Structural Insights on Microtubule Doublet Interactions in Axonemes

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Running title: Microtubule Doublet Structure

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Summary

Coordinated sliding of microtubule doublets, driven by dynein motors, produces periodic beating of the axoneme. Recent structural studies of the axoneme have used cryo-electron tomography to reveal new details of the interactions among some of the multitude of proteins that form the axoneme and regulate its movement. Connections among the several sets of dyneins, in particular, suggest ways in which their actions may be coordinated. Study of the molecular architecture of isolated doublets has provided a structural basis for understanding the doublet's mechanical properties that are related to the bending of the axoneme, and has also offered insight into its potential role in the mechanism of dynein activity regulation.

Introduction

Axonemes, whose structure is remarkably conserved from protists to mammals, play diverse roles in many physiological functions including cell motility and signal transmission. For example, respiratory cilia are responsible for airway clearance, and nodal flow generated by nodal cilia is critical to left-right axis development. Defects in their structure are associated with a number of clinical disorders.

The axoneme is one of the largest molecular machines, assembled from molecular devices that function collaboratively. More than 250 proteins are involved in forming the axoneme. The structure of the axoneme has been the subject of extensive study for more than half a century (for reviews, see [1-4]). In the axoneme, nine doublet microtubules are distributed around two singlet microtubules, termed the central pair. Radial spokes, dynein motors and nexin molecules brace and link them together to form a cylindrical structure.

Many components of the axoneme are involved in the regulatory process that maintains a regular beating wave. The dynein regulatory complex (DRC) [5-7] is involved in coordination of dynein arm activity. Flexible nexin molecules that link

adjacent doublets are also proposed to be part of the regulatory mechanism [8*]. The central pair, probably, acting through the radial spokes, plays a role that is not yet fully understood [9-11]. Mutations that delete the central pair usually, but not always, cause a lack of motility in sperm [8*,12-14]. Removal of the outer dynein arms produces a reduction in beat frequency but has little effect on the axoneme waveform [15,16]. It appears that the various components may have overlapping or complementary roles in both the mechanics and regulation of beating. Knowing the structures and interactions of all these components will clearly be essential for understanding the mechanisms of motion.

The basic structure of the axoneme has been elucidated through many electron microscopy studies that have mainly used embedded and sectioned specimens. The framework of the doublet consists of two tubules, a complete microtubule containing 13 tubulin protofilaments (A-tubule) and an incomplete tubule of 10 protofilaments (B-tubule). Locations of a number of the other components have been identified, although the nature of the specimen preparations has limited the extent to which we can understand their structure and function. Recently, significant advances have been achieved in describing the axoneme's molecular architecture in three dimensions by cryo-electron tomography [17*,18**,19**]. Studies of axonemes from sea urchin and *Chlamydomonas* [17*,18**] have clarified a number of the interactions among the axoneme's components and provided especially important insights on structural and functional features of the dyneins. In studies of isolated doublets, we have resolved details of several interprotein interactions and identified candidates for several of the components [19**].

Electron Tomography of the Axoneme and Doublets

There are some limitations in the cryo-tomography procedures that constrain our interpretations of the maps, principally the low exposures that the specimens can tolerate and the fact that the specimen can be tilted to only around 70 degrees, resulting in resolution that is worse in the direction perpendicular to the plane of the specimen than within the plane. Averaging images of similar structural motifs improves the low signal-to-noise ratio that results from the limited exposure. In the case of the intact axoneme,

averaging can be carried out over the nine differently oriented microtubule doublets, relieving the resolution anisotropy. Surface renderings of the averaged structures show a complete inventory of the dyneins as well as a surprising number of connections among them (Figure 1). The bending modulus of isolated doublets is anisotropic, favoring bending in the plane that contains the two tubules rather than perpendicular to it as one might expect from the cross section of the doublets. Because the doublets on a microscope grid are never completely straight, they tend to orient with the two tubules in the specimen plane. Thus, even though the doublet density map is obtained by averaging over a number of doublets in different orientations, the preferential orientation results in resolution that is better in the plane of the doublet than perpendicular. Still, the resolution in this map (Figure 2a) is sufficient to identify all of the tubulin protofilaments, so that a pseudo-atomic model of the tubulin component could be constructed (Figure 2b)[1]. Computing the density expected for the tubulin under the conditions of the tomography experiment then allowed calculation of a difference map that revealed the non-tubulin components (Figure 2c). Thus, the recent cryo-electron tomographic reconstruction of the doublet has given a picture of the location of these proteins and led to hypotheses about the identity of several of them.

