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An additional P_{II} in *Escherichia coli*: a new regulatory protein in the glutamine synthetase cascade

Wally C. van Heeswijk^{a,b,1}, Brenda Stegeman^{b,2}, Sjouke Hoving^{b,1},
Douwe Molenaar^{b,3}, Daniel Kahn^c, Hans V. Westerhoff^{a,b,*}

^a E.C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands

^b Division of Molecular Biology (H5), The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands

^c Laboratoire de Biologie Moléculaire des Relations-Plantes Microorganismes, CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex, France

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Abstract

The P_{II} protein in the glutamine synthetase cascade transduces the nitrogen signal, as sensed by uridylyltransferase, both to the NRII/NRI two-component system and to adenylyltransferase, to regulate the activity of glutamine synthetase. Here we describe the amplification of a chromosomal DNA fragment from *Escherichia coli* which contains the sequence of a P_{II} homologue. The derived amino acid sequence of this DNA fragment is 67% identical to *E. coli* P_{II} . It contains the conserved tyrosine residue which is known to be the site of uridylylation in P_{II} . *E. coli* is the first organism in which two different P_{II} proteins have been detected.

Keywords: P_{II} -protein; *GlnB*; Glutamine synthetase; Signal transduction; Ammonia assimilation; Nitrogen regulation; *Escherichia coli*

1. Introduction

In enteric bacteria, ammonia assimilation is regulated by the nitrogen status of the cell. The control of glutamine synthetase (GS) is important and involves

transcriptional regulation, covalent modification and metabolite levels. The nitrogen status of the cell is determined by the relative concentrations of glutamine and 2-oxoglutarate which is sensed by the enzyme uridylyltransferase (UTase). To regulate the transcription of the gene encoding glutamine synthetase, *glnA*, the signal is transduced via P_{II} and NRII to the transcriptional activator NRI. Via P_{II} the signal is also transduced to adenylyltransferase (ATase), which can modify glutamine synthetase from the native (active) form to the adenylylated (inactive) form and vice versa (see [1] for a review). In recent decades biochemical and genetic analyses have unravelled the system at the molecular level, and the regulatory network was considered to have been mapped completely. For many years it has

* Corresponding author. Tel: +31 (20) 444 7228; Fax: +31 (20) 444 7229; E-mail: hw@bio.vu.nl

¹ Present address: Department of Microbial Physiology, Faculty of Biology, Free University, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands.

² Present address: Department of Endocrinology, Wilhelmina Children Hospital, Postbus 18009, NL-3501 CA Utrecht, The Netherlands.

³ Present address: Kernforschungs-Anlage Jülich, IBT 1, Postfach 1913, D 52405 Jülich, Germany.

appeared as a textbook example of cascade regulation. We started to analyse the glutamine synthetase cascade in the light of the modular metabolic control analysis [2]. When attempting to quantify the control exerted by P_{II} on the adenylation of glutamine synthetase we were surprised to find that its control was very small (unpublished observation). This report describes the identification of an additional P_{II} protein which adds an extra regulatory component to this already sophisticated system.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli wild-type strains YMC10 or RB9010 and the *glnB*-deletion strain RB9060 [3] were grown in 22 mM glucose-40 mM MOPS medium [4] with 14 mM glutamine or 14 mM glutamine and 14 mM NH_4Cl as nitrogen source to an OD_{600} of 0.3.

2.2. Gel electrophoresis and Western analysis

Cell suspensions, equivalent to 10 μg of protein, were loaded onto SDS-PAGE (15% acrylamide) mini-gel (Biorad). For purified P_{II} (purification essentially as in [5]), 2.6 ng protein in 0.75 mg ml⁻¹ BSA, was used. The gel was blotted onto nitrocellulose. The result was probed with polyclonal P_{II} antibody and the bands were visualized using the ECL system of Amersham.

