Virus Particle Dynamics Derived from CryoEM Studies

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Summary: The direct electron detector has revolutionized electron cryo-microscopy (CryoEM). Icosahedral virus structures are routinely produced at 4Å resolution or better and the approach has largely displaced virus crystallography, as it requires less material, less purity and often produces a structure more rapidly. Largely ignored in this new era of CryoEM is the dynamic information in the data sets that was not available in X-ray structures. Here we review an approach that captures the dynamic character of viruses displayed in the CryoEM ensemble of particles at the moment of freezing. We illustrate the approach with a simple model, briefly describe the details and provide a practical application to virus particle maturation.

Introduction

It is well recognized that the dynamics of animal virus particles are critical for their infectivity[1]. Until recently, characterizing these dynamic properties required indirect detection, in many cases examining the transient exposure of lytic peptides associated with non-enveloped viruses[2]. The first evidence of the large-scale nature of these dynamic properties was suggested by studies of poliovirus. Discrete polypeptides were synthesized over the entire range of the Vp1 capsid subunit and antibodies were raised

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against these polypeptides. The antibodies were then tested for binding to and neutralization of virus particles. A significant number did bind and neutralize, suggesting the surface antigenic regions of poliovirus Vp1[3]. When the poliovirus structure was solved in 1985[4], there was excellent agreement between the peptide studies and the virus structure with one exception. Peptides near the N-terminus of Vp1 were clearly internal in the X-ray structure and yet antibodies to peptides in this region bound to the virus. Dynamic behavior of a virus particle that would transiently expose such a peptide was not expected and an explanation of the result was not put forward. Later studies indicated that interaction with the cell surface and the poliovirus receptor lead to the exposure of the N-terminus of Vp1 and the internal Vp4 [5] while the particle was still intact. Subsequent studies demonstrated that the exposure of the N-terminus of Vp1 did not require interaction with the cell surface and it was concluded that there must be a reversible, large-scale reorganization of this portion of Vp1 when virus particles were in solution[6,7].

A more quantitative approach for the study of particle dynamics based on mass spectrometry was developed later[8]. The experimental protocol was straightforward with particles of the insect Flock House Virus (FHV) exposed to trypsin in a time course and the positions of cleavage determined by the mass of the resulting peptides. FHV is a non-enveloped, bipartite, single stranded, RNA virus with a $T=3$, quasi-equivalent surface lattice. The single subunit type is composed of 407 amino acids. During maturation an autocatalytic cleavage occurs between residues 363 and 364. Residues 364-407 are referred to as the gamma peptide and it stays associated with the particle after cleavage and is internal in the X-ray structure of the virus[9]. Proteolytic studies showed that the gamma peptide and the N-terminal region of the subunit (also internal in the X-ray structure) were cleaved first indicating their transient exposure. Similar studies were performed with human rhinovirus 14 (HRV14; a picornavirus having structural homology with poliovirus) where Vp4 and the N-terminus of Vp1 were cleaved first by the protease[10]. The presence of the HRV14 antiviral drug Win52084 demonstrated that all cleavage was inhibited, consistent with the drug preventing particle dynamics and hence infectivity.

Understandably, the dynamic character of virus particles is not obvious from their crystallographic structures. The lattice nature of the ensemble requires that the 10^9 particles in a typical, well ordered, virus crystal must be closely similar with the intrinsic dynamics quenched by lattice forces or the selection of specific conformations during crystallization. The positive aspect of the crystal is the visualization of a detailed image of the particle (often at 3Å resolution or better) in its lowest energy form under the conditions of crystallization. Computational chemistry can provide accurate, short trajectory, dynamic descriptions of virus particles, but the amplitudes of biologically relevant motions are generally greater than those achievable by computation[11]. Thus, until recently, indirect methods comparable to those described have been the primary means of characterizing larger scale virus particle motions.

