Crystal structure of Rv1220c, a SAM-dependent O-methyltransferase from *Mycobacterium tuberculosis*

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Rv1220c from *Mycobacterium tuberculosis* is annotated as an O-methyltransferase (*Mtb*OMT). Currently, no structural information is available for this protein. Here, the crystal structure of *Mtb*OMT refined to 2.0 Å resolution is described. The structure reveals the presence of a methyltransferase fold and shows clear electron density for one molecule of S-adenosylmethionine (SAM), which was apparently bound by the protein during its production in *Escherichia coli*. Although the overall structure of *Mtb*OMT resembles the structures of O-methyltransferases from *Corynebacterium glutamicum*, *Coxiella burnetii* and *Alfa alfa*, differences are observed in the residues that make up the active site. Notably, substitution of Asp by His164 seems to abrogate metal binding by *Mtb*OMT. A putative catalytic His–Asp pair located in the vicinity of SAM is absolutely conserved in *Mtb*OMT homologues from all species of *Mycobacterium*, suggesting a conserved function for this protein.

1. Introduction

S-Adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs) catalyze the transfer of a methyl group from SAM to an acceptor substrate (Schubert *et al.*, 2003; Fontecave *et al.*, 2004). Within the SAM-dependent MT group, some enzymes specifically catalyze methylation of the O atom of the substrate. These enzymes are referred to as SAM-dependent O-methyltransferases (OMTs; Hou *et al.*, 2007). Substrates of OMTs include DNA, RNA, proteins, lipids and small molecules (Martin & McMillan, 2002).

Caffeoyl-CoA OMT (CCoAOMT) methylates the 3'-hydroxyl group of the phenolic moiety to produce feruloyl-CoA (Parvathi *et al.*, 2001). The enzyme requires a metal ion to deprotonate the hydroxyl group and has a narrow range of substrate specificity (Ferrer *et al.*, 2005). In contrast to the CCoAOMTs, the phenylpropanoid and flavanoid OMT (PFOMT) from *Mesembryanthemum crystallinum* has a promiscuous substrate specificity (Ibdah *et al.*, 2003). Comparison of the structure of PFOMT with that of the CCoAOMT from *Alfa alfa* suggests roles for the N-terminal region and the environment around the active site in imparting substrate specificity (Ibdah *et al.*, 2003). In mammals, the OMTs involved in catechol metabolism have been well studied (Vidgren *et al.*, 1994; Männistö & Kaakkola, 1999). The structure of catechol OMT from rat has been solved and the structural view of the active site supports an S_N2-like mode of reaction kinetics (Chen *et al.*, 2005; Lau & Bruce, 1998; Sipilä & Taskinen, 2004; Lautala *et al.*, 2001). Surprisingly, the physiological significance of OMTs from bacteria continues to remain obscure. A potential role for these enzymes in the synthesis of secondary metabolites such

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Table 1

Macromolecule-production information.

In the primers, the restriction sites are underlined. The additional residues introduced during construction are indicated in bold.

Source organism	<i>M. tuberculosis</i> (strain ATCC 25618/H37Rv)
DNA source	<i>M. tuberculosis</i> H37Rv
Forward primer	CCCATATGATGCCTGGCCAG
Reverse primer	CCCTCGAGTTAGTCCCGCAG
Cloning vector	pET-15b
Expression vector	pET-15b
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHSSGLVPRGSHMPGQPAPSRGESL-MPGQPAPSRGESLWAHAEGSISEDVILAGARE-RATDIGAGAVTPAVGALLCLLAKLSGGKVAE-VGTGAGVSGLLSGMRDDGVLTTIDIEPEHLRLARQAFAGAGIGPSRTRLISGRAQEVLRLLADSYDLVFIDADPIDQPDYVAEGVRLRLSGGVIVVHRAALGGRAGDPGARDAEVIAVREARLI-AEDERLTPALVPLGDGVLAARD
Total no. of residues	215

Table 2

Crystallization.

Method	Vapour diffusion
Plate type	Sitting-drop plate
Temperature (K)	295
Protein concentration (mg ml ⁻¹)	20
Buffer composition of protein solution	30 mM HEPES pH 7.5, 300 mM NaCl
Composition of reservoir solution	1.0 M (NH ₄) ₂ SO ₄ , 0.1 M HEPES pH 7.0, 0.5% (w/v) polyethylene glycol 8000
Volume and ratio of drop	1 µl:1 µl
Volume of reservoir (µl)	100

as antibiotics has been proposed. For example, *Myxococcus xanthus* OMT methylates precursors such as L-dihydroxyphenylalanine in the synthesis of saframycin (Nelson *et al.*, 2007). Interestingly, the crystal structures of bacterial OMTs such as those from *Leptospira interrogans*, *Synechocystis* spp., *Bacillus cereus* (Hou *et al.*, 2007; Kopycki *et al.*, 2008; Cho *et al.*, 2008) and *Staphylococcus aureus* (PDB entry 3ntv; W. Qiu, R. Lam, V. Romanov, K. Jones, E. F. Pai & N. Y. Chirgadze, unpublished work) all reveal a conserved topology that is shared across different species, suggesting an evolutionarily conserved function for these enzymes.

