Published in final edited form as:

J Struct Biol. 2008 September ; 163(3): 246–253. doi:10.1016/j.jsb.2008.03.013.

Multidisciplinary Studies of Viruses: The Role of Structure in Shaping the Questions and Answers

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Abstract

This contribution to the 50th anniversary issue of the Journal of Structural Biology traces a path in which the author evolved from seeing macromolecular structure as end in it self to a means of organizing and correlating data from many sources. The author looks at where we have been and where we are going in this enterprise and the role that structure plays in defining ever more ambitious biological questions and testing and refining models that incorporate data from many techniques. In this, essentially, personal account, the author reflects on 35 years of structural virology and the stages experienced; from “stand alone” crystallography of virus particles to the study of virus assembly and maturation *in vitro* and eventually into the entire virus infection process from particle cell entry to egress. In the process data from many sources were incorporated into reasonable and testable models based on structures ranging in resolution from near-atomic determined by crystallography, to nanometer, determined by electron cryo-microscopy and image reconstruction, to 5 nanometer tomographic studies in the cell. The technological development over this period, for structural studies at all resolutions and functional studies that were unimaginable three decades ago, has been astonishing. Here we look at an aspect of this development applied to virology.

Introduction

Large macromolecular complexes provide an opportunity to characterize the chemistry that defines biology. Since viruses lie at the “threshold of life”, they have been fruitful subjects for molecular and structural biology and as a driving force for technological development. Virtually all of the viruses analyzed to date display properties that are more complex than its parts (often multiple copies of a single gene product) would predict. The challenge of modern biology is to integrate information from as many methods and disciplines as possible to obtain insights into this emergent behavior. Structures at various resolutions play a key role in both defining the important questions and integrating the experimental data to create a meaningful model that can be further elaborated and tested.

As a mature, if not old, structural virologist I have personally experienced the astonishing technical advances in the arena of multi disciplinary studies during the last thirty-five years and found myself swept into the commotion, insights and pleasure afforded by them. This brief review traces my own experience from a hard-core macro-molecular crystallographer to its current breadth that includes most of the disciplines shown in Figure 1. I have chosen systems from my own experience to discuss the retrospective and prospective views. While specific to this investigator, it should be straightforward to generalize these reflections and projections to

a variety of macromolecular systems. Indeed, many senior investigators could write similar stories with very different systems.

Life Before Multidisciplinary Virology: southern bean mosaic virus

Beginning in 1972, I spent six years as a post doctoral fellow with Michael Rossmann mastering the crystallography associated with a 300Å plant virus called southern bean mosaic virus (SBMV). In 1980, two years after I left the group, the structure was published at 2.8Å resolution (Abad-Zapatero et al., 1980). This followed by less than two years the structure of tomato bushy stunt virus (TBSV) determined by Harrison and colleagues (Harrison et al., 1978). The astonishing thing about these two structures was their similarity in quaternary (i.e. the shape of a rhombic tri-icantahedron) and tertiary structure (an eight stranded anti parallel beta sandwich). Although Rossmann was an early advocate of structural comparisons and anticipated the repetitive nature of folding motifs starting with the discovery of the nucleotide-binding (Rossmann) fold (Rao and Rossmann, 1973), even he did not anticipate the striking similarity observed in these structures that did not have detectable (at the time) sequence similarity (Figure 2). A major goal of the work was to understand the molecular switching required for the same gene products to form both hexamers and pentamers in the T=3, quasi-equivalent, icosahedral surface lattice (Caspar and Klug, 1962). This feature was also maintained between the two viruses with an order-disorder element of a polypeptide obvious at the N-terminal region of the subunits. The structures generated significant data on protein-protein interactions and the phenomenon of quasi-equivalence, however, to our surprise and disappointment, there was little excitement generated in the virology community. The work was viewed as a tour-de-force of crystallography and generated little of what might be called “chemical virology”. A significant reason for this was the lack of supporting molecular genetics such as infectious clones for these and other plant viruses that later became available. In many ways the structures were ahead of the time when they would become useful. As plant virus capsids they were also relatively inert and lacked many of the interesting features that emerged with the study of animal viruses. It was a harsh lesson to learn that a structure with no multi-discipline background does not stand-alone. While the reaction to the initial studies was muted, it did not discourage continued crystallography of virus particles. As of today there are 211 crystallographic coordinate data sets in the Virus Particle Explorer (VIPER) web site with 96 unique viruses represented at near atomic resolution(Natarajan et al., 2005).

