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Different responses of the GlnB and GlnZ proteins upon *in vitro* uridylylation by the *Azospirillum brasilense* GlnD protein

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Azospirillum brasilense is a diazotroph found in association with important agricultural crops. In this organism, the regulation of nitrogen fixation by ammonium ions involves several proteins including the uridylyltransferase/uridylyl-removing enzyme, GlnD, which reversibly uridylylates the two PII proteins, GlnB and GlnZ, in response to the concentration of ammonium ions. In the present study, the uridylylation/deuridylylation cycle of *A. brasilense* GlnB and GlnZ proteins by GlnD was reconstituted *in vitro* using the purified proteins. The uridylylation assay was analyzed using non-denaturing polyacrylamide gel electrophoresis and fluorescent protein detection. Our results show that the purified *A. brasilense* GlnB and GlnZ proteins were uridylylated by the purified *A. brasilense* GlnD protein in a process dependent on ATP and 2-oxoglutarate. The dependence on ATP for uridylylation was similar for both proteins. On the other hand, at micromolar concentration of 2-oxoglutarate (up to 100 µM), GlnB uridylylation was almost twice that of GlnZ, an effect that was not observed at higher concentrations of 2-oxoglutarate (up to 10 mM). Glutamine inhibited uridylylation and stimulated deuridylylation of both GlnB and GlnZ. However, glutamine seemed to inhibit GlnZ uridylylation more efficiently. Our results suggest that the differences in the uridylylation pattern of GlnB and GlnZ might be important for fine-tuning of the signaling pathway of cellular nitrogen status in *A. brasilense*.

Key words: *Azospirillum brasilense*; Nitrogen fixation; PII-like protein; GlnD; GlnB; GlnZ

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Proteins from the PII family comprise a class of highly conserved regulatory proteins which are among the most widely distributed in nature. In Gram-negative bacteria, these proteins are homotrimers of about 12 kDa per monomer, and each subunit can be reversibly modified by uridylylation at the tyrosine 51 residue in response to fluctuation in the availably of ammonium ion in the external medium (1). The reversible uridylylation of PII is catalyzed by the GlnD protein, a bifunctional uridylyltransferase/uridylyl-removing enzyme (2).

When the external ammonium ion supply is low, the intracellular glutamine concentration is low, favoring the promotion of PII uridylylation by the uridylyltransferase activity of GlnD. Upon an ammonium ion increase, the intracellular glutamine concentration increases, triggering the uridylyl-removing enzyme activity of GlnD and leading to PII deuridylylation (3,4). Whether unmodified or uridylylated, the PII proteins signal high and low ammonium ion concentration, respectively, to specific regulatory target proteins such as AmtB, NtrB, and ATase (1,4), controlling their activities by protein-protein interactions. In addition to posttranslational modification, binding of small regulatory effectors such as adenine nucleotides and 2-oxoglutarate can also control PII protein activity (1).

Azospirillum brasilense, a diazotrophic α-proteobacterium associated with important agricultural crops (5), has

two genes coding for PII-like proteins, *glnB* and *glnZ* (6), which are predominantly expressed under nitrogen-limiting conditions (6,7). Their role in nitrogen fixation by *A. brasilense* has been studied in several laboratories and it has been shown that GlnB is absolutely required for NifA activity (8,9) and that both GlnB and GlnZ are involved in posttranslational control of nitrogenase (10,11).

In order to characterize the function of GlnD, GlnB, and GlnZ in signaling the external ammonium ion levels in *A. brasilense*, we analyzed the *in vitro* uridylylation of GlnB and GlnZ catalyzed by the GlnD protein.

For over-expression of GlnD, the *glnD* gene of *A. brasilense* strain FP2 was amplified using purified genomic DNA as a template and primers based on the sequence deposited in the GenBank database (Accession No. AF149716). Primers AbglnDN (ACGTTTTGCATATGCT CTCCAC) and AbglnDC (CTGTCCTGGATCCCGGCTTT) were aligned to the 5' and 3' ends, respectively, of the *glnD*-coding sequence with *Nde*I and *Bam*HI sites (underlined) introduced in the amplified fragment in order to facilitate cloning. This fragment was cloned into the pET28a vector (Novagen, USA), yielding the pALIpGT plasmid. The cloned fragment was completely sequenced to confirm its integrity. The *A. brasilense* GlnD protein was over-expressed as a His-tag protein (GlnD-His) in the *Escherichia coli glnBglnD*⁻ strain RB9065(λDE3) upon induction with 0.5 mM isopropyl ß-D-thiogalactopyranoside (IPTG) at 37°C for 3 h. Cells were harvested, resuspended in buffer S (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM dithiothreitol, 10% glycerol) and then lysed by sonication. The crude extract was then centrifuged at 10,000 rpm for 15 min and the supernatant was loaded onto a Hi-trap-chelating-Ni^{2+} column (GE Healthcare, USA) pre-equilibrated in buffer S. Two washing steps were performed using ten column volumes each of buffer W (50 mM Tris-HCl, pH 6.3, 500 mM NaCl) with 10 and 40 mM imidazole. The proteins were eluted by a stepwise increase of imidazole (50, 100, 300, and 500 mM) in buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, and 10% glycerol, using five column volumes at each concentration. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and those containing GlnD-His with purity over 97%, as determined by densitometric analysis, were stored at -70°C and used in our assays.

