Backbone resonance assignment for the N-terminal region of bacterial tRNA-(N⁰G37) methyltransferase

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Abstract

Bacterial tRNA (guanine³⁷-N¹)-methyltransferase (TrmD) is an important antibacterial target due to its essential role in translation. TrmD has two domains connected with a flexible linker. The N-terminal domain (NTD) of TrmD contains the S-adenosyl-l-methionine (SAM) cofactor binding site and the C-terminal domain is critical for tRNA binding. Here we report the backbone NMR resonance assignments for NTD of Pseudomonas aeruginosa TrmD. Its secondary structure was determined based on the assigned resonances. Relaxation analysis revealed that NTD existed as dimers in solution. NTD also exhibited thermal stability in solution. Its interactions with SAM and other compounds suggest it can be used for evaluating SAM competitive inhibitors by NMR.

Keywords TrmD · tRNA methyltransferase · Drug discovery · Antibacterial · Protein dynamics · Backbone assignment

Biological content

Bacterial tRNA (guanine³⁷-N¹)-methyltransferase (TrmD) catalyses methyl-transfer to the N¹ atom of G37 in tRNA using S-adenosyl-l-methionine (SAM) as a cofactor (O’Dwyer et al. 2004). This reaction is ubiquitous in bacteria and is important in maintaining translation fidelity by methylation at the G37 site, thereby preventing reading frame shifts (Bjork et al. 1989). TrmD has been proven to be essential for growth of several Gram-negative bacteria such as Pseudomonas aeruginosa (Jaroensuk et al. 2016), Escherichia coli (Persson et al. 1995) and some Gram-positive bacteria such as Streptococcus pneumoniae (Thanassi et al. 2002; O’Dwyer et al. 2004), thereby making it an attractive target for antibiotic development (Hill et al. 2013). As
human and bacterial methyltransferases have different SAM binding pockets and bind to SAM in distinct conformations, designing inhibitors of TrmD that compete with SAM binding represents an attractive strategy for the development of antibacterial molecules.

Structural studies conducted on bacterial TrmDs (Ito et al. 2015; Thomas et al. 2011) reveal two distinct domains: the N-terminal domain (NTD) and the C-terminal domain (CTD) that are connected by a short and flexible linker. TrmD forms homodimers in which the NTD is critical for dimerization. As the NTD comprises the main binding site for SAM and of some competitive inhibitors such as sinefungin (Holmes et al. 1995), studying this domain might be useful in antibacterial development. Indeed we found in the present study that the NTD can be produced in large quantities for structural investigations.

In this study, we carried out solution NMR studies on the NTD (residues 1–167) of P. aeruginosa TrmD. Backbone assignment and secondary structural analysis reveal that the solution structure of NTD is similar to that of the NTD determined in the context of the full-length P. aeruginosa TrmD crystal structure (PDB ID: 5WYQ). NTD contains a long loop, which was analysed by dynamic analysis. We found that the NTD interacts with SAM, S-adenosyl-l-homocysteine (SAH), methylthioadenosine (MTA) and sinefungin in solution. Our studies suggest that NTD is a useful construct to probe the molecular interactions with SAM competitive inhibitors, in the search for new molecules with antibiotic activity.

Materials and methods

Protein purification

The cDNAs encoding the N-terminal region (residues 1–167, NTD) of Pseudomonas aeruginosa TrmD were cloned into pET29b. The resulting plasmid was transformed into E. coli BL21(DE3) competent cells. To produce isotopically labeled proteins, the cells were grown in M9 medium supplemented with 1 g L⁻¹ ¹⁵NH₄Cl and 2 g L⁻¹ ¹³C-glucose. D₂O was used to replace the water in the M9 medium to generate deuterated samples. Protein expression was carried out at 18 °C overnight in the presence of 0.5 mM IPTG when the OD₆₀₀ of the cells reached 0.6–0.8. The cells were then harvested by centrifugation at 8000× g, 4 °C. The cells harboring recombinant protein were suspended in a suspension buffer that contained 20 mM phosphate, pH 7.8, 0.5 M NaCl, and 2 mM β-mercaptoethanol. Cells were lysed by sonication in an ice bath. The resulting cell lysate was cleared by centrifugation at 40,000×g for 20 min at 4 °C. Recombinant NTD was purified using Ni²⁺-NTA affinity resin in a gravity centrifugation at 40,000×g for 20 min at 4 °C. Recombinant protein were suspended in a suspension buffer that contained 20 mM phosphate, pH 7.8, 0.5 M NaCl, 1 mM MgCl₂, 1 mM DTT and 0.05% Tween-20 or assay buffer containing 0.9% DMSO was added in place of the test ligand. A linear 25 to 95 °C temperature gradient was applied on the samples.

