ARRANGEMENT OF HIGH MOLECULAR WEIGHT ASSOCIATED PROTEINS ON PURIFIED MAMMALIAN BRAIN MICROTUBULES

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ABSTRACT

The arrangement of the high molecular weight proteins associated with the walls of reconstituted mammalian brain microtubules has been investigated by electron microscopy of negatively stained preparations. The images are found to be consistent with an arrangement whereby the high molecular weight molecules are spaced 12 tubulin dimers apart, i.e., 960 Å, along each protofilament of the microtubule, in agreement with the relative stoichiometry of tubulin and high molecular weight protein. Molecules on neighbouring protofilaments seem to be staggered so that they give rise to a helical superlattice, which can be superimposed on the underlying tubulin lattice. In micrographs of disintegrating tubules there is some indication of lateral interactions between neighbouring high molecular weight molecules.

When the microtubules are depolymerized into a mixture of short spirals and rings, the high molecular weight proteins appear to remain attached to their respective protofilaments.

A group of high molecular weight polypeptides, with molecular weights within the range 270,000 to 400,000, varying according to different estimates, is found by the majority of workers to constitute the main non-tubulin component of microtubules isolated from mammalian brain (23, 6–8, 10, 13, 18, 22). Borisy et al. (6) have found these high molecular weight microtubule-associated proteins (HMW/MAP) to be present in stoichiometric amounts in reconstituted microtubules, and they estimate from SDS gels that there are 10–13 tubulin dimers per HMW/MAP molecule. The amounts of high molecular weight protein quoted by other authors tend to be somewhat lower. Microtubule-associated proteins of considerably lower molecular weight have also been reported (37). The results of Sloboda and Rosenbaum (32, 33) suggest that some of the smaller proteins may be proteolytic fragments of HMW/MAP. These workers found that the HMW/MAP gradually disappeared during storage of their microtubule protein and that smaller polypeptides left in the preparations cross-reacted with antibodies to HMW/MAP. This phenomenon could account for the reported ratios of HMW/MAP to tubulin, which are considerably lower than the value quoted by Borisy et al. (6).

Dentler et al. (11) and Murphy and Borisy (29) have shown by electron microscopy of fixed and sectioned pellets of purified microtubules that the HMW/MAP molecules form filamentous projections from the tubule walls. Murphy and Borisy found an approximate axial repeat distance of 320 Å between successive projections (that is, four times the basic 80 Å repeat of the tubulin dimers). Since there are not enough HMW/MAP molecules to attach at 320 Å intervals to the entire length of all 13 protofilaments of the tubule wall,
the relationship between the arrangement of HMW/MAP on the microtubule and the stoichiometry between tubulin and HMW/MAP is not immediately obvious. Solution of this problem is important for discussion of the role of HMW/MAP in microtubule assembly and function.

MATERIALS AND METHODS

Purification of Microtubules

Microtubules were prepared from pig or bullock brain by a method essentially similar to that of Shelanski et al. (31). Centrifugation and incubation times were kept to a minimum, especially during the first cycle of depolymerization and repolymerization, to minimize proteolysis. After homogenization in the cold (5°C) of 150 g of brain in 200 ml of MES buffer (40 mM 2-[N-morpholino]ethane sulfonic acid, 2 mM ethylene glycol-bis(β-aminoethyl ether)N,N',N'-tetraacetate (EGTA), 1.5 mM MgCl₂, 1 mM GTP, 1 mM dithiothreitol or 5 mM mercaptoethanol, 0.02% sodium azide, pH 6.5), debris was spun down at 25,000 rpm, 2°C, for 20 min. An equal volume of 8 M glycerol in MES buffer was added to the supernatant fluid, which was then incubated 20-30 min at 37°C and centrifuged 40 min at 35,000 rpm, 27°C. The pellet of repolymerized microtubules was resuspended in 80 ml of MES buffer and chilled on ice 20-30 min, before further centrifugation at 25,000 rpm, 2°C, for 20 min. Microtubules were repolymerized a second time by incubating the supernatant fluid at 37°C with or without glycerol (after the addition of an equal volume either of 8 M glycerol in MES buffer or of MES buffer alone) and collected by centrifugation at 35,000 rpm for 40-60 min. For storage, microtubule protein was kept in 8 M glycerol in MES buffer at -20°C.

