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Crystallization and preliminary crystallographic analysis of a viral RNA-silencing suppressor encoded by *Wuhan nodavirus*

Wuhan nodavirus (WhNV), which is a new member of the *Nodaviridae* family, encodes a viral protein, B2, that suppresses RNA silencing and host-cell RNA interference (RNAi)-mediated immunity. Although *Flock House virus* (FHV), another member of the *Nodaviridae* family, also produces a B2 protein with a similar function, the primary sequences of the B2 proteins from WhNV and FHV have no similarity. To gain a better understanding of the structural details and the mechanism of suppression of RNA silencing by WhNV B2 and to compare it with FHV B2, recombinant WhNV B2 protein has been overexpressed in *Escherichia coli*, purified and crystallized at 291 K using PEG 4000 as a precipitant. A 2.8 Å resolution data set has been collected from a single crystal at 100 K. This crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 27.3$, $b = 45.6$, $c = 133.9$ Å, $\alpha = \beta = \gamma = 90^\circ$. Assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient is $2.2 \text{ Å}^3 \text{ Da}^{-1}$.

1. Introduction

RNA interference (RNAi) plays an essential role in the cellular response to viral infection (Ding & Voinnet, 2007). In virus-infected cells, the viral genome and its double-stranded RNA replication intermediates can be processed by the RNase III enzyme Dicer into small interfering RNAs (siRNAs), which are 19–21 bp long with 5'-phosphates and 3' two-nucleotide overhangs. These siRNAs are then used to guide the RNA-induced silencing complex (RISC) to specifically degrade the viral genome.

To protect the virus against RNAi-mediated immunity, viruses encode viral suppressors of RNA silencing (VSRs) that target different components in the RNAi machinery. However, the molecular mechanism of this process is still unclear. Previous results revealed that nodaviruses encode a viral protein that acts as a VSR (Aliyari *et al.*, 2008; Galiana-Arnoux *et al.*, 2006; Li *et al.*, 2002). Nodaviruses are nonenveloped icosahedral viruses that have bipartite positive-sense RNA genomes, among which RNA1 encodes protein A (ProA), the catalytic subunit of the RNA-dependent RNA polymerase (RdRp), and RNA2 encodes coat precursor protein α (Pro α), which undergoes an autocatalytic maturation cleavage into two viral capsid proteins, β (Pro β) and γ (Pro γ) (Cai *et al.*, 2010). During virus replication, viral RdRp directs the production of an additional subgenomic RNA3 to encode one or two small nonstructural proteins: proteins B1 and B2 (Cai *et al.*, 2010).

Flock house virus (FHV) is a prototypic member of the *Nodaviridae* family. After infection, FHV encodes a 106-residue protein B2, a potent suppressor of RNA silencing, to protect FHV against host defence mediated by Dicer (Aliyari *et al.*, 2008). B2 protein is synthesized at high levels early in the viral replication cycle and helps FHV to escape the host immune defence (Li *et al.*, 2002). The crystal structure of the FHV B2 dimer with dsRNA revealed that FHV B2 forms a helix-bundle structure and binds tightly (K_d value of 1 nM) to dsRNA in a sequence-independent manner through interacting exclusively with the ribose-phosphate backbone of two successive minor grooves and the intervening major groove of the bound 18 bp dsRNA (Chao *et al.*, 2005). This structure revealed that FHV B2 blocks both cleavage of the FHV genome by Dicer and incorporation

Figure 1
Crystals of WhNV B2 grown in 25% PEG 4000, 200 mM ammonium acetate, 50 mM Tris-HCl pH 8.0.

of FHV siRNAs into the RNA-induced silencing complex (Chao *et al.*, 2005).

Wuhan nodavirus (WhNV) is a newly identified member of the *Nodaviridae* family which is isolated from the major economic pest *Pieris rapae* (Cai *et al.*, 2010). Sequence analysis of the WhNV genome shows that it is different from those of all known nodaviruses (Cai *et al.*, 2010). In particular, the B2 protein encoded by WhNV shows no primary sequence homologies to other reported nodaviral B2 proteins. Moreover, further functional analysis revealed that WhNV not only sequesters dsRNA but also directly contacts the RNase III and Piwi-Argonaut-Zwille (PAZ) domains of Dicer-2 (Dcr-2) and siRNA (Qi *et al.*, 2012). The binding of WhNV B2 to Dcr-2 blocks the activities of Dcr-2 in processing dsRNA and incorporating siRNA into the RISC.

To further elucidate the molecular mechanism of the suppression of RNAi by WhNV B2 and to compare the B2 proteins from WhNV and FHV, we performed crystallization and preliminary crystallographic analysis of WhNV B2 protein.

2. Materials and methods

2.1. Protein expression and purification

The gene encoding WhNV B2 protein was chemically synthesized and then inserted into the expression vector pGEX-6p-1 with *Bam*HI and *Xho*I restriction sites following a general protocol. The recombinant plasmid was transformed into *E. coli* strain BL21 (Rosetta 2) and the transformed cells were cultured at 310 K in Luria-Bertani broth medium containing 0.1 g l⁻¹ ampicillin. When the OD₆₀₀ reached 0.6, the culture was cooled to 289 K and expression of the recombinant protein was induced by supplementation with 1 mM isopropyl β-D-1-thiogalactopyranoside. After overnight induction, the cells were harvested by centrifugation. The cells were then resuspended in PBS buffer consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.5 followed by homogenization by sonication at 277 K. Insoluble material was removed by centrifugation at 20 000g. The fusion protein was first purified by GST affinity chromatography and treated with PreScission protease at 277 K overnight. The eluted target protein was collected and further purified using a Superdex 200 gel-filtration column (GE Healthcare, USA) in a buffer consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl. The purified WhNV B2 protein was >95% pure according to SDS-PAGE analysis. The purified protein was concentrated to 10 mg ml⁻¹ and stored at 193 K.

