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Post-translational modification of P_{II} signal transduction proteins

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The P_{II} proteins constitute one of the most widely distributed families of signal transduction proteins in nature. They are pivotal players in the control of nitrogen metabolism in bacteria and archaea, and are also found in the plastids of plants. Quite remarkably P_{II} proteins control the activities of a diverse range of enzymes, transcription factors and membrane transport proteins, and in all known cases they achieve their regulatory effect by direct interaction with their target. P_{II} proteins in the Proteobacteria and the Actinobacteria are subject to post-translational modification by uridylylation or adenylylation respectively, whilst in some Cyanobacteria they can be modified by phosphorylation. In all these cases the protein's modification state is influenced by the cellular nitrogen status and is thought to regulate its activity. However, in many organisms there is no evidence for modification of P_{II} proteins and indeed the ability of these proteins to respond to the cellular nitrogen status is fundamentally independent of post-translational modification. In this review we explore the role of post-translational modification in P_{II} proteins in the light of recent studies.

Keywords: p_{II} protein, post-translational modification, uridylylation, adenylylation, phosphorylation

THE P_{II} PROTEIN FAMILY

P_{II} proteins were first identified by B. M. Shapiro in the late 1960s when he was studying control of the activity of *Escherichia coli* glutamine synthetase (GS) by adenylylation/deadenylylation (Shapiro, 1969). Subsequent studies by Stadtman and co-workers characterized two proteins: P_I which was responsible for the adenylylation/deadenylylation of GS, and P_{II} which modulated these activities (Brown et al., 1971). Furthermore they observed that P_{II} itself existed in two forms, the interconversion of which appeared to involve the covalent attachment of a uridine derivative to P_{II}. In due course it was shown that P_{II} was encoded by *glnB*, that uridylylation occurred on residue Tyr51 in the T-loop of the protein, and that both uridylylation and deuridylylation were effected by the enzymatic activity of GlnD (Arcondéguy et al., 2001; Huergo et al., 2013).

Extensive genomic research has shown that P_{II} proteins are extremely widespread amongst bacteria, archaea, and plants (Sant'Anna et al., 2009). They are, however, not found in fungi or animals. Their presence in plants is considered to be a consequence of the cyanobacterial origin of the plastid and the protein, whilst encoded in the nucleus, is expressed in the chloroplast (Chellamuthu et al., 2013). Bacteria and archaea often encode multiple P_{II} proteins, e.g., proteobacteria typically encode two, designated GlnB and GlnK. However, cyanobacteria and plants usually encode just a single copy. All P_{II} proteins show a very high level of sequence conservation and protein crystallography studies of P_{II} proteins from bacteria, archaea, and plants indicates that their tertiary structure is also highly conserved (**Figure 1**). They are homotrimers with a core 12–13 kDal subunit. The trimer forms a compact cylindrical-shaped molecule from which three long exposed loops (the T-loops) protrude. Two smaller loops, the B- and C-loops, are located at the interface between adjacent subunits of the trimer such that the T- and B-loops of one monomer and the C-loop of the adjacent monomer form an inter-subunit cleft that constitutes a ligand binding site. Although there is now extensive structural data on P_{II} proteins from a range of organisms no structures have been solved for uridylylated or phosphorylated forms (Huergo et al., 2013).

Whilst originally identified as regulating the activity of the GS adenylyltransferase in *E. coli*, P_{II} proteins are now known to control the activities of a very diverse range of enzymes, a large number of transcription factors and some membrane transport proteins. In all known cases they achieve their regulatory effect by direct interaction with their target and in the majority of cases studied to date that interaction involves the T-loops of the P_{II} protein.