Gel Electrophoresis

SDS-gel electrophoresis of microtubule protein was performed on polyacrylamide slab gels, with or without a stacking gel, using the buffers described by Laemmli and Favre (26). For rapid assays of the various fractions, a micro-slab gel apparatus (3) was used to run small, thin slabs which can be electrophoresed, stained, and destained within an hour, without significant loss of resolution. Gels were stained with Coomassie Brilliant Blue dye. For quantitation of the bands, both normal-size and micro slabs were densitometered with a Joyce-Loebl microdensitometer (Joyce-Loebl Ltd., Gateshead, England).

Electron Microscopy

For electron microscopy, drops of dilute suspensions were applied to grids coated with holey carbon films and negatively stained according to the technique of Huxley (20). Microtubules polymerized in the absence of glycerol were normally used, since traces of glycerol often seemed to inhibit stain films from forming across the holes in the carbon. The stain used was either 1% aqueous uranyl acetate, unbuffered, or 1% phosphotungstic acid (PTA), adjusted to pH 7 with NaOH. Some samples were fixed 5 min with 1% glutaraldehyde before being stained. Electron microscopy was carried out on a Philips EM300, at 80 kV, at a nominal magnification of 57,000. Optical diffraction of selected micrographs was performed with the instrument described by DeRosier and Klug (12).

RESULTS

The following results were found to be applicable, whether material was obtained from pig brain or from bullock brain.

Gel Electrophoresis of Purified Preparations

Figure 1 shows a typical SDS gel pattern of microtubule protein after purification by two cycles of depolymerization and repolymerization. Densitometry of the band patterns (e.g. Fig. 1b) suggests that the apparent mass ratio of total HMW/MAP to total tubulin in the samples prepared as described above in reproducibly 20-25%, in good agreement with the results obtained by Borisy et al. (6). The ratio was the same whether or not glycerol was present during the second repolymerization; it did not vary significantly if further cycles of purification were carried out. The preparations investigated thus appeared to be fully saturated with HMW/MAP. Assuming molecular weights of between 300,000 and 400,000 daltons for HMW/MAP and 110,000 daltons for the tubulin dimer, the stoichiometry suggested by the SDS gel patterns is one HMW/MAP molecule to between 11 and 18 tubulin dimers. The pattern of peaks within the HMW/MAP band was found to be rather variable. Figure 1 shows the most common type of distribution. Sometimes, however, only a single HMW/MAP peak was observed, usually corresponding to the main HMW/MAP peak in Fig. 1; at other times, the band was resolved into three or even four different peaks.

Electron Microscopy of Microtubules

Negatively stained microtubules supported on a carbon substrate showed the usual beaded proto-
as shown in Fig. 2. In Fig. 2a the filamentous arms can be seen quite clearly. The stain films invariably break in the electron beam, producing great distortion of unfixed microtubules (Fig. 2a), but it was found that the structure could be stabilized quite satisfactorily by fixing the tubules briefly in 1% glutaraldehyde before staining them. After fixation, the arms appear as shorter, more compact projections (Figs. 2b and c), presumably as a result of intramolecular cross-linking.

The projections in many images give an impression of being in some kind of helical arrangement, although it is not immediately obvious what this arrangement is. Optical diffraction of such images was therefore carried out to see whether the projections would give rise to periodic reflections. Unfixed microtubules over holes gave very poor diffraction patterns, in which even the 40 Å tubulin reflections were smeared out. In the diffraction patterns of the majority of images of fixed tubules, only the 40 Å reflections and the equatorial peaks could be identified, as in the diffraction patterns of Erickson (13), but in some there appeared to be additional reflections close to the center of the pattern. The only additional layer line consistently observed was one at 320 Å, as shown in the example in Fig. 4a.

