available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

# *Azospirillum brasilense* $P_{II}$ proteins GlnB and GlnZ do not form heterotrimers and GlnB shows a unique trimeric uridylylation pattern

*Juliana Inaba, Luciano F. Huergo, Ana C. Bonatto, Leda S. Chubatsu, Rose A. Monteiro, M. Berenice Steffens, Giseli Klassen, Liu U. Rigo, Fábio O. Pedrosa, Emanuel M. Souza**

*Department of Biochemistry and Molecular Biology, Universidade Federal do Paraná, Francisco H. dos Santos s/n, CP 19046, Curitiba, PR, Brazil*

ARTICLE INFO

*Article history:*
Received 17 March 2008
Received in revised form
1 August 2008
Accepted 22 August 2008
Published online 1 October 2008

*Keywords:*
*Azospirillum brasilense*
GlnB
GlnZ
$P_{II}$-like protein,
Nitrogen fixation

ABSTRACT

In many organisms, nitrogen metabolism is co-ordinated by a class of highly conserved proteins from the $P_{II}$ family. In Gram-negative bacteria $P_{II}$ proteins are trimers that can be covalently modified by uridylylation according to the cellular nitrogen status. Several prokaryotes have more than one gene that code for $P_{II}$ proteins. In *Escherichia coli* it was shown that the two $P_{II}$ proteins (GlnB and GlnK) can form heterotrimers and it was suggested that heterotrimerization of $P_{II}$ proteins could be widespread in Bacteria. The nitrogen-fixing plant-associative bacteria *Azospirillum brasilense* code for two $P_{II}$ proteins, GlnB and GlnZ. The expression of *glnB* and *glnZ* genes are induced under nitrogen fixing conditions and these proteins control both the expression and the activity of the nitrogenase enzyme. Here we show that unlike *E. coli* $P_{II}$ proteins, *A. brasilense* GlnB and GlnZ, do not form heterotrimers *in vitro*. Our data also suggest that *A. brasilense* GlnB shows a unique uridylylation pattern.

© 2008 Elsevier Masson SAS. All rights reserved.

## 1. Introduction

The associative nitrogen-fixing bacteria *Azospirillum brasilense* has attracted considerable attention due to its capacity of colonize the roots and enhance the growth and yield of several economically important crops [8,27]. Inoculants of *Azospirillum* spp. have been used in many part of the world for crops such as rice and maize. The understanding of its nitrogen metabolism and the fine tuning of its regulatory network is essential for genetically engineering strains with improved agronomic efficiency.

This organism can only fix nitrogen under microaerobic conditions and when fixed nitrogen is limiting. Nitrogenase expression and activity in *A. brasilense* are controlled by the $P_{II}$ proteins GlnB and GlnZ [12,13,18,24,25]. In response to nitrogen levels, these proteins are reversibly modified by the bifunctional enzyme GlnD. *In vitro* assays showed that GlnB and GlnZ are uridylylated by GlnD in the presence of ATP and 2-oxoglutarate. The presence of glutamine inhibits uridylylation and stimulates deuridylylation of both GlnB and GlnZ [3]. The GlnB protein controls the activity of the transcriptional activator NtrC through its partner NtrB [13] and is required for

* *Corresponding author*. Tel.: +55 41 3361 1667; fax: +55 41 3266 2042.
E-mail address: souzaem@ufpr.br (E.M. Souza).
1164-5563/$ – see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejsobi.2008.08.006

the activity of NifA, the transcriptional activator of the *nif* genes [2]. The GlnZ protein cannot substitute GlnB in these functions [13]. GlnB and GlnZ also play specific roles in the control of the nitrogenase activity through the reversible ADP-ribosylation of dinitrogenase reductase (NifH) [19,24,25]. Under nitrogen fixing conditions GlnZ-$UMP_3$ interacts with the enzyme DraG (dinitrogenase reductase ADP-ribosyl-glycohydrolase), which activates nitrogenase by removing the ADP-ribosyl group from NifH [19]. After an ammonium shock, the deuridylylated form of GlnB interacts with the enzyme DraT (dinitrogenase reductase ADP-ribosyl-transferase), which inactivates nitrogenase by ADP-ribosylation of NifH. Under these conditions, GlnZ is deuridylylated and the GlnZ–DraG complex associates with the membrane protein AmtB, inhibiting nitrogenase activation by DraG [17,18].

