Review

The Leeuwenhoek lecture 2006. Microscopy goes cold: frozen viruses reveal their structural secrets

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The electron microscope provides a powerful tool for investigating the structure of biological complexes such as viruses. A modern instrument is fully capable of atomic resolution on suitable non-biological specimens, but biological materials are difficult to preserve, owing to their fragility, and to image, owing to their radiation sensitivity. The act of imaging the specimen severely damages it. Originally, samples were prepared by staining with a heavy metal salt, which provides a stable specimen but limits the amount of details that can be retrieved. Now particulate specimens, such as viruses, are prepared by rapid freezing of unstained material and observed in a frozen state with low doses of electrons. The resulting images require extensive computer processing to extract fully detailed three-dimensional information about the specimen. The whole process is referred to as single-particle electron cryomicroscopy. Using this approach, the structure of the human hepatitis B virus core was solved at the level of the protein fold. By comparing maps of RNA- and DNA-containing cores, it was possible to propose a model for the maturation and control of the envelopment of the virus during assembly. These examples show that cryomicroscopy offers great potential for understanding the structure and function of complex biological assemblies.

Keywords: electron cryomicroscopy; virus structure; hepatitis B virus; image processing

1. INTRODUCTION

The invention of the electron microscope in the 1930s gave scientists, for the first time, the potential to visualize biological particles, such as viruses, that were too small to be seen using light microscopy. Among the earliest specimens to be examined were bacterial viruses or bacteriophages, but little could be discerned from the images apart from the general size and shape of the particles. Many technical developments in the instrument itself, in specimen-preparation techniques and in image analysis were necessary for the full potential of this approach to determining biological structures to be realized. I shall describe here some of these developments and show, in particular, how they have led to an understanding of the structure and function of the human hepatitis B virus.

Three hundred years ago, Antoni van Leeuwenhoek, after whom this lecture is named, was at the forefront in applying microscopy to microbiology. A draper by trade, he was a respected member of the community in Delft, holding various municipal offices. At the age of 40, he took up microscopy. Over a period of 50 years, he reported a remarkable series of observations in more than 100 letters to the Royal Society, of which he was elected a Fellow in 1680. His letters were read at the meetings of the Society and many were published in *The Philosophical Transactions*. His small handheld instruments were simple microscopes with a single tiny spherical lens,

essentially a magnifying glass, but of such small focal length that he could achieve magnifications of up to approximately 300 times. This was sufficient for him to visualize bacteria for the first time. He employed a draftsman to make drawings of the various specimens viewed through the microscope, and the depictions, though not very detailed, are sufficiently accurate for some of the species to be identified. The significance of his investigations was rediscovered by Clifford Dobell (1932) and summarized in an earlier Leeuwenhoek Lecture (Hall 1989), so I shall not elaborate further. It suffices to say that as a microscopist of sorts myself (and sharing a first name), it is particular honour and pleasure to have been asked to give this lecture.

The fineness of details that can be visualized with the light microscope is limited by the wavelength of light itself. It was thus necessary to find some other way of probing matter, and in the 1930s, Ernst Ruska and colleagues in Germany invented the electron microscope. Ruska was belatedly awarded the Nobel Prize for his contributions to the development of the electron microscope, and he included in his Nobel lecture (Ruska 1986) an early example of a micrograph of bacteriophage, taken by his brother in the 1930s. A bright source, such as a pointed heated filament, provides electrons that are then accelerated by a high voltage and focused by magnetic lenses to make a collimated beam to illuminate the specimen. In order for the beam to propagate, the interior of the microscope column has to be under high vacuum, and the specimen is inserted

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through an airlock. An objective lens and projector lenses form a magnified image of the specimen, which can be viewed on a fluorescent screen or recorded on film or on an electronic detector. Typically for biological specimens, one works with a final magnification on the film of 30 000–60 000 $\times$. Thus, the image is at least 100 times more powerfully magnified than Leeuwenhoek could achieve, and I wonder what he would have made of such an instrument. Indeed, the resolving power of modern electron microscopes is sufficient to visualize individual atoms directly, but this depends on having a specimen, such as a metal or semiconductor, robust enough to withstand the damaging effects of the electron beam. Biological particles are far more fragile and require special methods of preparation, appropriate schemes for recording the images and then sophisticated computer processing to reveal the structure. Although it is not yet possible to see atomic details, we can now visualize the secondary structure of proteins in particulate biological specimens, such as viruses.