Microtubules stained with uranyl acetate were found to be generally better preserved than those stained with PTA. The latter stain frequently caused the tubules to disintegrate, even after partial fixation (Figs. 2c and 3a). The stain appeared to dissolve away parts of the microtubule wall, leaving a residue which shows evidence of periodicities longer than the tubulin lattice repeat. In Fig. 3a, the dissolved part of the microtubule has the appearance of a helical structure, with a minor pitch roughly equal to the flattened tubule diameter (i.e., 300–360 Å). Similar results were obtained with "aged" tubules that had sat at room temperature for over an hour. These tubules appear to disintegrate in a different manner than...
those induced to depolymerize in vitro by chilling or addition of divalent cations, whereby the protofilaments apparently roll up at one end of the microtubule to form short spirals which then break off (13, 14, 24, 25). Instead, aged microtubules seem to become limp and wavy (Fig. 3b), and the 40 Å tubulin lattice reflections disappear from the diffraction patterns of their images (Fig. 4b). Eventually, they apparently turn into irregular, twisted fibers. But in the intermediate stage shown in Figs. 3b and c, there are still vestiges of a long-range periodic structure, which sometimes gives rise to a 320 Å layer line in the diffraction patterns (Fig. 4b). This must represent some kind of periodic lattice in the microtubule wall which is not dependent upon the preservation of the 40 Å lattice (i.e., not just a regular perturbation of the tubulin lattice).

**Electron Microscopy of Rings and Spirals**

Microtubule protein was observed in the elec-
FIGURE 4  (a) Optical diffraction pattern of part of the image shown in Fig. 8d. The 40 Å layer line comes from the periodic arrangement of tubulin subunits. Layer lines corresponding to longer axial periodicities are not usually found in diffraction patterns of brain microtubules (13). In particular, an 80 Å periodicity corresponding to the tubulin dimer repeat is hardly ever observed, although it is present in the patterns from flagellar microtubules (1). The 320 Å layer line seen here was found in only a minority of patterns in the present work, but most probably arises from the arrangement of HMW/MAP. It is presumably not seen in all the patterns because the HMW/MAP projections tend to be rather disordered. (b) Optical diffraction pattern of a stretch of "aged" microtubule in Figure 3b. There is no sign of a 40 Å layer line, indicating that the tubulin subunits are disordered. However, the 320 Å layer line can still be identified.

electron microscope after depolymerization by chilling on ice or by the addition of 10 mM CaCl₂. Rings and spirals, of one, two or more turns, were observed whether or not glycerol was present in the buffer. Again, the high molecular weight proteins were not readily visible (presumably for the same reasons that they are rarely observed on negatively-stained microtubules), but in some images, especially of material on holey grids, filamentous projections could be seen attached to spirals and rings. It was not usually possible to identify more than two projections per particle. Examples of this appearance are shown in Fig. 5. Unfortunately, staining conditions which allow the projections to be seen do not seem to be ideal for showing the substructure of the spirals; particles suspended over holes are invariably badly shrunk and usually distorted.

DISCUSSION

Determination of the arrangement of HMW/MAP

The pattern of peaks in the HMW/MAP band of SDS gels of purified microtubules is variable; it is, therefore, not clear how many different species of polypeptide chain it represents. Since sometimes only one peak is observed, it is possible that the multiple peaks seen on other occasions are simply a result of different conformations of a single species, or that partial cleavage of the protein has occurred. Another possibility is that there are different species but that each one comes from a different population of microtubules, since the preparations have been obtained from a whole brain homogenate. The simplest assumptions for present purposes are that all of the HMW/MAP molecules are structurally equivalent and that in purified microtubules there is one such molecule specifically associated with each group of tubulin molecules, consisting of 11-18 dimers. We therefore expect an axial repeat of 880-1440 Å.

For a full analysis of a structure by optical diffraction, an undistorted stretch containing several unit cells is required, which in the present case would mean lengths of several thousand Angstroms. The particles on which the HMW/MAP projections are most visible come from holey grids, which do not give such long-range preservation. In a few favourable cases, as in Fig. 4, optical diffraction reveals an axial periodicity of 320 Å. Apart from this, we have analyzed the structure by...
FIGURE 5 Electron microscope images of tubulin spirals and rings suspended in uranyl acetate on holey carbon support films. Arrows indicate filamentous material apparently attached more or less at right angles to the coiled tubulin protofilaments and therefore thought to represent HMW/MAP. In a few cases, the projections appear inside the rings rather than outside. Bar, ~ 1,000 Å. × 360,000.

comparing the patterns seen locally in electron microscope images with those expected from model structures, such as those in Figs. 6 and 7. This approach has previously been highly successful in solving the structure of viruses, such as turnip yellow mosaic virus (17).

