Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary X-ray analysis of immunogenic virus-like particles formed by infectious bursal disease virus (IBDV) structural protein VP2

Cheng-Chung Lee,[a,b] Tzu-Ping Ko,[b] Meng-Shiou Lee,[a] Chia-Cheng Chou,[b] Su-Yuan Lai,[c] Andrew H.-J. Wang[b]† and Min-Ying Wang[a]*†

[a]Graduate Institute of Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan, [b]Institute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan, and [c]Department of Food Science, Chungtai Institute of Health Sciences and Technology, Taichung 40605, Taiwan

† AHJW and MYW contributed equally to the supervision of this work.

Correspondence e-mail: mywang@dragon.nchu.edu.tw

Received 1 December 2002
Accepted 15 April 2003

Infectious bursal disease virus (IBDV) causes a highly contagious disease in young chicks and leads to significant economic losses in the poultry industry. VP2 protein, which consists of 452 amino-acid residues, is the primary immunogen of IBDV and contains the epitopes responsible for eliciting neutralizing antibodies. When the chimeric VP2 protein (rVP2H) of a local IBDV strain P3009 was expressed alone using the baculovirus system, virus-like particles of approximately 23 nm in diameter formed spontaneously. Highly pure rVP2H particles, obtained using ammonium sulfate precipitation, immobilized metal-ion affinity chromatography and gel-filtration chromatography, were successfully crystallized using the vapour-diffusion method. These crystals, with a maximum dimension of 0.4 mm, diffracted X-rays to 4.5 Å resolution, but data were only collected to 6 Å. Preliminary analysis of the diffraction data showed that the rVP2H crystals belong to the cubic space group $P2_13$, with unit-cell parameter 323.1 Å. The icosahedral symmetry of the particles is clearly seen in the self-rotation function maps, with dyads and triads coincident with the crystallographic axes. Each asymmetric unit contains 1/3 of the particle, or 20 rVP2H subunits, and there are four particles in a unit cell, probably in a tetrahedral arrangement.

1. Introduction

The infectious bursal disease virus (IBDV) is the cause of an immunosuppressive disease in chickens that leads to mortality and causes significant economic losses in the poultry industry. The disease destroys the precursors of antibody-producing B cells in Fabricius' bursa and results in severe immune system depression (Narajan & Kibenge, 1995; Saif, 1998). IBDV belongs to the genus *Avibirnavirus* of the *Birnaviridae* family, which is characterized by two double-stranded RNA segments (Dobos *et al.*, 1979). The proteins VP2 and VP3 of apparent molecular masses 41 and 32 kDa, respectively, are derived from a 115 kDa polyprotein precursor encoded by the larger RNA segment (Hudson *et al.*, 1986). For the maturation of VP2, the polyprotein first generates a precursor protein, pVP2; this is then further processed to yield VP2. Although the cleavage of the polyprotein is well known, the maturation mechanism of VP2 and VP3 has only recently been delineated (Sanchez & Rodriguez, 1999; Lejal *et al.*, 2000; Chevalier *et al.*, 2002). The proteins VP2 and VP3 as well as peptides derived from pVP2 constitute the viral capsid, which has a diameter of 60–65 nm (Azad *et al.*, 1987; Jagadish *et al.*, 1988; Da Costa *et al.*, 2002).

VP2 protein is the primary host-protective immunogen and contains the epitopes responsible for eliciting the neutralizing antibodies (Azad *et al.*, 1987; Becht *et al.*, 1988; Fahey *et al.*, 1989; Heine *et al.*, 1991). Structural analysis of the IBDV virion showed that VP2 subunits are displayed as 260 trimeric clusters of outwardly protruding structures (Böttcher *et al.*, 1997). VP2 protein alone spontaneously forms particles of 20–26 nm in diameter (Wang *et al.*, 2000). A recent study by Castón *et al.* (2001) using cryoelectron microscopy showed that VP2 particles have dodecahedral morphology with a topography similar to that of wild-type IBDV. Because the immunogenicity of the VP2 particle is similar to that of the whole IBDV virion, elucidation of the structure of the VP2 particle will facilitate the design of alternatives to live attenuated IBDV vaccines against IBDV infection.

In a previous investigation, a chimeric recombinant IBDV P3009 (a local strain) VP2 protein with six extra histidine residues at the C-terminus (rVP2H) was successfully expressed using the baculovirus expression system and the particles formed by rVP2H protein were shown to be highly immunogenic (Wang *et al.*, 2000). We have recently obtained highly pure rVP2H particles by a combination of immobilized metal-ion affinity chromatography (IMAC) and gel-filtration chromatography (Cheng *et al.*, 2001). In this work, we crystallized the purified rVP2H particles and studied the higher order structure of these

immunogenic rVP2H particles using X-ray crystallography.