The recognition that the T-loops are potentially very flexible and would be able to adopt a variety of structures together with the knowledge that uridylylation of the protein in proteobacteria occurs within the T-loop, suggested early on that post-translational modification could play a key role in facilitating the regulatory activity of P_{II} proteins. However, these proteins are able to bind both ATP/ADP and 2-oxoglutarate (2-OG) and biochemical and crystallographic studies indicate that these effectors alone can have major effects on T-loop structure and on the interaction of P_{II} proteins with many of their targets (Conroy et al., 2007; Jiang and Ninfa, 2007, 2009; Truan et al., 2010). ATP and ADP compete for binding to the same site in the inter-subunit cleft but 2-OG can only bind in the presence of Mg-ATP and ADP does not support 2-OG binding. The ability of P_{II} proteins to bind 2-OG allows them to respond to aspects of the cellular nitrogen and carbon status whilst it has been proposed that the competitive binding of ATP and ADP also makes P_{II} proteins potential sensors of the adenylylate energy charge.

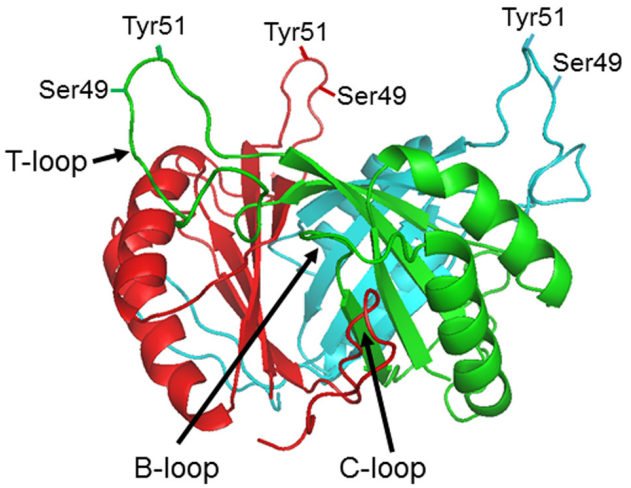

FIGURE 1 | P_{II} protein structure. Side view of the *Escherichia coli* GlnB trimer (PDB: $2P_{II}$) in the absence of ligands showing the positions of the B, C, and T loops and the residues within the T-loop (Ser49 and Tyr51) which can be subject to post-translational modification in different organisms.

The factors influencing effector binding and the physiological influences on their interactions with P_{II} are presently a matter of debate (Zeth et al., 2014). Changes of the effector pools *in vivo* under different physiological conditions have been studied in the case of the interaction of GlnK and AmtB in *E. coli* (Radchenko et al., 2010; Radchenko and Merrick, 2011). When the intracellular nitrogen status is high 2-OG levels in the cell are low and ADP is found bound to GlnK within the GlnK–AmtB complex (Conroy et al., 2007; Radchenko et al., 2013). The binding of ADP leads to concomitant change in the GlnK T-loop to form a structure in which the apex of the loop projects 28Å above the core of the protein facilitating complex formation with AmtB (Conroy et al., 2007). Conversely when the intracellular nitrogen status is low, 2-OG levels in the cell are high (Radchenko et al., 2013). Under these conditions GlnK is expected to bind 2-OG and Mg-ATP, the T-loops are relatively unstructured and complex formation is not promoted (Truan et al., 2010, 2014).

The switch between the ATP and ADP bound forms of GlnK has been ascribed by Radchenko et al. (2013) to an ATPase activity of the protein that is only manifest in low 2-OG. Other authors have proposed that the nucleotide occupancy of the effector binding site is solely a reflection of the cellular ATP/ADP ratio (Jiang and Ninfa, 2007, 2009; Fokina et al., 2011; Zeth et al., 2014). Radchenko et al. (2013) propose that a drop in the intracellular 2-OG pool promotes hydrolysis of ATP to ADP leading to a rearrangement of residues in the binding pocket, most notably Gln39 and K58, and a concomitant change in the T-loop structure. Such a model is consistent with the observed GlnK–AmtB and P_{II}–PipX structures and the mode of action of the P_{II} proteins in both these cases (see later). However, it is not consistent with *in vitro* studies of P_{II}–*N*-acetylglutamate kinase (NAGK) complex formation which indicate that this interaction is promoted by ATP alone and is inhibited by 2-OG (Fokina et al., 2011; Zeth et al., 2014).

