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# Atomic force microscopy investigation of a chlorella virus, PBCV-1

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## Abstract

A virus PBCV-1, which infects certain fresh water algae and has been shown by transmission and cryo-electron microscopy to exist as a triskaidecahedron, was imaged using atomic force microscopy (AFM). From AFM the particles have diameters of about 190 nm and the overall structure is in all important respects consistent with existing models. The surface lattice of the virion is composed of trimeric capsid proteins distributed according to p3 symmetry to create a honeycomb arrangement of raised edges forming quasi-hexagonal cells. At the pentagonal vertices are five copies of a different protein forming an exact pentagon, and this has yet another unique protein in its center. The apical protein exhibits some unusual mechanical properties in that it can be made to retract into the virion interior when subjected to AFM tip pressure. When PBCV-1 virions degrade, they give rise to small, uniform, spherical, and virus like particles (VLP) consistent with  $T=1$  or 3 icosahedral products. Also observed upon disintegration are strands of linear dsDNA. Fibers of unknown function are also occasionally seen associated with some virions.

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**Keywords:** Virus like particles; Degradation; Virus structure; Algae; Probe microscopy

## 1. Introduction

*Paramecium bursaria* chlorella virus type 1 (PBCV-1) is the type member of a large group of viruses (genus Chlorovirus, family Phycodnaviridae) that infect certain unicellular, eukaryotic, chlorella-like green algae, and are common in freshwater bodies worldwide (Van Etten, 2000). PBCV-1 is structurally similar to the large, icosahedral, iridoviruses (Simpson et al., 2003; Van Etten, 2003). The multilayered PBCV-1 virions are ~190 nm in diameter and have a lipid bilayered membrane located inside an outer glycoprotein capsid (Yan et al., 2000). Over 50 proteins are detected in highly purified, protease treated PBCV-1 particles by one-dimensional SDS-PAGE (Skrdla et al., 1984); SDS-PAGE combined with tandem mass spectrometry has identified ~125 virus

encoded proteins that are associated with PBCV-1 virions (Dunigan, Cerny, and Van Etten, unpublished results). A 54 kDa surface glycoprotein, Vp54, accounts for ~40% of the total protein mass (Skrdla et al., 1984). Its linear 330 kb dsDNA genome contains ~375 protein-encoding, non-overlapping genes and 11 tRNA genes; the genome termini consist of 2.2 kb identical, inverted repeats with 35 incompletely base paired hairpin ends (Van Etten, 2003). PBCV-1 infects its host, *Chlorella* NC64A, by rapidly attaching to the algal cell wall, possibly through a unique vertex, releases cell wall degrading enzymes, which breaks down the wall at the attachment point, and subsequently releases viral DNA into the cell (Meints et al., 1984).

Ultrastructural studies have been conducted on both intact and disrupted PBCV-1 virions using either negative staining (Becker et al., 1993) or cryo-electron microscopy with three-dimensional image reconstruction (Yan et al., 2000). This latter study was completed and extended by X-ray structure determination

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of the major capsid protein Vp54 and by fitting the protein model to the cryo-electron microscopy density maps. These results are detailed elsewhere (Nandhagopal et al., 2002; Simpson et al., 2003), along with the pertinent molecular biological properties of the virus (Van Etten, 2003) and will not be reiterated. However, certain features of the virus structure are particularly relevant to the work presented here and warrant comment.

PBCV-1 is icosahedral and can be deconstructed into triangular and pentagonal plates known as trisymmetrons and pentasymmetrons (Simpson et al., 2003) which are centered on the three- and fivefold axes, respectively. The symmetrons have an outer glycoprotein shell composed almost entirely of 1680 ( $h=7$ ,  $k=8$ ) pseudo-hexagonal, closely packed, trimeric capsomeres resulting in a  $T$  value of 169 (Yan et al., 2000). The capsid is constructed from 20 trisymmetrons and 12 pentasymmetrons. The trisymmetrons are triangular assemblies of 66 trimeric proteins (capsomeres) with approximate local  $p3$  plane-group symmetry, and have 11 capsomeres, along each edge. Adjacent trisymmetrons are related by icosahedral twofold axes that produce discontinuities of the  $p3$  lattice at the boundaries. The pentasymmetrons consist of five triangular faces, each with six trimeric capsomeres (Nandhagopal et al., 2002; Simpson et al., 2003).

