P$_{II}$ signal transduction proteins: pivotal players in post-translational control of nitrogenase activity

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The fixation of atmospheric nitrogen by the prokaryotic enzyme nitrogenase is an energy-expensive process and consequently it is tightly regulated at a variety of levels. In many diazotrophs this includes post-translational regulation of the enzyme's activity, which has been reported in both bacteria and archaea. The best understood response is the short-term inactivation of nitrogenase in response to a transient rise in ammonium levels in the environment. A number of proteobacteria species effect this regulation through reversible ADP-ribosylation of the enzyme, but other prokaryotes have evolved different mechanisms. Here we review current knowledge of post-translational control of nitrogenase and show that, for the response to ammonium, the P$_{II}$ signal transduction proteins act as key players.

Introduction

Biological nitrogen fixation, the reduction of atmospheric N$_2$ to NH$_3$ by nitrogen-fixing bacteria, is a key step in the nitrogen cycle. This process is catalysed by nitrogenase, the most common form of which is the molybdenum nitrogenase, composed of dinitrogenase (MoFe protein or NifDK), an $\alpha_2\beta_2$ tetramer encoded by the nifD and nifK genes, respectively, and dinitrogenase reductase (Fe protein or NifH), a $\gamma_2$ homodimer encoded by the nifH gene. The NifH protein is responsible for ATP-hydrolysis-driven electron transport to the NifDK protein, which contains the site for the reduction of dinitrogen to ammonium (Seefeldt et al., 2009). The reduction of N$_2$ to two molecules of ammonium is an energy-expensive process requiring the hydrolysis of 16 ATPs.

To avoid energy wastage, diazotrophs have evolved both transcriptional and post-translational mechanisms to shut-down nitrogen fixation when ammonium is available in the environment. Post-translational control of nitrogenase activity has been found in a range of diazotrophs and affords a rapid and reversible mechanism by which the organism can respond to transient changes in the environment. Here we review current knowledge of the different mechanisms of post-translational control of nitrogenase. We focus on the two best-described systems: ADP-ribosylation of NifH, which occurs in proteobacteria, and the interaction of NifI regulatory proteins with NifDK in archaea, which potentially also operates in some anaerobic diazotrophic bacteria.

Regulation of nitrogenase activity by reversible ADP-ribosylation

Historical perspective

The process of metabolic inactivation of nitrogenase in response to ammonium was first described in Azotobacter vinelandii (Burris & Wilson, 1946); this phenomenon was later identified in other prokaryotes and named nitrogenase ‘switch-off’ (Zumft & Castillo, 1978). Different mechanisms are used to regulate nitrogenase post-translationally depending on the organism. The best-studied mechanism operates through reversible ADP-ribosylation of NifH (reviewed by Nordlund, 2000). This system responds to not only the presence of ammonium but also a decrease in the availability of cellular energy, in response to either darkness in phototrophs such as Rhodospirillum rubrum or anaerobiosis as seen in Azospirillum brasilense.

Researchers at the University of Wisconsin demonstrated that nitrogenase switch-off in R. rubrum occurred due to the addition of an ADP-ribosyl group to the arginine 101 residue of one subunit of the NifH protein (Pope et al., 1985). This residue is located in the docking site between NifH and NifDK during the electron transfer cycle of nitrogenase (Schindelin et al., 1997). ADP-ribosylation of this residue presumably disrupts the contact between the nitrogenase components, blocking the electron transfer reaction necessary to reduce N$_2$.

The enzymes involved in nitrogenase modification in R. rubrum were purified and characterized in vitro (Pope et al., 1986; Saari et al., 1986). ADP-ribosylation of NifH is
A BLASTX analysis identified 29 different bacterial genera constitute an operon and are presumably co-transcribed. Some years later, the same group cloned, sequenced and analysed the function of the structural genes for these enzymes, draT and draG, from R. rubrum (Fitzmaurice et al., 1989).

Distribution of the nitrogenase ADP-ribosylation system in prokaryotes

The draTG genes have also been sequenced and characterized in A. brasilense (Zhang et al., 1992), Azospirillum lipoforum (Fu et al., 1990b; Inoue et al., 1996), Rhodobacter capsulatus (Masepohl et al., 1993) and in Azoarcus sp. (Oetjen & Reinhold-Hurek, 2009). The genes usually constitute an operon and are presumably co-transcribed.

A BLASTX analysis identified 29 different bacterial genera coding for DraT orthologues, including members of the alpha-, beta-, gamma- and deltaproteobacteria, verrucomicrobia, deferribacteres, chysiogenetes and some unclassified bacteria (Table 1). DraG orthologues are widespread in nature (see below). As all the species listed in Table 1 also have NifH orthologues, we predict that these organisms regulate nitrogenase through ADP-ribosylation. In most cases, draT and draG are adjacent to each other; in some organisms like Azoarcus sp. they are approximately 6 kb apart. draT is usually located near the nitrogenase structural genes or genes involved in nitrogenase co-factor biosynthesis.

Protein ADP-ribosylation has been reported to perform a variety of functions, and genes coding for ADP-ribosyltransferases are found in organisms ranging from viruses to humans. DraT is classified as part of the R-S-E ‘cholera toxin-like’ group of ADP-ribosyl-transferases, in which R-S-E identifies a conserved catalytic triad (Hottiger et al., 2010). However, DraT has low sequence similarity to other members of the R-S-E group and should therefore be considered as a particular subgroup. In contrast, DraG belongs to the ADP-ribosyl-hydrolase family, and shares a much higher sequence and structural similarity to all members of this family, including orthologues in archaea, bacteria and eukarya (Koch-Nolte et al., 2008). DraG orthologues are widely distributed in prokaryotes, including organisms that do not encode DraT or NifH proteins (Oetjen & Reinhold-Hurek, 2009), suggesting that these orthologues have evolved different functions.