2. Materials and methods

2.1. Preparation of rVP2H particles

rVP2H particles were expressed in High-Five cells (Invitrogen, Carlsbad, CA, USA) infected with a recombinant baculovirus, vP3009VP2H-5, as described previously (Cheng *et al.*, 2001). Cell pellets were collected from the growth medium and resuspended in a native binding buffer of 20 m*M* NaH_2PO_4, 0.5 *M* NaCl pH 7.8. The suspension was sonicated and centrifuged to remove the cell debris. Solid ammonium sulfate was added to the resulting cell lysate to a final concentration of 10% saturation. Following centrifugation, the pH value of the supernatant was adjusted to 7.8 and the supernatant was loaded onto a column (C 16 × 20, Amersham Pharmacia Biotech, Piscataway, NJ, USA) packed with 50 ml Ni^{2+}–NTA–Superflow agarose (Qiagen, Hilden, Germany). The column was washed with the native binding buffer and a similar wash buffer at pH 6.3. The bound proteins were then eluted with the same buffer adjusted to pH 4. The eluate was collected, concentrated and injected into a self-packed column (C 16 × 100) of Sepharose CL-6B resin (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The column was eluted with a buffer consisting of 20 m*M* NaH_2PO_4, 0.5 *M* NaCl, 0.1% NaN_3 pH 6.5. Fractions containing rVP2H particles were identified using the ELISA assay as described previously (Cheng *et al.*, 2001) and positive fractions were further checked using SDS–PAGE. The selected 'peak' fractions were then pooled and concentrated for subsequent use.

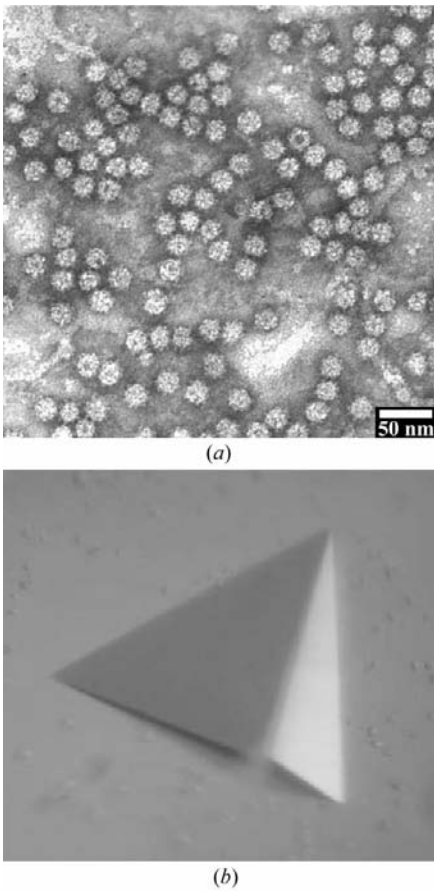

Figure 1
Pictures of rVP2H particles and rVP2H-particle crystals. In (*a*), the particles were stained with uranyl acetate and examined under an electron microscope. The size of the particle is about 23 nm in diameter. (*b*) is a photograph of the crystal used in data collection. The maximal dimension of the tetrahedron is about 0.4 mm.

2.2. Electron microscopy

The method of direct observation through negative staining was performed as described previously (Ho *et al.*, 1999). Briefly, 300-mesh grids covered with Formvar/carbon membranes were incubated with rVP2H particles in phosphate buffer pH 8. Following decantation, the grids were floated on an aqueous solution of 5% uranyl acetate to achieve contrast. After decantation and drying, the grids were observed through a transmission electron microscope (Jeol JEM 1200 EX-2) with a voltage acceleration of 120 kV.

2.3. Crystallization of rVP2H particles

The initial crystallization screening was performed with Hampton Research Crystal Screens (Laguna Niguel, CA, USA) using the hanging-drop vapour-diffusion method. In general, 2 µl of solution containing rVP2H particles was mixed with 2 µl of reservoir solution and the mixture was maintained at 300 K, while the reservoir contained 200 µl 0.1 *M* 2-(*N*-morpholino)-ethanesulfonic acid (MES) pH 6.5, with a precipitant concentration of 8–14% PEG 20 000. Crystallization was carried out with rVP2H particle concentrations of between 3 and 10 mg ml^{-1}. The preliminary crystallization conditions were optimized further by variation of the PEG size (8000–20 000), pH (5.0–7.0) and ionic strength (0.05–0.25 *M* NaCl) in order to obtain crystals suitable for subsequent diffraction analysis. The crystal was scooped up in a cryoloop and flash-frozen in liquid nitrogen with the addition of 21% glucose as a cryoprotectant. It was then mounted on the goniometer head in a nitrogen stream at 100 K.

