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Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly *in vitro*

(crossbridges/self-assembly/porcine brain tissue)

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ABSTRACT High-molecular-weight components (HMW) specifically associated with microtubule protein purified from porcine brain tissue were separated from tubulin by DEAE-Sephadex ion exchange chromatography. Analysis by viscometry, sedimentation, and electron microscopy of the unfractionated microtubule protein, separated HMW and tubulin fractions, and reconstituted mixtures showed that HMW promoted formation of ring structures at 5° and tubule polymerization at 37°. The HMW reassociated with tubulin and was identified in thin sections as 18.9 × 5.6 nm projections attached to the microtubules with a longitudinal periodicity of 32.5 nm. These studies: *(1)* indicate that the HMW fraction stimulates microtubule assembly by facilitating the formation of ring structures which are apparently intermediates in polymerization, and *(2)* demonstrate that the HMW associates with microtubules as a structural component projecting from the surface of the microtubule wall.

Cytoplasmic microtubules play important roles in cell elongation and motile processes such as mitosis and intracellular transport (1). For some of these functions microtubule-associated elements such as crossbridges have been postulated (2, 3), and structural components attached to microtubules have been observed in neurons (4), myoblasts (5), and the mitotic spindle (6, 7). Although a microtubule sidearm which produces the force for axonemal motility has been isolated from cilia and flagella (8), in no case have the structural components observed on cytoplasmic microtubules been characterized biochemically or had their exact functions defined.

Microtubule-associated proteins have been observed in preparations of purified microtubule protein obtained from brain tissue by an *in vitro* assembly procedure (9, 10). These components account for 15–20% of the total purified material and are typically resolved on 5% acrylamide gels as a pair of closely spaced bands (286,000 and 271,000 MW) together with a minor band of higher molecular weight (345,000 MW). We refer to these bands collectively as high-molecular-weight components (HMW). Bands of high molecular weight have also been observed by others using a similar assembly procedure (11–13), an assembly procedure using glycerol (14), and in purified tubule preparations stabilized in hexylene glycol (15). Previous results showed that the HMW components copurified in constant stoichiometry with tubulin through repeated cycles of assembly–disassembly (9, 10, 14). Agents that inhibited microtubule assembly also inhibited the sedimentation of the HMW under conditions that sedimented microtubules but not the unpolymerized material (10). Therefore, we concluded that these components were not contaminants but rather were specifically associated with microtubules. The HMW components were therefore further investigated to determine the nature of their association with microtubules and their effect on tubule assembly *in vitro*.

Abbreviations: PM, polymerization medium; MW, molecular weight; HMW, high-molecular-weight components.

MATERIALS AND METHODS

Preparation of HMW and Tubulin Fractions. Purified microtubule protein was obtained from porcine brain tissue by two cycles of a reversible temperature-dependent assembly procedure (10). Protein was prepared at 0° in polymerization medium (PM) [0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes) adjusted with NaOH to pH 6.94 at 23° containing 0.1 mM $MgCl_2$] supplemented with 0.1 mM GTP. HMW components were separated from tubulin on DEAE-Sephadex A-50 by stepwise elution with salt at 5°. Columns containing 4 or 16 ml of DEAE-Sephadex gel were equilibrated with 2–3 bed volumes of PM containing 0.1 mM GTP. Samples of microtubule protein (1 mg/ml) in PM containing 0.1 mM GTP were applied to the DEAE-Sephadex (3–4 mg of protein per ml of gel) and separated into three fractions by stepwise elution with 4 bed volumes each of: *(1)* PM containing 0.1 mM GTP; *(2)* the same solution containing 0.3 M KCl; *(3)* the same solution containing 0.8 M KCl. A control sample of unfractionated microtubule protein was made 0.8 M in KCl at the same time the 0.8 M KCl solution was applied to the column. The peak fractions were desalted by gel filtration on columns of Sephadex G-25 (coarse) equilibrated in PM containing 1.0 mM GTP. The desalted 0.3 M and 0.8 M KCl fractions were mixed either 1:1 (v/v) with each other or with PM containing 1.0 mM GTP and polymerized at 37°.