2. SPECIMEN PREPARATION

In the late 1950s, it was realized that detailed images of viruses could be obtained by embedding them in a coat of a heavy metal salt, such as uranyl acetate. The salt is relatively stable in the electron beam and scatters electrons strongly. The biological material is interpreted as holes in the stain, a bit like making a plaster cast. Sydney Brenner introduced the term negative staining for this technique, by analogy with a similar technique in light microscopy (Brenner 1997). You can see the molecular substructure mapped out on the surface of the virus and also any cavities into which the stain penetrates. However, the information obtained is pretty much limited to surface features, and the resolution set by the granularity of the stain is not much better than 20 Å. Nevertheless, this approach was very important in establishing the molecular architecture of viruses and is still useful as a quick and robust method of visualizing particulate specimens.

From the earliest electron micrographs, it emerged that simple viruses were either rod shaped or spherical. Crick & Watson (1956) presented a powerful hypothesis, based on genetic economy and protein interactions, to suggest that in simple viruses the coats would be made from multiple copies of a coat protein, symmetrically arranged. For rod-shaped viruses, this would imply helical symmetry, whereas for spherical or isometric viruses the symmetry would be cubic. For a spherical closed shell, the largest number of subunits that can be accommodated is 60, when the symmetry is that of an icosahedron. To build shells containing more than 60 subunits requires a relaxation of the condition of strict equivalence of subunits and, as shown by Caspar & Klug (1962), certain multiples of 60 are allowed. This formalism provided a powerful impetus to the interpretation of features seen in micrographs of negatively stained viruses.

Further progress had to await the invention of a new technique for preparing specimens. This was plunge freezing developed during the mid-1980s by Jacques Dubochet and his colleagues at the European Molecular Biology Laboratory in Heidelberg (Adrian *et al.* 1984;

Figure 1. Preparation of rapidly frozen grids. (a) The freezing apparatus, showing the plunger, forceps for holding the grid and container for the cryogen. A small quantity of virus suspension is placed on the microscope grid and blotted to leave a thin film. (b) Enlarged view showing the grid held in forceps, about to be plunged into ethane slush held at liquid nitrogen temperature. (c) The resulting frozen grid has a thin film of vitreous ice spread over holes in a holey carbon film, as seen in this low-magnification electron micrograph. (d) The virus particles are suspended in this vitreous layer, illustrated here in cross section, and the grid can now be transferred into the microscope.

Dubochet *et al.* 1988). A small drop of solution containing the virus is put onto a microscope grid, on which there is a holey carbon film. The excess liquid is blotted away leaving a very thin film of the virus suspension across the grid, which is then plunged into ethane slush held at liquid nitrogen temperature (figure 1). The film is so thin that it freezes in a tiny

fraction of a second, so rapidly that it cannot form ice crystals but rather becomes the so-called vitreous or glass-like ice. This obviously provides a nice water-like environment for the virus, with no heavy metal stains around. However, the microscopy now gets trickier, as the grid must not warm up or the vitreous ice will turn crystalline. The grid has to be kept at liquid nitrogen temperature while it is transferred into the microscope and during recording of the pictures. This is achieved by cryotransfer devices and special microscope cold stages. The specimen is now very radiation sensitive—the act of imaging with electrons destroys the specimen—so pictures have to be recorded with very low doses of electrons. The pictures are of low contrast, because there is now no heavy metal stain to enhance the scattering. This means that the images have to be deliberately taken out of focus to enhance the contrast. Extensive computer processing is then needed to correct the images and recover the details of the structure. However, an important point is that it is now the biological material itself, not the stain, that is being imaged, so there is some hope of seeing the internal details of the structure.

3. IMAGE PROCESSING

Owing to the large depth of focus of the electron microscope compared with the size of viruses, all the features at different heights in the virus are simultaneously in focus, and the recorded two-dimensional image is a projection of the three-dimensional scattering matter in the specimen. That is to say, features at the top and bottom and at all levels in between become superimposed in the image and are therefore difficult to unscramble. In a single projected view, a particular feature could have come from any height in the specimen. You can imagine therefore that to get back to the three-dimensional structure, you have to look at the specimen from different directions, because then the different features superimpose in different ways. You can get the different views either by tilting the specimen inside the microscope or by using particles sitting in different orientations on the grid or by using internal symmetry in the specimen. Another difficulty is that the images of the specimen are quite noisy, owing to the limited number of electrons used to record them, so the information from many particles has to be averaged to reinforce the signal coming from the specimen and cancel out the noise.