Interpretation of the Structures

Cryo-tomography has revealed a wealth of features of the inner and outer dynein arms that drive microtubule sliding in the axoneme [18**]. They are most closely associated with the A-tubule, and in some views the narrow stalks that reach the B-tubule

[1] Over the years there have been several schemes by which the protofilaments have been numbered. In describing the high resolution doublet structure, we developed a scheme based on structural features resolved in our map. In light of the fact that the literature on sea urchin doublets has been quite consistent in the use of a different scheme [20*], this may not have been the best choice, but we continue in this paper with the same scheme in order to retain continuity with our previous publication. See Figure 2 caption for correspondence of these schemes.

are also seen. The shapes of the dyneins are well enough resolved that their orientations, as well as differences among the several isotypes of dynein present, are clear. A number of physical connections both between outer dynein arms and between outer and inner arms are also resolved, leading to hypotheses about the flow of information that coordinates dynein actions. The conformation of the nexin that links doublets is seen as a zigzag structure, suggesting one mechanism for its great extensibility. The close relation seen between nexin and the DRC supports the notion that stretching of nexin may play a role in the dynein regulation and coordination [8*].

Our cryo-tomography map of the isolated doublets reached a resolution of about 2.9 nm, sufficient to clearly resolve the protofilaments of tubulin monomers and other proteins although not sufficient to resolve the small differences between alpha and beta tubulins [21-23]. Associated with the protofilament framework, there are a number of other proteins that appear to play roles either in connecting or stabilizing interactions between the tubules and the protofilaments within them, or as sites for docking of other structural components to the doublet.

There is an intimate connection between the A- and B-tubules toward the outside of the axoneme connecting protofilament B10 to A4 and A5. The protofilaments that separate the tubule interiors, A1-A4, are known as the "partition". Toward the inner side of the axoneme, there is a gap between the A-and B-tubules. This region, also seen as a gap between the B- and C-tubules of microtubule triplets in basal bodies [24*], has been seen with varying structure in previous studies, sometimes appearing as an eleventh protofilament of the B-tubule, but generally with a distinctive density. The 3-D map shows no continuous filament in the gap, but rather an apparently flexible link between the tubules, from protofilaments B2 and B1 on the inside of the B-tubule to the outside of the A-tubule at protofilaments A2 and A3, which we term the "linker density" (Figure 3a).

Inside the A-tubule, several non-tubulin protein densities contact groups of protofilaments, as seen in Figure 2. We term these proteins "inner-microtubule associated proteins" (iMAPs), in contrast to the conventional microtubule associated proteins (MAPs) that bind to the outside of the microtubule (with the possible exception of tau [25]). Nicastro et al. [18**] also reported two density domains inside A-tubule, termed MIP1 and MIP2. MIP1 corresponds to the density near protofilament A10 and

MIP2 to the density near protofilament A6. A density inside the B-tubule identified as MIP3 in the same report appears to correspond to the linker density.

One of the more prominent iMAP features seen in this reconstruction is a filament running along the partition protofilaments inside the A-tubule with lateral projections onto several protofilaments (Figure 3b) that, in the end view, form a structure termed the "bridge" density. Conspicuous density in this region has been previously reported in conventional preparations [20*]. On the other side of the partition, the linker density that connects the A- and B-tubules at the inner axoneme side also extends across several protofilaments of the A-tubule.

