Towards An Integrative Structural Biology Approach

Combining Cryo-TEM, X-ray crystallography, and NMR

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Cryo-electron microscopy and particularly single particle analysis is becoming an essential technique for structure determination of viruses and protein complexes. Single particle analysis is a structural technique where 2D Transmission Electron micrographs of individual, randomly orientated protein complexes or viruses can be mathematically aligned, through image processing techniques, to generate a 3D volume of the specimen [1,2,3,4,5]. The samples are typically either encased in a heavy metal stain (negative stain), such as uranyl acetate, or imaged at cryogenic temperatures with the sample embedded in vitreous ice, free of strain (Cryo-TEM) [6,7]. In order to prepare specimens for cryo-TEM, the specimen is applied to a carbon coated EM grid with a series of small holes (µm size range). The sample is blotted away, leaving a thin film with the specimen residing within the small holes. The grid is then rapidly plunged into liquid ethane or propane and cooled to liquid nitrogen temperatures. The low vapor pressure and high thermal capacity of the liquid ethane or propane results in extremely rapid freezing where ice crystals are not formed, rather water molecules are trapped in the same orientation they had in the aqueous phase. This forms amorphous, vitreous ice and preserves the specimen in a frozen hydrated state, which mirrors the native state. The specimen must then be maintained at cryogenic temperatures in order to prevent a phase transition, which would result in formation of crystalline ice and damage to the specimen [7]. The resulting sample is subsequently imaged in an electron microscope, and reconstruction software is used to create the 3D structure from the individual particle images. Although individual particles are imaged at low contrast, the resulting structure can be extremely high resolution due to averaging of thousands of particles. Classically single particle analysis, and particularly when applied to cryo-TEM studies, has been thought of as a very low resolution (~30Å resolution) structural technique that can be applied to only very rigid, megadalton sized protein complexes; such as the ribosome, or highly symmetric icosahedral viruses.

While large protein complexes and viruses are still being studied, cryo-TEM has undergone a revolution, aided by advancements in sample preparation, electron microscope technology, automation in data collection, detector technology, and image processing [2,3,4,5]. Taken as a whole, these advancements have allowed cryo-TEM researchers to determine protein structures at atomic resolution and addressed biological research questions previously thought

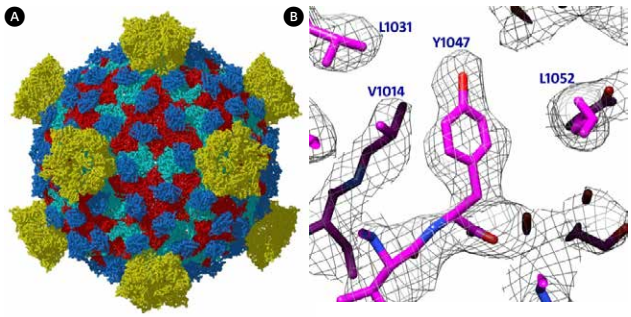

Figure 1. (A) 3.3Å. structure of a non-enveloped icosahedral virus using cryo-TEM. (B) Image of a selected region were individual side chains are clearly visible in the cryo-TEM reconstruction validating the high resolution of the reconstruction. Images kindly provided by Z. Hong Zhou Electron Imaging Center for Nanomachines (EICN) CNSI and Department of Microbiology, Immun & Mol. Genetics, UCLA, and adapted from [8].

unattainable by the method. For instance in 2009 Zhang et al. published the seminal atomic resolution 3.3Å aquareovirus structure in the journal Cell where individual side chains were clearly resolved [8] (**Figure 1**). Due to the extremely high resolution obtained in the structure, autolytic cleavages site were visualized which revealed a novel priming mechanism for cell entry for non-enveloped viruses. The same year Liu et al. published a 3.6Å structure of adenovirus in the journal Science [9]. In the same issue, an x-ray crystal structure of adenovirus at 3.5Å was also published [10]. Steven Harrison (Harvard) wrote in a commentary about the two structures, stating that while the x-ray structure was at higher resolution, the Cryo-TEM structure actually showed substantially better details on a more difficult and more biologically relevant type of the virus [11]. Cryo-TEM had unequivocally bested x-ray crystallography and is currently considered the premier method for icosahedral virus structure determination. This is further echoed in a recent publication (Confessions of an icosahedral virus crystallographer) by structural virologist, Jack Johnson (Scripps Research Institute), where he details his methodological change from using x-ray crystallography to study virus structure to solely using cryo-TEM [12].

