Influence of the ADP/ATP ratio, 2-oxoglutarate and divalent ions on Azospirillum brasilense PII protein signalling

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Proteins belonging to the PII family coordinate cellular nitrogen metabolism by direct interaction with a variety of enzymes, transcriptional regulators and transporters. The sensing function of PII relies on its ability to bind the nitrogen/carbon signalling molecule 2-oxoglutarate (2-OG). In Proteobacteria, PII is further subject to reversible uridylylation according to the intracellular levels of glutamine, which reflect the cellular nitrogen status. A number of PII proteins have been shown to bind ADP and ATP in a competitive manner, suggesting that PII might act as an energy sensor. Here, we analyse the influence of the ADP/ATP ratio, 2-OG levels and divalent metal ions on in vitro uridylylation of the Azospirillum brasilense PII proteins GlnB and GlnZ, and on interaction with their targets AmtB, DraG and DraT. The results support the notion that the cellular concentration of 2-OG is a key factor governing occupation of the GlnB and GlnZ nucleotide binding sites by ATP or ADP, with high 2-OG levels favouring the occupation of PII by ATP. Both PII uridylylation and interaction with target proteins responded to the ADP/ATP ratio within the expected physiological range, supporting the concept that PII proteins might act as cellular energy sensors.

INTRODUCTION

The PII family comprises a group of widely distributed trimeric signal transduction proteins found in nearly all Bacteria and also present in Archaea and in the chloroplasts of plants (Forchhammer, 2008). The archetypes of this family are the two Escherichia coli PII proteins, GlnB and GlnK, the functions and structures of which have been extensively characterized. PII proteins regulate the activity of a variety of target proteins through direct protein–protein interactions. These interactions are modulated by the allosteric binding of ATP, ADP and 2-oxoglutarate (2-OG) to PII altering its 3D structure (Forchhammer, 2008). The major structural rearrangements in response to the binding of these molecules occur in a flexible region of PII known as the T-loop (Forchhammer, 2008), and in some organisms PII activity is further modulated by post-translational modification of the T-loop. In Proteobacteria, PII is subjected to reversible uridylylation catalysed by the bifunctional uridylyltransferase/uridylyl-removing enzyme GlnD. The uridylylation status of PII is mainly regulated by the intracellular levels of glutamine which binds to GlnD and controls its opposing activities (Jiang et al., 1998).

Structural and biochemical analysis showed that ATP and ADP bind in a competitive manner to the lateral clefts between each monomer of the PII trimer (Conroy et al., 2007; Jiang & Ninfa, 2009; Xu et al., 1998). Therefore, PII proteins have the potential to act as sensors of the ADP/ATP ratio (D/T). Recent studies demonstrated that the regulation of NtrB and ATase by E. coli GlnB (Jiang & Ninfa, 2009) and the regulation of NAGK and PipX by Synechococcus elongatus GlnB (Fokina et al., 2011) were sensitive to the D/T in vitro, suggesting that PII might be able to sense the cellular energy.

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Abbreviations: 2-OG, 2-oxoglutarate; D/T, ADP/ATP ratio.

Four supplementary figures are available with the online version of this paper.
The binding mode of 2-OG to PII remained elusive until the recent resolution of the crystallographic structures of PII bound to this molecule. In the structures of *Azospirillum brasilense* GlnZ (Truan et al., 2010), *S. elongatus* GlnB (Fokina et al., 2010) and *Archaeoglobus fulgidus* GlnK3 (Maier et al., 2011), the 2-OG binding site was located in the vicinity of the ATP binding site where 2-OG contacts the Mg$^{2+}$ bound to the ATP phosphates. Another important contact is a salt bridge formed between the 5-carboxyl group of 2-OG and the side chain of the highly conserved K58 residue (Fig. 1a). These structural data explained the previous observation that MgATP and 2-OG bind to PII cooperatively (Truan et al., 2010). The binding of 2-OG to *S. elongatus* and *E. coli* GlnB in the presence of saturating MgATP exhibits strong anti-cooperativity such that the occupation of the first 2-OG binding site occurs within the low micromolar range, whereas the occupation of the second and third sites occurs with higher dissociation constants (Fokina et al., 2010; Jiang & Ninfa, 2009).