The $P_{II}$ family comprises highly conserved signal transduction proteins. These proteins are found in Archaea, Bacteria and Eukarya and control the activity of transcriptional activators, key metabolic enzymes and membrane transporters through direct protein–protein interactions [16,26]. In Proteobacteria, $P_{II}$ proteins undergo reversible uridylylation by the bifunctional enzyme GlnD [16]. The structural models of some $P_{II}$ proteins have been determined. The $P_{II}$ proteins from *Escherichia coli* (GlnB and GlnK) are homotrimers that form a compact barrel with a central and three lateral clefts [10,30]. Each subunit extends a flexible region known as the T-loop, which contains the uridylylation site, a highly conserved tyrosine residue (Tyr51). Uridylylation of *E. coli* $P_{II}$ proteins requires ATP and 2-oxoglutarate and these proteins have a binding site for each effector [6,22]. The ATP binding sites are located in the lateral clefts between the $P_{II}$ subunits [30,31]. The 2-oxoglutarate binding site of GlnB proteins from *E. coli* and *Herbaspirillum seropedicae* seems to be also in the lateral cleft [9,21]. Recent data have suggested that $P_{II}$ proteins can also bind ADP and AMP [20,23,29,32]. The ability of $P_{II}$ proteins to interact and thus regulate their targets depends on the $P_{II}$ uridylylation status and the effectors bound.

*E. coli* $P_{II}$ proteins (GlnB and GlnK) can form heterotrimers both *in vivo* and *in vitro* [15,28]. The formation of heterotrimers between these proteins seems to promote fine regulation of $P_{II}$ target activities such as glutamine synthetase [28]. It was suggested that heterotrimerization could be widespread in Bacteria. As both $P_{II}$ proteins coded by *A. brasilense* (GlnB and GlnZ) are induced under nitrogen limiting conditions [12,17], we decided to verify if $P_{II}$ heterotrimerization could also occur in this organism. Here we show that *A. brasilense* $P_{II}$ proteins, GlnB and GlnZ, do not form heterotrimers *in vitro*. The data also suggest that *A. brasilense* GlnB trimer is only stable when completely unmodified or when fully uridylylated.

## 2. Material and methods

### 2.1. Expression and purification of A. brasilense GlnB, GlnZ and His-GlnD proteins

The *A. brasilense* GlnB, GlnZ and His-GlnD (N-terminal 6xHis tagged version of the GlnD enzyme) proteins were expressed in *E. coli* BL 21 from plasmids bearing the T7 promoter and purified as described previously [3].

### 2.2. In vitro uridylylation of GlnB and GlnZ and purification of fully uridylylated forms

The uridylylation reactions were performed as described previously [3]. The products of the reactions were analyzed by native gel electrophoresis as described [3] and MALDI-TOF spectrometry (see below). Samples were collected at 0, 5, 10, 20, 30 and 60 min and the reaction stopped by addition of 1 mM EDTA and 5 μl of native electrophoresis sample buffer. $P_{II}$-UMP was purified after uridylylation by removing GlnD-His with HisMagnetics beads (Promega Co., Madison, WI) and the system was dialyzed against 50 mM Tris–HCl pH 7.5, 0.1 M KCl, 20% glycerol overnight at 4 °C to remove ATP and 2-oxoglutarate.

### 2.3. Heterotrimer formation of $P_{II}$

Purified GlnB, GlnB-$UMP_3$, GlnZ and GlnZ-$UMP_3$ proteins were mixed (final concentration of 0.5 μg/μl) at the indicated ratios in a buffer containing 100 mM Tris–HCl pH 7.5, 100 mM KCl and 25 mM $MgCl_2$. When indicated, samples were boiled for 5 min and kept on ice for 5 min. The samples were analyzed by native gel electrophoresis [3].

### 2.4. Native polyacrylamide gel electrophoresis (PAGE)

Samples were mixed with 5 μl of loading buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 0.01% bromophenol blue) and separated in a non-denaturing polyacrylamide gel electrophoresis (10%) as described [14].

### 2.5. MALDI-TOF analysis

The protein bands separated by native gel electrophoresis were excised, distained with a solution containing 50% of acetonitrile (ACN) and ammonium bicarbonate 25 mM. Samples were dehydrated in acetonitrile 100%, lyophilized in a SpeedVac, and digested with porcine trypsin (10 μg/ml) (Promega Co., Madison, WI) overnight at 37 °C. The peptides were extracted using 30 μl of ACN 50% and trifluoroacetic acid (TFA) 5% for 10 min in an ultrasonic water bath. The supernatant was collected, lyophilized in a SpeedVac and mixed with 2 μl TFA 0.1% and 1 μl of matrix (α-cyano 4-hydroxycinnamic acid). The samples were spotted on a MALDI plate (Bruker Daltonics) and the mass spectra obtained in a MALDI-TOF–MS Autoflex spectrometer (Bruker Daltonics, Bremen, Germany) using a positive reflector mode, accelerating voltage of 20 kV, delay time of 150 ns and acquisition mass-range 800–3200 Da. The spectra were analyzed by the FlexAnalysis 2.0 software (Bruker Daltonics, Bremen, Germany) and Mascot Program.

## 3. Results and discussion

### 3.1. In vitro GlnB uridylylation

The $P_{II}$ proteins are found in solution as trimers. The uridylylation of *A. brasilense* GlnZ by GlnD (Utase/UR) generates trimers containing one, two or three subunits linked to UMP that can be resolved in a non-denaturing gel electrophoresis: as uridylylation increases the protein mobility due to the

Download English Version:

https://daneshyari.com/en/article/4392300

Download Persian Version:

https://daneshyari.com/article/4392300

Daneshyari.com