Published in final edited form as:

Methods Enzymol. 2010 ; 483: 121–142. doi:10.1016/S0076-6879(10)83006-X.

Cryoelectron Microscopy Applications in the Study of Tubulin Structure, Microtubule Architecture, Dynamics and Assemblies, and Interaction of Microtubules with Motors

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Abstract

Cryo-EM is ideally suited for the study of cytoskeleton polymers and their interaction with cellular partners. Our understanding of microtubule (MT) structure and interactions has benefited tremendously from the application of different EM techniques, from the use of electron crystallography to determine the first high-resolution structure of tubulin, to electron tomographic reconstructions of unique MT-based organelles; from molecular details governing the regulated interaction of MTs with kinesin motors, to an atomic description of how antimetabolic agents bind to tubulin and affect MT stability. In this chapter, we review these structural findings with an emphasis on how cryo-EM enables our studies.

1. Introduction: The Role of Electron Microscopy in Tubulin Studies

Microtubules (MTs) are hollow, fibrous polymers present as part of the cytoskeleton in all eukaryotic cells. They play many essential roles, including intracellular transport, chromosome segregation, and cell movement. To carry out these functions, MTs should be very stable or highly dynamic, growing, and shrinking to allow the ends to explore the cell volume. In these activities, they interact with a large number of other proteins that affect their stability, act as motors using MTs as a track to move cargo, or make use of MT's dynamic character to localize or carry out work.

Electron microscopy (EM) has been intimately involved with many aspects of the study of MTs and their principal component protein, tubulin, and a great deal of what we know about structural aspects of MTs has come from EM. It is interesting to review how developments in EM technology have been frequently followed by new information about MTs. Soon after the development of ultramicrotomes for cutting resin sections suitable for EM, MTs were discovered by examination of sections of plant tissue (Ledbetter and Porter, 1963). The overall architecture containing 13 protofilaments of tubulin monomers was soon revealed (Ledbetter and Porter, 1964). Shortly after the development of techniques for deriving 3D models from EM images of helical structures, the arrangement of the subunits was resolved in negative stain (Amos and Klug, 1974). Soon after, it was discovered that 2D crystals

could be produced by polymerizing tubulin in the presence of zinc ions (Gaskin and Kress, 1977; Larsson *et al.*, 1976), and the nascent technique of electron crystallography was soon applied to obtain a slightly higher resolution map of the structure (Baker and Amos, 1978; Crepeau *et al.*, 1978). Further work on these crystals was limited by their small size and high disorder. As single-particle methods began to develop, methods were proposed for combining crystallographic and single-particle approaches to improve the data extracted from the tubulin crystals (Crepeau and Fram, 1981). This in turn led to the development of techniques for “lattice unbending” that proved to be key to extending EM resolution to the atomic level with protein crystals (Henderson *et al.*, 1986). At about this time, the technology of cryo-EM was being developed (Adrian *et al.*, 1984). The combination of these two advances led to the first protein structures solved by electron crystallography, including bacteriorhodopsin (bR; Henderson *et al.*, 1990) and the plant light harvesting complex (LHC; Kühlbrandt *et al.*, 1994). These successes inspired us to begin work on the tubulin crystals, starting with the demonstration that they could be prepared to produce electron diffraction to a resolution sufficient to build an atomic model (Downing and Jontes, 1992). At about the same time, cryo-EM was being used to study the structure and assembly/disassembly processes of MTs (Mandelkow *et al.*, 1991). The crystallographic approach eventually led to the full atomic structure of tubulin (Löwe *et al.*, 2001; Nogales *et al.*, 1998), and the characterization of the interactions of tubulin with various anticancer ligands (Nettles *et al.*, 2004; Snyder *et al.*, 2001). More recently, new approaches in cryo-EM have been developed to visualize MTs and other tubulin assemblies at improved resolution, and interacting with a variety of cellular partners, most remarkably kinesin (Sindelar and Downing, 2010). Electron tomography has been used to study larger and much more complex tubulin-based complexes (Bui *et al.*, 2009; Nicastro *et al.*, 2006; Sui and Downing, 2006). In this chapter, we focus on the various applications of cryo-EM in the crystallographic determination of the tubulin structure, the characterization of assembly and disassembly intermediates in MT dynamics, and on the interaction of MTs with motor proteins.