The first recognized function of PII proteins was to regulate nitrogen metabolism according to the cellular nitrogen status. In diazotrophs, both the synthesis and the activity of nitrogenase are regulated by PII (Dixon & Kahn, 2004; Dodsworth & Leigh, 2006; Huergo et al., 2012; Sarkar et al., 2012). In *A. brasilense*, GlnB binds to and activates the ADP-ribosyltransferase enzyme DraT promoting nitrogenase inactivation upon an ammonium shock. The second PII protein in this organism, GlnZ, regulates the activity of the enzyme catalysing the nitrogenase ADP-ribosyl removing reaction, DraG. The regulation of DraG occurs through the formation of a ternary complex between AmtB, GlnZ and DraG on the cell membrane (Huergo et al., 2012).

In order to further characterize the putative energy sensing function of PII proteins, we analysed the influence of the D/T, 2-OG levels and the metal ions Mg$^{2+}$, Mn$^{2+}$ and Ca$^{2+}$ on *in vitro* uridylylation of *A. brasilense* GlnB and GlnZ and on the *in vitro* interactions between GlnB, GlnZ and their protein targets AmtB, DraG and DraT. The results support the concept that the cellular concentration of 2-OG is a key factor governing the occupation of the GlnB and GlnZ nucleotide binding sites by ATP or ADP. The interactions of GlnB and GlnZ with their target proteins responded to the D/T within the physiological range, supporting the proposal that PII proteins might function as cellular energy sensors.

### METHODS

**Protein purification.** The *A. brasilense* GlnD, DraT, DraG and AmtB proteins were expressed and purified as N-terminally 6×His-tagged proteins, as described previously (Huergo et al., 2007, 2009). The *A. brasilense* GlnB, GlnZ, Q39K and Q39E proteins were expressed and purified as described previously (Moure et al., 2012; Rajendran et al., 2011). The GlnZ Q39E mutant was obtained using the Quick-Change site-directed mutagenesis kit (Agilent) using the pMSA4 plasmid (Moure et al., 2012) as a template. The HisDraT–GlnB complex was purified as described previously (Huergo et al., 2009). Proteins were quantified using the Bradford reagent (Sigma).

**Uridylation reaction.** Uridylylation assays were performed as described previously (Araújo et al., 2008). The reactions contained 10 mM Tris/HCl pH 7.5, 10 mM KCl, 25 mM MgCl$_2$, 1 mM UTP, 3 μM GlnB or GlnZ, 100 nM GlnD and the indicated concentration of ATP, ADP and 2-OG. ATP (>99%), ADP (>95%) and 2-OG (>98%) were purchased from Sigma. In some experiments, MgCl$_2$ was substituted with 5 mM MnCl$_2$. Reactions were incubated at 30 °C and stopped at the times indicated in each experiment by adding 20 mM EDTA. Samples were analysed by native-PAGE and gels were stained with SyPro-Ruby (Invitrogen).

**Protein pull-down assay.** In *vitro* complex formation was performed using MagneHis-Ni$^{2+}$ beads (Promega), as described previously (Huergo et al., 2009). Interaction reactions were conducted in buffer containing 50 mM Tris/HCl pH 8, 0.1 M NaCl, 10% glycerol, 20 mM imidazole and 5 mM MgCl$_2$ in the presence or absence of effectors (2-OG, ATP and ADP) as indicated in each experiment. In experiments using HisDraG or HisAmtB, 0.05% (w/v) lauryldimethylamine-oxide (LDAO) was used to keep AmtB soluble and reduce non-specific interaction of PII with the resin. For experiments using HisDraT, LDAO was substituted with 0.05% (v/v) Tween 20. Samples were analysed by using 12.5% SDS-PAGE gels stained with Coomassie blue. The data reported were confirmed in at least two independent experiments.

**ATP determination.** The contaminant levels of ATP in ADP solutions were quantified by using the ATP bioluminescence kit (HSLI (Roche)). Briefly, 50 μl samples or ATP standards diluted in the range of 10⁻⁶–10⁻¹⁵ M were mixed with 50 μl luciferase reagent. The luminescent signals were recorded in a 96-well plate reader (Tecan Infinity 200) using 1 s delay and integration from 1 to 10 s.