2.4. Data collection and analysis

Preliminary X-ray diffraction experiments were carried out with an R-AXIS IV image-plate detector (Molecular Structure Corporation, The Woodlands, TX, USA) using Cu $K\alpha$ radiation generated by a Rigaku RU-300 rotating-anode generator. Data at higher resolution were collected using synchrotron radiation and an ADSC Quantum 4 CCD camera at BL12B2 Taiwan beamline at SPring-8, Japan. Data were processed using the *HKL* software package (Otwinowski & Minor, 1997) and preliminary analysis employed the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. Crystallization

To obtain highly pure rVP2H particles, a purification process that included ammonium sulfate precipitation, IMAC and gel filtration was applied. As shown in Fig. 1(*a*), the purified rVP2H particles have a uniform morphology and are 20–25 nm in diameter. The regular compact arrays of rVP2H particles suggest the possible success of their crystallization *in vitro*. In addition, rVP2H particles were found to be very stable and maintained their morphology for up to three months in 20 m*M* NaH_2PO_4, 0.5 *M* NaCl, 0.01% NaN_3 pH 6.5 buffer at room temperature. Therefore, this protein solution was used for crystallization screening of rVP2H particles. A reservoir solution containing 12% PEG 20 000, 0.1 *M* MES pH 6.5 was the most favourable condition for obtaining crystals of rVP2H particles that were suitable for X-ray diffraction analysis, although crystals could also be obtained with other precipitants. In general, crystals grew to their maximal sizes within four weeks. Fig. 1(*b*) shows a photograph of a typical crystal. After crystallization, electron micrographs of redissolved rVP2H crystals also showed that the morphology of rVP2H particles did not change (data not shown), indicating that the integrity of the particles was maintained in the crystals.

3.2. Data collection and analysis

rVP2H-particle crystals with a maximum dimension of 0.4 mm were used in diffraction experiments. The crystals diffracted X-rays to higher than 4.5 Å resolution, but owing to the large size of the unit cell the intensities were only measured to 6 Å resolution. The wavelength of the synchrotron radiation used was 1.28 Å and the crystal-to-detector distance was 210 mm. The exposure

Table 1
Data-collection statistics for rVP2H-particle crystals.

Values in parentheses are for the highest resolution shell.

Space group	$P2_13$
Unit-cell parameter (Å)	323.12
Resolution range (Å)	50.0–6.0 (6.21–6.00)
No. of observations	433433 (43296)
Unique reflections	28251 (2815)
Completeness (%)	99.8 (100.0)
Average $I/\sigma(I)$	24.4 (8.8)
$R_{\rm merge}$ (%)	13.7 (39.8)

time was 60 s per frame. The oscillation angle of each frame was 0.50° and a total of 251 frames were recorded. Preliminary processing of the rVP2H-particle diffraction data suggested that the crystal has a primitive cubic unit cell with parameters $a = b = c = 323.1$ Å. The systematic absence of odd axial reflections indicated the space group to be $P2_13$ rather than $P23$. The data-collection statistics are summarized in Table 1.

Fig. 2 shows the self-rotation function map sections for twofold, threefold and fivefold symmetry. The map calculated with $\kappa = 90°$ does not show significant peaks, indicating a lack of fourfold symmetry. Apparently, the particles have a uniform orientation in the crystal. From previous results, rVP2H particles are approximately 200–250 Å in diameter. Assuming the particles to be nearly spherical, with a diameter of 225 Å, each particle will occupy about 6 000 000 Å^3. The cubic unit-cell volume is 33 700 000 Å^3. Based on crystallographic symmetry constraints, packing consideration suggests that the unit cell contains four rVP2H particles in a tetrahedral arrangement, with their centres placed at (1/4, 1/4, 1/4) and symmetry-related equivalents. The face diagonal of the unit cell is about 458 Å, just large enough to accommodate two particles. The tetrahedral arrangement of particles inside the crystal may be correlated with the morphology of the crystal (Fig. 1*b*). In such a case, rVP2H particles occupy about 70% of the unit-cell volume and each asymmetric unit contains 1/3 of the icosahedral viral particle or 20 rVP2H subunits. On the other hand, a specific volume of 3.42 Å^3 Da^{-1} suggests that the solvent content of the crystal is about 64%. Therefore, about 34% of the unit-cell volume corresponds to the central cavities of the particles, each having a volume of 2 850 000 Å^3 and a diameter of about 176 Å, consistent with the results of analysis by cryo-EM (Castón *et al.*, 2001).

