

# Interactions between P<sub>II</sub> proteins and the nitrogenase regulatory enzymes DraT and DraG in *Azospirillum brasilense*

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**Abstract** In *Azospirillum brasilense* ADP-ribosylation of dinitrogenase reductase (NifH) occurs in response to addition of ammonium to the extracellular medium and is mediated by dinitrogenase reductase ADP-ribosyltransferase (DraT) and reversed by dinitrogenase reductase glycohydrolase (DraG). The P<sub>II</sub> proteins GlnB and GlnZ have been implicated in regulation of DraT and DraG by an as yet unknown mechanism. Using pull-down experiments with His-tagged versions of DraT and DraG we have now shown that DraT binds to GlnB, but only to the deuridylylated form, and that DraG binds to both the uridylylated and deuridylylated forms of GlnZ. The demonstration of these specific protein complexes, together with our recent report of the ability of deuridylylated GlnZ to be sequestered to the cell membrane by the ammonia channel protein AmtB, offers new insights into the control of NifH ADP-ribosylation.

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**Keywords:** ADP-ribosylation; Nitrogenase; DraT; DraG; P<sub>II</sub> protein

## 1. Introduction

In some diazotrophs, including *Azospirillum brasilense*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum*, nitrogenase is post-translationally controlled by reversible ADP-ribosylation of the dinitrogenase reductase (NifH) homodimer [1]. In response to conditions unfavourable for nitrogenase activity, such as addition of ammonium to the medium, an ADP-ribose group from NAD<sup>+</sup> is covalently linked to an arginine residue in just one NifH subunit resulting in nitrogenase inactivation (switch-off) [2,3]. This process is catalyzed by dinitrogenase ADP-ribosyltransferase (DraT). When the added ammonium is exhausted by cellular metabolism, the ADP-ribose group is removed by dinitrogenase reductase activating glycohydrolase (DraG) leading to nitrogenase activation (switch-on). DraT and DraG activities are themselves regulated according to the cellular nitrogen status, but the signalling pathways that mediate this control have not been fully defined to date [1].

Several lines of evidence have indicated that proteins of the P<sub>II</sub> family and the ammonia channel protein AmtB are in-

involved in the regulation of DraT and DraG. P<sub>II</sub> proteins constitute a family of highly conserved trimeric proteins that act as sensors of cellular nitrogen status in prokaryotes and that are also present in plants [4]. Most prokaryotes encode at least two P<sub>II</sub> proteins, typically designated GlnB and GlnK, the latter being almost invariably encoded within the same operon as the ammonia channel protein AmtB [5]. *A. brasilense* encodes two P<sub>II</sub> proteins, GlnB and GlnZ, and the uridylylation/deuridylylation of both proteins are coordinated with NifH ADP-ribosylation [6]. GlnB is required for ammonium-induced NifH ADP-ribosylation, suggesting that GlnB is necessary for DraT activation [7], and GlnZ has been shown to be involved in reactivation of NifH [8]. *R. rubrum* encodes three P<sub>II</sub> proteins (GlnB, GlnJ and GlnK) and the absence of GlnB and GlnJ prevents modification of NifH in response to ammonium [9]. In *R. capsulatus*, the presence of AmtB [10] and either of its two P<sub>II</sub> proteins, GlnB or GlnK, [11] is required for ammonium-induced NifH modification; whilst in *Azoarcus* sp. all three proteins, GlnB, GlnK and AmtB, are required [12]. Interactions between P<sub>II</sub> proteins and DraT have been identified in *R. capsulatus* and *R. rubrum* using the yeast two-hybrid system.

In *E. coli*, AmtB sequesters deuridylylated GlnK to the cell membrane within seconds of the addition of ammonium to the extracellular medium [13,14]. Formation of the AmtB/GlnK complex inactivates AmtB and rapidly lowers the cytosolic GlnK pool [14]. This phenomenon has been shown in a number of bacteria and is potentially a common property of AmtB [15]. We have shown that, in *A. brasilense*, ammonium addition causes membrane sequestration in a manner co-ordinate with NifH modification, suggesting that DraG membrane association causes its inactivation [6]. The ammonium-induced membrane binding of DraG requires AmtB, GlnB and GlnZ. However the precise roles of GlnB and GlnZ were unclear due to the marked pleiotropic effects of the respective mutants [6]. DraG localization to the membrane in a GlnJ and AmtB-dependent manner also occurs in *R. rubrum* [16]. These studies suggest a means by which DraG could be regulated, but the question remains as to what triggers DraT activity in response to a rise in extracellular ammonium. Hence although genetic analysis has suggested a role for P<sub>II</sub> proteins in the regulation of DraT and/or DraG activities, these observations could not distinguish between direct and indirect effects of the various mutations in P<sub>II</sub>-encoding genes. In order to clarify the situation we have now used pull-down experiments to investigate whether DraT and/or DraG interact with GlnB and/or GlnZ in vivo in *A. brasilense*.

