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Overexpression, crystallization and preliminary X-ray crystallographic analysis of *Pseudomonas aeruginosa* MnmE, a GTPase involved in tRNA modification

Hyung Ho Lee[a]* and Se Won Suh[b,c]*

[a]Department of Bio and Nano Chemistry, Kookmin University, Seoul 136-702, Republic of Korea, [b]Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, Republic of Korea, and [c]Department of Biophysics and Chemical Biology, College of Natural Sciences, Seoul National University, Seoul 151-742, Republic of Korea

Correspondence e-mail: hhlee@kookmin.ac.kr, sewonsuh@snu.ac.kr

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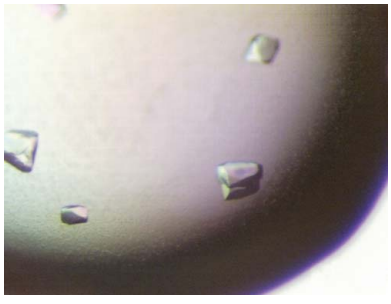
MnmE, an evolutionarily conserved GTPase, is involved in modification of the uridine base (U34) at the wobble position of certain tRNAs. Previous crystal structures of MnmE suggest that it is a dimer with considerable conformational flexibility. To facilitate structural comparisons among MnmE proteins, structural analysis of MnmE from *Pseudomonas aeruginosa* encoded by the *PA5567* gene was initiated. It was overexpressed in *Escherichia coli* and crystallized at 297 K using a reservoir solution consisting of 100 m*M* sodium ADA pH 6.5, 12%(*w*/*v*) polyethylene glycol 4000, 100 m*M* lithium sulfate, 2%(*v*/*v*) 2-propanol and 2.5 m*M* dithiothreitol. X-ray diffraction data were collected to 2.69 Å resolution. The crystals belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 96.74, b = 204.66, c = 120.90 Å. Two monomers were present in the asymmetric unit, resulting in a crystal volume per protein mass (V_M) of 2.99 Å^3 Da^{-1} and a solvent content of 58.8%.

1. Introduction

tRNAs are frequently modified in the anticodon region, where the wobble uridine of certain bacterial and mitochondrial tRNAs is modified at position 5 through a reaction pathway that utilizes the evolutionarily conserved MnmE (also known as TrmE) and MnmG (also known as GidA) proteins (Moukadiri *et al.*, 2009). The resulting modification plays a critical role in decoding NNG/A codons and reading-frame maintenance during mRNA translation (Moukadiri *et al.*, 2009). The lack of this tRNA modification produces a pleiotropic phenotype in bacteria and has been associated with mitochondrial encephalomyopathies in humans (Moukadiri *et al.*, 2009). MnmE, together with the protein MnmG (Brégeon *et al.*, 2001; Elseviers *et al.*, 1984), catalyzes the formation of a carboxymethylaminomethyl group at the 5 position of U34 (cmnm^{5}U). MnmE is a GTPase that is conserved in all three kingdoms of life.

The crystal structure of *Thermotoga maritima* MnmE revealed that MnmE is a three-domain protein (Scrima *et al.*, 2005). The N-terminal α/β domain (residues 1–118) is responsible for dimerization and the binding of 5-formyltetrahydrofolate. The central helical domain (residues 119–210 and 381–450) contains the catalytically important C-terminal C(I/L/V)GK motif and forms a four-helix bundle. The G domain (residues 211–380), which exhibits the canonical Ras-like fold, is inserted between two helices of the central helical domain. Cys451 of the C(I/L/V)GK motif in *Escherichia coli* MnmE (corresponding to Cys447 in *T. maritima* MnmE) is essential for the tRNA-modification activity of MnmE (Yim *et al.*, 2003). Subsequently, the structure of the MnmE G domain from *E. coli* determined in the presence of GDP-AlF$_x$ and potassium ions showed that the G domains of MnmE dimerize in a potassium-dependent manner (Scrima & Wittinghofer, 2006), which suggested a possible role for conformational change of the G domain in tRNA modification. More recently, the crystal structures of full-length MnmE from *Chlorobium tepidum* and *Nostoc* in the diphosphate and triphosphate states confirmed the high mobility of the G domain and demonstrated the



nature of the conformational changes of MnmE during its GTPase cycle (Meyer *et al.*, 2009). A combined approach consisting of X-ray crystallography and pulse electron paramagnetic resonance (EPR) spectroscopy using full-length MnmE showed that the G domains adopt an open conformation in the nucleotide-free/GDP-bound state and an open/closed two-state equilibrium in the GTP-bound state (Meyer *et al.*, 2009). In view of this high flexibility, further structural studies of MnmE from other sources would help in understanding the link between the structural mobility of the G domain and tRNA-modifying activity.