**EPR.** For the EPR analysis, 100 μl purified *A. brasilense* GlnZ at 120 μM (trimer concentration) was incubated on ice in 50 mM Tris/HCl pH 7.5, 100 mM KCl, 20% glycerol (v/v), 2 mM MnCl$_2$, with 2 mM ATP, ADP and 2-OG as indicated in each experiment. EPR experiments were carried out on a Bruker Elexsys E500 spectrometer equipped with a Bruker ERH122SHQE resonator. The experiments were performed at 77 K.

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**Fig. 1.** Comparison between the structures of GlnZ bound to MgATP 2-OG (PDB 3MHY) (a) and to ADP (PDB 305T) (b). Key: cyan, one GlnZ monomer; blue stick, 2-OG; black sphere, Mg$^{2+}$; dashed lines, octahedral coordination of Mg$^{2+}$. In the ADP-bound form the Q39 residue interacts with K58 (green sticks), whereas in the MgATP plus 2-OG structure, Q39 participates in the coordination of Mg$^{2+}$. The contaminant levels of ATP in ADP solutions were quantified by using the ATP bioluminescence kit (HSLI (Roche)). Briefly, 50 μl samples or ATP standards diluted in the range of 10⁻⁶–10⁻¹⁵ M were mixed with 50 μl luciferase reagent. The luminescent signals were recorded in a 96-well plate reader (Tecan Infinity 200) using 1 s delay and integration from 1 to 10 s.

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Fig. 2. Effect of ATP and ADP on the uridylylation of A. brasilense GlnZ and GlnB. The uridylylation reaction was carried out in the presence of 5 mM 2-OG and varying the ATP and ADP concentrations as indicated. (a) and (b), GlnZ; (c) and (d), GlnB. Samples were analysed by native PAGE.

**RESULTS**

**Effects of adenine nucleotides and 2-OG levels on in vitro uridylylation of A. brasilense PII**

We showed previously that in vitro uridylylation of the A. brasilense PII proteins by GlnD required ATP and 2-OG (Araújo et al., 2004, 2008). Given that PII can also bind ADP we investigated whether this molecule could promote or affect PII uridylylation in vitro. Using a saturating concentration of 2-OG (5 mM), ATP activated the uridylylation of both GlnZ and GlnB at a concentration as low as 1 μM. Nearly full uridylylation was observed using 1 mM ATP, confirming previous results (Fig. 2a, c). Note that partially uridylylated GlnB trimers are not detected in native PAGE, as reported previously (Araújo et al., 2008). When ATP was substituted with ADP, uridylylation of both proteins started to occur only at 10–25 μM ADP, and uridylylation was partial even when up to 1 mM ADP was used (Fig. 2b, d). These results are in accordance with those observed with E. coli GlnB, Rhodospirillum rubrum GlnJ and Herbaspirillum seropedicae GlnB; all these proteins were uridylylated less efficiently when ADP was the activating nucleotide in comparison with ATP (Jiang et al., 1998; Teixeira et al., 2008). One exception is H. seropedicae GlnK, which was efficiently uridylylated when ADP was used together with high 2-OG levels (Bonatto et al., in press).

We next tested the uridylylation response of PII in a fixed concentration of 1 mM ATP or ADP and varying 2-OG concentrations. Both GlnZ and GlnB were uridylylated at low micromolar concentrations of 2-OG when ATP was present (Fig. 3a, c). However, uridylylation only occurred in high micromolar 2-OG concentrations when ADP was used as the activating nucleotide (Fig. 3b, d). Interestingly, the ATP and 2-OG requirements for GlnB uridylylation were lower than those for GlnZ uridylylation, suggesting that GlnB and GlnZ have different affinities for binding these effectors.

The fact that ADP was a much less efficient effector of GlnB and GlnZ uridylylation could be explained by an inefficient binding of ADP to GlnB and GlnZ under the assay conditions. This hypothesis is excluded by the fact that interaction between PII, AmtB and DraG, which is dependent on the occupation of PII by ADP, was observed in the presence of 1 mM ADP and 2-OG up to 2 mM (see below). Reaction in the presence of 5 mM 2-OG and AMP in concentrations ranging from 1 μM to 1 mM did not result in detectable uridylylation (data not shown).