Assay Procedures for Microtubule Polymerization. Polymerization was examined by viscometry as described previously (16), by a quantitative sedimentation assay (17), and by electron microscopy. Protein concentrations were determined by the method of Lowry *et al.* (18) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis. Samples and gels (5% acrylamide, 0.6 × 8.0 cm) were prepared according to Shapiro *et al.* (19) and stained in Coomassie brilliant blue according to the method of Fairbanks *et al.* (20). The mass fractions of HMW and tubulin in the samples were determined by planimetry of densitometer tracings of the gels.

Electron Microscopy. Negative staining was performed as described previously (16). For thin sections approximately 2 mg of polymerized protein were placed in 8 ml polycarbonate tubes and centrifuged at 225,000 × *g* at 25° for 30 min. The pellets were fixed in 2.5% glutaraldehyde in PM containing 1.0 mM GTP, postfixed in 1% osmium tetroxide in

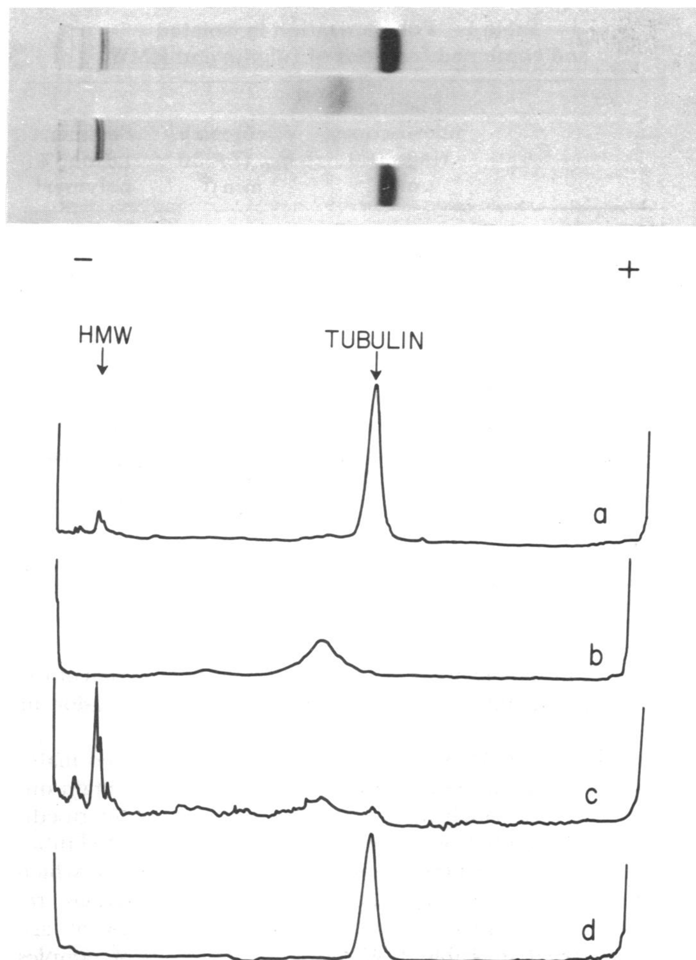

FIG. 1. Gel electrophoretic analysis of DEAE column fractions. Stained gels are at the top; densitometer tracings, below. (a) Unfractionated microtubule protein; (b) unbound protein eluted in equilibration buffer; (c) 0.3 M KCl fraction (HMW components: major doublet and minor band); (d) 0.8 M KCl fraction (tubulin).

the same buffer, dehydrated, and embedded in Araldite using standard procedures. The sections were stained in methanolic uranyl acetate and lead citrate and examined at a magnification of 40,200 as determined by calibration with a carbon replica grating with 21,600 lines/cm. The dimensions and periodicity of projections on the microtubule surface were measured directly from negatives using a Gaertner microcomparator (Gaertner Scientific Corp., Chicago, Ill.).