2.2. Crystallization

Crystallization was performed at 291 K using the hanging-drop vapour-diffusion technique. Crystals were obtained by mixing 1.5 μl protein solution with an equal volume of reservoir solution and equilibrating the drop against 500 μl reservoir solution. Initial trials

Figure 2
A typical diffraction pattern of the WhNV B2 crystals.

Table 1

Data-collection and processing statistics for WhNV B2.

Values in parentheses are for the outermost resolution shell.

Unit-cell parameters	
<i>a</i> (Å)	27.3
<i>b</i> (Å)	45.6
<i>c</i> (Å)	133.9
$\alpha = \beta = \gamma$ (°)	90.0
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Wavelength (Å)	1.0000
Resolution (Å)	50.00–2.80 (2.85–2.80)
Total No. of reflections	26883 (1320)
No. of unique reflections	4230 (189)
Completeness (%)	100.0 (99.9)
Average <i>I</i> / σ (<i>I</i>)	14.9 (5.6)
<i>R</i> _{merge} [†] (%)	7.8 (29.3)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the observations $I_i(hkl)$ of reflection hkl .

were carried out using the commercial crystallization screen kit Wizard 1 and 2 from Emerald BioSystems (USA). After optimization, large stick-shaped crystals with good diffraction quality were obtained from 25% (w/v) PEG 4000, 200 mM ammonium acetate, 50 mM Tris–HCl pH 8.0 and reached final dimensions of 50 × 50 × 400 µm after 7 d growth (Fig. 1).

2.3. X-ray diffraction analysis

The crystal was soaked in cryoprotectant consisting of 40% PEG 4000, 200 mM ammonium acetate, 50 mM Tris–HCl pH 8.0 and then mounted on a nylon loop for cooling and storage in liquid nitrogen. Native diffraction data for WhNV B2 were collected at 100 K using an ADSC Q270 CCD detector on beamline BL17A at the Photon Factory (PF), Japan. A total of 360 frames of diffraction were

collected with 0.5° oscillation range (Fig. 2). The intensity data were indexed, integrated and scaled with the *HKL-2000* package (Otwinowski & Minor, 1997). The crystal belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 27.3, *b* = 45.6, *c* = 133.9 Å, $\alpha = \beta = \gamma = 90^\circ$. We assume that there are two molecules in the asymmetric unit, which gives a Matthews coefficient (Matthews, 1968) of 2.2 Å³ Da^{−1} with 42% solvent content considering the 9 kDa molecular weight of the WhNV B2 protein. The final statistics of data collection and processing are summarized in Table 1.

3. Results and discussion

Initial needle-shaped microcrystals appeared after 7 d in a reservoir solution consisting of 50 mM Tris–HCl pH 8.0, 200 mM ammonium acetate, 30% (w/v) PEG 4000. We then performed several rounds of optimization, including changing variables such as temperature, pH, protein concentration, salt and PEG 4000 concentration, in 240 droplets. The results revealed that pH values in the range 6.5–8.5 and salt concentrations from 100 to 400 mM had a slight impact on crystal growth. However, the crystals were very sensitive to the PEG 4000 concentration. Large stick-shaped crystals with good diffraction quality were obtained from 25% (w/v) PEG 4000, 200 mM ammonium acetate, 50 mM Tris–HCl pH 8.0 and reached final dimensions of 50 × 50 × 400 µm after 7 d (Fig. 1). Additive screens and detergent screens from Hampton Research were also introduced to gauge their effect on crystal quality, but no significant effect could be observed.

Before data collection, we tested numerous cryoprotectants (*e.g.* PEG 4000, glycerol, sugar, oil *etc.*) and tried different methods of introducing the cryoprotectant into the crystal (*e.g.* directly moving the crystal into cryoprotectant, a gradient transfer of the crystal to the final cryoprotectant and equilibration against the final cryoprotectant). The crystals of WhNV B2 only survived in a cryoprotectant consisting of 40% PEG 4000, 200 mM ammonium acetate, 50 mM Tris–HCl pH 8.0.

To solve the structure of WhNV B2, we would ideally like to use the molecular-replacement (MR) method. However, sequence alignment gave no obvious homologue of WhNV B2. Since FHV is similar to WhNV and also produces a B2 protein as an RNA-silencing suppressor (Qi *et al.*, 2012), it is conceivable that WhNV B2 may share structural similarity with FHV B2, although their primary sequence identities are lower than 10%. Therefore, we performed MR with the crystal structure of FHV B2 (PDB entry 2az2; Chao *et al.*, 2005) in both monomeric and dimeric forms as initial search models using *Phaser* (McCoy *et al.*, 2007). Unfortunately, we could not obtain a correct MR solution according to the very low rotation and translation *Z*-scores (the rotation *Z*-score is lower than 2.1). Homodimerization was revealed to be required for the biological function of WhNV B2 (Qi *et al.*, 2012) and the structure of FHV B2 was also solved in a dimeric form (Chao *et al.*, 2005). However, we could not observe an obvious peak corresponding to a noncrystallographic twofold axis in the self-rotation function (Fig. 3). The structure of WhNV B2 is necessary to elucidate the mechanism for its dimerization and function. Heavy-atom and selenomethionine derivatization of WhNV B2 are under way in order to obtain the correct phases.

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Figure 3

$\kappa = 180^\circ$ section of the self-rotation function calculated for the WhNV B2 native data set using *MOLREP* (Vagin & Teplyakov, 2010) with an integration radius of 22.7 Å and data in the resolution range 40.0–2.9 Å.

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