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Crystallization and X-ray diffraction of virus-like particles from a piscine betanodavirus

Dragon grouper nervous necrosis virus (DGNNV), a member of the genus *Betanodavirus*, causes high mortality of larvae and juveniles of the grouper fish *Epinephelus lanceolatus*. Currently, there is no reported crystal structure of a fish nodavirus. The DGNNV virion capsid is derived from a single open reading frame that encodes a 338-amino-acid protein of approximately 37 kDa. The capsid protein of DGNNV was expressed to form virus-like particles (VLPs) in *Escherichia coli*. The VLP shape is $T = 3$ quasi-symmetric with a diameter of ~ 38 nm in cryo-electron microscopy images and is highly similar to the native virion. In this report, crystals of DGNNV VLPs were grown to a size of 0.27 mm within two weeks by the hanging-drop vapour-diffusion method at 283 K and diffracted X-rays to ~ 7.5 Å resolution. In-house X-ray diffraction data of the DGNNV VLP crystals showed that the crystals belonged to space group $R32$, with unit-cell parameters $a = b = 353.00$, $c = 800.40$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. 23 268 unique reflections were acquired with an overall R_{merge} of 18.2% and a completeness of 93.2%. Self-rotation function maps confirmed the fivefold, threefold and twofold symmetries of the icosahedron of DGNNV VLPs.

1. Introduction

After *in situ* observations in a fish hatchery in Kyushu, Japan for five years, Mori *et al.* (1992) published a report on a new piscine virus from infected larvae of striped jack (*Pseudocaranx dentex*). The virus was named *Striped jack nervous necrosis virus* (SJNNV) according to the symptoms of the disease caused. In 2001, the International Congress of Virology officially classified the piscine nodaviruses to be a new genus of *Betanodavirus*, in order to distinguish them from *Alphanodavirus*, which infects insects, although in Asia *Betanodavirus* was previously known as piscinodavirus (Munday *et al.*, 2002). *Betanodavirus* has been characterized as a non-enveloped, icosahedral, positive-stranded RNA virus. The genome of *Betanodavirus* is bipartite: RNA1 is presumed to encode the viral replicase and RNA2 encodes the capsid protein. The RNA2 of 1390 nucleotides (nt) contains a single open reading frame (ORF) which encodes a 338-amino-acid protein of approximately 37 kDa for the virion capsid.

Based upon partial sequences (T4 region) of RNA2 from more than 20 *Betanodavirus* RNA2 segments, Nishizawa *et al.* (1997) divided betanodaviruses into four groups: SJNNV, *Tiger puffer nervous necrosis virus* (TPNNV), *Barfin flounder nervous necrosis virus* (BFNNV) and *Redspotted grouper nervous necrosis virus* (RGNNV). Sequence alignment of dragon grouper nervous necrosis virus (DGNNV; isolated from *Epinephelus lanceolatus* in Taiwan; GenBank AF245004.1) showed that the RNA2 was 95% identical to those of RGNNV and Singapore greasy grouper nervous necrosis virus (GGNNV; Lin *et al.*, 2001). The RNA2 of DGNNV was 99% identical to that of MGNNV (malabar grouper nervous necrosis virus; GenBank AF245003.1).

Capsid ORFs in the RNA2 of DGNNV and MGNNV have been expressed to form virus-like particles (VLPs) in insect (*Spodoptera frugiperda* Sf21) cells and *Escherichia coli* JM109 cells (Lin *et al.*,

2001; Lu *et al.*, 2003). The DGNNV and MGNNV VLPs are non-enveloped, icosahedral and $T = 3$ quasi-symmetric (Lin *et al.*, 2001; Tang *et al.*, 2002; Lu *et al.*, 2003); the VLPs from the two viruses were structurally indistinguishable from the native virus particles based upon their binding affinity to a fish cell line (Liu *et al.*, 2006). The three-dimensional structure of the MGNNV VLPs was determined at a resolution of 23 Å from single-particle reconstruction images by cryo-electron microscopy (cryo-EM; Tang *et al.*, 2002). Prominent protrusions are clearly visible at the quasi-threefold symmetry axes. In cryo-EM, the maximum diameters of DGNNV (C.-H. Wang & C.-S. Lin, unpublished work) and MGNNV (Tang *et al.*, 2002) are approximately 380 Å, which is significantly larger than the particles in traditional EM with negative staining, because the protrusions from the shell surface are thin and indistinguishable from the background in negative-stained images. The inner shell is dense. Compared with the cryo-EM structure of the insect nodavirus *Pariacoto virus* (PaV; PDB entry 1f8v; Tang *et al.*, 2001), the interior space defined by the DGNNV and MGNNV is large enough to contain the genomic RNAs.