RESULTS

Polymerization of Purified Components. HMW was separated from tubulin by chromatography of purified microtubule protein* on columns containing DEAE-Sephadex. A small amount of protein (5%) did not bind to the column and eluted with the column equilibration buffer, while most of the bound protein eluted in two well-defined peaks at 0.3 M and 0.8 M KCl. The composition of these three fractions and the unfractionated sample as determined by gel electrophoresis is shown in Fig. 1a–d. The sample of microtubule protein contained approximately 75% tubulin and 15–20% HMW components. The unbound protein contained a species of approximately 70,000 MW but virtually no tubulin or HMW components. The 0.3 M KCl fraction consisted of 75% HMW (doublet band plus minor band), very little tubulin (3.5%) and other trace components. The 0.8 M KCl fraction was greater than 95% tubulin with only traces of other components observed on overloaded gels.

*In this paper microtubule protein refers to protein containing HMW as obtained from the reversible assembly procedure. Tubulin is used to indicate the HMW-free fraction obtained by ion exchange chromatography.

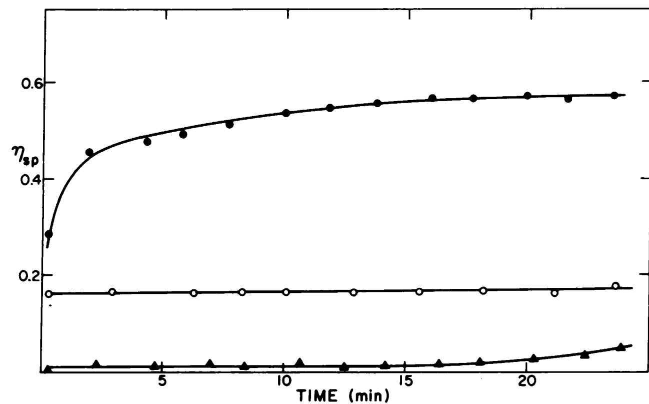

FIG. 2. Test of competency of DEAE-purified tubulin to polymerize. Tubulin subunits were mixed with microtubule fragments (0.25–2.0 μm in length) prepared by shearing a sample of polymerized microtubule protein in a syringe. The purified tubulin (2.0 mg/ml) and microtubule fragments (2.0 mg/ml) were mixed 1:1 (v/v) either with each other or with PM containing 1.0 mM GTP placed at 37°. Specific viscosities were measured at the indicated times. Tubulin (▲), microtubule fragments (○), and mixture (●).

Since monovalent cation concentrations above 0.2 M inhibit microtubule assembly (16, 21), it was necessary to remove the salt from the purified fractions before assaying them for polymerization. When purified tubulin (0.8 M KCl fraction) was desalted and brought up to 37° in PM containing 1.0 mM GTP, viscometry and sedimentation showed that little polymerization occurred at protein concentrations as high as 4.0 mg/ml. Similarly, the desalted 0.3 M KCl fraction containing purified HMW did not form microtubules upon incubation at 37°. To examine whether exposure to salt had irreversibly denatured the subunits, a sample of unfractionated microtubule protein at the same concentration was made 0.8 M in KCl, desalted on Sephadex G-25, and examined for its ability to polymerize. Samples treated with salt for 30 min and then desalted developed a specific viscosity at 37° of 80–90% that of microtubule protein not exposed to salt. These observations indicated that brief exposure to high salt did not irreversibly inactivate the tubulin.

To test whether the purified tubulin subunits were competent to add onto preexisting seeds and thus support microtubule growth, we mixed tubulin subunits with microtubule fragments which were prepared by shearing a sample of polymerized microtubules at 37° with a 2.5 inch (6.3 cm) 22 gauge syringe needle. As seen in Fig. 2 the specific viscosity of the fragments alone remained unchanged at 0.16 and that of the subunits alone reached only 0.05 after 25 min incubation. The mixture of subunits with fragments, however, polymerized rapidly and by 15 min the specific viscosity reached a plateau value of 0.56, three times higher than the sum of the viscosities of the fragments and subunits, indicating that appreciable subunit addition had occurred. Sedi-

