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Preliminary crystallographic analysis of RraB from *Escherichia coli*

RraB, an inhibitor of the essential endoribonuclease RNE in *Escherichia coli*, is essential in regulating the abundance of RNA by directly interacting with RNE. In this study, RraB from *E. coli* was cloned, expressed, purified and crystallized. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 58.59$, $b = 58.34$, $c = 156.95$ Å. X-ray diffraction data were collected to a resolution of 2.9 Å. Analysis of the native Patterson map revealed a peak of ~37% the height of the origin peak in the $\nu = 0.5$ Harker section, suggesting twofold noncrystallographic symmetry parallel to the b crystallographic axis. The Matthews coefficient and the solvent content were estimated to be 4.09 Å³ Da⁻¹ and 69.94%, respectively, assuming the presence of two molecules in the asymmetric unit.

1. Introduction

RNAse E (RNE) is an essential endoribonuclease that plays an important role in global mRNA metabolism as well as in the maturation of functional RNAs such as noncoding RNA (Carpousis, 2007), rRNAs, tRNAs, tmRNA and small regulatory RNAs E in *Escherichia coli* (Bouvier & Carpousis, 2011). The 1061 amino-acid *E. coli* RNAse E protein can be divided into an N-terminal region, a central region and a C-terminal region. The N-terminal region contains the endonuclease active site (McDowall & Cohen, 1996) and a zinc-finger motif, the central region performs the membrane-anchoring (Khemici *et al.*, 2008) and RNA-binding functions (McDowall & Cohen, 1996), and the C-terminal region is an unstructured scaffold domain that contains binding sites for the other core degradosome components, for example polynucleotide phosphorylase, the RhlB helicase and the glycolytic enzyme enolase (Coburn & Mackie, 1999).

The cellular level of *E. coli* RNAse E is controlled at a relatively stable level *via* autoregulation of its synthesis by modulating the decay of its own mRNA (Jain & Belasco, 1995). The activity of RNAse E in *E. coli* is also controlled by the protein inhibitors RraA and RraB (regulators of RNAse activity A and B; Yeom *et al.*, 2008). These two inhibitors can repress the endonucleolytic activity of RNAse E by connecting to the scaffold domains of the enzyme because the catalytic domain alone is not sufficient to bind the RNA substrates (Gao *et al.*, 2006). RraA and RraB have a conserved function in RNA metabolism and modulation of RNA stability in bacteria, but share only a very low sequence identity with each other (14.2% over 138 amino acids). This conserved function may play an important role in a common mechanism of global control of transcript abundance in bacteria in response to dynamic changes in the extra-cellular or the intracellular environment.

The present evidence indicates that RraA forms a ring-like trimer with a central cavity of approximately 12 Å in diameter (Monzingo *et al.*, 2003), and that it binds to and masks the RNA-binding domains within the C-terminal domain of RNAse E and also binds to the basic C-terminal extension of RhlB, which is required for RNA binding by the helicase (Chandran *et al.*, 2007). By occluding these binding sites in the degradosome, RraA represses the activity of the helicase and, indirectly, PNPase (Górna *et al.*, 2010).

E. coli RraB, previously annotated as YjgD in the NCBI database, is a 15.6 kDa protein which contains 138 amino acids and has a very low theoretical pI of 3.64.

In contrast to RraA homologues, which exist in numerous bacterial genomes, RraB is found only in gammaproteobacteria, suggesting that it may have a more specialized role in modulating RNA degradation (Yeom *et al.*, 2008). In addition, RraB exhibits key differences in its mode of action and its effects on the transcript profile (Zhou *et al.*, 2009). The previous study also revealed that amino acids 694–727 of RNase E were required for the binding of RraB (Gao *et al.*, 2006), similar to the major degradosome components which bind to specific continuous regions within the C-terminal region of RNase E. This region partially overlaps a stretch of amino acids extending from positions 685 to 712 which is predicted to be important for binding of the enzyme to structured RNAs (Callaghan *et al.*, 2004). In contrast to RraB, the binding region between RraA and RNase E could not be mapped to a single contiguous epitope, instead mapping to a long region from amino acids 585 to 1061 of RNase E.