The cryo-electron microscopy study indicates that the trimeric capsomeres have doughnut shapes with depressions in the centers and outside diameters of 7 nm (Yan et al., 2000). Each pentasymmetron has a pentavalent capsomer at its center that is likely to consist of a homopentamer of a unique protein; this protein(s) may be responsible for viral attachment and entry into host cells.

The atomic force microscopy (AFM) study undertaken here is intended to complement the electron microscopy and X-ray diffraction analyses that preceded it. AFM has several features that distinguish it from other approaches. With AFM, virus particles can be visualized in appropriate buffers that preserve structure over extended periods of time, however, because of the softness and deformability of the PBCV-1 virions, it was necessary to fix them with glutaraldehyde. Most important, AFM is not dependent on symmetry averaging as is cryo-electron microscopy, nor are particles or proteins physically averaged as they are when crystallized. Thus, AFM can reveal eccentricities of individual particles and their unusual defects and structural anomalies. AFM yields three-dimensional images and, unlike transmission electron microscopy, not projections of whole particles onto a plane. The resolution of AFM images is very good in the vertical direction, less than a nanometer, the most problematic direction for electron microscopy, however, the lateral resolution in this study is probably no better than 2–3 nm. While internal features of the virions cannot be seen directly with AFM, some features are revealed by examination of damaged or disrupted particles and their contents.

## 2. Materials and methods

Purified PBCV-1 samples were prepared as previously described (Van Etten et al., 1983) and stored in 50 mM Tris-HCl, pH 7.8 at 4 °C. The methods employed for AFM visualization of PBCV-1 are essentially the same as previously described for the analyses of HIV (Kuznetsov et al., 2003), MuLV (Kuznetsov et al., 2004), and vaccinia virus (Malkin et al., 2003). After appropriate dilution into fresh buffer, virions were spread on poly-L-lysine coated glass coverslips. Particles were then fixed with 0.5% glutaraldehyde for 30 min. Virions were also postfixed with  $\text{OsO}_4$ , but this addition did not improve the images. Specimens adhering to the glass cover slips after brief rinsing with buffer were mounted on a J-piezoscanner of a Nanoscope IIIa atomic force microscope (Digital Instruments, Santa Barbara, CA) and imaged under buffer. Oxide-sharpened silicon nitride tips were used in the 75  $\mu\text{l}$  fluid cell. AFM images were collected in tapping mode (Hansma and Hoh, 1994; Hansma et al., 1994) at frequencies of about 9.2 kHz with a scanning frequency of 1 Hz. The forces employed were similar to those used in previous virus studies.

Because of finite tip dimensions, isolated objects protruding above the substrate plane in AFM images appear broader than their true dimensions. For this reason, the dimensions of spherical and cylindrical objects are quantitated according to their heights above the background surface (Kuznetsov et al., 2001). The dimensions of objects in closely packed aggregates or arrays, particularly symmetrical arrays, can be evaluated from center to center distances.

## 3. Results

### 3.1. The virion shape

Fig. 1 contains low magnification images of PBCV-1 spread on mica in buffer after mild fixation with glutaraldehyde. The triangular faces and pentagonal vertices of the virions are suggested even here, consistent with the proposed model based on cryo-electron microscopy. The particles have a vertical height of 175 nm which implies compression of about 8%, and exhibit widths of 200 nm. The latter dimension is about 5% larger than deduced by other techniques and is exaggerated due to the convolution of the cantilever tip shape with that of the particle. In Fig. 1, however, center-to-center distances between particles in condensed masses are 195 nm and better agree. The particles are somewhat soft and readily deformed, even when fixed with glutaraldehyde, and this was immediately evident from the response of the AFM tip in contact with the particles. This is also apparent in the particle distortion observed in close packed arrays as in Figs. 1C and D. The softness of the

