Volume 330, number 1, 95–98 FEBS 12944 September 1993

Uridylylation of the P_{II} protein in *Rhizobium leguminosarum*

Sergio Colonna-Romano, Eduardo Jorge Patriarca, Mohamed Amar, Paola Bernard, Giuseppe Manco, Alessandro Lamberti, Maurizio Iaccarino, Roberto Defez*

International Institute of Genetics and Biophysics, CNR Via Marconi 10, 80125 Naples, Italy

Received 13 July 1993; revised version received 22 July 1993

Permeabilization with cetyl trimethyl ammonium bromide was used to study the post-translational modification of the P_{II} protein in *Rhizobium leguminosarum*. Upon incubation with radioactive UTP a single band was obtained after SDS-PAGE and autoradiography. RNase resistance and snake venom phosphodiesterase sensivity showed that radioactivity was bound through a phosphodiester bond to a protein which was absorbed by an antiserum specific for the P_{II} protein. Uridylylation of the P_{II} protein was shown to be dependent on the modifications of the glutamine/α-ketoglutarate ratio.

Uridylylation; GS cascade; P_{II} protein

1. INTRODUCTION

In enteric bacteria, transcription of *glnA* (encoding for glutamine synthetase, GS) and of the *ntr* (nitrogen regulation) regulon is activated in response to nitrogen limitation [1]. Regulation is achieved by a two-component system in which NtrC (NRI) is the transcriptional activator, while NtrB (NRII) catalyzes the phosphorylation and dephosphorylation of NtrC. The regulation of kinase and phosphatase activities of NtrB depends upon the availability of the *glnB* gene product, the P_{II} protein, which elicits the phosphatase activity of NtrB. Under conditions of nitrogen limitation, when phosphatase activity is not needed, P_{II} is inactivated by reversible uridylylation. Conversely, under conditions of nitrogen excess P_{II} interacts with NtrB and the resulting phosphatase activity causes inactivation of NtrC, thus preventing activation of σ^{54}-dependent promoters [1]. Moreover, in response to intracellular signals of nitrogen availability, P_{II} protein regulates the activity of GS through adenylylation/deadenylylation [2–4]. The uridylylated form of P_{II} promotes the adenylyltransferase (ATase)-dependent deadenylylation of GS, while the unmodified form promotes the ATase-dependent adenylylation [5,6]. Under conditions of nitrogen limitation, covalent attachment of UMP to a tyrosyl residue at each of the four P_{II} subunits [6,7] is catalyzed by uridylyltransferase (UTase), while under conditions of nitrogen excess, hydrolysis of the bound UMP is catalyzed by the uridylyl-removing enzyme (UR) [6,8]. Both UTase and UR activities are due to the same enzyme complex, encoded by *glnD* [9].

Bacteria of the genus *Rhizobium* contain genes coding for three different glutamine synthetase isozymes: GSI (the homologous of enteric GS), GSII and GSIII ([10] and references quoted therein), among which only GSI activity appears to be regulated by adenylylation [11]. A *glnB* gene has been found in *R. leguminosarum* biovar *viciae* (hereafter called *R. l. viciae*) tightly linked to *glnA*, the structural gene for GSI [12,13] and it is possible that its gene product may have a role analogous to that exerted in enteric bacteria.

In this paper we show that the P_{II} protein of *R. l. viciae* may be uridylylated and that uridylylation is modulated by the intracellular glutamine/α-ketoglutarate ratio. This evidence is obtained with permeabilized bacteria and therefore the results are closer to physiological conditions than what previously published with pure proteins [6,9]. The method described may probably be used in the study of post-translational modifications of other proteins.