No crystal structure of a fish nodavirus is currently available. One reason is the difficulty in preparing pure concentrated samples of these viruses for crystallogenesis. However, we can produce VLPs in sufficient quantities and concentrations for structural studies. The aim of this study was to grow crystals of DGNNV VLPs for X-ray diffraction in order to resolve the structure of the capsid protein at the atomic level. The structure of a fish nodavirus VLP will lead to greater understanding of viral assembly and infection.

2. Materials and methods

2.1. Purification of DGNNV VLPs

The plasmid pDA8 expressed DGNNV capsid protein to form VLPs that were purified as described elsewhere (Lu *et al.*, 2003). The pDA8-transformed *E. coli* JM109 cells were grown in 1 l of LB broth at 303 K containing 100 mg l⁻¹ ampicillin. When the cell density reached an optical density (OD_{600 nm}) of 0.2–0.3, a final concentration of 0.84 mM IPTG was added for induction. After induction for 4 h, the cells were harvested by centrifugation at 5150g for 20 min at 277 K. The cell pellet from a 1 l culture was resuspended in 30 ml 10 mM Tris–HCl pH 8.0 buffer (Amresco, USA) containing 0.5% (w/v) Triton X-100 (Sigma–Aldrich, USA) for 1 h at 277 K. The cells were broken by passing through a French press three times (Avestin Emulsiflex-C5). Cell debris was removed by centrifugation at 39 500g for 40 min at 277 K (Hitachi CR20B2). The supernatant was ultracentrifuged, with a cushion of 5 ml 20–35% (w/w) sucrose, at 141 000g for 3.5 h at 277 K in a swinging-bucket rotor (Hitachi SRP28SA). The VLP pellet was resuspended in 0.5 ml 10 mM Tris–HCl pH 8.0 at 277 K overnight. After sucrose gradients had been prepared using a gradient maker (BioComp Gradient Master 107ip), the VLP solution was gently layered onto 5 ml of a 10–40% (w/w) sucrose density gradient and centrifuged at 300 000g for 1 h at 277 K (Hitachi RPS55T-2). The fractions were collected from the top of the gradient by displacing the gradient from the bottom of the centrifuge tube with Fluorinert FC-40 Electronic Liquid (3M), with continuous monitoring of the absorbance at 254 nm, and fractions were collected in quantities of 150 µl per tube. A small aliquot of each fraction was analyzed by SDS–PAGE. The fractions containing VLPs were diluted fivefold with buffer to reduce the density of sucrose in the solution and were centrifuged at 300 000g for 1 h at 277 K; the VLP pellet was then resuspended in 50 µl 10 mM Tris–HCl pH 8.0. The resulting VLPs were ultracentrifuged once more in a 10–40% (w/w) sucrose gradient. To verify purity and homogeneity, purified DGNNV VLPs

were analyzed with a 10% (w/v) SDS–PAGE gel. Only one prominent band (about 37 kDa) was observed.

2.2. Electron microscopy

For electron microscopy of negatively stained particles, 10 µl VLPs were applied to carbon-coated grids and left for 1 min. After being washed three times with 10 µl 10 mM Tris–HCl pH 8.0, the samples were then rinsed twice with freshly prepared 2% (w/v) uranyl acetate (Sigma–Aldrich, USA) in 10 mM Tris–HCl pH 8.0 and stained for 90 s. The grid was blotted with a filter paper and then dried under vacuum overnight. Specimens were examined using a 200 kV electron microscope (Jeol JEM-2100, equipped with a Gatan-832 CCD camera) and images were taken at a magnification of 40 000× with a defocus of 3 µm.