The results depicted in Figs 2 and 3 suggest either that GlnB and GlnZ are better substrates for uridylylation when saturated with MgATP and 2-OG or that GlnD is activated by these molecules. In order to distinguish between these models we analysed the ability of two GlnZ variants, Q39K and Q39E, to be uridylylated in vitro. According to the crystal structure of GlnZ bound to MgATP and 2-OG, the side chain of Q39 participates in the coordination of Mg$^{2+}$ and is required for the formation of the 2-OG binding site (Fig. 1a). The addition of a positive (Q39K) or negative (Q39E) charge in this region would affect 2-OG binding. Indeed, an E. coli GlnB Q39E mutant is impaired in 2-OG binding (Jiang et al., 1997) and the same substitution in R. rubrum GlnJ inhibited GlnJ uridylylation (Teixeira et al., 2008). Both A. brasilense E. C. M. Gerhardt and others

Fig. 3. Effect of 2-OG on the uridylylation of GlnZ and GlnB. The reactions were carried out in the presence of 1 mM ATP (a and c) or ADP (b and d) and the 2-OG concentration indicated. (a) and (b), GlnZ; (c) and (d), GlnB. Samples were analysed by native PAGE.
Q39K and Q39E could not be uridylylated in vitro (Fig. S1, available with the online version of this paper), supporting the proposal that the occupation of the 2-OG binding site of PII is required for uridylylation.

Biochemical studies with *E. coli* and *S. elongatus* PII proteins showed that ADP has an antagonistic effect upon 2-OG binding to PII (Fokina *et al.*, 2010, 2011; Jiang & Ninfa, 2009), and this is supported by structural data showing that the ADP-bound conformation of PII occludes the 2-OG binding site (Truan *et al.*, 2010) (Fig. 1b). It is still not clear whether or how 2-OG binds to PII proteins when the PII nucleotide-binding site is occupied by ADP. Furthermore, calorimetric titration studies using *S. elongatus* GlnB and *H. seropedicae* GlnK failed to detect 2-OG binding in the presence of ADP (Fokina *et al.*, 2011; M. A. S. Oliveira and others, personal communication).

Strikingly, we noted that high levels of ADP and 2-OG combined were able to promote GlnB and GlnZ uridylylation (Figs 2 and 3), suggesting that 2-OG could interact with PII in the presence of ADP. An alternative explanation for these results would be that the uridylylation in the presence of ADP is caused by traces of ATP contaminating the ADP solutions. Measurement of ATP using luciferase indicated 1.6% contamination of ATP in the ADP solution used and, indeed, the GlnZ uridylylation profile in the presence of ADP correlates well with the level of contaminating ATP. For instance, GlnZ uridylylation using 250 μM ADP, which has about 5 μM contaminating ATP, was similar to that obtained using 5 μM ATP (Fig. 2). Hence, the uridylylation observed with ADP could be caused by contaminant traces of ATP which would outcompete ADP under high 2-OG levels because ATP and 2-OG bind to PII in a cooperative manner.

Recent studies suggested that PII proteins respond to the D/T in vitro (Fokina *et al.*, 2011; Jiang & Ninfa, 2009). To verify whether the uridylylation of *A. brasilense* GlnZ was responsive to the D/T, uridylylation assays were carried out under different D/Ts, keeping the total nucleotide concentration at 1 mM and using three different 2-OG levels (0.01, 0.1 and 2 mM) covering the physiological range (Fig. 4). As expected from the results using ATP and ADP separately, increasing the D/T reduced GlnZ uridylylation in all three 2-OG conditions (Fig. 4). However, the effect was relatively small at 0.01 mM 2-OG, presumably because the very low 2-OG limits the MgATP 2-OG form of GlnZ. Within the physiological 2-OG range (0.1–2 mM) an increasing D/T clearly reduced GlnZ uridylylation though there was relatively little influence on GlnZ uridylylation by varying the 2-OG concentration.

