Details of the Arrangement of the Outer Capsid of Rice Dwarf Phytoreovirus, as Visualized by Two-Dimensional Crystallography

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Two-dimensional crystals were obtained from purified P8, an outer capsid protein of rice dwarf phytoreovirus. A filtered image of the two-dimensional crystal, in combination with the results of biochemical analysis, revealed the unit formation of the capsid protein, a capsomere structure, which appeared to be an approximately equilateral triangle with sides of approximately 6 nm and which was composed of a trimer of P8 protein. Details of the arrangements of the outer capsid of the virus are described.

Rice dwarf virus (RDV) is a phytoreovirus that is a member of the family Reoviridae (4). It exists as icosahedral double-shelled particles 693 Å in diameter (9, 20). The core particle of the virus contains 12 segments of double-stranded RNA and four species of protein. The core is enclosed by an outer capsid layer composed of P2 and P8 proteins (21). The P2 minor outer capsid protein was recently shown to be essential for infection (21). The P8 major outer capsid protein also appears to be associated with infection, since neutralizing antibodies can be raised against this protein (9a). The appearances of viral particles with and without the P2 protein are indistinguishable (21), an observation that suggests that the basic morphological organization of the outer capsid of the virus depends on the arrangement of the P8 protein. The three-dimensional structure of virus particles suggests that the outer capsid of RDV is composed of 260 capsomer units (trimers), which consist of 780 protein subunits (8). From calculations of the volumes of unit structures, it has been proposed that capsomers are trimers of the P8 (46-kDa) protein (10). Detailed structural analysis of the virus is important if we are to understand the molecular organization of the particle. Such an understanding will provide insights into, for example, the molecular mechanisms of the entry of the virus into cells and the assembly of viral particles. Our eventual goal is the design and implementation of antiviral strategies, such as the generation of transgenic plants that express critical polypeptide domains which can function as protein-binding regions. We report here the assembly in a two-dimensional (2D) crystal of purified P8 protein and the arrangement of the capsid in the virus particle.

The O strain of RDV (5) was purified as described by Omura et al. (11). Infected rice leaves were macerated with a meat chopper, clarified by treatment with CCl4, and subjected to differential centrifugation and consecutive density gradient centrifugation in 10 to 40% and 40 to 60% sucrose. The final pellet after high-speed centrifugation of the materials in the band of viral particles was suspended in a 0.1 M solution of histidine that contained 0.01 M MgCl2, pH 6.2 (His-Mg).

High concentrations of MgCl2 have been used to remove P8 protein from intact RDV particles (19). The preparation of viral particles was adjusted to 0.8 M MgCl2 in His-Mg, incubated for 5 min at room temperature, and centrifuged for 10 min at 200,000 × g at 4°C in a model TL-100 rotor (Beckman, Palo Alto, Calif.). The supernatant contained P8 and a very small amount of P3 protein (Fig. 1B, lane 2; the band of P3 was too faint to be seen in this figure).

The protein was further purified by gel filtration fast-protein liquid chromatography on a column of Superdex 200 HR10/30 (Pharmacia, Uppsala, Sweden) in 0.8 M MgCl2 in His-Mg. Fractions corresponding to the major peak of protein (Fig. 1A) were pooled and concentrated with an Amicon diafiltration unit with a 10-kDa normal molecular mass cutoff membrane (Centricon; Amicon, Beverly, Mass.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified preparations on a 10% acrylamide gel (7) yielded a single band (Fig. 1B, lane 3).

For crystallization of the P8 protein, the concentration of MgCl2 in the preparation of P8 protein was varied by dialysis against different target concentrations of MgCl2. Purified P8 protein in 0.8 M MgCl2 was dialyzed against 0.4, 0.2, or 0.1 M MgCl2; or against distilled water at 4°C for 2 days. The resulting solutions were transferred to microtubes and stored on ice. Precipitation of P8 protein occurred at concentrations of MgCl2 below 0.1 M.

For observations of details of the molecular structure of P8, we dropped samples of purified protein onto carbon-coated 400-mesh copper grids for electron microscopy. Samples were negatively stained with 1% uranyl acetate and examined with an electron microscope (model JEM1010; JEOL, Tokyo, Ja-
operated at 100 kV. Images of crystals were recorded at a magnification of \(100,000\). The images of negatively stained crystals were digitized with a Leaf Scan-45 densitometer (a Scitex Company, Herzlia, Israel) and were processed on a DEC/Alpha 3000/400 (Maynard) with computer programs essentially as described by Henderson et al. (3). Uniform arrangements of a small-hole lattice were observed in noise-filtered images (Fig. 2). The capsomere, the unit formation of the capsid protein, resembled an equilateral triangle with sides of approximately 6 nm and a central hole roughly 2 nm in diameter. Two capsomeres were included in one lattice cell whose constant was about 11 nm. Six triangular capsomeres formed a hexagonal cavity about 5 nm in diameter. These cavities might be candidates for a path by which solutes can enter and viral mRNA can leave the virus particle without any treatment (6), as has been proposed for the core particles of rotavirus (12).

