Purification, crystallization and preliminary crystallographic analysis of the 16S rRNA methyltransferase RsmI from *Escherichia coli*

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Received 8 March 2014
Accepted 23 July 2014

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RsmI and RsmH are AdoMet-dependent methyltransferases that are responsible for the 2'-*O*-methylation and *N*⁴-methylation of C1402 of *Escherichia coli* 16S rRNA, respectively. Modification of this site has been found to play a role in fine-tuning the shape and function of the P-site to increase the decoding fidelity. It is interesting to study the mechanism by which C1402 can be methylated by both RsmI and RsmH. The crystal structure of RsmH in complex with AdoMet and cytidine has recently been determined and provided some implications for *N*⁴-methylation of this site. Here, the purification and crystallization of RsmI as well as its preliminary crystallographic analysis are reported. Co-crystallization of RsmI with AdoMet was carried out by the sitting-drop vapour-diffusion method and X-ray diffraction data were collected to 2.60 Å resolution on beamline 1W2B at BSRF. The crystal contained three molecules per asymmetric unit and belonged to space group *C*2, with unit-cell parameters *a* = 121.9, *b* = 152.5, *c* = 54.2 Å, β = 93.4°.

1. Introduction

Post-transcriptional modifications of specific bases in ribosomal RNA (rRNA) play important roles in fine-tuning protein synthesis *via* regulation of the ribosome (Grosjean, 2005). The majority of these modifications are located in and around the conserved decoding and peptidyltransfer centre (PTC) of the ribosome. Among these modifications, base methylation is the most frequent type of modification in bacteria, while pseudouridylation and 2'-*O*-methylation are common in archaea and eukaryota (Rozenski *et al.*, 1999; Ofengand & Del Campo, 2004).

There are 24 methylated nucleotides in *Escherichia coli* rRNA, among which only four 2'-*O*-methylated bases have been identified (Sergiev *et al.*, 2011). A set of 23 methyltransferases (MTases) responsible for the modification of 24 bases have been described (Golovina *et al.*, 2012). A recent study discovered that C1402 in *E. coli* 16S rRNA can be modified by the AdoMet-dependent MTases RsmI and RsmH, which are responsible for 2'-*O*-methylation and *N*⁴-methylation of this site (m⁴Cm1402), respectively (Kimura & Suzuki, 2010). The *rsmH* and *rsmI* genes are conserved in almost all species of bacteria, suggesting that this modification at the P-site is a common structural feature of bacterial 16S rRNA. C1402 directly interacts with the phosphate backbone of the P-site codon, and its dimethylation plays an important role in P-site function, especially in start-codon selection (Kimura & Suzuki, 2010). The P-site contains several conserved residues (positions 1400–1405 and 1496–1502) in the top part of helix 44 in the 16S rRNA of the 30S subunit (Berk *et al.*, 2006). Precise codon–anticodon pairing occurs in this site between the mRNA and a peptidyl-tRNA during the elongation cycle to maintain the reading frame (Berk *et al.*, 2006; Selmer *et al.*, 2006). Several point mutations in the P-site of *E. coli* 16S rRNA have been found to cause growth reduction (Jemiolo *et al.*, 1985; Meier *et al.*, 1986). Although we have determined the crystal structure of RsmH in complex with AdoMet and cytidine (Wei *et al.*, 2012), the mechanism of dimethylation of C1402 remains unclear and the structure and function of RsmI need to be studied. The molecular weight of RsmI is 31 kDa. Similar to RsmH and other RNA MTases, RsmI is most likely

to be composed of an N-terminal substrate-recognition domain and a C-terminal catalytic domain.

In this report, the expression, purification, crystallization and preliminary X-ray crystallographic analysis of RsmI are presented.

2. Materials and methods

2.1. Macromolecule production

The full-length *rsmI* gene from *E. coli* K-12 genomic DNA was amplified by PCR. The amplified insert of the *rsmI* gene was cloned into the pET-28a(+) expression vector, in which a His₆ tag followed by a cleavable *Tobacco etch virus* (TEV) protease site is introduced upstream, transformed into the *E. coli* DH5 α cloning strain and plated onto LB kanamycin plates. The plasmid was isolated and transformed into the *E. coli* BL21 (DE3) Star expression strain (Invitrogen).