In order to provide further structural data on MnmE proteins for the comparison of MnmE proteins and to provide clues about the function of G-domain movement in tRNA modification, we initiated the determination of the three-dimensional structure of MnmE from *Pseudomonas aeruginosa* (PA5567). Its 455-residue polypeptide shows a high level of sequence similarity to other members of the MnmE protein family, with 35% identity to *T. maritima* TrmE (TM0267). As the first step towards the structure determination of MnmE from *P. aeruginosa*, it was overexpressed in *E. coli* and crystallized. Here, its crystallization conditions and preliminary X-ray crystallographic data are reported.

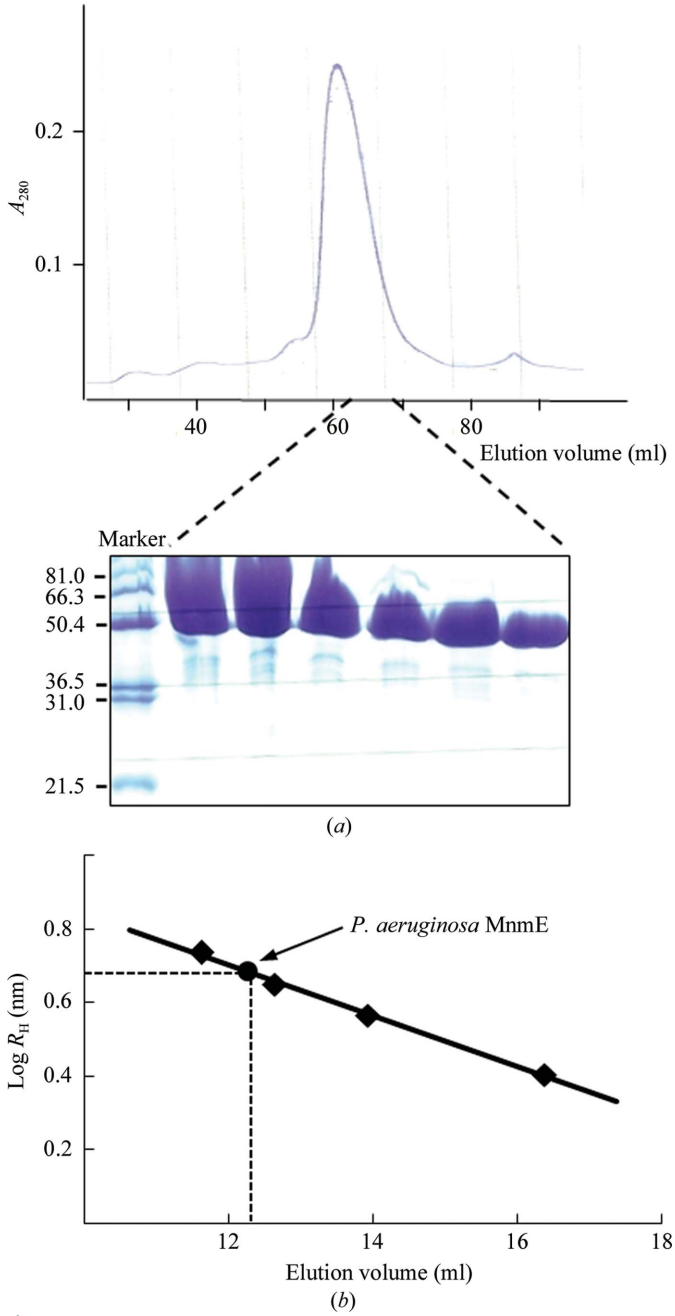


Figure 1
(*a*) Gel filtration and SDS–PAGE of *P. aeruginosa* MnmE. (*b*) Comparison of the *P. aeruginosa* Stokes radius (R_H) derived from the size-exclusion data obtained using a Superdex 200 (10/300) column. The standards consist of sweet potato β-amylase (200 kDa, R_H = 5.42 nm), *Saccharomyces cerevisiae* alcohol dehydrogenase (150 kDa, R_H = 4.45 nm), bovine serum albumin (66 kDa, R_H = 3.67 nm) and bovine erythrocyte carbonic anhydrase (29 kDa, R_H = 2.53 nm). The dotted line represents the position of *P. aeruginosa* MnmE.