**Effects of combinations of ATP, ADP, 2-OG and divalent metal ions on GlnZ–DraG, GlnZ–AmtB and GlnB–AmtB complex formation**

Biochemical and structural analysis showed that *A. brasilense* GlnZ interacts with DraG in vitro only when bound to ADP. This interaction is abolished when the GlnZ binding sites are empty or when bound only to ATP (Huerigo *et al.*, 2009; Rajendran *et al.*, 2011). A similar response has been observed for the AmtB–GlnZ and AmtB–GlnB complexes (Rodrigues *et al.*, 2011). Therefore, the capacity of PII to interact with DraG or with AmtB is an indirect method to analyse the competitive binding of ATP and ADP to PII.

Complex formation between His-tagged DraG and native GlnZ was assayed by pull-down in the presence of different D/Ts. The final nucleotide concentration was kept constant at 1 mM. As expected, in the presence of ADP alone, GlnZ co-eluted with His–DraG, indicating complex formation (Fig. 5a). There was no co-elution when using ATP only, confirming previous results. In the presence of combinations of ATP and ADP up to a 9:1 ratio, the DraG–GlnZ interaction was still detected, though the intensity of GlnZ co-eluted corresponded to 48% of that observed when using ADP only (Fig. 5a). Similar results were obtained with the AmtB–GlnZ and AmtB–GlnB complexes (Fig. S2). These data support the proposition that GlnZ and GlnB nucleotide binding sites are partially occupied by ADP even when ATP is nine times more abundant than ADP.

Addition of 2-OG to the reaction containing different D/Ts altered the results in a 2-OG-concentration-dependent manner (Figs 5b and S2). The higher concentration of 2-OG used (2 mM, which reflects the highest physiological level reported under nitrogen limitation) (Radchenko *et al.*, 2010; Senior, 1975) strongly reduced the DraG–GlnZ interaction in most of the D/Ts assayed (Fig. 5b). When ADP was the sole nucleotide, 2 mM 2-OG reduced only 23% of the DraG–GlnZ protein interaction (Fig. 5b, compare lanes 5 and 15). As we still do not know whether 2-OG can bind to GlnZ when the protein is saturated with ADP, this slight inhibition might be caused by contaminant ATP in the ADP solution as suggested by our uridylylation assays. The use of 0.01 mM 2-OG generated results similar to those observed without the addition of 2-OG, this is not surprising since this concentration is lower than the reported physiological range. An intermediate concentration of 2-OG (0.1 mM, which reflects the lowest physiological level reported under nitrogen abundance) (Radchenko *et al.*, 2010; Senior, 1975) resulted in an
Effects of Mg\(^{2+}\), Mn\(^{2+}\) and Ca\(^{2+}\) on P\(_{II}\) effector molecule binding

According to the structural data, high 2-OG would facilitate ATP binding only when Mg\(^{2+}\) is present (Fig. 1a). To confirm this we assayed DraG–GlnZ complex formation using a D/T of 1 in the absence or presence of 2 mM of 2-OG and in the absence or presence of 5 mM of the divalent metal ions Mg\(^{2+}\), Mn\(^{2+}\) or Ca\(^{2+}\). As expected, complex formation was sensitive to 2-OG in the presence of Mg\(^{2+}\) but not in its absence (Fig. 6a). Replacement of Mg\(^{2+}\) with Mn\(^{2+}\) resulted in a similar response, as observed with Mg\(^{2+}\), whilst Ca\(^{2+}\) had no effect.

In order to analyse whether the binding mode of Mn\(^{2+}\) to GlnZ was similar to that proposed for Mg\(^{2+}\) in the GlnZ structure (Fig. 1a) an EPR approach was used. We expected that the coordination of Mn\(^{2+}\) would change when GlnZ is simultaneously bound to MnATP and 2-OG and this change could be potentially detected by EPR (Fig. 1a). The EPR analysis indicates the appearance of a typical six line hyperfine pattern of Mn\(^{2+}\) species in the region of 150–200 mT when GlnZ was in the presence of Mn\(^{2+}\) in combination with both ATP and 2-OG. When ATP or 2-OG was omitted, this signal was not observed (Fig. S3). Furthermore, this signal was not observed in control solutions containing Mn\(^{2+}\), ATP and 2-OG but without the addition of GlnZ (data not shown). Similar responses were observed with A. brasilense GlnB (data not shown). These data indicate that the coordination of Mn\(^{2+}\) changes in the presence of GlnZ, ATP and 2-OG, suggesting that Mn\(^{2+}\) binds to P\(_{II}\) in a similar manner as Mg\(^{2+}\) (Fig. 1a).