Molecular-replacement searches with other viral particles of similar sizes, including *Galleria mellonella* densovirus (PDB 1dnv) and *Escherichia coli* phage MS2 (PDB 1e7x), did not yield satisfactory results. Calculation of initial phase angles by construction of a spherical model and 20-fold non-crystallographic symmetry averaging are in progress. For phasing by anomalous dispersion, we have also produced selenium derivatives of the rVP2H protein, which contains ten methionine residues. A more detailed model of the viral capsid will provide a better account for the immunogenic characteristics of IBDV and these can be employed in improving vaccine production.

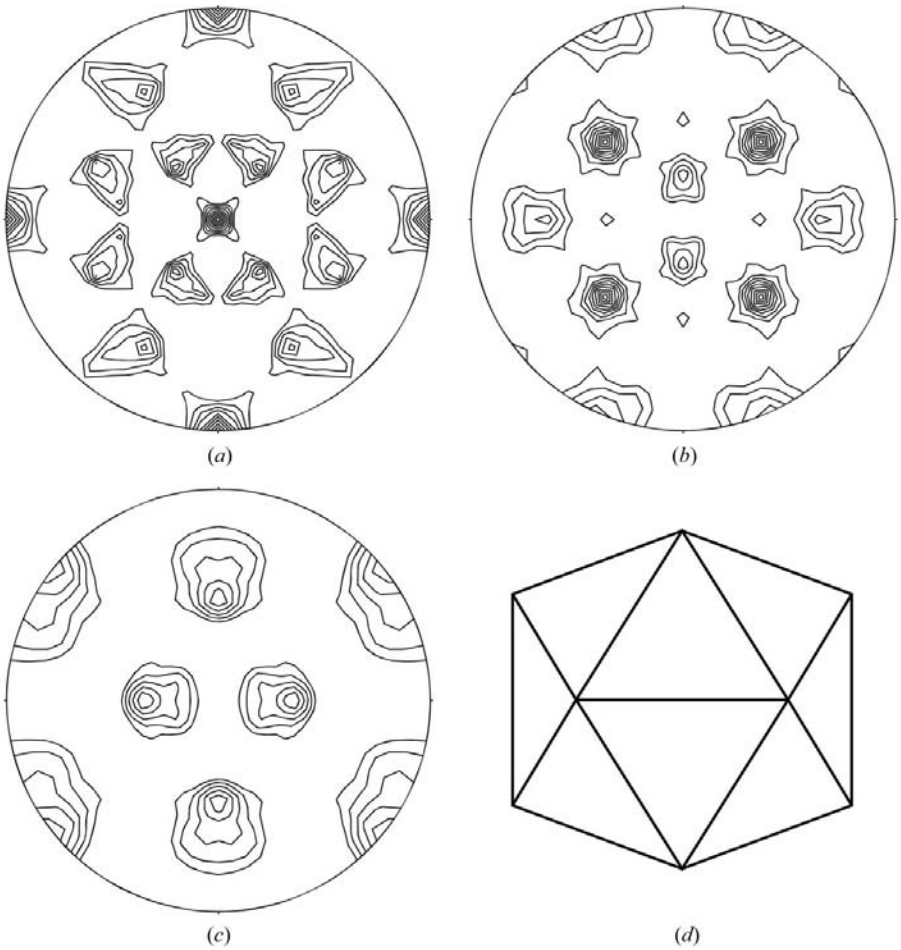

Figure 2
Self-rotation function maps of a rVP2H-particle crystal. The maps were calculated using the program *POLARRFN* from the *CCP*4 suite, using all data in the resolution range 50–6.0 Å. (*a*), (*b*) and (*c*) show maps with spherical angles of $\kappa = 180$, 120 and 72° for twofold, threefold and fivefold symmetry, respectively. The contour levels are at 10% steps from 10% to 100% height of the crystallographic symmetry. (*d*) shows a diagram of the icosahedron properly oriented to match the symmetry elements of the particle in the crystal.

This work is based upon research conducted at the National Synchrotron Radiation Research Center (NSRRC), Taiwan, which is supported by the National Science Council (NSC) of the Republic of China, Taiwan, using the Biological Crystallography Facility at SRRC (BioSRRC). The authors especially thank Dr Mau-Tsu Tang for his kind help with data collection at SPring-8 and Professor Chih-Ning Sun (Department of Entomology, National Chung-Hsing University) in reviewing this manuscript. This research was supported by grants from the NSC (NSC 89-2317-B-005-016 and NSC 90-2317-B-005-001 to MYW, NSC91-3112-P-001-020-Y to AHJW) and Academia Sinica to AHJW.

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