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Near-Atomic-Resolution Cryo-EM for Molecular Virology

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Abstract

Electron cryo-microscopy (cryo-EM) is a technique in structural biology that is widely used to solve the three-dimensional structures of macromolecular assemblies, close to their biological and solution conditions. Recent improvements in cryo-EM and single-particle reconstruction methodologies have led to the determination of several virus structures at near-atomic resolution (3.3 – 4.6 Å). These cryo-EM structures not only resolve the C α backbones and side-chain densities of viral capsid proteins, but also suggest functional roles that the protein domains and some key amino acid residues play. This paper reviews the recent advances in near-atomic-resolution cryo-EM for probing the mechanisms of virus assembly and morphogenesis.

Introduction

The key to understanding the mechanism of virus assembly is to know the structures involved. While X-ray crystallography provides atomic-resolution structural information for many of these components [1], the stringent requirement for crystallization often presents challenges for determining the structure of viruses at many functional states during assembly. Alternatively, the same systems that prove intractable for X-ray crystallography can be studied by electron cryomicroscopy (cryo-EM). A key advantage of cryo-EM is the ability to work with samples that have been suspended in vitreous ice [2], thus preserving them in specific functional or biochemical states prior to imaging in an electron microscope. Virus particles are particularly suitable for cryo-EM studies because of their high symmetry, molecular mass, stability, and solubility in aqueous buffers [3]. In addition, virus particles are the biological specimens that have driven technological developments in single-particle cryo-EM since its inception [4]. Recently, cryo-EM has shown the capability of resolving virus structures at near-atomic resolution (3.3 – 4.6 Å) [5**, 6–8, 9*, 10, 11**, 12–14, 15*, 16]. These results have been validated for cases where crystal structures of capsid proteins are known and have also generated new virus structures where crystallographic data does not exist.

The ability to determine the detailed structural features of a virus, through cryo-EM, is dependent on resolution [17]. As resolution improves, it is possible to extract more information from the density map. At low-resolutions (20 – 10 Å), general features of the viral capsid can be identified (e.g. shape, capsomere morphology). Improving the resolution of the density map to subnanometer resolutions (9 – 6 Å) allows for individual subunit

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boundaries to be approximated, and secondary structure elements (SSEs) identified [18]. At this resolution range, α -helices appear as long rod-like densities and β -sheets as thin continuous planes. When the resolution reaches approximately 4.5 Å or better, a complete C α trace can be modeled using *de novo* techniques [18] and key regions of biochemical interests can be determined. In addition, at or beyond 4.5 Å, the pitch of α -helices, separation of β -strands and some side-chain densities become evident, as seen in the 3.8 Å cryo-EM structure of rotavirus (Figure 1A–C). In this case, the fidelity of the structural features identified by cryo-EM was confirmed by the availability of the crystal structure for one of the viral proteins (VP6) of a single-shelled rotavirus [15*]. For cryo-EM maps at near-atomic resolution, where crystallographic data is lacking, it becomes possible to model side-chains densities, determine molecular interactions and predict functionally active regions, as was done for cytoplasmic polyhedrosis virus [14]. This review highlights several near-atomic-resolution cryo-EM reconstructions and new structural insights gained from these studies.

Discovering protein folds and previously unknown capsid proteins

Near-atomic resolution structures of viruses, previously studied by cryo-EM, offer a unique opportunity for new discoveries to be made in systems that were once described at low resolutions. One such example is the 9.5 Å resolution density map of the ϵ 15 phage, in which secondary structure elements could be identified (Figure 2A) [19]. Once this dataset was expanded to nearly 20,000 particle images, the resolution of the ϵ 15 phage density map was subsequently extended to 4.5 Å [9*]. At this resolution, the capsid proteins went from having a globular appearance (at 9.5 Å) to revealing the pitch of α -helices and the separation of β -strands. Using computational modeling methods and sequence analysis, the C α trace was constructed for each of the seven gp7 monomers in the asymmetric unit, revealing many conserved SSEs (Figure 2A). The fold of a large portion of gp7 was shown to match well with that of the previously resolved crystal structure of the HK-97 phage [20], even though the two proteins share no apparent sequence similarity.

After modeling gp7, there were extra densities unaccounted for on the exterior of the ϵ 15 phage capsid shell. This observation hinted at the presence of an additional protein in the capsid shell. Further biochemical analysis of the capsid protein compositions by gel electrophoresis and mass spectroscopy indeed confirmed the presence of gp10 (111 residues), the location and presence of which was previously unknown prior to this study. Using SSEhunter [21] to analyze this extra density in the map, it was suggested that a β -sheet and two small α -helices comprised the density. Due to limited resolvability in this β -sheet region and the lack of long α -helices, the backbone of gp10 could not be modeled with confidence [18]. Nevertheless, the location of this protein between the neighboring gp7 subunits and adjacent capsomeres (Figure 2B–C), suggest that gp10 may act as a staple to stabilize the capsid shell, similar to decorating proteins described for other phages [22,23].

