Structure and binding properties of a cameloid nanobody raised against KDM5B

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Received 11 June 2015

Accepted 17 August 2015

Edited by P. Dunten, Stanford Synchrotron Radiation Lightsource, USA

Keywords: nanobody; KDM5B.

PDB reference: NB17, 4zg1

Supporting information: this article has supporting information at journals.iucr.org/f

The histone demethylase KDM5B is considered to be a promising target for anticancer therapy. Single-chain antibodies from llama (nanobodies) have been raised to aid in crystallization and structure determination of this enzyme. The antigen-binding properties of 15 of these nanobodies have been characterized. The crystal structure of one of these (NB17) has been determined to a resolution of 1.85 Å. NB17 crystallizes in space group $P4_322$ with six molecules in the asymmetric unit. The six molecules in the asymmetric unit pack as an entity with approximate $D3$ symmetry with interactions mediated by the CDR loops, which could act as a crystallization nucleus. NB17 does not bind to monomeric KDM5B residues 1–820, but is found to bind to aggregates formed after incubation at 310 K.

1. Introduction

The reversible post-translational modification of N-terminal histone tails is a central mechanism in the regulation of gene expression (Mosammaparast & Shi, 2010). The histone demethylase (HDM) class of enzymes appear to play a key role in the regulation of known oncogenes (Chi *et al.*, 2010; Arrowsmith *et al.*, 2012). Lysine demethylase 5B (KDM5B) belongs to the HDM family and recognizes methylated lysine residues, an epigenetic marker, on N-terminal histone tails. Demethylation of these markers has been linked to both transcriptional activation and repression.

Overexpression of KDM5B has been shown to enhance cancer-cell proliferation and to reduce expression of tumour suppressor genes, thus indicating KDM5B to be a novel target in oncological drug discovery. For example, in a recent study, it has been found that depletion of KDM5B induces senescence in colorectal cancer (Ohta *et al.*, 2013).

Potent pharmacological agents that selectively inhibit KDM5B are of great interest. At present, potent inhibitors of KDM5B have been identified (Labelle *et al.*, 2014), but no selective high-affinity inhibitors of KDM5B are available. A three-dimensional structure of an intact KDM5B catalytic core would be of great value to guide drug design and shed light on the complex biology that KDM5B is involved in. Very recently, a fragment of the catalytic core of KDM5B has been deposited in the PDB (PDB entry 5a1f; Structural Genomics Consortium, unpublished work). Despite this, the structure of an intact catalytic core is desirable.

A crystallization strategy for KDM5B is to use nanobodies as crystallization chaperones. Nanobodies are monomeric single domains of cameloid antibodies (Rasmussen *et al.*, 2007). They are highly stable and small (10–15 kDa) and can provide both stability and solubility to their target antigen by binding

Table 1

Macromolecule-production information.

Source organism	<i>Lama glama</i> (B cells)
DNA source	<i>L. glama</i>
Forward primer†	5'-GATGTCCAGCTGCAGGAGTCTGGRGGRGAG-G-3'
Reverse primer†	5'-GGACTAGTGGCGGCGCTGGAGACGGTGACCT-GGGT-3'
Cloning vector	pHEN4c
Expression vector	pHEN6c
Expression host	<i>E. coli</i> strain WK6
Complete amino-acid sequence of the construct produced‡	MKYLLPTAAAGLLLLAAQPAMAQVQLQESGGGLV- QAGGSLRLSCAASGSTFGIRTMGWYRQAPGKQ- RDLVAITSSGSGSTDYADSVKGRFTISRDNAKN- TVYLYQMDSLKPEDTAIYYCNARVGTMLAHWG- QGTQVTVSSHSHHHH

† Underlined bases correspond to the PstI and BstEII cleavage sites; R indicates A or G. ‡ The underlined amino-acid sequence corresponds to the PelB signal peptide.

specifically to conformational epitopes with picomolar to nanomolar affinity. Binding to the target antigen might also increase the polar surface and enable better crystal growth. Cameloid nanobodies have an extended complementarity-determining region (the CDR3 loop) compared with, for example, human antibodies, which can penetrate deeply into the binding pockets of enzymes, suggesting that the raised nanobodies may also have inhibitory properties (Desmyter *et al.*, 1996).

