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Escherichia coli P_{II} protein: purification, crystallization and oligomeric structure

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Abstract

The *Escherichia coli* signal transduction protein P_{II}, product of the *glnB* gene, was overproduced and purified. The predicted molecular weight of the protein based on the correct nucleotide sequence is 12,427 and is very close to the value 12,435 obtained by matrix-assisted laser desorption mass spectrometry. Hexagonal crystals of the unuridylylated form of P_{II} with dimensions $0.2 \times 0.2 \times 0.3$ mm were grown and analysed by X-ray diffraction. The crystals belong to space group $P6_3$ with $a = b = 61.6$ Å, $c = 56.3$ Å and V_m of 2.5 for one subunit in the asymmetric unit. A low-resolution electron density map showed electron density concentrated around a three-fold axis, suggesting the molecule to be a trimer. A sedimentation equilibrium experiment of the meniscus depletion type was used to estimate a molecular weight of 35,000 ± 1,000 for P_{II} in solution. This result is consistent with the native protein being a homotrimer.

Key words: Signal transduction; Nitrogen metabolism; Crystallisation; *glnB*

1. Introduction

Protein P_{II} plays a critical role in regulation of nitrogen metabolism in *Escherichia coli* by controlling the level and activity of glutamine synthetase (product of *glnA*), which catalyses the ATP-dependent assimilation of ammonia with glutamate to form glutamine [1,2]. When the level of nitrogen is high (> 1 mM NH_3), P_{II} acts with adenylyl transferase (product of *glnE*) to covalently modify glutamine synthetase subunits to progressively inactivate the dodecameric enzyme. At high concentrations of NH_3, P_{II} also lowers the level of glutamine synthesis by repressing expression of the *glnALG* operon mediated by the regulatory proteins NR_{II} and NR_I (products of *glnL* and *glnG*, respectively) [3]. When the level of nitrogen is low (< 1 mM NH_3) P_{II} is uridylylated at tyrosine-51 to form P_{II}-UMP [4]. The interaction of P_{II}-UMP with adenylyl transferase reconverts the modified glutamine synthetase to the active unmodified enzyme. The absence of unuridylylated P_{II} also causes NR_{II} to phosphorylate NR_I and in turn activate the transcription of the *glnALG* operon from a strong promoter using the nitrogen regulated σ-54 factor for transcription [5]. The signalling role of P_{II} in the regulation of glutamine synthetase thus involves at least five different protein–protein interactions. We intend to examine the structure of P_{II} as a first step in understanding the interactions between it and the other proteins in the cascade regulation of glutamine synthetase.

P_{II} in other species is also thought to play a critical role via phosphorylated NR_I in transcriptional regulation of *nifLA*, whose products are required for the activation of proteins that carry out nitrogen fixation [6]. It was recently shown that P_{II} in the nitrogen fixing bacterium *Rhizobium leguminosarum* is uridylylated, which supports the notion that the protein is involved in an analogous signal transduction role in that species [7].

We have reported previously that the gene that encodes P_{II} (*glnB*) occurs downstream of *hmp*, the gene that codes for a haemoglobin-like protein with dihydropteridine reductase activity [8]. We corrected several errors in the *glnB* sequence of an earlier report [4]. In this work we have cloned the *glnB* gene in an expression vector to overproduce the protein, purified it by a convenient

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LB, Luria-Bertani (medium); PAGE, polyacrylamide-gel electrophoresis; PCMBS, 4-chloro-mercuriphenylsulfonic acid; SDS, sodium dodecyl sulfate.

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method, and grown crystals of unuridylylated P_{II} that are suitable for high-resolution structure determination by X-ray crystallography. In contradiction to a long held view that native P_{II} is a tetramer, we now show that it is a homotrimer.

2. Materials and methods

2.1. Construction of expression plasmid pCG646

The plasmid pCG646 was constructed by insertion of a 1338-bp *Eco*RI-*Sma*I fragment from pPL246 bearing the entire P_{II} coding sequence [8] between similar sites in the expression vector pPL450 [9]. This placed *glnB* under the transcriptional control of tandem bacteriophage λ promoters P_R and P_L that are repressed at 30°C by the temperature sensitive *cI857* gene product and derepressed at 42°C [9].