Interestingly, there is little similarity at the sequence level among the coat proteins in the same virus family (e.g. dsDNA phages). However, there are substantial similarities among them at the 3-D structure levels as demonstrated in the HK-97 [20] (Figure 2G–I), ϵ 15 [9*], T4 [24], P-SSP7 [10] (Figure 2D–F) and P22 [5**] (Shown later in Figure 4). Their structural similarities are probably attributable to their similar functions to package, contain and release the dsDNA. However, when examining the details of these structures through cryo-EM, we find that there are subtle but critical differences in interactions, within, and across the capsomere subunits that help maintain particle stability, either with (Figure 2C) or without accessory proteins (Figure 2F, 2I). In the case of HK97, cross linking between subunits across the capsomeres is used to stabilize the particle.

Discovering new interactions and networks between the capsid proteins

Advancing the cryo-EM technique and applying it towards one of the largest and most complex dsDNA human viruses, adenovirus, revealed atomic interactions of multiple capsid proteins. Using 31,815 individual adenovirus particle images, a 3.6 Å cryo-EM structure was reported [11**] side by side with a 3.5 Å crystal structure [25]. Both cryo-EM and the crystal structures resolved the two major capsid proteins: hexon trimers (protein II) and penton-base pentamers (protein III). When comparing the cryo-EM and X-ray models, the two major proteins have the same folds, with a C α RMSD of 1.34 Å and 1.26 Å for the protein II and III, respectively. However, substantial variations in small loop regions did exist. In addition, the cryo-EM density map revealed the N-arm region of the penton-base capsid protein (Figure 3A and B), which could not be resolved by X-ray crystallography. The N-arm interacts with two adjacent IIIa minor proteins, as well as the genome core. This newly resolved N-arm extension is analogous to the rotavirus N-terminal arm of VP7, a major outer shell protein whose interaction with the inner capsid protein VP6 was revealed by cryo-EM, but not by X-ray crystallography [6].

It is known that four minor proteins (proteins IIIa, VI, VIII, and IX) play key roles in cementing the major proteins on the T=25 icosahedral lattice of adenovirus [26, 27]. In cryo-EM [11], three minor proteins (protein IIIa, VIII and IX) were resolved enough in the density map (Figure 3C) for modeling a major portion of the proteins and detecting ~85% of side-chain densities (Figure 3D). Protein VI was seen in fragments, and thus no model could be built. In the X-ray map [25], only two minor proteins (protein IX and VIII) could be resolved with enough details to generate a partial model for each. Nevertheless, the X-ray map resolved less of the polypeptide density than the cryo-EM map for these two proteins. With the resolvability of these extra densities, the cryo-EM model [11] was able to reveal more interactions based on the modeling of a larger amount of the minor proteins.

The specific function of each minor protein is unique and dependent on its location within the virion (Figure 3C), and their interactions allow these proteins to work as a unit, providing capsid stability. Minor protein IIIa links the penton-base and the neighboring hexon trimers. Protein VIII is located on the inner capsid surface at both the 3 and 5-fold axes. Furthermore, this minor protein interacts with minor protein IIIa as well as hexon trimers. Minor protein IX interacts extensively with the hexon trimers. These resolved minor proteins act as a lattice, stabilizing the surrounding capsomeres, and allowing for the capsid to contain an increased genome core, while maintaining its structural properties.

Visualizing detailed conformational changes during virus morphogenesis

Many viruses undergo conformational changes during their maturation process [28]. It was not known if the changes occurred at localized domains or multiple sites in the capsid protein. While inferences to this process can be made biochemically, resolving the high-resolution structures of a virus in different morphogenetic states, as was done by cryo-EM for P22 phage [5**], allows this question to be answered.

Though numerous attempts have been made to crystallize the P22 phage, they have failed. Recently, cryo-EM was able to solve the structures in two unique states at near-atomic resolution. The P22 procapsid was resolved to 3.8 Å resolution with ~23,400 particle images, revealing a C α backbone trace for the coat protein (gp5) and the C-terminus end of the scaffolding protein (gp8). It was the first time that the interactions were directly visualized between the negatively charged N-arm region of the coat protein and the positively charged helix-loop-helix C-terminus of the scaffolding protein. Interestingly, the locations of many previously identified temperature-sensitive mutations, which results in failure of the procapsid formation [5**, 29–31], were mapped onto the extra-density (ED)

domain on the capsid surface and at the interface of the capsid proteins, within and across the capsomeres of the procapsid. This ED domain in P22 is unique among all known phage structures.