The Structure of Human Rhinovirus 14; a multidisciplinary study

By circumstance and nature, animal virus capsid structures offered many interesting features, dependent on multi disciplinary approaches, when they were determined in 1985(Hogle et al., 1985; Rossmann et al., 1985). This began exciting, “hybrid-method”, studies based on virus structure. Prior to the Human Rhino Virus 14 (HRV14) structure determination, the sequences of its capsid antigenic sites were mapped with virus mutants that escaped neutralization by monoclonal antibodies (Sherry et al., 1986). With the structure it was possible to show that the antigenic “hot spots” on the virus particle were on extended surface loops immediately suggesting that these animal viruses had regions of their structure that were readily changeable and functioned as decoys for the immune system. The presence of a deep “canyon” in the Vp1 subunits of HRV14 and poliovirus was proposed, and later shown, to be the site of receptor binding (Olson et al., 1993). Such complementarity suggested new anti-viral strategies that came into play shortly after the first structure determinations with the discovery of a class of compounds that inhibited picornavirus infection(Smith et al., 1986). Subsequent studies resulted in the refinement of the compounds by rational drug design eventually leading to clinical trials (Diana et al., 1989).

Combining crystallography and electron cryo-microscopy (CryoEM)

Hybrid methods involving multiple structural approaches began with the study of adenovirus. Burnett and colleagues determined the structure of the purified trimeric “hexon” capsid protein of adenovirus with crystallography and then used electron microscopy to demonstrate how the subunits were situated in the virus particle (Roberts et al., 1986; van Oostrum et al., 1987). Many studies have now been performed where crystallography and cryoEM were combined to reveal structural details of large viruses or dynamic character of particle intermediates. Often these particles cannot be crystallized due to their size or lack of homogeneity. In each case, either a viral subunit or the whole virus (in a stable form) was determined by crystallography, then the atomic coordinates were used to model into the cryoEM density of a large complex virus or into a less stable form of the dynamic virus. Examples from my own experience include mapping the dynamics of ssRNA viruses (Canady et al., 2000; Speir et al., 1995) and dsDNA phage capsids (Wikoff et al., 2006). The resulting coordinates describe a “pseudo-atomic model” of the structure determined by EM (Baker and Johnson, 1996).

Another blend of crystallography and cryoEM emerged from the study of Fab fragments bound to the surface of a plant virus. Monoclonal antibodies raised against cowpea mosaic virus were converted to Fab fragments by proteolysis, and then the antibody-virus complex was studied by cryoEM (Wang et al., 1992). The cryoEM density, combined with the crystallographic structure of the virus determined previously, allowed the mapping of the Fab “footprint” on to the virus surface, showing which viral residues interacted with the Fab (Porta et al., 1994). Like the crystallography of the plant viruses, the work generated little initial interest, but it did inspire fruitful studies of Fab and receptor interactions with picornaviruses (e.g. (Olson et al., 1993; Smith et al., 1993a; Smith et al., 1993b)). The approach is now commonly used for mapping receptor and antibody interactions with many different viruses.

Deriving function from structure: Multi-disciplinary studies of Noda and Tetraviruses

Nodavirus Studies

Nodaviruses have been the subject of multi discipline studies for decades. Like picornaviruses, there was a rich, virion-based, literature available from the laboratory of Roland Rueckert when the first Nodavirus crystal structure was determined. The Black Beetle Virus structure placed insect viruses into the continuum of other virus structures with the appearance of the beta sandwich topology found in other virus structures determined (Hosur et al., 1987). In the context of other data the structures of Flock House Virus (FHV) and Pariocoto virus (Figure 3) also revealed the multifunctional nature of the capsid protein (Fisher and Johnson, 1993; Tang et al., 2001). At least 6 functions for the viral coat protein were identified including 1) assembly to form a T=3 icosahedral particle 2) organization of RNA into a duplex at icosahedral 2-fold axes of the particle 3) autocatalytic cleavage 4) recognition of the receptor on the cell surface 5) generation of a dynamic capsid to transiently expose the gamma peptide 6) particle disassembly under mild conditions after entering the cell (Schneemann et al., 1998). Lynn Enquist of Princeton University recently summarized the features of FHV that make it an attractive system for study (Faculty of 1000 Biology, 5 Feb 2008 <http://www.f1000biology.com/article/id/1100174/evaluation>). *“Flock house virus is in the family Nodaviridae. If you don't know much about these viruses, you should take time to learn about them. They are amazing: first among their stunning molecular magic tricks is that they can replicate in insects, plants, yeast, and mammalian cells. They have a single capsid protein (180 copies) that cleaves itself into beta and gamma subunits after assembly. The tiny gamma subunit (4 kda) is hydrophobic and is essential to breach the host membrane enabling capsid entry”*.

