Journal of Structural Biology 191 (2015) 175–183

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Functional insights from high resolution structures of mouse protein arginine methyltransferase 6

Luc Bonnefond [a], Johann Stojko [b], Justine Mailliot [a], Nathalie Troffer-Charlier [a], Vincent Cura [a], Jean-Marie Wurtz [a], Sarah Cianférani [b], Jean Cavarelli [a,*]

[a] Département de Biologie Structurale Intégrative, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Université de Strasbourg, CNRS UMR7104, INSERM U964, 1 rue Laurent Fries, Illkirch, F-67404, France
[b] Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), IPHC-DSA, Université de Strasbourg, CNRS, UMR7178, 25 rue Becquerel, Strasbourg 67087, France

ARTICLE INFO

Article history:
Received 4 July 2014
Received in revised form 19 March 2015
Accepted 18 June 2015
Available online 18 June 2015

Keywords:
Methyltransferase
PRMT6
Epigenetics
Protein structure
S-adenosyl-L-methionine

ABSTRACT

PRMT6 is a protein arginine methyltransferase involved in transcriptional regulation, human immunodeficiency virus pathogenesis, DNA base excision repair, and cell cycle progression. Like other PRMTs, PRMT6 is overexpressed in several cancer types and is therefore considered as a potential anti-cancer drug target. In the present study, we described six crystal structures of PRMT6 from *Mus musculus*, solved and refined at 1.34 Å for the highest resolution structure. The crystal structures revealed that the folding of the helix αX is required to stabilize a productive active site before methylation of the bound peptide can occur. In the absence of cofactor, metal cations can be found in the catalytic pocket at the expected position of the guanidinium moiety of the target arginine substrate. Using mass spectrometry under native conditions, we show that PRMT6 dimer binds two cofactor and a single H4 peptide molecules. Finally, we characterized a new site of *in vitro* automethylation of mouse PRMT6 at position 7.

1. Introduction

PRMTs catalyze the transfer of the methyl group from S-adenosyl-L-methionine (SAM or AdoMet) to the side-chain N atoms of arginine residues of a target protein substrate to form methylated arginines and S-adenosyl-L-homocysteine (SAH or AdoHcy). The target arginine residues, mainly located within glycine- and arginine-rich patches, can be mono- or dimethylated. At least nine members of PRMTs (PRMT1 to PRMT9) have been identified and classified into three main classes. All classes catalyze the formation of monomethylarginine. In a second step, type I PRMTs (PRMT1, PRMT3, PRMT4/CARM1 PRMT6 and PRMT8) further form asymmetric dimethylarginine, whereas type II (PRMT5) form symmetric dimethylarginine. With few exceptions such as PRMT7 (Miranda et al., 2004), PRMTs are active as homodimer with each monomer containing a structurally conserved catalytic core module to which additional domains may be added (Antonysamy et al., 2012; Cheng et al., 2011; Cura et al., 2014a,b; Hasegawa et al., 2014; Ho et al., 2013; Sun et al., 2011; Troffer-Charlier et al., 2007; Wang et al., 2014b; Weiss et al., 2000; Yue et al., 2007; Zhang and Cheng, 2003; Zhang et al., 2000).

PRMT6 is a nuclear protein that typically targets glycine and arginine rich (GAR) motifs (Frankel et al., 2002). PRMT6 is involved in transcriptional regulation, human immunodeficiency virus pathogenesis, DNA base excision repair, and in cell cycle progression (Kleinschmidt et al., 2012; Phalke et al., 2012; Stein et al., 2012). It is responsible for the *in vitro* methylation of the arginine 2 residue of the histone H3 tail (H3R2) and of the arginine 3 of histone H4 (H4R3) (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008). Several other PRMT6 targets have been identified including histones H2AR29 (Waldmann et al., 2011) and H3R42 (Casadio et al., 2013) the high mobility group protein HMGA1a and HMGA1b (Miranda et al., 2005; Sgarra et al., 2006), HIV-1 trans-activator of transcription (Tat), regulator of virion (Rev) and nucleocapsid proteins (Boulanger et al., 2005; Invernizzi et al., 2007; Xie et al., 2007), and the DNA polymerase α (El-Andaloussi et al., 2006). Like other PRMTs, PRMT6 is overexpressed in several cancer types, such as bladder and lung cancers (Yoshimatsu et al., 2011). It is therefore considered as a potential anti-cancer drug

Abbreviations: PRMT, protein arginine methyltransferase; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; SFG, sinefungin; PDB, Protein Data Bank; rmsd, root square mean deviation; ESI-MS, electrospray ionization mass spectrometry; LC–MS/MS, liquid chromatography tandem mass spectrometry.

