Electron cryo-tomographic structure of cystovirus ϕ12

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Abstract

Bacteriophage ϕ12 is a member of the Cystoviridae virus family and contains a genome consisting of three segments of double-stranded RNA (dsRNA). This virus family contains eight identified members, of which four have been classified in regard to their complete genomic sequence and encoded viral proteins. A phospholipid envelope that contains the integral proteins P6, P9, P10, and P13 surrounds the viral particles. In species ϕ6, host infection requires binding of a multimeric P3 complex to type IV pili. In species ϕ8, ϕ12, and ϕ13, the attachment apparatus is a heteromeric protein assembly that utilizes the rough lipopolysaccharide (rlps) as a receptor. In ϕ8 the protein components are designated P3a and P3b while in species ϕ12 proteins P3a and P3c have been identified in the complex. The phospholipid envelope of the cystoviruses surrounds a nucleocapsid (NC) composed of two shells. The outer shell is composed of protein P8 with a $T=13$ icosahedral lattice while the primary component of the inner shell is a dodecahedral frame composed of dimeric protein P1. For the current study, the 3D architecture of the intact ϕ12 virus was obtained by electron cryo-tomography. The nucleocapsid appears to be centered within the membrane envelope and possibly attached to it by bridging structures. Two types of densities were observed protruding from the membrane envelope. The densities of the first type were elongated, running parallel, and closely associated to the envelope outer surface. In contrast, the second density was positioned about 12 nm above the envelope connected to it by a flexible low-density stem. This second structure formed a toroidal structure termed “the donut” and appears to inhibit BHT-induced viral envelope fusion.

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Introduction

The cystoviruses (ϕ6 to ϕ14) are a unique group of viruses that infect strains of the plant pathogen Pseudomonas syringae pv. phaseolicola and have been very useful research models for elucidating replication mechanisms of RNA viruses. In particular, the RNA-directed RNA polymerase (RDRP) is structurally and mechanistically related to the comparable enzyme of the flaviviruses and it has been used to study de novo initiation of viral RNA synthesis (Butcher et al., 2001; Lisal et al., 2004; Yang et al., 2003). The replicative mechanism and the multishell structure of cystoviruses are analogous to members of Reoviridae family (Mindich, 2004) and both families package their mRNA as precursors to the double-stranded RNA (dsRNA) genomic segments. Within the cystovirus family all species share a very similar genetic organization and encode a comparable set of proteins (Mindich et al., 1999). Nevertheless, there is considerable variability in the amino acid sequences of analogous proteins from the individual species thus producing a ready-made mutant library (Gottlieb et al., 1988, 2002a,b; Hoogstraten et al., 2000; McGraw et al., 1986; Mindich et al., 1988; Qiao et al., 2000).

A schematic depiction of the structure common to all of the cystoviruses species is shown in Fig. 1. A phospholipid
membrane constitutes the outer virus layer and contains virally encoded proteins P6, P9, P10, and P13 (Etten et al., 1976; Gottlieb et al., 1988; Sinclair et al., 1975). The P3 attachment protein complex is responsible for initial binding to the host bacteria during infection (Mindich et al., 1999). In the ϕ6 cystovirus, the multimeric P3 complex consists of a single polypeptide (P3) (Gottlieb, 1988) and associates specifically with the host cell pili. In contrast, ϕ12 attaches to the rough lipopolysaccharide (rlps) using a complex of at least two proteins (P3a and P3c) (Gottlieb et al., 2002b). This heteromeric complex is conserved in the ϕ8 and ϕ13 species that also recognize rlps as a host cell receptor (Hoogstraten et al., 2000; Mindich et al., 1999; Qiao et al., 2000). In all the cystoviruses, the P3 host cell complex is anchored to the membrane envelope by the integral membrane protein P6 (Stitt and Mindich, 1983). After P3 recognizes and binds to a host cell, the P6 protein also mediates membrane fusion, thus releasing the nucleocapsid (NC) into the periplasmic space (Bamford et al., 1987). Inside mediates membrane fusion, thus releasing the nucleocapsid (NC) into the periplasmic space (Bamford et al., 1987). Inside