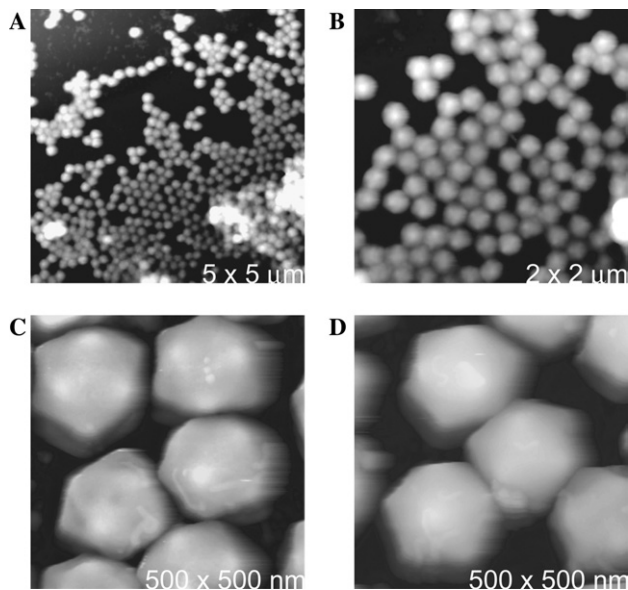


Fig. 1. In (A) through (D) are AFM images of PBCV-1 spread on poly-L-lysine coated glass cover slips and maintained under buffer. The virions are seen at increasing magnification. Clusters of PBCV-1 spread on mica show that some virions undergo compression and distortion under pressure from neighbors. This is only one indication of the softness and deformability of the particles. The large triangular faces and pentagonal vertices of the triskaidecahedron are evident.

virions along with the inclinations of the triangular faces made imaging somewhat problematic, nonetheless, with persistence, satisfactory images were obtained.

As magnification increases, the surface structure of the virions begins to emerge as illustrated in Fig. 2. It is

evident at these magnifications that the particles often exhibit defects or damage to their capsids that appear as pockmarks and pits on the surfaces. The trisymmetron plates of the icosahedron are more clearly evident but, because of the height of the particle, only those on the top portion of the virion that are contacted by the AFM tip are visible.

### 3.2. The surface lattice

Higher magnification images of individual virions, including those in Fig. 2 show that the particle surface appears as a very open network with large spaces, approximately hexagonal in shape. The trimeric coat proteins, which protrude from the surface and give definition to the spaces occupy the boundaries. Thus, the surface lattice of the trisymmetrons has a honeycomb appearance where the bottom of each “cell” is at least 15 Å below the tops of the surrounding edges.

The highest resolution AFM images of the lattice are shown in Fig. 3. The area shown in Fig. 3A comes from a large fragment of the virus capsid that had been flattened on the mica substrate and was seen in Fig. 2D. This provided a uniquely favorable opportunity for imaging the protein surface network. The individual trimeric capsid proteins can be visualized, as well as their subunits displayed about the central threefold axis. The trimer has a small hole in its center, but a distinctive triangular shape that is more angular and accentuated than the “doughnut” shape deduced from cryo-electron microscopy (Yan et al., 2000). Close examination of

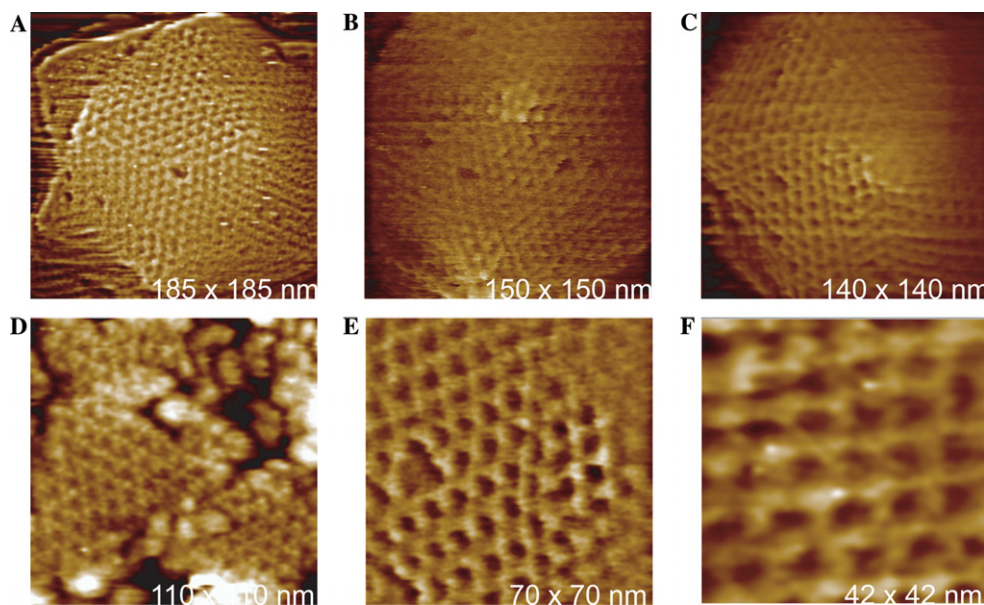


Fig. 2. At higher magnification, the honeycomb appearance of the surface lattice of PBCV-1 is evident. Scars and defects are common on the virion faces, and the unique pentagonal clusters at the vertices can be seen in some cases. In (D) a large fragment of a trisymmetron is flattened on the mica substrate, thereby allowing high-resolution imaging. In (E and F) are two higher magnification images of the network of trimeric proteins making up the honeycomb arrangement.