The dissociation of the R. rubrum GlnJ–AmtB1 complex showed different sensitivities to 2-OG whether Mg\(^{2+}\) or Mn\(^{2+}\) was present (Teixeira et al., 2008), suggesting that, when compared with MgATP, MnATP could increase the affinity of GlnJ for 2-OG binding, and hence that the relative concentrations of Mg\(^{2+}\) and Mn\(^{2+}\) could alter the P\(_{II}\) output signal. We did not observe any significant differences in the apparent affinity of nucleotides and 2-OG for the in vitro interaction assay between DraG and GlnZ when Mg\(^{2+}\) was substituted with Mn\(^{2+}\) (Fig. 6a). We also studied the effect of different metal ions on the in vitro dissociation of the A. brasilense DraT–GlnB complex. The DraT–GlnB complex is stable in the presence of MgATP but is readily dissociated by 2-OG in the presence of ATP and Mg\(^{2+}\) (Huergo et al., 2009). As expected, the complex was sensitive to 2-OG in the presence of Mg\(^{2+}\) but not in its absence. Mn\(^{2+}\) resulted in a similar response, whilst Ca\(^{2+}\) again had no effect (Fig. S4a). To investigate if the dissociation constant for 2-OG binding to GlnB could be altered by the ATP counter-ion we assessed the stability of the DraT–GlnB complex using a fixed concentration of ATP and variable 2-OG in the presence of Mg\(^{2+}\) or Mn\(^{2+}\). The results support the proposal that the affinity for 2-OG binding is similar whether Mg\(^{2+}\) or Mn\(^{2+}\) is used as the ATP counter-ion (Fig. S4b).

Even though Ca\(^{2+}\) can form octahedral complexes with oxygen, its larger ionic radius and increased coordination distances in comparison to Mg\(^{2+}\) and Mn\(^{2+}\) (Harding, 2000) could explain its inability to facilitate the binding of
2-OG to PII in the presence of ATP (Figs 6a and S4a). However, like Mg$^{2+}$, Ca$^{2+}$ relieved the inhibitory effect of ATP on S. elongatus NAGK–PII interaction (Maheswaran et al., 2004), suggesting that this ion can interact with the ATP phosphates in PII. Hence, Ca$^{2+}$ may have the potential to act as a modulator of the PII response to 2-OG, because Ca$^{2+}$ could compete with Mg$^{2+}$ for binding to the ATP phosphates, though only PII–MgATP and not PII–CaATP would be able to engage in interaction with 2-OG.

To verify this hypothesis, we analysed complex formation between DraG–GlnZ in the presence of a D/T of 0.33 and 0.1 mM 2-OG using different Mg$^{2+}$/Ca$^{2+}$ ratios. The results indicate that Ca$^{2+}$ is indeed able to abrogate the inhibitory effect of 2-OG when this ion is five times more abundant than Mg$^{2+}$ (Fig. 6b, lane 5). There is a precedent for a link between Ca$^{2+}$ signalling and regulation of nitrogen metabolism by PII (Leganés et al., 2009); however, the reported intracellular levels of Ca$^{2+}$ are within the nanomolar range whilst those of Mg$^{2+}$ are in the micromolar range (Dominguez, 2004). Consequently, according to the data in Fig. 6(b), Mg$^{2+}$ would outcompete Ca$^{2+}$ for ATP binding, and modulation of 2-OG binding to PII by Ca$^{2+}$ is unlikely to occur in vivo under regular physiological conditions. On the other hand, we cannot completely rule out that transient Ca$^{2+}$ upshifts could affect PII signalling in vivo.