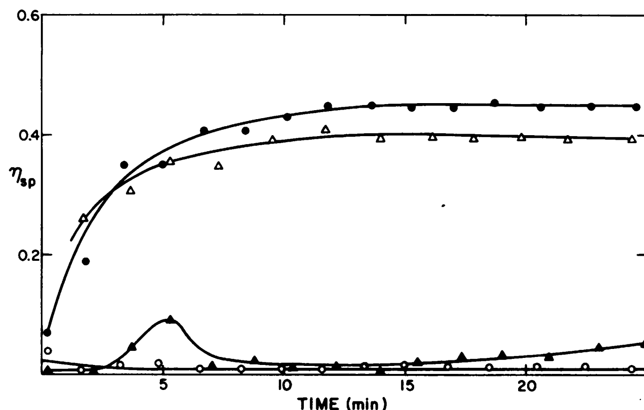

FIG. 3. Effect of HMW on microtubule polymerization. The kinetics of polymerization are shown for the isolated tubulin (▲) and HMW fractions (○), for tubulin reconstituted with HMW (●), and for unfractionated microtubule protein (△). The HMW and tubulin fractions were mixed 1:1 (v/v) with each other or with PM containing 1.0 mM GTP. Unfractionated material was adjusted to match the protein concentration in the mixture. Protein concentrations for HMW, tubulin, mixture, and unfractionated material were 0.3, 2.1, 2.3, and 2.4 mg/ml, respectively.

Table 1. Polymerization in isolated and combined fractions of tubulin and HMW

	Electron microscopy, rings/100 μm^2 *	Viscometry, η_{sp} (t = 30 min)†	Sedimentation, % polymer‡
MTP	323 ± 46	0.40 ± 0.15	100
HMW	0 ± 0	0.02 ± 0.02	7 ± 4
Tubulin	1 ± 0.7	0.08 ± 0.07	19 ± 11
Tubulin + HMW	148 ± 70	0.45 ± 0.08	78 ± 10

Ring formation is expressed as number of particles per 100 μm^2 area of grid surface in 5° samples negatively stained with 1% uranyl acetate. η_{sp} represents plateau specific viscosity at 37°. Amount of polymer is expressed as percent of polymer produced at 37° in unfractionated microtubule protein (MTP) which was designated 100%. Average protein concentrations were 2.5 mg/ml (MTP); 0.4 mg/ml (HMW); 2.2 mg/ml (tubulin); 2.6 mg/ml (tubulin + HMW).

* Standard error for three experiments.

† Standard deviation for four experiments.

‡ Standard deviation for five experiments.

mentation analysis showed that the microtubule growth resulting from the addition of purified tubulin subunits at 2.0 mg/ml was 75% of that which was obtained using unfractionated microtubule protein which had received a similar exposure to high salt. These data indicated that the DEAE-purified tubulin was competent to polymerize onto preexisting microtubule fragments but could not polymerize well on its own.

Effect of HMW on Microtubule Assembly. Since the difference between the purified tubulin and unfractionated material was the absence of the HMW components, the HMW fraction was added back to the tubulin fraction to determine if the ability to polymerize could be restored. HMW and tubulin fractions were desalted and combined 1:1 (v/v) at 5°, producing a mixture containing 2.2–2.6 mg/ml of the tubulin fraction and 0.3–0.4 mg/ml of the HMW fraction. We used viscometry, sedimentation, and electron microscopy to examine polymerization in the reconstituted mixture, the isolated fractions, and unfractionated microtubule protein which had received similar exposure to salt.

(1) Viscometry. As seen in Fig. 3, the isolated components showed little polymerization at 37°, whereas the mixture polymerized rapidly to levels comparable to those of the unfractionated material. The viscosity of the tubulin fraction typically showed a transient rise at approximately 5 min followed by a rapid decline to baseline levels. After 20–30 min the viscosity of the tubulin sample gradually increased but rarely rose above 0.10 even after 60 min. The results of four experiments are summarized in Table 1 and show that significant polymerization occurred in the reconstituted mixture but not in the isolated tubulin and HMW fractions.