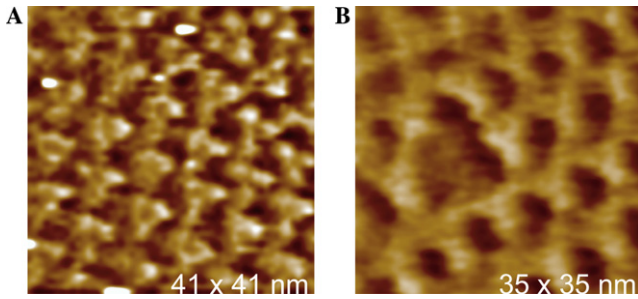


Fig. 3. The highest magnification AFM images of the surface lattice of PBCV-1 are seen here; the triangular nature of the trimeric coat protein is more evident. The trimers have a depression or central hole at the threefold axis. In (B) the triangular defect produced by loss of one coat trimer is shown.

many of the trimers in various patches of well-ordered lattice indicates that the shapes of the subunits and their arrangements are most consistent with that seen schematically in Fig. 4A. One end of each subunit is distinctly higher than the other in all of the AFM images, which means that it protrudes significantly more from the plane of the lattice than the other end. This is in accord with the crystal structure of Vp54 (Nandhagopal et al., 2002). These protruding ends appear responsible for most of the contact area between trimers.

If the trimers in Fig. 4A are extended in a p3 surface net, consistent with the model derived from cryo-electron microscopy, then the lattice in Fig. 4B is obtained. This agrees well with the AFM images. Support for this trimer

shape and distribution of the trimers is provided by inspection of the frequent surface lattice defects that seem to arise from loss of a single capsid trimer. One of these is simulated in Fig. 4B. The triangular gap in the lattice could be filled by addition of a single trimer shown in Fig. 4A.

In addition to the triangular facets of the icosahedron, we imaged the pentagonal vertices. The immediate neighborhood of the vertices is distinctly different than the trisymmetron lattice, and is clearly composed of different proteins. As shown in Fig. 5, there is a single globular protein (although we cannot say if it contains multi-subunits) exactly on the fivefold axis. Unless it is itself pentameric, it breaks the icosahedral symmetry of the particle. Surrounding the apical protein are five other unique globular proteins that form an exact pentamer. These five proteins are about the same size as the apical protein, and all are larger than the trimeric protein making up the trisymmetrons, VP54. This is apparent from the strong appearance of the proteins in the AFM images and their pronounced height that extends well above the surface of the virion.

We noted an unusual feature of the pentameric arrangement of proteins. When the vertex seen in Fig. 6A was scanned again with greater force, neither the surrounding trisymmetron lattice appeared affected, nor did the five proteins making up the pentamer. They remained unchanged, but, as seen in Fig. 6B, the apical protein was forced by tip pressure into the vertex and disappeared into the virion interior, leaving in its place a distinct hole. When