2. Tubulin Studies by Electron Crystallography

Our efforts to study tubulin by electron crystallography began with work to improve the crystals themselves and to prepare them for high-resolution study. It had long been known that under many conditions tubulin loses its ability to polymerize with a half life of just hours, so the early attempts to grow crystals used incubation times on the order of 20 min (Baker and Amos, 1978; Larsson *et al.*, 1976). The crystals were a few tenths of a micron in size, and apparently sufficiently disordered to limit the resolution even in negatively stained preparations. We eventually found that in the presence of salt and protease inhibitors and at slightly subphysiological temperatures incubation for up to 24 h was possible, yielding crystals over a micron in size (Nogales *et al.*, 1995). Since the time of the earlier experiments, Taxol had been discovered as the first of a series of small ligands that stabilize MTs. We found that Taxol also stabilizes the crystals, and it was routinely added after crystal formation to avoid any problems of crystals depolymerizing, for example, from decrease of the temperature during sample preparation. The first attempts to embed these crystals in glucose, simply following the approach that had been successful with bR, gave

somewhat promising results (Downing and Jontes, 1992). Inspired by the use of tannin embedment with catalase and LHC, we obtained even better diffraction patterns extending to 3.5Å with tannin. We also applied the “back injection” technique that had been worked out with bR and then applied to LHC, modified for a combination of tannin and glucose. A small square of carbon film was floated from mica onto a 1% tannin solution and picked up on a bare molybdenum grid. The crystal suspension was injected into the lens of liquid adhering to the grid. An equal volume of 1% glucose was then added and the solution thoroughly but gently mixed. The grid was then simply blotted, briefly air dried, and frozen in liquid nitrogen. The high sugar concentration avoids crystallization of the water on freezing, and samples prepared this way have shown diffraction spots up to 2.5Å. Figure 6.1 shows an example of a diffraction pattern from a tubulin crystal embedded in the tannin–glucose mixture. While this may not be considered to be a cryo-EM sample in the most common sense, all of our work has been done with the specimen at low temperature using all of the protocols that are generally applied with frozen-hydrated specimens.

As has been common with all the other samples studied by electron crystallography, the tubulin crystals have been plagued by issues of specimen flatness (see Chapter 11 in MIE volume 481). This problem is manifested with tilted crystals in the rapid loss of diffraction spot sharpness and intensity with increasing distance from the tilt axis (Glaeser *et al.*, 1991). The interpretation of this observation is that the specimen is not perfectly flat, and variations in tilt angle cause sampling of different parts of the reciprocal lattice rods in different areas of the crystal. Depending on the length scale of the variation in tilt, the diffraction spots may blur increasingly with increasing distance from the tilt axis or may simply disappear. The use of molybdenum grids rather than copper reduces the effect somewhat because molybdenum has a lower thermal expansion coefficient, closer to that of carbon, leading to less wrinkling of the carbon when the sample is frozen. While it is still not possible to generalize the complete solution to the problem, use of a particular type of carbon rod, evaporation conditions, age of the carbon film, and other factors seem to play critical roles in obtaining good diffraction. These factors may vary significantly from one type of specimen to another. We have found that, as with bR, it is important to use carbon films that are on the order of 1–2 weeks old for best results with tubulin. The “double carbon film” specimen preparation technique (Gyobu *et al.*, 2004), in which a second carbon film is placed on the droplet adhering to the grid before blotting and freezing, appears to provide a very substantial improvement in crystal flatness. For the tubulin crystals, the success rate dramatically increased by limiting the amount of blotting, leaving a meniscus of tannin–glucose on the grid with only small areas in each square of the right thickness, which eliminated the deleterious effect of the liquid removal and added mechanical strength to the grid.

One can estimate the number of diffraction patterns or images that are required to fill reciprocal space with sufficient sampling to reach a target resolution of d as $N = \pi D/d$, where D is the protein thickness. For tubulin at a resolution of 3.5Å, this number would be about 50. Factors such as the nonuniformity of angular sampling, limited range of tilt angles, and especially the noise level influence the number actually needed. The initial data set for diffraction amplitudes used about 100 diffraction patterns (Nogales *et al.*, 1998), and this

was subsequently expanded to 200 with most of the new patterns collected at higher tilts (Löwe *et al.*, 2001). About 130 images were used to obtain structure factor phases. Data collection and processing generally followed the protocols of the MRC image processing system, which for the images included several cycles of lattice unbending (Crowther *et al.*, 1996).