In addition to metal-ion-dependent OMTs, homologous enzymes that perform metal-ion-independent methyl transfers are also known to exist. In particular, OMTs that methylate caffeic acid and its derivatives rely on general acid/base catalysis or optimal positioning of the substrate in the proximity of the reactive methyl group of SAM to perform methyl transfers (Zubieta *et al.*, 2001). Rv1220c from *Mycobacterium tuberculosis* (*Mtb*) is annotated as an OMT (TB database; <http://www.tbdb.org/>). The physiological role of such an activity in *Mycobacterium* is currently unknown. To obtain clues about the *in vivo* function of the protein (referred to in the following as *Mtb*OMT), we initiated studies on structural characterization of the enzyme. A structural view of *Mtb*OMT reveals a typical methyltransferase fold with one molecule of SAM bound to the protein. A close inspection of the active site and its comparison with homologous structures reveals

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 17U, SSRF
Wavelength (Å)	0.97923
Temperature (K)	100
Detector	PILATUS 2M, Dectris
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	0.5
Space group	<i>P</i> ₄ ₁ ₂ ₁ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	57.70, 57.70, 158.16
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.5
Resolution range (Å)	32.62–2.00
Total No. of reflections	251504
No. of unique reflections	18783
Completeness (%)	99.1 (100)
Multiplicity	13.4 (13.7)
$\langle I/\sigma(I) \rangle$	14.2 (4.4)
<i>R</i> _{int}	0.131 (0.558)
Overall <i>B</i> factor from Wilson plot (Å ²)	38.9

key differences in the amino acids surrounding the SAM. The impact of these differences on the OMT activity of *Mtb*OMT is discussed.

2. Materials and methods

2.1. Macromolecule production

The gene coding for *Mtb*OMT (Rv1220c) was PCR-amplified from the genomic DNA of *M. tuberculosis* strain H27Rv and ligated into pET-15b vector to generate pET-*Mtb*OMT. *Escherichia coli* BL21 (DE3) cells harbouring pET-*Mtb*OMT were grown in Luria–Bertani (LB) medium at 37°C until the OD at 600 nm reached 0.5–0.6. To induce protein expression, 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the culture. After 18–20 h of induction at 16°C, the cells were harvested by centrifugation.

The cell pellet was resuspended in lysis buffer consisting of 50 mM Tris–HCl pH 8.0, 500 mM NaCl. After disruption by sonication, the lysate was clarified by centrifugation at 30 000g for 40 min at 4°C. The protein was purified by nickel-affinity chromatography. After buffer exchange, the protein was further purified by anion-exchange chromatography using a Resource Q column (Invitrogen), followed by gel-filtration chromatography using a Superdex 200 16/60 column (GE Healthcare). Pure *Mtb*OMT suspended in 30 mM HEPES pH 7.5, 300 mM NaCl was concentrated to 20 mg ml⁻¹ and stored at –80°C until further use. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Crystals were grown at room temperature (22°C) using the sitting-drop vapour-diffusion method. The best diffracting crystals grew in a solution consisting of 1.0 M ammonium sulfate, 0.1 M HEPES pH 7.0, 0.5% (w/v) polyethylene glycol 8000. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Crystals were cryoprotected by adding 20% glycerol to the crystallization buffer before flash-cooling them in liquid nitrogen. Raw data were collected to 2.0 Å resolution on beamline 17U at Shanghai Synchrotron Radiation Facility (SSRF). The data were processed using the *HKL-2000* suite of programs (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 3.

2.4. Structure solution and refinement

The structure of *MtbOMT* was solved by the molecular-replacement (MR) method with the program *Phaser* in the *CCP4* suite (Read, 2001; Winn *et al.*, 2011), using the structure of an *O*-methyltransferase from *Coxiella burnetii* (PDB entry 3tr6; Franklin *et al.*, 2015) as the search template. Manual adjustments to the model were made with *Coot* (Emsley *et al.*, 2010). Refinement was carried out using *phenix.refine* (Afonine *et al.*, 2012). The final model consisted of 198

residues, one molecule of SAM and 84 water molecules. The atomic model was refined to an R_{work} of 19.22 ($R_{\text{free}} = 21.57$) and was judged to have good stereochemistry according to the Ramachandran plot calculated by *MolProbity* (Chen *et al.*, 2010). Refinement statistics are summarized in Table 4.