The architecture of the viral particles

Results

Examination of a conventional projection image of a sample of frozen-hydrated ϕ12 viruses showed that intact particles are surrounded by a distinct envelope (Fig. 2A). In these images, it is just possible to discern weak densities protruding from this envelope (red and blue arrows in Fig. 2A). Some of the particles consisted only of the NC, either filled with RNA (black arrowheads in Fig. 2A) or are empty (white arrowheads in Fig. 2A). We believe that these isolated NC result from intact viruses that lost the membrane envelope during preparation, although it is also possible that the viruses never acquired their envelopes during assembly.

These features were much more evident in cryo-electron tomograms. In 7-Å-thick sections through the resulting 3D structures, the viral membrane was clearly delineated as well as projections from the membrane surface (Fig. 2B). Furthermore, a distinct gap is evident between the envelope and the surface of the NC. In certain sections, the bare NCs appeared hexagonal, as expected from characteristic views of their icosahedral symmetry. Also, there was a clear distinction between empty NC (white arrows) and those that contained RNA (black arrowheads in Fig. 2A). For further analysis, individual virions and their associated membrane projections were extracted from the tomograms.

Mass distribution of the ϕ12 virus shows that the NC is centered within the viral envelope

Fig. 3 represents the 3D structure of individual ϕ12 virion particles. Figs. 3A and B show a surface rendering of an individual virus defined by a simple density threshold. Although these maps are noisy, the distinction between the outer membrane and the inner NC shell is clear from the cutaway view (Fig. 3B). Also, two different types of surface features are visible as rings (colored red) or elongated densities (colored
blue) on the outside of the membrane envelope. The membrane envelope and the surface protrusions are also clearly visible in individual sections (0.7 nm thick) through two different virions shown in Figs. 3C and D. Segmentation offers an alternative representation, in which selected features are manually outlined in the noisy 3D density map and used for surface rendering. Such outlining of red and blue densities is illustrated in the individual section shown in Fig. 3C and the corresponding rendering is shown in Fig. 3E, with the outer membrane surface colored yellow.

In order to investigate the relative alignment of the NC within the membrane envelope, we centered each of 180

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Fig. 2. Electron tomography of ϕ12. (A) Conventional projection image of frozen-hydrated ϕ12 virus. Weak densities are visible protruding from these particles and are indicated by red and blue arrows. RNA-filled viral cores are indicated by black arrows and empty cores by white arrows. (B) A 7-Å-thick slice from the tomogram of frozen-hydrated viruses shows the membrane envelope, the nucleocapsid, and external densities protruding at different distances from the membrane. The closer external density is indicated by blue arrows and the further density (about 12 nm away from the surface) is indicated by red arrows. In the tomogram slice, viral cores are indicated by black arrows and those without RNA (having a uniform hexagon shape) are indicated by white arrows. Scale bars, 50 nm.