The native *A. brasilense* GlnB and GlnZ proteins were over-expressed in *E. coli* strain BL21(λDE3)pLysS upon plasmids pLMA4 (9) and pMSA-L1 (12), respectively, and induction with 0.5 mM IPTG at 37°C for 3 h. After harvesting, cells were resuspended in buffer SN (50 mM Tris-HCl, pH 7.5, 200 mM KCl, 1 mM EDTA, and 20% glycerol) and lysed by sonication. The crude extract was clarified by centrifugation at 10,000 rpm for 20 min, and the soluble fraction was loaded onto a Hi-trap-heparin column (GE Healthcare) pre-equilibrated in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM EDTA). After washing with five column volumes of buffer A, proteins were eluted by using a linear KCl gradient (0.1 to 1 mM) in buffer A. Fractions were analyzed by SDS-PAGE and purity was determined by densitometry. Our procedure efficiently purified the GlnB and GlnZ proteins using only one chromatographic step, reaching purity of 95% for GlnB and of 92% for GlnZ, as determined by densitometric analysis by SDS-PAGE. These fractions were used in our assays. We observed that ion-exchange chromatography and/or protamine sulfate pre-treatment, used regularly as procedure for PII purification, also eliminated a significant amount of PII proteins and were not essential for *A. brasilense* GlnB or GlnZ purification (data not shown).

Although *E. coli* BL21(λDE3)pLysS can produce endogenous GlnB and GlnK, allowing the formation of heterotrimers (13) this is unlikely to be the case, since the expression of *E. coli* GlnB is significantly low (14) compared to the high expression of the recombinant *A. brasilense* GlnB or GlnZ, and there is no significant expression of *E. coli* GlnK in the N-rich medium used for protein induction. Also, the purified GlnB and GlnZ migrated as sharp and homogenous bands by native gel electrophoresis, suggesting that most of the purified proteins were non-modified homotrimers (Figure 1A).

The uridylylation assay was performed as described (12) and analyzed by non-denaturing PAGE (Figure 1A). Densitometric analyses of SYPRO-Red-stained (Invitrogen, USA) gels were performed to determine the percentage of PII uridylylation. Given the trimeric nature of all PII proteins studied so far, we expected to observe up to four forms of these proteins: non-uridylylated PII, PII-UMP, PII-UMP_2, and PII-UMP_3 on native gels.

The effect of GlnD concentration on the uridylylation of purified GlnB and GlnZ is shown in Figure 1. During 30-min incubation, an increasing amount of purified GlnD produced an increase in uridylylation of the PII subunits, achieving total uridylylation of GlnB and near 96% of GlnZ with 100 nM of purified GlnD (Figure 1A,B). With lower amounts of GlnD we observed all four possible forms of GlnZ in the gel (i.e., GlnZ, GlnZ-UMP_1, GlnZ-UMP_2, and GlnZ-UMP_3) since, as expected, the addition of the negatively charged UMP group increased protein migration (Figure 1A). Interestingly, for GlnB only two forms were observed on the gels (Figure 1A) and this result was reproduced in different experiments using different GlnB preparations (data not shown).

To check the nature of the GlnB forms observed in the

native gels (Figure 1A) we performed mass spectrometry analyses (Maldi-ToF) of the GlnB samples before and after the uridylylation reactions by mixing them with sinapic acid matrix (1:9), spotting on a Maldi plate (Bruker Daltonics, Bremen, Germany) and allowing the sample to dry. Mass spectra were acquired using a Maldi-ToF-MS Autoflex spectrometer (Bruker Daltonics) in a positive linear mode with an accelerating voltage of 20 kV, delay time of 330 ns and acquisition mass range of 5-20 kDa. Peak lists were created with the sophisticated numerical annotation procedure algorithm using the FlexAnalysis 2.0 software (Bruker Daltonics). Before incubation with GlnD, the GlnB Maldi-ToF analyses yielded a single peak with an m/z ratio of 12,367, which was in good agreement with the predicted monomeric molecular mass of 12,371 kDa. After incubation with 100 nM GlnD, the GlnB Maldi-ToF analysis yielded a single peak with an m/z ratio of 12,672, corresponding to the GlnB monomer covalently bound to UMP. Maldi-ToF analyses of incomplete uridylylation reactions yield two peaks with an m/z ratio corresponding to the unmodified and modified forms of monomeric GlnB. These results suggest that the faster migrating band observed on the gel after incubation with 100 nM GlnD is the homogeneous trimeric modified form of GlnB, GlnB-UMP_3.

