Acta Cryst. (1998). D**54**, 996–998

Crystallization and preliminary X-ray analysis of *Escherichia coli* GlnK

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(Received 28 November 1997; accepted 30 January 1998)

Abstract

The trimeric signal-transduction protein GlnK, from *Escherichia coli*, has been over-expressed, purified to homogeneity and crystallized. The crystals belong to space group $P2_13$ with $a = 85.53$ Å and have two subunits in the asymmetric unit. The complex of GlnK with ATP crystallized in space group $P6_3$ with $a = 57.45$ Å and $c = 54.79$ Å. These crystals have a single subunit in the asymmetric unit. High-quality diffraction data from crystals of GlnK and the GlnK complex have been collected to 2.0 Å.

1. Introduction

GlnK is a signal-transduction protein which has recently been implicated in the regulation of glutamine synthetase (GS) in *Escherichia coli* (van Heeswijk *et al.*, 1996). It is a homotrimer with subunits of 112 amino acids and has 67% sequence identity with the P_{II} protein, whose role in the regulation of GS in enteric bacteria is well studied (Merrick & Edwards, 1995). Under nitrogen-limiting conditions the soluble receptor/sensor protein urdiylyl transferase/removase (UT/UR) attaches an urdiylyl monophosphate (UMP) to Tyr51 of P_{II}. P_{II}–UMP interacts with the bifunctional effector enzyme adenylyltransferase (ATase) to deadenylylate GS–AMP (adenosine monophosphate) and in doing so, activates it. At the same time, the loss of P_{II} leads to increased transcription of the *glnALG* operon from a strong nitrogen-regulated promoter. On the other hand, when nitrogen is in abundance, the UR activity of UT/UR converts P_{II}–UMP to P_{II} which stimulates the ATase adenylylation activity so that inactive GS–AMP is formed. In addition to this, P_{II} stimulates the phosphatase activity of NRII (NtrB) to inactivate the transcription factor NR1-P (phosphorylated NtrC). This represses the transcription of the *glnALG* operon. Apart from interacting with three bifunctional enzymes, P_{II} has recently been shown to bind two effector molecules: adenosine triphosphate (ATP) and α-ketoglutarate. ATP and α-ketoglutarate may be necessary for P_{II} to interact with other proteins.

Although P_{II} and P_{II}–UMP are referred to as indicators of nitrogen availability, strains which lack the gene encoding P_{II} (GlnB) retain the ability to regulate the adenylylation activity of GS. This effect has recently been shown to be due to GlnK (van Heeswijk *et al.*, 1996). The nitrogen-assimilation signalling is quite sophisticated but the recent discovery of GlnK points to possible further complexity that needs to be characterized. The crystal structure of *E. coli* P_{II} (Cheah *et al.*, 1994; Carr, Cheah *et al.*, 1996) together with associated structure and function studies (Jaggi *et al.*, 1996; Jiang, Zucker, Atkinson *et al.*, 1997; Jiang, Zucker & Ninfa, 1997) have revealed an interesting molecule with an intriguing signalling loop (*T* loop) which allows it to participate in at least three different protein–protein interactions. The analogous region in GlnK is identical except for changes in three of the 19 residues. A comparison of the structure of GlnK with P_{II} may provide valuable clues as to how these highly homologous molecules differ in their function.

2. Materials and methods

2.1. *Construction of plasmid pNV102*

The DNA sequence encoding GlnK was polymerase chain-reaction (PCR) amplified from template plasmid pWVH149 (van Heeswijk *et al.*, 1996) using a forward primer (5′-AATGGATCC**CATATG**AA-GCTGGTGACCGTG-3′) which introduced a convenient *Nde*I restriction site (CATATG) at the start and the M13 primer (5′-GTAAAACGCGACGGCCAGT-3′). PCR using standard conditions was carried out with Expand polymerase (Boehringer Mannheim). The amplified fragment was purified by phenol/chloroform extraction followed by ethanol precipitation and digested with restriction enzymes *Nde*I (CATATG) and *Sph*I essentially as described previously (Vasudevan *et al.*, 1991). The resulting DNA fragment (~380 bp) was cloned into similarly cut lambda promoter vector pND707 (Love *et al.*, 1996) to yield the plasmid pNV102 which expressed GlnK.

