden and Roberts, 1989). Recently, the P_{II} proteins have become excellent candidates for these effectors.

The first indication of this P_{II} role came from the heterologous expression of *draTG* from *A. brasilense* and *R. rubrum* in *K. pneumoniae,* which ordinarily lacks this regulatory system (Fu et al., 1990; Zhang et al., 2001a). Such *K. pneumoniae* strains displayed apparently proper regulation of nitrogenase activity in response to NH_4^+. This not only indicated that DRAT/DRAG is sufficient for the ADP-ribosylation, but also that *K. pneumoniae* must have functionally conserved regulatory pathways or molecules for the regulation of DRAT and DRAG activities. This regulation was altered in a *glnB* mutant and completely abolished in a *glnK* mutant, which strongly suggested a direct role for P_{II} in DRAT/DRAG regulation in *R. rubrum* as well.

Analysis of *R. rubrum* mutants with all possible combinations of *glnB, glnK* and *glnJ* mutations showed that either GlnB or GlnJ was sufficient for fairly normal posttranslational regulation of nitrogenase activity. The presence or absence of *glnK* had a negligible effect. Interestingly, the *glnB glnJ* double mutants also failed to respond to darkness, suggesting that in *R. rubrum* P_{II} homologs play a role in not only nitrogen regulation and sensing the carbon-nitrogen balance, but also in sensing the energy status (Zhang et al., 2001a; Zhang et al., 2001b). As mentioned above, P_{II} activity can be regulated by binding α-KG and it will be interesting to see if ATP levels are a direct energy signal for the P_{II} homologs in *R. rubrum.*

There are two inconsistencies that need to be resolved: First, *K. pneumoniae* mutants in *glnB* and in *glnK* result in different DRAT/DRAG responses, as if there is some functional specificity at the P_{II} level. It is therefore somewhat surprising that GlnB and GlnJ of *R. rubrum* appear to be roughly equivalent in their roles, at least at the cursory level thus far examined. Secondly, the *K. pneumoniae glnB* and *glnK* mutants seem to affect DRAG and DRAT activities differentially, yet such a differential effect is not yet apparent with *glnB* and *glnJ* of *R. rubrum* (Zhang et al., 2001a; Zhang et al., 2001b).

The DRAT/DRAG systems in *A. brasilense* and *R. capsulatus* appear to be generally rather similar, but there are some interesting differences. These include the rates at which nitrogenase activity is altered and the level of

residual activity (which reflects the unmodified population of dinitrogenase reductase) under "switch-off" conditions (Masepohl et al., 1993; Pierrard et al., 1993; Zhang et al., 1995a, 1996). In *A. brasilense* and *R. capsulatus*, there is also another regulatory system that responds to NH_4^+, but does not appear to involve covalent modification of the nitrogenase proteins (Pierrard et al., 1993; Zhang et al., 1996).

4. Other Roles of P_{II} Homologs in *R. rubrum*

Another phenotype of *R. rubrum glnB glnJ* mutants is that they grow slower than does wild type on both rich and minimal medium. This phenotype is not stable, with suppressor mutations arising at high frequency, consistent with loss-of-function mutations. At least some of these suppressor mutations have been found to lie in *ntrBC*, though the physiological implications of this are unclear (Zhang et al., unpublished data). Interestingly, although the presence or absence of *glnK* does not affect this phenotype, its overexpression can support good growth in a *glnB glnJ* background (Zhang et al., unpublished data). Although the mechanism for this poor growth is still unknown, it appears that P_{II} might have another target, perhaps also affected in some way by NtrBC, which is necessary for optimal growth. This effect is reminiscent of the lethality seen in *A. vinelandii* and *Nostoc punctiforme* when the single P_{II} homolog of these organisms is eliminated (Rudnick et al., 2002; Hanson et al., 1998).

Because none of the above phenotypes were affected by either the presence or absence of normal expression of *glnK*, we were concerned that this gene might either be cryptic or expressed only under unusual conditions. We therefore examined the requirements for the various P_{II} homologs in the regulation of the adenylylation of GS. While all single and double mutants (lacking various P_{II} homologs) behaved normally, the triple mutant was unable to significantly modify GS in response to NH_4^+ addition. This demonstrates that all three P_{II} homologs have this biochemical capability and that GlnK is expressed at sufficient level to support this regulation (at least in a strain lacking GlnB and GlnJ) (Zhang et al., 2001b). In *E. coli*, GlnB and GlnK display some distinct properties such as the ability of GlnB-UMP, but not GlnK-UMP, to strongly stimulate the deadenylylation of GS (van Heeswijk et al., 2000). GlnK is also less potent in activating the adenylylation of GS by ATase (Atkinson and Ninfa, 1999). However, these different properties of the P_{II} homologs are unknown in *R. rubrum*. The various functions, along with an indication of the P_{II} homologs involved, are summarized in Fig. 3.