**Discussion**

We have analysed the effects of the D/T, 2-OG levels and divalent metal ions on A. brasilense PII protein uridylylation and interaction with the target proteins in vitro. Our data support the view that occupation of the PII trimer by MgATP and 2-OG is a prerequisite for the A. brasilense PII uridylylation. Therefore, uridylylation is an indirect method to determine the status of PII occupation by MgATP 2-OG. Conversely, the interaction between PII and DraG or between PII and AmtB was used as an indirect method to determine the levels of PII bound to ADP. The combination of these analyses indicates that the cellular concentration of 2-OG is a key factor governing the occupation of the GlnB and GlnZ by ATP or ADP. High 2-OG favoured ATP binding to PII, as supported by the increasing uridylylation rates (Fig. 3), and reduction in the DraG–GlnZ (Fig. 5), AmtB–GlnZ and AmtB–GlnB interactions (Fig. S2). These same analyses indicate that low 2-OG levels favour the binding to PII of ADP over ATP.

Comparison between the structures of A. brasilense GlnZ bound to MgATP 2-OG and that bound to ADP (Fig. 1) provides a rational explanation for these results. ATP, 2-OG and the side chain of Q93 participate in the coordination of Mg$^{2+}$ in the GlnZ MgATP 2-OG structure and the 5-carboxyl group of 2-OG forms a salt bridge with the side chain of K58 (Truan et al., 2010) (Fig. 1a). When GlnZ is bound to ADP, the side chain of Q93 interacts with K58 and occupies the same space to which 2-OG binds (Rajendran et al., 2011) (Fig. 1b). Therefore, 2-OG binding would facilitate ATP binding but would perturb the ADP-bound conformation of GlnZ.

In agreement with the results reported here, high 2-OG stimulated ATP binding but either did not affect or inhibited the binding of ADP to E. coli GlnB and GlnK in vitro. This response was confirmed by interaction assays between AmtB and GlnK, in which a D/T of 0.08 (ATP 13 × more abundant than ADP) led to the same response as that observed when using ADP only (Rudchenko et al., 2010). Similar results were observed here for the A. brasilense AmtB–PII (Fig. S2). The addition of 2 mM 2-OG under this condition completely disrupted AmtB–PII complex formation (Fig. S2) and E. coli AmtB–GlnK complex (Rudchenko et al., 2010). Hence, the fact that increasing concentrations of 2-OG promote the exchange of the ADP bound to GlnZ for ATP could be a universal feature in proteobacterial PII.

In prokaryotes, 2-OG is the major carbon skeleton used for assimilation of inorganic nitrogen. Its pool decreases from
~1.4 to ~0.3 mM a few seconds after a nitrogen-deprived culture of \textit{E. coli} receives an ammonium shock (Radchenko et al., 2010). Such 2-OG reduction would facilitate the exchange of the ATP bound to PII for ADP, generating a 2-OG-controlled conformational switch in PII which, in turn, would regulate the interaction between PII and nitrogen-related proteins as AmtB and DraG.

Is this 2-OG-controlled conformational switch the only reason for the ability of PII to bind ATP and ADP competitively or could the PII structure also respond to variations in the available D/T in a fixed 2-OG concentration? The reported physiological D/T in bacteria varies from 0.06 to 1.48 (Bennett et al., 2009; Paul & Ludden, 1984; Radchenko et al., 2010; Upchurch & Mortenson, 1980). Variations in D/T within this range altered the DraG–GlnZ, AmtB–GlnZ and AmtB–GlnB interactions significantly, especially at 0.1 mM 2-OG (Figs S5 and S2). Hence, in principle, the \textit{A. brasilense} PII proteins have the potential to act as D/T sensors.

The best-documented functions of \textit{A. brasilense} PII are to control nitrogenase expression and activity and to regulate the global nitrogen transcriptional factor NtrC, which activates the expression of a plethora of genes under nitrogen deprivation (de Zamaroczy, 1998; Huergo \textit{et al.}, 2012). Both of these processes are energy demanding and it would be rational to use PII as a sensor to downregulate all these processes when energy is scarce. Further analysis correlating the PII activity with intracellular fluctuations in the D/T will be required to clarify the predicted energy-sensing function of PII proteins.

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