2.3. Crystallization

Over the past few years, we have made numerous attempts to crystallize DGNNV VLPs. Initial crystallization trials of DGNNV VLPs were performed using the hanging-drop vapour-diffusion method with either conventional or alternative reservoirs (Newman, 2005; Dunlop & Hazes, 2005). In order to find DGNNV VLP crystallization conditions, we surveyed 74 conditions that have been employed to crystallize other viruses, including some conditions from the VIPERdb website (<http://viperdb.scripps.edu>). Among the crystallization conditions for viruses, the salts found predominantly are ammonium sulfate, lithium sulfate, NaCl and CaCl₂; frequently used PEGs are PEG 3350, PEG 6000 and PEG 8000, and the buffers are Tris–HCl and HEPES. The salt concentrations are in the range 0.001–3.3 M, PEG concentrations are 0.05–24% (w/v) and buffers are at concentrations of 0.01–0.5 M with pH 3–9.5. We surveyed variations of these conditions with concentrations of purified DGNNV VLPs ranging from 5 to 50 mg ml⁻¹. The best results achieved were microcrystalline precipitates.

We also designed in-house screens suitable for crystallizing DGNNV VLPs using the sitting-drop vapour-diffusion method in 24-well Cryschem plates (Hampton Research) at 277–293 K. In all cases (sitting and hanging drops) 2 µl DGNNV VLP solution was mixed with 2 µl reservoir solution. The commercial kits included PEG/Ion (Hampton Research), PEG 3350 II (Qiagen), The PEGs II Suite (Qiagen) and SaltRx (Hampton Research). The initial crystallization trials were performed at 277 K using the hanging-drop vapour-diffusion method. The protein concentration of DGNNV VLPs used was 20 mg ml⁻¹. Conditions 7, 20, 25 and 28 of the PEG/Ion kit gave a few small crystal-like solids in the middle of brown precipitates after four weeks (Supplementary Table S1¹). The PEG effects on the crystallization were further studied using the PEG 3350 II and The PEGs II Suite (Supplementary Table S1). Condition No. 47 of the PEG 3350 II kit resulted in needle crystals of 0.1 mm in length, while condition No. 69 gave small diamond-shaped crystals. Small crystals from DGNNV VLPs were observed under the condition Nos. 31, 32, 35 and 56 of The PEGs II Suite. The crystals of DGNNV VLPs obtained using condition Nos. 31, 35 and 56 were 0.2 mm long and diamond-shaped, while those obtained using condition No. 32 were much smaller. It was noted that condition Nos. 31 and 35 also contained lithium sulfate. Some microcrystalline precipitates from DGNNV VLPs were observed under condition Nos. 38, 39, 42 and 45 of the SaltRx kit (Supplementary Table S1). The crystals of DGNNV VLPs obtained using condition Nos. 42 and 45 were 0.1 mm in length

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: RL5072).

Table 1
Optimum conditions for crystallizing DGNNV VLPs.

Name	Crystallization reagent composition	Crystallization description	Crystal size (mm)	Resolution† (Å)
LS	0.2 M lithium sulfate, 13%(w/v) PEG 4000, 0.1 M Tris-HCl pH 8.5	Small rhombohedral crystals	0.1–0.2	50.00–16.00
AN	2.3 M ammonium nitrate, 13%(w/v) PEG 4000, 0.1 M Tris-HCl pH 8.5	Small rhombohedral crystals	0.1–0.2	50.00–16.00
ASNC	0.3 M ammonium sulfate, 13%(w/v) PEG 4000, 0.1 M Tris-HCl pH 8.5, 2 mM CaCl ₂ , 1.9 M ammonium nitrate	Long rhombohedral crystals	0.1–0.2	50.00–16.00
AS	0.3 M ammonium sulfate, 13%(w/v) PEG 4000, 0.1 M Tris-HCl pH 8.5, 2 mM CaCl ₂ , 10%(v/v) glycerol	Rhombohedral crystals	0.22–0.27	7.85–7.50

† The diffraction images were collected under cryogenic conditions.

and diamond-shaped, while those obtained using condition Nos. 38 and 39 were much smaller. This result suggested that ammonium nitrate was also a useful salt for DGNNV VLP crystallization.

We had found previously that divalent ions under alkaline conditions stabilized the assembly of subunits into icosahedral VLPs (Wu *et al.*, 2008; Wang *et al.*, 2010). These additives were tested in an attempt to improve the crystal quality and quantity. Adding divalent metal ions to the crystallization conditions for DGNNV VLPs revealed that alkaline earth metals at 2 mM, including MgCl₂, CaCl₂, SrCl₂ and BaCl₂, shortened the crystallization course by about 3 d, while the effects of transition elements such as MnCl₂, CoCl₂, NiCl₂ and ZnCl₂ varied the crystal sizes from 0.1 to 0.4 mm (Supplementary Fig. S1).