The problem of unscrambling the projection data was solved by DeRosier & Klug (1968). They exploited a mathematical device called the Fourier transform, which splits the density in the image into all its two-dimensional spatial frequency components. This two-dimensional set of components of the projected density gives the corresponding central section of the three-dimensional Fourier transform of the object density. Thus, different projected views fill in different central sections of the transform, and once sufficient views have been included, the three-dimensional object density can be recovered by adding the components together in a Fourier synthesis. Fortunately, there are very efficient computer programs available for carrying out Fourier analysis and Fourier synthesis. Besides the projection/section relation, we can also exploit Fourier

transforms for finding the orientations of particles, correcting the defocus and for general quality control.

The overall scheme for processing icosahedral virus particles is shown in figure 2. The micrograph has first to be scanned with a film scanner to turn it into an array of numbers representing the blackness on the film. It is then in a suitable form to be processed by computer. After a particle has been selected, its approximate centre is found and then the orientation is determined, that is to say the direction you are looking at it relative to the icosahedral symmetry axes is established. For highly symmetrical particles, like viruses, this can be done using the so-called common lines in the Fourier transform (Crowther 1971). With an approximate orientation, the origin can be refined and the process can be repeated cyclically. Owing to the low contrast and high noise level, not all the parameters may be correct at this stage. By repeating this procedure for a number of different particles, a dataset is built up from which a preliminary three-dimensional map can be computed (using the section/projection relation). Assuming that most of the parameters were roughly correct, the map will be an approximate representation of the specimen but with a much reduced noise level compared with the original images. The computed projections of the map can then be used as a reference for determining and refining by cross-common lines the parameters of particles brought in from additional micrographs (Crowther *et al.* 1994). The determination of particle parameters in this way by cross-common lines against the current best model is much more reliable than using the self-common lines of individual images, owing to the reduced noise level in the computed projections and the greater number of common lines that can be used. (An alternative formulation of model-based refinement, which has the same advantages as cross-common lines, has been proposed by Cheng & Baker (1996).) In this way, more and more particles are brought in, their parameters become more accurately defined and the computed map shows finer and finer reliable structural details. The fineness of reliable details can be assessed by splitting the particle set in half and computing two independent maps from the half sets. The two maps are then correlated as a function of spatial frequency to determine to what extent the fine details of the structure have been reliably determined.

4. HEPATITIS B VIRUS

Hepatitis B virus is a major human pathogen, a member of the Hepadnaviridae, a family of viruses that replicate in the liver (Ganem & Schneider 2001). It is estimated that there are at least 350 million carriers of the virus worldwide, that is, 5% of the world's population, and that it causes in excess of one million deaths a year from liver disease. It is particularly prevalent in central and eastern Asia and in sub-Saharan Africa. Hepatitis B is transmitted by contaminated blood products and dirty needles, sexually and also perinatally from infected mother to baby. Infection as an adult generally leads to an acute disease, which is cleared by the immune system, leaving the person immune to further infection. Infection as a baby from

Figure 2. Outline of the scheme for processing the images of virus particles. The micrograph has first to be digitized on a film scanner to convert the blackness of the film into an array of numbers suitable for processing in a computer. Particles are selected and boxed out, and approximate centres and orientations relative to the icosahedral symmetry axes are found by self-common lines (Crowther 1971). A preliminary three-dimensional map is calculated from an initial set of images. From the map, a set of projections can be calculated, effectively simulating particle images but with a greatly reduced noise level. This set of projections provides a basis for determining by cross-common lines the centres and orientations of additional particles selected from further micrographs (Crowther *et al.* 1994). The whole process is iterated, increasing the number of particles included, refining their parameters more accurately and increasing the resolution of three-dimensional map.

a carrier mother often leads to chronic infection and carrier status. In this condition, cirrhosis and primary liver cancer can ensue. There is an effective vaccine but, as is often the case, there are logistical and economic problems in reaching those in need.

