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Received 7 December 2013

Accepted 5 March 2014

Purification, crystallization and preliminary crystallographic analysis of a ribosome-recycling factor from *Thermoanaerobacter tengcongensis* (TteRRF)

Ribosome-recycling factor (RRF) plays an essential role in the fourth step of protein synthesis in prokaryotes. RRF combined with elongation factor G (EF-G) disassembles the post-termination ribosome complex and recycles the protein synthesis machine for the next round of translation. A reductive-methylation-modified RRF from *Thermoanaerobacter tengcongensis* (TteRRF) has been crystallized using the vapour-diffusion method. The crystal grew in a condition consisting of 0.1 M citric acid pH 3.5, 3.0 M NaCl and 50 mg ml⁻¹ methylated protein solution at 289 K. A complete data set was collected from a crystal to 2.80 Å resolution using synchrotron radiation at 100 K. The crystal belonged to space group *P*6₁22/*P*6₅22 with unit-cell parameters *a* = *b* = 103.26, *c* = 89.17 Å. The asymmetric unit was estimated to contain one molecule of TteRRF.

1. Introduction

Protein biosynthesis is considered to be an important procedure in the life cycles of both prokaryotes and eukaryotes. Four consecutive steps designated as initiation, elongation, termination and recycling of the ribosomal complex have been accepted, along with the discovery of and detailed research on the small protein ribosome-recycling factor (Hirokawa *et al.*, 2006). During the termination step, the nascent peptide is formed when the release factors RF1 and RF2 recognize the stop codon and catalyze the hydrolysis of the peptidyl-tRNA (Scolnick *et al.*, 1968). RF3 then releases RF1 and RF2 from the biosynthesis machinery, leaving the post-termination complex consisting of 70S ribosome, deacylated tRNA and mRNA (Freistroffer *et al.*, 1997). Ribosome-recycling factor (RRF) in concert with elongation factor G (EF-G) and GTP catalyzes the decomposition of the post-termination complex (Janosi, Hara *et al.*, 1996; Rao & Varshney, 2001). It has been shown that RRF is essential for the growth of prokaryotes (Janosi *et al.*, 1994), but not eukaryotes (Kaji *et al.*, 1998). Deletion of RRF is lethal to *Escherichia coli* (Janosi *et al.*, 1994) and leads to the retention of binding of ribosome to mRNA as a result of unscheduled translation (Ryoji *et al.*, 1981). RRF is a multifunctional protein that can prevent errors throughout the translation process (Janosi, Ricker *et al.*, 1996).

The protein sequences of all of the reported RRFs from prokaryotes show great similarity, as do their three-dimensional structures. The crystal structures of RRFs from *Thermotoga maritima* (Selmer *et al.*, 1999), *E. coli* (Kim *et al.*, 2000) and its mutant R132G (Nakano *et al.*, 2002), *Thermus thermophilus* (Toyoda *et al.*, 2000), *Vibrio parahaemolyticus* (Nakano *et al.*, 2002), *Mycobacterium tuberculosis* (Saikrishnan *et al.*, 2005) and solution structures from *Aquifex aeolicus* (Yoshida *et al.*, 2001) and *E. coli* (Yoshida *et al.*, 2003) demonstrate that they have two distinct domains. Domain I, consisting of a three-helix bundle, and domain II, consisting of a three-layer $\beta/\alpha/\beta$ sandwich, are connected by a hinge. The RRF executes its function through the movement of the two domains. The gooseneck model or swinging-door model has been proposed according to its structural characteristics (Yoshida *et al.*, 2003). Domain swapping between *E. coli* RRF and TteRRF (*Thermoanaerobacter tengcongensis* RRF) (Guo *et al.*, 2006) revealed that RRF domain I binds to ribosomes and domain II plays a crucial role in the concerted action of RRF and EF-G in disassembly of the post-

Table 1

X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the outermost shell.