2. MATERIALS AND METHODS

2.1. *Antiserum preparation*

In order to raise a rabbit antiserum against P_{II}, a 435 bp *Pst*I–*Pst*I fragment obtained from a subclone of plasmid pMG10 [14] and containing 88 C-terminal codons of the *glnB* open reading frame, was cloned into the *Pst*I restriction site of pEX34C [15]. The resulting expression plasmid (pCS149) was used to transform *E. coli* strain M72 Sm^r *lacZ*am *Δbio-uvrB ΔtrpEA2* (*λN*am7-*N*am53*c*I857*Δ*H1) [16] containing a temperature-sensitive mutant of the cI λ repressor gene inserted into the chromosome. Plasmid pCS149 encodes a fusion protein between the N-terminal part of the MS2-polymerase and the P_{II} protein lacking the 23 N-terminal aminoacids, the expression of which is controlled by the heat inducible (3 h at 42°C) phage λ PL promoter. The MS2 polymerase-P_{II} fusion protein, m w. 22,000 Da, was recovered and purified as described [15] except that bacterial proteins were

*Corresponding author. Fax: (39) (81) 725 7202.

Abbreviations. CETAB, cetyl trimethyl ammonium bromide; SVPD, snake venom phosphodiesterase.

dissolved in 7 M urea and loaded on a reverse-phase HPLC (C8) column (Pharmacia), eluted with a gradient of 0 to 70% acetonitrile in 0.1% trifluoroacetic acid. The major peak corresponding to the fusion protein was collected and concentrated under vacuum. Purity was shown by SDS-PAGE to be greater than 98%. A rabbit was primed with 600 μg of protein suspended in Freund's complete adjuvant, boosted three times at intervals of 2 days with the same protein amount and finally with about 2 mg. The serum was recovered and used in immunoblot experiments essentially as described [17].

2.2. *Preparation of crude extracts*

R. l. viciae strain LPR1105 [18] was grown in RMM minimal medium [18] supplemented with KNO_3 or NH_4Cl as nitrogen sources at a concentration of 1 $g \cdot l^{-1}$. For metabolic shock experiments, bacteria were grown on KNO_3 up to $OD_{590} = 0.6$, then 1 $g \cdot l^{-1}$ of NH_4Cl was added and growth prolonged for 4 h. Bacteria were harvested by centrifugation, washed in 0.9% NaCl, divided into aliquots and then frozen at −20°C when used in immunoblot experiments, or immediately processed for detection of P_{II} uridylylation. Bacterial lysis was performed by sonic oscillation as described [11] and protein concentration was determined by the method of Lowry [19].

2.3. *Polyacrylamide gel electrophoresis*

Crude extracts were fractionated on 15% SDS-polyacrylamide gels. After electrophoresis gels were blotted for immunological analysis, or immediately vacuum dried and autoradiographed for labelling detection. After blotting, proteins were revealed by Coomassie blue on post-blotted gels or by the Ponceau red staining on nitrocellulose filters.

The molecular weight of the bands reported in Figs. 1 and 2 was calculated by interpolation of data obtained with SDS-PAGE standards (Bio-Rad): aprotinin (6,500 Da), lysozyme (14,400 Da), trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), ovalbumin (45,000 Da) and serum albumin (66,200 Da).

2.4. *Uridylylation*

To determine uridylylation, bacterial pellets from a 10 ml culture of *R. l viciae* grown on KNO_3, with or without NH_4Cl shock, were resuspended in 270 μl of 50 mM 2-methyl-imidazole buffer pH 7.6 containing 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM ATP and 10 μg/ml of cetyl trimethyl ammonium bromide (CETAB) and incubated on ice for 20 min in order to allow membrane permeabilization. Labelling was started by addition of 1 μl of [α-^{32}P]UTP (800 Ci/mmol) and 30 μl of H_2O, for 2 h at 30°C. In some samples H_2O was replaced with 30 μl of 180 mM α-ketoglutarate, while in other samples, after 2 h of incubation with α-ketoglutarate and [α-^{32}P]UTP, 30 μl of 200 mM L-glutamine were added and incubation was continued for a further 2 h. Reactions were stopped in ice, bacteria were collected by centrifugation and washed in 50 mM 2-methyl-imidazole buffer to remove unincorporated UTP. After lysis by sonic oscillation, samples were fractionated on 15% SDS-PAGE. Treatment of protein extracts either with snake venom phosphodiesterase (SVPD) (500 μg/ml) or with RNase (50 μg/ml) for 1 h was performed in some cases.