Hepatitis B virus has a layered structure (figure 3*a,b*). On the inside is an icosahedral core containing the genetic material, in this case partially double-stranded DNA, consisting of one complete copy of the genome plus one partial copy on the incomplete matching DNA strand. The core is surrounded by an envelope consisting of lipid membrane containing virally encoded surface proteins. Hepatitis B virus has a very compact genome approximately 3200 bases long (figure 3*c*). It codes for four different kinds of protein: the core protein itself and a secreted variant; the surface proteins that come in three sizes; the viral polymerase that copies the genetic material; and a mysterious protein X, whose function is not really understood. Remarkably, the surface protein genes entirely overlap the viral polymerase gene and are coded by DNA bases just one base out of phase from the polymerase. This is an extraordinarily compact genome.

For structural studies, it is difficult to work with the virus itself because it is a pathogen and because it is quite hard to make the virus in sufficient quantity. However, by cutting out the core gene and expressing it in bacteria, we can make a lot of the protein in a form

that is non-infectious and non-pathological. This is a standard approach now for studying pathogens and has been made possible by developments in molecular biology and genetic engineering. Some of the earliest applications of this approach to hepatitis B virus were described in a previous Leeuwenhoek lecture (Murray 1987). The core protein when expressed in bacteria assembles into icosahedral core shells (figure 3*d*), which are very similar to the cores in hepatitis B virus itself. Using the methods of cryomicroscopy outlined above, a three-dimensional map of the core shell was computed at 7.4 Å resolution (figure 4; Böttcher *et al.* 1997). This was a sufficiently good map for the fold of the protein, which is largely α -helical, to be deduced (figure 5*a*). Furthermore, it was possible, using biochemical and immunological data, to propose an amino acid numbering scheme for the protein chain. Subsequently, the structure of the core shell was solved to higher resolution by X-ray crystallography (Wynne *et al.* 1999), confirming and extending the results from microscopy (figure 5*c,d*). If suitable well-ordered crystals can be produced, then X-ray crystallography will give atomic details, as was the case here for the hepatitis B core. However, in many situations, well-ordered crystals of the particular specimen of interest cannot be made. Single-particle microscopy then gives the chance of studying such specimens at a level of detail finer than

Figure 3. Hepatitis B virus. (a) Schematic showing the inner nucleocapsid or core containing the DNA and viral polymerase, surrounded by the lipid-containing envelope with embedded viral surface proteins. (b) Cryoimage of a similar hepatitis virus from woodchuck showing the layered structure. (c) Diagram of the genome of the virus showing the various protein-coding regions. (d) Cryoimage of shells of the bacterially expressed core protein. Scale bar, (b,d) 500 Å.

Figure 4. Map of the hepatitis B core protein (Böttcher *et al.* 1997). (a) View of the whole shell. Map computed from approximately 6300 particle images from 34 micrographs. (b) Enlarged view of the boxed area indicated in (a). This form of the shell is made from 240 copies of the core protein, so after icosahedral averaging there are four computationally independent subunits, which are here coloured blue, red, yellow and green. The red–blue and yellow–green molecules form closely associated dimers. The positions of the icosahedral twofold, threefold, fivefold and local threefold axes are indicated (2, 3, 5, I3), as are the positions of the N- and C-termini of the protein. The fold of the four subunits is identical, and the thicker tubular regions correspond to α -helices. Bundles of four helices (an example is encircled) form the spikes protruding from the surface.

10 Å. Fitting crystallographic maps of subcomponents into lower-resolution maps from microscopy of molecular complexes can often give useful information about molecular interactions in the complex.

The core shells consist of 240 copies of the core protein arranged as 120 dimers. Each dimer makes a spike that protrudes from the shell and consists of two

α -helical hairpins, one from each molecule in the dimer. The spike thus consists of a four-helix bundle (Böttcher *et al.* 1997; Conway *et al.* 1997; Wynne *et al.* 1999). The shells that were analysed in these studies were made from core protein with the basic C-terminal region removed (figure 5b). This is the region of the protein that interacts with the packaged nucleic acid,

Figure 5. Fold of the core protein. (a) Fold deduced from the cryomicroscopy map (Böttcher *et al.* 1997; figure 4). (b) The amino acid sequence of the core protein, indicating where the protein was truncated after residue 149 to remove the very basic C-terminal region. (c) Fold determined by X-ray crystallography (Wynne *et al.* 1999). (d) The crystallographic structure of the core shell viewed down an icosahedral fivefold axis, with the four independent subunits coloured red, yellow, blue and grey.

and in its absence, the shells are empty. Functional studies require the full-length protein and packaged nucleic acid. General aspects of the structure and assembly of core protein shells have been reviewed by Steven *et al.* (2005).