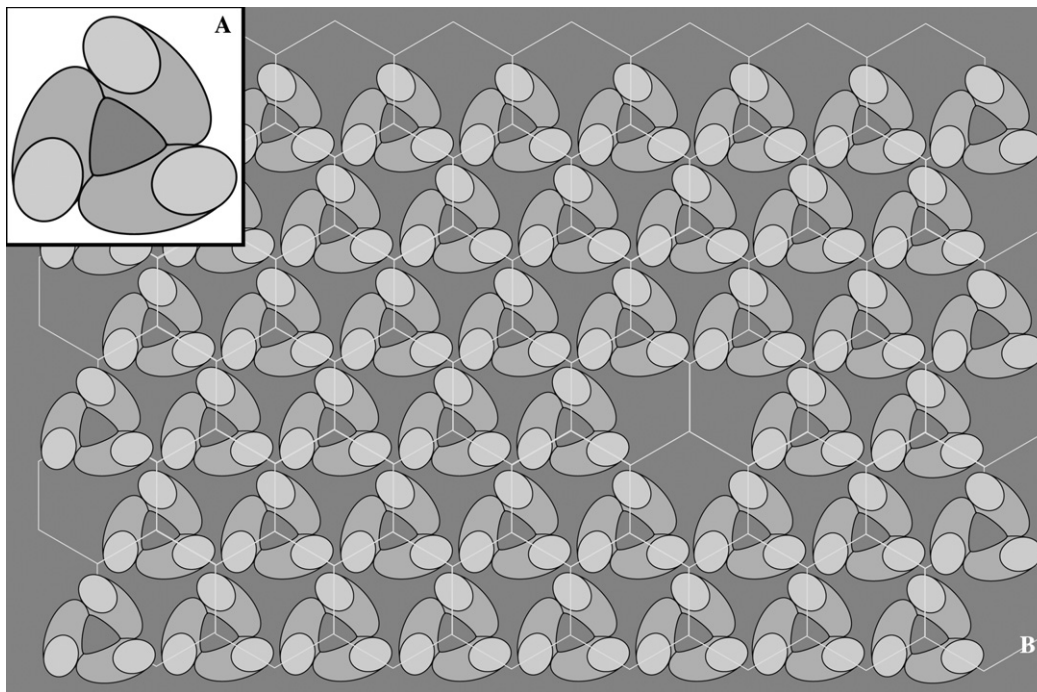


Fig. 4. A schematic drawing of the shape of the coat protein trimer that seems in best agreement with its appearance in the AFM images is in (A). If this trimer is repeated according to a close packed p3 plane lattice, the pattern seen in (B) is obtained. One coat trimer was omitted to simulate the defect seen in Fig. 3B.

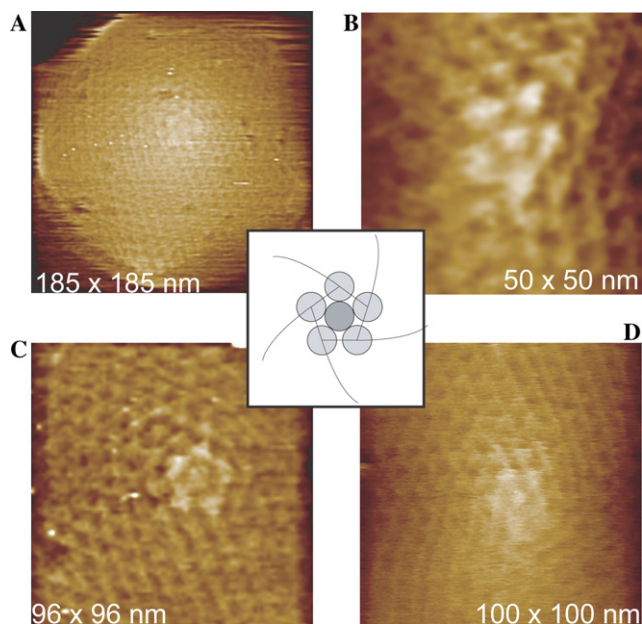


Fig. 5. AFM images of the surfaces of PBCV-1 virions showing the pentameric arrangements of proteins about the fivefold vertices, with a unique and different protein exactly on the vertex.

the AFM tip pressure was returned to its original value, then, as seen in Fig. 6C, the apical protein returned at the center of the pentameric cluster. We do not know the source of the pressure sensitivity of the apical protein, but it appears less integrated into the surface structure, unconnected physically to the pentagon of surrounding proteins, and free to move perpendicular to the virion surface.

### 3.3. Disintegration of virions

After standing in 50 mM Tris-HCl for several days, some virions undergo various degrees of degradation. The damaged particles and the products of degradation are interesting because they allow us to visualize with AFM some of the interior structure and degradation products that are released. In the case of PBCV-1 some of these products were unanticipated.

