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Crystallization and preliminary X-ray analysis of the tRNA thiolation enzyme MnmA from *Escherichia coli* complexed with $tRNA^{Glu}$

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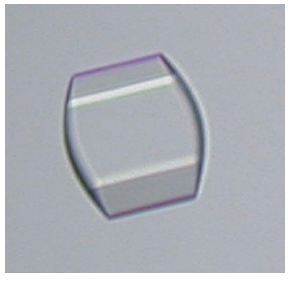


MnmA catalyzes a sulfuration reaction to synthesize 2-thiouridine at the wobble positions of $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Lys}$ in *Escherichia coli*. The binary complex of MnmA and $tRNA^{Glu}$ was crystallized in two different crystal forms: forms I and II. Cocrystallization of MnmA–$tRNA^{Glu}$ with ATP yielded form III crystals. The three crystal forms diffracted to 3.1, 3.4 and 3.4 Å resolution, respectively, using synchrotron radiation at SPring-8. These crystals belong to space groups $C2$, $I2_12_12_1$ and $C2$, with unit-cell parameters a = 225.4, b = 175.8, c = 53.0 Å, β = 101.6°, a = 101.5, b = 108.0, c = 211.2 Å and a = 238.1, b = 102.1, c = 108.2 Å, β = 117.0°, respectively. The asymmetric units of these crystals are expected to contain two, one and two MnmA–$tRNA^{Glu}$ complexes, respectively.

1. Introduction

In all organisms, transfer RNA (tRNA) contains a number of post-transcriptionally modified nucleosides (Björk, 1995). Many of these modifications frequently appear in the anticodon wobble position (position 34), where they play an essential role in the precise decoding of the genetic code by stabilizing the correct codon–anticodon interactions (Björk, 1995; Urbonavicius *et al.*, 2001; Yokoyama & Nishimura, 1995). Furthermore, wobble modifications generally serve as determinants for aminoacylation through recognition by the cognate aminoacyl-tRNA synthetases (Beuning & Musier-Forsyth, 1999; Sylvers *et al.*, 1993).

2-Thiouridine (s^2U34) is phylogenetically conserved in the anticodon wobble position of the tRNAs specific for Glu, Lys and Gln from all organisms (Björk, 1995; Yokoyama & Nishimura, 1995). Glu, Lys and Gln are encoded by two degenerate codons ending in purine (NNR). Therefore, the purine-specific recognition at the third base of the cognate codons by $tRNA^{Glu}$, $tRNA^{Lys}$ and $tRNA^{Gln}$ is essential for translational fidelity. In this regard, the 2-thiouridine at the wobble position of these tRNAs plays a crucial role in discriminating cognate from noncognate codons during the mRNA-decoding process by stabilizing the codon–anticodon interaction (Krüger *et al.*, 1998; Ashraf *et al.*, 1999; Urbonavicius *et al.*, 2001): the 2-thio modification allows the U34 nucleoside to adopt the C3′-*endo* puckering conformation of the ribose, which facilitates Watson–Crick base pairing only with purines (Yokoyama *et al.*, 1985). The s^2U34 modification thus enables the tRNAs for Glu, Lys and Gln to discriminate purines from pyrimidines to prevent the incorrect decoding of the NNY codon.

The S atom of s^2U34 is derived from L-cysteine (Lauhon, 2002). In *Escherichia coli*, many protein components mediate sulfur trafficking to synthesize 2-thiouridine: firstly, a cysteine desulfurase, IscS, catalyzes the transfer of sulfur from L-cysteine to its active-site Cys residue to form a persulfide intermediate and then the activated terminal sulfur of the persulfide is transferred to a catalytic Cys residue of TusA (Ikeuchi *et al.*, 2006). The activated S atom is in turn transferred to the Cys residues of the proteins that comprise the sulfur relay system (Ikeuchi *et al.*, 2006; Numata *et al.*, 2006). The S atom is finally transferred to the catalytic Cys residue of a thiouridylase, MnmA, which specifically recognizes $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Lys}$ and catalyzes the sulfuration reaction (Kambampati & Lauhon, 2003; Ikeuchi *et al.*, 2006). On the basis of the amino-acid sequence, MnmA is suggested to interact with ATP and to activate

the substrate by forming an adenylated intermediate. Since there is no structural information available for MnmA, the catalytic mechanism of RNA sulfuration as well as the mechanism by which MnmA discriminates the substrate tRNAs from other tRNAs remain to be elucidated. In the present paper, we report the crystallization and preliminary X-ray diffraction analysis of MnmA from *E. coli* in complex with $tRNA^{Glu}$.