Table 1. Organisms with DraT orthologues

The A. brasilense DraT was used as a query to search orthologues in the NCBInr database using BLASTX. All hits reported have an E-value <3 × 10⁻²¹.

<table>
<thead>
<tr>
<th>Alphaproteobacteria</th>
<th>Deltaproteobacteria</th>
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<tr>
<td>Azospirillum brasilense</td>
<td>Anaeromyxobacter sp. Fw109-5</td>
</tr>
<tr>
<td>Azospirillum lipoforum</td>
<td>Anaeromyxobacter sp. K</td>
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<td>Desulfobacterium autotrophicum HRM2</td>
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<td>Geobacter bemidientsi Bem</td>
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<td>Geobacter lovleyi SZ</td>
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<td>Geobacter metalloreducens GS-15</td>
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<td>Betaproteobacteria</td>
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<td>Sideroxydans lithotrophicus ES-1</td>
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<td>Rubrivivax benzoylactylus JA2</td>
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<td>Chrysiogenetes</td>
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<td>Desulfitospirillum indicum S5</td>
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The regulation of DraT and DraG activities

DraT and DraG activities are subject to opposing regulation in vivo according to the prevailing nitrogen or energy levels (Kanemoto & Ludden, 1984; Liang et al., 1991; Zhang et al., 1993). The model proposed by the Wisconsin group (reviewed by Zhang et al., 1997) suggested that under nitrogen-fixing conditions, DraT is inactive and DraG is active. When ammonium is added or when the energy levels decrease, DraT becomes active and DraG is inactivated; DraT remains active for a short period while DraG remains inactive until consumption of the ammonium by bacterial metabolism. After ammonium exhaustion or restoration of the energy levels, DraG is reactivated to restore nitrogenase activity (Fig. 1). The same regulation model was assumed to occur in darkness-induced nitrogenase switch-off.

The major question posed by this model was to understand how the signal of the prevailing environmental ammonium or cellular energy levels could be transduced to change DraT and DraG activities. The signalling pathway is likely to be similar in different organisms, as demonstrated by the fact that A. brasilense draTG genes in draTG mutants of R. rubrum re-establish NifH ADP-ribosylation in response to ammonium or energy depletion and vice versa (Zhang et al., 1995). Furthermore, heterologous expression of dra genes in organisms that do not regulate nitrogenase by ADP-ribosylation led to regulated ammonium nitrogenase switch-off (Fu et al., 1990a; Inoue et al., 1996; Yakunin et al., 2001). Hence, the ammonium-sensing components of the system were likely to be part of a ubiquitous bacterial nitrogen control system.

Role of PII and AmtB proteins in the ammonium-elicited nitrogenase ADP-ribosylation pathway

PII protein function

Nitrogen metabolism in prokaryotes is regulated by a highly conserved family of trimeric proteins named PII (Forchhammer, 2008; Leigh & Dodsworth, 2007). Many prokaryotes encode two PII proteins, usually named GlnK and GlnB, and some organisms encode a third PII named GlnJ. The glnK genes are defined by their transcriptional association with the ammonium transporter gene, amtB. The glnB genes are usually linked to the glutamine synthetase (glnA) or the NAD⁺ synthetase (nadE) genes (Sant’Anna et al., 2009). There are some exceptions to these rules, for instance in A. brasilense the glnZ gene (orthologous to glnK) is not linked to amtB (de Zamaroczy, 1998). A different subgroup of PII is the NifI1 and NifI2 proteins, which are found in methanogenic archaea and in strictly anaerobic bacteria. The nifI genes are located in the vicinity of nitrogenase structural genes (Sant’Anna et al., 2009).

PII proteins bind small molecules such as ATP, ADP and 2-oxoglutarate (2-OG), known as the PII effectors. ATP and ADP bind to the same site in the lateral clefts between each subunit of the PII trimers in a competitive manner (Xu et al., 1998, 2001; Jiang & Ninfa, 2007). Each trimer can bind up to three adenine nucleotide molecules, thus the in vivo population of PII proteins can have different combinations of bound ATP/ADP (Jiang & Ninfa, 2009; Jiang & Ninfa, 2007). Three 2-OG binding sites are also located in the lateral clefts between each subunit of PII (Truan et al., 2010; Fokina et al., 2010).

Many PII proteins are subject to reversible covalent modification in a flexible loop, the T-loop, that projects from the signal of the prevailing environmental ammonium or energy levels to the cellular energy status, and 2-OG availability. The intracellular glutamine pool. High intracellular glutamine indicates high nitrogen levels and triggers de-uridylylation of PII. Conversely, under low nitrogen, glutamine levels are reduced and PII is uridylylated (Jiang et al., 1998).

The signal transduction function of PII depends on conformational changes determined by its post-translational modification status and the effectors that are bound to the three lateral clefts of the trimer. The intracellular availability of each PII effector defines the prevailing conformation of PII and thereby regulates its interaction with a variety of different PII target proteins. The balance between ATP/ADP signals the cellular energy status, and 2-OG availability signals not only the cellular carbon status but also nitrogen availability, because 2-OG is a key intermediate in ammonium assimilation.