2.2 Purification of P_{II}

Escherichia coli strain AN1459 (*ilv thr leu supE recA srl*::Tn*10*) bearing plasmid pCG646 was grown in LB broth supplemented with 100 μg/ml ampicillin at 30°C to $A_{595} = 0.5$. Cultures were then treated for a further 2 h at 42°C to induce synthesis of P_{II}. Cells from 2 litres of culture were harvested by centrifugation, washed with 20 mM HEPES, pH 7.5, containing 1 mM β-mercaptoethanol and 1 mM EDTA (Buffer A), resuspended in the same buffer (3 ml/g wet weight of cells), and lysed by passage through a French press operating at 10,000–12,000 psi. Proteins in the soluble extract obtained following centrifugation (150,000 × *g*, 30 min, 4°C; Fraction I) were precipitated with ammonium sulfate (0.4 g/ml of extract) over 1 h at 4°C. The pellet obtained after centrifugation at 48,000 × *g* for 30 min at 4°C was dissolved in Buffer A and dialysed against 1 litre of the same buffer (two changes) at 4°C to yield Fraction II. Fraction II was then made 26% (v/v) in β-mercaptoethanol and stirred at room temperature for 20 min before being placed on ice. The suspension was centrifuged at 150,000 × *g* for 1 h at 4°C to separate a clear gelatinous pellet. The supernatant (Fraction III) was dialysed at 4°C against 1 l of 20 mM HEPES, pH 7.5, containing 1 mM EDTA (2 changes), then applied to a column (2.5 × 15 cm) of Fractogel TSK DEAE-650 (from Merck). The bound proteins were eluted in a linear gradient (1000 ml) of 0–300 mM NaCl in Buffer A. Fractions containing unmodified P_{II} (eluted near 200 mM NaCl) were pooled and dialysed against Buffer A, concentrated to 10 mg/ml using a Centricon C3 miniconcentrator (Amicon) and stored at −70°C, to give Fraction IV (88 mg protein). Fraction IV contained P_{II} that was > 95% pure as judged by Coomassie blue stained SDS-PAGE [10] (Fig. 1). Protein concentration was determined by the method of Bradford [11]. N-terminal amino acid sequencing was carried out using an Applied Biosystems 477A gas-liquid phase sequencer.

2.3. Mass spectrometry

The relative molecular mass of subunits of P_{II} protein was determined by matrix-assisted laser desorption mass spectrometry, using a VG TofSpec mass analyser. 2-(4-Hydroxyphenylazo)benzoic acid was used as matrix as described [12]. Spectra were standardized internally with bovine insulin (M_r 5733.8) and chicken egg white lysozyme (M_r 14,311.8). The quoted result for P_{II} is the mean (± 2 s) of three independent determinations.

2.4. Sedimentation equilibrium

P_{II} protein (Fraction IV) at a concentration close to 1 mg/ml was dialysed against 2 changes (1 litre of each) of Buffer A at 4°C. A meniscus depletion type [13] sedimentation equilibrium experiment was performed in a Spinco model E analytical ultracentrifuge at 25°C, 34,000 rpm using a double-sector cell with the dialysed P_{II} solution (0.1 ml) in one sector and the equilibrium dialysate (0.11 ml) in the other. Photographs of the concentration distribution at equilibrium were taken using the Rayleigh interference optical system and measured as suggested [13] with a Nikon microcomparator. The data was plotted in the form ln(fringe displacement in μm) versus the square of the radial distance from the axis of rotation (i.e. $ln\ y$ vs. r^2).

2.5. Crystallisation of unuridylylated P_{II}

P_{II} was crystallised using vapour diffusion with the hanging drop technique [14]. Crystals were grown from a 10 μl drop (8 mg/ml protein) that contained 1 M phosphate buffer at pH 7.0. The drops were placed over reservoirs that contained 2 M phosphate (pH 7.0) and left to equilibrate by vapour diffusion. At 4°C, hexagonal crystals with dimensions 0.2 × 0.2 × 0.3 mm appeared after 4 weeks.

2.6. Crystallographic methods

Precession photographs were collected using a precession camera mounted on a Rigaku RU200 generator running at 50 kV and 180 mA and producing CuKa radiation. X-Ray diffraction data were collected with a RAXIS-II image plate detector (Rigaku) mounted on a separate RU200 generator run at 50 kV and 100 mA and producing CuKa radiation from a fine focus filament. Data were reduced with Rigaku software provided with Raxis-II and scaled with the PROTEIN program of Steigemann [22]. This program was used to calculate difference Patterson functions, refine heavy atom positions and calculate Fouriers.