To optimize the crystallization conditions for DGNNV VLPs, we employed the combinatorial design method using different concentrations of ingredients in which the VLPs can form acceptable crystals: 10–40 mg ml^{−1} for VLP, 8–20%(w/v) for PEG, 0.1–0.3 M for ammonium sulfate and lithium sulfate, 1.5–6 M for ammonium nitrate and 0–60 mM for divalent ions in Tris-HCl pH 8–9. The optimal concentration of VLPs was in the range 20–30 mg ml^{−1}. For the PEG precipitant agents, the concentrations were in the range of 7.5–10%(w/v) for PEG 8000, 10–15%(w/v) for PEG 4000 and

15–20%(w/v) for PEG 1500 in 0.3 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5. In PEG 8000 and PEG 10 000, the protein formed spherulite-like clusters. When the VLP concentration was 40 mg ml^{−1}, the PEG 4000 concentration needed to be reduced to 8%(w/v). Therefore, we chose 13%(w/v) PEG 4000 as the appropriate concentration for VLP concentrations of 20 mg ml^{−1}.

When the pH was less than pH 7, DGNNV VLPs would not form crystals. At pH 7–7.5, the crystal shape was irregular. When the pH was between pH 8 and 8.5, the crystals formed large diamonds. Trials of temperature showed that at 277 K crystals grew over a period of 7 d into irregular crystals, and at 283 K better crystals were produced but more slowly over 14 d. X-ray diffraction data also showed that crystals grown at 283 K were of the highest quality.

X-ray diffraction under cryogenic temperature is a commonly used method because it can decrease the damage to proteins caused by free radicals, which undermines the internal protein structure (Garman & Doublé, 2003; Pflugrath, 2004). To avoid ice crystals, glycerol, PEG 400 and PEG 600 were selected as antifreeze agents, and the X-ray diffraction results showed that 10%(v/v) glycerol was a suitable cryoprotectant. The crystals under the conditions outlined in Table 1 were preliminarily tested for X-ray diffraction at cryogenic temperature.

Figure 1
Crystals of DGNNV VLPs. (a) When grown in a reservoir solution consisting of 0.3 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5, 2 mM CaCl₂, 10%(v/v) glycerol, 13%(w/v) PEG 4000 by vapour diffusion, the crystals have a longest dimension of 0.27 mm. The scale bar represents 100 µm. (b) Mounting the cryoloop onto the goniometer to maintain the crystal under cryoconditions at 100 K. The scale bar represents 100 µm. (c) Diffraction images of DGNNV VLPs. The X-ray diffraction pattern was obtained with a Rigaku FR-E SuperBright X-ray generator using 30 s exposure and 0.2° oscillation range. X-ray diffraction was performed under cryogenic conditions.

Crystals of many viruses, such as *Foot-and-mouth disease virus* (FMDV) and *Satellite tobacco necrosis virus* (STNV), diffract to high resolution at room temperature (Fox *et al.*, 1987; Fridborg *et al.*, 1965), indicating that noncryogenic conditions are sometimes promising in viral crystallography. To prepare crystals for room-temperature *in situ* diffraction screening, an 80 mm quartz capillary (Hampton Research; catalogue No. HR6-132) was trimmed to 30 mm. A 3 μ l volume of the DGNNV VLPs was siphoned into shortened quartz capillaries with internal diameters ranging from 0.1 to 1 mm, and 48 μ l of the precipitant solutions shown in Table 1 were

layered over the top. The quartz capillary ends were sealed using superglue. These counter-diffusion experiments were performed at 277 K. Crystals in capillaries can be directly transferred onto the goniometer at the synchrotron, which may prevent crystal deformation when they are picked from a hanging drop.

2.4. X-ray data collection and analysis

Initially, crystals of DGNNV VLPs were analyzed on an in-house Rigaku FR-E SuperBright microfocus X-ray generator (Cu $K\alpha$,

Figure 2 Diffraction images of DGNNV VLPs. (a) A typical X-ray diffraction pattern from a crystal of DGNNV VLPs obtained in a hanging drop. The diffraction images were collected under cryogenic conditions. The oscillation range was 1°. (b) An enlarged image of the area indicated in (a). (c) A typical X-ray diffraction pattern from a crystal of DGNNV VLPs in a quartz capillary. The diffraction images were collected at room temperature. (d) An enlarged image of the area indicated in (c). The crystals were grown in 0.3 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5, 2 mM CaCl_2 , 13% (w/v) PEG 4000, 10% (v/v) glycerol at 277 K.