In summary, changes in T-loop structure in response to changes in cellular N status as reflected by the 2-OG pool, or possibly in response to changes in the cellular ATP/ADP ratio, are sufficient to explain the ability of P_{II} proteins to interact with their cognate targets. Consequently the role of post-translational modification of P_{II} proteins is a facet of P_{II} biology that needs to be re-evaluated and studied in much more detail.

URIDYLYLATION AND ADENYLYLATION OF P_{II} PROTEINS

P_{II} uridylylation and deuridylylation are carried out by a single enzyme, encoded by *glnD,* that has been characterized from a number of proteobacteria. GlnD proteins have a molecular mass of about 100 kDal and contain a conserved nucleotidyltransferase superfamily motif. They have at least four domains of which the N-terminal domain encodes the uridylyltransferase (UTase) activity and the adjacent HD domain encodes the uridylyl-removing (UR) activity (Jiang et al., 1998; Zhang et al., 2010): hence, the two activities are not thought to share an active site. GlnD has a single glutamine-binding site and its activity is regulated by the intracellular glutamine level such that UTase activity predominates in low glutamine and UR activity is stimulated by high glutamine levels. P_{II} is the only known substrate for GlnD and the regulation of GlnD activity by glutamine means that in organisms where P_{II} is subject to uridylylation both the 2-OG and the glutamine pools influence P_{II} activity. The metabolic links between 2-OG and glutamine mean that usually the levels of these two effectors change in a reciprocal manner but there may be physiological conditions where the two are at least partially uncoupled.

The *glnD* gene is found ubiquitously in the proteobacteria and the actinobacteria, and sporadically in a few other diverse genera (Huergo et al., 2013). In the actinobacteria *glnD* is part of the *amtB, glnK, glnD* operon, whereas in the proteobacteria it is usually encoded elsewhere in the genome. Furthermore, studies of P_{II} modification in two actinobacteria, *Streptomyces coelicolor* and *Corynebacterium glutamicum*, found that in both these organisms the activity of GlnD is to adenylylate the single P_{II} protein, GlnK, rather than to uridylylate it (Hesketh et al., 2002; Strosser et al., 2004). The modification takes place on the equivalent Tyr51 residue of the T-loop and the exact basis for transfer of an adenyl rather than an uridyl group has not been determined. Hence, it is clear that within the P_{II} family post-translational modification by uridylylation/adenylylation is relatively restricted (**Figure 2**), and as with the P_{II} proteins (Sant'Anna et al., 2009) the distribution of *glnD* also suggests that horizontal gene transfer may have played a part in its present distribution.

PHOSPHORYLATION OF P_{II} PROTEINS

An alternative form of post-translational modification of P_{II} occurs in the cyanobacteria. In this case the T-loop is subject to phosphorylation on residue Ser49 and, as with uridylylation and adenylylation, the modification occurs in response to nitrogen limitation. T-loop phosphorylation has been observed in *Synechococcus elongatus* and in *Synechocystis* but despite conservation of residue Ser49 in the T-loop phosphorylation is not found in other cyanobacteria such as *Prochlorococcus* and *Anabaena* (Forchhammer et al., 2004). Furthermore, there is no evidence for post-translational modification of P_{II} in plants (Smith et al., 2004).