The purified protein was further purified using size-exclusion chromatography with a HiPrepTM 16/60 sephacryl™ S-200 HR column in the NMR buffer that contained 20 mM sodium phosphate, pH 7.2, 150 mM KCl, 0.5 mM EDTA and 1 mM DTT. Purified protein samples were concentrated to 0.8–1.0 mM using an Amicon Centrifugal Filter unit with a molecular weight cutoff of 3 kDa for NMR data acquisition.

Backbone resonance assignment

¹³C, ¹⁵N, ²H-labeled NTD was prepared in the NMR buffer and used for data acquisition. The following experiments namely 2D-¹H, ¹⁵N-HSQC, transverse relaxation-optimized spectroscopy (TROSY) (Pervushin et al. 1998; Salzmann et al. 1998)-based 3D-HNCA,CB, HNCA, HN(CO)CA, HN(CO)CACB, HNCO, and NOESY-TROSY (mixing time 120 ms) experiments were used for the backbone resonance assignment (Li et al. 2016, 2010; Kim et al. 2013). All the experiments were carried out at 37 °C on a Bruker Avance spectrometer with a proton frequency of 600 MHz or 700 MHz. The data were acquired using Topspin (version 2.1) from Bruker and processed with NMRPipe (Dela-glio et al. 1995). Topspin (version 2.1) and visualized using NMRView (Johnson 2004) and CARA. Secondary structure predictions of the NTD were carried out using TALOS+ (Shen et al. 2009).

Relaxation experiments

¹⁵N-spin-lattice relaxation rate (T₁), spin–spin relaxation rate (T₂), and steady-state heteronuclear NOEs (hetNOE) (Kay et al. 1989) were obtained to probe NTD dynamics in solution. The relaxation experiments were carried out using 0.4 mM ¹⁵N-labeled NTD on a spectrometer with a proton frequency of 600 MHz (Zhang et al. 2016; Gayen et al. 2012). The experiments for obtaining T₁ and T₂ were collected using pseudo-3D experiments. The data were processed using NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson 2004).

Thermal stability assay

The thermal stability analysis was performed in a 96-well PCR plate (Bio-Rad) with 50 µl per reaction containing 5 × SYPRO Orange dye (Invitrogen), 4 µM of test protein and 4 mM of test ligand(s) (SAM, SAH, and MTA) or 1 mM sinefungin. 0.9% (v/v) DMSO was present when sinefungin was tested for optimal solubility of the test compound. To serve as negative controls, assay buffer only (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM DTT and 0.05% Tween-20) or assay buffer containing 0.9% DMSO was used. The fluorescence was measured with a FluoStar Omega Plate Reader (BMG Labtech).
in an i-Cycler iQ5 real-time PCR (Bio-Rad). Thermal stability curves and the temperature midpoint $T_m$ for protein-unfolding transitions were analysed using the Bio-Rad iQ5 software.

**Assignments and data deposition**

The NTD of *Pa*TrmD (Fig. 1a) was produced in milligram quantities from a 1-l culture of *E. coli* cells. This recombinant protein yield was sufficient for several hetero-nuclear NMR studies and NMR ligand binding experiments. Recombinant NTD has a theoretical molecular mass of 19.1 kDa (Fig. 1b) while gel filtration chromatography indicated that it was in a dimeric form (Data not shown). We collected the $^1$H-$^{15}$N-HSQC spectrum of $^{15}$N-labeled NTD. NTD exhibited well dispersed cross peaks in the spectrum with proton resonances ranging from 7 to 10 ppm (Fig. 1c) indicative of a well folded NTD protein in solution, containing β-strand structures.