One enhancement for image recording developed during the course of this work was the implementation of a procedure for dynamic focus correction with spot-scan imaging. All of the images were collected using a spot-scan protocol, in which the beam is focused to a diameter of 300–1000 Å and stepped over the image in a 2D raster rather than illuminating the entire area at once (Downing, 1991). This approach had been shown to give greatly improved data quality, presumably by reducing beam-induced specimen movement during imaging. With tilted specimens, the beam was scanned in the direction of the tilt axis and the focus was adjusted by an amount between scan lines to keep the entire image at the same focus (Downing, 1992). This greatly reduced the effort involved in image processing by removing the focus ramp that normally occurs across the image of a tilted sample, in addition to eliminating any loss of quality that might occur in areas of high defocus.

The initial 3D density map was calculated at a nominal resolution of 3.7 Å, and while the quality was at least as good as that of a typical X-ray map at the same resolution, fitting the polypeptide into the map was a challenge. Nonetheless, a full atomic model was built which left very little ambiguity and revealed essentially all the details of the long-sought tubulin structure (Nogales *et al.*, 1998). With additional diffraction data and application of structure refinement procedures from X-ray crystallography, the quality of the model improved along with extension of the resolution to 3.5 Å (Löwe *et al.*, 2001). Figure 6.2 is a section of the density map used to derive the final structure, showing the high quality of the data and strong constraints that it provided for structure building.

Figure 6.3 is a ribbon diagram of the tubulin dimer structure. The alpha- and beta-tubulin monomers share a high degree of structure similarity, with a core of beta sheet surrounded by alpha helices. The N-terminal part of the chain forms a Rossmann fold, having alternating strand and helix segments quite similar in topology to the fold found in proteins such as GAPDH. Each of the loops connecting a beta strand to the following helix has some contact with the nucleotide, a significant departure from many other GTP- and ATP-binding proteins, which coordinate most of the nucleotide via the so-called “P-loop.” A second domain is formed by a series of beta strands and helices, with the “core helix” providing a strong interface between the domains. A third domain is identified as containing a pair of helices running along the surface of the monomer on the side opposite from the core helix. The essential C-terminal extension of tubulin, containing the last 12–15 residues in alpha and beta-tubulin was unstructured.

It is important to realize that the zinc-induced sheets we refer to here as “crystals” are in fact a polymer form of tubulin, involving large intradimer and interdimer contacts along protofilaments that are conserved with the MT (see later). Just like MTs, these polymers can go through cycles of assembly and disassembly with temperature, they depend on GTP, and they are stabilized by Taxol. Thus the tubulin electron crystallographic structure goes

beyond defining the fold of the tubulin molecule and carries functional information with direct relevance to our understanding of MT assembly.

Important for understanding the properties of nucleotide-binding, hydrolysis, and exchange, which are central to the process of MT dynamic instability, the structure shows that the nucleotide sits at the surfaces of the monomers that are buried in intermonomer contacts along protofilaments. The GTP bound to alpha-tubulin within the alpha-beta dimer is firmly trapped at the intradimer interface. While the GDP bound to beta-tubulin appears just as trapped within the protofilament, its position at the inter-dimer interface renders it exchangeable at the surface of a free dimer. This arrangement thus explains the differences in exchangeability of the nucleotide in the tubulin monomers and the change in exchangeability upon polymerization: the nucleotide in beta is exchangeable only in the dimer state but becomes buried and thus nonexchangeable, like in alpha, within the polymer. The position of the exchangeable nucleotide and the structure of the interdimer interface readily tells us about how hydrolysis of the nucleotide in beta is stimulated by residues from alpha concomitant with polymerization.

The biggest difference between alpha and beta-tubulins is found in the loop between helices 9 and 10, which is 10 residues shorter in beta-tubulin. Most interestingly, filling the space left by this deletion is the binding site of the broadly used cancer therapeutic Taxol. This location of Taxol suggested ways in which it might stabilize MTs, either by favoring contacts between protofilaments or by inhibiting relative movement of the nucleotide-binding and intermediate domains (Amos and Löwe, 1999; Nogales *et al.*, 1998). Smaller structural differences between the GDP-bound beta-tubulin and the GTP-bound alpha-tubulin are located in the T5 loop (involved in ribose binding), its neighboring helix H6, helix H10, and the M and T7 loops. These are all essential regions in longitudinal (T5, H6–H7 loop, T7) and lateral (M-loop, H10).