The last five years has seen an unprecedented revolution in structural biology in general[12] and, for the sake of this article, structural virology in particular[13] due to advances in electron microscopy. Electron microscopes with extraordinary stability and equipped with recently developed “direct electron” detectors, have produced many virus structures at 3.5 Å resolution or better when the sample is prepared by “flash freezing”. Achieving such resolutions with CryoEM is notable and the effort and amount of material required to do this has decreased dramatically compared to that required for virus crystallography. However, an equally exciting aspect of the technology is the unconstrained dynamic nature of the sample. At the moment of freezing (which occurs in a few milliseconds) the virus particles occupy the full range of dynamic states that are intrinsically available in solution. In the extreme case these can be clustered into individual categories and structures determined for discrete states that the particles occupy. However, the most common experimental result is a distribution of particle states that is continuous and it is impossible to distinguish individual states with clustering algorithms. We have proposed an approach to characterize both discrete and continuous heterogeneity that is described in the following section. There are at least two alternatives that are post-processing approaches, which are applied after a traditional reconstruction has been computed. One approach is resampling [14] in which new image stacks are created by randomly selecting images from the original stack. Then a reconstruction is computed from each new image stack and the sample variances of the voxels in the reconstructions are computed and used to characterize the heterogeneity. Since multiple reconstructions are computed, computational requirements are substantial. The variance of each voxel is the typical goal because covariance between pairs of voxels would require even more computing. A second approach is to estimate the covariance of the voxel values from the sample covariance of the images[15]. Resolution has been restricted in this approach since the entire map is limited to 16 voxels on each side, and it is uncertain how to incorporate the fact that there are other sources of variability in the images beyond the heterogeneous nature of the particles. The “opinion” presented here describes a novel method for the analysis of continuous particle distributions and the derived results that gives direct insight into virus dynamics and associated biological implications.

Seeing the “invisible”

Figure 1 describes a variety of Gaussian distributions characterized by different standard deviations (sigma values). A typical cryoEM experiment can be viewed as a distribution of this type with an ensemble of virus particles (typically 10-20 thousand) imaged. Barring any irregularities, different experiments with the same virus under the same conditions should give the same distribution. Thus, the distribution is a characteristic of the particle population for a given virus under a given condition. Ensembles in which the particles are closely similar will have a small standard deviation and be tightly bunched (red line, figure 1), while particles that are more heterogeneous (e.g., particles that were raised to a higher temperature) will have a broader distribution with a larger standard deviation (blue or pink lines, figure 1). Traditional reconstruction algorithms[16,17] as well as state of the art approaches[18] generally ignore the nature of these distributions orienting and averaging the particles to arrive at a composite electron scattering density.

A specific virus under different conditions may display different distributions, but if the average structure is the same the average scattering density will be similar for all the distributions, although the larger standard deviations may reduce the resolution of the reconstruction, providing evidence of the heterogeneity.

We employed an approach described schematically in Figure 2 and illustrated in Figure 3 that explicitly determines the continuous distribution of the particle ensemble while determining the averaged scattering density[19]. This provides a three-dimensional map of the variance over the population as well as the cryoEM structure that is comparable to that obtained by other methods. It is instructive to illustrate the value of such information with a simple example. Figure 3a shows the one-dimensional scattering density that would be typical of an RNA virus particle. The region of the capsid protein is well ordered and has low variance, while the region in the less ordered RNA has higher variance, but the average density is closely similar to that of the protein (3c), a situation frequently found in RNA viruses [20]. Model data were created to make the point. A small random variation was assigned to the region of the capsid and a larger variation was assigned to the RNA. 200 “particles” with this behavior were created (individual particle scattering densities are shown in figure 3b) and the averaged scattering density and variance computed (3c). If only the average electron scattering density is considered, the particle density appears uniform. If, however, the density variance is evaluated the difference between the capsid and the RNA is readily discernible and the RNA envelope is well defined. Thus features that are invisible in the scattering density are clearly defined in the variance map.

Variance Analysis of *Nudaurelia capensis* Omega Virus (NøV) Maturation

NøV shares similar structural and functional features with FHV. It is a ssRNA bi partite virus with a 644 amino acid subunit that undergoes an autocatalytic cleavage at position 570 to create a gamma peptide corresponding to residues 571-644 that remains with the particle after cleavage and is internal in the X-ray structure[21]. NøV, however, is a T=4 virus with 240 subunits in the capsid. Expression of the capsid protein in a baculovirus system produces a procapsid, purified at pH 7.6, that can be matured *in vitro* by lowering the pH to 5[22]. Figure 4 summarizes the NøV maturation process that is characterized by two major events. The first is a fast (ms) conformational change driven by acidification and consequent protonation of negatively charged aminoacid residues found in the intersubunit contacts[23]. The other event is the progressive and slow (min – hours) autocatalytic cleavage triggered by intrasubunit structural transitions that follows capsid rearrangement, for recent reviews see [24,25].