Although the mechanism for the effects of GlnB and GlnJ on the DRAT/DRAG regulatory system is unknown, it is likely caused by the altered

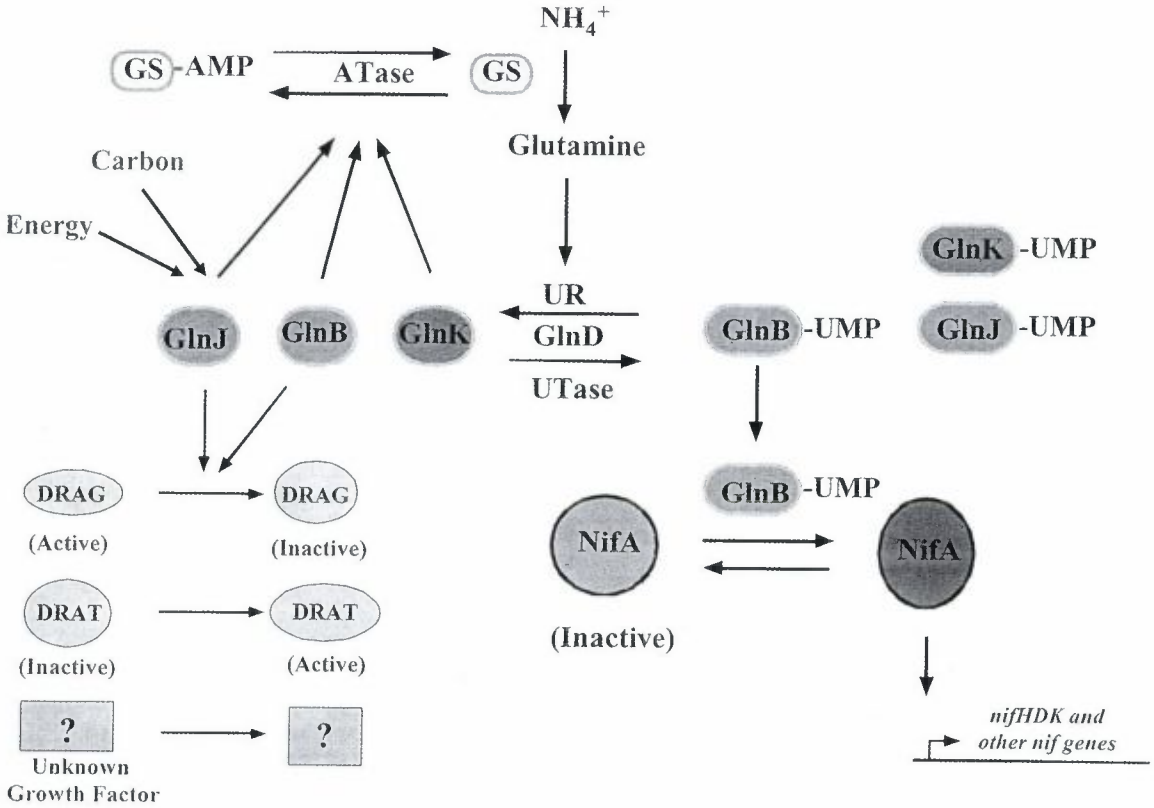

Figure 3. The various roles of GlnB, GlnK and GlnJ in *R. rubrum*.

posttranslational regulation of DRAT/DRAG activities, rather than by the alteration of expression levels of these proteins. This regulation is presumably through the direct interaction of DRAT/DRAG with P_{II}. Both GlnB and GlnJ are regulated by uridylylation in response to NH_4^+ or by binding α-KG in response to carbon levels, and probably by binding ATP in response to energy status. By analogy with the P_{II} interaction with NtrB (Atkinson and Ninfa, 1999), the different forms of GlnB or GlnJ might interact with DRAT/DRAG differently. For example, the unuridylylated form of GlnB might activate DRAT activity under nitrogen-excess conditions, and the uridylylated form of either protein might activate DRAG activity.