Early work aimed at describing the components of the doublets revealed that stable ribbons, formed by three or four protofilaments, remained after disruption of most of the doublet by detergent treatment. While it was clear that these protofilaments came from the A-tubule adjacent to the inner junction with the B-tubule, controversy developed about which protofilaments they actually were. Some researchers interpreted them as arising from the partition, protofilaments A1-A3 or A2-A4 [26], while there was also the suggestion that the ribbons comprise protofilaments A13, A1 and A2 or A12, A13 and A1 [20*,27*]. The protein composition of the ribbons is fairly simple. In addition to the alpha and beta tubulins, they contain three proteins known as tektins A, B and C, as well as proteins originally named Sp77 and Sp83 in the sea urchin. The tektins are predicted to form an extended α-helical rod that is interrupted by short non-helical linkers. The α-helical rods are believed to form a filamentous structure of coiled coils, similar to that of intermediate filament proteins, although their amino acid sequence identity is less than 30% [28-32]. Electron micrographs of the stable ribbons often show a filamentous extension, which was identified as tektin by immuno-EM. Further work even suggested that this tektin filament formed one of the protofilaments in the ribbon [33*].

A number of features in our map support the interpretation that the ribbons arise from the partition region and that all of the doublet protofilaments are formed of tubulin. We interpreted the filamentous structure associated with the bridge density as the tektin filament [19**]. The lateral projections, which form a pattern that repeats with a 16-nm spacing along the filament, appear to bind the filament tightly to protofilaments A1 – A4. Additional proteins associated with the partition on the outside of the A-tubule appear to

give the partition protofilaments a particularly high degree of stability. Within the limits of our resolution, it appears that all of the protofilaments of the doublet have essentially the same cross section, as well as similar bumpy surfaces that fit on the helical lattice of the microtubule. This similarity of all the doublet protofilaments is consistent with previous electron micrographs, particularly of thin sections in which tannic acid was used to accentuate the tubulin contrast. However, this interpretation does not account for the clearly distinctive protofilament seen both in stained and frozen-hydrated ribbons containing four protofilaments [33*]. This issue will be resolved once higher resolution reconstructions are available.

The position and 16-nm repeat of the linker density, along with published results of biochemical and immunolabeling experiments, led us to extend the hypothesis that the partition forms the stable ribbon by suggesting that the linker density seen in the 3D map is composed of proteins Sp77 and Sp83 [19**]. Immunoelectron microscopy shows that anti-Sp77/Sp83 labels the densities that decorate the ribbons [34] and that antibodies against Rib72, a *Chlamydomonas* homologue of Sp77 [35*], heavily label the ribbons with a repeat of 16 - 24nm [36*]. Rib72 contains three DM10 domains and two EF hand motifs that are predicted to bind Ca^{2+} in a regulatory process [36*,*37]. Proteolysis of native Rib72 *in situ* revealed a significant difference in sensitivity to digestion in the presence and absence of Ca^{2+}, suggesting that Rib72 undergoes conformational changes upon Ca^{2+} binding [37*] and supporting its involvement in Ca^{2+} regulation of cilia/flagella motility.

Nexin links connect protofilaments A9-A11 of the A-tubule in one doublet with protofilament B2 of the B-tubule in the adjacent doublet, near where the linker joins the B-tubule protofilaments [19**, 18**], as shown schematically in Figure 4. Our model suggests that the regulatory mechanism could involve the Sp77/Sp83 linker between the A- and B-tubules through an interaction between the linker and nexin. Previous studies showed that removal of most of the tubulin from an axoneme by incubation at around 40°C leaves the ribbons still in a 9-fold array, apparently held together by the inter-doublet nexin links and probably also inner dynein arms and radial spokes [38]. As suggested in Figure 4, this phenomenon may result from a direct interaction between nexin and the linker proteins Sp77 and Sp83.