3. Microtubule Structure

From the birth of cryo-EM, application of this technique to the study of MTs has been highly revealing about the structure and behavior of these polymers. As early as 1985, the MT lattice was resolved by cryo-EM at a resolution at least as good as the earlier work with negative stain, allowing a 3D reconstruction that showed the arrangement of monomers (Mandelkow and Mandelkow, 1985). In the course of this work, MTs sometimes disassembled due to cooling of the sample prior to freezing. Depolymerization occurred with peeling of protofilaments away from the MT into spirals and rings with a narrow distribution of diameters. Such rings had been seen earlier in stain and characterized by X-ray scattering, and the cryo-EM work now suggested that they represented the natural, curved form of protofilaments containing GDP. This in turn led to models for storage of the energy of hydrolysis as strain within the MT, where the protofilaments are constrained by lattice contacts to be straight (Caplow *et al.*, 1994). A series of papers used the improved preservation of the cylindrical shape of frozen-hydrated MTs to characterize variations in protofilament numbers and the associated surface lattice (Chrétien and Wade, 1991; Song and Mandelkow, 1995; Wade *et al.*, 1990). Furthermore, helical reconstruction techniques were being applied to the study of naked and kinesin-bound MTs, with resolutions

approaching 20Å. By the time the tubulin crystal structure became available, reconstructions were thus available to dock the crystal structure into the MT density.

The protofilaments in the zinc-induced crystals are antiparallel, while those in MTs are parallel. Thus, while the crystal structure provided a clear view of the interactions within dimers and between dimers along the protofilaments, the actual interaction between protofilaments, as well as the orientation of the dimer in the MT, was still unknown. The asymmetry of the tubulin dimer was sufficient to allow docking into an MT map having a resolution around 20Å with enough precision to clearly define the general orientation and regions of contacts between protofilaments. The C-terminal domain faces the outside of the MT, defining a crest for motor binding, while the Taxol binding site faces the lumen of the MT, near the site of lateral contact between protofilaments (Nogales *et al.*, 1999). The loop connecting H7 to S9 along the side of the monomers, named the M-loop, plays a major role in lateral contacts between protofilaments via its interaction with the loop between H1 and S2 in the N-terminal domain of the adjacent subunit.

In an effort to improve understanding of the MT structure, we developed a protocol for MT image processing based more on a combination of single-particle and crystallographic methods than on helical reconstruction. The MT images were boxed into small regions, typically about 10 dimers in length, and each subsection was aligned to a reference. We focused our analysis on 13-protofilament MTs, which are not strictly helical because of a discontinuity or “seam” in the surface lattice where alpha and beta monomers meet, but are amenable to averaging along the axis. With a target resolution in the 5–10Å range the differences between alpha and beta could be ignored, giving a structure which is an average of the two monomers. This approach gave better tolerance to the distortions that are inevitable in a structure like that of the MT and allowed extension of the resolution to about 8Å, sufficient to visualize most of the secondary structure (Li *et al.*, 2002). This improved resolution led to a far greater precision in docking the crystal structure into the density map and showed more clearly the interaction of the M-loop with loops from the adjacent monomers. This work has recently been extended to the derivation of maps with comparable resolution of MTs with 11, 12, 13, 14, 15, and 16 protofilaments (Sui and Downing, 2010). These new results confirm that the variation in protofilament number is accommodated by changes in the loop regions involved in interprotofilament contacts. Figure 6.4 shows a comparison of the six 3D density maps from this work.

While our approach for processing MT images and the inclusion of more data than in previous work certainly contributed much to the resolution extension, it may also be that the nature of the specimen preparations provided a substantial benefit as well. All of the bare MT images, as well as those of kinesin-decorated MTs described later, have been recorded at 400 kV. While this decreases the contrast below what is more commonly obtained at 120 or 200 kV, it allows working with thicker ice, which provides a more rigid structure and may produce images with higher inherent resolution. On the other hand, there are now well over a dozen reconstructions of MT complexes that all reach the 8–9Å resolution range but not beyond, suggesting that the inherent flexibility of MTs may currently limit resolution. Conformational heterogeneity could easily arise from distortions that produce an oval cross-section. If these distortions are in fact the source of the resolution limit, it should not be too

difficult to use classification techniques to identify them and computationally achieve significantly higher resolution.

4. Structure of Microtubule Assembly/ Disassembly Intermediates

A very interesting facet of MT dynamics is that the nucleotide-regulated tubulin assembly and disassembly processes occur via structural intermediates, rather than individual tubulin subunits directly adding to or leaving the MT lattice (Figs. 6.5A and 6.6A). These structural intermediates have been directly observed at the ends of MTs using cryo-EM. Mandelkow and colleagues first characterized the peeling of depolymerizing MTs via “ram’s-horn” intermediates (Mandelkow *et al.*, 1991). These structures are outwardly curved protofilaments that break down into ring-like structures closely resembling the double-rings formed de novo from GDP-tubulin (Frigon and Timasheff, 1975). On the other hand, Chrétien *et al.* (1995) described the presence of open sheet structures with slight outward curvature at the end of rapidly growing MTs. How do these distinct oligomeric states of tubulin relate to the structure and interactions of tubulin subunits?

