Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Preliminary X-ray characterization of the ribonuclease P (C5 protein) from *Escherichia coli*: expression, crystallization and cryoconditions

Hui-Woog Choe,[a,b]* Dae-Gwin Jeong,[a] Jung Hee Park,[a,b] Ramona Schlesinger,[c] Jörg Labahn,[c] Klaus Peter Hofmann[b] and Georg Büldt[c]

[a]Department of Chemistry, College of Natural Science, Chonbuk National University, 561-756 Chonju, South Korea, [b]Institut für Medizinische Physik und Biophysik, Humboldt-Universität zu Berlin, Universitätsklinikum Charité, 10098 Berlin, Germany, and [c]Institut für Biologische Informationsverarbeitung, Forschungszentrum Jülich, 52425 Jülich, Germany

Correspondence e-mail: hwchoe@moak.chonbuk.ac.kr

Received 17 July 2002
Accepted 13 November 2002

The gene for *Escherichia coli* ribonuclease P (RNase P) protein (also known as C5 protein) and its mutant C5-C113A have been expressed as GST fusion proteins in *E. coli* at a high level. After cleavage of the fusion protein, highly purified functional C5 protein is obtained that can be crystallized with 2.5–2.6 *M* $(NH_4)_2HPO_4/(NH_4)H_2PO_4$ pH 7.0 at room temperature. These crystals are suitable for X-ray analysis, belong to the space group $P3_121$ or $P3_221$ (unit-cell parameters $a = b = 66.67$, $c = 142.09$ Å) and diffract to 2.9 Å at 100 K using sorbitol and glycerol as cryoprotectants. For three molecules in the asymmetric unit a V_M of 2.17 Å^3 Da^{-1} was calculated.

1. Introduction

Ribonuclease P (RNase P; EC 3.1.26.5) holoenzyme is a ribonucleoprotein that catalyzes the removal of the 5′-leader elements of precursor tRNAs and generates the mature 5′-end of tRNAs. This step is essential for the formation of functional tRNA molecules in bacteria, archaea and eukarya (Kurz & Fierke, 2000; Altman & Kirsebom, 1999; Frank & Pace, 1998; Li & Deutscher, 1996; Pace & Brown, 1995). In bacteria, in which RNase P is well characterized, the holoenzyme is composed of two subunits: an RNA component and a protein subunit. In *Escherichia coli*, RNase P holoenzyme consists of M1 RNA (377 nucleotides encoded in the *rnpB* gene) and the C5 protein (119 amino-acid residues encoded in the *rnpA* gene) (Altman & Kirsebom, 1999; Frank & Pace, 1998). While both subunits are essential for *in vivo* activity, the RNA component from bacterial RNase P is sufficient to catalyze the cleavage of pre-tRNA *in vitro* in the presence of high salt concentrations (Guerrier-Takada *et al.*, 1983), indicating that the RNA component is the essential determinant for binding and cleaving pre-tRNA. The protein moiety, however, facilitates cleavage of pre-tRNA under physiological salt concentrations (Kurz *et al.*, 1998; Reich *et al.*, 1988) and is required for efficient tRNA processing *in vivo* (Kole *et al.*, 1980). RNase P is the only known ribozyme that catalyzes multiple turnovers and is unchanged during catalysis (Beebe & Fierke, 1994)

The three-dimensional structure of RNase P protein from *Staphylococcus aureus* has been determined by nuclear magnetic resonance (NMR) spectroscopy in solution (Spitzfaden *et al.*, 2000) and that from *Bacillus subtilis* has been analyzed by X-ray crystallography (Stams *et al.*, 1998). Both known structures display a distinctive topology containing a left-handed $\beta\alpha\beta$-crossover connection.

Here, we report some biophysical properties of the recombinant wild-type *E. coli* RNase P protein, which is the most characterized RNase P protein, and the preliminary X-ray analysis of crystals of the mutated *E. coli* C5-C113A.

2. Materials and methods

2.1. High-level expression of C5 and mutant (C5-C113A) in *E. coli*

Cloning of C5 protein as a glutathione *S*-transferase (GST) fusion and generation of C5-C113A were performed as follows. A synthetic *rnpA* (kindly provided by Dr Younghoon Lee, KAIST, Taejon, South Korea) was amplified by PCR using oligonucleotides which introduced a *Bam*HI site at the 5′-end and a *Xho*I site at the 3′-end of the gene. The resulting PCR product was digested with *Bam*HI/*Xho*I and cloned into corresponding sites of the vector pGEX-4T-1 (Pharmacia), leading to an in-frame fusion to the GST gene. On cleavage of the expressed fusion protein with the protease thrombin, full-length C5 protein is released. It contains two additional residues at the amino-terminus before methionine (Gly−2, Ser−1, Met1, ..., Ser119), giving a protein of about 14 kDa. In order to generate the point mutation of C113A, a gene internal oligonucleotide was used to exchange the triplet TGT for GCT by PCR. *E. coli* strain BL21::DE3 was used for the expression of fusion proteins.