3. Results

3.1. Overall structure of *MtbOMT*

MtbOMT was overexpressed as a soluble protein in *E. coli*, with a yield of approximately 1 g per litre of culture. The purified recombinant *MtbOMT* migrated as a 25 kDa protein band on SDS-PAGE (Fig. 1a). During gel-filtration chromatography using a Superdex 200 16/60 column, the absorbance of the protein peak at 254 nm wavelength was unusually high and was almost equal to the absorbance at 280 nm wavelength (Fig. 1a). The reason underlying this optical behaviour of the protein became clear during the subsequent structure determination of the protein. The absorbance at 254 nm for the

Figure 1

Overall structure of *MtbOMT*. (a) Elution curve of *MtbOMT* during size-exclusion chromatography. Inset: SDS-PAGE gel showing purified recombinant *MtbOMT* migrating as a 25 kDa protein. (b) Cartoon representation of the structure of *MtbOMT* bound to SAM. (c) Superimposition of the C^α atoms of the structures of homologues of *MtbOMT*. The structures are shown in ribbon representation. Ligands are shown as sticks. (d) Cartoon representation of a dimer of *MtbOMT*. In (a)–(c), the termini of the protein are marked N and C. The disordered loop is shown as a dotted line. SAM is represented as sticks.

Table 4

Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	32.617–1.997 (2.10–1.99)
Completeness (%)	98.2
σ Cutoff	$F > 1.34\sigma(F)$
No. of reflections, working set	18619 (2416)
No. of reflections, test set	922 (125)
Final R_{cryst}	0.192 (0.2359)
Final R_{free}	0.216 (0.2530)
No. of non-H atoms	
Protein	1444
Ligand	27
Water	84
Total	1555
R.m.s. deviations	
Bonds (Å)	0.007
Angles (°)	0.975
Average B factors (Å ²)	
Protein	53.8
Ligand	44.0
Water	54.3
Ramachandran plot	
Favoured regions (%)	97.94
Additionally allowed (%)	2.06

protein originated from the cofactor SAM, which was bound by the protein during its expression in *E. coli*.

The structure of recombinant *Mtb*OMT was solved by molecular replacement and the final model encompassing residues 11–168 and 176–215 was refined to 2.0 Å resolution (Table 4). Initial analysis of the structure of *Mtb*OMT revealed that the overall fold resembles that of a typical methyltransferase (Fig. 1*b*). The structure consists of eight α -helices and seven β -strands. A central β -sheet is flanked by α -helices on either side. Six of the strands from the sheet run in parallel, while the seventh strand is positioned antiparallel. With the exception of helix α 1, which is observed to protrude out of the protein, the rest of the structural elements assemble together in a group to form a compact globular structure. A search of the Protein Data Bank for structural homologues retrieved the structures of OMTs from *Corynebacterium glutamicum*

(*Cgl*OMT; PDB entry 3dr5; sequence identity 36%; Z-score 27.8; r.m.s.d. of 1.8 Å for 196 matching C α atoms; Northeast Structural Genomics Consortium, unpublished work), *Coxiella burnetti* (*Cbu*OMT; PDB entry 3tr6; sequence identity 31%; Z-score 27.3; r.m.s.d. of 1.7 Å for 195 matching C α atoms; Franklin *et al.*, 2015) and *Alfa alfa* (*Aal*OMT; PDB entry 1sui; sequence identity 26%; Z-score 25.5; r.m.s.d. of 2.1 Å for 195 matching C α atoms; Ferrer *et al.*, 2005) as matches (Fig. 1*c*; Holm & Rosenström, 2010). Similar to its homologues, *Mtb*OMT crystallized as a dimer. The N-terminus of the protein is observed to mediate dimerization (Fig. 1*d*).