Bacterial cells were grown to mid-log phase in LB medium at 310 K in the presence of 0.05 mg ml⁻¹ kanamycin. Induction of the culture was then carried out with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 293 K. Cells were pelleted after 20 h by centrifugation at 11 260g for 10 min at 277 K. The cell pellet was resuspended in buffer A [20 mM Tris, 500 mM NaCl, 5%(v/v) glycerol, 2 mM β -mercaptoethanol, 1 mM PMSF pH 8.5] and lysed by ultrasonication on ice. The cell debris and membranes were pelleted by centrifugation at 28 620g for 60 min at 277 K. The soluble N-terminally His₆-tagged RsmI was purified by affinity chromatography with nickel-nitrilotriacetic acid resin (Bio-Rad). Untagged proteins were removed with buffer A containing 30 mM imidazole. Recombinant RsmI was then eluted with buffer A containing 250 mM imidazole. After dialysis, the resulting protein was digested by TEV protease (added to the protein at a mass ratio of 1:40) at 277 K for 6 h to remove the His₆ tag and obtain native protein in buffer C [20 mM Tris, 200 mM NaCl, 5%(v/v) glycerol, 2 mM DTT pH 8.5]. Only one amino acid (glycine) remained fused to the N-terminus after TEV protease cleavage.

The untagged protein was further purified using a heparin column (GE Healthcare) pre-equilibrated with buffer B (50 mM Tris-HCl pH 8.5, 80 mM NaCl). The protein was eluted from the column with a gradient of NaCl (0.08–0.5 M). It was then purified by gel filtration on Superdex 200 (GE Healthcare) equilibrated with buffer C using an ÄKTApurifier system (GE Healthcare). Highly purified RsmI fractions were pooled and were then concentrated to 13 mg ml⁻¹ by ultrafiltration in an Amicon cell (Millipore, California, USA). The protein concentration was determined using the Bio-Rad protein assay kit.

The gene for the truncated protein Gly12–Pro258 was also cloned into *NdeI/XhoI*-digested pET-28a(+) expression vector and the truncated protein was expressed and purified in essentially the same way as full-length RsmI.

2.2. Limited trypsin-proteolysis assay

5 μ l of protein solution (1 mg ml⁻¹) was incubated with trypsin in buffer C overnight at 277 K. The trypsin (1 mg ml⁻¹) was used at dilutions of 1:1, 1:5, 1:25, 1:125 and 1:625. The reactions were stopped by the addition of SDS–Coomassie sample-loading buffer for analysis by gel electrophoresis.

2.3. Crystallization

Full-length RsmI consisting of 286 residues (0.42 mM or 13 mg ml⁻¹) and AdoMet (Sigma, USA; 180 mM stock solution prepared with buffer C) were mixed in a molar ratio of 1:5 and incubated on ice for 6 h before performing co-crystallization experiments. Initial crystallization conditions for full-length RsmI were screened by the utilization of several sparse-matrix screens (Hampton Research, USA) with the sitting-drop vapour-diffusion method at room temperature, but no crystals were obtained.

For the truncated protein (Gly12–Pro258), several crystallization conditions were obtained for the mixture with AdoMet. The best crystal, with a rod-like shape (Fig. 1*a*), was obtained in using 0.2 M DL-malic acid pH 7.0, 20% PEG 3350 at 291 K and was used for data collection.

Figure 1
A crystal of RsmI as grown by the sitting-drop method (*a*) and an X-ray diffraction image obtained from the crystal using a MAR165 CCD detector (*b*).

2.4. Data collection and processing

Diffraction data were collected from a single crystal on beamline 1W2B at Beijing Synchrotron Radiation Facility (BSRF), Beijing, People's Republic of China using a MAR165 CCD detector at a wavelength of 1.0 Å (Fig. 1*b*). The total oscillation was 250°, with 1° rotation per image, and the exposure time was 100 s per image. The sample-to-detector distance was set to 160 mm. Before data collection, crystals were cryoprotected in paraffin oil and then flash-cooled in liquid nitrogen. The temperature was maintained at 100 K by a cold nitrogen-gas stream during data collection. The data were processed with *HKL-2000* (Otwinowski & Minor, 1997) and the data statistics are listed in Table 1.