Cryo-TEM is also rapidly being applied to smaller (sub-megadalton) protein complexes. For instance cryo-TEM has been proven the superior method for studying the trimeric HIV surface spike and has been instrumental in proving that the spike can undergo dramatic conformational changes upon binding to the target host cell proteins [13,14,15] (**Figure 2**). Visualization of these conformational states could prove critical in future drug design. Currently, numerous research groups are now applying cryo-TEM methods to sub 500kDa sized proteins complexes, and recently Wu et al. published a sub-nanometer structure of HIV integrase bound to Fabs resulting in a total molecular weight of 110kDa [16].

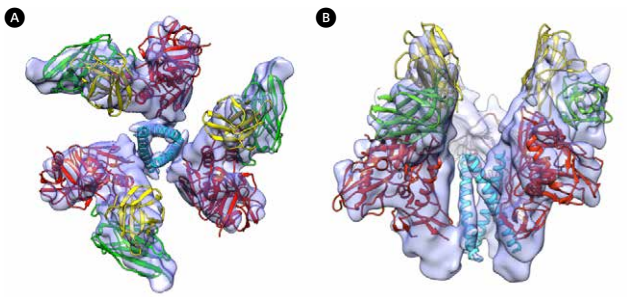

Figure 2. 9Å three-dimensional reconstruction of soluble gp140 HIV envelope glycoprotein trimers bound to 3 copies of the Fab fragment from 17b, a neutralizing antibody whose binding mimics that of the co-receptor. The structure revealed the presence of a previously unknown "activated" intermediate state, where three buried helices become exposed and potentially accessible to binding by entry inhibitors. X-ray crystallographic and NMR-based structural studies have not been able to determine intact trimeric HIV envelope glycoproteins structures. Determination of the structure of the envelope glycoproteins is critical to understanding HIV entry and infection and for rational vaccine design. (A) is a top view of the trimer and (B) is a side view of the trimer. images kindly provided by Sriram Subramaniam, Lab of Cell Biology, National Cancer Institute, National Institutes of Health. Adapted from [15].

One of the more common specimens applied to cryo-TEM research is the ribosome. While some of the very early ribosome structures solved by single particle analysis were at very modest resolutions <50Å, currently researchers are able to routinely solve ribosome structures at nearly atomic resolution. For instance, Bai et al. developed and published a rapid data collection and image processing scheme where, within a week, a ~4Å ribosome structure can be solved [17]. The process is so rapid that it is considered "in silico purification" which can assess the presence bound co-factors and ribosomal accessory proteins. Recent advancements in the use of complementary metal-oxide-semiconductor (CMOS) direct electron based detectors to record images have been essential to these methods.

Not only has cryo-TEM been able to generate very high resolution reconstructions of the ribosome, it has proved invaluable to visualizing dramatic structural changes that occur during RNA translation. Using "4D" or time resolved cryo-TEM, Fischer et al. have been able to reconstruct numerous intermediate structural states of ribosome within a single dataset using advanced image processing algorithms [18] (**Figure 3**). Combining these structural intermediates resulted in a movie of the process of translational. Additionally, by determining the occupancy of each conformational state it was possible to calculate the entire thermodynamic landscape through the whole dynamic process.

Single particle analysis has resulted in numerous biological insights, however as is the case with several other research methods, it is the combined use of various techniques that often result in the most significant scientific advancements, where the whole is greater than the sum of its individual parts. There is strong belief that hybrid methods are the future of structural biology, with cryo-TEM being an