2. Materials and methods

2.1. Protein preparation

The gene encoding MnmA (Kambampati & Lauhon, 2003) was PCR-amplified from *E. coli* genomic DNA and cloned into the pQE80L expression vector (Qiagen). The selenomethionyl recombinant protein with a His tag, MRGSHHHHHHGS, attached to the N-terminus of MnmA was produced in the *E. coli* methionine auxotroph B834(DE3). After sonication of *E. coli* cells in 20 m*M* Tris–HCl buffer 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 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). MnmA was eluted with buffer *B* (the same as buffer *A* but 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, 100 m*M* NaCl, 1 m*M* DTT). Solid ammonium sulfate was added to the protein solution to a final concentration of 1.5 *M* and the supernatant was loaded onto a Resource ISO column (16 × 30 mm) previously equilibrated with buffer *C* containing 1.5 *M* ammonium sulfate. A linear gradient was developed from 1.5 to 0 *M* ammonium sulfate in buffer *C*. The fractions rich in the protein were pooled and dialyzed against buffer *C* and the protein solution was loaded onto a Resource Q column (16 × 30 mm) previously equilibrated with buffer *C*. MnmA was eluted with a linear gradient of 100–750 m*M* NaCl in buffer *C*. The fractions enriched in the protein were combined and dialyzed against buffer *D* (10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* DTT) and MnmA was concentrated to 22 mg ml^{-1} in buffer *D* and stored at 193 K.

2.2. tRNA preparation

The gene encoding *E. coli* $tRNA^{Glu}$ with the UUC anticodon (NCBI GeneID 948510) 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. $tRNA^{Glu}$ was purified under denaturing conditions by polyacrylamide gel electrophoresis and was eluted from the gel pieces. After ethanol precipitation, $tRNA^{Glu}$ was dissolved in buffer *E* (20 m*M* Tris–HCl pH 7.6, 8 m*M* $MgCl_2$) and passed through a PD-10 column (Amersham) to completely remove the residual urea. $tRNA^{Glu}$ was further precipitated with ethanol, dissolved in buffer *E* and stored at 193 K.

2.3. Crystallization

$tRNA^{Glu}$ was denatured by heat treatment at 343 K for 10 min and was then renatured by gradual cooling of the solution to 298 K prior to formation of the MnmA–$tRNA^{Glu}$ complex. MnmA was mixed with $tRNA^{Glu}$ in a MnmA:$tRNA^{Glu}$ molar ratio of 1:1.2 at a final protein concentration of 5 mg ml^{-1}. Crystallization conditions were screened using the sparse-matrix sampling method by hanging-drop vapour-diffusion at 293 K. Hanging drops were prepared by mixing 1 µl reservoir with 1 µl of the MnmA–$tRNA^{Glu}$ complex solution described above and were equilibrated against 400 µl reservoir solution. Two different crystal forms (forms I and II) grew in 90 m*M* Na HEPES buffer pH 7.5 containing 40 m*M* ammonium dihydrogen phosphate, 1.8% PEG 400 and 1.7–1.9 *M* ammonium sulfate (form I) and in 100 m*M* Na HEPES buffer pH 7.5 containing 1.3–1.5 *M* lithium sulfate (form II). The form II crystals prepared as described above only diffracted to resolutions of ∼4.5 Å with high mosaicity (0.5–1.0°). To improve the diffraction qualities of the form II crystals, the MnmA–$tRNA^{Glu}$ complex was crystallized by the sitting-drop vapour-diffusion technique combined with the following methods recently developed by Adachi and coworkers (Adachi *et al.*, 2003, 2004): an aliquot of the MnmA–$tRNA^{Glu}$ solution (3–7 µl) was mixed with an equal volume of the crystallization solution for the form II crystals and was equilibrated with 50 µl reservoir solution by shaking the plates on a rotary shaker for 2 d. The drop solution was then subjected to femtosecond laser irradiation to promote the generation of crystal nuclei, followed by a further incubation of the crystallization plates with stirring at 293 K. As a result, we succeeded in obtaining larger form II crystals with low mosaicity (0.28–0.45°). To obtain further structural insights into the catalytic mechanisms of MnmA, we attempted to determine the structure of the reaction intermediate MnmA in complex with $tRNA^{Glu}$, in which the wobble uridine is adenylated. Soaking the form I crystals in reservoir solution containing 2.5 m*M* ATP and cocrystallization of MnmA–$tRNA^{Glu}$ with ATP under the form I crystallization conditions were not successful. On the other hand, cocrystallization of MnmA–$tRNA^{Glu}$ with 2.5 m*M* ATP under the optimized form II crystallization conditions produced crystals (form III crystals) in which the space group was fundamentally different from that of the form II crystals, despite their similar size and shape to the form II crystals.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