Space group	$P6_122/P6_522$
Wavelength (Å)	0.97928
Resolution range (Å)	50.00–2.80 (2.85–2.80)
Unit-cell parameters (Å, °)	$a = b = 103.26$, $c = 89.17$, $\alpha = \beta = 90.00$, $\gamma = 120.00$
No. of molecules in asymmetric unit	1
No. of observed reflections	54418
No. of unique reflections	6965
$\langle I/\sigma(I) \rangle$	21.4 (4.3)
Completeness (%)	94.3 (84.8)
$R_{\text{merge}}^\dagger$ (%)	8.4 (49.7)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the observations $I_i(hkl)$ of reflection hkl .

termination complex. In contrast, *TteRRF* C-terminal mutants lacking one to four amino-acid residues are inactive, those lacking five to nine amino-acid residues are reactivated to a level that is similar to or a little higher than that of wild-type *TteRRF*, and those lacking ten to 12 amino-acid residues are inactivated again gradually (Zhang *et al.*, 2007). The *TteRRF* variants seem more complicated with respect to activity and truncation (Zhang *et al.*, 2007). In order to shed light on these phenomena from their structures, it is necessary to determine the structure of *TteRRF*. In this paper, we report the expression, purification, crystallization and preliminary X-ray analysis of *T. tengcongensis* RRF.

2. Materials and methods

2.1. Protein expression and purification

The RRF protein was expressed and purified as described by Zhang *et al.* (2003) with minor changes. Briefly, the amplified *TteRRF* gene containing *NcoI* and *BamHI* restriction sites was digested and ligated into pET-DB (Zhang *et al.*, 2003), resulting in the vector pET-DB-*TteRRF*. The vector was transformed into *E. coli* BL21 (DE3) pLysS competent cells. Transformants were grown in LB medium containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 25 $\mu\text{g ml}^{-1}$ chloramphenicol overnight at 310 K for a small-scale culture. 20 ml aliquots of the overnight culture were subcultured into 1 l fresh LB medium containing kanamycin (50 $\mu\text{g ml}^{-1}$) and chloramphenicol (25 $\mu\text{g ml}^{-1}$). When the OD₆₀₀ reached 0.8 at 310 K, 0.5 ml of 0.8 mM IPTG was added to 1 l culture and protein expression was induced at

Figure 1
Hexagonal prism crystal of *TteRRF*.

310 K for 4 h. The cell pellet was collected by centrifugation at 4000 rev min⁻¹ for 30 min at 277 K, resuspended in 80 ml buffer A (20 mM sodium phosphate pH 7.4, 500 mM NaCl, 20 mM imidazole). 800 μl of 0.1 M PMSF was added to the buffer to a final concentration of 1 mM. The bacterial pellet was ultrasonicated on ice for 10 min. The clear supernatant (80 ml), after centrifugation at 16 000 rev min⁻¹ at 277 K for 30 min, was applied directly onto a 5 ml Ni Sepharose Fast Flow column (GE Healthcare) equilibrated with buffer A. The contaminant proteins were washed with washing buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl, 80 mM imidazole). Finally, the target protein was eluted with elution buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl, 250 mM imidazole).

The major protein peak was collected and concentrated to 2 ml, and then applied onto a Superdex G200 (GE Healthcare) size-exclusion chromatography (SEC) column equilibrated with buffer consisting of 20 mM HEPES pH 7.5, 100 mM NaCl. The target peak was collected and concentrated to 10 mg ml⁻¹ for chemical modification.

2.2. Reductive methylation

Reductive methylation of RRF was carried out using a previously described protocol (Shaw *et al.*, 2007). In brief, 20 μl 1 M dimethylamine-borane complex (DMAB) and 40 μl 1 M formaldehyde were added to 1 ml of 10 mg ml⁻¹ protein solution. The reaction mixture was incubated in the dark with shaking. The addition of chemicals was repeated twice at 2 h intervals. Finally, 10 μl DMAB was added and the reaction mixture was incubated overnight. The excess amount of chemicals was removed by SEC using 20 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT buffer solution. The methylated protein was concentrated to 50 mg ml⁻¹ and screened for crystallization.