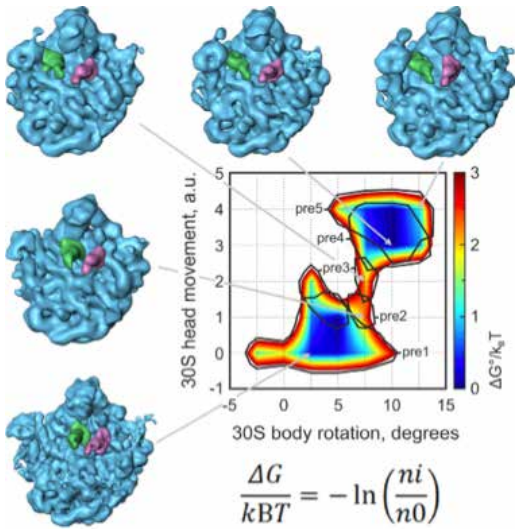

Figure 3. Use of time-resolved cryo-TEM to visualize ribosome dynamics and tRNA movement. Furthermore, using the ratio particles residing in each state, the entire thermodynamic dynamic landscape of the transition between states was determined. This is first example of how using cryo-TEM structural information to calculate thermodynamic parameters. Image kindly provided by Niels Fischer and Holger Stark, MPI Gottingen. Adapted from [19].

essential method in these studies. In this context, two studies in particular, combining cryoTEM, x-ray crystallography, and molecular dynamics simulations have resulted in groundbreaking results. For instance, a recent publication combined cryo-TEM structural studies of the areolysin pore with x-ray crystallography and molecular dynamics analysis [19]. Areolysin is a member of an important and widely distributed pore forming protein. There were several essential questions in the pore forming field, such as how areolysin like proteins oligomerize and form pores in the membrane. To address these questions Degiacomi et al., used cryo-TEM single particle analysis to generate 3D reconstructions in three intermediate states ranging from a heptameric prepore state to a transition to the pore state (**Figure 4**).

The monomeric crystal structure in combination with earlier EM analysis resulted in a perplexing issue, where sugar moieties present in glycosylphospatidylinositol-anchored proteins face away from the membrane, as well as having receptor binding sites positioned 10 nm above the membrane surface. Both of these observations were in direct conflict with biochemical information. Cryo-TEM provided elegant clarity to this issue as well as a mechanism for pore insertion into the membrane. Visualization of the prepore states and intermediate states showed an unexpected and dramatic conformational change due to a major twisting motion where areolysin converts the prepore state into the pore state by folding inside out. This allows the pore to insert into the membrane in the functional pore state. When combined with

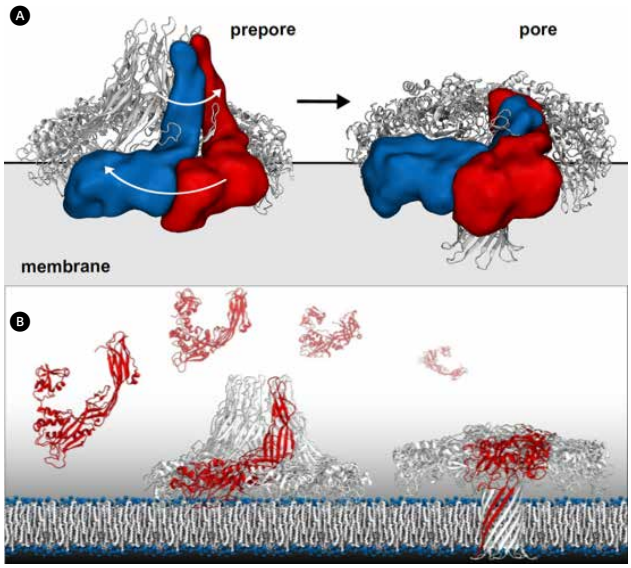

Figure 4. (A) Cryo-TEM reveals various conformational states of areolysin pore formation where a prepore states transitions to a functional toxin pore inserted in the target membrane. Combining X-ray crystallography, Cryo-TEM, and molecular dynamics modeling revealed a novel swirling membrane insertion mechanism to form the pore. (B) Combining these methods allowed for an atomic interpretation of the transition from monomer to prepore to the functional pore state. Images kindly provided by Matteo Dal Peraro, Biomolecular Modeling – LBM Institute of Bioengineering, School of Life Sciences École Polytechnique Fédérale de Lausanne - EPFL & Swiss Institute of Bioinformatics - SIB Adapted from [20].

