Identity and Polymerization-Stimulatory Activity of the Nontubulin Proteins Associated with Microtubules†

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ABSTRACT: Microtubule protein purified by an in vitro assembly procedure contains several accessory proteins which copurify with tubulin and are required for efficient tubulin assembly. When purified microtubule protein was critically examined by sodium dodecyl sulfate gel electrophoresis, two principal high-molecular-weight bands and 33 additional nontubulin components were identified in addition to tubulin. These proteins were isolated and characterized with respect to their binding affinity for microtubules, their relative stimulatory activity and the specificity of the stimulatory effect with the following results: (1) although 35 nontubulin components were identified after two cycles of purification, only a few proteins, including the high-molecular-weight components, bound to microtubules with high affinity and maintained a constant stoichiometry to tubulin through six cycles of purification; (2) from analysis of an activity profile of the fractionated components, 60% of the stimulatory activity in our preparations of microtubule protein was attributed to the high-molecular-weight material and the remaining 40% was attributed to a spectrum of other nontubulin proteins; and (3) the stimulatory effect was not restricted to the HMW or other accessory proteins but was also exhibited by cationic substances and by glycerol, suggesting that many factors that bind to tubulin also stimulate microtubule polymerization. Since the kind and amount of nontubulin protein detected by other workers appear to depend on the methods employed for their purification, details are also presented for the ion-exchange procedures we have developed for preparing nontubulin proteins from porcine brain microtubules.

Microtubules perform important roles in the determination of shape and generation of motility in eukaryotic cells, and it has been proposed that these functions may be regulated by components that associate with the microtubule surface (for reviews, see Tilney, 1971; Bardele, 1973; Roberts, 1974). Microtubule protein[1] purified from porcine brain tissue by an in vitro assembly procedure contains approximately 25% nontubulin components which consist primarily of a doublet band of 286 000 and 271 000 MW as determined by sodium dodecyl sulfate gel electrophoresis (Borisy et al., 1974, 1975). These components, referred to as HMW 1 and 2 due to their high-molecular-weight nature,[2] have been isolated and identified as a structural component that specifically binds to the tubule wall (Murphy and Borisy, 1975). Other workers (Dentler et al., 1975) have reported similar microtubule-associated proteins (designated MAPS) derived from chick brain tissue.

Analysis of microtubule assembly in isolated and combined fractions of HMW and tubulin showed that, whereas purified tubulin failed to polymerize by itself, tubulin reconstituted with HMW polymerized rapidly (Murphy and Borisy, 1975). Therefore, HMW appeared to be required for efficient polymerization. However, as described by Weingarten et al. (1975), a fraction of microtubule-associated proteins (designated tau factor) prepared from microtubule protein containing very little HMW also stimulated polymerization, suggesting that more than one factor can promote assembly in vitro. Both of these preparations of microtubule-associated proteins were reported to contain numerous components; however, the relative amounts of these components were not determined nor were the minimal levels of factor required to stimulate polymerization specified. Consequently, it has neither been possible to distinguish conclusively between those components that stimulated assembly and those that were associated with the tubules nonspecifically, nor to determine the relationship between the HMW fraction and the tau fraction. This report is a more detailed examination of the role of the nontubulin components in microtubule assembly in vitro.

In this paper, HMW and all of the trace components present after two cycles of in vitro assembly were identified by sodium dodecyl sulfate gel electrophoresis. To determine which of these proteins had a high affinity for microtubules, the mass ratio of these components to tubulin was assayed through six cycles of assembly-disassembly. The nontubulin proteins were compared for their ability to stimulate tubulin assembly in order to determine if a specific stimulatory factor was present and to assess the role of the trace components in assembly. Since microtubule protein contains many nontubulin components in addition to tubulin, it was necessary to first segregate the components in order to assay their stimulatory activity. This paper therefore also presents the details of the procedures we have developed in preparing nontubulin proteins from porcine brain microtubules. A brief account of an earlier version of one of these procedures has previously been reported (Murphy and Borisy, 1975). From these studies, the HMW components were determined to comprise most of the nontubulin material and were concluded to be the principal factors

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[1] 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.

[2] Abbreviations used: HMW, high-molecular-weight components; PM, polymerization medium; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MW, molecular weight; MAPS, microtubule-associated proteins.

responsible for promoting tubulin assembly in our preparations of microtubule protein. In addition, several nonspecific factors were found to stimulate polymerization, indicating that the microtubule-associated proteins were not an absolute requirement for assembly in vitro.