(2) Sedimentation. Following incubation at 37° for 30 min, we assayed 0.5 ml aliquots of the same samples by sedimentation to determine the extent of polymerization. Under the conditions of this assay (28,000 × g for 20 min at 37°) all of the microtubule polymer but less than 5% of the unpolymerized material was sedimented (17). These data are given in Table 1. The mixture produced 78% as much polymer as the unfractionated material, and this was four times the amount of pellet formed in the isolated fractions alone. These results confirm the observations made by viscometry which showed extensive polymerization in the fraction of tubulin reconstituted with HMW but little polymerization in either of the isolated fractions.

(3) Electron Microscopy. At 5° the unfractionated material and reconstituted mixture were observed to contain numerous rings which have been postulated to be intermediates in microtubule assembly (10), whereas the isolated tubulin and HMW contained few or none. Thus, rings, which were not observed in either of the isolated fractions, reformed in the mixture. The results of three experiments are summarized in Table 1. Electron micrographs of samples after incubation at 37° also showed that both the unfractionated material and reconstituted mixture contained numerous microtubules, whereas the tubulin and HMW fractions contained only a few.

Reassociation of HMW onto Microtubules. These results strongly suggested that the HMW fraction promoted microtubule assembly, but it was not known if the HMW had physically reassociated with the microtubules. To determine if reassociation could be demonstrated biochemically, we determined the HMW/tubulin ratio from electrophoretic analysis of the polymerized and unpolymerized fractions obtained from the sedimentation assay described above. The results of two experiments are presented in Table 2. Under conditions which promoted polymerization of microtubules, 80% of the tubulin and HMW was sedimented. When polymerization was inhibited by low temperature or 100 μM colchicine, the sedimentation of HMW fell to low levels, indicating that HMW did not sediment in the absence of tubule polymerization. In fractions of tubulin reconstituted with HMW, however, 69% of the added HMW was sedimented, indicating that a large proportion of the HMW reassociated with the microtubule polymer.

To determine if the reassociation and presence of HMW on microtubules could be detected morphologically, we sedimented the polymerized samples and examined sections of pellets by electron microscopy. As seen in Fig. 4a and d, sections of the unfractionated material showed hairlike periodic projections or sidearms decorating the microtubules. As determined from 50 measurements taken from regions where the surface decoration was clearly distinguishable and not obscured by adjacent material, the projections measured

Table 2. Dependence of HMW sedimentation on tubulin polymerization

Polymerization conditions	HMW sedimented (%)
MTP (37°C)	80
MTP (0°C)	3
MTP (37°C), 100 μM colchicine	3
Tubulin + HMW (37°C)	69

HMW and tubulin were prepared as described in *Materials and Methods*. Microtubule protein (MTP) in this experiment was not exposed to high salt. Samples were incubated for 30 min prior to sedimentation. Unincubated samples and supernatants and pellets after sedimentation were analyzed for total protein content and for HMW and tubule composition by quantitative gel electrophoresis. Amount of HMW is expressed as percent of total HMW in sample before sedimentation.

18.9 ± 5.5 nm in length and 5.6 ± 2.5 nm in width. These structures, therefore, were morphologically distinct from the thicker dynein crossbridges seen in flagellar axonemes or the globular-headed myosin molecules present in muscle. In transverse sections the projections arose at all azimuthal angles; in longitudinal sections the projections exhibited a periodic spacing of 32.5 ± 9.4 nm along the microtubule surface, which is approximately the interval occupied by four 8.0 nm tubulin dimers. In some cases the hairlike projections appeared to interconnect adjacent, parallel microtubules.

Although the tubulin fraction did not generate much polymer, sufficient material was obtained for electron microscopy. After incubation at 37°, the purified tubulin fraction contained microtubules which were entirely free of adhering material (Fig. 4b). The microtubules in these pellets were observed to pack much closer together and frequently touch one another, whereas microtubules bearing projections were generally separated from one another.

