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Crystallization and preliminary X-ray diffraction analysis of an archaeal tRNA-modification enzyme, TiaS, complexed with $tRNA^{Ile2}$ and ATP

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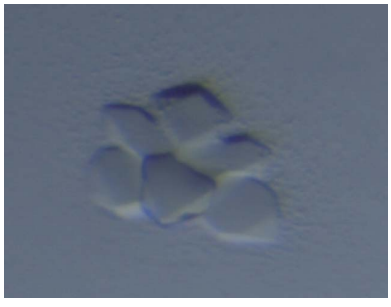

The cytidine at the first anticodon position of archaeal $tRNA^{Ile2}$, which decodes the isoleucine AUA codon, is modified to 2-agmatinylcytidine (agm^2C) to guarantee the fidelity of protein biosynthesis. This post-transcriptional modification is catalyzed by $tRNA^{Ile}$-agm^2C synthetase (TiaS) using ATP and agmatine as substrates. *Archaeoglobus fulgidus* TiaS was overexpressed in *Escherichia coli* cells and purified. $tRNA^{Ile2}$ was prepared by *in vitro* transcription with T7 RNA polymerase. TiaS was cocrystallized with both $tRNA^{Ile2}$ and ATP by the vapour-diffusion method. The crystals of the TiaS–$tRNA^{Ile2}$–ATP complex diffracted to 2.9 Å resolution using synchrotron radiation at the Photon Factory. The crystals belonged to the primitive hexagonal space group $P3_221$, with unit-cell parameters $a = b = 131.1$, $c = 86.6$ Å. The asymmetric unit is expected to contain one TiaS–$tRNA^{Ile2}$–ATP complex, with a Matthews coefficient of 2.8 $Å^3$ Da^{-1} and a solvent content of 61%.

1. Introduction

Post-transcriptional modifications at the first position (wobble position or position 34) of the tRNA anticodon play an important role in the precise decoding of the genetic code (Björk, 1995; Suzuki, 2005; Yokoyama & Nishimura, 1995). In most archaea, the wobble cytidine (C34) of the AUA codon-reading isoleucine tRNA ($tRNA^{Ile2}$) is modified to 2-agmatinylcytidine (agm^2C or agmatidine), an agmatine-conjugated cytidine derivative (Ikeuchi *et al.*, 2010; Mandal *et al.*, 2010). The precursor form of $tRNA^{Ile2}$ with unmodified C34 is misaminoacylated by methionyl-tRNA synthetase and deciphers the methionine AUG codon (Ikeuchi *et al.*, 2010; Köhrer *et al.*, 2008), because $tRNA^{Ile2}$ bears the CAU anticodon sequence that also appears in $tRNA^{Met}$. In contrast, $tRNA^{Ile2}$ with the agm^2C modification precisely deciphers the isoleucine AUA codon instead of AUG (Ikeuchi *et al.*, 2010). In addition, mature $tRNA^{Ile2}$ is charged with isoleucine by isoleucyl-tRNA synthetase *in vitro* owing to the presence of the agm^2C modification (Köhrer *et al.*, 2008). Thus, the agm^2C modification switches the codon specificity from AUG to AUA and enables $tRNA^{Ile2}$ to be correctly aminoacylated with isoleucine. Therefore, the agm^2C modification is crucial for accurate decoding of the genetic code.

The formation of agm^2C is catalyzed by $tRNA^{Ile}$-agm^2C synthetase (TiaS) using ATP and agmatine as substrates (Ikeuchi *et al.*, 2010). In its primary structure, TiaS has three conserved motifs: DUF1743, an OB-fold and a zinc finger-like motif (Ikeuchi *et al.*, 2010). A mutational analysis of conserved residues in each motif revealed that all of these motifs are essential for agm^2C formation (Ikeuchi *et al.*, 2010). As observed in many RNA-binding proteins (Krishna *et al.*, 2003; Schmitt *et al.*, 1998), both the OB-fold and zinc finger-like motifs in TiaS would be involved in tRNA recognition. TiaS requires ATP during agm^2C formation, but there is no clear ATP-binding motif in its primary structure. In addition to the three conserved motifs, TiaS has several conserved residues in the N-terminal region upstream of DUF1743. Thus, the uncharacterized DUF1743 and/or the N-terminal region of TiaS might serve as an ATP-binding motif for agm^2C formation. Several crystal structures of ATP-utilizing tRNA-modification enzymes have been reported (Chimnaronk *et al.*, 2009;

