Crystallization and preliminary X-ray
crystallographic analysis of a bacterial
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Most canonical aminoacyl-tRNAs are synthesized directly by their cognate aminoacyl-tRNA synthetases (aaRSs), but glutaminyl-tRNA^{Gln} and asparaginyl-tRNA^{Asn} are synthesized indirectly by two-step processes. These processes are catalyzed by the transamidosome, a large ribonucleoprotein particle composed of GatA, GatB, GatC, aaRS and tRNA. In this study, the Asn-transamidosome from *Pseudomonas aeruginosa* was reconstructed and crystallized by mixing purified GatCAB complex, AspRS and tRNA^{Asn}. The crystal of the Asn-transamidosome belonged to space group $P2_1$, with unit-cell parameters $a = 93.3$, $b = 186.0$, $c = 287.8$ Å, $\beta = 93.3^\circ$, and diffracted to 3.73 Å resolution. Preliminary X-ray crystallographic analysis showed that the asymmetric unit contained two Asn-transamidosomes, each composed of two GatCABs, one AspRS dimer and two tRNA^{Asn}s, indicating that the construction of the current Asn-transamidosome differs from that of *Thermus thermophilus*.

1. Introduction

Accurate synthesis of aminoacyl-tRNA is essential for translational fidelity. 17 of the 20 canonical aminoacyl-tRNAs are synthesized directly by aminoacyl tRNA synthetases (aaRSs), whereas glutaminyl-tRNA, asparaginyl-tRNA and cysteinyl-tRNA (Gln-tRNA^{Gln}, Asn-tRNA^{Asn} and Cys-tRNA^{Cys}, respectively) are synthesized indirectly by two-step processes in some bacteria and archaea (Wilcox & Nirenberg, 1968; Curnow *et al.*, 1996; Sauerwald *et al.*, 2005). In the case of Asn-tRNA^{Asn} formation, aspartic acid (Asp) is first mischarged on tRNA^{Asn} by non-discriminate AspRS, and Asp-tRNA^{Asn} is then amidated to Asn-tRNA^{Asn} by a heterotrimeric amidotransferase GatCAB composed of GatA, GatB and GatC (Curnow *et al.*, 1996, 1997). During the two-step processes, GatCAB, AspRS and tRNA^{Asn} assemble into a ribonucleoprotein particle designated the transamidosome (Bailly *et al.*, 2007). A similar ribonucleoprotein particle is also formed for Gln-tRNA^{Gln} synthesis, in which GatCAB, GluRS and tRNA^{Gln} participate. Crystal structures of the Asn-transamidosome from *Thermus thermophilus* (Blaise *et al.*, 2010) and the Gln-transamidosome from *Thermotoga maritima* (Ito & Yokoyama, 2010) have been reported, in which two GatCABs, two archaeal-type AspRS dimers and four tRNA^{Asn}s assembled in the Asn-transamidosome, whereas one GatCAB, one GluRS and one tRNA^{Gln} are present in the Gln-transamidosome.

Bacterial AspRSs commonly possess an additional ~16 kDa insertion domain named the GAD domain within the catalytic domain compared with archaeal or eukaryotic counterparts (Wolf *et al.*, 1999). Superposition of bacterial AspRS on the archaeal Asn-transamidosome causes steric hindrance between the GAD domain and GatCAB, suggesting that the presence of the GAD domain may cause marked structural changes. Furthermore, the precise function of the GAD domain is still unclear. The structure of the Asn-transamidosome may provide insight into the significance of the GAD domain.

In this study, we overexpressed, purified and crystallized the Asn-transamidosome from *Pseudomonas aeruginosa*. The results of preliminary X-ray crystallographic analyses showed that the crystal contained all five components of the Asn-transamidosome, *i.e.* GatA (51.9 kDa), GatB (53.1 kDa), GatC (10.5 kDa), AspRS (66.2 kDa) and tRNA^{Asn} (24.7 kDa).

2. Materials and methods

2.1. Expression and purification of proteins

The genes for the GatCAB operon and AspRS from *P. aeruginosa* PAO1 (*Pa*) were amplified by PCR from genomic DNA. The DNA fragments encoding *Pa*GatCAB and *Pa*AspRS were then cloned between *Nde*I and *Xho*I restriction sites in a modified pET-28b vector in which the thrombin site and extra residues upstream of the N-terminal His tag were deleted.