A feature of FHV immediately investigated was the auto-catalytic cleavage that releases residues 364–407 (referred to as the gamma peptide) from residues 1–363 (referred to as the beta peptide). Although covalently independent, gamma remained associated with the particles (Fisher and Johnson, 1993). The structure revealed an amphipathic helix for residues 364–385, with the remaining residues invisible in the electron density. This observation, together with the fact that the cleavage is required for infectivity (Schneemann et al., 1992), triggered a fascinating hybrid approach to function. First, what is the mechanism of the auto-catalytic cleavage? This was elucidated by referring to a large body of peptide research showing that both cleavage and de-amidation can occur at ASN residues. Analysis of the structure revealed that the active site for cleavage had the ideal geometry and chemistry for the ASN side chain to make a nucleophilic attack of its own carbonyl carbon leading to a cyclic imide formation. Hydrolysis of this intermediate leads to the cleavage. The fact that the cleavage depends on assembly was clear from the structure. While all the residues required for cleavage are on the same polypeptide chain, their position is dependent on inter subunit interactions that occur when assembly is complete (Reddy et al., 2004). The cleavage requires metal ions, as purification of the particles in the presence of EDTA dramatically reduces cleavage (Schneemann, 1992; Schneemann et al., 1994). The role of the metal ions in this chemistry is still under investigation, however, the observation revealed a potential advantage of the metal dependence. Since the free metal ion concentration is much greater outside of the cell than in the cell, only release from the cell generates the infectious particle, preventing interactions of the free peptide within an infected cell.

What is the role of the cleaved off peptide? Studies by limited proteolysis and mass spectrometry revealed that the peptides were transiently exposed, although they are internal in the X-ray structure (Bothner et al., 1998). This confirmed the dynamic nature of the viral capsid, first indicated for poliovirus (Roivainen et al., 1991; Roivainen et al., 1993). The amphipathic nature of the visible portion of the gamma peptide suggested that it might interact with membranes, an interesting possibility because it is still not clear how non enveloped viruses breach cellular membranes to deliver their genome to the cytoplasm. A series of in vitro studies strongly supported that hypothesis, as the synthetic polypeptide corresponding to residues 364–385 had a dramatic effect on synthetic membranes, causing dye release from artificial liposomes, thinning of the membrane bilayer and generating pores in the membranes. Polarized infra red spectroscopy showed that the helices formed on interacting with the membranes and that they were oriented with the helix axis parallel to the bi-layer (Bong et al., 1999; Janshoff et al., 1999). A significant body of work now indicates that most, if not all, non-enveloped animal viruses require a polypeptide similar to gamma to enter cells (Banerjee and Johnson, 2008). Recently, in vivo studies showed that the infectivity of non-cleaved FHV particles can be rescued by providing gamma in trans through a non-infectious virus-like-particle made in an expression system (Walukiewicz et al., 2008). A cartoon level model of the FHV infection is shown in Figure 4.

The intriguing meta-stability of animal virus particles has not been explained by structural studies, but has been elucidated by a variety of multi-discipline investigations including cell biology, calorimetry, kinetics and biochemistry. Hogle and co-workers have shown that binding of the virus to the receptor initiates a destabilization that is required for infection (Huang et al., 2000; Tsang et al., 2001). Observations of entry intermediates in FHV confirm that this is a convergent behavior in animal virus entry regardless of capsid type (Walukiewicz et al., 2006). The mechanism for such destabilization is still a subject of intense interest that has been aided by studies of WIN-type picornavirus anti-virals that stabilize the capsid and prevent the formation of disassembly intermediates (Tsang et al., 2000).