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## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*A. brasilense* strains FAJ310, *amtB::km* [17], UB2, *draT::km* and UB4, *draG::km* [18] and plasmids pLHdraThisGMP, *ptac* 6HisdraT, *draG* and pLHdraGhisMP, *ptac* 6His *draG* [19] have all been described earlier.

### 2.2. Media and growth conditions

Growth of *A. brasilense* and preparation of cell extracts was as described by Huergo et al. [6].

### 2.3. Electrophoresis and Western blotting

This was performed as described by Huergo et al. [6]. Signals on the X-ray film were quantified using the LabWorks program (UVP).

### 2.4. In vivo interactions between DraG or DraT and GlnB or GlnZ

Control cells and cells expressing His-DraT (from plasmid pLHdraThisGMP) or His-DraG (from plasmid pLHdraGhisMP), were grown under nitrogen fixing conditions as described [6]. A 30 ml aliquot of a 110 ml nitrogenase derepressed culture was withdrawn before, or 5 min after, the addition of 200  $\mu$ M NH<sub>4</sub>Cl; cells were immediately cooled in liquid nitrogen (10 s) and harvested by centrifugation (5000  $\times$  g for 5 min at 4 °C). The cell pellet was re-suspended in 1 ml of binding buffer (40 mM K<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 5 mM imidazole, pH 7.2), sonicated, and clarified by centrifugation (16000  $\times$  g for 10 min at 4 °C). His-tagged proteins were pulled-down from the cell extract using His Mag™ Agarose Beads (Novagen) following the manufacturers' instructions. The imidazole concentration was 15 mM in the wash buffer and 500 mM in the elution buffer. Samples were analysed by Western blotting with appropriate antibodies.

## 3. Results

### 3.1. DraT interacts with deuridylylated GlnB

Cell extracts were prepared from nitrogenase derepressed cells before, and 5 min after, addition of 200  $\mu$ M NH<sub>4</sub>Cl to the external medium. The strains used were UB2 (*draT::km*) as a negative control, and UB2 carrying plasmid pLHdraThisGMP that expresses both His-DraT and native DraG from the *ptac* promoter [20]. In cells carrying pLHdraThisGMP, His-DraT is functional [19] and NifH is normally regulated in response to ammonium [6] (Fig. 1A). To avoid any potential titration effect of a regulator of DraT and/or DraG activities expression from *ptac* was not induced.

His-DraT was pulled-down from cell-free extracts using His-Mag™ agarose beads and was recovered only in the elution fraction of cells carrying the His-DraT plasmid (Fig. 1B). GlnZ was not detected in any elution fractions (Fig. 1C) suggesting that DraT does not interact with GlnZ in vivo. However, GlnB was detected in the elution fraction of cells expressing His-DraT and subjected to an ammonium shock (Fig. 1D). We conclude that DraT interacts with GlnB in vivo and that the interaction is controlled by the cellular nitrogen status. Native electrophoresis showed that GlnB was almost fully deuridylylated after the ammonium shock (Fig. 1E), suggesting that DraT only interacts with deuridylylated GlnB.

### 3.2. DraG interacts with GlnZ independently of the GlnZ uridylylation status

Experiments to investigate possible interactions of DraG with GlnZ and/or GlnB were performed using cell extracts of strains UB4 (*draG::km*) as a negative control, and UB4 carry-