2.5. *Immunoabsorption*

Aliquots (20 μl) of antiserum or preimmune serum were reacted overnight at 4°C with 100 μl bed volume of preswollen protein A-Sepharose CL-4B and 400 μl of a solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5%, Na deoxycholate, 0.1% ovoalbumine and 0.1% methionine. After washing with 1 ml of the above solution to remove unbound material, the Sepharose-bound antibodies were incubated with 500 μg of extract labelled with [α-^{32}P]UTP in a total volume of 600 μl for 2 h at 4°C. After centrifugation an aliquot of the supernatant was electrophoresed on 15% SDS-PAGE.

2.6. *Strains overexpressing the glnB gene*

R. l. viciae mutants carrying a constitutive copy of the *glnB* gene were constructed by subcloning an *Eco*RV–*Bam*HI 720 bp DNA fragment obtained from a subclone of pMG10 [14], containing the *R. l viciae glnB* gene devoid of its promoter, into the *Bam*HI site of the Tn*5* insert of the suicide plasmid pSUP1011 [20]. Restriction map experiments on several of these plasmids demonstrated that one of these, named pCS147, carried the *glnB* gene fragment in the same orientation as the *kan* gene [21]. After conjugation from *E. coli* strain S17-1 [20] into *R. l. viciae*, ten independent neomycin resistant (Neo^r) chloramphenycol sensitive (Cm^s) colonies were isolated. The Neo^r and Cm^s phenotypes indicate that these strains contain the Tn*5* transposon, but not the suicide pSUP1011 plasmid. DNA hybridization experiments show that these strains contain an extra copy of *glnB* at different locations on the chromosome. One of these was named CS101 and used for further studies.

3. RESULTS

3.1. *Immunodetection of the P_{II} protein*

When crude extracts from the *R. l. viciae* wild type strain grown on KNO_3 or NH_4Cl were fractionated on 15% SDS-PAGE, blotted and tested with the antiserum against the P_{II} fusion protein a single band with an apparent molecular weight of 11,500 Da was observed (Fig. 1, lanes 1 and 2). No signal was detected with an extract of strain BS11, a *R. l. viciae* mutant altered in the *glnB* gene (Fig. 1, lane 3). Strain CS101, carrying a second copy of the *glnB* gene under the control of the *kan* gene promoter of Tn*5* inserted on the chromosome (see section 2) showed a more intense signal, even if 10-fold less proteins were used (Fig. 1, lane 4). No immunoreaction was evident in parallel experiments using either the preimmune serum or an antiserum raised against the MS2-polymerase protein (not shown).

It should be noted that the relative amount of immunoreactive material revealed by the antiserum on protein extracts from *R. l. viciae* grown on NH_4Cl is one third as compared to the signal obtained when bacteria are grown on KNO_3 (Fig. 1, lanes 1 and 2). The signal does not decrease further when *R. l. viciae* is grown in NH_4Cl plus 1 $g \cdot l^{-1}$ glutamine (not shown). To increase sensitivity, in the experiments described below P_{II} labelling was performed on KNO_3 grown bacteria.

3.2. *Uridylylation of the P_{II} protein*

Fig. 2 shows the autoradiography of *R. l. viciae* protein extracts fractionated on 15% SDS-PAGE after [α-^{32}P]UTP incubation of cells permeabilized with CETAB under the conditions described in section 2.

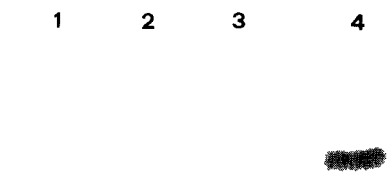

Fig. 1. Immunodetection of P_{II} protein. Lanes 1 and 2, 300 μg of protein from wild type *R. l viciae* grown on KNO_3 or NH_4Cl; Lane 3, 300 μg of protein from *R. l viciae* strain BS11 (*glnB*$^-$) grown on glutamate; Lane 4, 30 μg of protein from *R. l viciae* strain CS101 grown on NH_4Cl.