It is believed that the most ancient protein target regulated by PII proteins is the ammonium transport protein AmtB (Javelle & Merrick, 2005). Some prokaryotes encode more than one Amt protein, and in nearly all cases amt genes are co-transcribed with PII coding genes (Sant’Anna et al., 2009). AmtB is a trimeric integral membrane protein with each monomer forming a membrane-spanning pore which conducts ammonia (Khademi et al., 2004). Depending on the uridylylation status of PII, and the intracellular levels of ATP, ADP and 2-OG, PII can form a complex with AmtB on the membrane, the T-loop of PII acting as a plug to block ammonia flux through the transporter (Conroy et al., 2007; Gruswitz et al., 2007; Radchenko et al., 2010). Interaction between Amt and PII is widespread in prokaryotes and has been described in several bacteria and also in archaeal species (Tremblay & Hallenbeck, 2009).

Genetic evidence identified PII and AmtB proteins as part of the ammonium signalling pathway

Given the key role of PII proteins in nitrogen metabolism, these proteins were obvious candidates to control DraT and DraG activities. The first indication that PII could regulate DraT and DraG was reported in R. capsulatus where a glnB mutant showed defective ammonium-induced nitrogenase inactivation (Hallenbeck, 1992).
Further studies supported the participation of P II proteins in the regulation of DraT/DraG activities in *R. rubrum* (Zhang *et al.*, 2001b), *A. brasilense* (Klassen *et al.*, 2001, 2005; Huergo *et al.*, 2005), *R. capsulatus* (Drepper *et al.*, 2003) and *Azoarcus* sp. (Martin & Reinhold-Hurek, 2002). All these studies reported defects in nitrogenase switch-off and/or ADP-ribosylation in P II or *glnD* mutant backgrounds.

An important breakthrough in the field was the observation that the *amtB* gene was necessary for ammonium-induced ADP-ribosylation of nitrogenase in *R. capsulatus* (Yakunin & Hallenbeck, 2002). Ammonium uptake was not affected in the *amtB* mutant owing to the presence of alternative uptake pathways, and hence it was suggested that AmtB might play a role in the ammonium sensing mechanism (Yakunin & Hallenbeck, 2002). The same response was later observed in other organisms.

The ammonium regulation model in *A. brasilense*

*A. brasilense* encodes only two P II proteins (GlnB and GlnZ) and one Amt protein (AmtB), and therefore provides a simpler genetic background than *R. capsulatus* and *R. rubrum* in which to study the role of P II and Amt proteins in NiFH ADP-ribosylation. Below we describe the current knowledge of the ammonium signalling pathway in this organism and then discuss how the resulting model can be applied to other bacteria.

The first insight into the ammonium signalling pathway in *A. brasilense* came from the observation that *ntrBC* mutant
strains do not ADP-ribosylate NifH in response to ammonium (Zhang et al., 1994). In this organism, $\text{amtB}$, $\text{glnZ}$ and $\text{glnB}$ are all under NtrC transcriptional control (de Zamaroczy et al., 1993; de Zamaroczy, 1998; van Dommelen et al., 1998; Huergo et al., 2003), suggesting that alteration in the expression of these genes could be the cause of the observed phenotype in an ntrC mutant. A $\text{glnZ}$ mutant showed only partial recovery of ammonium-induced switch-off (Klassen et al., 2001) and NifH modification persisted for a longer period after ammonium addition (Huergo et al., 2006b). A $\text{glnB}$ mutant showed neither ammonium-induced nitrogenase switch-off (Klassen et al., 2001) nor NifH ADP-ribosylation (Huergo et al., 2006b), whilst GlnB overexpression resulted in NifH modification under nitrogen-fixing conditions (Huergo et al., 2005). These results supported the hypothesis that GlnB and GlnZ might be involved in the regulation of DraT and DraG activities, respectively.

As had been shown previously in $E.\ coli$ (Coutts et al., 2002), P$_II$ proteins in $A.\ brasilense$ are sequestered to the cytoplasmic membrane in response to an ammonium shock in an AmtB-dependent fashion (Huergo et al., 2006b). A time-course analysis of GlnB/GlnZ uridylylation, membrane sequestration and NifH ADP-ribosylation cycles in response to ammonium showed that these processes are synchronized (Huergo et al., 2006b). An $A.\ brasilense$ $\text{amtB}$ mutant did not ADP-ribosylate NifH in response to ammonium, but showed wild-type GlnB/GlnZ uridylylation cycles in response to ammonium, suggesting that ammonium uptake was not affected in the absence of AmtB. However, as expected, the $\text{amtB}$ mutant failed to sequester GlnB and GlnZ to the membrane, supporting the notion that this event could be part of the NifH modification signalling pathway (Huergo et al., 2006b).

Cellular localization studies showed that DraG behaves exactly as the P$_II$ proteins do, in that upon an ammonium shock it is also reversibly associated with the membrane in an AmtB-dependent manner. The movement of DraG to the membrane is synchronized with NifH modification, supporting the idea that membrane binding could inactivate DraG after ammonium addition (Huergo et al., 2006b). This model would explain why NifH is not modified in an $\text{amtB}$ mutant, as DraG remains in the cytosol and thus active after ammonium addition. So why is membrane sequestration of DraG AmtB-dependent? One possibility is that DraG interacts with AmtB indirectly through the formation of a ternary complex involving a P$_II$ protein.

To study whether DraT and/or DraG could interact with P$_II$ in vivo, strains expressing His-tagged versions of these enzymes were constructed and used to perform pull-down experiments in which cells were collected under nitrogen-fixing conditions and after an ammonium shock (Huergo et al., 2006a). DraT interacted with de-uridylylated GlnB, and DraG interacted with both uridylylated and de-uridylylated GlnZ, though the DraG–GlnZ interaction was stronger with non-uridylylated GlnZ (Huergo et al., 2006a). The roles of the P$_II$ effectors and P$_II$ uridylylation in the regulation of DraT–GlnB or DraG–GlnZ complex formation were studied in vitro. Both complexes were more stable when P$_II$ was de-uridylylated and when the ATP/ADP ratio and the 2-OG concentrations were low (Huergo et al., 2009). The same response was observed for the AmtB–GlnB and AmtB–GlnZ complexes. A ternary complex between AmtB–GlnZ–DraG could be formed in vitro in the presence of ADP, explaining the role of AmtB in DraG sequestration to the membrane after an ammonium shock (Huergo et al., 2007).