It is unlikely that the absence of other GlnB intermediates in the native gels (i.e., containing 1 or 2 UMP) is a result of co-migration of the mono-, di- and tri-uridylylated forms as a single band on the gels. As the migration profile on native gels depends on the protein charge and conformation, the superimposition of GlnB-UMP_1, GlnB-UMP_2, and GlnB-UMP_3 as a single band would imply major conformational changes to counterbalance the effect of the presence of more negatively charged UMP groups. This scenario seems unlikely since the site of PII uridylylation (Tyr51) is located in the T-loop which seems very flexible and extends away from the core structure of the PII trimer (1).

The presence of only two GlnB forms was also observed in native gels when the *A. brasilense* GlnB was assayed for *in vivo* deuridylylation and re-uridylylation (11), suggesting that this could be the case not only for uridylylation but also for re-uridylylation.

The physiological role of the different pattern of GlnB modification as compared to GlnZ is still unclear. Several known PII-target interactions are controlled by the PII uridylylation status and such response might be necessary especially for a trimer-to-trimer interaction as that occurring with *A. brasilense* AmtB-GlnB (15). The AmtB protein acts as a trimer containing three ammonia channels. Binding of a PII trimer to the cytoplasmic surface of AmtB inhibits channel activity because the non-uridylylated PII T-loop from each monomer blocks one AmtB channel pore (16,17). The partially modified GlnB (i.e., GlnB-UMP_1 and GlnB-UMP_2) could compete with fully deuridylylated GlnB for AmtB binding but only the latter would effectively block channel activity. In this case a full or null interaction might be essential.

It has been shown that PII uridylylation is controlled by the effectors ATP and 2-oxoglutarate in many organisms (1,3). To analyze the effect of these effectors on the uridylylation of *A. brasilense* PII proteins, reactions were conducted in the presence of varying concentrations of ATP and 2-oxoglutarate (Figure 2A,B). In the absence of ATP or 2-oxoglutarate, purified *A. brasilense* GlnD was

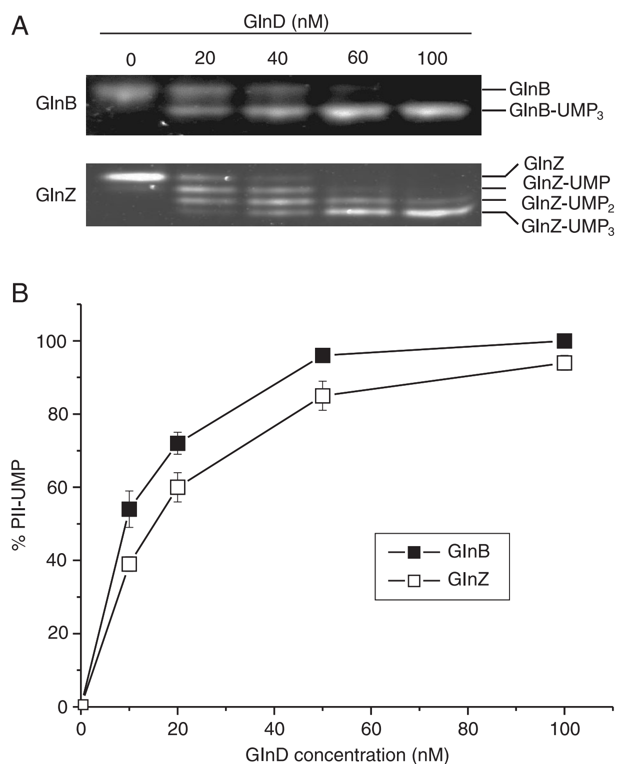

Figure 1. *In vitro* uridylylation of the GlnB and GlnZ proteins (PII) using *Azospirillum brasilense* GlnD. Reactions were performed in uridylylation buffer (100 mM Tris-HCl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, and 0.1 mM dithiothreitol) using 3 µM of each PII protein, 0.1 mM ATP, 10 mM 2-oxoglutarate, 1 mM UTP, and the indicated concentration of GlnD. Samples were incubated for 30 min at 30°C and the reactions were stopped by adding 10 mM EDTA and 5 µL electrophoresis sample buffer (0.5 mM Tris-Cl, pH 6.8, 10% glycerol, and 0.05% (w/v) bromophenol blue). Samples were kept on ice before loading for non-denaturing 10% polyacrylamide gel electrophoresis. *Panel A*, the gel shows a representative experiment stained with SYPRO Red. Lines indicate the unmodified and modified PII species. *Panel B* shows a densitometric analysis of at least three independent experiments. Data are reported as means ± SD.