Microtubules formed in reconstituted mixtures had projections indistinguishable from those of microtubules formed in the unfractionated material (Fig. 4c). When the HMW/tubulin ratio in the reconstituted mixtures was adjusted to match the ratio in the unfractionated material, the extent of tubule decoration by the projections was comparable. Therefore, the projections can be dissociated from the tubulin, purified, and reassociated. Since tubules formed in tubulin fractions containing no detectable HMW were free of projections and since tubules formed in tubulin reconstituted with a fraction containing 75% HMW both bound HMW and showed numerous projections, we conclude that the HMW components are the projections attached to microtubules.

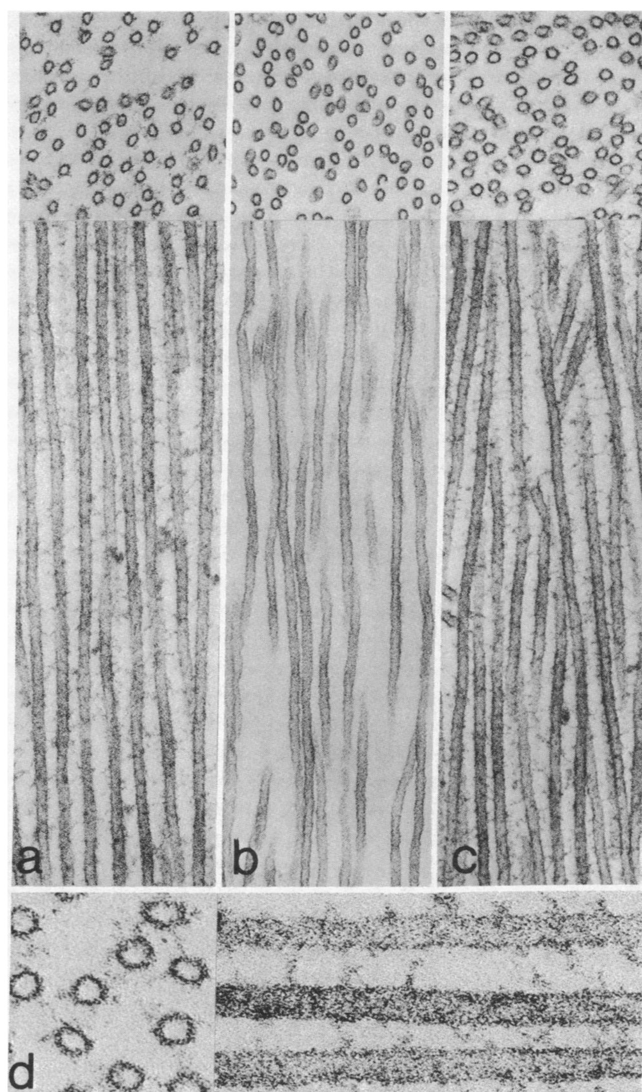

FIG. 4. Reassociation of HMW onto microtubules. Transverse and longitudinal sections of microtubules obtained from (a) unfractioned microtubule protein; (b) purified tubulin; (c) tubulin reconstituted with HMW. The HMW/tubulin ratio in the reconstituted mixture was adjusted to match the ratio in the unfractionated material; note that the pattern and extent of decoration are comparable. ×60,000. (d) Transverse and longitudinal sections of microtubules obtained from unfractionated microtubule protein. HMW, detectable as a 18.9 × 5.6 nm component, projects at 32.5 nm intervals from the microtubule wall. ×177,000.

DISCUSSION

By means of ion exchange chromatography using DEAE-Sephadex and stepwise elution with salt, microtubule protein was separated into two fractions containing HMW and tubulin. The purified HMW fraction has been defined electrophoretically as a mixture of three primary species: a doublet band (85%) of 286,000 and 271,000 MW and a minor band (15%) of 345,000 MW and residual trace components. The separation of HMW from tubulin has made it possible to examine polymerization in preparations of purified tubulin, to determine the effect of HMW on microtubule assembly, and to characterize HMW both morphologically and biochemically.