Materials and Methods

Preparation of Tubulin and Purification of Microtubule-Binding Proteins. Microtubule protein was purified from porcine brain tissue by two cycles of a reversible, temperature-dependent assembly procedure (Borisy et al., 1975). The protein was prepared at 0 °C in polymerization medium (PM) (0.1 M Pipes adjusted to pH 6.94 at 23 °C containing 0.1 mM $MgCl_2$), supplemented with 1.0 mM GTP.

HMW and other microtubule-binding proteins were isolated by chromatography of purified microtubule protein on DEAE-Sephadex using stepwise elution with salt. Typically, 16 mL of DEAE-gel was placed in a column of 2.5 × 45.0 cm, equilibrated with 2–3 bed volumes of PM containing 0.1 mM GTP and eluted at a flow rate no greater than 1.0 mL/min. All operations were performed at 0–4 °C. Fractions of 0.8 mL were collected, absorbance at 285 nm was determined, and the peak fractions were pooled to give samples of 3.0 mL. These samples were immediately desalted by gel filtration at 5 °C on columns containing Sephadex G-25 (coarse) equilibrated in PM containing 1.0 mM GTP. Desalted samples were used within 2 h of preparation (unless otherwise stated) in order to assure maximal activity of the protein. In some experiments, desalted samples were frozen in liquid N_2 and thawed immediately before use (see Results).

To obtain all of the microtubule-binding proteins in one fraction (designated the nontubulin fraction), salt (KCl) was added to the sample of microtubule protein (10–20 mg/mL) to a final concentration of 0.3 M, and the sample was applied to a column containing DEAE-Sephadex equilibrated with the same salt solution. Under these conditions, only tubulin bound to the column and all of the nontubulin proteins were collected together as the fraction of unbound protein.

To separate HMW proteins from other microtubule-associated proteins referred to as tau factor by Weingarten et al. (1975), a variation of the above procedure was employed. In this procedure samples of microtubule protein at relatively low concentration (1 mg/mL) in PM solution containing 0.1 mM GTP were applied to the DEAE-Sephadex at low loadings (3–4 mg protein/mL gel). Protein species that did not bind to the column eluted with the column equilibration buffer. This material (unbound fraction) did not contain HMW components or tubulin but did contain other nontubulin components (tau factor). A fraction containing HMW plus other trace components was subsequently eluted with buffer containing 0.3 M KCl (HMW fraction); tubulin was eluted with buffer containing 0.8 M KCl (tubulin fraction). In this procedure the fraction of unbound protein was concentrated before use by ultrafiltration in a Diaflo cell with a PM30 filter (Amicon Corp., Lexington, Mass.).

Polyacrylamide Gel Electrophoresis. Samples and gels (5.0 and 7.5% acrylamide, 0.6 × 8.0 cm) were prepared and run according to the methods of Shapiro et al. (1967). Gels were stained for protein with Coomassie brilliant blue according to the procedure of Fairbanks et al. (1971), and scanned at 560 nm with a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Staining by Coomassie blue was demonstrated previously to be quantitative for amounts of protein from 0.5 to 5.0 μg (Borisy et al., 1975). Below 0.5 μg the stain density was less than predicted, thus leading to the possible underestimation of the levels of trace components. Therefore, samples were applied to gels over a range of concentrations such that the trace components fell in the linear region of the Coomassie stain response. For determinations of the purity of tubulin fractions, gels were run both with normal loadings (4 μg) and overloadings (100 μg). A contaminant present at the 1% level would then be quantitatively detectable on the overloaded gels. Using this procedure, purities exceeding 99% were assigned to the tubulin fractions. Peak areas from the densitometer tracings of the gels were quantitated by planimetry or by weighing the peaks cut out from photocopies of the original gel tracings. For studies in which numerous components were followed through several cycles of purification, peak area was estimated by multiplying the peak height by the peak width measured at half the height of the peak.

For determinations of molecular weight using sodium dodecyl sulfate gel electrophoresis, cross-linked oligomers of bovine serum albumin were prepared by the method of Payne (1973) and run with microtubule protein both separately and together on the same gel.