3.2. SAM-binding site of *Mtb*OMT

In the structure of *Mtb*OMT, clear electron density for one molecule of SAM is observed (Fig. 2*a*). SAM binds in a cavity surrounded by helices α 2, α 5, α 6 and α 7. The cavity lies just above the central β -sheet. While the adenosine moiety sits on the surface of the protein, the tail of the ligand is buried deep inside a hydrophobic cavity. Residues such as Gly66, Asp90, Ile91, Arg119, Ala120, Gln121, Ala139, Asp140 and Tyr147 are involved in entrenching the adenine moiety firmly on the protein (Fig. 2*b*). Notably, Gln121 and Tyr147 form hydrogen bonds to the N atoms of the adenine ring. Furthermore, the O2 and O3 hydroxyl O atoms of the ribose sugar of SAM form hydrogen bonds to the carboxyl O atoms of Asp90. Other hydrogen bonds observed between the ligand and the protein include those mediated by the N1 atom of SAM. This N atom forms two hydrogen bonds: (i) a 2.7 Å hydrogen bond to the carboxyl OD1 atom of Asp138 and (ii) a 2.8 Å hydrogen bond to the hydroxyl O atom of Ser72 (Fig. 2*b*). The hydroxyl O atom of Ser72 further forms a hydrogen bond to the carboxyl O atom of SAM. These interactions probably assist in orienting and optimally positioning SAM for catalysis.

3.3. Comparison of the active site of *Mtb*OMT with those of its structural homologues

The active sites of OMTs were compared by superimposing the structures of *Cgl*OMT, *Cbu*OMT and *Aal*OMT on the structure of *Mtb*OMT bound to SAM. *Mtb*OMT bound SAM in a similar location as SAH bound to *Cbu*OMT and *Aal*OMT. However, differences are observed in the residues interacting with the ligand. Specifically, Arg119 of *Mtb*OMT that interacts with the adenosine moiety of SAM is replaced by a proline in *Cbu*OMT and *Aal*OMT. In addition, Gln121 of *Mtb*OMT located in the vicinity of SAM is replaced by a leucine in *Cgl*OMT and *Aal*OMT. The equivalent position is occupied by a lysine in *Cbu*OMT. Furthermore, His95 of *Mtb*OMT, which is observed to engage the ribose of SAM, is replaced by a serine in *Cbu*OMT and an asparagine in *Aal*OMT.

The most significant difference between the active sites of the OMTs is observed around the metal ion that is bound by *Cbu*OMT and *Aal*OMT. A divalent metal ion such as Ca²⁺ is essential for the activity of *Aal*OMT. The metal ion bound to *Cbu*OMT occupies a similar position to that of Ca²⁺ in *Aal*OMT. Remarkably, Asp170 of *Cbu*OMT and Asp189 of *Aal*OMT that coordinate the catalytically critical metal ion are

Figure 2
SAM-binding site of *Mtb*OMT. (a) $2F_o - F_c$ electron density for SAM contoured at 1.0σ using the 2.0 Å resolution electron-density map. (b) Residues (green sticks) interacting with SAM (magenta sticks).

replaced by a histidine in *Mtb*OMT (Fig. 3a). Interestingly, *Cgl*OMT has neither aspartate nor histidine at this position. Instead, an alanine residue is observed at the equivalent position. In addition, Thr45, Gln49 and Asn171 of *Cbu*OMT (Thr63, Glu67 and Asn190 of *Aal*OMT), which possibly participate in octahedral coordination of the divalent metal ion, are replaced by Val42, Val46 and Arg165, respectively, in *Mtb*OMT (Fig. 3b). These differences indicate that unlike *Cbu*OMT and *Aal*OMT, *Mtb*OMT probably cannot bind positively charged divalent metal ions. Inspection of the structure of *Cgl*OMT seems to indicate that the side chain of Gln136 could possibly sterically hinder the binding of metal ions by *Cgl*OMT.

In order to confirm the structural observation that *Mtb*OMT may not be able to bind a metal ion in the putative active site, we performed metal-ion analysis on *Mtb*OMT using inductively coupled plasma atomic emission spectroscopy (ICP-AES). In agreement with the structural observation, the

results of the metal-ion analysis revealed that the protein did not bind Ca^{2+} , Zn^{2+} , Mg^{2+} or Mn^{2+} ions (Supplementary Table S1). We attempted co-crystallization of the protein in the presence of Mg^{2+} ions as well as the soaking of crystals of *Mtb*OMT in a solution containing Mg^{2+} ions prior to data collection. However, no electron density for any metal ion was observed in the active site of the enzyme. Thus, *Mtb*OMT does not bind metal ions.