The helical surface lattice of tubulin dimers in the microtubule wall, with a basic axial repeat of 80 Å, has already been established (19, 1, 13) and the hand of the lattice determined (28, 13, 9). The results of Bibring et al. (5) suggest that alternate dimers may be chemically different, which would make the basic axial repeat of the tubulin lattice 160 Å, but this would not alter the following arguments. If the HMW/MAP molecules are arranged in a completely symmetrical fashion on the tubulin lattice, the possible arrangements are fairly limited. For each possible axial repeat distance along the tubulin lattice, only one closed superlattice will fit around 13 protofilaments. We can reduce the number of possibilities by requiring that 320 Å be a subperiod of the lattice (cf. Figure 4). Within the range of possible ratios of tubulin dimers to HMW/MAP molecules, axial repeats of 12 and 16 dimers satisfy this requirement, and, of these two, an axial repeat of 12 dimers (960 Å) has been found to agree best with the electron micrographs. This lattice in fact corresponds to the 960 Å helical superlattice discussed by Amos and Klug (1) in connection with the longer axial periodicities of flagellar microtubules. Figure 6a represents the opened-out 960 Å helical lattice, as viewed from the outside surface, with equivalent sites for the HMW/MAP represented by the black circles. The two sets of thicker lines drawn connecting the circles represent two helical families, which have been chosen fairly arbitrarily. The lattice could equally well be represented by other helical families. Successive circles along the 1-start 192 Å pitch helix have been lettered A to N, to distinguish sites associated with different protofilaments. Figures 6b and c show projected views of the lattice when a is rolled up to form a cylinder. Figures 6b and c represent two extremes of the range of different projected views; it can be seen that the variation of appearance with view angle is rather slight, so that all views can be adequately represented by these two projections or their mir-
FIGURE 6  (a–c) The 960 Å superlattice of microtubules. This is a unique superlattice with 960 Å axial spacing which can be superimposed on the basic tubulin lattice of the microtubule (1, 28, 13). Vertical lines delineate the 13 tubulin protofilaments. Equivalent sites along each protofilament are shown schematically as black circles or projections. It is not known, of course, exactly how the HMW/MAP projections are attached to the tubulin protofilaments. Fig. 6 a represents the opened-out lattice as viewed from the outside. The underlying tubulin lattice is shown by dumb-bell shapes, which are meant to represent tubulin dimers. The grouping of the tubulin subunits into possible tetramers (5) is indicated by additional vertical connections. There are 12 tubulin dimers within each unit cell of the 960 Å lattice. Figs. 6 b and c represent two different projections of the lattice when a is rolled up to form a complete cylinder. Lattice lines on the back of the cylinder are shown dotted, and projections situated at the back are shown with white centers. End-on views of the two orientations are shown above. In Fig. 6 b, protofilaments on the front of the cylinder are exactly superimposed on protofilaments at the back; the result is a projected view with horizontal lines of mirror symmetry at levels labeled A and halfway between them. In Fig. 6 c, as a result of a small rotation (π/13), protofilaments at the back project exactly between those at the front. This rotation destroys the mirror symmetry; projection K now sticks out more than E and B more than N on one side, and F more than J on the other. A similar rotation in the opposite direction would have the reverse effect—i.e., N and E would protrude more than B and K, while J would protrude more than F. Note that on one side of Fig. 6 b and both sides of Fig. 6 c the arms projecting sideways have an average axial spacing of 320 Å.
FIGURE 7 Comparison of the projected views of various different superlattices which can be superimposed on the tubulin lattice. The figure under each image refers to the number of tubulin dimers in the axial repeat of each lattice. There seems to be no symmetrical superlattice which repeats after 13 tubulin dimers. Although the projections of several of the superlattices appear fairly similar to each other, only the lattices repeating after 12 and 16 dimers have the 320 Å subperiod (a second order of a 640 Å periodicity in the latter case), which optical diffraction patterns (see Fig. 4) show to be a property of the real lattice. Careful comparison of the model projections with electron micrographs (see Figs. 2 and 8) shows that the 12-dimer lattice provides the best interpretation of the images.

ror images. Similar projections for superlattices with different axial repeat distances are compared in Fig. 7.