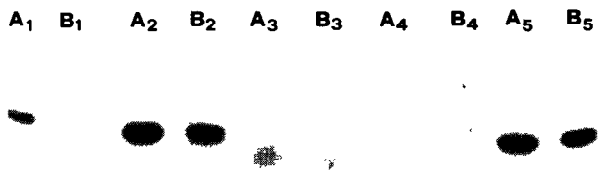

Fig. 2. Autoradiogram of *R. l viciae* extracts after labeling with [α-^{32}P]UTP on permeabilized cells. Lanes A, bacteria pre-grown on KNO_3; lanes B, after growth on KNO_3 bacteria were shocked for 4 h on NH_4Cl; lanes 1, [α-^{32}P]UTP plus H_2O; lanes 2, [α-^{32}P]UTP plus α-ketoglutarate; lanes 3, [α-^{32}P]UTP plus α-ketoglutarate followed by glutamine; lanes 4, as in lanes 2, followed by a treatment with SVPD; lanes 5, as in lanes 2, followed by a treatment with RNase.

When [α-^{32}P]UTP incubation was performed with bacteria permeabilized after growth on KNO_3 a single band of about 11.5 kDa was revealed (lane A_1). This band was not detected if the cells were subjected to a metabolic shock with NH_4Cl before permeabilization (lane B_1). No labelling was observed when the incorporation was performed in the presence of an excess of cold UTP (660 μM), or when the extracts were immunoabsorbed with the antiserum raised against the P_{II} protein (not shown).

When [α-^{32}P]UTP incorporation was performed in the presence of 18 mM α-ketoglutarate the band was more intense (lane A_2) and a signal of similar intensity was observed even with bacteria pretreated with NH_4Cl (lane B_2). No labelling was observed when α-ketoglutarate treated bacteria were incubated for further two hours in the presence of 18 mM glutamine (lanes A_3 and B_3). After [α-^{32}P]UTP incubation, treatment with SVPD (lanes A_4 and B_4) abolished labelling, while treatment with RNase (lanes A_5 and B_5) was ineffective.

4. DISCUSSION

Results reported in this paper with the combined use of a proper labelled precursor and an antiserum show that the P_{II} protein of *R. l. viciae* is uridylylated in permeabilized bacteria and that this post-translational modification is under the control of the intracellular concentration of glutamine and α-ketoglutarate.

The expression of P_{II} in extracts of *R. l. viciae* grown on KNO_3 is 3-fold higher than in extracts from bacteria grown on NH_4Cl. Thus, the *glnB* gene, which is involved in the regulation of nitrogen metabolism (M. Amar et al., submitted for publication) is itself nitrogen regulated, while in *E. coli* it is constitutive [22]. Since P_{II} in enteric bacteria is essential for inactivation of the NtrC transcriptional activator we suggest that the high level of P_{II} in bacteria grown in KNO_3 is useful for a rapid inactivation of NtrC when a rich nitrogen source is added to a nitrogen poor medium.

The *R. l. viciae* and *E. coli* P_{II} proteins show a high degree of similarity and Tyr-51, the site of uridylylation, is conserved [12] suggesting that the *R. l. viciae* P_{II} protein may be uridylylated. We find that incubation with radioactive UTP gives a band of the expected size that is chased out by an excess of cold UTP and removed by immunoabsorption. Radioactivity is removed by phosphodiesterase and it is resistant to RNase. We conclude that the radioactive band is P_{II}-UMP. Therefore, the permeabilized cells permit to use the bacterial enzymatic machinery without purification of the proteins involved. It may be seen that preincubation with NH_4Cl prevents uridylylation, while addition of α-ketoglutarate overcomes the NH_4Cl effect. Finally, further incubation with glutamine abolishes labelling. We believe that the band obtained after incubation with radioactive UTP of bacteria pregrown in KNO_3 (lane A_1) is due to uridylylation turnover and that the NH_4Cl shock (lane B_1) causes deuridylylation, while α-ketoglutarate preincubation gives maximum uridylylation and/or turnover. In conclusion, uridylylation of P_{II} in *R. l. viciae* is controlled by the glutamine/α-ketoglutarate ratio, thus suggesting the presence of a *glnD*-like gene product. This regulatory mechanism should be of value in the *Rhizobium*–legume symbiosis since the carbon and nitrogen availability change as a consequence of the day/night period.