The curved protofilaments at the ends of shortening MTs constitute a structural intermediate in the disassembly process where GDP-tubulin is in its relaxed state, clearly distinct from its constrained state in the body of the MT wall. We used the fact that high concentrations of divalent cations stabilize tubulin ring assembly, likely by a charge shielding mechanism that may be shared with MAPs (microtubule-associated proteins), to form tubular assemblies of GDP-tubulin in which ring closure does not occur and the protofilaments form a tight, double-layer helix (Fig. 6.5B). This helix, which can form a tube containing tens of turns, appears to recapitulate the shape of the horn-like protofilament structures at depolymerizing MT ends, and its intrinsic order facilitated its structural characterization by cryo-EM. The double-layer nature of these tubes results in systematic overlap of the Bessel terms from the inner and outer layers on all the layer lines, making impossible to obtain the orientation relationship between different images of the tubes directly using traditional helical reconstruction methods (see Chapter 5 in MIE volume 482). To overcome this issue and still take advantage of the helical symmetry of the tubes, we generated a modified Fourier space-based, helical reconstruction algorithm to deal with Bessel order overlap and the need of multiple projection views of the structure (Wang and Nogales, 2005a). This method relies on an iterative approach to generate accurate values for the relative orientations between different projection images. The overall procedure is as follows: first, we calculate initial reconstructions for the inner-layer and outer-layer helices using an initial input for the relative orientations of all the projection images (we demonstrated that these could be arbitrary values). We then use those initial 3D reconstructions as references, calculate their sum with different orientation combinations (for the inner and outer layers), and cross-correlate them with the raw projection images to find a new set of orientations for each of the projection images. At this point, we apply the new set of orientations to produce an improved 3D reconstruction of the inner and outer helices. The cycle of cross-correlation search and reconstruction is repeated until the result converges, that is, the orientations do not change and the resolution of the reconstruction does not improve.

We used the above methodology to produce independent 3D reconstructions of the inner and outer layers of the tube, using data up to 10Å resolution (Wang and Nogales, 2005b). Our structures showed, for the first time, distinctive intra- and interdimer interactions and thus a distinction between the GTP and GDP interfaces (Fig. 6.5B). While both interfaces are kinked, the bending angles are clearly different, and one interface is dramatically more flexible than the other (Wang and Nogales, 2005b). The cryo-EM, in agreement with X-ray studies of disassembled tubulin bound to the RB3 stathmin fragment (Ravelli *et al.*, 2004), showed that, irrespective of the presence or absence of a depolymerizer, the bending of the intra- and interdimer interfaces in GDP-tubulin (Fig. 6.5C) is incompatible with the formation of the lateral contacts which are present in MTs.

A two-step MT assembly process, involving a structural intermediate, was directly suggested by the cryo-EM studies of Chrétien and colleagues about a decade ago. They showed that under conditions of fast tubulin assembly, growth occurs via open sheets at the ends of MTs that later close into a cylinder (Chrétien *et al.*, 1995). A model of this process is shown in Fig. 6.6A. As a stable, surrogate assembly of those sheets we studied a tubulin polymer that forms in the presence of the nonhydrolysable GTP analogue GMPCPP when MT assembly is inhibited by low temperatures. These structures showed protofilaments to be slightly and smoothly curved, with small indistinguishable intra- and interdimer kinks between tubulin monomers (Wang and Nogales, 2005b; Fig. 6.6C). Most importantly, the structure showed the presence of alternating lateral contacts between protofilaments, which otherwise preserved the precise stagger between proto-filaments seen in the MT (Fig. 6.6B). This means that the structures would be able to convert into MTs without the need of longitudinal sliding between protofilaments, but simply by a rotation of the protofilaments. This type of arrangement, involving alternative lateral contacts without longitudinal displacements between protofilaments, is fully compatible with the extended sheets observed by Chretien and colleagues at the growing end of MTs, and their direct conversion into MTs by closure.

Altogether, the studies above support the separation of the process of straightening from the curved, depolymerized state to the straight proto-filaments in MTs into two stages: one, nucleotide-dependent, that allows for lateral association into a curved sheet, and a later one that occurs upon MT closure.