Equivalent sites on the superlattice in Fig. 6 are separated axially by spacings of 960 Å. In sideview, however, projections from three or even four neighboring protofilaments might be expected to protrude from each side, giving an apparent axial spacing of much less than 960 Å. This could account for the average axial spacing of 320 Å observed by Murphy and Borisy (29). As shown in Figs. 6b and c, the successive axial spacings between sideways projections of the 960 Å lattice would really be 370, 370, and 220 Å, in turn. But, since the projecting filaments seem to be rather long and flexible, a regular variation in successive spacings would be masked by movement of the free ends. The superlattice which repeats after 16 dimers (Fig. 7) does not give projected images which could account for an apparent axial spacing of 320 Å.

Comparison of the microtubule images with the model 960 Å lattice is illustrated in Fig. 8, where the electron microscope images are printed with reversed contrast to show the features more clearly. HMW/MAP molecules have been identified as projections from the sides of the tubules or as black patches superimposed on the tubules. Since these images are those of microtubules suspended over holes in the carbon, the structures have unfortunately been subjected to a certain amount of anisotropic shrinkage. Furthermore, the projections appear to be somewhat flexible, so that the free ends may not accurately represent the points of attachment. However, allowing for these distortions, it is possible to match features in different images of fixed tubules, as indicated. Where the black patches and projections appear to correspond with projections from the three or four protofilaments nearest to each side, they have been lettered using the same convention as in Fig. 6. There are also a few unlettered projections on some of the images which can be accounted for as the ends of molecules attached further round the microtubules.

Both extreme views of the proposed lattice (Figs. 8a and g) are shown for comparison with the electron microscope images. Some stretches
correspond more closely to 8a and others to 8g (or its mirror image – mirrored about a line perpendicular to the axis). In most cases, the microtubule appears to twist very slightly (that is, the protofilaments follow a small, but variable, angle relative to the axis of the tubule), so that the effective view changes gradually along its length. Thus, different stretches of the same tubule may correspond to different views of the model, making it difficult to follow the pattern of projections over very long lengths of microtubule. Also, the superlattice of HMW/MAP may not necessarily extend continuously over more than a limited stretch of reconstituted tubule. Under the conditions described here, a small proportion of tubulin dimers inserted between stretches of protofilament associated with HMW/MAP could cause occasional dislocations in the HMW/MAP lattice.

The question arises of what happens to the superlattice of HMW/MAP molecules on depolymerization of the microtubules. It seems to be well established that reversible in vitro depolymerization involves the breaking of interprotofilament bonds and a rolling up of the tubulin protofilaments, to form short spirals (24, 25, 13, 14). All the evidence from studies of microtubule polymerization and depolymerization suggests that the HMW/MAP molecules remain associated with the spirals, and the results shown here support this. The projections have not been observed in previous electron microscope studies of the structure of the rings. However, the preparations studied by Kirschner and colleagues (24) contained no high molecular weight protein but only the much smaller tau factor, while Erickson’s (14) preparations seem to have contained a relatively small proportion of HMW/MAP.

One would expect the HMW/MAP molecules to be incorporated into the spirals at the same intervals of 12 tubulin dimers as they occur along the microtubules, and there is no evidence to suggest that this is not so. Most of the spirals appear to consist of about two turns and may have one or two visible projections. The results are not inconsistent with the measurements of Kirschner and collaborators (24, 25, 36) on their 36s particles, which indicate that a two-turn spiral should consist of twenty to thirty tubulin dimers (i.e., approximately two 12-dimer repeats). It remains to be discovered whether or not the spirals consistently break at specific points relative to the HMW/MAP attachment sites, which would lead to quantization of the spirals in units of 12 tubulin dimers.

Do Associated Proteins Help Determine Microtubule Structure?

The results shown here indicate that when brain microtubules are saturated with a full complement of HMW/MAP molecules, the filamentous projections tend to be arranged symmetrically on the microtubule wall, with an axial repeat distance of 12 tubulin dimers, or 960 Å. The HMW/MAP molecules thus have the effect of introducing a longer periodicity in the microtubule wall, which is apparently not inherent in the tubulin lattice alone. It is interesting that the axial repeat distance is the same as that found in flagellar doublet tubules (1, 2). It is possible that a similar 960 Å nontubulin superlattice on the A tubules of flagellar doublets underlies the arrangement of the various flagellar accessory proteins.

