Yong Liu,^{a,b} Geng Meng,^{a,b} Ming
 Luo^c and Xiaofeng Zheng^{a,b*}

^aState Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing, 100871, People's Republic of China, ^bDepartment of Biochemistry and Molecular Biology, School of Life Sciences, Peking University, Beijing, 100871, People's Republic of China, and ^cDepartment of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Correspondence e-mail: xiaofengz@pku.edu.cn

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Crystallization and X-ray crystallographic analysis of the cap-binding domain of influenza A virus H1N1 polymerase subunit PB2

PB2 is one of the subunits of the influenza virus heterotrimeric polymerase. By its cap-binding domain (PB2_{cap}), PB2 captures the 5' cap of the host pre-mRNA to generate a capped 5' oligonucleotide primer for virus transcription. The crystal structure of influenza A virus H3N2 PB2_{cap} with bound cap analogue m⁷GTP has been reported previously. To show the substrate-free structural details of PB2_{cap} and clarify whether obvious conformational changes exist between the substrate-free and substrate-bound cap-binding domain, we have successfully obtained the crystal of substrate-free H1N1 PB2_{cap}. The crystal of H1N1 PB2_{cap} diffracted to a high resolution of 1.32 Å. The crystal symmetry belongs to space group *P*1 with unit-cell parameters *a* = 29.49, *b* = 37.04, *c* = 38.33 Å, α = 71.10, β = 69.84, γ = 75.85°. There is one molecule in the asymmetric unit.

1. Introduction

The influenza virus is a negative-stranded RNA virus which consists of eight viral genomic RNAs (vRNAs). Transcription and replication of influenza viral vRNA take place in the nucleus of infected cells. The viral RNA-dependent RNA polymerase (RdRp) is composed of PB1, PB2 and PA subunits. The viral messenger RNA (mRNA) is transcribed by the heterotrimeric RdRp using a cap-snatching mechanism (Plotch *et al.*, 1981). After the nucleocapsid–RdRp complexes enter the nucleus following virus entry, the PB2 cap-binding activity is activated through PB1–PB2 interaction and then PB2 captures the 5' cap of the host pre-mRNA (Li *et al.*, 2001; Guilligay *et al.*, 2008; Sugiyama *et al.*, 2009). Subsequently the cap-dependent endonuclease of PA cleaves off the cap together with 10–13 nucleotides downstream of the cap of the pre-mRNA and generates the new short-capped primer for the initiation of viral transcription by PB1 (Yuan *et al.*, 2009; Dias *et al.*, 2009; Jung & Brownlee, 2006).

PB2 captures the cap structure of the host pre-mRNA by the cap-binding domain and residues 318–483 are identified as the cap-binding domain (PB2_{cap}) (Tarendeau *et al.*, 2007; Guilligay *et al.*, 2008). This domain was crystallized with 7-methylguanosine 5'-triphosphate (m⁷GTP), a cap analogue to imitate the 5'-end 7-methyl-guanosine (m⁷G) cap of the host pre-mRNA. Determination of this ligand-bound crystal structure provided the detailed substrate-binding information of the cap-binding pocket in this domain (Guilligay *et al.*, 2008). The conserved PB2 cap-binding pocket shows structural differences in comparison to other human cap-binding proteins, such as eIF4E and nuclear cap-binding complex (Mazza *et al.*, 2002; von der Haar *et al.*, 2004; Marcotrigiano *et al.*, 1997). The structural differences in the cap-binding pocket between PB2_{cap} and human cap-binding proteins provide the basis for designing a new anti-influenza competitive inhibitor targeting viral transcription (Hooker *et al.*, 2003; Hsu *et al.*, 2012).

The reported complex structure of PB2 with bound cap analogue m⁷GTP (PDB code: 2vqz, Guilligay *et al.*, 2008) described the main features of influenza PB2 cap-binding domain and identified the key residues essential for cap binding. To clarify the conformational change of PB2 during the cap-snatching process and provide the

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Figure 1

Amino-acid sequence alignment of PR8-H1N1 PB2_{cap} (labelled as PR8-H1N1) with influenza A/Victoria/3/1975 (H3N2) PB2_{cap} (labelled as 2vqz). The key residues interacting with m⁷GTP are labelled with a blue triangle at the bottom. Sequence alignment was performed using *ESPrpt* (Gouet *et al.*, 1999).

substrate-free structural details of the PB2 cap-binding pocket for drug design, we have cloned the PB2 cap-binding domain (residues 318–483, PB2_{cap}) from influenza A/Hong Kong/1/68 (H3N2) and influenza A/Puerto Rico/8/34 (H1N1), and successfully obtained the substrate-free crystal of PR8-H1N1 PB2_{cap} for the first time. PB2 is highly conserved in all subtypes of influenza A virus. Amino-acid sequence alignment of PR8-H1N1 PB2_{cap} and the reported influenza A/Victoria/3/1975 (H3N2) PB2_{cap} (PDB code: 2vqz) showed that the amino-acid sequence identity is 93.4% and the key residues interacting with m⁷GTP are completely conserved (Fig. 1). Therefore, determination of the substrate-free PB2_{cap} structure will show the conformational change of PB2 during the cap-snatching process and provide more information about the cap-binding pocket as an important drug target.