5. DRAT/DRAG Regulatory System in Other Bacteria

ADP-ribosylation has been extensively studied in large part because the bacterial toxins for cholera and pertussis catalyze the ADP-ribosylation of G-proteins (Jacobson and Jacobson, 1989; Moss and Vaughan, 1990). Endogenous ADP-ribosyltransferases have also been found in many animal tissues, consistent with a regulatory role in normal cells. Although very low primary sequence similarity was found among these eukaryotic ADP-ribosyltransferases, bacterial toxins, and DRAT, some conserved residues have been identified (Domenighini and Rappuoli, 1996). ADP-ribosylarginine hydrolases have also been purified from animal and human tissue and showed similarity to DRAG in some conserved regions (Moss et al., 1992). However, neither the target proteins nor the physiological roles of these regulatory proteins are known in these eukaryotes.

In recent years the explosion in bacterial genomic sequencing has led to the discovery of DRAT/DRAG homologs in many diverse nitrogen-fixing or non-nitrogen-fixing Proteobacteria, Actinobacteria and Archaebacteria. A partial list of organisms with DRAT/DRAG homologs is shown in Table 1.

The DRAT/DRAG regulatory system has only been well characterized in *R. capsulatus* and *A. brasilense*, in addition to *R. rubrum*. Although the mutations in two P_{II} homologs (P_{II} and P_Z) have been constructed in *A. brasilense*, their effect on DRAT/DRAG regulation is unknown. A mutant lacking both P_{II} homologs also showed poor growth on both rich and minimal media (de Zamaroczy, 1998). In *R. capsulatus* two P_{II} homologs, GlnB and GlnK, have been identified, and they play a significant role in the regulation of DRAT/DRAG activity. In *glnB glnK* mutants, regulation of nitrogenase activity in response to NH_4^+ is altered, but unlike the *glnB glnJ* case in *R. rubrum*, the nitrogenase activity in this double mutant is still regulated in response to darkness (Masepohl et al., 2002). A modification of dinitrogenase reductase and "switch-off" of nitrogenase activity in response to NH_4^+ or NO_3^- has been found in an

Table 1. List of organisms containing DRAT/DRAG, nitrogenase systems, as well as PII homologs

Organisms	PII	Nitrogenase system	DRAT/DRAG
Eubacteria			
Acidithiobacillus ferrooxidans	PII-1, 2, 3, 4	+	DRAT, DRAG
*Aquifex aeolicus***	PII-1, 2	–	DRAG*
Azoarcus sp. BH72.	GlnB, GlnK, GlnY	+	Unknown
Azospirillum brasilense	PII, PZ (GlnK)	+	DRAT, DRAG
*Clostridium acetobutylicum***	PII-1, 2	+	DRAG*
*Deinococcus radiodurans***	PII	–	DRAG*
Geobacter metallireducens	PII-1, 2	+	DRAT, DRAG
Geobacter sulfurreducens	PII-1, 2, 3, 4	+	DRAT, DRAG
*Listeria monocytogenes***	PII	–	DRAG*
Magnetococcus sp. MC-1	PII-1, 2	+	DRAT, DRAG
Magnetospirillum magnetotacticum	PII-1, 2, 3, 4	+	DRAT, DRAG
*Mesorhizobium loti***	GlnB, GlnK	+	DRAG*
Methylococcus capsulatus	PII-1, 2	–	DRAT (DRAG?)***
Nostoc sp. PCC7120	PII	+	DRAG*
Pseudomonas fluorescens	PII-1, 2	–	DRAG
Rhodobacter capsulatus	GlnB, GlnK	+	DRAT, DRAG
Rhodopseudomonas palustris	PII-1, 2, 3	+	DRAT, $DRAT_2$, DRAG
Rhodospirillum rubrum	GlnB, GlnK, GlnJ	+	DRAT, $DRAT_2$, DRAG
*Streptomyces coelicolor***	PII	–	DRAG*
Synechococcus sp. PCC7002	PII	+	DRAG*
Archaea			
*Archaeoglobus fulgidus***	PII-1, 2, 3	–	DRAG*
*Methanococcus jannaschii***	PII-1, 2	+	DRAG*
Methanosarcina barkeri	PII-1, 2, 3, 4	+	DRAG*
Methanosarcina mazei	PII-1, 2, 3, 4	+	DRAG*

*Low similarity to DART or DRAG. **Complete genomic sequence. ***It is unclear if *Methylococcus capsulatus* lacks DRAG. No *draG* homolog has been found in unfinished genomic sequence.

associative N_2-fixing bacterium *Azoarcus* sp. BH72, although the mechanism is unknown. Three P_{II} homologs, GlnB, GlnK and GlnY, have been identified in *Azoarcus* sp. BH72, and some play important roles in the regulation of nitrogenase activity; a *glnB glnK* mutant lacks the modification of

dinitrogenase reductase and inhibition of nitrogenase activity in response to both NH_4^+ and anaerobiosis (Martin and Reinhold-Hurek, 2002).