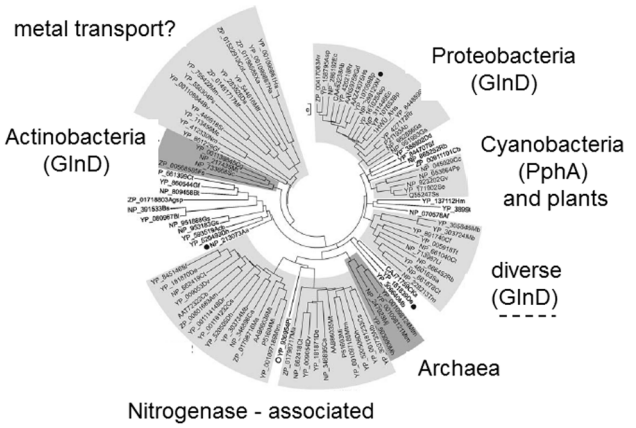

FIGURE 2 | Phylogenetic tree of P_{II} proteins showing those groups in which T-loop modification occurs. Phylogenetic analysis of the P_{II} protein family (Sant'Anna et al., 2009) identified at least seven distinct groups four of which are associated with specific taxonomic groups of organisms. Two groups appear to be associated with specific processes in taxonomically diverse organisms: one group control nitrogenase in certain diazotrophs (Leigh and Dodsworth, 2007) and the other group is often genetically linked to metal transport genes. Post-translational modification has only been described in four groups within the tree. Uridylylation (mediated by GlnD) is found in the proteobacteria and some "diverse" bacteria. Adenylylation (also mediated by GlnD) is found in the actinobacteria. Phosphorylation (associated with the presence of the phosphatase PphA) is found in some cyanobacteria but not in plants.

A completely novel modification, namely nitration of Tyr51, has been reported for *Anabaena* (Zhang et al., 2007).

The mechanism of P_{II} modification in cyanobacteria is also not fully understood. Dephosphorylation is driven by a specific phosphatase, PphA, the activity of which is inhibited by 2-OG in concert with Mg-ATP (Irmler and Forchhammer, 2001; Ruppert et al., 2002). Hence, 2-OG plays a key role as an effector, both by binding to P_{II} in conditions of N-limitation and also by inhibiting the phosphatase and ensuring that P_{II} remains phosphorylated in this situation. However, despite considerable efforts, the kinase responsible for phosphorylation of P_{II} has yet to be identified and consequently the factors regulating its activity are also still unknown (Chellamuthu et al., 2013). Potentially the kinase could offer another signal transduction route into the system, for example if glutamine was to inhibit the kinase in an analogous mode to the inhibition by glutamine of GlnD UTase activity, but at present such hypotheses await discovery of the kinase.

THE ROLE OF P_{II} MODIFICATION

An assessment of the various taxonomic groups of P_{II} proteins identified by Sant'Anna et al. (2009) makes it clear that many members of the family are probably not subject to any form of post-translational modification (**Figure 2**). There are to date no reports of P_{II} modification in archaea, nor in the Firmicutes such as *Bacillus subtilis*. The novel group of P_{II} proteins that function specifically to regulate activity of the nitrogenase enzyme in a variety of bacteria have also not been found to be controlled by post-translational modification and, as discussed above, modification is scattered amongst groups such as the cyanobacteria. So it would appear that P_{II} modification has arisen more than once in evolution and that many P_{II} proteins function effectively without such modification.

To assess the role of P_{II} modification in those organisms where it occurs it is necessary to examine model systems in which the interaction between a P_{II} protein and its target has been characterized in some detail, preferably both biochemically and structurally.

GlnK–AmtB

Regulation of the ammonium transporter AmtB by the P_{II} protein GlnK is widespread in both bacteria and archaea. The structural genes for these proteins are invariably linked in a single operon (*glnK amtB* or *amtB glnK*) and it has been suggested that this is the evolutionary origin of the P_{II} protein family (Thomas et al., 2000; Sant'Anna et al., 2009). The interaction of GlnK with AmtB has been studied in considerable detail both *in vivo* and *in vitro* and the structure of the complex from *E. coli* has been solved.

