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Purification, crystallization and preliminary crystallographic analysis of the 23S rRNA methyltransferase RlmM (Cm2498) from *Escherichia coli*

RlmM is an AdoMet-dependent methyltransferase that is responsible for 2'-*O*-methylation of C2498 in the peptidyl-transferase loop of bacterial 23S rRNA. This modification occurs before assembly of the 50S ribosomal subunit, and lack of C2498 methylation can cause a slight reduction in bacterial fitness. Here, the purification and crystallization of RlmM from *Escherichia coli* as well as its preliminary crystallographic analysis are presented. Cocrystallization of RlmM with AdoMet was carried out and X-ray diffraction data were collected to a resolution of 2.30 Å on beamline BL17U at the SSRF. However, electron density for AdoMet cannot be observed by comprehensive crystallographic analysis, indicating that it is not bound by RlmM during the cocrystallization process. The structure was solved by molecular replacement and refinement is in progress. The crystal contained one molecule in the asymmetric unit and belonged to space group $P2_1$, with unit-cell parameters $a = 56.07$, $b = 59.38$, $c = 54.35$ Å, $\beta = 94.84^\circ$, which differs from the $P3_1$ or $P3_121$ space groups of previously reported RlmM structures (PDB entries 4auk, 4atn and 4b17).

1. Introduction

Methylation of ribosomal RNA (rRNA) is the process in which the methyl group of *S*-adenosyl-L-methionine (AdoMet) is transferred to an O or N atom of the target RNA catalyzed by AdoMet-dependent methyltransferases (MTases). These modifications play an important role in fine-tuning the process of protein synthesis (Grosjean, 2005). Most of the modifications are located in or around the conserved decoding and peptidyltransferase active sites of the ribosome. Methylation is also closely related to the fine-tuning of local rRNA structure, antibiotic resistance and many other crucial modifications of ribosome activity (Ofengand & Fournier, 1998; Connolly *et al.*, 2008; Douthwaite *et al.*, 2005; Xu *et al.*, 2008). Base methylation is the most frequent type of rRNA modification in bacteria, while pseudouridylation and 2'-*O*-methylation are common in archaea and eukaryota (Rozenki *et al.*, 1999; Ofengand & Del Campo, 2004).

Only four 2'-*O*-methylated nucleotides have been identified to date in the rRNAs of *Escherichia coli* (Purta *et al.*, 2009). RlmM, an AdoMet-dependent 2'-*O*-ribose methyltransferase, is specific for C2498 methylation of the peptidyl-transferase loop of 23S rRNA in *E. coli* and other bacteria (Purta *et al.*, 2009). C2498 is located within a highly conserved and heavily modified rRNA sequence and the activity of RlmM is influenced by other modification enzymes that target this region. Recombinant RlmM can methylate C2498 in naked 23S rRNA but not in assembled 50S subunits or ribosomes, indicating that this modification occurs before assembly of the 50S ribosomal subunit. The *rlmM*-knockout ($\Delta rlmM$) strain showed slower growth compared with wild-type *E. coli*, indicating that a defect in this modification results in a slight reduction in bacterial fitness (Purta *et al.*, 2009).

Although its physiological function has been comprehensively studied, little is known about the process by which RlmM binds the methyl-group donor AdoMet, specifically recognizes the nucleotide C2498 and then transfers a methyl group to the target. Determination of three-dimensional structure is a powerful means of functional characterization; therefore, an attempt was made to study the catalytic mechanism of RlmM by X-ray crystallography. Very recently,

crystal structures of *E. coli* RlmM in the apo form at 1.9 Å resolution and in complex with AdoMet at 2.6 Å resolution have been reported (Punekar *et al.*, 2012). An N-terminal THUMP domain and a C-terminal catalytic Rossmann-like fold MTase domain are found in a novel arrangement in RlmM. In this report, the expression, purification, crystallization and preliminary crystallographic analysis of RlmM are presented. Compared with the structure of RlmM reported by Punekar *et al.* (2012), the crystal structure presented here has a similar resolution (2.30 Å) but belongs to a different space group.

2. Materials and methods

2.1. Cloning

The full-length *rlmM* gene was amplified from *E. coli* K-12 genomic DNA by PCR using a forward primer containing a *Bam*HI restriction site (5'-CGCGGATCCATGAATAAGGTTGTATTGCTGTG-3') and a reverse primer containing an *Xho*II restriction site (5'-CCGCTCGAGTTAGCGGCGGACGTGCA-3'); the restriction sites are indicated in bold. The amplified insert of the *rlmM* gene was

(a)

(b)

Figure 1
(a) A crystal of RlmM grown by the sitting-drop method. (b) An X-ray diffraction image obtained from the crystal of RlmM using an ADSC Q315r CCD detector.

cloned into the pET28at_plus expression vector, in which a His₆ tag followed by a cleavable tobacco etch virus (TEV) protease site is introduced upstream of the *Bam*HI site, and transformed into the *E. coli* DH5α cloning strain and plated onto LB kanamycin plates. The plasmid was isolated and transformed into the *E. coli* BL21 (DE3) Star expression strain (Invitrogen).