2. Methods and results

2.1. Protein expression and purification

The gene encoding PR8-H1N1 PB2_{cap} was amplified from the cDNA library of influenza A/Puerto Rico/8/34 (H1N1), digested by *NdeI* and *XhoI*, and then inserted into the pET28a vector (Novagen) with an N-terminal His₆ tag followed by a thrombin protease cleavage site.

The recombinant plasmid was confirmed by DNA sequencing, then transformed into *Escherichia coli* strain Rosetta (DE3) and incubated on a Luria–Bertani broth (LB) agar plate with 50 mg l⁻¹ kanamycin at 310 K overnight. A single transformant was cultured in 50 ml LB medium containing 50 mg l⁻¹ kanamycin overnight and subsequently transferred into 1 l of fresh LB medium. The expression of PR8-H1N1 PB2_{cap} was induced with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside; Sigma) for 16 h at 291 K. The cells were harvested by centrifugation at 5000g for 15 min at 277 K, and the pellet was resuspended and sonicated in sonication buffer consisting of 50 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 mM imidazole. The lysate was centrifuged twice at 40 000g for 30 min at 277 K. The supernatant containing the recombinant protein was applied onto a 5 ml nickel-affinity HiTrap chelating HP column (GE Healthcare) and then eluted in the same buffer with a gradient of 5 to 500 mM imidazole. The fractions of target protein were harvested in 500 mM imidazole, concentrated to 1 ml and then diluted with 10 ml sonication buffer to reduce the imidazole concentration. The diluted frac-

Figure 2

SDS-PAGE analysis of purified PR8-H1N1 PB2_{cap} obtained after a four-step purification (as described in §2) for crystallization.

tion was concentrated to about 2 ml followed by thrombin treatment for 0.5 h at room temperature. A second nickel-affinity purification was performed to remove the free His₆-tag fragment. The protein fraction without the His₆ tag was successfully eluted in buffer containing 50 mM Tris–HCl pH 8.0, 500 mM NaCl and 5 mM imidazole, and concentrated to 1 ml for further purification on a Superdex 75 column (GE Healthcare) which was equilibrated with buffer consisting of 10 mM Tris–HCl pH 8.0, 500 mM NaCl. The peak fraction from the Superdex 75 column was pooled and concentrated, and desalted with a 5 ml HiTrap desalting column (GE Healthcare) to keep the protein in buffer consisting of 10 mM Tris–HCl pH 8.0, 200 mM NaCl. The protein concentration was measured and the final yield was 16 mg l⁻¹ culture with a purity over 99% (Fig. 2).

2.2. Protein crystallization

The purified protein was concentrated to 10 mg ml⁻¹ for crystallization screening. PEG/Ion, Crystal Screen, Crystal Screen 2, Index

Figure 3
(a) Initial screened crystal of PR8-H1N1 PB2_{cap}. (b) Optimized crystal of PR8-H1N1 PB2_{cap} for diffraction data collection.

Figure 4
Diffraction pattern of the PR8-H1N1 PB2_{cap} optimized crystal. The crystal diffracted to a resolution of 1.32 Å.

and Matrix (Hampton Research) were used for initial screening by the sitting-drop vapour-diffusion method at 293 K. Micro-crystals were obtained under the condition consisting of 0.2 M potassium nitrate, 20% (w/v) polyethylene glycol 3350 (Fig. 3a). Further crystal optimization was performed using the hanging-drop vapour-diffusion method at 293 K. The crystal (0.05 × 0.08 × 0.16 mm) that yielded the best diffraction quality was obtained from 0.2 M potassium nitrate, 0.1 M HEPES pH 7.5, 20% (w/v) polyethylene glycol 3350, 8% (v/v) 1,4-butanediol (Fig. 3b).