3. Results and discussion

We have previously reported the nucleotide sequence of *glnB*. The open reading frame coded for a polypeptide of 112 amino acids [8]. The predicted protein sequence was identical to the sequence of *Klebsiella pneumoniae* P_{II} [15] except that the threonine residue at position 66 is substituted by proline in the *E. coli* protein. P_{II} from other species such as *Synechococcus* and *Bradyrhizobium japonicum* are around 65% identical and are polypeptides of the same length [16]. A recombinant strain containing plasmid pCG646 directed the synthesis of a 12.5-kDa polypeptide upon induction at 42°C, as judged by analysis on a 15% SDS-PAGE. P_{II} constituted about 20% of total protein (Fig. 1). Purification of P_{II} was greatly aided by the high level of overproduction and the unconventional step of treatment of Fraction II with 26% (v/v) β-mercaptoethanol to selectively precipitate contaminating proteins [4]. The protein was almost pure at this stage. Since P_{II} can be covalently modified by uridylylation, it was important to separate the uridylylated protein from the unmodified form. Snake venom phosphodiesterase has been utilised to obtain homogeneous unmodified P_{II} by other workers [17]. In this work P_{II} was conveniently resolved as two peaks by anion-exchange chromatography, where the leading peak was unuridylylated as judged by its UV absorption spectrum [4]. The lagging P_{II} peak on the other hand showed absorption at 260 nm consistent with it being at least partly uridylated (data not shown).

The sequence of the N-terminal 10 amino acids of the purified protein was identical to the predicted P_{II} sequence; the N-terminal methionine residue was present. The relative subunit mass of purified unmodified P_{II} was determined by matrix-assisted laser desorption mass spectrometry to be 12,435 ± 16, which compared favourably with the calculated value (12,427).

Hexagonal crystals of unmodified P_{II} were found to belong to space group $P6_3$ from inspection of precession

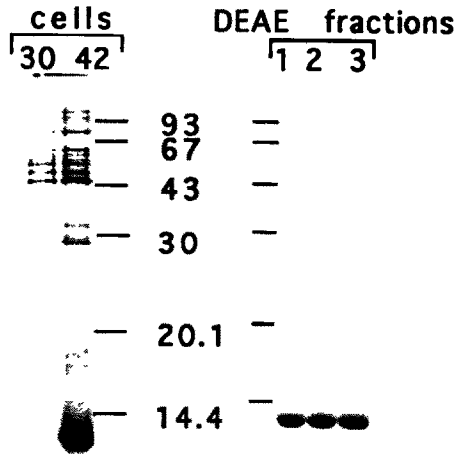

Fig. 1. Overproduced and purified P_{II} analysed by SDS-PAGE (15% gel). A sample of AN1459 bearing plasmid pCG 646 grown at 30°C was withdrawn at OD_{595} of ~ 0.5 and a second sample was withdrawn after the remaining culture was treated at 42°C for 2 h. The cells were suspended in SDS-gel loading buffer to OD_{595} = 10 and heated at 95°C for 2 min. A 20 μl aliquot of each sample was applied to the gel. Three fractions containing P_{II} (15 μg each) eluted from DEAE fractogel that were pooled to give fraction IV were analysed by SDS-PAGE (15% gel). The molecular weight markers were phosphorylase *b* (93 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), α-lactoglobulin (20.1 kDa) and soybean trypsin inhibitor (14.4 kDa).

photographs of the *hko* and *hol* layers. Accurate cell dimensions were determined by analysis of oscillation images obtained on the RAXIS-II. The cell dimensions were $a = b = 61.7$ Å and $c = 56.3$ Å. Assuming that there is one subunit in the asymmetric unit, then a value of V_m (solvent content parameter) of 2.5 Å^3/Da is obtained; this is close to the average observed for protein crystals [18]. There has been a report of another crystal form of unuridylylated P_{II} [17]. For that work, unmodified P_{II} was prepared by treatment of a mixture of forms with snake venom phosphodiesterase. Crystals were cubic and belonged to the space group I23. However, the data presented in that work suggested that the cubic crystals did not diffract well.

A native data set that showed X-ray diffraction to a resolution of 1.8 Å and four heavy-atom derivative data sets were collected using conditions summarised in Table 1. A low resolution (5 Å) electron density map was calculated using the phases from the heavy-atom derivatives with an overall mean figure of merit of 0.84. This map (Fig. 2) showed density to be crowded around a three-fold axis, strongly suggesting that the native protein is a trimer. In contrast, a molecular weight determination by gel filtration [19] had previously indicated a native molecular weight near 50,000, suggesting P_{II} to be a tetramer. Analytical equilibrium centrifugation was used to resolve the issue of oligomeric structure. A plot of the data ($ln\ j$ vs. r^2) obeyed a linear relationship and was fitted by linear least squares (correlation coefficient = 0.999) with slope value of 2.260 (data not shown). This showed that the protein solution was homogeneous with respect to molecular weight.