Variance analysis of partially cleaved particles at the different time points are shown in figure 5a with large variance at 1 minute and 30 minutes and small variance at four hours. The observation suggests that there is protein frustration at low pH in particles that have not undergone cleavage and that this is relieved as maturation cleavage occurs. Indeed, particles unable to cleave due to mutation show large variance at low pH that is not relieved with time indicating that the particles are sampling many structures when a cleaved ground state cannot be achieved[26].

The variance analysis method was extended further to characterize the order of cleavages in the different subunits. The average variance was determined for voxels within 10Å of the cleavage site in the A, B, C, and D subunits. These are plotted in Figure 5b. It is clear that at 3 and 30 minutes after acidification the A and D subunit cleavage sites have significantly lower variance than B and C, while they are all equal and low after 4 hours. A straightforward interpretation of the data implies that the A and D cleavage sites form uniformly rapidly and lead to early cleavage, while B and C vary significantly over the ensemble and are still in the process of forming at 30 minutes, resulting in slower cleavage. These data corroborates early conclusions obtained by time resolved analysis of NøV maturation by Cryo-EM difference density maps around the cleavage site of the four quasi-equivalent positions after lowering the pH from 7.6 to 5. The partially cleaved capsid generated extra densities that were not observed in the mature capsid and provided qualitative evidence for increased dynamics. Those followed exactly the same trend that observed by variance analysis[27].

More than confirming the order of cleavage, the variance properties of the capsid helped to establish different roles for gamma peptides generated in different structural contexts of the capsid. NøV gamma, similarly to peptides generated during FHV, reovirus or picornaviruses maturation [28], can disrupt membrane bilayers and are, therefore, required for infectivity. We have previously demonstrated that maturing NøV capsids acquire maximum lytic activity within the first 30 minutes of cleavage, suggesting that only a special subset of gamma peptides is able to interact with membranes. A cutway view of completely cleaved NøV capsids colored by variance values shows that the center of the capsid fivefolds contacts are more dynamic than other regions of the capsid shell, including the sixfolds, where D gamma resides (figure 5c). Therefore, the function of the gamma peptides in the fast cleaving A and D subunits are different, with those in A subunits forming dynamic helical bundles able to interact with membranes, while those in D subunits form a portion of the molecular switch that leads to the formation of the T=4 capsid, further stabilized by gamma derived from B and C subunits. [29].

Conclusion

The maximum likelihood method described explicitly considers the distribution of the ensemble of particles in the cryoEM experiment allowing the variance of each voxel to be plotted on its three dimensional location. This is reminiscent of temperature factors in crystallography except that they are not derived from a model and they characterize much larger, biological relevant, dynamic motions. Future work will couple these motions to mechanical models that exploit not only the variance, but also the correlations among different regions of the particle.

Figure Captions

Figure 1. The panel shows a plot of Gaussian distributions with various values of variance (sigma). Regarding the cryoEM experiments described, the vertical axis is the probability of measuring a particular density value at a particular voxel in an ensemble of particles and the horizontal axis is the value measured. For simplicity the plots are centered at zero, but in the experiment the maximum probability would be centered at non-zero values characteristic of each voxel. The figure illustrates that without regarding the sigma value all the distributions give the same average value for the voxel.

Figure 2. The figure describes the algorithm used for simultaneously computing the structure and the characterization of ensemble heterogeneity by a maximum likelihood (ML) estimator. The particle is described by the mean and covariance of its Fourier coefficients, which are assumed to be Gaussian. The mean determines the structure while the covariance characterizes the heterogeneity. The ML estimator functions by computing a cost that measures the difference between (1) the structure (determined by the mean of the Fourier coefficients) and the heterogeneity (determined by the covariance of the Fourier coefficients) that is predicted by the current description of the particle (top row) and (2) the experimental images. The cost is then used to update the current description of the particle (bottom row) until convergence is achieved.