![Fig. 1](image)

**Fig. 1** Protein production and NMR analysis of NTD. a Diagram of *Pa*TrmD. The N-terminal domain (amino acids 1-167) was prepared for NMR studies. b SDS-PAGE analysis of purified $^{15}$N-labeled NTD. c Assignment of the $^1$H-$^{15}$N-HSQC spectrum of NTD. Cross peaks in the spectrum are labelled with residue name and sequence number. d Secondary structure of NTD. The secondary structure of NTD in solution was obtained based on the assigned chemical shifts of backbone atoms. Residues predicted to form α and β structures are shown with black and purple bars, respectively. The secondary structures of NTD in solution were compared with those of the X-ray structure of full-length TrmD (PDB ID: 5WYQ).
Backbone resonance assignments for the NTD are useful to predict secondary structural elements of the NTD and also aid in the mapping of NTD-binding inhibitors of TrmD. The assignment of the cross peaks in the $^1$H-$^{15}$N-HSQC spectrum was obtained (Fig. 1c). The NTD construct used in this study contains 172 amino acids including eight proline amino acids and a histidine tag at the C-terminus. Most amide protons and amide resonances of the NTD were assigned based on the connectivity of the Cα and Cβ chemical shifts. In total, 92% of non-proline amide and amide protons were identified and assigned. Amino acid residues for which assignments could not be made are M1, D2, T38, N41, R49, H50, Q51, G61, G63, L71, E121, G166 and H167. Excluding the five histidine residues at the C-terminus, 95% (159 out of 167) of $^{13}$Cα, 92% (137 out of 148) of $^{13}$Cβ and 90% (151 out of 167) of $^{13}$C were assigned. The chemical shift assignments have been deposited in the BioMagResBank under accession number 27566.

Using TALES+ (Shen et al. 2009), secondary structural elements of the NTD could be predicted based on assigned chemical shifts of backbone resonances. The secondary structure predictions of the NTD in solution are in good agreement with structures derived from X-ray crystallography (PDB accession code: 5WYQ). The NTD contains five β-strands and six α-helices (Fig. 1d). These β-strands are β1 (spanning residues L5–S11), β2 (L35–W40), β3 (A87–L92), β4 (A112–A117), and β5 (E134–W136). Predicted α-helices include α1 (spanning residues E15–D22), α2 (I25–Q32), α3 (L71–A82), α4 (Q101–E107), α5 (E125–H131), and α6 (G146–L160) (Fig. 1d). With the assignment in hand, protein–ligand interactions could be evaluated using solution NMR spectroscopy and their binding sites could be mapped onto the structure of the NTD.

We obtained $^{15}$N-$T_1$, $T_2$ and hetNOEs values of NTD to understand its dynamics in solution. Overall, the dynamics of NTD are consistent with its secondary structure. Structured regions exhibited higher $T_1$ and hetNOEs values than those residues from unstructured regions. NTD contains a long loop in which residues 45–66 are highly dynamic in solution (Fig. 2). Residues 138–142 form a loop region connecting a helix and a strand in the crystal structure (Ito et al. 2015). This loop in NTD has similar $T_1$, $T_2$ and hetNOEs values to those of residues in the structured regions, which might be due to its location at the dimeric interface. Based on the averaged $T_1/T_2$ ratio of residues from structured regions, the correlation time of NTD in solution is approximately 19 ns. Compared to the values of other proteins, the molecular weight of NTD in solution is close to 40 kDa, confirming that it forms dimers.

The NTD contains the SAM binding region and structural studies have revealed that this domain binds to SAM, SAH and active-site inhibitors such as the SAM analog, sinefungin (PDB access code: 5WYR). However, it was still unknown whether NTD in its dimeric form would retain the same binding capacity for these ligands. By performing fluorescence-based thermal shift assays, we first assessed the thermal stabilities of NTD in the presence of various active-site ligands such as SAM, SAH, MTA and Sinefungin (Fig. 2b). Metabolites such as SAM, SAH and MTA enhanced the thermostability of NTD by increasing its melting temperature ($T_m$) (Fig. 2b), suggesting that NTD on its own is able to recognize and bind SAM binding-pocket ligands.

In summary, we demonstrate here that the NTD of PaTrmD is amenable to solution NMR studies and presents a workable and useful system for the identification and validation of SAM-binding site ligands.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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