The surface lattice of the 2D crystals was similar to those observed on virus particles (8, 10, 20). Furthermore, the 2D crystals were formed under the same conditions as those under which virus particles can be reconstructed from their core and outer capsid components (19). These observations suggest that a local lattice of P8 in an intact RDV particle is not very different from that in our 2D crystals.

A triangular capsomere with a small central hole can be formed reasonably from three units. Such a model, together with calculations of volume (10), indicates that the capsomere is a trimer of the P8 protein. To confirm that the P8 protein has the capacity to form trimers, the material used for 2D crystallization that has been dialyzed against 0.1 M MgCl\(_2\) was subjected to SDS-PAGE in a 10% polyacrylamide gel. A faint band of a protein with an estimated molecular mass of 140 kDa, in addition to the band of P8, was detected (data not shown). This band was recognized specifically by antiserum against the P8 protein (Fig. 3), a result that indicated that the 140-kDa protein consisted of three 46-kDa P8 proteins. The fact that the 140-kDa protein was generated from material that has eluted as a single peak during gel filtration also indicated that the 140-kDa protein originated from monomers of the P8 protein or that the P8 protein was the product of dissociation of the 140-kDa protein. In any case, it is clear that P8 has the ability to form trimers.

To characterize in further detail the features of the P8 trimer, we treated samples with 2-mercaptoethanol (2-ME) or 6 M urea as described by Sabara et al. (17). Because the band of the 140-kDa protein was too weak to be recognized upon analysis of urea-treated protein that had been obtained from a peak fraction after gel filtration, purified viral particles without P2 (21) were subjected to the analysis. The sample buffers contained the following components: (i) 0.0625 M Tris-HCl (pH 6.8), 4% SDS, and 5% 2-ME; (ii) 0.0625 M Tris-HCl (pH 6.8) and 4% SDS; and (iii) 0.0625 M Tris-HCl (pH 6.8), 4% SDS, and 6 M urea. A new band of a protein of 140 kDa that was not detected in the presence of 2-ME was observed in the presence of 6 M urea (Fig. 4). The results were identical when

![FIG. 1. Purification of the P8 protein of RDV. (A) The P8-containing supernatant fraction (see panel B, lane 2) was applied to a column of Superdex 200 HR 10/30 (Pharmacia). The fractions in the peak were collected, concentrated, and used as P8 protein. (B) Analysis by SDS-PAGE (10% polyacrylamide) of RDV particles (lane 1), the supernatant fraction collected after centrifugation of MgCl\(_2\) (0.8 M)-treated RDV that had been purified after treatment with CCl\(_4\) (P2 free) (lane 2), and P8 protein after it was purified by gel filtration (lane 3). Molecular masses (in kilodaltons [K]) of the component proteins of RDV are based on values deduced from nucleotide sequence analysis (18).](http://jvi.asm.org/)

![FIG. 2. (A) Fourier noise-filtered, magnified view of an array, which is displayed in gray levels, with the protein shown as black areas. A capsomere is outlined by short dashes. The area corresponding to one face of a virus particle as shown by Lu et al. (8) is surrounded by long dashes. (B) Contour map of part of the array shown in panel A. Bars, 11 nm.](http://jvi.asm.org/)
samples were boiled in the same solutions for 3 min at 100°C (data not shown). The 140-kDa protein reacted with the antiserum against the P8 protein on a Western blot (data not shown). All these results demonstrate that the unit structure of the outer capsid of RDV is composed of trimers of P8 protein linked by disulfide bonds. This result is consistent with the data that RDV is composed of capsomeres, which consist of three protein subunits (8). In all viruses that belong to the family Reoviridae and have been studied to date, the second layers of the virus particles are all composed of trimers. Thus, μ1, a major outer capsid protein of reovirus (2); VP6, a major protein of the single-shelled rotavirus (13, 14); and VP7, a core surface protein of bluetongue virus (16), all form trimers. The packaging of the symmetrical clusters in the trimer of RDV P8 protein is distinct from that of VP6 of rotavirus, whose clusters are packaged asymmetrically when they are assembled in vitro (1, 15). The morphological and biological implications of this common structure in reoviruses that infect plants, insects, and mammals remain to be clarified.

The area surrounded by dashes in Fig. 2A corresponds to one face of a virus particle (T=13), shown in a reconstructed three-dimensional figure of RDV (8). P8 capsid protein by itself did not form icosahedral particles under our conditions. Therefore, it seems that the triangular clusters need another protein to support interactions between them.

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REFERENCES


FIG. 3. Immunochemical of the crystallized P8 protein. The molecular mass markers used were myosin (H chain; 200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (25.7 kDa). The position of P8, a 46-kDa protein, is indicated by an arrowhead labeled P8. The 140-kDa protein that reacted with the antiserum is indicated by the other arrowhead.

FIG. 4. Protein profiles of (P2-free) RDV after SDS-PAGE (10% polyacrylamide). Lanes: 1, preparation of virus treated with 2-ME-containing buffer; 2, preparation of virus treated with buffer without 2-ME; 3, preparation of virus treated with urea-containing buffer. The components of each buffer are given in the text. A new band is indicated by an arrowhead on the right. Positions of virus component proteins are shown on the left (refer to Fig. 1).