Here, we present the crystal structure of a nanobody (NB17) raised specifically against the catalytic core of KDM5B (ccKDM5B; residues 1–820). Investigation of the binding of NB17 to ccKDM5B reveals that binding is dependent on temperature and consequently on the aggregation state of KDM5b. This suggests that the nanobody might bind to a hydrophobic epitope of a partially unfolded ccKDM5b. The binding properties of 15 nanobodies raised against ccKDM5B are also presented.

2. Materials and methods

2.1. Protein expression and purification

2.1.1. Cloning, expression and purification of ccKDM5B for the generation of nanobodies. PCR-amplified DNA corresponding to human ccKDM5B (residues 1–820) was cloned into a pOPINF vector (Berrow *et al.*, 2007), providing an N-terminal 3C protease-cleavable His₆ purification tag. Recombinant baculovirus was generated as described previously (Kristensen *et al.*, 2012) in Sf9 insect cells by co-transfecting the ccKDM5B construct and linearized AcNPV-derived DNA (BD Biosciences). His₆-tagged ccKDM5B was expressed in 3 l suspension cultures of Hi5 cells adapted to BD Baculogold Max-XP Insect Cell Medium (BD Biosciences) supplemented with 3.5% foetal bovine serum. Hi5 cells at density of $1.8 \times 10^6 \text{ ml}^{-1}$ were infected at an MOI of 5 and were harvested after 40 h. The cells were resuspended in lysis/equilibration/wash (LEW) buffer [50 mM HEPES pH 7.7, 300 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 5 mM imidazole, 70 mg DNase I, 1 mM PMSF and one cOmplete protease-inhibitor cocktail tablet (Roche) per 50 ml] and lysed by

sonication, followed by centrifugation at 50 000g for 120 min. The filtered supernatant was combined with Talon metal-affinity resin (Clontech) equilibrated with LEW buffer and incubated on a rotating wheel for 2 h at 279 K. The resin was washed intensively with LEW buffer and ccKDM5B was eluted from the resin following on-column cleavage of the 3C protease-cleavable His₆ purification tag with human rhinovirus 3C protease (His-GST-tagged, produced in-house; data not shown). The eluted ccKDM5B was concentrated by ultrafiltration (Amicon) and subjected to size-exclusion chromatography on a HiLoad 16/600 Superdex 200 column (GE Healthcare Life Sciences) equilibrated with 50 mM HEPES pH 7.7, 300 mM NaCl, 10% glycerol, 1 mM DTT and stored at 173 K.

2.1.2. Generation of nanobodies against ccKDM5B. The nanobodies were produced by VIB Nanobody Service Facility, Brussels, Belgium following their standard protocols (Pardon *et al.*, 2014). Briefly, a llama was injected subcutaneously with 200 µg His₆-tagged ccKDM5B protein six times over one month. One week after the final injection, RNA extracted from blood lymphocytes was used as a template to create a cDNA library representing the repertoire of heavy-chain variable-domain antibodies present after immunization. PCR-amplified V_HH genes were cloned into the pHEN4C phagemid vector and expressed using standard nanobody phage display. Three consecutive rounds of panning were performed on a solid phase coated with His₆-tagged ccKDM5B; ccKDM5B-specific binders were identified by ELISA and the expressed V_HH genes were subjected to DNA sequencing. 22 nanobodies were selectively raised against ccKDM5B.