	Form I	Form II	Form III
Wavelength (Å)	0.97927	1.00	1.00
Temperature (K)	30	100	100
Space group	*C*2	$I2_12_12_1$	*C*2
Unit-cell parameters (Å, °)	*a* = 225.4, *b* = 175.8, *c* = 53.0, β = 101.6	*a* = 101.5, *b* = 108.0, *c* = 211.2	*a* = 238.1, *b* = 102.1, *c* = 108.2, β = 117.0
Resolution (Å)	50–3.1 (3.21–3.1)	50–3.4 (3.52–3.4)	50–3.4 (3.52–3.4)
Measured reflections	141141	159562	148561
Unique reflections	33063	16030	30355
Completeness (%)	89.8 (41.9)	96.8 (97.0)	94.5 (91.8)
$I/\sigma(I)$	11.2 (1.8)	34.4 (4.0)	24.6 (2.3)
R_{merge}†	0.118 (0.363)	0.053 (0.281)	0.061 (0.334)

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

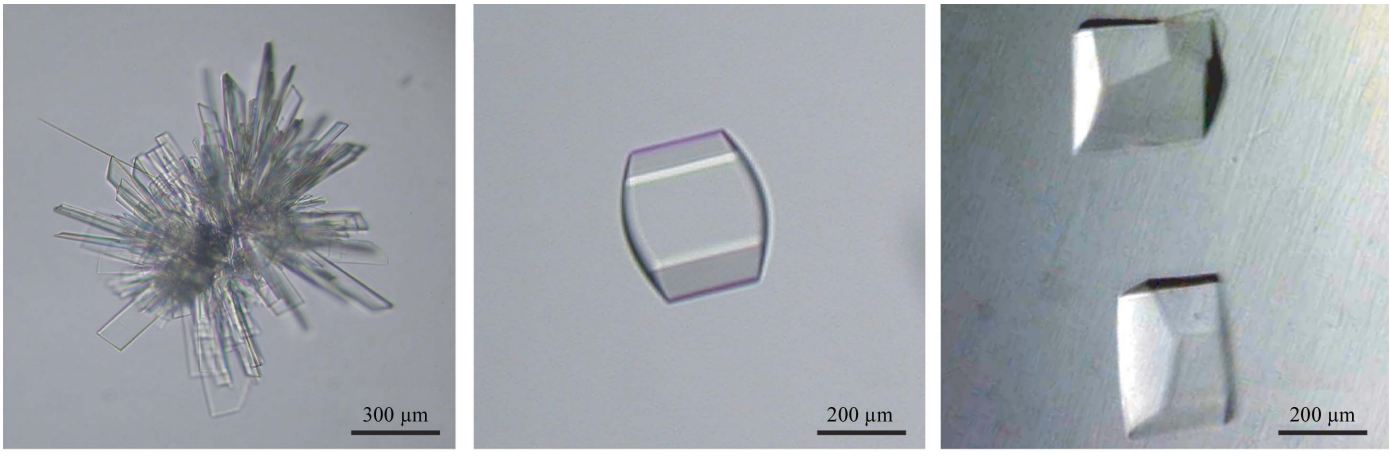
2.4. X-ray data collection

For data collection under cryogenic conditions, the form I, II and III crystals were transferred into individual cryoprotectant solutions in which the ethylene glycol concentration was gradually increased up to 20% in each reservoir solution and were flash-cooled in a nitrogen stream at 100 K. X-ray diffraction data for the form I crystals were collected at 30 K in a helium cryostream, whereas the data sets for the form II and III crystals were measured at 100 K in a nitrogen cryostream. The helium cryostream at 30 K considerably reduced the X-ray damage to the form I crystals compared with the nitrogen cryostream at 100 K. All data were collected at the BL41XU station of SPring-8 (Harima, Japan) using an Area Detector System Quantum 315 charge-coupled device detector. Diffraction data were