Based on metabolomics data from $E.\ coli$, it is expected that the 2-OG concentrations will drop and that the glutamine levels will increase seconds after ammonium addition to a nitrogen-starved culture (Yuan et al., 2009; Radchenko et al., 2010). The increase in glutamine concentration promotes de-uridylylation of GlnB and GlnZ, catalysed by GlnD (Araújo et al., 2008), whilst the drop in 2-OG levels facilitates the exchange of ATP for ADP in the nucleotide-binding sites of the P$_II$ proteins (L. F. Huergo, unpublished data), thereby stabilizing the DraT–GlnB and the DraG–GlnZ–AmtB complexes (Huergo et al., 2009) (Fig. 2). In this scenario, DraT is activated by binding to de-uridylylated ADP-bound GlnB, and DraG is inactivated by binding to the membrane-bound AmtB–GlnZ complex (Fig. 2) (Huergo et al., 2009).

According to this model (Fig. 2), DraG would be inactivated when bound to the AmtB–GlnZ complex on the membrane, and hence DraG should not be inactivated in a glnB mutant. Surprisingly, NifH ADP-ribosylation upon ammonium addition was observed in this mutant despite the fact that DraG was not sequestered to the membrane (Huergo et al., 2006b). It is possible that, in the absence of GlnZ, GlnB could partially substitute for GlnZ function. Indeed, GlnB and GlnZ are 67 % identical in sequence and the structure of the DraG–GlnZ complex shows that only three of the nine GlnZ amino acid residues involved in polar contacts with DraG differ in GlnB (Rajendran et al., 2011).

Structural analysis of the DraG–GlnZ complex revealed that upon GlnZ binding, the active site of DraG would be spatially hindered from binding to ADP-ribosylated NifH. However, a simple model where formation of a DraG–GlnZ complex would inactivate DraG is not supported by the lack of DraG regulation in an $\text{amtB}$ mutant, indicating that DraG inactivation requires the membrane-binding event. Our current hypothesis is that the DraG–GlnZ complex is not stable enough to effectively inactivate DraG; however, the binding of the DraG–GlnZ complex to AmtB further stabilizes the DraG–GlnZ interaction and provides effective DraG inactivation by steric hinderance of the active site (Rajendran et al., 2011).

Previous studies using purified $R.\ rubrum$ DraT and DraG showed that both enzymes are active in vitro, suggesting that the enzymes would be regulated by loosely bound negative effectors in vivo (Saari et al., 1986; Lowery & Ludden, 1988). Whilst our model supports the proposal that DraG is negatively regulated, we suggest that DraT is
activated by interaction with de-uridylylated ADP-bound GlnB. Interestingly, SDS-PAGE analysis of purified DraT from R. rubrum indicated a contaminant band with the molecular mass expected for a P II monomer. The authors also noted that attempts to remove this contaminant resulted in loss of DraT activity. Furthermore, DraT was only active if ADP was present in all purification steps, which, according to our model, would stabilize the active DraT–GlnB complex (Lowery & Ludden, 1988).

**Ammonium regulation in R. rubrum**

R. rubrum has three PII and two AmtB coding genes, named glnB, glnKamtb1 and glnJamtB2; the latter two pairs are organized in operons (Zhang et al., 2001b).

Ammonium-induced NifH ADP-ribosylation analysis indicated a normal response in glnB, glnK and glnBK mutants, but little modification was seen in glnBJ and glnBKJ mutants, suggesting that GlnB or GlnJ are necessary for the correct regulation of DraT and DraG (Zhang et al., 2001). Expression of a constitutively de-uridylylated GlnB (Y51F mutation) led to DraT activation under nitrogen-fixing conditions (Zhang et al., 2000), and DraT–GlnB interaction was detected using the yeast two-hybrid system (Zhu et al., 2006). These data are consistent with the A. brasilense model (Fig. 2) where DraT is activated by forming a complex with de-uridylylated ADP-bound GlnB.

![Fig. 2. Role of the AmtB and P II proteins in the regulation of DraT and DraG activities in response to ammonium. (a) Under nitrogen-fixing conditions GlnB and GlnK are fully uridylylated, located in the cytoplasm and presumably saturated with ATP and 2-OG (denoted by +), therefore are not complexed to DraT and DraG. Under this condition, DraT is inactive and DraG is located in the cytoplasm and active; consequently NifH is not modified, allowing nitrogenase activity. (b) Upon an ammonium shock, ammonium assimilation by the glutamine synthetase/glutamate synthase increases the intracellular glutamine and decreases the 2-OG pools. The rise in glutamine activates the uridylyl-removing activity of GlnD, thus GlnB and GlnK are de-uridylylated. The decrease in the 2-OG pool facilitates the exchange of ATP bound to P II to ADP; consequently, GlnB and GlnK are bound to ADP (denoted by *). GlnK* binds avidly to both AmtB and DraG blocking the ammonium transport and promoting DraG inactivation. At the same time, DraT is activated by interaction with de-uridylylated ADP-bound GlnB.](http://mic.sgmjournals.org)
required the presence of AmtB1 and GlnJ or GlnB (Wang et al., 2005; Zhang et al., 2006). Similarly, NifH ADP-ribosylation in this bacterium required the presence of those proteins. *In vitro* assays showed that all three PΠ proteins interact with AmtB1-containing membranes though the interaction was stronger with GlnJ and was stabilized by low ATP/ADP ratio and low 2-OG concentrations (Wolfe et al., 2007; Teixeira et al., 2008). Cross-linking experiments using membrane preparations of *R. rubrum* and purified DraG showed the presence of an adduct cross-reacting with DraG, AmtB and PΠ antibodies (Akentieva, 2008), suggesting that *R. rubrum* DraG is targeted to the membrane via the AmtB1–GlnJ complex and, similarly to the model proposed for *A. brasilense*, it is thereby inactivated (Fig. 2).