In N-limited conditions GlnK is fully uridylylated and free in the cytoplasm with Mg-ATP and 2-OG bound in the effector binding pocket as described earlier. An increase in the cellular N status leads to deuridylylation of GlnK and its sequestration to the inner membrane by AmtB (Radchenko et al., 2010). The crystal structure of the *E. coli* AmtB–GlnK complex shows that GlnK interacts with AmtB almost exclusively via the T-loop, the tip of which inserts deeply into the cytoplasmic pore exit of AmtB, thus blocking ammonia conduction into the cell (Conroy et al., 2007). The AmtB-bound GlnK has ADP bound in all three effector binding pockets. It is clear from the structure of the complex that uridylylation of GlnK will prevent complex formation and that deuridylylation is therefore a prerequisite for binding to GlnK. However, studies using a Tyr51Ala variant of GlnK that cannot be uridylylated have shown that this protein still responds to changes in cellular nitrogen status in a manner almost identical to the wild-type GlnK (Radchenko et al., 2014). As might be expected, when cells are subject to an ammonium shock the Tyr51Ala variant binds to AmtB slightly faster than the wild-type, presumably because the protein does not have to undergo deuridylylation prior to complex formation. Hence regulation of ammonia flux through AmtB by GlnK is not dependent on a functional T-loop modification and this is consistent with the fact that the GlnK–AmtB system is found in many organisms that do not exhibit post-translational modification of P_{II}.

P_{II}–PipX

This complex has been studied in the cyanobacterium *S. elongatus* where PipX is a co-activator of the transcription factor NtcA. P_{II} interacts with PipX to antagonize PipX–NtcA complex formation (Espinosa et al., 2006). In N-limited conditions de-phosphorylated P_{II} binds to PipX thereby freeing NtcA to activate transcription of genes required for growth in N-limitation. Complex formation between P_{II} and PipX is strongly reduced by ATP in concert with 2-OG, conditions that occur under N-sufficiency (Llacer et al., 2010). The complex has a 3:3 stoichiometry such that one trimer of P_{II} binds three molecules of PipX that are "caged" between the extended T-loops of the P_{II} trimer (Llacer et al., 2010; Zhao

et al., 2010). The T-loop conformation is similar to that seen in the GlnK–AmtB complex and consistent with this ADP has been shown to increase the affinity of P_{II} for PipX although no bound nucleotide was present in the structure of the complex. Residue Ser49 of the T-loop in P_{II} is positioned such that phosphorylation of Ser49 would not disrupt the complex and studies with a Ser49Glu variant that mimics phosphorylation confirm this (Llacer et al., 2010). Hence control of P_{II}–PipX association and dissociation appears to be independent of post-translational modification of P_{II}.

P_{II}–NAGK

N-acetylglutamate kinase is regulated by interaction with P_{II} in both cyanobacteria and plants (Burillo et al., 2004; Heinrich et al., 2004). NAGK is a key enzyme in arginine biosynthesis and arginine is used as a nitrogen store in these organisms. In conditions of N-sufficiency P_{II} binds to NAGK and enhances the enzyme's catalytic activity thereby increasing cellular arginine levels and allowing arginine to be used for nitrogen storage in the form of cyanophycin (Forchhammer, 2008).

Structures of the complex have been solved for both *S. elongatus* and *Arabidopsis thaliana* and both structures are very similar (Llacer et al., 2007; Mizuno et al., 2007). In each case a hexamer of NAGK is sandwiched between two P_{II} trimers and the T-loops constitute a major interface with NAGK. The *S. elongatus* complex had no bound nucleotide but the very similar *A. thaliana* complex had bound Mg-ATP. In *S. elongatus* ADP has been reported to inhibit P_{II}–NAGK complex formation (Maheswaran et al., 2004).