2.3. Diffraction data collection and analysis

Preliminary X-ray diffraction of the crystal in different conditions was performed at the Beijing Synchrotron Radiation Facility (BSRF,

Table 1

Crystallographic parameters and data-collection statistics of PR8-H1N1 PB2_{cap}.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	0.9795
Resolution (Å)	15–1.32 (1.37–1.32)
Completeness (%)	94 (91.4)
$R_{\text{merge}}^{\dagger}$ (%)	5.1 (11.5)
$\langle I/\sigma(I) \rangle$	28.3 (10.4)
Space group	<i>P</i> 1
Unit-cell parameters (Å)	$a = 29.49$, $b = 37.04$, $c = 38.33$
No. of observed reflections	232216
No. of unique reflections	31393
Molecules per asymmetric unit	1
V_M (Å ³ Da ^{−1})	2.03
Solvent content (%)	39.59

$$^{\dagger} R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl).$$

beamline 1W2B, China). The final data set for structure elucidation was collected on a MAR CCD 225 detector at the Shanghai Synchrotron Radiation Facility (SSRF, beamline BL17U, China).

Crystals were directly flash-cooled in liquid nitrogen and then immersed into a nitrogen stream at 100 K immediately; the crystal-to-detector distance was 120 mm. The complete data set of 720 frames of 1° oscillation was collected at a wavelength of 0.9795 Å (Fig. 4) and processed to 1.32 Å using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The crystal belongs to the *P*1 space group with unit-cell parameters $a = 29.49$, $b = 37.04$, $c = 38.33$ Å, $\alpha = 71.10$, $\beta = 69.84$, $\gamma = 75.85^\circ$. The asymmetric unit contains one molecule. The structure was preliminarily solved by the molecular-replacement (MR) technique using *MOLREP* in the *CCP4* suite (Vagin & Teplyakov, 2010; Winn *et al.*, 2011). The 2.3 Å resolution X-ray structure of influenza A/Victoria/3/1975 (H3N2) PB2_{cap} (PDB code: 2vqz, Guilligay *et al.*, 2008) was used as a search model. The *R*-factor and correlation coefficient of the best MR solution are 0.258 and 0.895, respectively. The detailed data-collection statistics are presented in Table 1.

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References

- Dias, A., Bouvier, D., Crépin, T., McCarthy, A. A., Hart, D. J., Baudin, F., Cusack, S. & Ruigrok, R. W. (2009). *Nature (London)*, **458**, 914–918.
- Gouet, P., Courcelle, E., Stuart, D. I. & Metoz, F. (1999). *Bioinformatics*, **15**, 305–308.
- Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R. W., Ortin, J., Hart, D. J. & Cusack, S. (2008). *Nature Struct. Mol. Biol.* **15**, 500–506.
- von der Haar, T., Gross, J. D., Wagner, G. & McCarthy, J. E. (2004). *Nature Struct. Mol. Biol.* **11**, 503–511.
- Hooker, L., Sully, R., Handa, B., Ono, N., Koyano, H. & Klumpp, K. (2003). *Biochemistry*, **42**, 6234–6240.
- Hsu, J. T., Yeh, J. Y., Lin, T. J., Li, M. L., Wu, M. S., Hsieh, C. F., Chou, Y. C., Tang, W. F., Lau, K. S., Hung, H. C., Fang, M. Y., Ko, S., Hsieh, H. P. & Horng, J. T. (2012). *Antimicrob. Agents Chemother.* **56**, 647–657.
- Jung, T. E. & Brownlee, G. G. (2006). *J. Gen. Virol.* **87**, 679–688.
- Li, M. L., Rao, P. & Krug, R. M. (2001). *EMBO J.* **20**, 2078–2086.
- Marcotrigiano, J., Gingras, A. C., Sonenberg, N. & Burley, S. K. (1997). *Cell*, **89**, 951–961.
- Mazza, C., Segref, A., Mattaj, I. W. & Cusack, S. (2002). *EMBO J.* **21**, 5548–5557.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Plotch, S. J., Bouloy, M., Ulmanen, I. & Krug, R. M. (1981). *Cell*, **23**, 847–858.
- Sugiyama, K., Obayashi, E., Kawaguchi, A., Suzuki, Y., Tame, J. R., Nagata, K. & Park, S. Y. (2009). *EMBO J.* **28**, 1803–1811.
- Tarendeau, F., Boudet, J., Guilligay, D., Mas, P. J., Bougault, C. M., Boulo, S., Baudin, F., Ruigrok, R. W., Daigle, N., Ellenberg, J., Cusack, S., Simorre, J. P. & Hart, D. J. (2007). *Nature Struct. Mol. Biol.* **14**, 229–233.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Yuan, P., Bartlam, M., Lou, Z., Chen, S., Zhou, J., He, X., Lv, Z., Ge, R., Li, X., Deng, T., Fodor, E., Rao, Z. & Liu, Y. (2009). *Nature (London)*, **458**, 909–913.