2.3. P_{II} antibody

5 mg of purified P_{II} (5 mg ml⁻¹) stored in 50% glycerol and 50 mM Tris-HCl, pH 7.6 was dialysed against 0.1 M $NaHCO_3$, pH 8.3 and 0.5 M NaCl for 16 h at 4°C. The dialysed P_{II} protein was covalently bound to CNBr-activated Sepharose-4B according to the manufacturer (Pharmacia). Polyclonal P_{II} antibody diluted in PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.2) [6] was bound to the P_{II} -sepharose column. On a Western blot the eluent no longer stained a band at the position of P_{II} , yet showed many cross-reacting bands at other locations (data not shown). After washing

the column with PBS, the P_{II} antibody was eluted with 10 mM NaOH. The eluent was directly neutralized with 0.1 M HCl and dialysed against PBS for 16 h at 4°C. Staining of bands at other locations than P_{II} , was greatly reduced as compared with the unpurified antibody (data not shown).

2.4. DNA amplification and Southern analysis

Degenerated oligonucleotides: 1, 5'-AT(T/C)AT(T/C)AA(A/G)CC(A/G)TT(T/C)AA(A/G)CT-GGA(T/C); 2, 5'-CA(T/C)AC(G/C)GA(A/G)C-TGTA(T/C)CG(T/C)GG(T/C); 3, 5'-(A/G)AA-(A/G)AT(T/C)TT(A/G)CC(A/G)TC(A/G)CC-(A/G)AT; and 4, 5'-(T/C)TC(A/G)CC(T/G)GT-(A/G)CG(A/G)AT(A/G)CG(A/G)AT (Isogen Bioscience) were synthesized. PCR (annealing temperature 37°C, oligonucleotide 150 ng/reaction), employed chromosomal DNA isolated from strain RB9010 or from the *glnB* deletion strain RB9060 [7]. The PCR incubations were loaded onto a 1.5% agarose gel including ethidium bromide, blotted with 0.4 M NaOH on a Hybond-N⁺ filter (Amersham) and the result was probed with a digoxigenin (Boehringer Mannheim) labeled *glnB* fragment (a 915 bp *SacII* and *SalI* fragment of pDK601 [8]). Hybridisation was performed in a 6^{*} SSC incubation buffer [7] at 50°C. The filter was washed three times with 6^{*} SSC and 0.1% SDS at 50°C and the DNA fragments were visualized with the chemiluminescent substrate CSPD (Tropix) using a Kodak X-OMAT AR film.

2.5. Cloning of the amplified fragment and sequence analysis

The amplified DNA fragment obtained from strain RB9060 by using oligonucleotide number 1 and 4, was cut from a 1.5% low melting point (LMP) agarose gel and re-amplified using new degenerated oligonucleotides with an extension of a *XbaI* or *EcoRI* restriction site (5'-GCTCTAGAGAT(T/C)A-T(T/C)AA(A/G)CC(A/G)TT(T/C)AA(A/G)C-TGGA(T/C) and 5'-CGGAATTCC(T/C)TC(A/G)CC(T/G)GT(A/G)CG(A/G)AT(A/G)CG(A/G)AT respectively). The PCR reaction was as described above with 1 μl of DNA containing melted LMP-agarose. The re-amplified DNA fragment was

cloned into pBluescript-II-SK + (Stratagene) digested with *Xba*I and *Eco*RI resulting in plasmid pWVH140. The cloned fragment was sequenced using the Applied Biosystems 373A DNA sequenator.