Hepatitis B is a pararetrovirus, which means that at different stages of its life cycle (figure 6) its genome may be in the form of RNA or DNA, and that reverse transcription of RNA to DNA is an essential part of that cycle (Summers & Mason 1982). The virus enters the hepatocyte via an unknown cellular receptor. The partially double-stranded DNA passes into the nucleus, where it is turned into completely double-stranded circular DNA. Various messenger RNAs are then transcribed from the DNA and these go on to direct the synthesis of viral proteins using the host cell machinery. When the viral polymerase is translated from an RNA message that corresponds to the whole viral genome, it stays associated with this copy of the message, and then the core proteins, which have been separately synthesized, assemble around the complex to form an immature core particle. The viral polymerase then gets to work inside the core copying the RNA genome into the first strand of DNA, degrading the RNA as it goes. The second matching strand of DNA is then started, and by the time one-half to

three-quarters of the second strand has been made, the core becomes mature. It can then interact with surface proteins, which have been separately synthesized and inserted into an inner cellular membrane, through which the core buds. The core thus gets enveloped and subsequently secreted from the cell to complete the viral cycle (for a review of envelopment, see Bruss 2004). Budding does not occur before second-strand DNA synthesis, so it has been hypothesized that a maturation signal must be propagated from the interior of the core, where the DNA is located, to the surface of the core where interaction with the surface proteins and membrane takes place (Summers & Mason 1982).

In order to investigate the nature of the maturation signal, it was necessary to compare the structure of immature RNA-containing cores, made by expressing the full-length core protein in bacteria, with the structure of mature DNA-containing cores, extracted from viruses isolated from infected blood samples (Roseman *et al.* 2005). The two structures (figure 7) are very similar to each other and to the empty core particle made from truncated protein (figure 4). In the cut-away parts of the maps, the additional material corresponding to the nucleic acid can be clearly seen, particularly in the RNA case. There are, however, small but significant differences between the two maps,

Figure 6. Life cycle of hepatitis B virus. After infecting a liver cell, the viral DNA passes to the nucleus where it is repaired and transcribed by cellular enzymes. The viral messages are translated, and when the viral polymerase is synthesized it stays associated with the full-length message from which it was translated, forming a complex around which the core protein assembles. The viral polymerase reverse transcribes the RNA into single-stranded DNA and then into partially double-stranded DNA. By this stage, the core has become mature and able to interact with the surface proteins that have been inserted into an intracellular membrane (ER). The core thus gets enveloped and the assembled virus is secreted, completing the cycle.

distributed over much of the protein shell (figure 8a). There are clear changes in the shape of the spike, showing that the interactions between helices in the four-helix bundle are different in the two structures, but there are also small changes in other places in the shell. It is as if the shell were acting as a mechanism by which changes on the inside are propagated through the protein to the outside, causing changes in the spike. One of the most significant changes is in a hydrophobic pocket that opens on the side of the spike in the DNA structure (figure 8b).

The probable functional importance of this region of the core protein is highlighted by the clustering there of various mutations that affect viral secretion (Ning & Shih 2004). A frequent natural mutation, observed in chronically infected patients, of isoleucine 97 to leucine (I97L) leads to the secretion of immature virus particles containing single-stranded DNA (Yuan *et al.* 1999). Two other natural mutations, proline 5 to threonine (P5T) and leucine 60 to valine (L60V), give very low secretion of virus (Le Pogam *et al.* 2000), and, strikingly, the P5T mutation corrects for the immature secretion of the I97L mutant when present as the double mutant I97L, P5T (Chua *et al.* 2003). Two other residues close by (leucine 95 and lysine 96) when changed to alanine result in cores in the cytoplasm that do not become enveloped or secreted (Ponsel & Bruss 2003). These amino acids in the core protein (P5, L60, L95 and I97) create a hydrophobic pocket on the side of the spike (figure 8b) that appears to be more open in the cores containing DNA than in the cores containing RNA. Most importantly, a mutation of alanine 119 to phenylalanine (A119F) in the surface protein can also

compensate for the effect of the I97L mutation in the core protein (Le Pogam & Shih 2002), indicating a probable site of molecular interaction between the core and surface proteins during assembly.