The P22 virion was modeled from the 4.0 Å resolution map reconstructed from ~18,300 particle images. The virion is ~100 Å wider and appears more angular than the procapsid. The skewed hexamers in the procapsid become more 6-fold symmetric in the virion, with smaller openings at the centers of the hexamers (Figure 4A–B). A careful comparison of the models between the procapsid and the virion shows multiple changes throughout the gp5 capsid protein responsible for these gross structural changes. These include the unkinking of the long-helix, the 20° rotation of N-arm, the flexing of the A-domain tip, and movements of D-loop and E-loop (Figure 4C). Such changes result in alterations of protein-protein interactions between the subunits within the capsomere (Figure 4A–B) and across the capsomeres (Figure 4D–E).

In addition to the two Cα backbone models of the procapsid and the virion, a subnanometer-resolution cryo-EM map of the P22 procapsid was reconstructed without imposed symmetry. This map showed the unexpected and non-equivalent interactions between the 12 copies of portal proteins and the surrounding 10 scaffolding proteins at one of the twelve 5-fold vertices [5**]. All of these structural details have led to a working model for P22 phage assembly: the capsid nucleation is initiated at the portal complex and the coat proteins are added to this nucleation site as a monomer or dimer with the assistance of the scaffolding proteins until a full procapsid of the proper size is assembled. Once the T=7 procapsid is formed, DNA is driven by the terminase complex into the capsid shell through the portal complex and the scaffolding proteins are released through the hexamer holes (~18 × 42 Å). Though the timing of these events is not known, it is conceivable that the negatively charged DNA may generate a repulsion against the negatively charged N-termini of the scaffolding proteins, which are located around the barrel-domain of the portal complex [5**,32]. This could subsequently disrupt the interactions of the scaffolding protein with the N-arm and A-domain tip of the coat protein. The scaffolding protein release allows the N-arm to rotate to further interact with P-domain loop of the neighboring coat protein subunit in an adjacent capsomere at the 3-fold symmetry axes (Figure 4F, 4G). The disassociation of the A-domain tip with the scaffolding protein subsequently allows the A-domain to flex towards the center of the hexamer to close up the hexamer opening, and to generate an electrostatic interaction between the two helices (Helix 2 and Helix 3) from the two neighboring subunits within one capsomere [5].

Without studying these P22 in two morphogenetic states at near-atomic resolution by cryo-EM, these small, yet significant changes upon maturation would have gone unnoticed. Previous lower-resolution studies did characterize general differences between the two states [33–35], such as capsid size and hexamer skewing, but specific interactions that stabilize both the procapsid and the virion could not be correctly resolved without the Cα backbone models. While solving individual states is important and can gain functional information, this research paves the way for new virus structural studies, and exhibits the ability to interpret mechanisms based on the cryo-EM structural differences at near-atomic resolution.

Conclusion

As resolution of cryo-EM for complete virus structures improves, it will enrich our knowledge of their structure-function relationship. Resolving complete structures in multiple states will be the key to discovering molecular mechanisms. It is clear that more intricate questions will demand even higher resolution structures, and the quest for these details will continue to drive technological improvements in cryo-EM.

While symmetry-based reconstructions lead the structural determination in terms of resolution, solving the structures of protein components, with low or no symmetry and at equivalent resolutions, still remains a challenge for cryo-EM. While difficult to produce at even moderate resolutions, these symmetry-free reconstructions have revealed unique interactions at the portal complex in the phage that could not be seen in the reconstructions with imposed icosahedral symmetry [5**,10,19,36–38].

Finally, analyzing virus structures in the cellular context is another frontier in structural virology. The ability of cryo-EM to preserve virus structures in their native environment (e.g. inside or infecting a cell [10,39,40]), puts virus-cell interactions well within the scope of study, and electron cryo-tomography has already been implemented as such [41,42]. Limitations in how thick of a sample the electron beam can penetrate makes studying virus-bacteria interactions ideal, given the currently available technology. Insights to how residues, domains, proteins, and the complete virus interact with individual cells will aid in understanding fundamental cellular processes undertaken by viruses. The structural knowledge gained from these studies will prove invaluable for translational research in viral gene delivery and anti-viral drug design.

Highlights

Cryo-EM resolves structures of viruses in native state with fine detail and high fidelity

Cryo-EM uncovers protein folds of virus capsid shells

Cryo-EM correlates structure and function of protein domains and key amino acid residues

Cryo-EM resolves structures of some regions which can't be seen in X-ray crystallography

Cryo-EM detects tertiary structural changes of capsid shells during virus morphogenesis

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Figure 1.