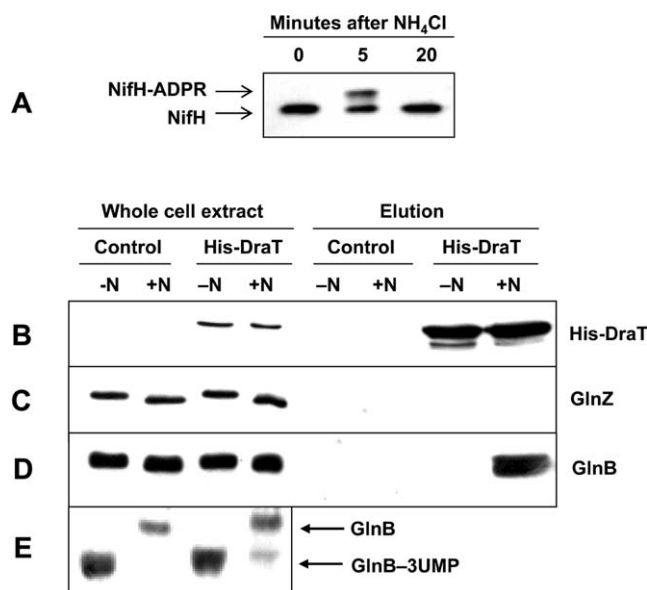


Fig. 1. DraT interacts with deuridylylated GlnB in vivo. Strain UB2(pLHThisGMP) expressing His-DraT were subjected to an ammonium shock at time zero. Samples were collected at 0, 5 and 20 min after addition of 200  $\mu$ M NH<sub>4</sub>Cl and analysed by SDS-PAGE and Western blotting using anti-NifH antibody (A). For His-DraT pull-down experiments, samples from strain UB2(pLHThisGMP), with strain UB2 (*draT::km*) as control, were collected before (–N) and 5 min after (+N) addition of 200  $\mu$ M NH<sub>4</sub>Cl and analysed by SDS-PAGE followed by Western blotting using anti-DraT (B), anti-GlnZ (C), or anti-GlnB antibodies (D). The uridylylation state of GlnB was analysed by native-PAGE of whole cell extracts revealed with anti-GlnB antibodies (E).

ing pLHdraGhisMP. This plasmid expresses His-DraG from the *ptac* promoter but again, expression from *ptac* was not induced. His-DraG is functional in vivo [19] and under the conditions used NifH was normally regulated in response to ammonium (Fig. 2A).

His-DraG was only pulled-down from extracts of cells carrying the His-DraG plasmid (Fig. 2B). In contrast to the pull-down of His-DraT, GlnB was not detected in any of the elution fractions (Fig. 2D), suggesting that DraG does not interact with GlnB in vivo. However the GlnZ protein was detected in the elution fraction of cells expressing His-DraG, though only before the ammonium shock (Fig. 2C), indicating that GlnZ can interact with DraG in vivo. The signal for DraG in fractions from cells collected after ammonium addition was significantly lower than that from –N cells (the signal in +N was 28% of that in –N) and this was reproducible (Fig. 2B). We have previously shown that DraG is sequestered to the membrane in an AmtB-dependent manner after an ammonium shock and is almost completely membrane-associated 5 min after addition of 200  $\mu$ M ammonium [6]. Therefore a possible explanation for reduction in the DraG signal after ammonium addition is that the membrane-associated His-DraG is less accessible to pull-down by His-Mag™ agarose beads.

To avoid ammonium-induced DraG membrane association, and thereby potentially increase the amount of DraG available to bind to the beads, we repeated the experiment described above using an *amtB* mutant strain. The strains used were FAJ310 (*amtB::km*) [17] as the negative control, and FAJ310(pLHdraGhisMP). In the *amtB* background the signal

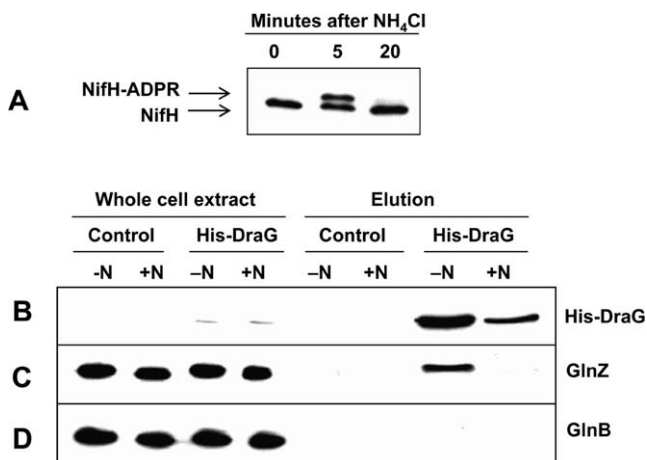


Fig. 2. DraG interacts with GlnZ in vivo. Strains UB4(pLHGhisMP) expressing His-DraG was subjected to an ammonium shock at time zero and analysed as described in Fig. 1 (A). For His-DraG pull-down experiments, samples from strain UB4(pLHGhisMP), and strain UB4 (*draG::km*) as control, were analysed as described in Fig. 1 using anti-DraG (B), anti-GlnZ (C), or anti-GlnB (D) antibodies.