Nakanishi *et al.*, 2009; Numata *et al.*, 2006; Waterman *et al.*, 2006), but TiaS lacks sequence similarity to these enzymes. Therefore, the ATP-binding mode and the catalytic mechanism of agm^{2}C formation by TiaS remain to be elucidated. The structural analysis of the complex containing TiaS and its substrates is indispensable for revealing the mechanism of agm^{2}C formation. In the present paper, we report the crystallization and preliminary X-ray diffraction analysis of *Archaeoglobus fulgidus* TiaS in complex with tRNA^{Ile2} and ATP.

2. Materials and methods

2.1. Protein preparation

The DNA fragment encoding *A. fulgidus* TiaS was produced by PCR amplification and cloned into the expression vector pET15b (Novagen). Recombinant TiaS with an N-terminal His tag was produced in *Escherichia coli* strain C41 (DE3) (Lucigen). After sonication of the *E. coli* cells in 20 m*M* Tris–HCl pH 8.0 containing 300 m*M* NaCl, 10 m*M* imidazole, 7 m*M* β-mercaptoethanol, 1 m*M* PMSF and 1 m*M* benzamidine, the clarified lysate was incubated at 338 K for 20 min. The heat-treated lysate was centrifuged at 8000 rev min^{-1} for 30 min and the supernatant was loaded onto an Ni–NTA (Qiagen) column equilibrated with buffer *A* (20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole, 7 m*M* β-mercaptoethanol). TiaS was eluted with buffer *B* (buffer *A* containing 250 m*M* imidazole). The eluate from the Ni–NTA column was dialyzed against buffer *C* (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 20 m*M* imidazole pH 7.0, 7 m*M* β-mercaptoethanol). The protein solution was then loaded onto a HiTrap Heparin column (16 × 25 mm; GE Healthcare) previously equilibrated with buffer *C*. A linear gradient from 200 to 1000 m*M* NaCl was developed in buffer *C*. The fractions enriched in the target protein were combined and dialyzed against buffer *D* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 7 m*M* β-mercaptoethanol). The protein solution was further purified on a Superdex 200 column (1.6 × 60 cm; GE Healthcare) previously equilibrated with buffer *D*. The fractions enriched in TiaS were combined and concentrated to 1.0–1.9 mg ml^{-1}. Selenomethionine-labelled (SeMet-labelled) TiaS was purified using the same procedure as was used for the native protein.

2.2. tRNA preparation

The gene encoding *A. fulgidus* tRNA^{Ile2} was PCR-amplified and cloned into the pUC18 vector. This plasmid was digested with the *Fok*I restriction enzyme and the digest was used as a template for *in vitro* transcription with T7 RNA polymerase. The transcribed tRNA^{Ile2} was purified under denaturing conditions by polyacrylamide gel electrophoresis and was eluted from the gel pieces. After ethanol precipitation, tRNA^{Ile2} was dissolved in water and then passed through a PD-10 column (GE Healthcare) to completely remove the residual urea. tRNA^{Ile2} was further precipitated with ethanol, dissolved in water and stored at 193 K.