2.1.3. Cloning, expression and purification of the nanobodies. The cloning, expression and purification followed the protocol provided by the manufacturer (Pardon *et al.*, 2014). Briefly, the nanobody gene, V_HH, was subcloned into the pHEN6C vector in frame with an N-terminal PelB signal peptide and a C-terminal His₆ tag (Table 1). Freshly transformed *Escherichia coli* cells (strain WK6) were grown in Terrific Broth (TB) medium containing 1% glucose and 100 µg ml⁻¹ ampicillin until an optical density of 0.9 was reached. Protein expression was then induced by adding IPTG to a final concentration of 1 mM and continued by incubation for 18 h at 301 K. The proteins were extracted from the periplasmic space by cold lysis in TES buffer (0.2 M Tris, 0.5 mM EDTA, 0.5 M sucrose pH 8.0). The cell lysate was subjected to immobilized metal affinity chromatography on a 5 ml HisTrap column (GE Healthcare) equilibrated with PBS buffer and eluted using PBS buffer supplemented with 0.5 M imidazole. The NB17 protein was further purified and the buffer was exchanged to 50 mM HEPES, 150 mM NaCl pH 7.44 by gel filtration on a Superdex 75 10/300 GL column (GE Healthcare). The collected fractions were pooled and were concentrated by ultrafiltration (Ultracel-3K, Amicon) to 21.6 mg ml⁻¹.

2.1.4. Thermal shift assay. The thermal denaturation of ccKDM5B and nanobodies was tested indirectly by a fluorescence thermal shift assay (TSA). ccKDM5B and nanobody

Table 2
Crystallization.

Method	Sitting drop
Plate type	Crychem, 24-well, Hampton Research
Temperature (K)	279
Protein concentration (mg ml ⁻¹)	21.6
Buffer composition of protein solution	50 mM HEPES pH 7.4, 150 mM NaCl
Composition of reservoir solution	14% PEG 4000, 0.133 M NH ₄ NO ₃
Volume and ratio of drop	1 µl:1 µl
Volume of reservoir (µl)	200

(65 µM) were mixed with buffer (50 mM HEPES, 150 mM NaCl pH 7.44) and SYPRO Orange at a final concentration of 4 µM. Using an Mx3005P qPCR (Agilent Technologies), the plate was heated from 298 to 368 K with a 1 K min⁻¹ increment. Fluorescence was measured using 492/590 nm filters.

2.1.5. Complex formation and binding. Analytical gel filtration was undertaken at a constant temperature of 298 K using a Superdex 200 10/150 GL column (GE Healthcare) on an Agilent 1200 HPLC system to confirm the binding by isocratic elution at 0.45 ml min⁻¹ (50 mM HEPES, 150 mM NaCl pH 7.44). The column was calibrated with protein standards (ovalbumin, conalbumin, ribonuclease A and aprotinin) under these conditions. NB17 and ccKDM5B were mixed in a molar ratio of 1:2 at final concentrations of 20 and 40 µM, respectively. Prior to chromatography, samples were incubated for 30 min at either 278 or 310 K. Separate samples of NB17 and ccKDM5B were also included as controls for this size-exclusion experiment. Identical experiments with incubation at 278 K were performed with the remaining nanobodies.

2.2. Crystallization

The purified and concentrated NB17 (21.6 mg ml⁻¹) was subjected to crystallization screening using a commercial 96-experiment kit (PEG/Ion HT, Hampton Research). 100 and 200 nl sitting-drop experiments were set up using robotics (Cartesian MicroSys). Reservoir solution and NB17 were mixed in a 1:1 ratio and equilibrated against 50 µl reservoir solution at 279 K. After 1 d, several conditions yielded crystals (screen conditions B7, B11, C2, C12, E3, E10, F1, F9, F10 and F12). Crystal sizes varied from microcrystals to large single crystals (<200 µm) (Table 2).

One condition (B7; 0.2 M ammonium nitrate, 20% PEG 3350) was optimized in a 4 × 6 grid screen using the *Mimer* software (Brodersen *et al.*, 2013) and a liquid handler (Gilson 215) to prepare solutions for 2 µl sitting-drop experiments. The grid screen spanned a matrix of 15–25% PEG 4000 and 0.05–0.3 M ammonium nitrate. PEG 4000 was thus used instead of PEG 3350. Again, several conditions resulted in the formation of crystals but with varying sizes and morphologies: microcrystals, rosettes, single bipyramids, plates and needles were all observed. After two weeks, 30 crystals were harvested from 13 different conditions; they were cryoprotected by swift transfer into a solution containing 40% PEG 4000, flash-cooled and stored in liquid nitrogen until data collection.