The regulation of nitrogenase activity has also been reported in some archaea, such as *Methanococcus maripaludis* and *Methanosarcina barkeri*, and might involve P_{II} as well (Kessler and Leigh, 1999; Lobo and Zinder, 1990). In *M. maripaludis* two *glnB* homologs, *nifI*$_1$ and *nifI*$_2$, have been found in the *nif* cluster and deletion of either of these genes affects posttranslational regulation of nitrogenase activity in response to NH_4^+, but not *nif* expression (Kessler et al., 2001; Kessler and Leigh, 1999). However, the mechanism for the posttranslational regulation of nitrogenase activity is unknown in *M. maripaludis* and no modification of dinitrogenase reductase was detected in response to NH_4^+. *draTG* homologs have not been identified in *M. maripaludis*, although *draG* homologs have been found in other diazotrophic Archaea such as *Methanococcus jannaschii*, *Archaeoglobus fulgidus*, *Methanosarcina mazei*, and *M. barkeri*.

As seen in Table 1, both *draT* and *draG* homologs have also been found in magnetotactic bacteria (*Magnetospirillum magnetotacticum* and *Magnetococcus* sp. MC-1), non-sulfur phototrophic bacteria (including *Rhodopseudomonas palustris*), a chemolithoautotrophic bacterium (*Acidithiobacillus ferrooxidans*), and metal-metabolizing bacteria (*Geobacter metallireducens* and *Geobacter sulfurreducens*). Because all of these bacteria fix N_2, their DRAT/DRAG systems are excellent candidates for regulation of nitrogenase activity. Some microbes (even non-N_2-fixing bacteria) have a *draG* homolog, but lack a *draT* homolog, suggesting that DRAG might have other functions in the cell. Interestingly, two *draT* homologs have been found in both *R. rubrum* and *R. palustris*. The function of the "second" *draT* is unknown, but certainly cannot replace the function of DRAT in *R. rubrum*, since the regulation of nitrogenase activity is completely abolished in a *draT* mutant.

Recently, it was reported that an *amtB* mutant of *R. capsulatus* also altered the DRAT/DRAG regulation in response to NH_4^+, but that darkness response is unaffected in this mutant (Yakunin and Hallenbeck, 2002). AmtB has long assumed to be an NH_4^+ transporter, but recent results suggest it might be an NH_4^+ sensor (Coutts et al., 2002; Yakunin and Hallenbeck, 2002). An *amtB* mutant in *Azoarcus* sp. BH72 also affects the response to NH_4^+, but not to anaerobiosis (Martin and Reinhold-Hurek, 2002). While the molecular basis of these effects is unknown, AmtB can bind P_{II} (mainly GlnK) in *E. coli* and *A. vinelandii* in response to the intracellular nitrogen status (Coutts et al., 2002). This membrane sequestration of GlnK by AmtB may be a common form of regulation of the activity of P_{II} homologs. Because P_{II} homologs regulate other proteins through direct interaction, it is possible that AmtB affects these other proteins indirectly through its direct interaction with P_{II}. It is interesting to note that DRAG has been shown to display a loose membrane association under

certain cell growth conditions, which can readily be rationalized through the above model.

6. Conclusion

There are three P_{II} homologs in *R. rubrum*. As shown in Fig. 3, these P_{II} homologs have both distinct and overlapping functions in the cell: 1) Only the uridylylated form of GlnB is able to activate NifA. GlnK and GlnJ do not appear to be involved in that process. 2) Either GlnB or GlnJ can serve as a critical element in the regulation of the reversible ADP-ribosylation of dinitrogenase reductase. Strains lacking both of these proteins (and irrespective of the presence or absence of GlnK) show an extremely poor response to either exogenous NH_4^+ or to energy deprivation. 3) The absence of both GlnB and GlnJ in *R. rubrum* causes poor growth, but overexpression of GlnK can suppress this phenotype. 4) The modification of GS can be regulated by any of the three P_{II} homologs in *R. rubrum*.

Recent discoveries of the involvement of P_{II} homologs in sensing nitrogen, carbon, and energy signals, as well as in the regulation of NifA activity and DRAT/DRAG activities, indicate the importance of this signal transduction protein in a broad range of regulatory systems in the cell.

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