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Expression, crystallization and preliminary X-ray crystallographic analysis of DNA-directed RNA polymerase subunit L from *Thermococcus onnurineus* NA1

RNA polymerase (RNAP) plays a crucial role in gene expression in all organisms. It is a multiprotein complex that produces primary transcript RNA. Generally, the basal transcription apparatus in archaea is simpler than the eukaryotic RNA polymerase II counterpart. To understand the structure and function of archaeal RNAP, the *TON-0309* gene encoding DNA-directed RNA polymerase subunit L (ToRNAP_L) from *Thermococcus onnurineus* NA1 was cloned and the protein was overexpressed in *Escherichia coli*, purified and crystallized. The purified protein was crystallized using the hanging-drop vapour-diffusion method and the crystal diffracted to 2.10 Å resolution. The crystal belonged to the hexagonal space group *P*6₁22, with unit-cell parameters $a = b = 42.3$, $c = 211.2$ Å. One molecule was present in the asymmetric unit, with a corresponding V_M of 2.5 Å³ Da⁻¹ and a solvent content of 50.0%.

1. Introduction

RNA polymerase (RNAP) is one of the central enzymes of gene transcription. In all life forms, RNAP functions as a multi-subunit protein complex. The subunit compositions differ depending on the domain of life (Ebright, 2000). Bacterial RNAP has the simplest form, being composed of the minimal five subunits $\beta'\beta\alpha_1\alpha_2\omega$. Eukaryotic RNAP II consists of 12 subunits and synthesizes all messenger RNAs in the cell. Archaeal RNAP is more similar to eukaryotic RNAP than to bacterial RNAP (Hirata *et al.*, 2008) and has 13 subunits (Korkhin *et al.*, 2009). The crystal structure of archaeal RNAP from *Sulfolobus solfataricus* (SsRNAP) has been determined, and the D/L subunits of SsRNAP possessed an iron-sulfur (Fe-S) cluster which plays a key structural role in RNAP assembly and does not exist in eukaryotic RNAPs. The residues of the Fe-S cluster were not conserved in the D/L subunits of *Thermococcus onnurineus* NA1. The D/L subunits have sequence homology to the Rpb3/Rpb11 subunits of eukaryotic RNAP II. Subcomplexes of both the D/L subunits and the Rpb3/Rpb11 subunits provide a platform for the assembly of the two largest catalytic subunits of SsRNAP and RNAP from *Saccharomyces cerevisiae* RNAP, respectively (Hirata *et al.*, 2008).

Recently, the complete genome sequence of the archaeon *T. onnurineus* NA1 has been determined (Lee *et al.*, 2008). Members of the genus *Thermococcus* are sulfur-reducing hyperthermophilic archaea and ubiquitously exist in various deep-sea hydrothermal vent systems. We are interested in the structure and function of the D/L subunits of RNAP from *T. onnurineus* NA1. The *TON-0309* gene encoding RNA polymerase subunit L from *T. onnurineus* NA1 (ToRNAP_L) encodes 95 amino acids that share 36% sequence identity with the L subunit of SsRNAP and 29% sequence identity with Rpb11 from the yeast *S. cerevisiae* (Fig. 1). In this study, we cloned the *TON-0309* gene and expressed ToRNAP_L in *Escherichia coli*. We also purified and crystallized the protein and performed a preliminary X-ray crystallographic study of the ToRNAP_L crystal.

Figure 1

Amino-acid sequence alignment of ToRNAP_L from *T. onnurineus* NA1, SsRNAP_L from *Sulfolobus solfataricus* and Rpb11 from the yeast *Saccharomyces cerevisiae*.

(N-terminus)

1-MRIEVIKREENVLEFYLEGEDHTFANLLNEVLHENEHVTFAGYTIEHPVLMARKPKFRVVTDGKITPEQV

LEKAAQKIFDRARAVLEAWKEVLGE-95

(C-terminus)

Figure 2

Full-length protein sequence of ToRNAP_L used in crystallization (95 amino acids).