* Corresponding author.
E-mail address: cava@igbmc.fr (J. Cavarelli).

http://dx.doi.org/10.1016/j.jsb.2015.06.017
1047-8477/

target. So far only one specific inhibitor has been reported, AMI1, that can inhibit PRMT6 to block arginine methylation of Rev (Invernizzi et al., 2006). The catalytic mechanism of PRMT6 has been thoroughly studied and two mechanisms have been proposed: a sequential ordered mechanism (Lakowski and Frankel, 2008) and a rapid equilibrium random mechanism (Obianyo and Thompson, 2012). Dissociation constant (K_d) for PRMT6 dimer dissociation has been measured by fluorescence resonance energy transfer (FRET) and isothermal calorimetry (ITC) in the absence and presence of SAH (Thomas et al., 2010). Crystal structures of *Trypanosoma brucei* PRMT6 (TbPRMT6) have been reported recently (Wang et al., 2014a). They revealed Trypanosoma-specific structural features, as well as subtle active site rearrangement upon SAH binding. Crystals structures of human PRMT6 (HsPRMT6) are also available (PDBID 4HC4, 4QQK, and 4QPP). In the present study, we described six crystal structures of PRMT6 from *Mus musculus*, solved and refined at 1.34 Å for the highest resolution structure.

2. Material and methods

2.1. PRMT6 cloning, expression and purification

The *M. musculus* gene *prmt6* (UP Q6NZB1) was cloned in the pnEA-vHis vector (Romier et al., 2006) between the *Nde*I and *Xho*I restriction sites and used to transform *Escherichia coli* BL21 (DE3) pRARE2 cells. The resulting recombinant protein harbors an amino-terminal hexahistidine tag followed by a Tobacco etch virus (TEV) protease cleavage site. The protein also contains a F315L point mutation, reported for the entry as a natural variant. The protein expression was induced for 4 h at 37 °C, with 0.1 mM IPTG in LB medium. The cells were harvested by centrifugation and resuspended in 10 ml per pellet g of buffer A [50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 7 mM 2-Mercaptoethanol (2-ME), 20 mM imidazole) supplemented with 1 mM phenylmethylsulfonyl fluoride]. After sonication, the lysate was centrifuged at 40,000×*g* for 25 min at 4 °C. The supernatant was mixed with 0.5 ml of NiNTA superflow resin (Qiagen) per liter of culture, incubated for 1 h at 4 °C, and then loaded on an Econo column (Bio-Rad). After washing the column with 10 column volumes (c.v.) of buffer A, the bound protein was eluted with 5 c.v. of buffer A supplemented with 500 mM imidazole. The fractions containing the recombinant protein were detected by a Bradford assay, pooled and supplemented with TEV protease. The sample was then dialyzed in a 10,000 MWCO dialysis membrane (Spectrum) against 1 l of buffer B [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 7 mM 2-ME and 1 mM Ethylenediaminetetraacetic acid] for 16 h at 4 °C. The dialyzed sample was loaded onto a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare), equilibrated in buffer C [20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM Dithiothreitol (DTT)]. The protein was finally concentrated on an Amicon Ultra-4 10,000 MWCO filter (Millipore) to a 5 mg ml^{-1} final concentration. Samples were either directly used for crystallization or flash-frozen as 25 µl aliquots in liquid nitrogen and stored at −80 °C.