The crystal structure of *R. rubrum* DraG was solved in both unbound and ADP-ribose-bound forms (Berthold et al., 2009). This work revealed the structure of a putative reaction intermediate analogue and suggested a mechanism of catalysis for ADP-ribose removal (Berthold et al., 2009). The structure of *R. rubrum* DraG is very similar to the *A. brasilense* orthologue (Li et al., 2009), further supporting that they share similar regulatory mechanisms.

**Ammonium regulation in *R. capsulatus***

*R. capsulatus* has two PΠ proteins encoded by *glnB*, which is co-transcribed with the glutamine synthetase gene (*glnA*), and *glnK*, which is co-transcribed with *amtB* (Zinchenko et al., 1994; Drepper et al., 2003). A *glnB*Δ*kn* double mutant failed to inactivate and ADP-riboseylate NifH upon an ammonium shock (Drepper et al., 2003). Later, it was shown that both PΠ proteins were necessary for the proper regulation of ammonium-induced nitrogenase switch-off and NifH ADP-ribosylation (Tremblay et al., 2007). Furthermore, interactions between the two PΠ proteins and DraT were detected by yeast two-hybrid analysis (Pawlowski et al., 2003).

Expression of a non-uridylylatable form of GlnK (Y51F mutant) in the *glnK* mutant was sufficient to re-establish ammonium-induced NifH ADP-ribosylation, suggesting that GlnK uridylylation is not critical to its function (Tremblay et al., 2007). This is consistent with the *in vitro* analysis of the GlnZ–DraG, GlnB–DraT interactions in *A. brasilense* where the major regulators of these interactions were shown to be the ATP/ADP ratio and 2-OG levels rather than PΠ uridylylation (Huergo et al., 2009). Both *R. capsulatus* GlnB and GlnK are sequestered to the membrane after an ammonium shock in an AmtB-dependent manner (Tremblay et al., 2007). Interestingly, *amtB* is required for ammonium-induced NifH ADP-ribosylation but not for ammonium uptake (Yakunin & Hallenbeck, 2002), and AmtB variants that do not form a complex with GlnK fail to complement NifH ADP-ribosylation in the *amtB* mutant (Tremblay & Hallenbeck, 2008). All these data support the concept that movement of PΠ to the membrane is critical for NifH regulation, presumably to inactivate DraG by membrane sequestration as proposed in *A. brasilense* (Fig. 2).

Strikingly, transport-incompetent AmtB variants that form the AmtB–GlnK complex upon an ammonium shock fail to properly regulate nitrogenase. This result was interpreted as an indication that ammonia transport and/or the occupation of the AmtB pore by ammonia could be essential for nitrogenase regulation (Tremblay & Hallenbeck, 2008). It is possible that ammonia transport through AmtB, whose mechanism is still under debate, could alter the intracellular levels of metabolites such as ATP/ADP, thus eliciting the protein–protein interactions required for NifH ADP-ribosylation.

**Ammonium regulation in *Azoarcus* sp. strain BH72**

*Azoarcus* sp. strain BH72 has one DraT gene and two unlinked draG genes, and the nitrogenase NifH protein is subject to ADP-ribosylation in response to either ammonium addition or oxygen deprivation (Oetjen & Reinhold-Hurek, 2009). However, whilst a DraT mutant fails to ADP-riboseylate NifH, it still shows nitrogenase switch-off in response to either ammonium addition or anaerobiosis. Hence, nitrogenase switch-off is independent of ADP-ribosylation and is presumed to be mediated by a second unknown mechanism (Oetjen & Reinhold-Hurek, 2009). *Azoarcus* sp. strain BH72 has three PΠ and two AmtB-coding genes – *glnB*, *glnKamtB* and *glnYamtY* – the latter two pairs are organized in operons (Martin et al., 2000). Ammonium-induced nitrogenase switch-off requires AmtB and GlnK, but not GlnB or GlnY, whilst NifH ADP-ribosylation requires the presence of GlnB, GlnK and AmtB (Martin & Reinhold-Hurek, 2002).

Surprisingly, GlnK was found associated with the membrane irrespective of the nitrogen status of the cell, and PΠ membrane binding also occurred independently of AmtB (Martin & Reinhold-Hurek, 2002). As already discussed, in all other organisms studied, PΠ interaction with the membrane is controlled by nitrogen levels and is AmtB-dependent. Hence, despite the fact that in *Azoarcus* sp. strain BH72 PΠ and AmtB participate in both metabolic nitrogenase inactivation and NifH ADP-ribosylation, it is possible that this organism has evolved a distinct nitrogen signalling system.