Polymerization Assays. Polymerization was monitored by examining the development of viscosity or turbidity at 37 °C and by electron microscopy. For viscometry, Ostwald capillary viscometers were employed using the methods described previously by Olmsted and Borisy (1973). Changes in turbidity were followed spectrophotometrically at 320 nm. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. For electron microscopy, samples were placed for 30 s on 400-mesh grids coated with Formvar and carbon and were successively displaced with 4 drops of the following solutions: 1 mg/mL cytochrome *c*; distilled water; 1% aqueous uranyl acetate. The excess stain was removed with a filter paper and the sample was air dried and examined with a Philips 300 electron microscope at 80 kV.

To determine the relative stimulatory activity of the nontubulin proteins, a fraction containing all of the nontubulin proteins (15 mg) was fractionated by molecular sieve chromatography on a column containing Bio-Gel A-15m equilibrated in PM buffer, and the column fractions were assayed for their ability to promote microtubule assembly. A tubulin fraction prepared in PM containing 2.0 mM GTP was frozen and stored during the chromatography to arrest the decay in the assembly competency of the tubulin (see Results). A 0.15-mL aliquot of each fraction was combined 1:1 (v/v) with the tubulin fraction, and the turbidity of the fractions was determined at 320 nm after incubation at 37 °C for 45 min. Since the voided peak itself was turbid after elution from the column, the turbidity was also recorded after depolymerization at 0 °C and the difference in the two readings was taken as a measure of cold-reversible polymer.

Biochemicals. E. coli tRNA was a gift from the laboratory of Dr. Robert Bock. Dextran sulfate was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. DEAE-Dextran, polylysine, protamine chloride, calf thymus histone, chicken ovalbumin, bovine serum albumin, and urease were obtained from Sigma Chemical Co., St. Louis, Mo.

Results

Identification of Microtubule-Binding Proteins and Determination of Their Affinity for Tubulin. It was known from previous studies that the HMW fraction contained numerous trace components. Furthermore, microtubule-binding proteins in addition to HMW have been demonstrated to have stimu-

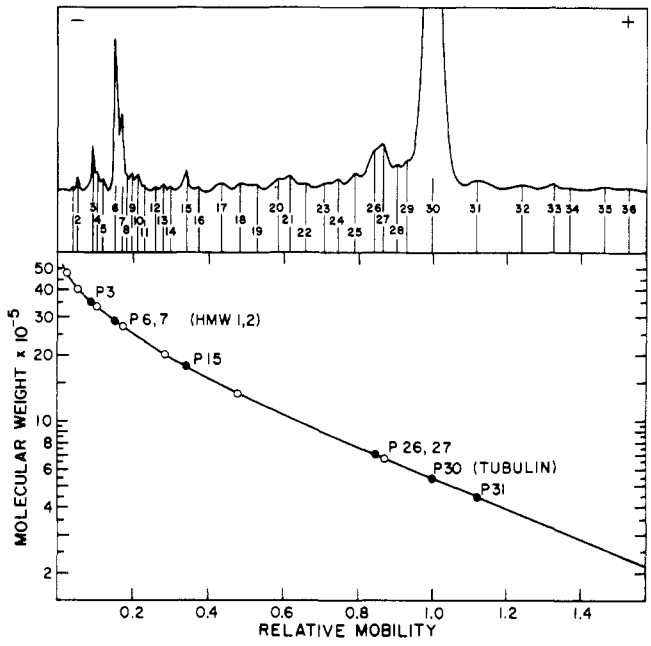

FIGURE 1: Gel electrophoretic analysis of microtubule protein purified by in vitro assembly. A densitometer tracing of a 5% acrylamide gel containing microtubule protein prepared by two cycles of assembly-disassembly. Tubulin (P30) represents 75% of the total material; 35 nontubulin components are also observed and are numbered sequentially beginning with those of high molecular weight. The molecular weight of any component can be determined directly from the accompanying plot of molecular weight vs. mobility relative to tubulin which is designated 1.0. The plot is based on a series of oligomers of bovine serum albumin cross-linked with glutaraldehyde (open circles) as described in Materials and Methods. In addition to tubulin (P30), the 345 000 MW component (P3), HMW 1 and 2 (P6, 7), and components of 180 000 MW (P15), 70 000 MW (P26, 27), and 45 000 MW (P31) are also indicated (filled circles).