2.2. Purification of C5 protein and its mutant (C5-C113A)

Isolated colonies were grown at 310 K overnight in 50 ml of dYT medium containing 100 µg ml^{-1} ampicillin. The culture was diluted 1:20 in the same medium and grown at 310 K to an approximate A_{600} of 0.5. Protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 m*M* with further incubation at 301 K for 3 h. Cells were harvested, resuspended in 50 ml buffer *A* [1 *M* NH_4Cl, 10 m*M* $Mg(OAc)_2$, 50 m*M* Tris–HCl pH 8.2] and sonificated on ice (Labsonic L from Braun M; 4 × 1 min, 90 W output). The extract was centrifuged (15 min at 16 000*g*; 291 K) and the supernatant filtered through a 0.45 µm filter. This filtrate was loaded onto a syringe column (1.5 × 15 cm) with glutathione Sepharose (Pharmacia). The column was washed with buffer *A* and the fusion protein eluted with 50 ml buffer *A* containing 30 m*M* reduced glutathione. All fractions containing protein were concentrated with a Centricon concentrator (Amicon; 30 kDa molecular-weight cutoff membrane) and incubated at 277 K overnight with 50 units of thrombin (Pharmacia). The completeness of digestion was checked by SDS–PAGE. Samples were further purified by gel filtration (Sephadex G50, 1.6 × 90 cm; equilibrated and eluted with buffer *B*: 0.1 *M* Tris–HCl pH 7.0, 150 m*M* NaCl).

2.3. Assay of RNase P functionality

For the assay, M1 RNA was prepared as described by Talbot & Altman (1994). RNase P holoenzyme was reconstituted from M1 RNA and C5 protein in the assay buffer (50 m*M* Tris–HCl pH 7.6, 10 m*M* $MgCl_2$ or 60 m*M* NH_4Cl) as described by Vioque *et al.* (1988) and incubated with 5′-end-labelled pre-tRNA^{Glu} (Baer *et al.*, 1990). The RNase P reaction was carried out in 100 µl of assay buffer. The reaction mixture was incubated at 310 K for 1 h and quenched by extracting with phenol/chloroform/isoamylalcohol (25:24:1). RNA was precipitated with ethanol. The reaction products were analyzed on 6% polyacrylamide gels containing 7 *M* urea. After electrophoresis, gels were dried and the autoradiograms were scanned using a Molecular Dynamics Image Quant imaging system.

Figure 1
E. coli RNase P C5-C113A crystals. The maximum dimensions of the crystals were 0.15 × 0.2 × 1.8 mm.

2.4. Crystallization of the wild-type C5 protein and the mutant C5-C113A protein

For crystallization, the purified C5 proteins were dialyzed against 10 m*M* PIPES buffer pH 7.0 and concentrated with 10 kDa Centricons (Amicon). The sparse-matrix crystallization method (Jancarik & Kim, 1991) was applied to screen the crystallization point of wild-type C5 protein and C5-C113A using protein droplets of 2–4 µl with the Hampton Research crystallization kit. The cover slips for the hanging-drop experiments were sealed with Baysilone vacuum grease (Bayer). To obtain large single crystals suitable for X-ray analysis, we had to change the crystallization method to sitting-drop vapour diffusion (droplet size 20 µl) using Crystal Clear Tape (Hampton Research) as a sealant. Protein solutions were equilibrated against 1 ml of reservoir solution.

3. Results and discussion

In *E. coli*, high-level expression of the wild type and mutant C5-C113A was obtained by processing GST fusion constructs. Both of the purified proteins are functionally active (data not shown). About 10 mg of purified wild-type C5 protein could be obtained from a litre of culture. In the case of C5-C113A, the yield was even higher (15–18 mg per litre of culture). Particularly for the wild-type C5 protein, the phenomenon of aggregation was found to be the major obstacle to crystallization. The wild-type C5 protein precipitates over time at a concentration of 5 mg ml^{-1} even after addition of dithiothreitol (DTT) and detergent (β-D-octylpyranoside). C5 protein from *E. coli* has a single cysteine residue that may allow intermolecular disulfide bridges to form between molecules of the wild-type C5 protein. For C5-C113A we did not observe any aggregation even at a concentration of 10 mg ml^{-1}. Only this mutant could be crystallized.

In conditions No. 3 [0.4 *M* $(NH_4)H_2PO_4$] and No. 48 [0.1 *M* Tris–HCl pH 8.5, 2.0 *M* $(NH_4)H_2PO_4$] of the crystallization kit (Hampton Research), we observed tiny needle crystals after eight weeks. However, in condition No. 48 the crystals redissolved with time. Therefore, these two sets were re-screened by systematically varying the concentration of protein (5–10 mg ml^{-1}), buffers (MES, PIPES, Tris–HCl; 10–100 m*M*), pH (6.5–8.2) and precipitating agents [NaH_2PO_4/K_2HPO_4 or $(NH_4)H_2PO_4/(NH_4)_2HPO_4$]. The best results were obtained with phosphate concentrations of 2.4–2.8 *M* at neutral pH. C5 protein is a basic RNA-binding protein (calculated pI of 11.9). Therefore, it was necessary to compensate the positive charges of C5 protein with a high concentration of negative ions in order to induce nucleation and crystal growth. It is possible that the phosphate mimics the effect of the nucleic acid on the C5 protein in the holoenzyme.