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Fig. 3. 3D representations of virion structure. (A) The surface of an individual virion has been rendered using a simple density threshold of 0.5 × sigma. The two types of protruding densities characterizing ϕ12 have been artificially colored red and blue, respectively. (B) Half of the virion in (A) has been cut away to reveal its interior. (C) A 7-nm-thick section through the tomographic volume from an individual virion shows strong density for the membrane as well as two classes of protruding densities. Red and blue lines around these protruding densities illustrate the process of segmentation, the result of which is displayed in panel E. Note that the red densities appear to be connected to the envelope by low density flexible stem structures (white arrows). (D) Section through a different virion illustrating densities connecting the inner face of the membrane envelope to the nucleocapsid (e.g., at arrow). (E) Surface rendering of a single virion based on segmentation shown in panel C. The yellow sphere corresponds to the outer surface of the membrane; the blue and red structures correspond to the two classes of surface spikes. (F) Average of 180 particles after translational alignment of their membrane centers. Subunits of the nucleocapsid are not visible because the SNR was insufficient for aligning these higher resolution features. A nearly identical structure was obtained by averaging the same particles aligned based on their nucleocapsid. Scale bar, 50 nm. (G) Radial density distributions and variance profiles for averaged virions centered via their membrane envelope or their nucleocapsid; the similarity of these profiles indicates that the nucleocapsid was well centered relative to the membrane. The variances are similar, with minima occurring at the location of the NC and membrane. The highest variance is observed outside the membrane, which is where the surface projections are observed.
individual virion particles relative to an idealized version of either a membrane or a NC. This centering involved only \(x\), \(y\), and \(z\) translations, because the low signal-to-noise ratio of these individual structures prevented a rotational alignment of the NC relative to an icosahedrally symmetric shell. Thus, after averaging, the resulting structure appears rotationally symmetric (e.g., Fig. 3F). Nevertheless, these structures were used to determine the radial mass distribution for the two independent centers. If the NC were eccentrically located within the membrane envelope, one would expect a broadened profile of the NC peak for the average based on the membrane center, and a broadened membrane peak for the average based on the NC. Fig. 3G shows that these profiles were comparable, as were plots of the image variance for the two populations. It is noteworthy that the variances were minimal at radii corresponding to the NC and the membrane envelope and maximal just outside the membrane envelope, which represents the region of the irregularly positioned membrane projections. These results indicate that the membrane was precisely centered around the NC. Furthermore, the radial density profile indicates that the membrane envelope had a radius of \(\sim 35\) nm with a thickness of \(\sim 4\) nm, whereas the NC had a radius of \(26\) nm with a shell thickness of \(\sim 5\) nm. The gap visible between the NC surface and the inner envelope was \(\sim 5\) nm. Although they were not segmented, the nucleocapsid surface appears to be connected to the inner envelope by low-density bridges, which can be seen in Fig. 3D which seem likely to be responsible for keeping the elements of the virus concentric.

Two types of densities are found on the outer viral envelope

As mentioned, two types of densities were observed protruding from the outer surface of the viral envelope (Figs. 2 and 3). One density is centered \(\sim 6\) nm from the outer membrane surface while the second density is roughly twice as far from this surface. When considered in three dimensions, the closer density forms an elongated, cigar shape on the surface of the virion. The further density appears as two circular components in cross-section (red highlighted Fig. 3C); however, when viewed in a surface representation, it is evident that these circles fuse in three dimensions to form a donut (Figs. 3A and E). Fig. 3E shows that the donut-shaped densities adopt a uniform orientation relative to the envelope and Fig. 3C shows that these donuts appear to be connected to the envelope by a low-density, flexible stem (white arrows). The number of donuts attached to each virus ranges from four to six. In contrast, the blue highlighted, cigar-shaped densities appear to lie directly on the viral envelope surface. There are typically one or two cigar-shaped densities per virus and no evidence of any connecting densities (Figs. 3A and C) suggesting that they may be multimers of one of the \(\phi12\) envelope proteins (Gottlieb et al., 2002a,b).

In order to better define the structure of the donut-shaped projections, \(\sim 1300\) individual donut structures were masked from the tomogram, aligned, and averaged using SPIDER (Fig. 4). Examples of individual donuts are shown in this figure (A–C) with the average shown in E (top view) and F (side view). The angular distribution of donuts extracted from tomograms is represented by the plot of Euler angles (\(\phi\) vs. \(\theta\)) above and (\(\psi\) vs. \(\theta\)) below. The side view in panel F reveals a weak connecting density between the donut and the membrane surface of the virion, which lies just below. (G) Contour plot through the central 0.7-nm section of the global average model. (H) FSC for the global average model indicated \(\sim 4.6\) nm resolution. Scale bar, 10 nm.
Discussion

Electron cryo-tomography has proven to be a powerful technique for the study of pleiomorphic structures lacking internal symmetry. With respect to viruses, it has recently been used to study the distribution and trimeric substructure of surface glycoproteins in SIV and HIV (Zhu et al., 2006; Zanetti et al., 2006). In the current work, we describe the overall architecture of the cystovirus φ12 using this method. In the resulting tomograms, we could readily distinguish the nucleocapsid and thus documented that it is concentric with respect to the surrounding membrane envelope. We have also detected densities that bind the NC to the surrounding inner envelope as well as two distinct asymmetric surface features, one of which has a toroidal or donut-like shape.