To investigate the structural characteristics of *E. coli* RraB in regulating RNA degradation and RNase E binding, a crystallization and preliminary X-ray crystallographic study was carried out and is reported here.

2. Materials and methods

2.1. Cloning and expression

Primers of sense strand 5'-GGCGCGCATATGGCAAACCCGG-AACAAGTGG-3' and antisense strand 5'-GACACTCGAGTT-AGTGGCGAACTCCGTCATCGTC-3' (Invitrogen) were used to amplify the *rraB* gene by polymerase chain reaction (PCR) from *E. coli* BL21 (DE3) cells (Novagen). The PCR fragment was digested by restriction endonucleases *Nde*I and *Xho*I and then inserted into expression vector p28 (Novagen; a modification of pET28a) with a hexahistidine tag (MGHHHHHH) at the N-terminus of the recombinant RraB. After sequencing, the plasmid was transformed into *E. coli* BL21 (DE3) cells (Novagen). The transformant was grown in 1.6 l Luria–Bertani (LB) medium containing 50 µg ml⁻¹ kanamycin at 310 K. When an OD₆₀₀ of 0.6–0.8 was reached, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction. After 20 h induction at 289 K, the cells were harvested by centrifugation at 6000g for 10 min.

2.2. Purification

The harvested cells were suspended in buffer A (20 mM Tris–HCl pH 7.5, 500 mM NaCl) and lysed by sonication on ice. The soluble portion obtained after centrifugation at 14 000g for 30 min was then applied onto an Ni–NTA column (Qiagen) pre-equilibrated with buffer A. The bound protein was eluted with buffer A containing 500 mM imidazole and dialyzed against buffer B (20 mM Tris–HCl pH 8.0, 50 mM NaCl). After ultrafiltration to 5 ml using a Millipore 10 kDa centrifugal device, the target protein was purified using a HiTrap Q FF column (GE Healthcare) pre-equilibrated with buffer B. The collected fractions were concentrated by centrifugal ultrafiltration (Millipore, 10 kDa cutoff) and the target protein was finally purified using a HiLoad 16/60 Superdex 200 (GE Healthcare) gel-filtration chromatography column previously equilibrated with buffer A. All purification steps were performed at 288 K. The protein is totally soluble and the final yield was as high as 7.8 mg per litre of culture. The purity of the target protein was estimated by SDS–PAGE gel (Fig. 1a).

The identity of the protein was further confirmed by LC–MS/MS peptide-mapping experiments. Briefly, the band from the SDS–PAGE was in-gel digested with trypsin (digested overnight at 310 K with 50 µl 0.01 µg µl⁻¹ trypsin solution) and the digested peptides were chromatographically separated (Jupiter 5 µm C18 300A column; Phenomenex) using a Surveyor high-performance liquid chromatography system (Thermo Fisher Scientific, California, USA) connected to an LTQ mass spectrometer (Thermo Fisher Scientific). The chromatographic method used a flow rate of 90 nl min⁻¹ with a step gradient from mobile phase A consisting of 0.1% formic acid in water to mobile phase B consisting of 0.1% formic acid in acetonitrile. MS/MS fragmentation was performed using collision-induced dissociation (CID) with an activation *Q* of 0.250, an activation time of 30.0 ms, 35% normalized collision energy and an isolation width of 1.0 Da. MS/MS data were compared using the *SEQUEST* software (Thermo Fisher Scientific).

Figure 1
(a) SDS–PAGE analysis of RraB. (b) Size-exclusion chromatography of RraB. The elution volume of RraB is 14.85 ml, which is consistent with a homodimer state (red curve); the black curve indicates the elution positions of standard molecular-weight protein markers (labelled in kDa).