unable to uridylylate the GlnB or GlnZ proteins (Figure 2A,B). Maximum uridylylation was achieved with our purified GlnB and GlnZ at 0.1 mM ATP (Figure 2A) and 1 mM 2-oxoglutarate (Figure 2B). The requirement for ATP in the uridylylation reaction was similar for both proteins. On the other hand, the dependence on 2-oxoglutarate seems to be different for GlnB and GlnZ in the micromolar range. At 0.1 mM 2-oxoglutarate or less, the uridylylation of GlnB was considerably higher than that of GlnZ (Figure 2B). On the other hand, at millimolar concentrations of 2-oxoglutarate, a similar response was observed for both proteins. Although the intracellular concentration of 2-oxoglutarate in *A. brasilense* is unknown, in *E. coli* the levels of 2-oxoglutarate are known to vary from 0.1 to 0.9 mM (18), suggesting that the uridylylation state of GlnB and GlnZ may also translate the cell carbon status.

ATP and 2-oxoglutarate are required for the uridylylation of *A. brasilense* GlnB and GlnZ, probably by the binding of

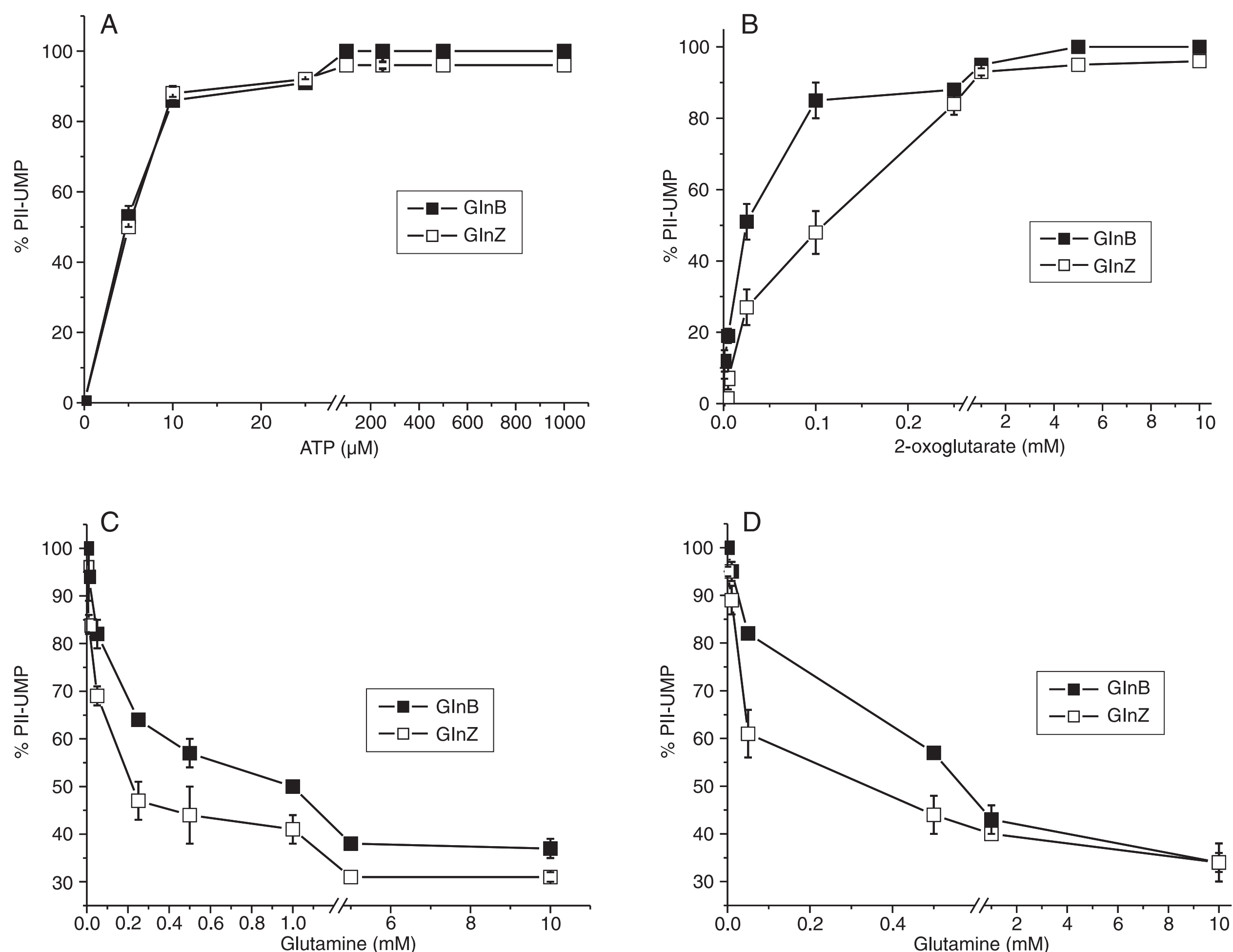

Figure 2. Effect of ATP (panel A), 2-oxoglutarate (panel B) and glutamine (panel C) on the *in vitro* uridylylation and the effect of glutamine on the *in vitro* deuridylylation (panel D) of purified *Azospirillum brasilense* GlnB and GlnZ proteins by purified *A. brasilense* GlnD. For the uridylylation assay, reactions were carried out with 3 µM PII proteins, 1 mM UTP, 100 nM GlnD, and 0.1 mM ATP (panels B and C) or 10 mM 2-oxoglutarate (panels A and C). Samples were incubated for 30 min at 30°C and loaded for non-denaturing 10% polyacrylamide gel electrophoresis. For the deuridylylation assay (panel D), samples were first fully uridylylated for 30 min under optimum uridylylation conditions, and then loaded onto a gel filtration column (GS25, GE Healthcare) pre-equilibrated with uridylylation buffer. The elution was carried out using the same buffer, and the eluted GlnB-UMP_3 and GlnZ-UMP_3 proteins were then used in the deuridylylation reaction. PII-UMP_3 proteins (3 µM) were incubated with 1 mM UTP, 2 mM 2-oxoglutarate, 0.1 mM ATP and 100 µM GlnD and the indicated concentrations of glutamine in uridylylation buffer for 30 min at 30°C. Reactions were then loaded for non-denaturing 10% polyacrylamide gel electrophoresis. Densitometric analyses were performed with SYPRO red-stained gels and indicate the percentage of modified PII protein. Results show the average of at least two independent experiments.