**The elusive energy depletion ADP-ribosylation signalling pathway**

ADP-ribosylation of nitrogenase can be triggered by energy depletion, in response to either darkness in phototrophs such as *R. rubrum* or anaerobiosis as seen in *A. brasilense*. However, very little is known about the signalling mechanism responsible for regulation of DraT and DraG in response to energy depletion. Pioneer studies in *R. rubrum* indicated that DraG was located in the membrane fraction of the cells and could be removed by salt washes (Ludden & Burris, 1976). DraG purification used membrane
preparations as the starting material (Ljungström et al., 1989; Saari et al., 1984) and because large volumes were used, the cells were probably subjected to anaerobic/darkness conditions, and hence energy depletion, during cell harvesting. Conversely, when small volumes of A. brasilense are collected and quickly cooled, thereby avoiding energy depletion, DraG is predominantly found on the cytosol (Huergo et al., 2006b). These data led to the hypothesis that DraG is negatively regulated by membrane binding after energy depletion, though this has not been proved by systematic experiments.

In R. rubrum, either GlnB or GlnJ is necessary for darkness-induced NifH ADP-ribosylation (Zhang et al., 2001b). Analysis of nitrogenase activity in a glnD mutant suggested that DraT is activated in response to darkness but DraG is not reactivated upon exposure to light. Hence, DraG reactivation requires uridylylation of P II proteins (Zhang et al., 2001). An R. rubrum DraG N100K variant did not show inactivation by darkness (Kim et al., 2004), and as the structure of the A. brasilense DraG–GlnZ complex (Rajendran et al., 2011) showed that N100 is part of the P II interaction surface, this supports the hypothesis that DraG–P II interaction might play a role in darkness-induced switch-off.

Another study showed that in the absence of amtB1, R. rubrum had only a partial response to darkness (Zhang et al., 2006); analysis of an amtB1draG double mutant led to the conclusion that DraG is not properly inactivated in the amtB background. Western blot analysis revealed that in response to darkness, GlnJ and GlnB are de-uridylylated and GlnJ associates with the membrane in an AmtB-dependent manner (Zhang et al., 2006; Teixeira et al., 2010). The signal triggering P II protein de-uridylylation in response to darkness is not known, though a slight increase in glutamine levels has been reported (Kanemoto & Ludden, 1987).

The data reviewed so far support the proposal that darkness-induced NifH ADP-ribosylation in R. rubrum might share at least some of the same signalling mechanisms as ammonium-dependent inactivation (Fig. 2). However, this hypothesis is challenged by several sets of experimental data. In R. rubrum, neither GlnB nor GlnJ are de-uridylylated under darkness switch-off when cells are cultivated using N2 as nitrogen source instead of glutamate (Teixeira et al., 2010). Hence, as fully uridylylated P II cannot interact with AmtB (Rodrigues et al., 2011), AmtB–P II complex formation seems dispensable for the darkness response in R. rubrum (Teixeira et al., 2010). Furthermore, AmtB is not required for darkness-induced NifH ADP-ribosylation in R. capsulatus (Yakunin & Hallenbeck, 2002) or for anaerobically induced NifH ADP-ribosylation in A. brasilense (L. F. Huergo, unpublished data) and Azoarcus sp. strain BH72 (Martin & Reinhold-Hurek, 2002).

So, it is possible that distinct signalling mechanisms might operate to transduce the levels of ammonium and energy to the DraT/DraG system. The DraT and DraG enzymes have opposite specificities for NifH bound to MgADP or MgATP: DraT is more active against the MgADP–NifH form and DraG against the MgATP–NifH (Saari et al., 1984; Lowery & Ludden, 1988). Consequently, a decrease in the ATP/ADP ratio could lead to the energy depletion switch-off response. However, measurements suggest that darkness-induced ATP/ADP fluctuations are probably insufficient to account for the regulation (Paul & Ludden, 1984), and furthermore a reduction in the cellular ATP levels did not significantly affect the post-translational regulation of nitrogenase in R. rubrum (Zhang et al., 2009).

The activities of R. rubrum DraT and DraG are also affected by the redox status of NifH in opposing ways. DraT is more active against oxidized NifH whilst DraG is more active using reduced NifH as substrate (Halbleib et al., 2000a, b). Cellular GTP and NAD+ levels have also been suggested to regulate nitrogenase switch-off but neither of these signals has been subjected to a systematic evaluation (Norén & Nordlund, 1994, 1997; Norén et al., 1997; Halbleib & Ludden, 1999).

In conclusion, although some studies in R. rubrum suggest that AmtB and P II proteins participate in energy depletion switch-off, this dependence seems to occur only when cells are cultivated using glutamate but not using N2 as nitrogen source. In A. brasilense, the data available so far suggest that AmtB and P II are not involved in anaerobic switch-off.

**Alternative nitrogenase switch-off mechanisms in bacteria**

Whilst NifH ADP-ribosylation is the best characterized and probably the major ammonium-induced switch-off mechanism in bacteria, amongst the model organisms studied, it is only in R. rubrum that it appears to be the only form of nitrogenase inactivation. In that organism, substitution of the arginine residue at the ADP-ribosylation site of NifH by tyrosine (R101Y) abolishes nitrogenase inactivation (Zhang et al., 1996).

By contrast, ammonium-induced nitrogenase inhibition without detectable NifH ADP-ribosylation has been reported in many diazotrophs, including R. capsulatus (Pierrard et al., 1993), A. brasilense (Zhang et al., 1993), A. vinelandii (Laane et al., 1980), Azospirillum amazonense (Hartmann et al., 1986), Rhodobacter sphaeroides, Methylo sinus trichosporium (Yoch et al., 1988), Herbaspirillum seropedicae (Fu & Burris, 1989), Gluconacetobacter diazotrophicus (Burris et al., 1991), Pseudomonas stutzeri A1501 (Desnoues et al., 2003) and Azoarcus sp. strain BH72 (see above). Despite these observations, little is known about the mechanism(s) involved in these organisms. Several authors have suggested that the addition of ammonium could decrease the ATP pool and/or divert electrons from nitrogenase, thus reducing its activity. However, systematic studies to examine such hypotheses are lacking.