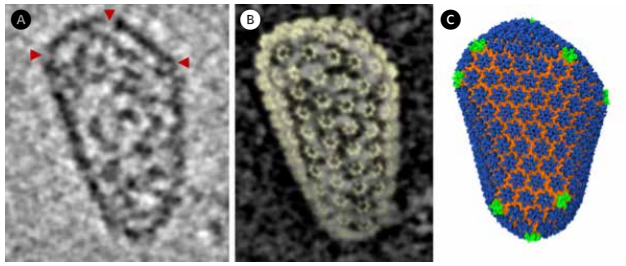

Figure 5. Using cryo-tomographic analysis of individual, native "fullerene" cone HIV-1 capsid, combined with the high resolution cryo-TEM hexamer structures; an all-atom molecular dynamics HIV-1 capsid model was created. This model highlights the three-fold capsid protein C-terminal domain as an attractive therapeutic target. (A) Slice through cryo-tomographic volume of an individual mature capsid. Red arrows highlight CA pentamers. (B) Yellow stars highlight the locations of CA pentamers. (C) The tomographic density matches the shape and size of the capsid, shown by the overlay of densities from the segmented capsid and the fullerene model (yellow). (C) Final molecular dynamics equilibrated all-atom capsid model. Images kindly provided by Peijun Zhang Univ. of Pittsburgh. Adapted from [21].

the crystal structure and molecular dynamic simulation this entire process was modeled and understood at the atomic scale and resulting in a very satisfying correlation between biochemical information and structural information. This study is truly an excellent example of the synergy of hybrid methods resulting in a major breakthrough in this field of research. Moreover, while still impressive, the single particle reconstructions were solved at modest resolution (16-18Å), yet yielded an incredible amount of biochemical insight.

Similar to the aerolysin research, hybrid methods were applied to analysis of HIV capsid formation [20]. Zhao et al. applied specialized cryo-TEM methods to address capsid formation: helical analysis and cryo-Electron Tomography (CET). Whereas single particle analysis is typically applied to monodispersed protein complexes randomly oriented in vitreous ice; Helical analysis is applied to periodic helical or tubular structures. While there are several examples of helical filaments in biology, it is also possible to induce proteins to form 2D periodic sheets or tubes [21]. Zhao et al. were able to form tubular arrays of hexameric capsid proteins and generate an 8Å reconstruction of the capsid protein. While the x-ray crystal structure of the capsid protein was previously solved, the tubular reconstruction revealed novel contact sites between capsid pentamers that are essential in supporting the formation of the full viral capsid. These same contacts supported formation of the periodic tube. After docking of the x-ray crystal structure into the cryo-TEM density map an all atom molecular dynamic simulation was performed. Without the novel contact site discovered by cryo-TEM, it would not have been possible to perform the simulation.

In the same publication the structure of the mature viral capsid was also determined. The viral capsid forms a fullerene cone comprised of pentamers and hexamers. However, it is a highly variable, pleomorphic structure and is therefore intractable to structural techniques that require averaging of structurally homogeneous particles. However CET is ideally

suited to study such structures. CET is analogous to an CT scan, where a scanner rotates around a specimen (i.e. a person), collecting images at each tilt allowing for generation of a 3D reconstruction of the specimen. In CET instead of tilting the microscope, the specimen is tilted within the column of the electron microscope [4,22,23,24]. With this method 3D tomographic volumes of individual mature capsids were generated. Combining a lower resolution tomographic volume as a constraint, the structures of pentamers, hexamers, and novel contact sites determined by tubular analysis, allowed for creation of an atomic model of the entire HIV capsid and subsequently a whole atom molecular dynamic simulation of the entire capid as well (**Figure 5**). To date it is the largest molecular dynamics simulation on a protein and was truly a heroic effort. More importantly the model highlighted the three-fold capsid protein C-terminal domain as an attractive therapeutic target and provided extremely useful biochemical information.

It is becoming abundantly clear that cryo-TEM is an essential research technique in structural biology, whose application and functionality will only grow into the future. The pace of research and number of publications are increasing rapidly each year. More importantly the complexity and variation of experiments is increasing as well. Several studies currently undertaken by cryo-TEM researchers would not have been considered possible even a year or two ago. The use of robotics in sample loading, improvements in TEM optical stability, automated data acquisition software packages and the use of direct electron detectors have revolutionized the cryo-TEM research field [25,26,27,17,28]. Not only have these technologies significantly advanced this field of research, they have also dramatically improved the ease of use of cryo-TEM, particularly through automation, and lowered the threshold for researchers in adjacent fields to adopt this technology. There is truly a great level of excitement in the structural biology research community concerning the future of research using this method.

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