5. Mechanism of Kinesin Movement Along Microtubule

During the time that the crystal structure was being determined, substantial progress was being made in the study of motor-decorated MTs in order to understand the interaction of these essential motor proteins with MTs. A series of papers resolved the basic pattern of monomer and dimer binding of conventional kinesins and the minus end-directed *ncd*, most using helical reconstruction and leading to a resolution around 20Å (Arnal *et al.*, 1996; Hirose *et al.*, 1995; Hoenger *et al.*, 1995; Kikkawa *et al.*, 1995). Conformational changes in the motors were identified as a consequence of altering the nucleotide state (Rice *et al.*, 1999). To some extent, these changes could be correlated with structural changes in the motors seen by X-ray crystallography. However, there was no absolute correlation of the X-

ray structure conformations with the nucleotide, so it remained unclear how ATP binding and hydrolysis were associated with MT binding.

Following our determination of the 8-Å MT structure, we applied the same basic methods to the study of kinesin-decorated MTs. One enhancement of the approach was required by the fact that we could not average over the seam, so identification of the seam location in the 13-protofilament MTs became necessary. Again, a reference-based approach allowed identification of the proper orientation. Plotting the correlation between each segment of the MT image and a series of projections calculated for a full rotation of the reference model produces 13 peaks as the tubulin protofilaments align. The height of the peaks increases with increasing alignment of the kinesins, so that the proper orientation is identified from the highest peak. The series of correlation peaks provides a particularly robust determination of the angle along with a measure of confidence in its determination. Using this approach we reached the 8–9Å resolution range, allowing precise docking of a kinesin crystal structure into the map and identification of structural rearrangements that occur upon binding of the motor to the MT (Sindelar and Downing, 2007). Subsequent cryo-EM work with kinesin in a series of nucleotide states that reflect the full hydrolysis cycle has led to a fairly complete understanding of the conformational changes that drive motor stepping (Sindelar and Downing, 2010). Figure 6.7 shows density maps for the nucleotide-free and ATP states with the fitted atomic model, along with a cartoon representation of the conformational changes associated with nucleotide-binding and the power stroke.

One essential feature revealed by this work is that binding to the MT causes the stabilization of an extension of the switch-II helix of kinesin. This extension is absent in almost all of the dozens of X-ray crystal structures of kinesins in the relevant nucleotide states. While the conformational changes that had been seen in the previous cryo-EM studies could be correlated with different conformations seen in the X-ray structures, the lack of correlation between the conformation and the nucleotide state in these structures made it difficult to obtain much functional insight. However, from the cryo-EM work the paradigm became clear that formation of the helix extension stabilized components of the switch-I and -II regions which complete the binding pocket for the nucleotide. Binding of ATP then triggers tilting of the core of the motor with respect to the MT-attached domain, which in turn exposes the neck-linker binding region on the opposite side of the molecule. Docking of the neck-linker to this region then pulls the lagging motor head forward in a mechanical step along the MT.

An interesting finding of the higher resolution cryo-EM work is that the switch-I regions of the kinesin X-ray structures were not compatible with the experimental density of the cryo-EM maps, because the structure in this region changes upon MT binding and formation of the switch-II extension. However, the corresponding region from the actin-associated motor myosin in the ATP-bound state fits very well, as might have been predicted based on the evolutionary relation between the two motors.

6. Drug Binding Studied by Diffraction and Modeling

The tubulin–Taxol complex of the zinc-induced crystals served as an excellent starting point for understanding the interaction and mechanism of a number of potential anticancer agents and other ligands that affect MTs' roles in the cell cycle. The Taxol conformation itself and its binding mode have been the subject of intense interest as a number of groups have worked toward development of novel derivatives with improved properties. The conformation derived from electron crystallography differs substantially from conformations resolved by NMR measurements in either polar or nonpolar solvents. Because of the limited resolution of our data, there were also limits on the confidence in details of the conformation. It was thus of interest to synthesize a Taxol derivative that was locked in the conformation of the crystals to see if it would in fact bind and to identify differences in binding constants. A compound with this conformation did in fact yield higher activity than the native Taxol molecule, lending support to the crystallographic result (Ganesh *et al.*, 2007).

Diffraction methods have been well worked out in X-ray crystallography for identifying small differences in ligands or structure between related protein structures. Briefly, one can use differences in diffraction amplitudes to identify differences in two closely related structures. A change in structure will, of course, change both amplitudes and phases of structure factors, but for sufficiently small changes the differences in either one can be ignored. The method has been widely used in X-ray diffraction for studying changes in ligand binding or structure. Some success has also been obtained in electron crystallography, for example, in characterizing changes in bR during its photocycle, where tilts in alpha helices could be visualized at moderate resolution (Subramaniam *et al.*, 1993; Vonck, 1996). In the case of MT-stabilizing ligands bound to the tubulin sheets, we found that difference maps based on data sets of about 200 electron diffraction patterns could indeed confirm that several of the compounds bind in the same location. However, the data quality, including resolution, completeness, and signal-to-noise ratio, were not sufficient to unambiguously determine the orientation and conformation of the highly flexible compounds.