these effectors to the PII protein as described for *E. coli* PII (19). Glutamine is also a regulator of PII protein uridylylation. The activity of *E. coli* GlnD is directly controlled by the levels of glutamine (20), which has a dual function: it inhibits the uridylyltransferase activity and stimulates the uridylyl-removing activity of GlnD (7,20). We tested the effect of glutamine on the uridylylation and deuridylylation of GlnB and GlnZ (Figure 2C,D). In the absence of glutamine, a situation indicating nitrogen deficiency, the GlnB and GlnZ uridylylation rate occurs at its maximum level (Figure 2C). However, by increasing the amounts of glutamine in the reaction, an inhibition of uridylylation was observed. At 10 mM glutamine, less than 40% uridylylation of GlnB and GlnZ occurred. This inhibition of uridylylation by glutamine seems to be higher for the GlnZ than the GlnB protein (Figure 2C).

We also analyzed the deuridylylation of GlnB-UMP_3 and GlnZ-UMP_3 by purified *A. brasilense* GlnD in response to the presence of glutamine (Figure 2D). At 1 mM glutamine, both proteins were deuridylylated up to 65%. Interestingly, at lower glutamine concentrations, the extent of deuridylylation of GlnZ-UMP_3 was higher than that of GlnB-UMP_3 (Figure 2D).

Furthermore, partial deuridylylation of GlnZ resulted in up to 4 bands representing the GlnZ(UMP), GlnZ$(UMP)_2$, GlnZ$(UMP)_3$, and unmodified forms. On the other hand, GlnB had only 2 forms probably corresponding to the unmodified and GlnB$(UMP)_3$, consistent with the observation *in vivo* (10). The results suggest that the levels of glutamine define the levels of PII uridylylation in *A. brasilense* and, therefore, the cellular nitrogen status.

We have examined *in vitro* the uridylylation of GlnB and GlnZ carried out by the *A. brasilense* GlnD protein. Purified *A. brasilense* GlnB and GlnZ proteins were uridylylated by purified GlnD in a process dependent on ATP and 2-oxoglutarate and glutamine inhibits uridylylation and promotes deuridylylation.

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