2.6. Glutamine synthetase adenylation assay

The level of adenylation state (n) of glutamine synthetase was determined by the γ -glutamyl transferase assay [9], performed in microtiterplates. Cultures were grown overnight in 35 ml MOPS-glucose-glutamine medium to an OD₆₀₀ of 0.3 and the cells were exposed to 30 mM NH₄Cl for 15 min or grown overnight in 300 ml MOPS-glucose-glutamine-ammonia medium. Cells were chilled on ice for 10 min, harvested by centrifugation and washed twice in icecold MOPS-14 mM glutamine medium (without glucose). The pellet was resuspended in 100 μ l MOPS-glutamine. For time zero 5 μ l of this suspension was added to 0.8 ml icecold MOPS-glutamine, mixed and pipetted in liquid nitrogen. The remainder of the cell suspension was added to 30 ml prewarmed (37°C) MOPS-22 mM glucose medium (without nitrogen) at time zero. An aliquot of 0.8 ml was pipetted in liquid nitrogen after 45 s. Samples were stored at –80°C. To inactivate adenylyl-transferase, 3.2 ml 0.67 mg ml^{–1} CTAB (hexadecyltrimethylammoniumbromide) in PBS was added to the cells and thawed in ice-water with occasional vortexing. Cells were centrifuged and the pellet was washed with 1 ml icecold imidazole buffer (10 mM imidazole pH 7.3, 0.5 mM MgCl₂ and 0.1 mg ml^{–1} CTAB). After centrifugation the cells were resuspended in 50 μ l imidazole buffer.

3. Results

3.1. Identification of a P_{II}-like protein

Unexpectedly, when the *glnB* deletion strain RB9060 was grown in minimal medium with glutamine as nitrogen source, a Western blot of a cell extract did show a band cross-reacting with the P_{II} antibody, with a molecular weight similar to that of P_{II} (Fig. 1). As shown in Fig. 1, the P_{II} antibody shows abundant cross-reactivity with other proteins. The P_{II} antibody was purified via a P_{II} affinity

Fig. 1. Western blot, probed with P_{II} antibody, after SDS-PAGE of whole cell lysates of wild-type strain RB9010 (lane 2) and the *glnB* deletion strain RB9060 (lane 3). Lane 1, purified P_{II}.

column. After this purification, probing similar Western blots showed again a cross-reacting band, with the same molecular weight as P_{II} (data not shown). The impossibility of removing the cross-reactivity of the polyclonal P_{II} antibody by affinity purification suggests the existence of a second P_{II}-like protein in *E. coli*.

3.2. Identification of a *glnB*-homologous gene fragment

To detect a possible *glnB* homologue in *E. coli*, polymerase chain reactions (PCR) were performed with chromosomal DNA isolated from wild-type cells and from the *glnB* deletion strain RB9060. For the PCR reaction, two forward and two reverse degenerated oligonucleotides were used which covered the most conserved amino acid regions among the P_{II} proteins of different bacteria [10,11]. The reverse primers should hybridize at the 3'-coding part of the *glnB* gene which is absent from strain RB9060 [8]. With all four primer combinations, PCR generated a DNA fragment on gel, both with chromosomal DNA from wild-type cells and with DNA of the *glnB* deletion strain (data not shown). With every primer combination the amplified fragment from strain RB9060 had the same size as the amplified fragment from the wild-type strain. To confirm the authenticity of the PCR fragments from RB9060, the gel was Southern blotted and probed with a labeled *glnB*

Fig. 2. Southern blot, probed with a *glnB* fragment, after electrophoresis of the amplified fragments of wild-type strain RB9010 (lane 2) and the *glnB* deletion strain RB9060 (lane 3). Lane 1, PCR without DNA. PCR with oligonucleotide number 1 and 4.

fragment. The hybridizing DNA fragments from strain RB9060 and RB9010 were similar in size using the same set of primers (Fig. 2). This indicates that the gene encoding the putative P_{II} -homologue is of a similar length as the *glnB* gene. The DNA fragment from strain RB9060, amplified using primers 1 and 4, was re-amplified using oligonucleotides with an extension of a *Xba*I or *Eco*RI restriction site. The resulting PCR DNA fragment was cloned into pBluescript-II-SK+. Sequence analysis of the cloned fragment (Fig. 3) revealed that

Table 1

GS adenylylation state (n) after removal of ammonia

	Medium without NH_4^+		Medium with NH_4^+	
	YMC10	RB9060	YMC10	RB9060
$t = 0$ s	11.4	11.4	8.1	10.5
$t = 45$ s	6.7	8.5	4.2	9.7

the derived amino acid sequence is identical to that of *E. coli* P_{II} protein for 67%.