Such an arrangement requires some kind of specific interaction, not only between tubulin and HMW/MAP, but also between different HMW/MAP molecules on neighboring protofilaments of the microtubule, either directly or transmitted through the intervening tubulin dimers. The pattern of disintegration of aged microtubules (Fig. 3) suggests that the former possibility is the more likely, with direct connections between nearby HMW/MAP molecules. The strength of the 320 Å reflection in some optical diffraction patterns (cf. Fig. 4) suggests that one set of connections may be along the 2-start left-handed 320 Å pitch family of helices. The shallower set of lines in Fig. 3a probably represents this family. The steeper lines in Fig. 3a appear to correspond to the left-handed family of 960 Å pitch helices (A to F to L etc., see Fig. 6) and may therefore represent connections between molecules on adjacent protofilaments.

The demonstration that HMW/MAP molecules are arranged in a specific symmetrical way on the brain microtubules is strong additional evidence that these are true microtubule-associated proteins rather than molecules which accidentally co-purify with tubulin, but their exact role is still unclear. They do not appear to be essential components of in vitro assembled microtubules. Lee and Timasheff (27) have found conditions under which tubulin will assemble without any associated proteins. Under buffer conditions similar to those used in the present work, tubulin apparently will not readily assemble alone, but addition of microtubule-associated proteins strongly promotes both the initiation and growth stages of assembly (37, 29, 22, 11). However, the latter effect can be
mimicked by a number of nonspecific polycationic substances, such as RNase A, protamine and DEAE dextran (21, 4, 35, 15, 16), although these substances tend to produce abnormal assemblies, such as double-walled tubules. Erickson and Voter (16) have shown that DEAE dextran, for example, combined with purified tubulin from which all native associated proteins had been removed, gave rise to double-walled microtubules, in which the inner wall consisted of 16 longitudinal protofilaments rather than the normal 13. In the outer wall, protofilaments lay at an angle of 38° to the tubule axis. In both layers, the local arrangement of tubulin dimers was identical to that in normal tubules: only the overall curvature of the structures was different. Similarly, Jacobs et al. (21) and Tsunprun et al. (35), with the aid of various polycations, have induced the formation of a second layer of tubulin around the outside of normal 13-protofilament tubules. Again, the tubulin lattice appeared to be conserved locally, but the curvature of the sheet was abnormal. These variations can be contrasted with microtubules assembled from tubulin and native associated proteins which have the same single-walled 13-protofilament structure as observed in microtubules in vivo (34). It is not yet known whether the microtubules assembled from pure tubulin by the method of Lee and Timasheff (27) always have the correct number of protofilaments. However, the conclusion which can be drawn from the above observations is that, while the properties of tubulin uniquely specify the pattern of interactions involved in the formation of tubulin sheets, the precise curvature taken up by the sheets in forming tubules can be strongly influenced by the associated proteins.

HMW/MAP appears to be specifically designed to be a cofactor in microtubule assembly, in that the symmetry requirements of a closed HMW/MAP superlattice would serve as a check that the correct 13-protofilament tubule is formed.

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FIGURE 8 Comparison of electron-microscope images of negatively stained microtubules on holey carbon films b-f with projections (a and g) of the 960 Å superlattice proposed in Fig. 6. To show the projections to best advantage, the images are printed with reversed contrast compared with Figs. 2 and 3 (i.e. protein is black and the stain appears white here). In the model images (a) and (g), projections from the three or four protofilaments nearest each side are lettered (as in Fig. 6), and corresponding projections in the electron microscope images have also been labeled. A few other projections in the electron microscope images appear to correspond to sites on protofilaments farther from the edges. Figs. 8 b-f x 340,000-420,000: magnifications were adjusted slightly to compensate for varying degrees of shrinkage and stretching in the axial direction (as judged by measurements of the positions of the 40 Å layer lines in optical diffraction patterns of each image). Allowing for these distortions, the axial spacing between equivalently labeled points on the same microtubule should be 960 Å.

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