Tetravirus Studies

The structure of a T=4 insect virus called Nudaurelia Capensis Omega virus (NWV) was solved in 1996 (Munshi et al., 1996). There was virtually no complementary data available for the virus other than the subunit sequence (Agrawal and Johnson, 1992) and that it contained a bipartite RNA genome like nodaviruses. The fold of the subunit was strikingly similar to the FHV fold; however, an entire Ig domain of over 100 residues was inserted between two strands of the beta sandwich. The structure also revealed that the subunit underwent a cleavage similar to FHV, releasing residues 571–644 that are internal and remain associated with the particle. Although there was no detectable sequence similarity, the helix following the cleavage site (residues 571–595) formed a bundle at the 5-fold axes that was strikingly similar to the Nodaviruses. The cleavage site was ASN-PHE, with residues very similar to those in FHV surrounding the cleavage site, suggesting that it was also autocatalytic. The molecular switching generating the T=4 surface lattice was novel in that it was modulated by the C-terminal portion of the subunits (corresponding to the analogue to the gamma peptide). As a stand alone structure the NWV structure had little more impact than the SBMV structure. However, when it was discovered that virus-like-particles (VLPs) could be made with a baculovirus system expressing the NWV coat protein gene, the system became exceptionally interesting (Figure 5). It was possible to isolate intermediates in the maturation of the virus when particles were made in the expression system. These were studied with cryoEM, solution-x ray scattering, biochemistry, mass spectrometry and molecular genetics. The results have revealed the remarkable dynamics of these particles and the hierarchy of morphological and biochemical maturation (Bothner et al., 2005; Canady et al., 2001; Canady et al., 2000; Taylor et al., 2003; Taylor and Johnson, 2005; Taylor et al., 2002).

Into the cell; structural studies of Flock House Virus assembly in vivo

Structural studies of FHV have moved into the cell with traditional cellular fixation methods (Kopeck et al., 2007) and high pressure frozen/freezing substituted methods (Lanman et al., 2007) being used for tomographic studies of RNA replication and virus assembly. Ahlquist and colleagues have shown that the FHV polymerase is localized to the mitochondria outer membrane (Figure 4) and that structures called spherules are formed in these membranes (Miller and Ahlquist, 2002; Miller et al., 2001). The spherules display remarkable homogeneity and order on these membranes and are currently under study with different staining methods (Sosinsky et al., 2007) and post tomographic processing. The current working hypothesis posits that RNA replication occurs in the spherules where ~100 polymerase molecules reside. The mitochondria of infected cells undergo dramatic morphological change with the entire mitochondria convoluting to make a chamber that contains a single opening. One side of this chamber has spherules pointing into it, with spherules pointing out on the outside of the chamber. It appears that spherules on the membrane facing into the chamber provide RNA for new virions. The mitochondrial chambers may be incubators for virus assembly, creating a confined environment where RNA replication, translation, assembly and RNA packaging occur in an assembly “factory”. Freshly made particles form large three-dimensional crystals that eventually grow to such a size that they break the plasma membrane, releasing the virions.

The Future of Multi-disciplinary Studies

Multidisciplinary structural studies will probably progress in two ways. The first approach considers a large supra molecular structure as center stage, such as the nuclear pore, and seeks to combine a wide variety of information, obtained by multidisciplinary methods, to develop a detailed model of this structure. Andrej Sali and his colleagues have taken this approach with considerable success (Alber et al., 2007a; Alber et al., 2007b). The second approach is a logical extension of what has occurred in the last twenty-five years where a biological system is attacked piecemeal from many different directions with ever growing sophistication and

integration. Such an effort eventually generates a “systems biology” approach describing, for instance, a virus life cycle. In this scenario the structure of a single species has given up the center stage to multiple structures that correlate and contribute components toward the overall understanding of biological function. In reality, the structure or structures still provide a unique means for correlating large quantities of data, but there will probably be many structures to be considered as well as the interrelationship among these structures. Since the author is only an observer of the first approach, the reader is referred to the papers describing the state of the art for the nuclear pore cited above.

The second approach will be discussed in the context of Nodaviruses as we move toward an understanding this virus life cycle. A multidisciplinary approach will include cell biology, molecular genetics, various imaging methods, biophysics and proteomics of infected cells to address the total virus life cycle. Methods that are emerging for *in vivo* structural analysis will play an important role in this respect and will depend on both high pressure frozen samples of whole cells e.g.(Lanman et al., 2007), flash frozen samples of either cellular components or small cells e.g. (Henderson et al., 2007) and correlated fluorescence and electron microscopy (Gaietta et al., 2002; Sartori et al., 2007). The future will depend on post tomographic processing to extract the maximum amount of information from the images and will incorporate automated correlation analysis of features in the cell, as pioneered by Baumeister and his colleagues(Best et al., 2007; Lucic et al., 2005; Ortiz et al., 2006).