Figure 3. A cartoon representation of the expected density distribution of an RNA virus shown in cross section, the red circle highlights the RNA. (A) The one-dimensional representation expected for the particles in the data set with the center at $X=0$. The radius of the entire particle is R and the diameter goes from $-R$ to R . The radius of the RNA is R_{RNA} and the diameter of the RNA goes from $-R_{RNA}$ to R_{RNA} . The Y axis corresponds to the scattering density. The region of the protein shell (with icosahedral symmetry) is plotted in blue and the variation. RNA is represented by the red region with large variation over the data set because it does not have the icosahedral symmetry of the shell. (B) The electron scattering density of 24 model particle images with low variance for the protein and higher variance for the RNA. The coordinates of the protein shell are highlighted in red (bottom graphs) and goes from $-R=-0.5$ to $-R_{rna}=-0.25$ and from $R_{rna}=0.25$ to $R=0.5$. (C, left) The average electron scattering density for 200 “particles” as represented in B. The electron scattering density is essentially the same for the entire region between $-R$ and R with no distinction between protein and RNA. (C, right) The standard deviation at each voxel for the 200 particles. It is small for the protein shell, but much larger for the RNA allowing the observation of the envelope for the RNA from the variance in the data set.

Figure 4. Summary of N ω V maturation. (a) N ω V assembles as procapsids that show no autocatalytic activity. The procapsid conformation is stable at pH 7.6 but acidification drives the transition from the procapsid state (490 Å diameter) towards the compact capsid state (410 Å diameter). The change in quaternary structure triggers the autocatalytic cleavage reaction of protein α , producing the main capsid protein β (residues 1-571) and the γ peptide (571-644) that remains non-covalently associated with the capsid. Although the conformational transition from procapsid to capsid is fast (ms) the autoproteolytic reaction is slow taking days to complete. The plot of the cleavage kinetics was derived from SDS-PAGE analysis of the maturation time-course. Note the

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4 unusual kinetics, with fast and slow components. (b) The tertiary structure of one of the
5 subunits of N ω V showing the location of the cleavage site between residues N570 and
6 W571. The zoom-in view shows that the cleavage site is closely similar for the 4
7 asymmetric units of the N ω V capsid (shown in blue-A, red-B, green-C and yellow-D). (c)
8 The arrangement of the four asymmetric units in N ω V T=4 mature capsid. A-subunits
9 form pentamers, while B, C and D form pleated hexamers. Thick black lines represent
10 bent contacts ($\sim 138^\circ$) and dashed lines highlight the flat contacts of the capsid. Black
11 crosses indicate the locations of the cleavage sites inside the capsid. An inside view of the
12 capsid is showed on the right, highlighting the differences in the γ peptide structure for
13 each of the subunits. γ peptides are depicted with strong colors and N570 is shown as
14 spheres. The flat interfaces of the capsid are stabilized by the c-terminal helix of γ
15 peptides coming from C and D subunits, shown as rods. Residues forming the switch
16 helices in the C and D subunits are not ordered in A and B.
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21 Figure 5. Subunit-specific dynamic properties of N ω V maturation intermediates (a) Time
22 resolved N ω V maturation data sets were used to extract dynamic information from Cryo-
23 EM reconstruction using the variance method. The structural models of capsids at each
24 maturation state were solved de novo using the maximum likelihood algorithm that
25 explicitly takes into account the continuous variability of the particles present in the data
26 set. The result describes the heterogeneity of the particle as a variance plotted at every
27 voxel of the reconstructed density. This new methodological approach allowed the
28 analysis of the whole particle dynamics during maturation, as shown by the surface
29 coloring of the capsids. (b) The graphs show that the variance computed for voxels
30 corresponding to 10 Å around the cleavage site of the four quasi-equivalent positions.
31 This analysis confirmed that maturation is clearly asymmetric. A and D subunits cleavage
32 sites are the first to be stabilized, accounting for 50% of the subunits cleaved after 30
33 minutes in pH 5.0 (see cleavage kinetic in figure 4a). B and C take longer to get in
34 position and form the slow component of the kinetic curve. (c) The cut-way view of the
35 mature capsid (3 days) was colored with a different heat-map scale to reveal the relatively
36 high dynamic region in the center of the capsid pentamers. Compare the position of
37 gamma peptides shown in ribbon on the same cut way view of the protein capsid shell
38 derived from the crystal structure reconstruction.
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20 describes the linear transformation. $C_y = LC_xL^T$ is itself a linear equation: if C_y (C_x) is arrayed as a vector v_y (v_x)
21 then there is a matrix W such that $v_y = Wv_x$. Since cryo EM is a linear imaging system, if the image is y and the
22 structure is x then statistics of the image (i.e. C_y or equivalently v_y) are related to statistics of the structure (i.e., C_x or
23 equivalently v_x) by $v_y = Wv_x$. After estimating the sample statistics of the image, this equation is solved for the
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Figure 1
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Ensemble distributions with various values of sigma

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