2.3. Crystallization, data collection and processing

The initial crystallization condition was screened at 289 K using commercially available kits from Hampton Research (Crystal Screen, Crystal Screen 2 and Index). 1 μl of 50 mg ml⁻¹ methylated protein solution was mixed with an equal amount of reservoir solution and equilibrated against 400 μl reservoir solution. Clusters of needle-shaped crystals appeared after 3 d in a condition consisting of 0.1 M citric acid pH 3.5, 3.0 M NaCl (Index condition No. 7) at 289 K. Fine-tuning the pH in the range 2.5–4.5 and the precipitant concentration

Figure 2
(a) 15% SDS-PAGE of native and chemically modified *TteRRF* stained with Coomassie Brilliant Blue. Lane 1, chemically modified *TteRRF*; lane 2, marker; lane 3, native *TteRRF*. (b) Purification profile of *TteRRF*, which eluted as a symmetrical peak from the SEC Superdex G200 column (blue peak). The vertical coordinate is the absorbency value (mAU) and the horizontal coordinate is the volume of solution (ml).

in the range 2.5–4 M did not produce crystals suitable for diffraction. Streak-seeding was used to improve the quality of the crystals. Seeds obtained by crushing the needle crystals in stabilizing buffer consisting of 0.1 M citric acid pH 3.5, 3.5 M NaCl were directly applied to the pre-equilibrated 2 µl drop. A suitable single crystal for data collection was finally obtained at 289 K (Fig. 1).

The harvested crystals were soaked in cryoprotectant consisting of 0.1 M citric acid pH 3.5, 3.0 M NaCl, 15% (v/v) glycerol for several seconds, and quickly mounted on the goniometer in a nitrogen stream at 100 K. Data were collected using a MAR 165 mm CCD detector on beamline 3W1A at the BSRF synchrotron radiation source, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing, People's Republic of China. Data collected were indexed, integrated

and scaled with *HKL-2000* (Otwinowski & Minor, 1997). The data-collection statistics are listed in Table 1.

3. Results and discussion

TteRRF was purified to homogeneity, eluting as a symmetrical peak from the SEC Superdex G200 column (Fig. 2*b*), and was shown to be monodisperse by dynamic light scattering (DLS; Fig. 3).

To our great dismay, *TteRRF* failed to crystallize during the initial screening even when the protein concentration reached 100 mg ml⁻¹ or even higher; most clear drops remained clear except several severe conditions. It is believed that crystallization is a surface phenomenon. Lys, Glu and Gln, which have high conformation entropy, seldom form crystal contacts owing to their disordered long side chains. Proteins with an abundance of Lys, Glu and Gln residues on the surface are recalcitrant to crystallization. Reductive methylation of Lys has been reported as a good method to assist the crystallization process. Two methyl groups were bound to the free amine of Lys. These methyl groups form cohesive (NZ)CH₃-O contacts with the neighbouring electron-negative carboxyl and carbonyl O atoms, which localizes the side chain of lysine residues in space, leading to reduction of the surface entropy (Shaw *et al.*, 2007). Reductive methylation of Lys may also reduce the solubility of the protein, resulting in a lower concentration being required to reach the super-saturated state. Amino-acid composition analysis revealed that there were 22 Lys and 24 Glu residues in the *TteRRF* of 158 amino acids in length. Therefore, chemical modification of *TteRRF* is the first choice. The modified protein showed a little larger molecular weight than that of the native protein on SDS-PAGE (Fig. 2*a*). The clusters of needle-shaped crystals were not improved after fine-tuning using a gradient of precipitant and pH. Detergent and additives from Hampton Research were also used to ameliorate its morphology. Although some detergents were able to produce larger crystals, the crystal surface still had defects. Finally, seeding proved to be successful.

The hexagonal prism crystal belonged to space group *P*6₁22/*P*6₅22, with unit-cell parameters *a* = *b* = 103.264, *c* = 89.173 Å, α = β = 90.00, γ = 120.00° (Fig. 4). The asymmetric unit is estimated to contain one molecule of ribosome-recycling factor, with a corresponding Matthews coefficient of 2.9 Å³ Da⁻¹ and a solvent content of 57.4% (Matthews, 1968). The crystal structure determination of *TteRRF* by molecular replacement is in progress.

We thank Professor Yuhui Dong and coworkers at the BSRF and Professor Jianhua He and coworkers at the SSRF for their help in data collection, and Dr Zhengqiang Gao for his help in data processing. This work was financially supported by the National Natural Science Foundation of China (30970578, 31070651) and New Century's Elitists' Program of Education Administer (NCET-08-0731).

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Figure 3
DLS regularization histogram analysis of *TteRRF*. The vertical coordinate axis corresponds to the relative dynamic light-scattering intensity and the horizontal axis corresponds to the hydrodynamic radius (on a logarithmic scale).

Figure 4
An image of the *TteRRF* diffraction pattern at a resolution 2.80 Å.

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