2. Materials and methods

2.1. Cloning

Genomic DNA from *T. onnurineus* NA1 was obtained from Korea Ocean Research and Development Institute. The *TON-0379* gene (288 bp) coding for RNA polymerase subunit L from *T. onnurineus* NA1 (ToRNAP_L) was amplified by PCR using the genomic DNA isolated from *T. onnurineus* NA1 as a template. The sequences of the oligonucleotide primers used for gene cloning were based on the DNA sequence of the *TON-0379* gene (GenBank accession No. NC_011529.1). Forward (5'-CCCCCCC**ATATG**AGGATTGAGGT-CATCAAGCGT-3') and reverse (5'-CCCGGT**TACCT**CACTCGCC-CAGGACCTCTTCCA-3') primers were designed to introduce *Nde*I and *Kpn*I restriction sites (bold), respectively. The PCR-amplified DNA fragments were purified using a PCR purification kit (Bioneer) and inserted into the same restriction enzyme-digested pETDuet1 expression vector at multiple cloning site 2 (MCS2). In this construct, the C-terminal S-tag is not fused to ToRNAP_L owing to the presence of a stop codon before the S-tag sequence of the vector. The expression vector was transformed into *E. coli* BL21(DE3) cells and plated on Luria-Bertani agar containing 50 µg ml⁻¹ ampicillin. An ampicillin-resistant colony was selected and plasmid DNA from the transformant was isolated using a plasmid purification kit (Favorgen). DNA sequencing to confirm the cloning was carried out at the Macrogen facility (Seoul, Republic of Korea).

2.2. Overexpression and purification

E. coli BL21 (DE3) cells containing pETduet1-ToRNAP_L were grown at 310 K to an OD₆₀₀ of 0.6 in Luria-Bertani medium supplemented with 50 µg ml⁻¹ ampicillin. Expression of ToRNAP_L protein (Fig. 2) was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. The cells were cultured at 310 K. After overnight growth, the cells were harvested by centrifugation at 9780g (Vision VS24-SMTi V5006A rotor) for 10 min at 277 K. The resultant cell pellets were resuspended in ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 15 mM NaCl, 3 mM β-mercaptoethanol) and disrupted using a sonicator (Sonomasher). The crude cell extract was centrifuged for 30 min at 39 300g (Vision VS24-SMTi V5008A rotor) at 277 K to remove cell debris. The supernatant was filtered using a 0.45 µm membrane (Minisart). All protein-purification steps were carried out at 277 K. ToRNAP_L was purified on a pre-equilibrated 5 ml HiTrap Q anion-exchange column

(GE Healthcare) equilibrated in buffer A (25 mM Tris-HCl pH 7.5, 15 mM NaCl, 3 mM β-mercaptoethanol). ToRNAP_L was washed and eluted with a gradient of 0–100% buffer B (25 mM Tris-HCl pH 7.5, 1 M NaCl, 3 mM β-mercaptoethanol). The homogeneity of the purified protein was checked via SDS-PAGE (Fig. 3). For crystallization, the protein solution was concentrated using centrifugal filters (Amicon Ultra-15, MWCO 10 kDa) to a final concentration of 15 mg ml⁻¹ in a buffer consisting of 25 mM Tris-HCl pH 7.5, 15 mM NaCl, 3 mM β-mercaptoethanol. The protein concentration was determined by the Bradford protein assay.

2.3. Crystallization and X-ray data collection

Initial crystallization screening was carried out at 283 K using a Hydra II e-drop automated pipetting system (Matrix) on a 96-well Intelli-Plate (Art Robbins). Drops consisted of 0.5 µl protein solution (15 mg ml⁻¹) and 0.5 µl reservoir solution and were equilibrated against 70 µl reservoir solution at 287 K. The initial crystallization conditions tested were from Morpheus (Molecular Dimensions), Wizard Precipitant Synergy (Emerald Bio) and Crystal Screen Cryo, Crystal Screen Lite, Crystal Screen HT, Index HT and SaltRX HT

Figure 3

Purified ToRNAP_L is shown on a 16% SDS-PAGE gel. Lane M, molecular-mass markers (labelled in kDa); lane P, ToRNAP_L.