Studies of this phenomenon in model organisms are limited to date. R. capsulatus strains expressing mutant
forms of the NifH protein in which the ADP-ribosylation site (R102) was substituted, still showed nitrogenase inactivation in response to ammonium (Pierrard et al., 1993). In A. brasilense, the substitution of the equivalent arginine residue (NifH R101) by tyrosine, or knock out of the draT gene, also displayed partial ammonium-induced switch-off without NifH ADP-ribosylation (Zhang et al., 1993). In A. brasilense, neither P11 norAmtB seem to be necessary for ADP-ribosylation-independent switch-off, since an ntrC knockout strain still shows partial inactivation of nitrogenase in response to ammonium at levels comparable to that found in a draT mutant (Zhang et al., 1994).

In H. seropedicae, knockout of either glnK or amtB partially abolished ammonium-induced nitrogenase switch-off (Noindorf et al., 2006). In this organism, GlnK is sequestered to the cell membrane by AmkB a few minutes after ammonium addition (Huergo et al., 2010). These results suggest that the ADP-ribosylation-independent nitrogenase switch-off in H. seropedicae could be controlled by formation of an AmtB–GlnK complex in the membrane (Noindorf et al., 2011).

Post-translational control of nitrogenase activity in archaea by the NifI proteins

Metabolic inactivation of archaeal nitrogenase in response to ammonium was first described in Methanosarcina barkeri (Lobo & Zinder, 1988). The inactivation was reversible and dependent on the amount of ammonium added. However, no electrophoretic alteration in NifH migration was detected by Western blotting, suggesting a mechanism other than NifH ADP-ribosylation (Lobo & Zinder, 1990; Kessler et al., 2001).

Most studies on nitrogenase activity control in archaea have been performed in Methanococcus maripaludis. This organism has five P11 protein-coding genes, two of which, nifI1 and nifI2, are located between the nifH and nifD genes (Kessler & Leigh, 1999). Deletion of M. maripaludis nifI1 or nifI2 abolished reversible inactivation of nitrogenase by ammonium (Kessler et al., 2001), and the nitrogenase activity in cellular extracts of a ΔnifI1nifI2 mutant was higher than that in wild-type cell extracts. Addition of 2-OG increased nitrogenase activity in M. maripaludis wild-type, but not in ΔnifI1nifI2, cell extracts, and the intracellular levels decreased after an ammonium shock, supporting the hypothesis that 2-OG is sensed by NifI proteins which, in turn, regulate nitrogenase activity (Dodsworth et al., 2005).

The inhibitory effect of NifI1 and NifI2 on nitrogenase occurs via direct interaction of a NifI1/NifI2 heteromer with the nitrogenase NifDK component. This interaction is disrupted in the presence of 2-OG, which causes further oligomerization of the NifI1/NifI2 heteromer (Dodsworth & Leigh, 2006). The binding of the NifI1/NifI2 heteromer to NifDK prevents the association of the latter with NifH, thereby disrupting the nitrogenase electron transfer mechanism (Dodsworth & Leigh, 2007). Based on these results, a model for regulation of nitrogenase activity in M. maripaludis was proposed (Leigh & Dodsworth, 2007) (Fig. 3). Under nitrogen-fixing conditions, the intracellular concentration of 2-OG is high; 2-OG binds to NifI forming higher order NifI1/NifI2 oligomers, possibly a dodecamer, which cannot interact with NifDK. Upon ammonium shock, the intracellular 2-OG concentration drops; the NifI1/NifI2 heteromer is converted into a lower mass oligomer (possibly an hexamer), which interacts avidly with NifDK abrogating the interaction of the latter with NifH and resulting in nitrogenase inactivation (Fig. 3).

The occurrence of nifI genes adjacent to nif genes is observed in all nitrogen-fixing archaea and in several anaerobic bacteria including members of the gamma-proteobacteria, firmicutes, chlorobi and chloroflexi (Table 2). This distribution suggests that the nitrogenase regulatory mechanism described in M. maripaludis is probably wide-spread among diazotrophs (Dodsworth & Leigh, 2006).

The presence of nifI genes in both archaea and bacteria suggests an early origin of the nifI genes. Hence, the NifI1/NifI2-dependent nitrogenase control is likely to be the most ancient mode of nitrogenase post-translational regulation. An analysis of the molecular evolution of nif clusters carrying nifI1 and nifI2 showed that in the nif operons of the firmicute Heliovibacterium chlorum and of the deltaproteobacterium Desulfotobacterium hafniense, the nifI genes are located upstream of nifH. These organisms are placed in an intermediate position between organisms with nif operons carrying nifI genes downstream of the nifH1 gene (found in archaea and anaerobic bacteria) and those with nif operons lacking nifI (typically found in aerobic bacteria) (Enkh-Amgalan et al., 2006).