The structure of the linker may undergo significant conformational changes that may, in turn, be related to regulation of the axoneme movement. Displacements between adjacent doublets can be a significant fraction of a micrometer [39,40], but the doublets themselves are subject to only the elastic deformations that accompany bending of the axoneme. In contrast to the tight interaction between the A- and B-tubules at the outside, the structure of the linker at the inner side would appear to provide freedom for displacements between the A-tubule and the edge of the B-tubule. If the direction of the bend is such that the A-and B-tubules are at different radii and the linker is tightly attached to both the A-and B-tubules, this displacement could be comparable to the tubule diameter, perhaps more than 10 nm, resulting in a substantial conformational distortion of the linker. Thus the apparent Ca^{+2} binding of Sp77/Rib72 is of particular interest, as the flexibility to accommodate this distortion might be regulated by local calcium concentration, or conversely, the distortion might affect calcium binding.

The shape of the tubules, as well as associated proteins, may play a role in the doublet's bending properties. As seen in Figures 1 and 2, the A- and B-tubules are compressed in the circumferential direction of the axoneme. The connection between protofilaments appears to be flexible enough to easily accommodate the range of inter-protofilament angles and spacings seen in the reconstruction [23]. What stabilizes the distortion in the doublets is not entirely clear, but presumably has to do with the other associated proteins. The tubule shape and these other proteins also most likely account for the anisotropic bending modulus of the doublets. Deformation and mechanical properties of microtubules have been the subject of several studies [41-43*], and it will be important to extend work to microtubule doublets in order to fully understand how their structure and resultant mechanics relate to their function.

Conclusion:

Cryo-electron tomography has produced 3-D density maps of intact axonemes and of isolated doublets in which many of the protein components are clearly resolved. Hypotheses about the identity of some of these proteins suggest novel connections between the structure and function of the doublet and other complexes that comprise the

axoneme. Inner-microtubule associated proteins bound to the inner wall of the doublet A-tubule may stabilize the protofilament framework and the docking of extra-tubule complexes, such as dynein arms and nexin links. Flexible links between the various parts offer new ideas on mechanisms that regulate and coordinate axoneme movement. We can look forward to continuing improvements in the resolution in structural studies that will complement the wealth of biochemical data on this subject in testing these hypotheses and extending our understanding of this movement.

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Figure Legends

Figure 1. 3-D map obtained by averaging tomograms of intact *Chlamydomonas* axonemes [18**]. (a) View from the distal end of the axoneme. (b) View seen from the plane of the dashed line in (a), with the proximal end at the left. The single two-headed dynein complex in *Chlamydomonas*, I1, is shown in red. Components and their boundaries are identified by correspondence with previous electron microscope images: A_t and B_t, A-and B-tubules of microtubule doublets; ODA, outer dynein arms; IDA, inner dynein arms; RS, radial spokes; N, nexin; IL, dynein intermediate chain/dynein light chain-tail complex; DRC, dynein regulatory complex; α and β, alpha-1 and beta-1 motor domains of the I1 complex; white arrows indicate linkers between inner and outer dynein arms. Used with permission.

Figure 2. 3-D tomographic density map of sea urchin doublet microtubule and its interpretation. (A) Isosurface view of the map seen from the proximal end of the axoneme, with the outer edge of the axoneme at the top. (B) Backbone representation of the tubulin model built into the experimental map. Protofilament numbers are as in [19**]. Note that the scheme used in previous work on sea urchins [20*] numbers protofilaments counter-clockwise, with the same position for A1, but A2 and A13 interchanged, and so on; in the B-tubule, B1 and B10 are interchanged, and so on. (C) 3-D density computed for the tubulin model component of the doublet under conditions of the experimental images (blue), and non-tubulin densities (green) defined by subtracting the model density from the experimental density.

Figure 3. Interior views of the doublet structure. The density from the tubulin model (blue) and difference map (green) are shown. (A) View looking into the B-tubule showing structure of the linker protein. (B) View of the filamentous density inside the A-tubule, interpreted as the tektin filament.

Figure 4. Schematic of interactions among microtubule doublets and other proteins in the axoneme. Locations of tubulin and other proteins within the doublets, as well as nexin, are as determined in [19**] Shapes and positions of the radial spokes, dyneins and DRC are drawn from [2], [7] and [18**].