The plasmid carrying *Pa*GatCAB was transformed into *Escherichia coli* strain B834 (DE3)-pRARE2 by electroporation, and a 3 l culture was grown in LB medium containing 25 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol at 310 K until the OD₆₀₀ reached 0.6. The culture was then induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 10 μM and shifted to 298 K for approximately 20 h before harvesting. The cells were harvested by centrifugation at 4500g for 25 min at 277 K and were resuspended in buffer A [50 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM β -mercaptoethanol (BME), 0.5 mg ml⁻¹ lysozyme, 0.1 mg ml⁻¹ DNase]. After sonication and centrifugation at 40 000g for 30 min, the His₆-tagged protein was purified by immobilized metal-ion affinity chromatography using a HisTrap HP column (GE Healthcare). The column was washed with 15 mM imidazole and eluted with a 20–250 mM imidazole gradient in buffer B (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM BME). The collected fractions were then dialyzed against buffer C (25 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 10% glycerol, 1 mM BME) and loaded onto a HiTrap Q HP column (GE Healthcare) which was eluted with a 0–800 mM NaCl gradient in buffer C. The collected fractions were diluted twice with buffer D (25 mM HEPES-NaOH pH 7.5, 5 mM MgCl₂, 5% glycerol, 2 M ammonium sulfate, 1 mM BME), further purified using a HiTrap Phenyl column (GE Healthcare) and eluted with a gradient of 1–0 M ammonium sulfate. Finally, the protein was loaded onto HiLoad 26/60 Superdex 200 pg (GE Healthcare) equilibrated with buffer E (25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT). The protein was concentrated to 27 mg ml⁻¹ using an Amicon Ultra 30 000 MWCO (Millipore), flash-frozen and stored at 193 K.

As *Pa*AspRS is toxic to the *E. coli* expression system, causing the depletion of free tRNA^{Asn} by mischarging with Asp (Bernard *et al.*, 2006), we used the co-expression method for preparing *Pa*AspRS. A plasmid encoding *Pa*AspRS was co-transformed into *E. coli* strain B834 (DE3)-pRARE2 by electroporation with a plasmid encoding GatCAB from *Staphylococcus aureus* (*Sa*) in pCDFDuet-1 vector without any tag. The culture was grown according to a method similar

to that described for *Pa*GatCAB with LB medium containing 25 $\mu\text{g ml}^{-1}$ kanamycin, 34 $\mu\text{g ml}^{-1}$ chloramphenicol and 50 $\mu\text{g ml}^{-1}$ streptomycin. After sonication and centrifugation at 40 000g for 30 min, the His₆-tagged protein was purified by immobilized metal-ion affinity chromatography on a HisTrap HP column (GE Healthcare). The sample was washed with 20 mM imidazole and eluted with a gradient of 20–250 mM imidazole in buffer B. The collected fractions were then diluted to 75 mM NaCl with buffer C, subjected to HiTrap Heparin HP column (GE Healthcare) and eluted with a gradient of 75–800 mM NaCl in buffer C. Finally, the protein was loaded onto HiLoad 16/60 Superdex 200 pg (GE Healthcare) equilibrated with buffer E. The protein was concentrated to 16 mg ml⁻¹ using an Amicon Ultra 30 000 MWCO (Millipore), flash-frozen and stored at 193 K.

2.2. Preparation of tRNA^{Asn}

*Pat*tRNA^{Asn} was transcribed *in vitro* as described previously (Nakamura *et al.*, 2006). The DNA oligonucleotides used to construct *P. aeruginosa* tRNA^{Asn} were as follows: 5'-primer, **TAATACGACTCACTATATCCGCGATAGCTCAGTC** (forward); middle template, **CACTATATCCGCGATAGCTCAGTCGGTAGAGCAAATGACTGTTAATCATTGGGTCCCTGGTTCGAGTCC** (forward); 3'-primer, TGMGCTCCGCGACCTGGACTCGAACCAGGGAC (reverse); in which the T7 promoter sequence is shown in bold. Gm represents the 2'-O-methyl modification, which is used for manipulating the uniform 3'-end of transcribed products (Sherlin *et al.*, 2001). Transcribed tRNAs were purified by denaturing urea-PAGE and extracted with an Elutrap Electroelution system (Whatman). Pooled tRNAs were precipitated with ethanol and resuspended in buffer F (20 mM Tris-HCl pH 7.6, 100 mM NaCl, 20 mM MgCl₂). tRNAs were heated at 353 K for 5 min and then slowly cooled at room temperature over 5 min, purified using a Resource Q column (GE Healthcare) and eluted with a gradient of 300–700 mM NaCl in buffer G (20 mM Tris-

Figure 1
Crystal of Asn-transamidosome from *P. aeruginosa*.

Figure 2
X-ray diffraction image of an Asn-transamidosome crystal collected to 3.73 Å resolution.

HCl pH 7.6, 8 mM MgCl₂). Pooled fractions were precipitated with ethanol and stored at 243 K.

2.3. Crystallization and data collection

Asn-transamidosome was prepared by mixing purified AspRS dimer, GatCAB and tRNA^{Asn} at a final concentration of 4.8 μM each in buffer *H* (20 mM HEPES–NaOH pH 7.5, 10 mM MgCl₂, 5% glycerol). For crystallization, 500 μl Asn-transamidosome solution was concentrated to 120 μl using an Amicon Ultra-0.5 Centrifugal Filter Device (Millipore).