The molecular weight (M) was evaluated from the expression:

$$M = 2\ RT/(1-\bar{v}\rho)\omega^2 \cdot (\mathrm{d}\ln j/\mathrm{d}r^2)$$

where R is the gas constant, T the absolute temperature, $\bar{v}$, the partial specific volume of the protein, and ρ the solution density. The value of $\bar{v}$ was calculated from the amino acid content of P_{II} to be 0.74 ml/g which is marginally higher than the average assumed value of 0.73 ml/g for globular proteins [20]. Substitution of these values in the above expression indicated the molecular weight of native P_{II} to be 35,000 ± 1000. This value is clearly representative of a trimeric molecule within the limits of experimental errors [21].

In conclusion, the low-resolution electron density map of P_{II} suggests that the native molecule is a homotrimer. This observation was confirmed by molecular weight determination by equilibrium sedimentation analysis.

Table 1
Summary of data collection, derivative preparation and map calculation at 5 Å

Label	PII33	PII11	PII25	PII79	PII23
Derivative	Native	PCMBS	Hg/Ir[a]	Hg/Pt[b]	Na_2IrCl_6
Concentration	–	saturated	50% sat. + 10 mM	50% sat. + 10 mM	20 mM
Soak time	–	7 h	2 h/1.25 h	2 h/19.5 h	5.5 h
R_{merge} (%)[c]	2.50	5.60	5.80	4.60	3.70
Resolution (Å)	1.8	2.8	4.5	2.8	3.0
No. of sites	–	1	2	2	1
Mean F_h/E[d]	–	3.4	4.5	3.2	3.3

[a] 50% sat. PCMBS for 3 h followed by 10 mM Na_2IrCl_6 for 1.25 h.
[b] 50% sat. PCMBS for 2 h followed by 15 mM $K_2Pt(CN)_4$ for 19.5 h.
[c] $R_{merge} = \{\Sigma\Sigma\ |\ F^2(i) - < F^2(h) > |\} \div \Sigma\ F^2(i)$. $F^2(i) = i^{th}$ integrated intensity of a reflection. $< F^2(h) >$ = averaged intensity of a reflection with Miller indices h = *h*, *k*, *l*.
[d] F_h = heavy atom contribution and E = lack of closure error.

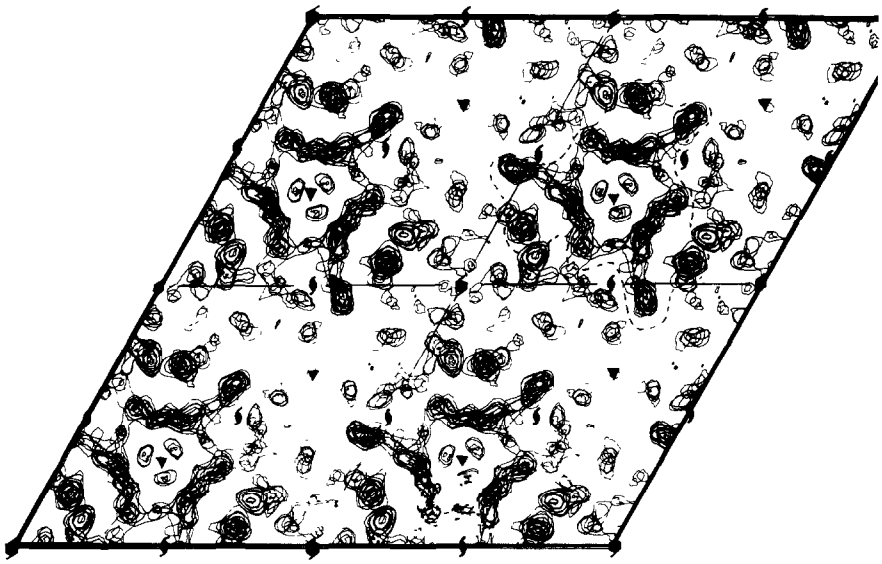

Fig. 2. Four sections of the low-resolution electron density map of P_{II} protein. The map shows 4 unit cells with standard symmetry symbols. The electron density has been contoured at 1 σ intervals starting at 1 σ.

which gives an estimate that is independent of the shape of the molecule [20,21]. Solution of the high-resolution structure of the protein is in progress.

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