2.3. Crystallization

The purified tRNA^{Ile2} in 10 m*M* Na HEPES pH 7.5 and 5 m*M* $MgCl_2$ was denatured by heat treatment at 343 K for 10 min and was then refolded by gradual cooling of the solution to 298 K. TiaS was mixed with tRNA^{Ile2} in a 1:1.2 molar ratio of TiaS:tRNA^{Ile2} at a final protein concentration of 0.9–1.8 mg ml^{-1} and the mixture was incubated at 310 K in the presence of 5 m*M* $MgCl_2$, 1 m*M* DTT and 1 m*M* ATP. After the sample solution had been placed on ice for 15 min, crystallization conditions were screened by the sitting-drop vapour-diffusion method at 293 K. Sitting drops were prepared by mixing 1 µl reservoir solution with 1 µl sample solution and were equilibrated against 100 µl reservoir solution. Crystals grew using a reservoir solution consisting of 25.5% PEG 4000, 0.17 *M* sodium acetate, 0.085 *M* Tris–HCl pH 8.5, 15% glycerol. We subsequently optimized the crystallization conditions by the hanging-drop vapour-diffusion method. Hanging drops were prepared by mixing 2 µl reservoir solution with 2 µl sample solution and were equilibrated against 500 µl reservoir solution. Consequently, crystals grew to dimensions sufficient for X-ray diffraction analysis using a reservoir solution consisting of 26.5–28% PEG 4000, 0.17 *M* sodium acetate, 0.085 *M* Tris–HCl pH 8.5, 15% glycerol. Under the optimized reservoir conditions, SeMet-labelled TiaS was also cocrystallized with tRNA^{Ile2} and ATP by the hanging-drop vapour-diffusion method. To determine whether the crystals contained the TiaS–tRNA^{Ile2} complex, the crystals were harvested, washed well, dissolved in Milli-Q water and examined by SDS–PAGE and denaturing PAGE.

Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

Wavelength (Å)	0.97898
Space group	$P3_221$
Unit-cell parameters (Å, °)	$a = b = 131.1$, $c = 86.6$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution (Å)	50–2.90 (2.95–2.90)
Measured reflections	295793
Unique reflections	19261
Multiplicity	15.4 (8.2)
Completeness (%)	99.9 (99.9)
$\langle I/\sigma(I)\rangle$	29.3 (2.7)
R_{merge}†	0.100 (0.407)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity over symmetry-equivalent measurements.

2.4. X-ray data collection

The TiaS–tRNA^{Ile2}–ATP complex crystals were polycrystals. For X-ray data collection, the polycrystals were carefully separated into single crystals using a needle. The separated single crystals were mounted in a cryoloop and then directly flash-cooled in a nitrogen stream at 95 K. X-ray diffraction data were collected on beamline BL-17A of KEK (Ibaraki, Japan) using an ADSC Q270 CCD detector. Diffraction data were integrated and scaled with the program *HKL*-2000 (Otwinowski & Minor, 1997). The processing statistics are summarized in Table 1.

3. Results and discussion

The TiaS protein, with a His tag attached to its N-terminus, was produced in *E. coli* strain C41 (DE3) using the pET15b expression vector and was purified to homogeneity through several chromatographic steps. The amount of recombinant TiaS obtained by this procedure was 1.0 mg from 8 l culture. tRNA^{Ile2} was prepared by *in vitro* transcription using T7 RNA polymerase. The purified TiaS and tRNA^{Ile2} were mixed in a 1:1.2 molar ratio and the mixture was used for crystallization screening by the sitting-drop vapour-diffusion method at 293 K. The crystals were obtained under conditions consisting of 25.5% PEG 4000, 0.17 *M* sodium acetate, 0.085 *M* Tris–HCl pH 8.5, 15% glycerol. Electrophoretic analyses of the obtained crystals by SDS–PAGE and denaturing PAGE demonstrated that the crystals contained both TiaS and tRNA^{Ile2} (data not shown). We subsequently optimized the crystallization conditions by the hanging-