Crystallization was carried out by the sitting-drop vapour-diffusion method in 96-well plates at 293 K. Initial screening for the crystallization conditions of Asn-transamidosome was performed with The JCSG+ Suite and The PACT Suite (Qiagen) screening kits by mixing 1 μl of Asn-transamidosome solution with an equivalent volume of reservoir solution. In initial crystallization screening, microcrystals were obtained with reservoir solution No. 93 of The JCSG+ Suite consisting of 100 mM bis-tris pH 5.5, 200 mM lithium sulfate, 25% (w/v) PEG 3350. The crystallization conditions were optimized by changing the type of buffer, the pH and the precipitant concentration and adding various additives. Crystals of Asn-transamidosome which were suitable for diffraction experiments were obtained using additives in one week.

For the X-ray diffraction experiment, the crystals were soaked with cryoprotectant consisting of reservoir solution containing 15% glycerol before flash-cooling in a cryostream. Diffraction data were collected on beamline BL41XU of SPring-8 (Harima, Japan) (proposal Nos. 2011A1062 and 2011B1227). A total of 180 images were collected with 1.0° oscillation and 1 s exposure time per image. The data were indexed, integrated, scaled and merged using *XDS* (Kabsch, 2010). The statistics of data collection are summarized in Table 1.

Figure 3
2F_o – F_c electron-density map around the anticodon stem region of tRNA^{Asn} contoured at 1.0σ (magenta). GatCAB (blue) and AspRS (yellow) are represented as ribbon models, while tRNA (grey) is shown as a stick model.

Table 1

Statistics of data collection.

Values in parentheses are for the highest resolution shell.

Beamline	BL41XU, SPring-8
Detector	MX225HE
Crystal-to-detector distance (mm)	320
Wavelength (Å)	1.0000
No. of images	180†
Oscillation range (°)	0–180
Resolution range (Å)	48.89–3.73 (3.96–3.73)
Total No. of reflections	281233 (35593)
No. of unique reflections	94516 (13239)
Space group	<i>P</i> 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 93.3, <i>b</i> = 186.0, <i>c</i> = 287.8, β = 93.3
Completeness (%)	92.7 (81.2)
<i>R</i> _{meas} ‡ (%)	22.7 (78.3)
Multiplicity	2.98 (2.69)
<i>I</i> /σ(<i>I</i>)	5.28 (1.71)
Mosaicity (°)	0.514
Wilson <i>B</i> factor (Å ²)	66.13

† 37 out of 180 frames were omitted in data processing. ‡ $R_{\text{meas}} = \sum_{hkl} [N(hkl) / (N(hkl) - 1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively.

3. Results and discussion

To overcome the problem of toxicity in overexpressing *Pa*AspRS in *E. coli*, we expressed *Pa*AspRS using a co-expression method with *Sa*GatCAB. The Asn-transamidosome complex was reconstructed by mixing purified GatCAB, AspRS dimer and tRNA^{Asn} in a 1:1:1 ratio. After optimization of crystallization, plate-like crystals measuring ~0.4 × 0.2 × 0.1 mm were grown in the presence of 100 mM MES pH 6.0, 200 mM lithium sulfate, 25% (w/v) PEG 3350, 10 mM hexamine cobalt(III) chloride (Fig. 1). Diffraction data were collected to 3.73 Å resolution (Fig. 2) and the crystal belonged to space group *P*2₁, with unit-cell parameters *a* = 93.3, *b* = 186.0, *c* = 287.8 Å, β = 93.3°.

The phases for the Asn-transamidosome complex were determined by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007). The coordinates of GatCAB from *Staphylococcus aureus* (PDB entry 2g5h; Nakamura *et al.*, 2006), the AspRS dimer from *E. coli* (PDB entry 1eqr; Rees *et al.*, 2000) and tRNA^{Asp} from the AspRS–tRNA^{Asp} complex from *E. coli* (PDB entry 1c0a; Eiler *et al.*, 1999) were used as search models. Four solutions for GatCAB and two solutions for the AspRS dimer were successfully obtained with a final TFZ score of 28.9, although no solution was obtained for the tRNA molecules. The models of GatCAB and AspRS were subjected to rigid-body refinement using *phenix.refine* (Afonine *et al.*, 2012) followed by several cycles of jelly-body refinement with local NCS restraints in *REFMAC5* (Murshudov *et al.*, 2011). The resultant 2F_o – F_c and F_o – F_c electron-density maps clearly showed four tRNA molecules, and models of tRNA (PDB entry 1c0a) were placed at each site (Fig. 3). Model building and refinement are currently in progress. The results of preliminary structural analysis showed that the crystal contains two Asn-transamidosomes composed of two GatCABs, one AspRS dimer and two tRNA^{Asn}s in an asymmetric unit, with a V_M value of 3.02 Å³ Da^{–1} and a solvent content of 59.2% (Matthews, 1968), indicating that the construction of the present Asn-transamidosome is different from that of *Thermus thermophilus*.

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