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline I911-3, MAX-lab
Wavelength (Å)	0.9199
Temperature (K)	110
Detector	CCD
Crystal-to-detector distance (mm)	217.88
Rotation range per image (°)	0.5
Total rotation range (°)	100
Space group	<i>P</i> ₄ ₃ ₂ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	91.52, 91.52, 184.59
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.240
<i>Z</i>	6
Resolution range (Å)	41.21–1.85 (1.90–1.85)
Total No. of reflections	510190 (5674)
No. of unique reflections	67728 (896)
Completeness (%)	100.0 (99.8)
Multiplicity	7.5 (5.6)
$\langle I/\sigma(I) \rangle$	12.200 (2.6)
<i>R</i> _{r.i.m.}	0.118 (0.6179)
<i>R</i> _{p.i.m.}	0.043 (0.306)
Overall <i>B</i> factor from Wilson plot (Å ²)	20.150

2.3. Data collection and processing

Only one out of around 30 crystals tested diffracted to an acceptable resolution. 200 diffraction images were collected on MAX-lab beamline I911-3. The data were processed with *xia2* (Winter, 2010) from the *CCP4* suite (Winn *et al.*, 2011). This encompassed indexing and integration using *XDS* (Kabsch, 2010), finding symmetry from unmerged intensities with *POINTLESS* and finally scaling and merging of intensities in *SCALA* (Evans, 2006). The crystal diffracted to a resolution of 1.85 Å and belonged to space group *P*₄₃₂₂. Data-collection statistics and crystallographic data are given in Table 3.

2.4. Structure solution and refinement

Chain *B* of PDB entry 3ezj (81% sequence identity), a published nanobody structure (Korotkov *et al.*, 2009), was used as a search model for molecular replacement in *Phaser* (McCoy *et al.*, 2007). Automated model building was carried out using *ARP/wARP* (Langer *et al.*, 2008). Missing or poorly built residues were manually built utilizing *Coot* (Emsley & Cowtan, 2004). Owing to poor electron density, it was not possible to model the His₆ tag in any of the chains and Ser117 in chain *D*. Refinement was performed with automatic TLS group determination in the *PHENIX* suite (Adams *et al.*, 2010), resulting in 56 TLS groups. Individual isotropic displacement parameters were used. No sigma cutoff was applied to the structure-factor amplitudes during refinement. A total of 29 residues were modelled with alternate conformations; these residues were unevenly distributed throughout the six chains.

Data-refinement statistics are given in Table 4. Atomic coordinates, structure factors and other experimental information from the structure have been deposited in the PDB as entry 4zg1. The PDB file was checked for higher symmetry

Table 4
Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	41.2–1.85 (1.87–1.85)
Completeness (%)	100.0
No. of reflections, working set	64347 (2643)
No. of reflections, test set	3308 (134)
Final R_{cryst}	0.186 (0.2553)
Final R_{free}	0.235 (0.3006)
No. of non-H atoms	
Protein	5451
Solvent	730
Total	6181
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.156
Average B factors (Å ²)	
Protein	27.01
Ramachandran plot	
Most favoured (%)	98.33
Allowed (%)	1.67
Outliers (%)	0.00
MolProbity scores	
Rotamer outliers (%)	0.9
Overall score	1.22
PDB code	4zg1

Figure 1
Crystals of NB17. A picture of well diffracting crystals with characteristic multi-crystal morphology.

using *LABELIT* from the *PHENIX* package; however, no higher symmetry was found.

3. Results and discussion

The immunizations were carried out using ccKDM5B, a protein slightly larger than the previously published KDM5B catalytic core protein with enhanced expression levels (Kristensen *et al.*, 2012). The investigations of NB17 are part of the characterization of the 22 nanobodies raised against KDM5B. Of the 22 nanobodies raised to date, 15 have been successfully cloned, expressed and purified in milligram amounts. TSA and analytical gel-filtration (SEC) experiments have also been undertaken on all 15 of these nanobodies.