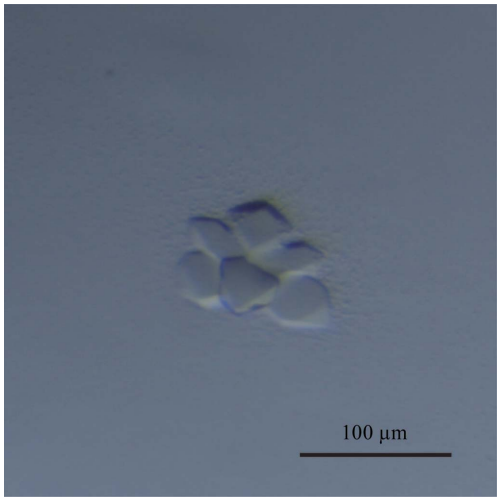

Figure 1
Crystals of the TiaS–tRNA^{Ile2}–ATP complex.

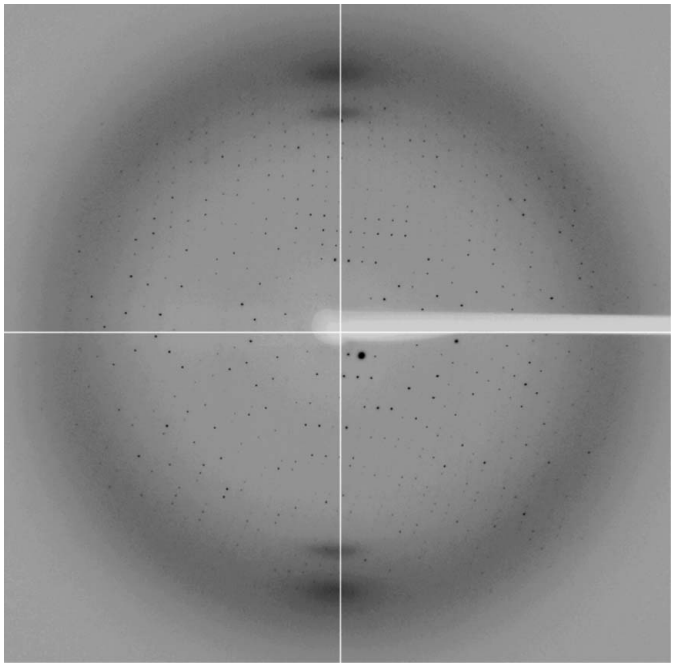

Figure 2
Diffraction pattern of a TiaS–tRNA^{Ile2}–ATP complex crystal.

drop vapour-diffusion method and obtained complex crystals suitable for X-ray diffraction experiments using the reservoir conditions of 26.5–28% PEG 4000, 0.17 *M* sodium acetate, 0.085 *M* Tris–HCl pH 8.5, 15% glycerol. The complex crystals grew to maximum dimensions of 40 × 20 × 20 µm within a week (Fig. 1). We also obtained complex crystals containing SeMet-labelled TiaS, tRNA^{Ile2} and ATP by the hanging-drop vapour-diffusion method. The complex crystals diffracted to 2.9 Å resolution (Fig. 2) and belonged to the primitive hexagonal space group $P3_221$, with unit-cell parameters $a = b = 131.1$, $c = 86.6$ Å. On the basis of the molecular mass of the recombinant TiaS (50 kDa) and tRNA^{Ile2} (25 kDa), the complex crystals are expected to contain one TiaS–tRNA^{Ile2} complex per asymmetric unit, which corresponds to a solvent content of 61% and a Matthews coefficient of 2.8 Å^3 Da^{-1}. Quite recently, we have succeeded in determining the TiaS–tRNA^{Ile2}–ATP ternary complex structure by the single-wavelength anomalous dispersion (SAD) method using the SeMet-labelled crystals. In the SAD experimental electron-density map we observed clear density for ATP in addition to that for the TiaS protein and tRNA^{Ile2}, demonstrating that the crystals definitely contain an ATP molecule and shedding light on the structural aspects of the mechanism of agm^{2}C formation. The structural analysis of the TiaS–tRNA^{Ile2}–ATP ternary complex will be discussed elsewhere in the future.

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