$\lambda = 1.54178 \text{ \AA}$) equipped with an R-Axis HTC area detector and an X-stream cryohead (Rigaku MSC). X-ray diffraction data were collected from crystals that had been cryoprotected with 10% (v/v) glycerol in the precipitant solution containing 13% (w/v) PEG 4000. Crystal-to-detector distances of 450 mm were used to record 0.2° oscillation images at exposure times of 30 s. The crystals were mounted in a cryoloop (Hampton Research) and immediately flash-cooled in a cold nitrogen-gas stream maintained at 100 K. The measured diffraction intensities were indexed, integrated, scaled and merged with the *HKL-2000* suite of programs (Otwinowski & Minor, 1997), and preliminary analysis employed the *CCP4* program suite (Winn *et al.*, 2011). Data at higher resolution were collected using synchrotron radiation on SPXF beamlines BL13B1 and BL13C1 at the NSRRC, Taiwan and SP12B2 at SPring-8, Japan. The beamlines were equipped with an ADSC Q315r CCD detector, a Rayonix MX300HE CCD detector and a MAR Research Mar345 image-plate detector, respectively.

3. Results and discussion

3.1. Screening for crystallization conditions

The single capsid polypeptide from DGNNV RNA2 had previously been cloned and expressed to form VLPs (Lu *et al.*, 2003). Attempts to crystallize DGNNV VLPs using 74 virus crystallization conditions described in the literature, including those used for insect nodaviruses, did not result in good crystals, only microcrystalline-like precipitates. After examining the conditions resulting in precipitates, we found that salts and PEG were crucial. Therefore, we employed commercial kits containing a variety of salts and PEGs to screen the crystallization conditions of the DGNNV VLPs. The results of these trials suggested that the optimal molecular weight of PEG was in the range 1500–8000, and that lithium sulfate, ammonium nitrate and ammonium sulfate were suitable salts. The salts lithium sulfate, ammonium sulfate and ammonium nitrate were also factorially combined in various concentrations to improve the crystal quality. The four optimal conditions for crystallizing DGNNV VLPs are summarized in Table 1. The crystals from ammonium nitrate and lithium sulfate solutions resulted in low-resolution X-ray diffraction images, with the best diffraction being from DGNNV VLP crystals grown at 283 K using a precipitant consisting of 0.3 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5, 2 mM CaCl_2 , 13% (w/v) PEG 4000, 10% (v/v) glycerol (Figs. 1a and 1b).

3.2. Diffraction of DGNNV VLP crystals

A total of 299 useful images were collected from 60° of in-house diffraction for a single VLP crystal with an oscillation angle of 0.2° and an exposure time of 30 s per image (Fig. 1c). This crystal of recombinant DGNNV VLPs diffracted X-rays to $7.5 \text{ \AA}$ resolution. The data were indexed in a rhombohedral crystal system, which was suggested by autoindexing in the *HKL-2000* package (Otwinowski & Minor, 1997). Diffraction data statistics and DGNNV VLP crystal parameters are given in Table 2. In-house data were processed and the crystal belonged to space group *R*32, with unit-cell parameters $a = b = 353.00$, $c = 800.40 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$. The 60 035 observed reflections were reduced to 23 268 unique reflections with an overall R_{merge} of 18.2% and a completeness of 93.2%.

The synchrotron diffraction data ($50\text{--}7.85 \text{ \AA}$; Figs. 2a and 2b) also showed that the crystal belonged to space group *R*32, with unit-cell parameters $a = b = 352.468$, $c = 801.445 \text{ \AA}$. 122 638 observed reflections were reduced to 38 941 unique reflections with an overall R_{merge}

Table 2

Data-processing statistics for DGNNV VLP crystals.