DEAE-purified tubulin polymerized poorly under conditions that gave optimal assembly for unfractionated microtubule protein containing HMW. Since the ability of purified tubulin to polymerize was restored by adding back the HMW fraction, we conclude that this fraction stimulates microtubule assembly. The molecular basis for this enhancement is not fully understood. Previous studies on the equilibrium nature of microtubule polymerization suggested a mechanism of condensation-polymerization characterized by distinct phases of nucleation and growth (17, 22). Since purified tubulin subunits were capable of rapid addition onto preexisting microtubule fragments and because HMW-free tubulin did polymerize slowly, these results and the work of Kirschner *et al.* (23) demonstrate that HMW is not an absolute requirement for the growth or elongation of

polymer. However, the addition of HMW to tubulin at 0° resulted in the formation of numerous rings; since rings have been postulated to be intermediates in microtubule assembly (10), HMW may promote polymerization by facilitating the formation of an essential intermediate.

Indistinct structures (projections or crossbridges) have been reported previously on cytoplasmic microtubules in nerves (4), the spindle apparatus (6, 7), and numerous other sources (3), but to date these structures have not been demonstrated biochemically. In this report the HMW components have been morphologically identified as sidearms projecting from the microtubule wall. Since the HMW fraction consists primarily of the doublet band we infer that the projections are comprised principally of this material. Whether the minor electrophoretic band is also a component of these sidearms or represents a separate class of tubule-associated structures remains to be determined.

The role of the HMW component has not yet been established. One possibility is that, in analogy to cilia and flagella, HMW serves a motility function and is required for tubule-dependent particle transport; however, the relationship between the HMW component and dynein, the ATPase crossbridge in cilia and flagella, is not clear. On the basis of electrophoretic mobility, Gaskin *et al.* (14) and Burns and Pollard (24) reported the presence of a dynein-like protein associated with microtubules from brain tissue, although little or no ATPase activity was detectable in their preparations. Our results differ in that the predominant high-molecular-weight doublet associated with microtubules purified by *in vitro* assembly has an electrophoretic mobility distinct from that of dynein; however, the minor band (Fig. 1) does have a mobility close to that of dynein. The structural appearance of HMW is also similar to but distinct from that of flagellar dynein. The HMW projection measures 18.9×5.6 nm and displays a longitudinal periodicity of 32.5 nm along the length of the microtubule, whereas the dynein sidearm measures approximately 20 to 25×8 to 10 nm (25) and displays a longitudinal periodicity of 23.0–24.0 nm (3) along the A fiber of outer doublet tubules. ATPase activity has been detected in our preparations of microtubule protein (0.1 μM P_i/mg per min in 30 mM Tris-HCl buffer at pH 7.8 at 22°), but this activity has not yet been definitely identified with the HMW components.

Rather than functioning as a mechanochemical component, HMW may instead perform a role as a structural crossbridge. Recent observations on the length of microtubules in the spindle of crane fly spermatocytes (26) and in developing myoblasts (5) indicate that these arrays consist primarily of short, overlapping tubule fragments. These studies suggest that the structural integrity of the overall microtubule array requires lateral interaction between paraxial tubules. Such a lateral interaction could be provided by the HMW component serving as a structural, intertubule bridge.

A third possibility is that since HMW is required for rapid polymerization, the HMW components are involved in the control of microtubule assembly. Since HMW was not required for growth of tubulin onto microtubule fragments but did promote the formation of ring structures, HMW may serve a role in tubule nucleation rather than elongation. However, since HMW associates with microtubules along their entire length, the tubulin-HMW interaction is clearly not restricted to the initiation step. A reasonable suggestion is that in addition to its effects on initiation, HMW can reversibly associate with periodic binding sites on formed microtubules. Demonstration of this suggestion would require titration binding analyses of HMW to HMW-free microtubules.

Since the HMW fraction is a mixture of three primary electrophoretic components plus some trace species, it is not yet clear whether in solution these components represent a single molecule or several molecules and whether they serve a single function or several functions. Clearly further work is required to distinguish among these possibilities.

Note Added in Proof. Recently the identification of high-molecular-weight proteins as filaments attached to microtubules has also been reported by Dentler *et al.* (27).

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