4. Discussion

The enzymatic activity of *Aal*OMT has been well characterized (Ferrer *et al.*, 2005). This OMT requires a divalent cation for enzymatic activity. In the structure of *Aal*OMT, Asp163 and Asp189 are observed to coordinate a Ca^{2+} ion. The replacement of Asp189 by a histidine in *Mtb*OMT probably perturbs metal binding by *Mtb*OMT. The inability of *Mtb*OMT to bind metal ions was confirmed by ICP-AES analysis of

Figure 3

Differences between the active sites of *Mtb*OMT and its homologues. (a) The structures of homologues such as *Cgl*OMT (PDB entry 3dr5; grey), *Cbu*OMT (PDB entry 3tr6; green) and *Aal*OMT (PDB entry 1sui; magenta) were superimposed on the structure of *Mtb*OMT (blue). A key substitution of Asp by His in *Mtb*OMT is shown in sticks. The metal ion in *Cbu*OMT and *Aal*OMT is depicted as a sphere. The ligand SAM/SAH is shown as sticks. (b) Residues of the active site of OMTs (*Cgl*OMT, grey; *Cbu*OMT, green; *Aal*OMT, magenta) superimposed on those of *Mtb*OMT (blue sticks) to highlight the differences. Metal ions are shown as spheres. (c) Asp138 hydrogen-bonded (dotted line) to His164 interacts (dotted line) with the reactive methyl group of SAM shown as green sticks. Placement of substrate, modelled as the phenolic ring of CCoA (blue), between the reactive methyl group of SAM and His164 could possibly result in methyl transfer. Interactions of <4 Å are depicted as dashed lines. (d) The active site of *Clarkia breweri* CAOMT (PDB entry 3reo) contains a His–Asp pair similar to that seen in *Rv1220c*. Binding of SAM and the substrate caffeic acid would bring the interactions (shown in dotted lines) within a distance of 4 Å. The protein was crystallized with the products SAH and ferulic acid (both shown as sticks).

MtbOMT and by performing structural studies. The presence of a histidine at an equivalent position to Asp189 in *AalOMT* results in the formation of a His–Asp pair in *MtbOMT*. A catalytic role for histidine has been proposed for other caffeic acid OMTs (CAOMTs; Green *et al.*, 2014; Zhang *et al.*, 2015). For instance, the CAOMTs from *Sorghum bicolor* (*SbCAOMT*) and *Streptomyces virginiae* (*SvCAOMT*) perform catalysis in a metal-ion-independent manner using the catalytic histidine to deprotonate the hydroxyl group of the substrate. An aspartate residue is also observed within hydrogen-bonding distance of the catalytic histidine of *SbCAOMT*. In the structure of *MtbOMT*, the ND1 atom of His164 is hydrogen-bonded by the OD1 carboxyl O atom of Asp138 (Fig. 3c). It overlaps with the position of the Ca²⁺ ion bound by *AalOMT* when the two structures are superimposed. This N atom of His164 is 6.0 Å away from the reactive methyl group of SAM. Thus, the hydroxyl group of an acceptor substrate can be accommodated between the histidine and the reactive methyl group of SAM (Fig. 3c). The methyl-transfer reaction is then likely to proceed *via* activation of His164 by Asp138. This is supported by the 2.8 Å hydrogen bond formed between the two side chains. His164 abstracts a proton from the hydroxyl group of the substrate and primes it for accepting the methyl group from SAM (Fig. 3c). Although further structural and functional studies are required to support this mechanism of catalysis, the preliminary structural work presented here seems to suggest that *MtbOMT* shares structural similarity with metal-ion-dependent OMTs such as *AalOMT* (Fig. 1c), but its catalytic machinery is more similar to those of metal-ion-independent OMTs such as *SbCAOMT* (Green *et al.*, 2014), *SvCAOMT* (Zhang *et al.*, 2015), *Lolium perenne* CAOMT (PDB entry 3p9c; Louie *et al.*, 2010), *Medicago sativa* CAOMT (PDB entry 1kyw; Zubieta *et al.*, 2002) and *Clarkia breweri* CAOMT (PDB entry 3reo; M. W. Bhuiya & C. J. Liu, unpublished work) (Fig. 3d).

Alignment of the primary sequences of homologues of *MtbOMT* (Supplementary Figs. S1, S2 and S3), which is annotated as an OMT, reveals two important features: (i) Asp138 and His164, which make up the His–Asp pair, are strictly conserved across all species of *Mycobacterium* that have been analyzed (Supplementary Fig. S2) and (ii) some homologous proteins, for instance the proteins from the genera *Nocardia*, *Pilimelia*, *Smaragdicoccus*, *Rhodococcus*, *Williamsia* and *Microbispora*, all carry Asp138 and His164 (Supplementary Fig. S3). Thus, it is quite likely that these homologous proteins probably perform a conserved function. Elucidation of the exact function and its physiological significance could further increase our understanding of methyl-transfer reactions, which occur routinely in all living organisms.

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