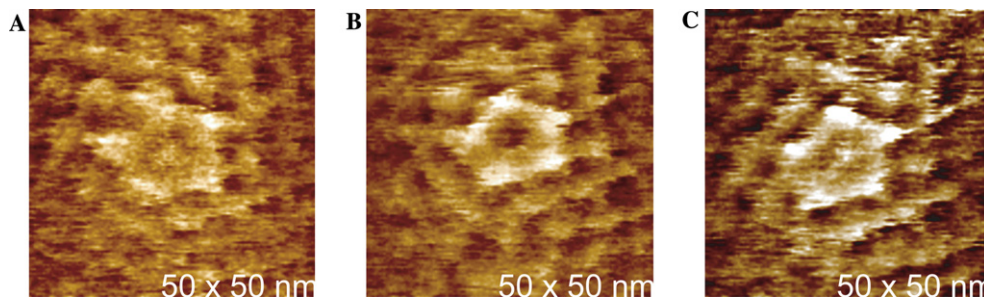


Fig. 6. In (A) is a high magnification AFM image of a PBCV-1 fivefold vertex showing the five protein units making up the pentagon, and the protein at the center. This image was obtained at normal scanning pressure. If the tip pressure is increased the five protein units of the pentagon remain in place, but the protein unit at the center disappears, replaced by a circular hole (B). If tip pressure is then reduced to its original value, the central protein reappears in (C).

If virions are dehydrated before fixation and imaged, they have the appearance of shriveled fruit. The virions collapse and the capsids become highly crenulated, revealing deep folds and fissures. Virions that are degraded more naturally in solution and then fixed and imaged, however, produce quite different products. On the substrate surrounding damaged particles, strands, and fragments of strands of genomic DNA were frequently visualized, as illustrated in Fig. 7. The DNA is linear and not circularized, consistent with its known structure (Rohozinski et al., 1989). In addition to the DNA, large amounts of protein and non-identifiable debris are often seen, and occasionally patches of surface lattice, as in Fig. 2D.

Most unexpected, however, was that virion degradation was accompanied by the appearance of many discreet, spherical particles of uniform size having diameters of about 24 nm (Fig. 8). They are widely abundant in slightly aged samples, and their numbers increase with time. The diameter of the particles resembles those of  $T=1$  or 3 plant viruses and are likely to be icosahedral virus like particles (VLP) derived from the major capsid protein Vp54. Imaging of the VLP by AFM, as in Fig. 8D, indicates their surfaces have a granular structure characteristic of VLP of other viruses and satellite viruses, though no icosahedral symmetry is evident. This,

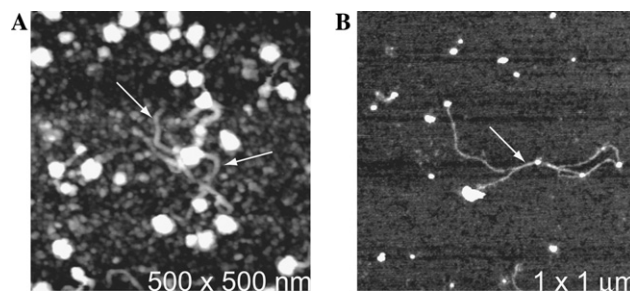


Fig. 7. In (A) several PBCV-1 virions spread on the glass substrate appear as intense white objects because of their height above background. Some of the virions are disrupted and strands, and tangles of strands of linear dsDNA are observed. In (B) a virion has broken open and a portion of its genomic dsDNA is displayed on the mica.



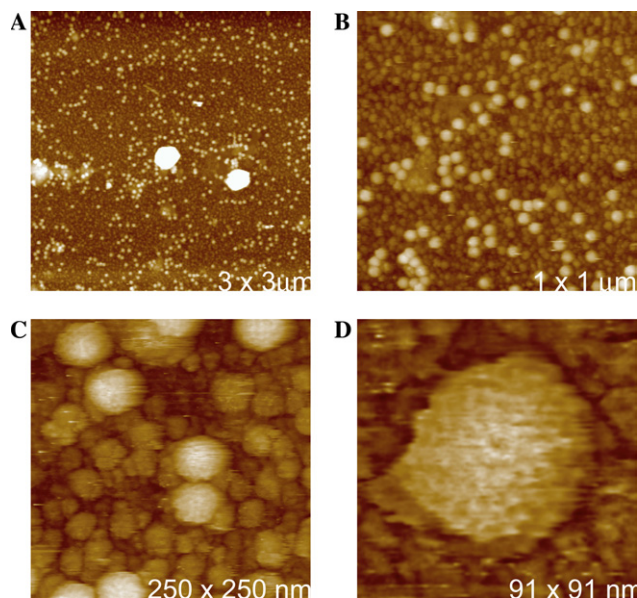


Fig. 8. In (A) the two intense white objects are intact, PBCV-1 virions that are probably in the initial stages of degradation. In the background, the substrate is covered by hundreds of small, uniform, spherical particles having a diameter of about 24 nm. These small virus like particles (VLP) are seen at higher magnification in (B and C), and some surface substructure is apparent in (D).

however, does not mean that such symmetry is absent, as we frequently fail to see symmetrical protein distributions on the surfaces of such small particles (Kuznetsov et al., 2000, 2001; Lucas et al., 2001a,b).