These observations suggest a model (Roseman *et al.* 2005) in which DNA synthesis inside the core causes changes in the core protein shell that propagate to the outside and alter the hydrophobic pocket in the spike. In the immature RNA-containing core, the pocket is in a closed state that cannot interact with the surface proteins, but after DNA synthesis the open pocket is able to interact with the surface proteins, and envelopment and secretion ensue. Mutations in the core protein may both modulate the affinity for surface protein and affect the way changes in structure propagate through the core shell. Thus, mutants P5T and L60V may either create a pocket that does not interact well with surface protein or form structures in which the pocket does not open properly, resulting in low secretion. In the I97L mutant, the pocket may be constitutively open or more easily opened by changes inside the shell, leading to premature secretion before DNA synthesis has proceeded. I97L and P5T could mutually compensate by creating a pocket with appropriate affinity or time of opening. The diverse nature and position of these functional mutations suggest that the core structure is a finely tuned mechanism for controlling appropriate envelopment.

What could be acting to trigger the observed changes? A possible answer to this question comes from the study of the assembly of a bacterial virus, a bacteriophage called $\phi 29$. In this phage, as in many others, the preformed empty head is filled with the double-stranded DNA genome by a packaging motor situated at a special point on the head. By single molecule measurements, it has been shown that, as the head is filled with double-stranded DNA, the packaging motor has to work increasingly hard against the bending, electrostatic and entropic forces generated by the close packing of the double-stranded DNA within the head (Smith *et al.* 2001). We have a somewhat similar situation in the hepatitis B core, in that single-stranded and therefore flexible RNA is being reverse transcribed within the limited volume of the core, first into single-stranded DNA, with the degradation of the RNA, and then into stiffer double-stranded DNA. There will be a corresponding change in charge density and nucleic acid flexibility that will have the effect of greatly increasing the force exerted on the inner surface of the capsid. We believe it is this increasing force that triggers the change in the core structure. Analysis of individual DNA molecules from virions shows a range of lengths for the partial second strand from about one-half to three-quarters of the genome, with a minimum length for the single-stranded region of 650–700 bases (Delius *et al.* 1983). It thus appears that the force generated by double-strand synthesis is sufficient to trigger change by the time between one-half and three-quarters of the genome has become double-stranded and envelopment will ensue. This idea is supported by the observation that in a mutant lacking the RNA-degrading activity of the native polymerase, the cores containing the resulting RNA–DNA double-stranded hybrid molecule become enveloped and

Figure 7. Hepatitis B core maps (Roseman *et al.* 2005). (a) Bacterially expressed full-length core protein containing RNA. (b) Mature DNA-containing core extracted from virus. The additional nucleic-acid-associated material (coloured grey) is apparent on the inside, where the outer protein shell has been cut away.

Figure 8. Differences between RNA and DNA maps. (a) Strongest significant differences between the RNA and DNA maps superimposed on part of the RNA map, viewed from outside the core shell. Red areas indicate where the RNA map is stronger and blue where the DNA map is stronger. There are red regions on the outside of the spikes and a blue region inside the spike between the helical hairpins. (b) Closer view of the extra density (grey surface) in the DNA map, between the helical hairpins. The extra density marks a pocket formed by various functionally important and mainly hydrophobic residues (P5, L60, L95, K96 and I97; see text). The two chains forming the dimer are coloured yellow and green, with side chain of I97 in red.

secreted, whereas in a mutant lacking polymerase activity and unable to synthesize DNA, no secreted single-stranded RNA containing virus-like particles could be detected (Gerelsaikhon *et al.* 1996). Production of double-stranded DNA is a very direct way of controlling the whole secretion pathway but still allows a role for mutations in the core protein to modulate the structural transitions associated with maturation and envelopment.