Figure 2
SDS-PAGE analysis of limited trypsin digestion of RsmI. The trypsin dilution ratios are shown above the gel. Lane *M* contains molecular-weight marker (labelled in kDa).

Figure 3
Plots of the self-rotation function of RmsI at $\chi = 180^\circ$ (left) and 120° (right).

Table 1
Data collection and processing.

Values in parentheses are for the outer shell.	
Diffraction source	1W2B, BSRF
Wavelength (Å)	1.0
Temperature (K)	100
Detector	MAR165 CCD
Crystal-to-detector distance (mm)	160
Rotation range per image (°)	1.0
Total rotation range (°)	250
Exposure time per image (s)	100
Space group	C2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	121.9, 152.5, 54.2
α , β , γ (°)	90.0, 93.4, 90.0
Resolution range (Å)	50–2.6 (2.64–2.60)
Total No. of reflections	122229
No. of unique reflections	30327 (1500)
Completeness (%)	100.0 (100.0)
Multiplicity	4.0 (3.8)
$\langle I/\sigma(I) \rangle$	21.1 (2.0)
$R_{\text{merge}}^\dagger$	0.05 (0.51)
Overall <i>B</i> factor from Wilson plot (Å ²)	44.9

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *hkl* indicates unique reflection indices and *i* indicates symmetry-equivalent indices.

3. Results and discussion

According to secondary-structure prediction using the ExPASy server (Slabinski *et al.*, 2007) and the results of a limited trypsin-digestion assay (Fig. 2), the truncation Gly12–Pro258 of RsmI was constructed and used for co-crystallization screening with AdoMet. The N-terminus (amino acids 1–11) is disordered according to the secondary-structure prediction. The protein bands of digested RsmI ran about 2.5 kDa lower than full-length RsmI on SDS-PAGE. This suggests that RsmI was digested from the Lys260 site because trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine.

The final yield of the pure truncated protein (Gly12–Pro258) was about 25 mg per litre of cell culture. RsmI in complex with AdoMet

was crystallized in PEG 3350-containing conditions after removing the disordered regions at both the N-terminus and the C-terminus. Crystals suitable for X-ray diffraction were obtained after 3 d. The size of the crystal used for data collection was $0.7 \times 0.1 \times 0.1$ mm. A total of 122 229 measured reflections were merged into 30 327 unique reflections with an R_{merge} of 5.0%. The crystal belonged to space group $C2$, with unit-cell parameters $a = 121.9$, $b = 152.5$, $c = 54.2$ Å, $\beta = 93.4^\circ$.

Preliminary crystallographic analysis showed there were three molecules in the asymmetric unit with a B factor of 44.9 Å² estimated from the Wilson plot and a solvent content of 59.7%. The structure was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the crystal structure of a putative methyltransferase from *Lactobacillus brevis* (PDB entry 3kwp; Midwest Center for Structural Genomics, unpublished work) with a sequence identity of 40.9% as the search model. *Phaser* found an initial solution with TFZ (translational Z -score) values of 13.7, 21.1 and 24.0, respectively, for three molecules in the asymmetric unit, with an increasing LLG (log-likelihood gain) of 181, 524 and 762. The self-rotation function (Fig. 3) was calculated using *MOLREP* (Vagin & Teplyakov, 2010) with x , y and z aligned with the crystallographic a , b and c^* axes, respectively. The resolution limits of the data used were 50–2.8 Å and the radius of integration was 27 Å. This shows that RsmI is a dimer not a trimer. The behaviour of RsmI in size-exclusion chromatography also demonstrates this result. Therefore, we deduce that the three molecules per asymmetric unit should form one dimer with a noncrystallographic twofold axis and a monomer that forms a dimer by crystallographic symmetry. Co-crystallization screening of RsmI with both AdoMet and a substrate mimic is being investigated.

The authors would like to gratefully acknowledge the staff of BSRF beamlines 1W2B and 3W1A for diffraction data collection.

This work was supported by grants from the National Natural Science Foundation of China (10979005, 31200552 and 31270791) and the National Basic Research Program of China (2012CB917203 and 2012CB911103).

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