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$
Wavelength (Å)	0.979
Unit-cell parameters (Å)	$a = 58.59, b = 58.34, c = 156.95$
Resolution limits (Å)	50.0–2.9 (3.00–2.90)
No. of observed reflections	74721
No. of unique reflections	12532
Completeness (%)	96.7 (99.8)
Multiplicity	6.2 (6.8)
$R_{\text{merge}}^{\dagger}$ (%)	7.6 (46.7)
Mean $I/\sigma(I)$	16.77 (6.00)
V_M (Å ³ Da ^{−1})	4.09
No. of subunits per asymmetric unit	2
Solvent content (%)	69.94

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over all equivalent and symmetry-related reflections.

2.3. Crystallization

The recombinant RraB was concentrated to 16 mg ml^{−1} in buffer A (calculated from the OD₂₈₀ using a molar absorption coefficient of 11 585 M^{−1} cm^{−1}; Eppendorf BioPhotometer Plus) by centrifugal ultrafiltration (Millipore; 10 kDa cutoff) prior to crystallization trials. Initial crystallization trials were performed using the hanging-drop vapour-diffusion method by mixing 1 µl protein solution and 1 µl reservoir solution and equilibrating against 200 µl reservoir solution using the Crystal Screen, Crystal Screen 2, Index, PEG/Ion and PEG/Ion 2 reagent kits (Hampton Research) at 287 K. Crystals were observed in several conditions within 2 weeks. Single crystals suitable for X-ray diffraction measurements grew in drops containing 1 M NaCl, 0.1 M citric acid pH 4.0.

2.4. Data collection and processing

For data collection, crystals were first flash-cooled in liquid nitrogen with a cryoprotectant solution consisting of 1 M NaCl, 0.1 M citric acid pH 4.0, 20% (v/v) glycerol and then transferred to the liquid-nitrogen stream. X-ray diffraction data were collected on beamline 17U1 of the Shanghai Synchrotron Radiation Facility (SSRF) using a Jupiter CCD detector. All frames were collected at 100 K using a 1° oscillation angle. The crystal-to-detector distance was set to 300 mm. The complete diffraction data set was subse-

quently processed using *HKL-2000* (Otwinowski & Minor, 1997). The detailed data-processing statistics are shown in Table 1.

3. Results and discussion

Full-length recombinant *E. coli* RraB was cloned, overexpressed and purified as a dimer in solution (as calculated by gel-filtration chromatography; Fig. 1*b*). The ~20 kDa band was identified as *E. coli* RraB based on mass-spectrometric detection of 44 amino acids of RraB covering 31.88% of the 138 amino-acid sequence. The rectangular prismatic crystals grew in 2 weeks (Fig. 2). A total of 180 diffraction images were recorded from a single crystal. The diffraction data were processed to 2.9 Å resolution. The RraB crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 58.59, b = 58.34, c = 156.95$ Å. The Matthews coefficient calculated using the *CCP4* suite (Winn *et al.*, 2011) suggested that the presence of two to four monomers in an asymmetric unit would give a reasonable V_M (2.04–4.09 Å³ Da^{−1}; Matthews, 1968). Calculation of a native Patterson map revealed a peak of ~37% the height of the origin peak in the $v = 0.5$ Harker section (Fig. 3*b*), consistent with the presence of two molecules per asymmetric unit with a solvent content of 69.94% (Winn *et al.*, 2011).

Figure 2

Crystals of recombinant RraB grown under the condition 1 M NaCl, 0.1 M citric acid pH 4.0.

Figure 3

(a) A diffraction image of the *E. coli* RraB crystal. (b) $v = 0.5$ Harker section of the native Patterson map of RraB crystal calculated using *FFT* from the *CCP4* suite.

Structure determination of recombinant *E. coli* RraB will be attempted by the molecular-replacement method using the solution structure of *Vibrio cholerae* protein VC0424 as a search model (PDB entry 1nxi; 44.2% amino-acid sequence identity; Ramelot *et al.*, 2003) and experimental phasing will be carried out if necessary (selenomethionine-derivative RraB crystals have already been prepared).

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