Acknowledgements. We thank C. Sole and N. Indaco for technical assistance; G. Blasi for preparing the manuscript M. A. was the recipient of a UNIDO-ICGEB fellowship. This study was partially supported by grants from MAF-DPGA, EEC CII-0408 and BIOT-0166-C(EDB) and RAISA-CNR Subproject 2 (paper no. 1090).

REFERENCES

[1] Magasanik and Neidhardt (1987) in: *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umbarger, H.E., Eds.) pp 1318–1325. American Society for Microbiology, Washington, DC.

[2] Mecke, D., Wulff, K., Liess, K. and Holzer, H. (1966) Biochem Biophys. Res. Commun. 24, 452–458.

[3] Kingdon, H.S., Shapiro, B.M. and Stadtman, E.R. (1967) Proc. Natl. Acad. Sci. USA 58, 1703–1710.

[4] Holtel, A. and Merrick, M. (1989) Mol. Gen. Genet. 217, 474–480.

[5] Chock, P.B. and Stadtman, E.R. (1977) Proc. Natl. Acad Sci. USA 74, 2766–2770.

[6] Adler, S.P., Purich, D. and Stadtman, E.R (1975) J. Biol. Chem. 250, 6264–6272.

[7] Kingdon, H.S., Hubbard, J.S. and Stadtman, E.R. (1968) Biochemistry 7, 2136–2143.

[8] Magnum, J.H., Magni, G. and Stadtman, E.R. (1973) Arch. Biochem. Biophys. 158, 514–525.

[9] Rhee, S.G., Huang, C.Y., Chock, P.B. and Stadtman (1978) Anal. Biochem. 90, 752–766.

[10] Chiurazzi, M., Meza, R., Lara, M., Lahm, A., Defez, R., Iaccarino, M. and Espin, G. (1992) Gene 119, 1–8.

[11] Rossi, M., Defez, R., Chiurazzi, M., Lamberti, A., Fuggi, A. and Iaccarino, M. (1989) J. Gen Microbiol. 135, 629–637.

[12] Colonna-Romano, S., Riccio, A., Guida, M., Defez, R., Lamberti, A., Iaccarino, M., Arnold, W., Priefer, U. and Pühler, A. (1987) Nucleic Acids Res. 15, 1951–1963.

[13] Holtel, A., Colonna-Romano, S., Guida, M., Riccio, A., Merrick, M.J. and Iaccarino, M (1989) FEMS Microbiol Lett. 58, 203–208

[14] Filser, M.M.K., Moscatelli C., Lambertı, A., Vincze, E., Guıda, M., Salzano, G. and Iaccarıno M (1986) J. Gen. Microbiol. 132, 2561–2569.
[15] Strebel, K., Beck, E., Strohmaier, K and Schaller, H. (1986) J. Virol 57, 983–991.
[16] Bernard, H.-V., Remaut, E., Hershfield, M.V., Das, H.K., Helinskı, D.R., Yanofsky, C. and Franklin, N. (1975) Gene 5, 59–76.
[17] Patrıarca, E.J., Chiurazzi, M , Manco, G., Rıccio, A., Lamberti, A., De Paolis, A., Rossı, M., Defez, R. and Iaccarino, M. (1992) Mol. Gen. Genet 234, 337–345.
[18] Hooykaas, P.J.J., Klapwijk, P M , Nutı, M.P, Schilperoort, R.A. and Roersch, A. (1977) J. Gen. Mıcrobiol. 98, 477–481.
[19] Lowry, D., Rosebrough, N., Farr, A. and Randall, R. (1951) J. Biol. Chem. 193, 265–275.
[20] Simon, R., Priefer, U. and Pühler, A (1983) Biotechnology 1, 784–791.
[21] Mazodıer, P., Cossart, P, Giarud, E. and Gasser, F. (1985) Nucleıc Acıds Res. 13, 195-205.
[22] Van Heeswijk, W.C., Rabenberg, M., Westerhoff, H.V and Kahn, D (1993) *Mol Mıcrobiol*, ın press.