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

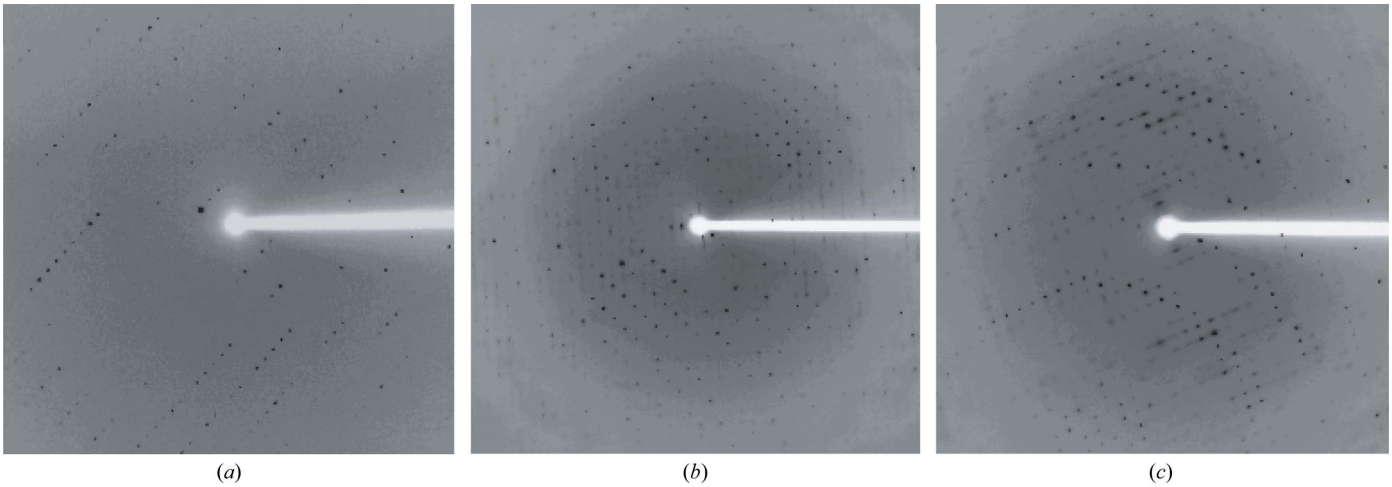
3. Results and discussion

The MnmA–tRNA^{Glu} binary complex was crystallized under two different crystallization conditions. These crystals were confirmed to contain both MnmA and tRNA^{Glu} by polyacrylamide gel electrophoresis (data not shown). The form I crystals grew within two weeks to maximum dimensions of 300 × 300 × 10 µm (Fig. 1*a*) and diffracted to a resolution of 3.1 Å (Fig. 2*a*). The completeness and the value of $I/\sigma(I)$ in the last shell (3.21–3.1 Å resolution) of the form I crystal are slightly low because of the anisotropy, caused by its thin shape, in the diffraction data of this crystal (Table 1). The form I crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 225.4$, $b = 175.8$, $c = 53.0$ Å, $\beta = 101.6^\circ$. On the basis of the molecular weight of the MnmA–tRNA^{Glu} complex (66 kDa; the molecular weight of MnmA is 41 kDa and that of tRNA^{Glu} is 25 kDa), the form I crystals are expected to contain two MnmA–tRNA^{Glu} complexes per asymmetric unit, which corresponds to a solvent content of 68.1% and a Matthews coefficient of 3.9 Å^3 Da^{-1}. The diffraction-quality form II crystals grew to dimensions of 250 × 250 × 200 µm over the course of 10 d (Fig. 1*b*) and diffracted to 3.4 Å resolution (Fig. 2*b*). The form II crystals belong to the orthorhombic space group $I2_12_12_1$, with unit-cell parameters $a = 101.5$, $b = 108.0$, $c = 211.2$ Å, and are indicated to contain one MnmA–tRNA^{Glu} complex in the asymmetric unit, which is consistent with a solvent content of 71.7% and a Matthews coefficient of 4.4 Å^3 Da^{-1}. Cocrystallization of MnmA–tRNA^{Glu} with ATP yielded the form III crystals (Fig. 1*c*) and these crystals produced diffraction data to 3.4 Å resolution (Fig. 2*c*). The form III crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 238.1$, $b = 102.1$, $c = 108.2$ Å, $\beta = 117.0^\circ$, and are suggested to contain two MnmA–tRNA^{Glu} complexes in the asymmetric unit, consistent with a Matthews coefficient of 4.4 Å^3 Da^{-1} and a solvent content of 72.0%. It is intriguing to note that the crystallization conditions of the form II and III crystals were identical to each other, except for the presence or absence of ATP. The difference in the space groups of these two crystal forms therefore indicates that the conformational change of the MnmA–tRNA^{Glu} complex is accompanied by the binding of ATP and by the subsequent formation of the reaction intermediate. Attempts to solve the cocrystal structure of MnmA and tRNA^{Glu} by the MAD method with selenium as the anomalous scattering atom are in progress.



(*a*) (*b*) (*c*)

Figure 1
Three crystal forms of *E. coli* MnmA complexed with tRNA^{Glu}. (*a*) Form I crystals, belonging to space group *C*2, of MnmA complexed with tRNA^{Glu}. (*b*) Form II crystals, belonging to space group $I2_12_12_1$, of MnmA complexed with tRNA^{Glu}. (*c*) Form III crystals, belonging to space group *C*2, of MnmA complexed with tRNA^{Glu} and ATP.



(*a*) (*b*) (*c*)

Figure 2
Diffraction patterns of form I crystals (*a*), form II crystals (*b*) and form III crystals (*c*) of the MnmA–tRNA^{Glu} complex.

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