Initial high-throughput co-crystallization screens of KDM5B with all of the produced nanobodies were attempted at the High Throughput Crystallization Laboratory at EMBL, Grenoble. Co-crystallization of KDM5B and NB17 resulted in a single crystal which grew out of a clear solution. The crystal diffraction was sufficient to allow indexing, integration and unit-cell determination. An in-house crystallization screening experiment on NB17 alone was performed in parallel as a control for the co-crystallization experiment, and the crystal structure of NB17 was subsequently solved. This revealed that the crystal that appeared in the co-crystallization screen with KDM5B and NB17 comprised the nanobody only. As there are only a very limited number of unbound nanobody structures in the PDB, it was decided to complete the structure determination, as the structure will have potential use in more theoretical studies of single-chain antibody binding.

The best diffracting crystals are shown in Fig. 1. As is evident from the reentrant angles, the crystals all consist of more than one individual lattice. However, it was possible to index and integrate the spots originating from a single lattice despite the presence of other spots.

The structure of NB17 is shown in Fig. 2(a). It displays the expected immunoglobulin fold: a β -sandwich formed by two antiparallel β -sheets. The complementarity-determining

Figure 2
Structure of NB17 and the six NB17 molecules in the asymmetric unit. CDR1, CDR2 and CDR3 are shown in blue, yellow and red, respectively. (a) Structure of NB17. (b) Six NB17 monomers forming the asymmetric unit with D_3 symmetry. (c) As (b) but rotated 90°. This figure was generated using PyMOL (DeLano, 2002)

regions 1–3 (CDR1–3; residues 26–31, 52–54 and 96–107) appear to have average lengths (Harmsen *et al.*, 2000).

NB17 crystallized with six monomers in the asymmetric unit. The six molecules in the asymmetric unit pack as an entity with approximate D_3 symmetry, as shown in Figs. 2(b) and 2(c). The formation of the D_3 oligomer also brings together six Arg residues in the centre of the moiety. This feature is remarkable because this takes place without charge compensation. Water molecule 208 in chain *C* is centred between four of the six arginine residues with tetrahedral coordination. The closest distances to the water molecule are commensurate with hydrogen bonding.

In the refined model, the B factor of water 208 in chain *C* is $52 \text{ \AA}^2$, a value similar to those of the surrounding arginines. As chloride is present in the protein buffer, water 208 in chain *C* was replaced by a chloride ion and three additional rounds of refinement were undertaken. The B factor of the refined Cl atom increased to $85 \text{ \AA}^2$. Based on this, a water molecule was retained at this position in the final model.

CDR1, CDR2 and CDR3 are seen to be important for the intermolecular interactions of the D_3 moiety. Even though NB17 clearly migrates as a monomer in the gel-filtration experiments, the *PISA* server (Krissinel & Henrick, 2007) reports that two of the three strongest intermolecular interactions are found in the D_3 entity interface, and the server also classifies the entity as possibly stable in solution. The two interfaces have average areas of 460 and $420 \text{ \AA}^2$, and both involve twofold-symmetric interactions between two CRD3 regions and between two CDR1 regions. This could suggest that the D_3 entity acts as crystallization nuclei. The only large interface responsible for the packing of the D_3 entity has an area of comparable size ($420 \text{ \AA}^2$).

The binding of NB17 to ccKDM5B was indicated by ELISA during phage panning; however, the co-crystallization experiment did not suggest significant interaction. We next undertook a TSA (Niesen *et al.*, 2007), and in agreement with the abovementioned results this did not suggest any stabilization of ccKDM5B on nanobody binding (Fig. 3). In contrast, two separate transitions were clearly visible. The melting

Figure 3
TSA temperature profiles of ccKDM5B (red) and of a mixture of NB17 and ccKDM5B (blue). Two separate transitions are clearly visible in the mixture.

Figure 4
Analytical gel filtration and complex formation. Chromatograms of ccKDM5B, NB17 and a mixture of ccKDM5B and NB17 are coloured red, black and blue, respectively. (a) Chromatography at 298 K after incubation at 278 K for 30 min. No ccKDM5B–NB17 complex formation is observed. (b) Chromatography at 298 K after incubation at 310 K for 30 min. Some ccKDM5B–NB17 complex formation is observed. (c) SDS-PAGE of the corresponding chromatogram peaks. From left: ccKDM5B, 97 kDa; NB17, 13 kDa. Lane 1, NB17 appears to migrate with aggregated species, as do some degraded species, at 310 K. Lane 2, NB17 does not bind to monomeric ccKMD5B. Lane 3, NB17 stock solution (peak 3). Lane 4, at 278 K monomeric ccKDM5B migrates as at 310 K and shows no binding of NB17. Lane *M*, molecular-mass markers (labelled in kDa).