Extending the diffraction approach by incorporation of computational modeling led to a plausible model for the complex of epothilone-A with tubulin (Nettles *et al.*, 2004). An extensive library of candidate conformations of the epothilone molecule was derived, starting with a set of conformations that had been determined by crystallography and NMR. Each of these conformations was computationally docked onto tubulin in an initial energy-minimized orientation, and the complex was then refined against the electron diffraction data using the crystallographic programs *refmac* and *cns*. Only a very small subset of the trial complexes could be successfully refined, and these converged to one structure that was then further refined. The resultant structure is consistent with a wealth of structure–activity data that has been collected from variants of the epothilone motif (Nettles and Downing, 2009). On the other hand, it differs from a number of previous proposals derived by different computational modeling methods as well as subsequent models derived by NMR and SAR-based modeling (Reese *et al.*, 2007). Increasing the quality of the diffraction data to more strongly constrain the modeling should help to resolve whatever ambiguities remain.

7. Tomography for Larger Structures

As with the other aspects of cryo-EM, the development of cryoelectron tomography has paralleled advances in our studies of tubulin and the complexes it forms. Tomography gives access to some larger MT-based superstructures, and one of the more intensively studied of these is the ubiquitous axoneme found in eukaryotic flagella and cilia. It had long been recognized that this structure is formed by nine MT doublets—themselves complexes of one complete and one incomplete MT—along with (usually) two singlet MTs and a host of other proteins. It has been estimated that axonemes are made of over 600 proteins. By far the best characterized of these are the dyneins, motor proteins providing the forces that give the axoneme its characteristic undulating movement seen, for example, in the tails of swimming sperm and the flagella of *Chlamydomonas*. Tomography of relatively intact axonemes has been particularly productive in identifying the locations and interactions of several isoforms of dyneins that bridge adjacent MT doublets and cause them to move with respect to each other, as well as some of the closely associated subunits (Bui *et al.*, 2009; Heuser *et al.*, 2009). Because the axoneme is such a large structure, on the order of 2500Å in diameter, the resolution in such tomographic studies is somewhat limited. The signal-to-noise ratio, and thus the resolution, can be substantially enhanced by volume averaging of segments along the axoneme. Figure 6.8 shows the results of averaging subvolumes from tomograms of *Chlamydomonas* axonemes, revealing the locations and interactions of dyneins and other identifiable components (Bui *et al.*, 2009).

Studies of smaller components, notably the isolated doublets, have the potential to reach higher resolution. Again, the use of averaging is essential to boost the SNR to take advantage of the resolution inherent in the tomogram. In studies of sea urchin sperm doublets, the resolution was sufficient to construct a model of the tubulin arrangement within the doublet and then to use this model to identify features of the 3D density map corresponding to some of the nontubulin components (Sui and Downing, 2006). Figure 6.9 shows a section of the density map derived from tomography of isolated doublets, along with the pseudoatomic model of the tubulin component and nontubulin components identified by difference mapping. As in much of the other work currently underway using EM to study tubulin-based structures, the focus is now on the mechanisms and functional aspects of the interactions of tubulin with the vast array of its binding partners.

Electron tomography also gives access to studies of macromolecules in their native environment of the cell. This often involves specimen areas that are much too thick for EM even with intermediate voltage microscopes. Thus sectioning of vitreous frozen materials is required. While this has proven to be a difficult technology to master, recent developments have led to some notable achievements (see Chapter 8). MTs are among the most easily visualized substructures in typical eukaryotic cells. One of the surprises in several studies that focused on MTs in such sections was the appearance of material within the lumen of the MT (Cyrklaff *et al.*, 2007). In stained resin sections, densely staining material is often seen inside MTs, but it was never entirely clear whether or not this was a staining artifact. Observation of material in cryosections removed this doubt, since these specimens should be free of such artifacts. The appearance of material with periodic spacings within the MTs has

been particularly intriguing, although there is as yet no clear evidence of what such material might be.