Phylogenetic analysis of the nifDK operon confirmed that the nifDK genes from deltaproteobacteria and Heliovibacterium are indeed placed between the group of organisms including archaea and anaerobic bacteria listed in Table 2 and those proteobacteria carrying draT listed in Table 1 (Hartmann & Barnum, 2010). Interestingly, some deltaproteobacteria contain nifI orthologues (Table 2), while others contain draT orthologues (Table 1) and Desulfotobacterium autotrophicum contains both nifI and draT. It is therefore tempting to speculate that DraT/DraG nitrogenase regulation evolved in an anaerobic proteobacteria ancestor containing nifI which was lost during the evolution to aerobic nitrogen fixation, while draT was kept in the proteobacteria lineage as a result of vertical inheritance combined with gene loss and/or lateral gene transfer. One apparent advantage of DraT/DraG over NifI is that the former system can also respond to other signals such as the cellular energy levels. Further extensive phylogenetic analysis is necessary to clarify the evolution of these nitrogenase regulatory systems.
Nitrogenase post-translational modification in cyanobacteria

Analysis of the electrophoretic migration of NifH in cyanobacteria revealed that it could be resolved into two bands, and the larger form was assumed to be modified, possibly by ADP-ribosylation. However, systematic studies using $^{32}$P in vivo labelling and anti-ADP-ribose antibodies showed that the larger NifH band is not ADP-ribosylated in *Anabaena variabilis* or *Gloeoece* (Durner et al., 1994; Gallon et al., 2000). Furthermore, draT orthologues have not been found in any cyanobacteria to date. The appearance of the larger form of NifH was shown to be regulated by light in *Synechococcus* sp. and was inversely correlated with nitrogenase activity, suggesting that it could be a modified inactive form of NifH (Chow & Tabita, 1994). Incubation of cultures of *Gloeoece* with $^3$H-labelled palmitic acid resulted in the accumulation of radioactivity in the larger form of NifH, suggesting palmitoylation; however, the functional significance of this modification was not investigated (Gallon et al., 2000). Furthermore, draT orthologues have not been found in any cyanobacteria to date.

Two isoforms of NifH were present in 2D gels of an unidentified cyanobiont of *Azolla*, and MS analysis suggested that one NifH isoform is modified, by an unidentified group, in the same portion of NifH that is subjected to ADP-ribosylation in proteobacteria. Thus, it is likely that the modified NifH represents an inactive form of the protein (Ekman et al., 2008). Three isoforms of NifH were identified in 2D gels of the *Nodularia spumigena*, two lighter forms with different pIs and one heavier form. The putative modification of the lighter form varied under light and darkness regimes but did not vary in response to different nitrogen regimes. The heavier form appeared to be constantly modified and membrane-bound; the nature and the function of the modifications remains unknown (Vintila et al., 2011). More studies are necessary to determine the functional significance of these reported NifH modifications and whether PII proteins participate in the regulation of nitrogenase activity in cyanobacteria.

Concluding remarks

Several nitrogen-fixing prokaryotes have evolved mechanisms to reversibly inactivate nitrogenase when the environmental conditions are not favourable for nitrogen fixation. These mechanisms are diverse and include (i) covalent modification of NifH in proteobacteria, (ii) interaction of the NifI inhibitory proteins with NifDK in archaea and possibly also in other anaerobic bacteria, and (iii) other unknown mechanisms that have been reported in diazotrophs. Such diversity suggests that these mechanisms appeared independently during evolution, supporting the concept that nitrogenase post-translational regulation prevents unnecessary use of ATP to drive N$_2$ reduction when ammonium is available, thereby offering a metabolic advantage. Despite this diversity, PII proteins, which are now recognized as key signal transduction proteins in the regulation of prokaryotic nitrogen metabolism, are emerging as the universal regulators of ammonium-induced nitrogenase inactivation in those systems where it has been well studied.
Table 2. Organisms with nifI orthologues adjacent to the nitrogenase structural genes

Organisms carrying nifI orthologues adjacent to the nitrogenase structural genes were retrieved from the paper by Sant’Anna et al. (2009) and also by using the String 9.0 gene neighbourhood analysis tool (http://string-db.org).

<table>
<thead>
<tr>
<th>Archaea</th>
<th>Firmicutes</th>
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<tbody>
<tr>
<td>Methanobacterium ivanovii</td>
<td>Alkaliphilus metalliredigenes</td>
</tr>
<tr>
<td>Methanococcus aeolicus Nankai-3</td>
<td>Caldicellulosiruptor saccharolyticus</td>
</tr>
<tr>
<td>Methanococcus maripaludis S2</td>
<td>Clostridium acetobutylicum ATCC 824</td>
</tr>
<tr>
<td>Methanococcus maripaludis C5</td>
<td>Clostridium beijersnickii</td>
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<tr>
<td>Methanococcus maripaludis C7</td>
<td>Clostridium beijersnickii NCIMB 8052</td>
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<td>Methanococcus vannielli SB</td>
<td>Clostridium beijersnickii NCIMB 8052</td>
</tr>
<tr>
<td>Methanosarcina acetivorans C2A</td>
<td>Clostridium kluyveri DSM 555</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>Desulfitobacterium hafniense Y51</td>
</tr>
<tr>
<td>Methanosarcina Barkeri str. Fusaro</td>
<td>Desulfitobacterium hafniense DCB-2</td>
</tr>
<tr>
<td>Methanothermobacter marburgensis</td>
<td>Desulfuraidus audaxviator</td>
</tr>
<tr>
<td>Methanothermococcus thermolithotrophicus</td>
<td>Helobacterium chlorum</td>
</tr>
</tbody>
</table>

Clorobi

| Chlorobaculum parvum | Clostridium dyhmostatum CaD3 |
| Chlorobium chlorochromatii CaD3 | Chlorobium phaeobacteroides BS1 |
| Chlorobium ferrooxidans | Chlorobium phaeovibrioides |
| Chlorobium tepidum TLS | Chlorobium limicola DSM 245 |
| Pelidictyon luteolum DSM 273 | Chlorobium phaeoclorovorans BS1 |
| Pelidictyon phaeolathratiforme BU-1 | Chlorobium tepidum TLS |
| Prosthecochloris aestuarii DSM 271 | Chlorobium phaeoaciditrophicus TLS |
| Prosthecochloris vibrioformis DSM 265 | Chlorobium tepidum TLS |

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