2. Experimental

2.1. Protein expression and purification

The *PA5567* gene was amplified by the polymerase chain reaction using the genomic DNA of *P. aeruginosa* strain PAO1 as template. The forward and reverse oligonucleotide primers designed using the published genome sequence are 5′-G GAA TTC **CAT ATG** CAA GCC GCC ACC GAA ACC-3′ and 5′-ATA GTT TAG **CGG CCG** CTT ACT TGC CGA TGC AGA AAC TCG AG-3′, respectively. The bases in bold represent *Nde*I and *Not*I restriction-enzyme cleavage sites, respectively. The amplified DNA was inserted into the *Nde*I/*Not*I-digested expression vector pET-28b(+) (Novagen). This vector construction yields the recombinant enzyme with a 20-residue tag (MGSSHHHHHHSSGLVPRGSH) in front of the amino-terminal methionine of the recombinant MnmE to facilitate purification. The protein was overexpressed in *E. coli* C41 (DE3) cells. The cells were grown at 310 K to an OD_{600} of 0.5 in Terrific Broth medium containing 50 µg ml^{-1} kanamycin and protein expression was induced by 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG). We continued to grow the cells at 291 K for 24 h after IPTG induction and harvested them by centrifugation at 5600*g* (8000 rev min^{-1}; Sorvall GSA rotor) for 10 min at 277 K.

The cell pellet was resuspended in ice-cold lysis buffer [20 m*M* Tris–HCl pH 7.9, 500 m*M* sodium chloride, 5 m*M* imidazole, 5%(*v*/*v*) glycerol and 1 m*M* phenylmethylsulfonyl fluoride] and homogenized with an ultrasonic processor. The crude lysate was centrifuged at 70 400*g* (30 000 rev min^{-1}; Beckman 45 Ti rotor) for 1 h at 277 K and the recombinant protein in the supernatant fraction was purified in four chromatographic steps. The first step utilized the hexahistidine tags by metal-chelate chromatography on Ni–NTA resin (Qiagen). Next, gel filtration was performed on a HiLoad XK 16 Superdex 200 prep-grade column (GE Healthcare) previously equilibrated with buffer *A* (50 m*M* Tris–HCl pH 7.0) containing 100 m*M* sodium chloride and 1 m*M* β-mercaptoethanol. The protein was subjected to a Mono S column (GE Healthcare) previously equilibrated with buffer *A* and the protein was eluted with a linear gradient of 0–1.0 *M* NaCl in buffer *A*. Final purification was performed by gel filtration on a HiLoad XK 16 Superdex 200 prep-grade column (GE Healthcare) previously equilibrated with buffer *A* containing 100 m*M* sodium chloride and 1 m*M* β-mercaptoethanol. The homogeneity of the purified protein was assessed by SDS–PAGE (Laemmli, 1970). The protein solution was concentrated using a YM10 ultrafiltration membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing a calculated extinction coefficient of 9970 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

Table 1
Stokes radii of MnmE proteins.

	R_H (nm)
C. tepidum MnmE (PDB code 3gee)	4.67 (crystal structure)
P. aeruginosa MnmE	4.84 (gel filtration)

The purified protein was subjected to analytical gel filtration on a Superdex 200 (10/300) column. A standard curve was obtained using molecular-weight markers (Sigma). The Stokes radius of MnmE from *C. tepidum* was calculated from the crystal structure (PDB code 3gee; Meyer *et al.*, 2009) using the *HYDROPRO* program (García De La Torre *et al.*, 2000).

2.2. Crystallization and dynamic light scattering

Crystallization was performed at 297 K by the hanging-drop vapour-diffusion method using 24-well VDX plates (Hampton Research). Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screens I and II, SaltRX, Index I and II, PEG/Ion and MembFac) and Emerald BioStructures (Wizard I and II). Each hanging drop was prepared on a siliconized cover slip by mixing 2 µl protein solution (17 mg ml^{-1} in a buffer consisting of 50 m*M* Tris–HCl pH 7.0, 100 m*M* NaCl and 1 m*M* β-mercaptoethanol) with 2 µl reservoir solution. The hanging drop was placed over 0.48 ml reservoir solution. Dynamic light-scattering experiments were performed using a DynaPro-801 instrument (Wyatt, Santa Barbara, California, USA). The data were measured at 297 K with the protein at 1 mg ml^{-1} concentration in 50 m*M* Tris–HCl pH 7.0, 100 m*M* NaCl and 1 m*M* β-mercaptoethanol.