Previous work on φ6 suggests that it does not have the donut complex that we observed on φ12. Rather, cryo-electron micrographs of φ6 reveal only the horizontal spikes that view). The entire population provided a good angular coverage of 3D space, as illustrated by their Euler angles plotted in Fig. 4D. The averaged structure had a resolution of 4.6 nm, as judged by the 0.5 cutoff of the Fourier Shell Coefficient (Fig. 4H). The diameter for the averaged donut was ~15 nm (containing a center hole of diameter ~3.5 nm) with a thickness of ~5 nm encompassing a volume corresponding to a total mass of 400–550 kDa, depending on the contour level chosen (3 and 2σ, respectively). This large size implies the existence of a large oligomeric assembly. A contour plot through the middle 0.7-nm section of the average model (Fig. 4G) reveals four peaks with apparent twofold symmetry. Indeed, the twofold character was judged by comparing the structure with itself after a 180° rotation; the 0.5 cutoff for the resulting Fourier Shell Coefficient was 4.2 nm, consistent with the resolution of the reconstruction and supportive of twofold symmetry. Fig. 4F also shows the donut structure in relation to the virus particle surface, thus illustrating a weak connecting density. The visibility of this connecting density was likely hampered by the fringe appearing at the boundary of high contrast features, such as the membrane envelope. Although we applied a Wiener filter to reduce this fringe, it was impossible to eliminate it. Importantly, the presence of the membrane surface at the bottom of the averaged structure shown in Fig. 4F indicates that these donuts have a fixed orientation relative to this membrane, which certainly implies the presence of some sort of connecting density to maintain its spatial orientation.

Viral particles treated with BHT undergo envelope fusion

We tested the effect of BHT treatment on φ12 samples, which for φ6 has been shown to remove P3 surface spikes and induce fusion (Bamford et al., 1987). Initially, we tested the effect of 1 mM BHT on infectivity of φ12 and showed that, in contrast to the high sensitivity of φ6 (Wanda et al., 1976), φ12 showed maximal inactivation only after 30 min and even then maintained a residual infectivity of 23%. These BHT-treated φ12 samples were then prepared for electron tomography. The resulting 3D structure showed that, like φ6, many virus particles had fused, producing multiple nucleocapsids within a common membrane envelope (Fig. 5A). However unlike φ6, BHT treatment of φ12 produced a distinctive barrier between some areas of the opposing viral envelopes (Figs. 5A and B). The area of the outer viral envelope that carries these barriers seems to be blocked toward further fusion. Visual examination shows that the barrier is particulate and is closer to one of the viral surfaces. In fact, the particles composing the barrier are ~12 nm from the closer surface and appear to be connected to it by weak densities (Figs. 5A and B). We therefore hypothesized that the barrier represents donuts that have coalesced on one side of the viral particle and blocked further fusion in this area. Envelope areas lacking the barrier appeared to still possess fusion activity. In addition to fusion, this BHT treatment also appeared to destabilize the P8 shell, as evidenced by the preponderance of uncovered PX visible within the fused membrane envelopes. The uncovered PX structures appear as a hexagon-shaped frame within the expanded membrane envelope (Fig. 5A), whereas the intact NC has a rounder and slightly larger shape. SDS–PAGE analysis of the viral proteins showed that, unlike φ6, BHT treatment of φ12 did not result in the significant loss of any of the constituent proteins (Fig. 5C).
constitute the host cell attachment apparatus (Bamford et al., 1987). More recently, electron cryomicroscopy has been utilized to compare the architecture of both φ6 and φ8 (Jaalinjoa et al., 2007). Examination of densities in projection images indicated that φ6 has spikes protruding 2 nm from the envelope surface while those of φ8 protrude 7 nm from the viral surface. Preliminary results from our laboratories show that the φ6 spikes are elongated and closely associated with the surface of the envelope. The data presented here demonstrates that φ12 has two types of structures on the envelope surface, one elongated and directly contacting the membrane surface and another composing a torroid ~12 nm above this surface.