Often, we imaged PBCV-1 particles undergoing dissociation (Fig. 9). The significant feature of these virions is

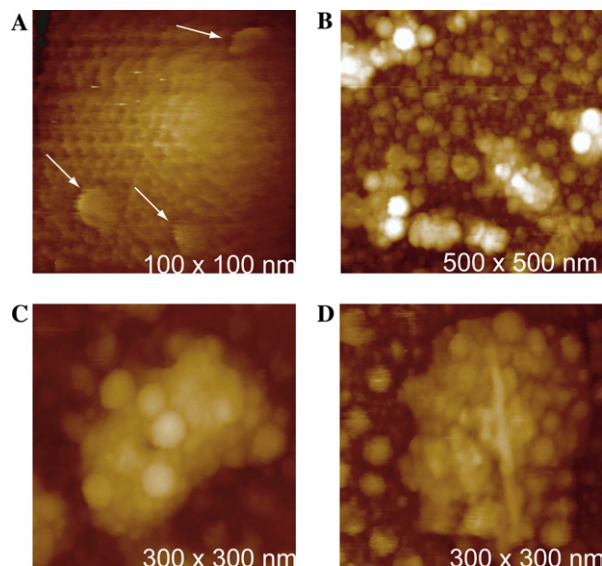


Fig. 9. In (A) three VLP of about 24 nm diameter, marked by arrows, are seen budding from the surface of a PBCV-1 virion in initial stages of degradation. In (B) the spherical particles are seen associated with debris from disintegrating virions. In (C and D) virions in advanced stages of disintegration are seen literally boiling off VLP.

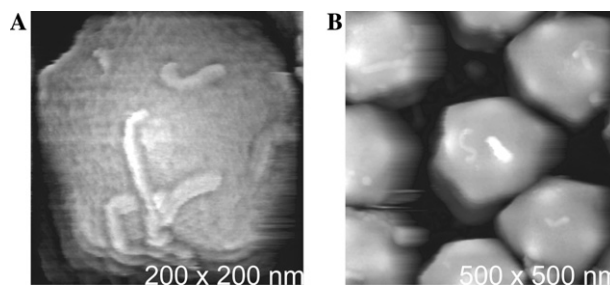


Fig. 10. In (A) is a single PBCV-1 virion showing several tubes, or fibers associated with its surface. In (B) is a cluster of virions, most of which have only one such fiber either on, or attached to their surfaces. These fibers do not appear to arise or be associated with pentagonal vertices, but are located at arbitrary sites on the trisymmetrons.

that they appear to be bursting into masses and clusters of the VLP spherical particles described above. Indeed, such particles can be seen budding from the surfaces of virions just beginning to degrade, while in more advanced states, the VLPs almost appear to be boiling from the remains of the disintegrating virions.

A final component of the PBCV-1 virions, whose origin is less certain, are fibers or tubes, apparently flexible, that are occasionally observed in association with what appear to be intact, or relatively intact virions. Examples of these are seen in Fig. 10. Sometimes there are multiple fibers associated with (we are reluctant to say attached to) a single virion. Sometimes we see one, but most generally we see none. While we do not know the source or function of the fibers, we mention their presence for two reasons. First, they were previously observed in association with degraded virions of another chlorella virus by transmission electron microscopy (Becker et al., 1993). Second, it has been postulated that attachment to host cells may occur through fiber structures (see below).

#### 4. Discussion

Chlorella virus PBCV-1 has been imaged under buffer using AFM after mild fixation with glutaraldehyde. The images are, for the most part, consistent with the model deduced from cryo-electron microscopy of the intact particles (Simpson et al., 2003; Yan et al., 2000), and X-ray diffraction analyses of the coat protein (Nandhagopal et al., 2002). The only refinements to that model that we would suggest are some modification to the overall shape of the trimeric capsid protein and the manner in which it is organized to produce the surface lattice. We see it as having less of a doughnut shape and being more of a triangular arrangement with a depression at its center. The surface lattice itself is a honeycomb affair with the coat protein trimers creating distinctive edges, or borders between indented cells.