for His-DraG in the eluate was similar regardless of the cellular nitrogen status (Fig. 3A). Again, GlnB was not co-eluted with His-DraG (Fig. 3C). GlnZ was detected in the elution fraction of cells expressing His-DraG, both before and after ammonium addition, although the intensity of the GlnZ signal in the elution fraction from ammonium-shocked cells was three-fold greater than that of nitrogenase derepressed cells (Fig. 3B). GlnZ was fully uridylylated when cells were collected under nitrogen fixing conditions and fully deuridylylated after the ammonium shock (Fig. 3D), indicating that DraG interacts with GlnZ independently of the uridylylation status.

The interaction between deuridylylated GlnZ and DraG might explain why, in the AmtB<sup>+</sup> strain after ammonium addition, His-DraG is poorly recovered and no GlnZ signal is observed (Fig. 2B and C). After ammonium addition DraG is membrane associated in an AmtB-dependent fashion, possibly via GlnZ–AmtB interaction [6]. Consequently His-DraG may well be less accessible to pull-down by His-Mag<sup>TM</sup> agarose beads as observed (Fig. 2B) and the DraG which is pulled



Fig. 3. DraG interacts with GlnZ in vivo independently of the cellular nitrogen status. For His-DraG pull-down experiments, samples from strain FAJ310(pLHGhisMP), and strain FAJ310 (*amtB::km*) as control, were analysed as described in Fig. 1 using anti-DraG (A), anti-GlnZ (B), anti-GlnB (C) antibodies. The uridylylation state of GlnZ was analysed by native-PAGE of whole cell extracts revealed with anti-GlnZ antibodies (D).

down may not bring down GlnZ as this is more tightly associated with AmtB.

#### 4. Discussion

These results demonstrate for the first time under physiologically relevant conditions that DraT and DraG form specific complexes with the P<sub>II</sub> proteins GlnB and GlnZ, respectively. Consequentially they provide significant new insights into the signaling pathway controlling ADP-ribosylation of nitrogenase.

##### 4.1. The role of DraT–GlnB interaction

The observation that DraT forms a specific complex with GlnB in *A. brasilense* is consistent with studies in *R. capsulatus* and *R. rubrum* where, using a yeast two-hybrid system, GlnB was found to interact with DraT [21,22]. Moreover our results indicate that deuridylylation of GlnB plays a pivotal role in GlnB/DraT complex formation. The complex is only formed when the external ammonium levels rise, i.e. under the conditions where DraT is activated, and consequently it seems likely that GlnB/DraT complex formation results in activation of DraT (Fig. 4). This hypothesis is corroborated by genetic analysis in *A. brasilense* showing that GlnB is required for ammonium-induced NifH-ADP ribosylation [6,7]. Furthermore, when GlnB is overexpressed, and thus not completely uridylylated due to titration of the uridylyltransferase, NifH is partially modified even under nitrogen-limiting conditions [20]. These observations in *A. brasilense* are consistent with data from *R. rubrum*, where the expression of a constitutively deuridylylated form of GlnB (GlnB Y51F) leads to NifH modification under nitrogen-fixing conditions in a *draG*<sup>-</sup> background [23]. More recently, physiological and biochemical analysis of mutant forms of the GlnB protein of *R. rubrum* also suggested that interaction with DraT is regulated by its uridylylation state [22].

##### 4.2. The role of DraG–GlnZ interaction

We have recently shown that DraG and the deuridylylated P<sub>II</sub> proteins GlnB and GlnZ are co-ordinately membrane sequestered after an ammonium shock [6]. In *A. brasilense* membrane sequestration of DraG is impaired in *amtB*, *glnB* or *glnZ* mutants, suggesting that AmtB, GlnB and GlnZ might be involved in this process, and in our working model DraG is inactivated through membrane binding [6]. Our new finding that DraG specifically interacts with GlnZ (Fig. 3) suggests that when the extracellular ammonium concentration rises DraG could be targeted to the membrane by formation of an AmtB–GlnZ–DraG ternary complex (Fig. 4). Such a mechanism would imply that AmtB may not only sequester P<sub>II</sub> proteins to the membrane but that it might also form ternary complexes involving both a P<sub>II</sub> protein and one of its targets, in this case DraG. The relative higher recovery of GlnZ from ammonium shocked cells (compare lanes –N and +N in Fig. 3B) suggests that the uridylylated form of GlnZ may bind less tightly to DraG resulting in a reduced recovery in the pull-down experiments from –N extracts.