2.2. *Purification of GlnK*

GlnK was over-expressed in *E. coli* strain RB9040 (UT/UR-deficient strain) bearing plasmid pNV102. Three 1 l cultures of Luria broth, containing 50 µg ml^{-1} each of ampicillin and thymine, were inoculated with 40 ml of overnight cultures and grown at 303 K. When the OD_{595} reached 0.5–0.6, the cultures were induced by raising the temperature quickly to 315 K. Growth continued for a further 2 h. The cells were harvested by centrifugation at 8300*g* for 10 min. The pellets were resuspended in a lysis buffer (20 m*M* Hepes pH 7.5, 1 m*M* β-mercaptoethanol, 1 m*M* EDTA and 20 m*M* spermine) and lysed in a French pressure cell. Cell debris was removed by centrifugation at 39000*g* for 30 min, and the proteins in the supernatant were precipitated with 0.25 g ml^{-1} $(NH_4)_2SO_4$. The precipitated protein was resuspended and dialysed against buffer *A* (20 m*M* Tris pH 8.8, 1 m*M* β-mercaptoethanol and 1 m*M* EDTA). The protein was then loaded onto a DEAE column previously equilibrated in buffer *A*. After washing the column with 5% buffer *B* (buffer *A* containing 1 *M* NaCl) the GlnK protein was eluted with a linear gradient of 50–500 m*M* NaCl. GlnK eluted between 200–300 m*M* NaCl and gave a prominent peak on the chromatogram. Sodium dodecyl sulfate

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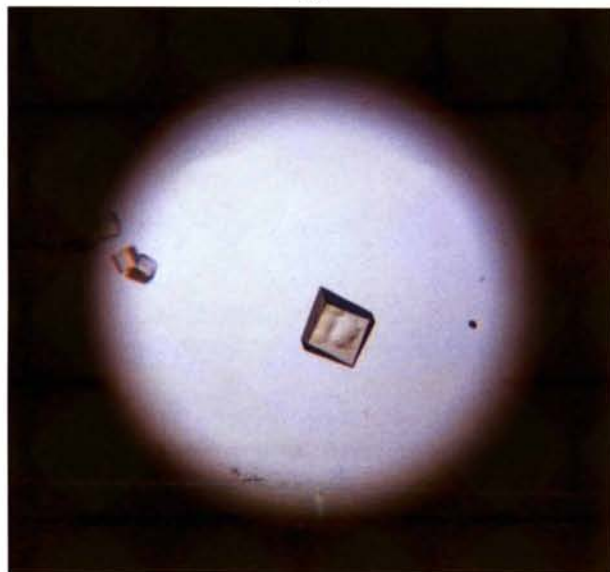
(1%)/polyacrylamide gel (20%) electrophoresis (SDS–PAGE) confirmed that this peak contained the partially purified protein. The peak fractions were dialysed against buffer *A* and loaded on to a Blue Sepharose column which had been previously equilibrated with buffer *A*. The protein was eluted from the Blue Sepharose column at 550–650 m*M* NaCl with a 0–1 *M* NaCl gradient. The peak fractions were pooled and dialysed against buffer *A*. The purified protein was concentrated using an Amicon Centricon 10 to 13.3 mg ml^{-1} for crystallization trials. Protein concentration was determined by the Bradford method (Bradford, 1976) using reagents provided by BioRad with γG as a protein standard.

(*a*)



(*b*)

Fig. 1. (*a*) Cubic crystals of GlnK. (*b*) Tapered hexagonal crystals of GlnK with ATP. The size of the larger crystals and the conditions used to grow the crystals are described in the text.