To achieve a comprehensive view of the virus life cycle the cellular location and mechanism of action must be established for each of the events characteristic of infection (Figure 4). Initial studies should involve particle entry and localization within the cell. Reagents described above are excellent for this purpose. Expression of the FHV coat protein gene in a baculovirus system results in particles that are virtually indistinguishable from authentic virions by structure, but they are more dynamic and expose the gamma peptide more readily than virions (Bothner et al., 1999). These particles, in which the gamma peptides and beta-coat protein are each labeled with the tetra-cysteine motif, are ideal for the studies of entry(Machleidt et al., 2007). Fluorescence and EM correlated microscopy employing the ReASH reagent that fluoresces only when bound to the tetra-cysteine motif (Gaietta et al., 2002) can be performed to localize the gamma peptides released from the VLPs, as well as the beta chain associated with the particles. This procedure allows the location of the labeled molecule to be determined by the attached fluorescent reporter (ReASH) with light microscopy, followed by a photo-conversion of the reporter to a form that has a high affinity for osmium, making it visible in the electron microscope. Previously mentioned eluted particles can be made with VLPs potentially allowing the cellular compartments to be identified where the particle transition occurs. In addition it will be possible to identify areas of co-localization of VLPs and immature authentic particles by employing separately labeled particles used in the “gamma in trans assay” described above. These approaches will allow a complete characterization of the early events in cellular attachment and entry.

The next stage of viral infection with FHV requires release of the viral genome into the cytoplasm and initial translation to produce the first newly synthesized viral protein. Open questions to be addressed include the participation of translation in the uncoating of the viral genome. Does ribosome progression on the RNA molecules drag the genomes from the weakened particle (corresponding to the “eluted particle”), across membranes and into the cytoplasm to generate the initial pools of protein(Hiscox and Ball, 1997)? If so, there must be exposure of the capped, 5' end of the RNA.

It is well established that the RNA polymerase known as Protein A is targeted to the mitochondria and that RNA replication occurs in the spherules created by this protein in the outer mitochondrial membrane(Miller and Ahlquist, 2002; Miller et al., 2001). Open questions

associated with this activity correspond to the earliest RNA replication. Logically it must initially occur prior to spherule formation as there are about 100 protein A molecules per spherule (Kopeck et al., 2007). Does that require different stages of RNA replication? Once spherules start to form, they make well-ordered arrays in the outer mitochondrial membrane. Are these arrays based structural proteins already in the membrane, or does Protein A create a lattice in the membrane? What is the structure of the spherules? They are approximately 60 nm in diameter and in high pressure frozen cells they appear remarkably uniform. It is known that the Protein A molecules are in close proximity within the membranes (Dye et al., 2005a; Dye et al., 2005b), potentially forming a shell. Ahlquist proposed that the cellular RNA replication environment for ssRNA viruses may be similar to that within the capsid of dsRNA viruses (Ahlquist, 2005; Ahlquist, 2006), suggesting the possibility that Protein A may create shells, anchored in the spherule membranes, that resemble the dimeric T=1 shells found in dsRNA viruses e.g. (Naitow et al., 2002). If this were the case, Protein A would contain a shell forming domain and an RNA replicase domain, potentially explaining its unusually large size for an RNA polymerase. Post tomographic data processing of the spherules may be able to determine if this hypothesis has merit.

What happens to the expression profile of cellular proteins during FHV infection of drosophila cells? This question was recently addressed by comparing expression profiles of uninfected and infected drosophila line 1 cells (Go et al., 2006). The largest increase in expression in infected cells was a mitochondrial elongation factor (mEF-G) that was raised more than 150 fold compared to uninfected cells. A protein that inhibits programmed cell death was dramatically decreased in infected cells. Similar studies performed with purified mitochondria may be more informative now that the role of the mitochondria in RNA replication is clear.