2.2. Protein expression and purification

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 carried out with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 203 K. Cells were pelleted after 20 h by centrifugation at 8000 rev min⁻¹ for 10 min at 277 K. The cell pellet was resuspended in buffer A [20 mM Tris-HCl, 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 16 000 rev min⁻¹ for 60 min at 277 K. The soluble N-terminally hexahistidine-tagged RlmM was purified by affinity chromatography with nickel-nitrilotriacetic acid resin (Bio-Rad). Untagged proteins were removed with buffer A containing 30 mM imidazole. Recombinant RlmM was then eluted with buffer A containing 250 mM imidazole. The resulting protein was digested by TEV protease (which was added to the protein at a 1:40 mass ratio) at 277 K for 6 h to remove the His₆ tag and obtain native protein.

The untagged protein was further purified on a heparin column (GE Healthcare) pre-equilibrated with buffer B (50 mM Tris-HCl pH 8.5, 80 mM NaCl) followed by gel filtration on a Superdex 75 column (GE Healthcare) equilibrated with buffer C [20 mM Tris-HCl, 100 mM NaCl, 5% (v/v) glycerol, 2 mM DTT pH 8.5] using an ÄKTApurifier System (Amersham). Highly purified RlmM fractions were pooled and concentrated by ultrafiltration in an Amicon cell (Millipore, California, USA) to 15 mg ml⁻¹ as quantified using the Bio-Rad protein assay kit.

2.3. Protein crystallization

Crystallization conditions for full-length RlmM were screened by the sitting-drop vapour-diffusion method using several sparse-matrix screens (Hampton Research, USA) at room temperature; however, no crystals were obtained. According to secondary-structure prediction using the ExPASy server, a loop composed of 13 amino-acid residues in the C-terminus is likely to influence crystallization. Moreover, these residues are not conserved on sequence alignment and their function has not previously been characterized. Therefore, a truncated version of RlmM without the 13 C-terminal residues was constructed. The protein (0.366 mM or 15 mg ml⁻¹) and AdoMet (Sigma, USA; 180 mM stock solution prepared in buffer C) were mixed in a molar ratio of 1:5 and incubated on ice for 6 h before performing cocrystallization experiments.

Initial crystallization conditions were obtained using the mixture of truncated RlmM (Met1–Arg353) and AdoMet in a solution consisting of 16% (w/v) polyethylene glycol 3350, 0.05 M citric acid, 0.05 M bis-tris propane pH 5.0 (PEG/Ion 2; Hampton Research). The crystal quality was optimized by adjusting the concentration of the precipitant and the buffer and by the addition of additives. The best crystal with a frustum shape (Fig. 1a) was obtained from the mixture of RlmM and AdoMet by the addition of 1% (w/v) benzidine from Silver Bullets (Hampton Research, USA) to the initial crystallization condition described above and was used for data collection.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9791
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 56.07$, $b = 59.38$, $c = 54.35$, $\beta = 94.84$
Resolution (Å)	2.30 (2.34–2.30)
Unique reflections	15558 (786)
Completeness (%)	98.7 (99.7)
Multiplicity	3.6 (3.7)
Mean $I/\sigma(I)$	17.68 (5.49)
Molecules in asymmetric unit	1
R_{merge} (%)	7.1 (27.1)

2.4. Data collection

Diffraction data were collected from a single crystal using an ADSC Q315r detector on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China) at a wavelength of 0.9791 Å (Fig. 1*b*). The total oscillation was 360° with 1° per image and the exposure time was 1 s per image. The crystal-to-detector distance was set to 120 mm. Before data collection, crystals were soaked for 5 s in a cryoprotectant consisting of 20% (v/v) glycerol in the crystal mother liquor and were then flash-cooled in liquid nitrogen. The temperature was maintained at 100 K in a cold nitrogen-gas stream during data collection. The data were processed using *HKL-2000* (Otwinowski & Minor, 1997) and the statistical results are listed in Table 1.

3. Analysis and discussion of the preliminary X-ray diffraction results

Crystals suitable for X-ray diffraction were obtained after 7 d. Although our purified RlmM and the previously reported RlmM were both crystallized in PEG 3350-containing conditions, there was no salt in our condition, which gives frustum-shaped crystals (Fig. 1*a*), while ammonium tartrate was present as the salt fraction in the previous condition, which gave rod-shaped crystals (Punekar *et al.*, 2012). Moreover, our crystals were obtained using a truncated version of RlmM (Met1–Arg353), while the previous crystals were obtained using its full length (Met1–Arg366). Analysis of the structure showed that the C-terminal residues 358–366 were disordered (Punekar *et al.*, 2012), which is consistent with our prediction that there is a flexible loop in this region.

Preliminary crystallographic analysis showed that there was one molecule in the asymmetric unit, with a Matthews coefficient of

$2.35 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 47.8%. The structure was solved by molecular replacement with the program *Phaser* (McCoy *et al.*, 2007) using the structure of RlmM (PDB entry 4auk; Punekar *et al.*, 2012) as the search model. Although our crystals were obtained by cocrystallization, electron density for AdoMet could not be observed even after comprehensive crystallographic analysis, and identical results were obtained from several sets of diffraction data. This may be caused by the open and shallow AdoMet-binding site of RlmM, as observed by Punekar *et al.* (2012), in which it appears that AdoMet cannot bind as tightly as other RNA MTases, or by the loss of AdoMet during the cocrystallization process.

The clear difference between our crystal and the previously reported crystals is that our crystal belonged to space group $P2_1$, with unit-cell parameters $a = 56.07$, $b = 59.38$, $c = 54.35$ Å, $\beta = 94.84^\circ$, while the latter belonged to space group $P3_1$ or $P3_121$ (PDB entries 4auk, 4atn and 4b17; Punekar *et al.*, 2012). Therefore, the crystal represents a different crystal packing.

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