5. ANTI-VIRAL STRATEGIES

So what can we do to fight back against viruses? The situation is more complicated than for disease-causing bacteria, where quite a wide range of antibiotics with bacteria-specific targets is now available. Since viruses use the cellular machinery to replicate, targeting this machinery is likely to do more harm than good. Most viruses do have a limited number of their own enzymes, but using these as anti-viral targets can quickly lead to resistance as the virus mutates, and combination therapies simultaneously targeting several enzymes are most effective. In the case of hepatitis B, a compound called lamivudine that targets the viral

Figure 9. Identification of the binding sites on the core of a peptide that blocks viral assembly. The positions of the peptide, determined from the differences between maps of the core with and without bound peptide (Böttcher *et al.* 1998), are indicated by the red areas at the tips of the spikes.

Figure 10. Maps of core shells with and without hantavirus insert. (a) Cryomicrograph of core shells without insert, showing predominantly the smaller form of the shell containing 180 subunits. (b) Core shells with hantavirus insert have additional material around the outside of the shell. (c) Map of the shell without insert. (d, e) Map of the shell with hantavirus insert viewed from the outside (d) or from the inside with half of the shell cut away (e). Note the additional material sticking out from the tips in (d) and (e) compared with (c), corresponding to the hantavirus insert (Geldmacher *et al.* 2004). Scale bar, (a, b) 500 Å.

polymerase has had limited success, but again resistant forms of the polymerase emerge.

Another possible approach to antiviral therapy might be through compounds that block some essential step in the complex viral assembly pathway. One such example is the compounds that misdirect the assembly of the core protein, so that it does not form properly closed shells (Deres *et al.* 2003; Hacker *et al.* 2003). Another example is the selection of a small peptide that binds to the core protein and blocks the interaction between the core protein and the surface protein (Dyson & Murray 1995). Using cryomicroscopy, we have shown that the peptide binds right at the tips of the spikes on the core (figure 9), a position very likely to interfere with the interactions between the core and surface proteins (Böttcher *et al.* 1998). This result demonstrates the power of cryomicroscopy for finding differences between structures amounting to only a few amino acids. The presence of the peptide significantly reduced the production of virus in a cellular transfection experiment, demonstrating proof of principle for using small molecules to inhibit viral assembly (Böttcher *et al.* 1998).

The structure of the core protein itself can be turned to advantage in the fight against disease. It has been shown that the core shell forms a very suitable framework for displaying parts of other proteins with a view to raising a strong immune response against the displayed parts (for review, see Pumpens & Grens 2001). The foreign pieces can be attached to the N- or C-terminus of the core protein or, more effectively,

inserted into the sequence at the tip of the spike. In many cases, the modified protein will still assemble into well-ordered shells when expressed in bacteria. These genetically engineered shells thus form the basis for a potential vaccine against whatever pathogen the inserted sequence comes from. For example, hantaviruses are an emerging group of viruses endemic in various rodent populations in different parts of the world, which can cause serious disease if humans become infected. When part of the hantavirus capsid protein is inserted into the tip of the spike, immunization of mice with the chimeric core particle produces a strong antibody response against the hantavirus protein (Geldmacher *et al.* 2004). Cryomicroscopy shows that the flexibly linked hantavirus fragment forms an extra shell of partially ordered material on the outside of the core protein (figure 10). Modified hepatitis B core particles may thus provide the basis for vaccines against hantavirus and other infections, such as malaria (Oliveira *et al.* 2005) or lyme disease (Skamel *et al.* 2006). For hepatitis B itself, there is already a very effective vaccine based on expressed surface protein particles.

6. CONCLUSIONS

The work described in this lecture demonstrates that it is possible for electron cryomicroscopy to contribute in a significant way to the understanding of the structure and function of viruses. In the case of the hepatitis B virus core protein, it was possible to

determine the fold of the protein and propose an amino acid numbering scheme. This was a particularly favourable case, as the protein has a large α -helical content, which can be visualized in a map at 7.4 Å resolution. Beyond this, it was possible to detect small but significant changes in the core structure related to maturation and the signalling for envelopment to take place. Small molecules consisting of a few amino acids could be detected when bound to the core, where they act to block envelopment. Single-particle microscopy is therefore a powerful tool for investigating viruses and other biological complexes and will become even more powerful, if some of the factors currently limiting the attainable resolution can be overcome (Henderson 2004).

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