8. Conclusion

Over the last 35 years, EM and particularly cryo-EM have proven crucial for our structural and functional knowledge of tubulin, its assembly into MTs and higher order structures, as well as for our understanding of how cellular factors interact with MTs and how anticancer agents bind to tubulin and affect its assembly properties. As in the study of other polymers of biological relevance, EM has offered the unique possibility of studying the large, functionally active assemblies that constitute the biologically relevant unit. With structural information spanning from the high-resolution electron crystallographic studies of tubulin to the tomographic analysis of the cellular MT cytoskeleton, there is no question that EM has provided, and will continue to provide, vivid, direct information on the complex and essential MT system and the molecular mechanisms that underlie its cellular functions.

Acknowledgments

This work was supported by NIH grant GM51487 and by the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. E. N. is a Howard Hughes Medical Institute Investigator.

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Figure 6.1.

Diffraction pattern from a tubulin crystal embedded in a mixture of tannin and glucose. Diffraction spots are frequently seen well beyond 3.5 Å.

Figure 6.2.

Section of the refined electron crystallographic density map used to derive the final structure of the tubulin dimer. This section includes the GTP bound to alpha-tubulin, which is shown as a ball-and-stick model, along with a sphere representing the associated Mg ion. The atomic model of the protein is shown as a wire structure, with several of the residues in the neighborhood of the nucleotide marked.

Figure 6.3.

Ribbon diagram of the tubulin dimer structure. This view shows the surface facing the lumen of the microtubule, with the plus end toward the top. Several of the secondary structure features and cofactors are marked.

Figure 6.4.

Three-dimensional reconstruction of microtubule structures. This figure shows the inner (left) and outer (right) surfaces of six 3D density maps obtained from microtubules with 11, 12, 13, 14, 15, and 16 protofilaments described in Sui and Downing (2010).

Figure 6.5.

Structure of microtubule disassembly intermediates and their relationship to tubulin conformation and nucleotide state. (A) Artistic rendition of the microtubule depolymerization process via peeling of protofilaments concomitant with the relaxation of GDP-tubulin into its low energy state. (B) Cryo-EM reconstruction of GDP-tubulin assembled into double-layered tubes closely corresponding to GDP-tubulin rings and a surrogate for depolymerizing protofilaments. (C) Schematic of the kinked dimer conformation observed in the cryo-EM reconstruction of GDP-tubulin.

Figure 6.6.

Structure of microtubule assembly intermediates and their relationship to tubulin conformation and nucleotide state. (A) Artistic rendition of the microtubule polymerization process via open sheets that later close into a tube. (B) Cryo-EM reconstruction of GMPCPP-tubulin assembled at low temperatures as a surrogate for the assembly sheets. All the protofilaments run in the same direction, but every other one is slightly rotated around its axis. (C) Schematic of the partially straightened dimer conformation observed in the cryo-EM reconstruction of GMPCPP-tubulin.

Figure 6.7.

Kinesin-microtubule interaction. *Top*: Density maps of the kinesin-microtubule complex with no nucleotide (left); with ADP and aluminum fluoride as an analogue of the ATP-bound state (right). Atomic models have been docked into the density. The most notable difference between these states is a tilt of the core of the motor with respect to the microtubule. *Bottom*: Cartoon representation of the conformational changes associated with nucleotide-binding and the power stroke. In unbound kinesin, the core is free to tilt back and forth independent of nucleotide. Once bound to a microtubule, the switch-II helix forms a base for residues that either prop the core to the right, with no nucleotide, or pull it to the left with ATP. Reprinted from Sindelar and Downing (2010).

Figure 6.8.

Tomography of *Chlamydomonas* axoneme. A section of a microtubule doublet obtained by averaging densities around and along axonemes is shown, encompassing the basic 96 nm repeat of dyneins and associated proteins. Views are as seen from the center of the axoneme (left) and tangential to the doublets (right). Densities attached to the microtubule are mainly components of the inner dynein arms, aside from the radial spoke components seen pointing down in the view at right. ODA, outer dynein arms (light blue in color figure); IDA, (red) inner dynein arms; DRC (green), dynein regulatory complex; LC (yellow), light chain of dynein 7; RS (blue), radial spokes. This view is similar to that in Bui *et al.* (2009) but represents the majority of dynein arrangements, not the uncommon one between doublets 1 and 9. Figure kindly provided by Drs K. H. Bui and T. Ishikawa.

Figure 6.9.

Tomographic reconstruction of sea urchin microtubule doublet. *Left:* Isosurface view of a section of the doublet reconstruction determined in Sui and Downing (2006). *Right:* Pseudoatomic model of the tubulin component is shown as a wire structure, and the component of the difference map showing nontubulin components is shown as a solid surface.