	In-house data	Synchrotron data	
		Low resolution	High resolution
Space group	<i>R</i> 32	<i>R</i> 32	<i>R</i> 32
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = b = 353.00$, $c = 800.40$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 352.47$, $c = 801.45$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 357.74$, $c = 807.39$, $\alpha = \beta = 90$, $\gamma = 120$
No. of observations	60035	122638	4983
Unique reflections	23268	38941	4776
Resolution ($\text{\AA}$)	50.00–7.50 (7.77–7.50)	50.00–7.85 (8.08–7.85)	30.00–4.00 (4.07–4.00)
$R_{\text{merge}}^\dagger$ (%)	18.2 (53.1)	17.1 (43.8)	15.8 (0)
Multiplicity	2.6	3.1	1.0
$R_{\text{p.i.m.}}^\ddagger$ (%)	14.4 (46.6)	11.8 (33.6)	‡
$\langle I \rangle / \langle \sigma(I) \rangle$	7.2 (2.6)	9.0 (2.8)	2.1 (0.2)
Completeness (%)	93.2 (91.9)	94.4 (41.1)	2.9 (0.1)

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$ and $R_{\text{p.i.m.}} = \frac{\sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity for all measurements including symmetry equivalents. ‡ The $R_{\text{p.i.m.}}$ is not yet available for the partially complete high-resolution data.

of 17.1% and a completeness of 94.4%. Statistics are also given in Table 2.

We also tried *in situ* X-ray diffraction at room temperature. For these tests, crystals of DGNNV VLPs were grown in quartz capillaries using counter-diffusion (Supplementary Fig. S2). The best diffracting crystals were obtained with a reservoir solution consisting of 0.3 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5, 2 mM CaCl_2 , 10% (v/v) glycerol, 13% (w/v) PEG 4000. Under this condition, the crystals grew to a maximal dimension of about 0.3 mm within two weeks. The diffraction data showed that the crystal belonged to space group *R*32, with unit-cell parameters $a = b = 357.740$, $c = 807.385 \text{ \AA}$. *In situ* data collection from capillary-grown crystals is still ongoing (4776 unique reflections were observed and only 2.9% of the data has been collected so far), but is promising for solution of the atomic structure of DGNNV, as these crystals show diffraction to $\sim 4 \text{ \AA}$ resolution (Table 2; Figs. 2c and 2d). Some of the conditions described here will be applicable to other betanodaviruses.

3.3. Analysis of crystal diffraction for the icosahedral symmetry of the DGNNV

Self-rotation function maps of in-house X-ray data were calculated using *POLARFN* from the *CCP4* suite, with spherical angles $\kappa = 72$, 120 and 180° for fivefold, threefold and twofold symmetry axes, respectively (Fig. 3). The map calculated with $\kappa = 90^\circ$ did not show significant peaks, indicating a lack of fourfold symmetry. The particles appear to have a uniform orientation in the crystal. From previous results, DGNNV VLPs are approximately $380 \text{ \AA}$ in diameter with an $\sim 80 \text{ \AA}$ protrusion. Assuming the particles to be nearly spherical with a diameter of $380 \text{ \AA}$, the volume of one particle will be about $28\,716\,300 \text{ \AA}^3$. The unit-cell volume of the *R*32 cell was $86\,377\,400 \text{ \AA}^3$. It is most likely that two VLPs were present per unit cell because the volume of $86\,148\,900 \text{ \AA}^3$ for three VLPs may be very tight to place in the lattice cell. The volume per molecular weight (V_M) would be 6.48 or $4.32 \text{ \AA}^3 \text{ Da}^{-1}$ for two and three viruses, respectively (Matthews, 1968); these are higher than the value of $3.91 \text{ \AA}^3 \text{ Da}^{-1}$ for *Flock house virus* crystals (PDB entries 4ftb and 4fsj; J. A. Speir, Z. Chen, V. S. Reddy & J. E. Johnson, unpublished work), indicating that a greater solvent content was present in the DGNNV crystals (Fisher *et al.*, 1992).

Figure 3

Self-rotation function maps of a DGNNV VLPs crystal. The maps were calculated using *POLARFN* from the *CCP4* suite using in-house X-ray diffraction data in the resolution range 20–8.0 Å. (a)–(d) show maps with spherical angles of $\kappa = 72, 90, 120$ and 180° for fivefold, fourfold, threefold and twofold symmetry, respectively.

In summary, we can produce DGNNV VLPs in sufficient quantity and grow crystals for X-ray diffraction. To resolve the structure of the capsid protein at the atomic level, we used suitable cryoprotectants and collected high-resolution diffraction data at synchrotron facilities or collected data from crystals grown *in situ* at room temperature. In the next step, at room temperature or under cryoconditions, the X-ray diffraction data from DGNNV VLPs with selenomethionine replacement will be used to determine phases for an atomic model. The MGNNV cryo-EM reconstruction maps can also be used as a search model for X-ray crystal structure determination by molecular replacement *via* cycling density averaging, phase extension and solvent flattening.

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