2.3. X-ray diffraction

The crystals were flash-frozen using a cryoprotectant solution consisting of 100 m*M* sodium ADA pH 6.5, 12%(*w*/*v*) polyethylene glycol 4000, 100 m*M* lithium sulfate monohydrate, 2%(*v*/*v*) 2-propanol, 2.5 m*M* dithiothreitol and 20%(*v*/*v*) glycerol. Crystals were soaked in 5 µl cryoprotectant solution for 10 s before being flash-cooled in liquid nitrogen. X-ray diffraction data were collected at 100 K using an Area Detector Systems Corporation Quantum 210 CCD detector at the experimental station NW12A of Photon Factory, Japan. The crystal was rotated through a total of 121° with a 1.0° oscillation range per frame. The raw data were processed and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997).

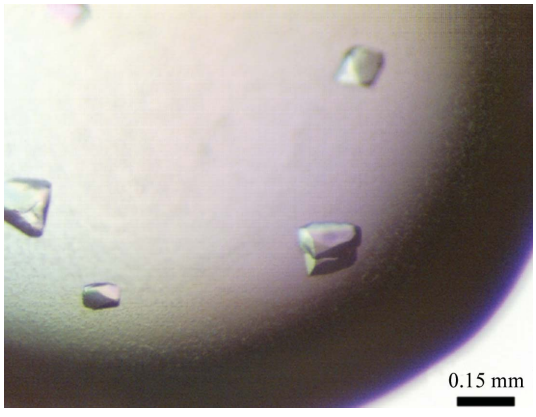


Figure 2
Crystals of *P. aeruginosa* MnmE encoded by the *PA5567* gene.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	NW12A, Photon Factory
X-ray wavelength (Å)	1.0000
Temperature (K)	100
Space group	$C222_1$
Unit-cell parameters (Å)	a = 96.74, b = 204.66, c = 120.90
Resolution range (Å)	50–2.69 (2.79–2.69)
Total/unique reflections	139550/30731
R_{merge}† (%)	3.7 (20.3)
Data completeness (%)	89.1 (92.5)
Average $I/\sigma(I)$	37.7 (8.4)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the mean value of $I(hkl)$ for all i measurements.

3. Results

MnmE from *P. aeruginosa* was overexpressed in *E. coli* in a soluble form, with a yield of ∼20 mg homogeneous protein per litre of culture. The molecular mass of the recombinant MnmE was estimated to be ∼150 kDa by dynamic light-scattering analysis, indicating that the enzyme exists as a compact homotrimer or an elongated homodimer in solution (calculated monomeric mass of 50 907.4 Da). To further confirm the oligomeric state of MnmE, we measured the molecular mass by performing analytical gel filtration. The apparent molecular mass of MnmE was estimated to be 156 kDa, which agrees well with the molecular mass from the dynamic light-scattering analysis (Fig. 1*a*). Using the gel-filtration data, the Stokes radius of *P. aeruginosa* MnmE was estimated to be 4.84 nm, which is similar to the calculated Stokes radius (4.67 nm) of the *C. tepidum* MnmE dimer in the crystal (Meyer *et al.*, 2009; Fig. 1*b*; Table 1). This result strongly suggests that MnmE from *P. aeruginosa* exists as an elongated dimer like the *C. tepidum* MnmE dimer in the crystal.

The best crystals were grown using a reservoir solution consisting of 100 m*M* sodium ADA pH 6.5, 12%(*w*/*v*) polyethylene glycol 4000, 100 m*M* lithium sulfate, 2%(*v*/*v*) 2-propanol and 2.5 m*M* dithiothreitol. Crystals grew to dimensions of 0.15 × 0.15 × 0.10 mm within 2 d (Fig. 2). A set of X-ray diffraction data was collected to 2.69 Å resolution at 100 K. A total of 139 550 measured reflections were merged into 30 731 unique reflections, giving an R_{merge} of 3.7% and a completeness of 89.1%. The space group was determined to be $C222_1$ on the basis of systematic absences and the symmetry of diffraction intensities. The unit-cell parameters were a = 96.74, b = 204.66, c = 120.90 Å. Table 2 summarizes the statistics of data collection. If a dimeric molecule is assumed to be present in the crystallographic asymmetric unit, the Matthews parameter V_M is 2.9 Å^3 Da^{-1} (Matthews, 1968) and the solvent content is 58.8%. Initial molecular-replacement trials gave a solution which could not be refined to an R_{free} of below 45%. This may be a consequence of differing conformations in *P. aeruginosa* MnmE and the starting model (PDB code 1xzp; Scrima *et al.*, 2005). We plan to solve the structure by collecting multiwavelength anomalous diffraction data.

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