As indicated above, there is some evidence from EMT of infected cells that the reformed mitochondria may create a chamber where freshly made protein and RNA are isolated for virus assembly. The generation of such “virus factories” would greatly reduce the required level of specificity between protein and RNA, since the viral RNA would be dominant within such a compartment. In such an environment the correspondence of RNA replication, protein synthesis and assembly could be highly tuned for the maximum production of virus. Improved resolution of tomograms and potentially correlated microscopy will be required to visualize the assembly of virus particles.

Conclusions

The challenge and excitement of multidisciplinary studies should be clear from the example discussed. FHV contains only two RNA segments that encode three proteins. The coat protein and RNA polymerase were discussed in some detail. An aspect not touched upon is the role of the third gene product, Protein B. It is synthesized from a subgenomic RNA that overlaps with the gene encoding Protein A and it inhibits the RNAi response of the drosophila cells (Chao et al., 2005). Investigation of this protein corresponds to an entirely different area of investigation from those described.

FHV is a minimalist replicating system, yet the level of complexity revealed in studies to date demonstrates the extraordinary complications associated with this virus infection. The functional density of viral gene products makes their study exceptionally interesting because of the interrelated and, in some cases, overlapping activities, making it difficult to probe one aspect of function without affecting another. Structure continues to play a key role in our understanding of mechanism in FHV and other systems, but it is certainly not an end in itself. Indeed, at virtually all the resolutions discussed, structure has answered some of the questions, but more importantly has raised questions that focus the effort to understand the biological system. The continued development in technology places today's investigator in a position in

which the limitations are defined by their creativity and imagination. The possibility of understanding fundamental aspects of the emergent behavior of biological systems is seemingly accessible to the current generation of scientists. It will be interesting to see where we are in the process when the Journal Structural Biology celebrates its 60th anniversary.

Acknowledgments

The work described from the author's laboratory was supported by grants from the National Institutes of Health. Gabriel Lander and Jason Lanman prepared the figures and their help is gratefully acknowledged.

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Figure 1.

A representative summary of methods commonly combined with structural studies to fully understand biological function.

Figure 2.

Comparison of the subunit tertiary structure (ramped in color from blue at the N-terminus to red at the C-terminus) and T=3 particle quaternary structure of Southern Bean Mosaic Virus and Tomato Bushy Stunt Virus. Although there was no detectable sequence homology, the structures of the shell-forming domain were virtually super imposable. TBSV has an additional domain at the C-terminus of the subunit that adds the surface protrusions to the particle. The quaternary structure of the contiguous shell domains in SBMV and TBSV are also nearly identical. The maximum dimension of the SBMV particle is approximately 300Å.

Figure 3.

The structure of Pariocota virus, a typical Nodavirus. At left is the fold of the A subunit (blue subunit in the quaternary structure and clustered about the 5-fold symmetry axes of the icosahedron). The structure is ramped in color as in figure 2. The T=3 quaternary structure has characteristic protrusions at the quasi 3-fold symmetry axes (middle). At right is a cutaway of the capsid showing the ordered duplex RNA and those portions of the subunit polypeptide that interact with the RNA. The gamma peptides are in blue and can be seen to lie in cavities within the protein shell, shown as density from a cryoEM reconstruction (Tang et al., 2001).

The FHV Lifecycle.

Figure 4.

A cartoon-level representation of the Flock House Virus life cycle within a *Drosophila* line 1 cell is shown in the figure. As discussed in the manuscript, it is believed that gamma peptides assist in the translocation of RNA across the endosome membrane and that ribosomes may participate in the process when the 5' end of the RNA genome has breached the membrane (Hiscox and Ball, 1997). RNA replication occurs in the mitochondria (brown) and the mitochondria are depicted early in infection (top) and after transformation by Protein A. Assembled virus particles form large arrays within the cytoplasm and the arrays grow to such a size that they rupture the plasma membrane and release the virions.

Figure 5.

The subunit tertiary structure (color ramped as in Figure 2) for Nudaurelia Omega Capensis Virus is shown at left (Helgstrand et al., 2004). The transition from procapsid to capsid for the T=4 particle is depicted on the right. The color for the quaternary structure is ramped by radius from red to blue. The procapsid particle is about 490 Å in diameter with the subunits clustered as dimers. After maturation at pH 5.0, the particle size decrease to 410 Å and the subunits are clustered as trimers. An auto-catalytic cleavage is activated when the particles compact (Canady et al., 2000).