Crystals suitable for X-ray analysis were grown by the sitting-drop vapour-diffusion method with tape as a sealant. 20 µl of 5–10 mg ml^{-1} C5-C113A protein was mixed with the same volume of reservoir solution [2.5–2.6 *M* $(NH_4)_2HPO_4/(NH_4)H_2PO_4$, 10 m*M* PIPES pH 7.0]. Hexagonal rods appeared within two weeks and grew for a further four weeks (see Fig. 1) to maximal dimensions of 0.15 × 0.2 × 1.8 mm. When no further growth could be observed, crystals were harvested. The phosphate concentration of the reservoir solution was almost saturated because of evaporation of water through the sealing tape. Rapid crystallization of phosphate buffer in the reservoir solution took place when the crystallization system was opened. Capillary-mounted crystals did not yield a complete data set because of X-ray damage during data collection. Room-temperature diffraction did not exceed 3.4 Å.

For data collection, cryoconditions had to be screened. However, conventional cryoprotectants (PEGs, MPD, glycerol) were incompatible with the high phosphate concentration of the mother liquor. Exchange of the mother liquor with lithium sulfate or ammonium sulfate led to crystal dissolution, even with saturated solutions of these salts. Mounting the crystals in oil failed, as indicated by an immediate loss of polarization under the microscope and subsequent dissolution of the crystals. A slow decrease in phosphate concentration by replacing 1/20 of the volume with a 40%(*w*/*v*) sorbitol solution twice a day allowed lowering of the phosphate concentration by 12–16%. Crystals in this solution can be frozen (Oxford Cryosystems) after dipping them into the new mother liquor containing ∼13%(*w*/*v*) glycerol.

Table 1
Data-collection statistics of C5-C113A crystals.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	36.5–3.10 (3.26–3.10)
Total reflections	48342 (6860)
Unique reflections	7053 (999)
Completeness (%)	99.4 (98.9)
$I/\sigma(I)$	6.2 (2.4)
$R_{\rm merge}$†	0.096 (0.257)

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$, where $\langle I \rangle$ is mean intensity.

For the X-ray studies, single crystals of C5-C113A were mounted in a cryoloop. These crystals diffracted to 2.9 Å (MAR Research image plate; Enraf–Nonius FR571 operating at 40 kV, 60 mA) at 100 K, but show strong anisotropy. The space group and unit-cell parameters were determined from a native data set (Table 1). Crystals are trigonal (space group $P3_121$ or $P3_221$; unit-cell parameters $a = b = 66.67$, $c = 142.09$ Å). For three molecules in the asymmetric unit, a $V_{\rm M}$ of 2.17 Å^3 Da^{-1} was calculated.

This work was supported by Korea Research Foundation Grant (KRF-99-015-DP0244). We thank Drs U. Heinemann and N. Krauss for allowing us to use their X-ray generators for parts of this study. Dr Y. H. Jeoung is greatly acknowledged for the functional assay of the wild-type C5 protein and C5-C113A.

References

Altman, S. & Kirsebom, L. (1999). *The RNA World*, edited by R. F. Gesteland, T. Cech & J. F. Atkins, pp. 351–380. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
Baer, M. F., Arnez, J. G., Guerrier-Takada, C., Vioque, A. & Altman, S. (1990). *Methods Enzymol.* **181**, 569–582.
Beebe, J. A. & Fierke, C. A. (1994). *Biochemistry*, **33**, 10294–10304.
Frank, D. N. & Pace, N. R. (1998). *Annu. Rev. Biochem.* **67**, 153–180.
Guerrier-Takada, C., Gardiner, K., Marsch, T., Pace, N. & Altman, S. (1983). *Cell*, **35**, 849–857.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kole, R., Baer, M. F., Stark, B. C. & Altman, S. (1980). *Cell*, **19**, 881–887.
Kurz, J. C. & Fierke, C. A. (2000). *Curr. Opin. Chem. Biol.* **4**, 553–558.
Kurz, J. C., Niranjanagumari, S. & Fierke, C. A. (1998). *Biochemistry*, **37**, 2393–2400.
Li, Z. & Deutscher, M. P. (1996). *Cell*, **86**, 503–512.
Pace, N. R. & Brown, J. W. (1995). *J. Bacteriol.* **177**, 1919–1928.
Reich, C., Olsen, G. J., Pace, B. & Pace, N. R. (1988). *Science*, **239**, 178–181.
Spitzfaden, C., Nicholson, N., Jones, J. J., Guth, S., Lehr, R., Prescott, C. D., Hegg, L. A. & Eggleston, D. S. (2000). *J. Mol. Biol.* **295**, 105–115.
Stams, T., Niranjanakumari, S., Fierke, C. A. & Christianson, D. W. (1998). *Science*, **280**, 752–755.
Talbot, S. J. & Altman, S. (1994). *Biochemistry*, **33**, 1399–1405.
Vioque, A., Arnez, J. & Altmann, S. (1988). *J. Mol. Biol.* **202**, 835–848.