The genetic classification of the cystoviruses showed that there are two types of host cell attachment apparatus: those that bind host cell rlps and those that bind host cell pili (Mindich et al., 1999). In the virus φ12 genome there are three open reading frames in the middle dsRNA segment encoding the attachment proteins, designated P3a, P3b, and P3c (Gottlieb et al., 2002b). This contrasts with the one P3 protein that is encoded by a reading frame in the φ6 m dsRNA segment (Gottlieb et al., 1988). The φ12 P3 proteins were also found to have a significant sequence identity with the corresponding proteins located on the m dsRNA segment of virus φ13 (Gottlieb et al., 2002b). In addition, the location and conformation of the membrane associated, elongated density is similar to that reported for the P3 attachment apparatus of virus species φ6 (Bamford et al., 1987) (Hu et al., unpublished observations).

Interestingly, φ8 has two genes encoding the P3 apparatus, the proteins designated P3a and P3b. The P3c open reading frame is absent from its m dsRNA segment (Hoogstraten et al., 2000). Notably, φ8, φ12, and φ13 all utilize the rlps as the host cell receptor, which may account for the larger distance of surface projections seen on φ8 and φ12. In contrast, φ6 recognize bacterial pili and would be expected to have a rather different attachment apparatus. Given the genomic structure of φ12, we hypothesize that either the donuts or the lower, elongated horizontal surface structures are the attachment proteins that have been specifically adapted for efficient interaction with the pseudomonas cell surface. Alternatively, the donut and the elongated surface structures could function in a cooperative mechanism to initiate virus attachment.

The receptor binding protein is the viral component most likely altered in response to host change. Indeed spontaneous host range mutations in P3 proteins from φ6 have been observed as adaptations to allow the virus infection of pseudomonas strains other than HB10Y (Duffy et al., 2006). Thus, one might expect morphological differences in the surface proteins of the different species of Cystoviridae.

Tomogram images of φ12 particles treated with BHT provided an additional perspective on the structure of the virus’ surface. BHT is known to remove P3 from φ6, causing P6 mediated particle fusion accompanied by loss of infectivity (Li et al., 1993; Wanda et al., 1976). In φ12, this reagent facilitated fusion, yet only partially inhibited infectivity as measured by plaque assays. There are two interpretations to these results. First, it is possible that the φ12 host cell attachment apparatus remains intact after BHT treatment and that the observed fusion was mediated by a process unrelated to P3. This notion is supported by SDS–PAGE indicating that the levels of P3a and P3c proteins are not affected by BHT treatment. We favor a second interpretation that involves a rearrangement of φ12 surface structures, as observed in our tomograms. Such rearrangement would only partially inhibit infectivity but could still facilitate viral fusion. In particular, our tomograms showed that after BHT treatment, fusion produced membrane envelopes containing multiple viral cores, many of which appeared to lose their shell of P8 proteins as well as their close association with the membrane envelope. Nevertheless, the membrane surface of these fused structures appeared to contain a protein assembly that formed a physical barrier towards further fusion. The distance of this barrier from the membrane and the presence of a weak connecting density suggested that the barrier might be formed from a clustering of the donuts into a linear (or 2D) array. Thus, while a dispersed distribution of donuts on the surface of the normal φ12 virion might be responsible for preventing spontaneous fusion, BHT-mediated aggregation would produce a large surface devoid these structures, thus accounting for the preponderance of fused viruses observed in this preparation.