2.2. PRMT6 crystallization

Crystallization conditions were screened using commercially available kits by the sitting-drop vapor-diffusion method in 96-well MRC2 plates (Swissci), and employing a Mosquito robot (TTP Labtech) with 200 nl protein solution + 200 nl mother liquor drops. The plates were placed in a Rock Imager (Formulatrix) at 293 K and monitored periodically. The PACT and JCSG+ screens (Qiagen) were tested for MmPRMT6 concentrated from 3 to 4 mg ml^{-1}. Initial hits were obtained for several conditions in both screen with mother liquor composed of a buffer at pH 7.0–8.0, a salt at 200 mM and PEG as a precipitant, mostly PEG 6000 and PEG 8000. These conditions were refined using the hanging drop diffusion method with 2 µl drops (1 µl + 1 µl). Large crystals ($0.4 \times 0.2 \times 0.2$ mm^3) of bipyramidal habit were obtained within 3 weeks from drops with crystallization solution containing either 100 mM of Tris–HCl, pH 7.5 or HEPES-NaOH, pH 8.0 for the buffer, 200 mM $MgCl_2$ or $CaCl_2$ as a salt and 20% PEG 1500, PEG 3350 or PEG 6000 as a precipitant. Co-crystallizations were performed with S-adenosyl-L-homocysteine (SAH) and sinefungin (SFG) at 1 mM final concentration. For the reduced form of PRMT6, the concentration of DTT in the protein solution was increased to 5 mM, changing the shape of the crystals to thin plates.

2.3. Data collection and structure solution

Crystals were soaked in the crystallization solution supplemented with 10–20% PEG 400 and flash-frozen in liquid nitrogen. Datasets were collected either at the SOLEIL PROXIMA1 beamline, using a PILATUS 6 M detector (Dectris) or at the ESRF ID23-2 beamline, using a MAR CCD-225 detector (Mar Research) and processed with autoPROC (Vonrhein et al., 2011), XDS (Kabsch, 2010) and HKL-2000 (Otwinowski and Minor, 1997). Six structures have been solved and refined. The crystals belong to the $I4_1$ space group with one PRMT6 molecule in the asymmetric unit forming a disulfide bond with the symmetric molecule, or to the $P2_12_12$ space group with two PRMT6 molecules in the asymmetric unit and all cysteine residues reduced. The structures were solved by molecular replacement using mouse CARM1 structure (PDBID 3B3F), deprived of its dimerization arm, as the search model. Iterative cycles of model building and refinement were carried out using Coot (Emsley et al., 2010), PHENIX (Adams et al., 2010) and Buster (Bricogne et al., 2011). A summary of the data statistics for the six solved and refined structures is provided in Table 1 and S1. The atomic coordinates and experimental data (PDBID 4C03, 4C04, 4C05, 4C06, 4C07 and 4C08) are available in the Protein Data Bank. All structure figures were prepared with Cuemol (http://www.cuemol.org).

2.4. Native mass spectrometry analysis

The $H4_{1\text{-}21}$ peptide and the SAH cofactor were prepared in 150 mM ammonium acetate (NH_4Ac), pH 8.0. Prior to any mass spectrometry experiment, MmPRMT6 buffer was exchanged twice against a 150 mM NH_4Ac solution at pH 8.2 using gel filtration columns (NAP-5 columns, GE Healthcare Life Sciences). Protein concentration was determined spectrophotometrically after buffer exchange. NanoESI-MS measurements were carried out on an electrospray quadrupole-time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Manchester, UK) equipped with an automated chip-based nanoESI source (Triversa Nanomate, Advion Biosciences, Ithaca, NY) operating in the positive ion mode. External calibration was performed with the multiply charged ions produced by a 2 µM horse heart myoglobin solution diluted in a 1:1 (v:v) water: acetonitrile mixture acidified with 1% formic acid. Purity and homogeneity of samples were first assayed in denaturing conditions by diluting the proteins to 2 µM in a 1:1 (v:v) water:acetonitrile mixture acidified with 1% formic acid. Analyses in native conditions were then performed by diluting analytes in NH_4Ac buffer 150 mM, pH 8.2 (adjusted with ammonia) after 10 min of incubation. Experiments were realized following careful optimization of instrumental parameters. Particularly, the pressure in the first pumping stage was raised up to 6 mbar using a throttling valve and the acceleration voltage applied on the sample cone was set to 120 V. Data analyses were performed with