Rotavirus VP6 structure generated by cryo-EM and single-particle reconstruction method (EMDB ID: 1461) [15]. (A) cryo-EM map of VP6 shown at 3.8 Å resolution along with the corresponding crystal structure (PDB ID: 1QHD) [43]. At this resolution, α -helices and β -sheets are well defined, and bulky side-chains are seen protruding from the density. (B) Individual stands in the β -sheet region begin to separate and the loops connecting the strands are defined. Docking the crystal structure into the density map reveals residues that correspond to the bulky side-chains. (C) Helix (amino acids 75-100) showing helical grooves along with bulky densities where individual residues from the X-ray model fit into the density. In addition, the loop region (residues 61-74; shown at the bottom) between SSEs has well defined density trace and bulky side-chain densities that match the corresponding X-ray structure.

Figure 2.

New proteins and individual folds are discovered with high-resolution structures. (A - Left) At 9 Å resolution (EMDB ID: 1176) [19] globular domains are revealed for the structure and larger SSEs can be identified in the map. SSEs were identified for ϵ 15 revealing α -helices (green cylinders) and β -sheets (blue planes). (A - Right) Improving the 9 Å map to 4.5 Å in resolution (EMDB ID: 5003) [9], reveals the α trace for the capsid protein (shown inside grey density) (PDB ID: 3C5B), as well as density for the newly resolved auxiliary protein gp10 (magenta density). This density was previously thought to be part of the capsid protein. (B) The complete ϵ 15 density map generated with the gp7 models shown with gp10 colored in magenta. (C) The location of gp10 is circled in magenta, and acts as a staple across the two fold, assisting in capsid stability. (D) P-SSP7 capsid protein model with 363 amino acids resolved (PDB ID: 2XD8) [10]. (E) The complete P-SSP7 capsid built with the generated models revealing extending loops and interacting subunits. (F) Interacting P-domains highlighted in red, purple, and green for individual asymmetric units. (G) HK-97 capsid protein model with 292 amino acids resolved (PDB ID: 1OHG) [20]. (H) The complete HK-97 capsid protein built with generated models, revealing the complexity of the chainmail interactions, used for stabilizing the capsid. (I) Chainmail interactions located at the 3-fold, highlighted in blue, orange, and green for individual asymmetric unit.

Figure 3.

The cryo-EM 3.6 Å structure of adenovirus reveals new regions as well as a network of interactions [11]. (A) The cryo-EM model (PDB ID: 3IYN) of the penton-based monomer (red) is similar to the X-ray structure (PDB ID: 1VSZ) (gray) [25], however, the cryo-EM structure reveals 14 unseen residues (shown in blue). (B) These 14 newly resolved residues (amino acids 37-51) are part of the N-arm and have multiple bulky side-chains that fit well in the cryo-EM density. (C) Inside view and outside view of adenovirus revealing minor and major proteins and their interactions resolved by cryo-EM (EMDB ID: 5172). (D) Selected regions of the three minor proteins (IIIa, IX, and VIII) reveal side-chain densities, thus allowing for specific interactions to be discovered.

Figure 4.

Conformational changes identified by resolving the cryo-EM structures of the P22 procapsid and the virion to near-atomic resolution [5]. (A) Three procapsid asymmetric units are shown to reveal interactions, in addition to spatial arrangements of individual subunits. Skewing of the hexamer subunits results in a large hexamer opening which allows scaffolding protein to exit the virion upon DNA packaging (PDB ID: 2XYY). (B) Three virion asymmetric units are shown to reveal interactions, in addition to spatial arrangements of individual subunits (PDB ID: 2XYZ). (C) Subunit differences between the procapsid (cyan) and virion (magenta) are shown with an outside view (top) as well as a tangential view (bottom). Multiple domains such as the A-domain, long-helix, D-loop, N-arm and E-loop undergo large conformational changes upon capsid maturation. (D) The 2-fold axis of the procapsid shows two neighboring subunits interacting through electrostatic charges. The D-loop of both subunits sits in a pocket of opposite charged residues beside the adjacent ED-

domain. (E) Upon maturation, the D-loops pull out of the pocket, become closer and interact to maintain capsid stability. (F) The 3-fold axis reveals 6 subunits (numbered on sides) that play a role in capsid stabilization for the procapsid. This location contains interactions between the P-domains (shown in red) and E-loops (shown in black). (G) The interactions at the 3-fold axis for the virion. The P-domains (red) still remain vital in capsid stabilization, interacting with N-arm regions (shown in blue) across capsomeres, while the E-loops are pulled out from the 3-fold location to interact with the long-helix and β -sheet within capsomeres (reproduced from [5]).