It is therefore conceivable that the φ12 species utilizes the donut as an adaptation to prevent particles from spontaneously fusing together and compromising the viral population’s infectivity. Our tomograms and projection images also appear to show that after BHT treatment, many viral cores inside the fused envelopes lacked the NC’s P8 shell. This is further evidence that the viral envelope and NC surface may be physically linked. Weak densities bridging the NC and the envelope are indeed evident in tomograms of normal viruses and would provide a mechanism for the precise centering of the envelope around the protein shell in cystoviruses. Such centering is also a feature of flaviviruses, but in that case they have an icosahedral shell of glycoproteins on the exterior that undoubtedly plays a role in maintaining the position of the membrane (Kuhn et al., 2002). Therefore, the flavivirus envelope is encased in a glycoprotein shell making this virus radically different from the cystoviruses where the membrane constitutes the outermost shell carrying only sparse projections. Other enveloped viruses lacking an exterior shell, such as HIV, have irregularly shaped membrane envelopes that do not maintain a close association with their icosahedral NC. Detergent isolation and cryo-electron microscopy studies have both shown that the P8 protein of the φ8 virus does not form a shell around the PX. Indeed P8 is a minor component of the viral envelope (Jaalinjoa et al., 2007; Sun et al., 2003). In φ8 virion reconstructions, a protein of 11 kDa was found attached to the PX at a site created by two P1 monomers. This unidentified protein was also envelope associated and was postulated to anchor the membrane to the PX. In φ12, it is possible that P8 performs a similar function to the φ8 11-kDa protein as well as assembling the outer shell of the NC. Thus, BHT treatment appears to interfere with this membrane anchoring, resulting in the loss of P8 association with the NC. This protein would nonetheless be retained within the membrane envelope, accounting for its continued presence in
SDS–PAGE analysis of BHT-treated preparation. In the physiological interaction with the host cell, a coupling of P8 shell disassembly with the removal of the viral envelope could be an adaptation for rapid deployment of the PX.

Materials and methods

**Bacterial strains**

*P. syringae* pv. *phaseolicola* HB10Y (HB) is the host of phage φφ and was utilized as a phenotypic screen based on its noninfectivity by φ12. *P. syringae* pv. *phaseolicola* strain LM2333 is a mutant of HB which φ12 productively infects (Mindich et al., 1999).

**Media and chemicals**

The media used to grow the HB host strain was LB supplemented with 50 μg/ml ampicillin. Buffers ACN (10 mM KPO₄ (pH 7.5), 1 mM MgSO₄, 200 mM NaCl, and 0.5 mM CaCl₂) and P (20 mM Tris–HCl (pH 7.5) 150 mM NaCl, 0.5 mM CaCl₂, and 1 mM MgCl₂) were used for the suspension of purified bacteriophage. Butylated hydroxytoluene (BHT) was purchased from Sigma (MP Biomedicals, Eschwege, Germany).

**Preparation of pure virus**

φ12 was plated into soft agar with a culture of LM2333 that had been grown overnight. A total of 40 plates were incubated overnight at room temperature. The top layer of agar, which contained the φ12 bacteriophage, was then collected and the cell debris and agar was removed by centrifugation in a Beckman TI270 rotor at 33,000 rpm for 1 h at 4 °C. The resulting pellet was resuspended in 6 ml of buffer P. This bacteriophage sample was next layered on a 10–60% sucrose gradient and centrifuged at 23,000 rpm for 1 h at 23 °C using a Beckman SW 50.1 rotor. The band of bacteriophage was detected by light scattering and was collected by needle puncture. After pelleting and resuspension in buffer P, final purification of the bacteriophage or donut-shaped densities were viewed with the specimen. Images were binned twofold, thus producing an effective pixel size of 7 Å on the specimen.

**Image processing**

Tomographic image reconstruction was performed using marker-free alignment as implemented by Winkler and Taylor (2006). Alignment of the original projection images was iterated until relative image shifts were less than 1 pixel and geometrical correction factors were within 1%. Prior to reconstructing the 3D structure by backprojection, the original projection images were corrected for the effects of the contrast transfer function. For this correction, a single defocus value was determined for the entire tilt series by calculating power spectra from 512×512 pixel areas along the tilt axis of each image and producing an incoherent average of the resulting amplitudes. The defocus was then determined from the spacing of the Thon rings in this incoherent average (Fernandez et al., 2006). Based on this empirically determined defocus, a Weiner filter was applied to each individual image using SPIDER (Frank et al., 1996) and assuming a signal-to-noise ratio of 5. Although this correction was not necessarily effective in recovering high-resolution data, it reduced the fringe that appears around high-contrast features, such as the membrane envelope, which primarily resulted from low-resolution effects of the contrast transfer function. Two-dimensional slices through the tomograms were visualized using IMOD (Kremer et al., 1996), whereas 3D subvolumes including virions or donut-shaped densities were viewed with Chimera (Pettersen et al., 2004). For visualization of raw tomographic densities, a 40-Å low-pass filter was applied to the reconstruction followed by a median filter (3×3). Amira (TGS, Inc.) was used for manual segmentation of densities. In particular, the surface representation of a single virion shown in Fig. 3E resulted from such segmentation, where the membrane envelope was outlined in yellow, the individual donut-shaped densities where outlined in red, and the elongated densities in blue. In order to differentiate and provide a clear assignment of a given protruding density to a specific virion, we utilized