temperature (T_m) of ccKDM5B is 310 K and is decreased by approximately 1 K in the presence of NB17. In order to further investigate the formation of a ccKDM5b–NB17 complex, gel-filtration experiments were performed. 40 μ M ccKDM5B was mixed with 20 μ M NB17 at 278 K for 30 min. At this ratio, and assuming a K_d of 1 μ M or lower, 95% or greater of the nanobody should be bound to ccKDM5B, and consequently no UV peak corresponding to unbound NB17 should be observable. Integration of the UV traces after incubation at 278 K, however, showed no reduction in the peak area at a retention time corresponding to NB17 (Fig. 4a). This indicates that NB17 has very low affinity for ccKDM5B, at least under these conditions. As the TSA assay revealed a melting temperature of around 310 K for ccKDM5B, we decided to investigate the antigen–antibody affinity after incubation at this temperature. Fig. 4(b) shows chromatograms of NB17, ccKDM5B and their combination after incubation for 30 min at 310 K. Notably, the size-exclusion profile of ccKDM5B changes and a higher molecular-weight aggregate is apparent. The retention time of the aggregate is close to that of the void volume of the column. Therefore, it is not possible to conclude anything about the size and homogeneity of the induced soluble aggregates. Combining NB17 and ccKDM5B decreases the areas of the ccKDM5B monomer and NB17 peaks and increases the area and shifts the position of the aggregate peak. Interestingly, the nanobody shifts the position of the aggregate peak towards a lower molecular weight.

Fig. 4(c) shows the corresponding SDS–PAGE of the size-exclusion peaks in the chromatogram of the mixture of KDM5B and NB17. NB17 was detectable in the chromatogram at 5.4 min, but was too dilute in the fractions to be detected on the SDS–PAGE gel. The peak at 2.3 min contains NB17 and aggregated ccKDM5B species. Additionally, there are bands of slightly lower molecular weight than ccKDM5B,

suggesting that both degradation and aggregation is occurring. Notably, NB17 is not evident in the monomeric peak of KDM5B (retention time 3.5 min) in either of the experiments.

These experiments may suggest that temperature-induced ccKDM5B degradation and aggregation exposes patches that are not accessible on monomeric ccKDM5B and that NB17 can bind to these patches. It is clear that NB17 is not suitable for KDM5B co-crystallization experiments. In general, however, understanding the details of the temperature-induced changes will require further studies.

Analytical gel-filtration experiments at 278 K and TSA were also performed on the remaining nanobodies raised against ccKDM5B (Fig. 5). Surprisingly, almost half of the nanobodies did not show any binding after incubation for 30 min. The experiments further show that changes in melting temperature and binding affinity do not appear to correlate.

ELISA and phage panning have previously been shown to induce unfolded or partially denatured states of proteins (Nizak *et al.*, 2005). The findings above emphasize that when identifying conformational binders (such as nanobodies) to relatively unstable intracellular proteins, standard panning and selection experiments may give false-positive results. Therefore, unless panning and selection are performed under conditions (buffer, pH and temperature) that maintain the native conformation of the protein, further characterization of binding affinities is required.

Acknowledgements

We thank MAX-lab for access to beamline 911-3. Access to the synchrotron was supported by DANSCATT (<http://www.danscatt.dk>). The University of Copenhagen program CoNeXT (<http://www.conext.ku.dk>) is acknowledged for financial support. Guillaume Hofmann from the High Throughput Crystallization Laboratory at EMBL, Grenoble is acknowledged for collecting a preliminary data set for NB17.

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Figure 5
 ΔT_m and binding properties of 15 nanobodies raised against ccKDM5B. Temperatures are relative to the ccKDM5B T_m of 310 K. Green indicates binding as determined by analytical gel filtration; red indicates no complex formation. Seven of the 15 nanobodies did not form complexes at 278 K.

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