This AFM study also provides new information on the structure of the PBCV-1 fivefold vertices that are involved in virus infection. A distinctive pentamer of five

unique proteins, probably a homopentamer, occurs at each fivefold vertex, along with another distinctive protein exactly in the center of the homopentamer. This apical protein is unusual in that it appears sensitive to mechanical force and retracts into the interior of the virion under AFM tip pressure. In the cryo-electron microscopy model, a hole was assigned to the pentagonal apex (Yan et al., 2000), and indeed we can produce such a hole by vertical displacement of the apical protein. It should be noted that PBCV-1 particles negatively stained with uranyl acetate have a distinctive 20- to 25-nm spike structure that extends from one vertex of the particle (Fig. 3C in Van Etten et al., 1991). Perhaps the retractable protein noted in this study and the spike structure are the same protein.

Despite the fact that several microscopic procedures have been used to examine PBCV-1 virions and/or PBCV-1 attached to its host, no unifying model covering infection agrees with all of the experimental results. Micrographs of thin-sections indicate PBCV-1 attaches to the surface of the host via one of its vertices and the cell wall is degraded at the point of attachment (Meints et al., 1984). However, isolated virus particles subjected to the quick-freeze, deep-etch procedure (Heuser, 1980) suggest flexible fibers with terminal swollen structures are attached to the virions (Fig. 3B in Van Etten et al., 1991) and that these hair-like fibers are involved in the initial attachment of PBCV-1 to the host cell wall (see Figs. 7 and 8 in Van Etten et al., 1991). Although three-dimensional image reconstructions of PBCV-1 from cryo-electron micrographs at 2.6 nm resolution suggested that all 66 capsomers in a virion trisymmetron are identical (Yan et al., 2000), more refined reconstructions indicate that three regularly spaced capsomers in each PBCV-1 trisymmetron have a kidney-shaped surface structure (Figs. 1D and F in Van Etten, 2003). Combining the information from these last three microscopic studies led us to propose that the following events occur during virus infection (Van Etten, 2003). We assume that the kidney-shaped surface structures and the hair-like “attachment” fibers are the same thing and the kidney-shaped structures resemble “coiled springs” that extend from the virus upon contact with its host (Van Etten, 2003). These fibers orient the virus so that contact occurs between a virus vertex and the cell wall to initiate infection. This contact presumably produces a conformational change in the virus vertex that leads to the release of cell wall digesting enzymes. Following digestion of the wall, the internal membrane of PBCV-1 presumably fuses with the host membrane to translocate virus DNA and probably associated proteins to the inside of the host, leaving an empty capsid on the surface.

The current study suggests that this model may need modification because no kidney-shaped surface structures or hair-like fibers were seen associated with the

capsomers in a trisymmetron. Although large virion-associated fibers were observed occasionally in the present study (Fig. 10), they are much larger than what one would predict from the previous studies and one questions their significance.

Recently, Onimatsu et al. (2004) have reported that a protein (called Vp130) in another chlorella virus (named CVK2) closely related to PBCV-1, specifically interacts with *Chlorella* NC64A cell walls. Vp130 is a homolog of PBCV-1 protein A140/145R (because of DNA sequencing mistakes, this ORF was originally listed as two separate ORFs). Using a different experimental approach, we also believe that the A140/145R protein is involved in PBCV-1 attachment to its host (manuscript in preparation). Therefore, A140/145R, which has a predicted molecular weight of 121 kDa and is considerably larger than the 54 kDa major capsid protein Vp54, could be one of the two vertex proteins.

The most unexpected finding in the current study is that degradation of PBCV-1 virions give rise to several interesting products. These include strands of linear dsDNA, unidentifiable debris, and a large number of small, uniform, and spherical particles. We often see these small particles budding from, and even boiling off the disintegrating virions in large numbers. The small particles have a size of about 24 nm. This value falls in the size range of  $T=1$  and 3 icosahedral viruses, and their size, shape, and uniformity suggest they are probably formed from the PBCV-1 major coat protein Vp54. No other viral protein is present in sufficient abundance to explain their number. Indeed, numerous VLPs or icosahedra of lower  $T$  number have been produced from the coat proteins of larger viruses, though usually after proteolytic cleavage of an amino or carboxy terminal polypeptide (Choi et al., 2000; Cuillel et al., 1981; Lucas et al., 2001a,b). Such cleavage, however, might well have occurred here during the process of virion disintegration.

## Acknowledgments

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