2.3. *Crystallization*

Crystals of GlnK were initially grown at 277 K using vapour diffusion employing the hanging-drop technique (McPherson, 1976). Crystals were obtained with reagent 26 of the Hampton Crystal Screen I (Jancarik & Kim, 1991) which contained 2-methyl-2,4-pentanediol (MPD), ammonium acetate and sodium acetate at pH 5.2. Optimal conditions for crystallization were achieved by mixing equal volumes of protein solution and a reservoir solution consisting of 30% MPD, freshly prepared 0.2 *M* ammonium acetate and 0.1 *M* cacodylate pH 6.5. Crystals of GlnK protein as a complex with ATP were also grown. In these experiments, ATP was present in a fourfold molar excess. Diffraction-quality crystals of the complex were obtained by mixing one part of protein solution with one part of a reservoir solution consisting of 30% MPD and 0.1 *M* sodium acetate pH 5.2.

2.4. *X-ray characterization*

X-ray data to 2.0 Å were collected with the GlnK crystals as well as the complex of GlnK with ATP. In both cases, the crystals were transferred to the reservoir solution which served as a cryobuffer. The crystals were flash cooled to 100 K in a nitrogen stream produced by a modified MSC cooling system as described by Carr, Barlow *et al.* (1996). X-ray data were collected with an R-AXIS II detector mounted on a Rigaku generator producing Cu $K\alpha$ radiation at a power of 5 kW (50 kV, 100 mA). The data were processed using the *DENZO* and *SCALEPACK* (Otwinowski, 1993) programs.

3. Results and discussion

The construction of plasmid pNV102 led to high-level expression of the GlnK protein. GlnK purification was based on that used for P_{II} (Vasudevan *et al.*, 1994). However, the β-mercaptoethanol precipitation step used with P_{II} was ineffective with GlnK and a new purification step was required to obtain pure protein. The presence of the ATP binding site in P_{II} prompted the successful use of Blue Sepharose in the purification of GlnK. The purified protein migrated as a single band on a 20% SDS gel consistent with a molecular mass of about 12 kDa. The final yield was approximately 97 mg of protein from 7 g of cells. Cubic crystals of GlnK (Fig. 1*a*) grew to 0.28 × 0.28 × 0.28 mm in four weeks. Their diffraction pattern could be indexed with a primitive cubic lattice with a = 85.53 Å. Axial reflections displayed absences consistent with a 2_1 symmetry. For space group $P2_13$, a total of 97474 observations were reduced to 14384 unique intensities with an R_{merge} of 4.7%. Crystals of the GlnK complex (Fig. 1*b*) grew in 6 weeks to a size of 0.88 × 0.18 × 0.18 mm and had a hexagonal cross section with tapered ends. The diffraction pattern produced by the crystals of GlnK complexed with ATP could be indexed with a hexagonal lattice with a = 57.45 and c = 54.79 Å. The absences along the c axis were consistent with a 6_3 screw. For space group $P6_3$, a total of 44483 observations were reduced to 6913 unique intensities with an R_{merge} of 3.9%.

Like P_{II}, the GlnK molecule is a trimer. In the GlnK crystals, two subunits in the asymmetric unit gives a V_m of 2.4 Å^3 Da^{-1} which is close to the mean observed for protein crystals (Matthews, 1968). This suggests that for the $P2_13$ space group there are two GlnK molecules on threefold axes. For the hexagonal cell formed by the GlnK ATP complex, a V_m of 2.2 is obtained for a single subunit in the asymmetric unit. The

crystals of the GlnK complex belong to the same space group and have similar cell dimensions to native P_{II} (Vasudevan *et al.*, 1994). The cell dimensions of P_{II} are larger by 4.1 and 1.5 Å in the *a* and *c* directions, respectively. The similarity of the two crystal forms is probably fortuitous.

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