3.3. When P_{II} is absent, the P_{II} -like protein may control adenylyl-transferase

The similarity of the new P_{II} -like protein to P_{II} suggests that the two may carry out the same or similar functions. Indeed we were first attracted to the possible redundancy of P_{II} when studying the deadenylylation of glutamine synthetase upon deprivation of ammonia. In the wild-type strain YMC10 glutamine synthetase was deadenylylated, as expected. In the *glnB* deletion strain (RB9060) grown on minimal medium with glutamine as nitrogen source and exposed to ammonia for 15 min, glutamine synthetase was still deadenylylated when the ammonia was removed (Table 1). This suggests that the P_{II} -like protein, which was indeed still present (Fig. 1) could substitute for P_{II} in transducing the

A

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GAGCTTCGTG AAGCGTTATC TTCCATTGGT ATTCAGGGCC TGACCGTCAC CGAAGTGAAA GGTTTCGGGC GTCAGAAAGG GCATGCCGAG CTGTACCGGG 100
D V R E A L S S I G I Q G L T V T E V K G F G R Q K G H A E L Y R G

GGGCGGAATA CAGCGTCAAT TTCTGCGCAA AAGTAAAAAT TGATGTGGCG ATTGCTGATG ACCAACTCGA TGAAGTGATC GATATCGTCA GTAAGCGCGG 200
A E Y S V N F L P K V K I D V A I A D D Q L D E V I D I V S K A A

TTACACCGGA AAAATTGGCG ACGGCAAAAT CTTCGTCGCT GAATTGCAAC GCGTC 255
Y T G K I G D G K I F V A E L Q R V

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B

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                                UMP
                                |
PII      DVREALNEVG I N S L T V T E V K G F G R Q K G H E L Y R G A E Y V D F L P K V K I E I V V P D D I V D T C V D P I I R T A L T G K I G D G K I F V D V A R V
PII-homologue DVREALSSIG I S L E V T E V K G F G R Q K G H E L Y R G A E Y V N F L P K V K I D V A I A D D Q L D E V I D I V S K A A E L Q R V

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Fig. 3. (A) Nucleotide sequence of a PCR fragment corresponding to the P_{II} homologue and its deduced amino acid sequence. (B) Amino acid alignment of the P_{II} homologue and the *E. coli* P_{II} protein. Amino acids identical in the two sequences are boxed. The uridylylatable tyrosine in P_{II} is marked with UMP.

signal of ammonia deprivation to the ATase. When the *glnB* deletion strain was grown on ammonia containing medium, the P_{II} -like protein was absent (data not shown). Accordingly, when those cells were deprived of ammonia, GS deadenylation was not activated (Table 1).

4. Discussion

The nucleotide sequence of the PCR fragment demonstrates the existence of a P_{II} -like protein in *E. coli* in terms of amino acid sequence homology. The derived amino acid sequence of the amplified fragment also contains the conserved tyrosine residue, which is known from P_{II} to be the site of uridylation depending on the nitrogen status of the cell [5,12]. The high amino acid sequence identity between the P_{II} homologue and P_{II} might explain why it was impossible to purify the polyclonal P_{II} antibody to P_{II} specificity. The P_{II} -like protein, of which the gene has been cloned here, is not only homologous at the amino acid sequence level but is also a functional P_{II} homologue, the expression of which depends on the growth medium (unpublished observation). As far as we know *E. coli* is the first organism in which two P_{II} proteins have been shown to function. Although the glutamine synthetase regulatory cascade as described in the textbooks is an elegant system to respond to the nitrogen status of the cell, it is apparently not the whole story.

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