A Ser49Glu variant of P_{II} is unable to form a complex with NAGK (Heinrich et al., 2004) suggesting that phosphorylation inhibits the interaction and the structure of the complex clarifies how this occurs. Upon phosphorylation, one donor hydrogen bond formed with NAGK by the Ser49 OH group is lost. Furthermore, the bulkiness and negative charge of the phosphate triggers a steric and electrostatic clash with NAGK binding. However, it would appear that P_{II} phosphorylation is likely to occur after dissociation from NAGK and that, as with the other characterized P_{II} complexes, modification does not directly control complex formation and dissociation. It should also be noted that *A. thaliana* P_{II} is not subject to phosphorylation suggesting that, at least in this plant, NAGK regulation by P_{II} can function in the absence of post-translational modification.

GlnZ–DraG

In the diazotrophic proteobacterium *Azospirillum brasilense* the NifH subunit of nitrogenase is subject to post-translational modification by ADP-ribosylation and this modification is mediated by an ADP-ribosyltransferase (DraT) and an ADP-ribosylhydrolase (DraG; Huergo et al., 2012). The antagonistic activities of both DraT and DraG are regulated by P_{II} proteins; DraT by GlnB and DraG by GlnZ. In N-limited conditions both GlnB and GlnZ are uridylylated and do not interact with DraT and DraG. However, in N-sufficient conditions both P_{II} proteins undergo deuridylylation and then interact with their respective target proteins. The structures of DraT and of the GlnB–DraT complex have yet to be solved so the role of T-loop modification in regulating DraT activity is not known. However, the structures of both DraG and of the GlnZ–DraG complex have been solved (Berthold et al., 2009; Li et al., 2009; Rajendran et al., 2011). The complex is unique amongst those P_{II} complexes for which structural information is presently available because the interface between the two proteins does not involve the T-loops. Hence GlnZ uridylylation will not apparently have a major influence on its interaction with DraG. Structural modeling indicates that DraG is inactivated when bound to GlnZ due to steric hindrance of the DraG active site (Rajendran et al., 2011).

CONCLUSION

Whilst post-translational modification has been recognized as a key feature of P_{II} protein biology since its recognition in *E. coli* in the early 1970s, subsequent studies have determined that it is not a universal feature of this large protein family. In the case of uridylylation mediated by GlnD in the proteobacteria (and probably the equivalent adenylylation in archaea) this modification appears to serve to allow integration of sensing of the glutamine pool, through the regulation of GlnD activity by glutamine, with sensing of the 2-oxoglutarate pool by direct binding to P_{II}. In those cases where detailed biochemical and structural studies are available post-translational modification does not appear to be essential for regulation of complex formation, at least in the physiological conditions studied. However, it may influence the dynamics of the process.

The phosphorylation of P_{II} proteins seen in some cyanobacteria also has the potential to facilitate additional sensory input through regulation of the P_{II}-specific kinase but as the kinase has yet to be identified and characterized this concept remains hypothetical at present. Where cyanobacterial P_{II} systems involving phosphorylation have been characterized post-translational modification again does not appear to be essential for regulation of complex formation, and this is supported by the fact that P_{II} phosphorylation is not ubiquitous in the cyanobacteria (Forchhammer et al., 2004).

In summary, from the studies undertaken to date there appears to be no unifying role for post-translational modification of P_{II} proteins. Interaction of P_{II} proteins with their targets is predominantly controlled by effector binding (MgATP, ADP, and 2-OG) and consequent changes in T-loop conformation (Truan et al., 2010; Radchenko and Merrick, 2011; Radchenko et al., 2013). In a number of cases post-translational modification appears to have the potential to operate as a check-point by providing another signal transduction input that has to be accommodated before complex formation between P_{II} and at least some of its targets can proceed.

There is clearly a need for much more information both with respect to studies of many more P_{II} interactions and in a more varied range of physiological conditions. Both types of study may reveal further important roles for P_{II} modification. It is also the case that the majority of studies to date have been of steady state situations and there is a definite need for more studies of P_{II} behavior under conditions of physiological transition because these could well be the situations where the influence of P_{II} modifications are most apparent.

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