Our data regarding DraG are consistent with recent studies on DraG localization in *R. rubrum* [16] and with studies on regulation of *R. rubrum* DraT and DraG activities in a heter-

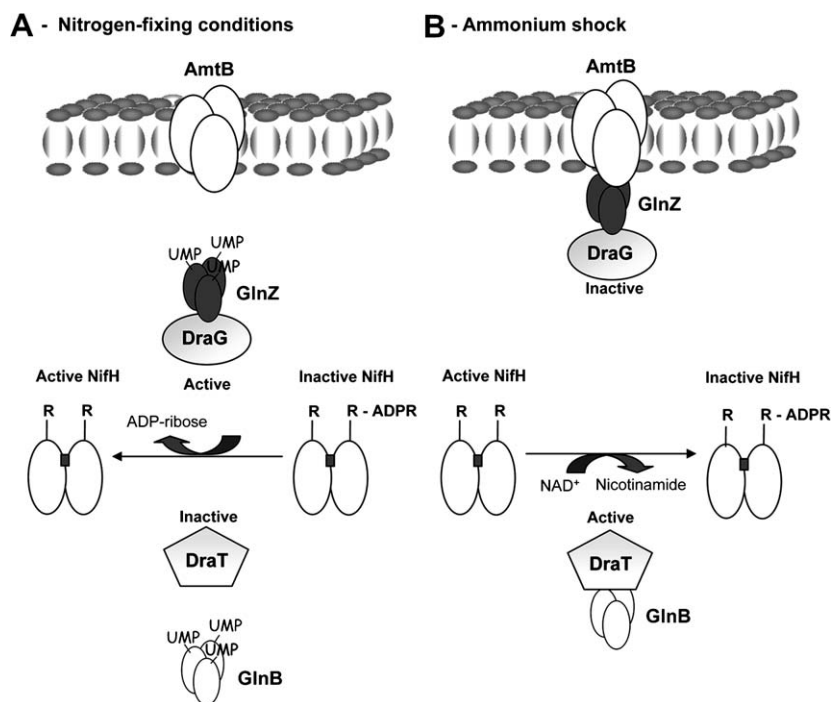


Fig. 4. Model for the regulation of DraT and DraG activities in response to ammonium in *Azospirillum brasilense*. (A) Under nitrogen-fixing conditions the  $P_{II}$  proteins GlnB and GlnZ are fully uridylylated and located in the cytosol. DraT and DraG are also located in the cytosol; DraG being in a complex with fully uridylylated GlnZ. In this condition DraG is active and DraT is inactive, thus NifH is unmodified and active. (B) As the extracellular ammonium concentration rises, GlnB and GlnZ become deuridylylated. Deuridylylated GlnB associates with DraT triggering DraT activity; NifH becomes modified and nitrogenase is inactivated. At the same time, deuridylylation of GlnZ causes the DraG–GlnZ complex to associate with the membrane protein AmtB. The location of DraG physically separates it from its cytoplasmic substrate, NifH, thereby inhibiting the ADP-ribosyl-removing process.

ologous background [24]. In a *K. pneumoniae glnK* mutant, *R. rubrum* DraG remained active after ammonium addition, which would be expected if GlnK were functioning as a GlnZ orthologue to sequester DraG to the membrane.

Although both deuridylylated GlnB and GlnZ are membrane sequestered in an AmtB-dependent manner [6] and form specific complexes with DraT and DraG, respectively (Figs. 1 and 3), only DraG, and not DraT, is membrane targeted after an ammonium shock [6]. Given that the GlnB–DraT and the putative GlnB–AmtB interactions occurs only when GlnB is deuridylylated (Fig. 1 and [6]), this suggests that DraT and AmtB may bind to the same face of the GlnB protein (probably the T-loop). If this is the case then these interactions would be exclusive and a ternary complex could not be formed. On the other hand, the DraG–GlnZ complex formation occurs independently of the GlnZ uridylylation status (Fig. 3), suggesting that in this case the T-loop face of GlnZ may not be involved in the interaction. Consequently DraG and AmtB might recognize different regions of the GlnZ protein, allowing the formation of a ternary complex and thus targeting DraG to the membrane after an ammonium shock. We are currently investigating the formation of the putative ternary complex between AmtB, GlnZ and DraG.

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