(Hampton Research). An initial hit for ToRNAP_L appeared in the Wizard Precipitant Synergy kit after 1 d with a precipitant solution

(a)

(b)

Figure 4

Crystals of ToRNAP_L. (a) Initial crystals obtained after 1 d using a reservoir solution consisting of 0.2 M lithium sulfate, 25%(w/v) PEG 3350, 15%(v/v) MPD, 0.1 M imidazole-HCl pH 6.5 from the Wizard Precipitant Synergy kit (Emerald Bio). (b) Optimized crystal of ToRNAP_L with dimensions of $0.3 \times 0.05 \times 0.05$ mm obtained using 0.2 M lithium sulfate, 21%(w/v) PEG 3350, 15%(v/v) MPD, 0.1 M imidazole-HCl pH 6.0. The scale bar represents 0.05 mm.

consisting of 0.2 M lithium sulfate, 25%(w/v) PEG 3350, 15%(v/v) MPD, 0.1 M imidazole-HCl pH 6.5 (Fig. 4). After further optimization, crystals were observed and reproduced in hanging drops consisting of 0.2 M lithium sulfate, 21%(w/v) PEG 3350, 15%(v/v) MPD, 0.1 M imidazole-HCl pH 6.0. The fully grown crystals were flash-cooled at 100 K in liquid nitrogen using a cryoprotectant solution consisting of 0.2 M lithium sulfate, 21%(w/v) PEG 3350, 15%(v/v) MPD, 0.1 M imidazole-HCl pH 6.0, 20%(v/v) glycerol.

X-ray data were collected at 100 K using an ADSC Q270 detector on beamline 7A of the Pohang Light Source (PLS), Republic of Korea. X-ray diffraction data were collected to 2.10 Å resolution from the ToRNAP_L crystal (Fig. 5). Diffraction data were collected from a single crystal with 1° oscillation per frame over a total of 360° at a crystal-to-detector distance of 250 mm and with 1 s exposure per frame. The raw data were processed and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997).

3. Results and discussion

The purification protocol for the recombinant form of ToRNAP_L consistently yielded more than 15 mg ToRNAP_L per litre of culture medium, and the protein exhibited a single band on SDS-PAGE at approximately 11 kDa (Fig. 3). Crystals were obtained by mixing 0.5 µl protein solution with 0.5 µl reservoir solution consisting of 0.2 M lithium sulfate, 21%(w/v) PEG 3350, 15%(v/v) MPD, 0.1 M imidazole-HCl pH 6.0. After two weeks, crystals grew to maximum dimensions of approximately $0.3 \times 0.05 \times 0.05$ mm (Fig. 4). X-ray diffraction data were collected on beamline 7A of Pohang Light Source (PLS), Republic of Korea. The space group was selected using the auto-indexing program in *HKL-2000* (Otwinowski & Minor, 1997) and data-collection statistics are provided in Table 1 (Karplus & Diederichs, 2012; Weiss, 2000; Diederichs & Karplus, 1997). The ToRNAP_L crystal belonged to the crystallographic space group *P*6₁22, with unit-cell parameters $a = b = 42.3$, $c = 211.2$ Å. According to the calculation of the Matthews coefficient (Matthews, 1968), one monomer was present in the asymmetric unit, with a corresponding V_M of $2.5 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 50.0%. Molecular

Figure 5

X-ray diffraction image of a ToRNAP_L crystal. The diffraction resolution is marked as a blue circle. The spindle axis was vertical.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Beamline 7A, PLS
Wavelength (Å)	0.97935
Detector	ADSC Q270
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Resolution range (Å)	36.03–2.10 (2.18–2.10)
Space group	<i>P</i> 6 ₁ 22
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 42.3, <i>c</i> = 211.2, <i>α</i> = <i>β</i> = 90.0, <i>γ</i> = 120.0
Mosaicity (°)	0.5
Total No. of reflections	277252
No. of unique reflections	12265
Multiplicity	22.6
Mean <i>I</i> / <i>σ</i> (<i>I</i>)	46.2 (9.0)
Completeness (%)	99.9 (100.0)
<i>R</i> _{p.i.m.} † (%)	1.4 (9.5)
No. of molecules in the asymmetric unit	1
Solvent content (%)	50.0
<i>V</i> _M (Å ³ Da ^{−1})	2.5

† $R_{p.i.m.} = \sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

replacement using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* program package (Winn *et al.*, 2011) with RNAP_L from *Sulfolobus solfataricus* (PDB entry 2pa8, sequence identity 36%; Hirata *et al.*, 2008) as the starting model was carried out. One molecule was found in the asymmetric unit. After a few rounds of

refinement using the *CCP4* program *REFMAC5* (Murshudov *et al.*, 2011), the values of *R*_{work} and *R*_{free} were 25.7 and 32.8%, respectively. Further refinement is being carried out. The structural details will be described in a separate paper.

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