Cryo-electron tomography

A suspension of φ12 bacteriophage was placed onto glow-discharged, perforated Quantifoil grids and plunge-frozen in liquid ethane. Images were recorded at 43,000× magnification with a defocus of 8 μm (±0.5 μm) using a Tecnai F20 electron microscope (FEI, Inc., Hillsboro, OR) operating at 200 kV. Such a high defocus was necessary for the fiducial-free alignment procedure for reconstruction (see below). Tilt series were recorded using the serialEM program (Mastronarde, 2005) on a 4000×4000 CCD camera (TVIPS) at specimen angles between ±70° with 2° steps according to the cosine rule. The low-dose imaging mode was used with a total specimen dose of 30–40 electrons/Å² for the entire tilt series. Images were processed using IMOD (Kremer et al., 1996), whereas 3D subvolumes including virions or donut-shaped densities were viewed with Chimera (Pettersen et al., 2004). For visualization of raw tomographic densities, a 40-Å low-pass filter was applied to the reconstruction followed by a median filter (3×3). Amira (TGS, Inc.) was used for manual segmentation of densities. In particular, the surface representation of a single virion shown in Fig. 3E resulted from such segmentation, where the membrane envelope was outlined in yellow, the individual donut-shaped densities where outlined in red, and the elongated densities in blue. In order to differentiate and provide a clear assignment of a given protruding density to a specific virion, we utilized
tomogram files where individual particles were not densely packed. Selection of the relevant densities was done on a slice-by-slice basis and a representative slice is shown in Fig. 3C.

To determine the relative centers of the capsid and the membrane envelope, 250 individual virions were isolated from the tomogram in subvolumes that measured 131 × 131 × 131 pixels. Two alternative masks were then applied to each of these 250 subvolumes, which excluded either the membrane or the capsid. These masked subvolumes were then aligned to a reference, consisting of a sphere with a diameter equivalent to either the membrane or the capsid. Attempts to align individual virions to an icosahedral reference were unsuccessful and this centering therefore involved only translations to match the center of the reference sphere. Thus, two independent centers were determined for each virion and, after inspecting the population, 180 virions were selected that appeared to be well centered within the volume. From these, the mean radial density profile was determined for each set of centers by averaging all 180 virions and performing a circumferential sum of density as a function of radius.

To determine an average structure for the donut-shaped densities, 1300 subvolumes were extracted from the tomograms with dimensions of 41 × 41 × 41 pixels. Upon selection, the donut-shaped densities were roughly centered within the subvolumes, which were large enough to include the associated viral membrane. One of these subvolumes was chosen as an initial reference for alignment, which was then iterated to refine both the rotational and translational alignments with the average structure serving as the reference for the next round of alignment. Steps of rotational and translational alignment were performed sequential with the OR 3Q and CC commands of SPIDER, respectively. The refinement was iterated until there was no significant change in the orientational parameters. Initially, a mask was applied to eliminate the viral envelope from the alignment, but this mask was ultimately relaxed to assure a consistent orientation of the donuts relative to the viral membrane. Although twofold symmetry was not applied to the map, the presence of this symmetry in the final structure was tested by rotating the structure by 180° and calculating an FSC map, the presence of this symmetry in the final structure was assured.

Intermediates in the assembly pathway of the double-stranded RNA virus phi6. EMBO J. 16 (14), 4477–4487.