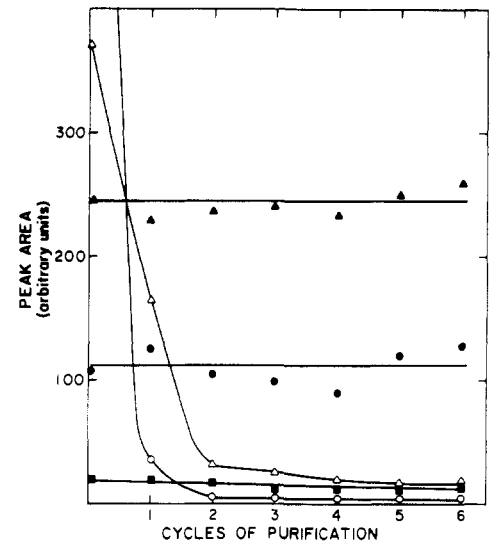

FIGURE 2: The copurification of microtubule-binding proteins with tubulin. Microtubule protein was examined by quantitative gel electrophoresis to determine the stoichiometry of microtubule-binding proteins to tubulin through six cycles of assembly-disassembly. The amount of various components was determined from densitometer tracings of the gels and is expressed as peak area in arbitrary units. The peak area for tubulin was the same for all six cycles. The values for the various species in the brain extract prior to purification are shown at 0 cycles. The proteins indicated are: the 345 000 MW component, P3 (●); HMW 1 and 2, P6, 7 (▲); 180 000 MW component; P15 (■); components of 70 000 MW, P26 and P27 (△); and 45,000 MW component P31 (○). The value for P31 at 0 cycles was 830 area units.

latory activity. To define these proteins more precisely, the composition of the nontubulin components associated with microtubules purified by two cycles of assembly-disassembly was analyzed by gel electrophoresis. To determine the specificity of the association of these components with microtubules, the mass ratio of these components to tubulin was assayed through six cycles of in vitro assembly-disassembly. In this way those proteins possessing the highest affinity for tubulin under the conditions employed would be identified.

A densitometer tracing of a sodium dodecyl sulfate gel containing microtubule protein after two cycles of purification is shown in Figure 1. Below the gel tracing is a plot of molecular weight vs. mobility relative to tubulin so that the molecular weight of any band can be determined. In addition to tubulin (P30), 35 bands were observed in the eight preparations that were analyzed. Most of the nontubulin material (60%) was composed of high-molecular-weight components that were visualized as bands in the uppermost portion of the gel (P1-11). We refer to all of the components in this region collectively as HMW. They consist chiefly (70%) of HMW 1 and 2 (P6, 7), a component of 345 000 MW (P3) and several other trace species. A densitometer tracing of microtubule protein is also shown in Figure 4 for convenient comparison with the gel patterns of the isolated ion-exchange fractions.

The affinity of the trace components for tubulin was examined by determining their stoichiometry to tubulin through six cycles of assembly-disassembly. The results of the gel electrophoretic analysis are shown in Fig. 2. Most proteins including a component of 45 000 MW having the mobility of actin (P31) were eliminated after two cycles of purification. Several proteins, including components of approximately 70 000 MW (P26, 27), were rapidly depleted after two cycles, but then remained at low levels during additional cycles of purification. After six cycles of assembly, HMW 1 and 2 and the 345 000 MW component were the only significant components present, and only traces of nine other bands could be detected. HMW 1 and 2 and the 345 000 MW components were maintained in constant stoichiometry to tubulin through all six cycles, and this stoichiometry was the same after purification as it was in the original brain extract. Other components, including one of 180 000 MW (P15), also maintained constant stoichiometry to tubulin but these were present in only minute amounts. Thus, while many cellular proteins bound to microtubules, only a few proteins, namely, HMW 1 and 2, the 345 000 and 180 000 HMW components, and some fraction of the 70 000 MW material, showed a high affinity for tubulin.

Preparation of Microtubule-Binding Proteins. The nontubulin proteins associated with microtubules after purification by in vitro assembly include, in addition to the HMW species, a number of other components. It was therefore necessary to fractionate these components in order to assay their relative stimulatory activity in microtubule assembly. Since chromatography on DEAE-Sephadex had previously been used to isolate tubulin in high purity from extracts of brain tissue (Weisenberg et al., 1968), we applied this method to assembly-purified microtubule protein to produce a fraction greatly enriched in HMW and a fraction of highly purified tubulin (Murphy and Borisy, 1975). Data are first presented for the purification of the nontubulin proteins and the compositional analysis of various protein fractions; these fractions are then analyzed for their relative ability to stimulate assembly. Chromatography of microtubule protein by gradient elution in salt showed that tubulin, HMW, and other microtubule-

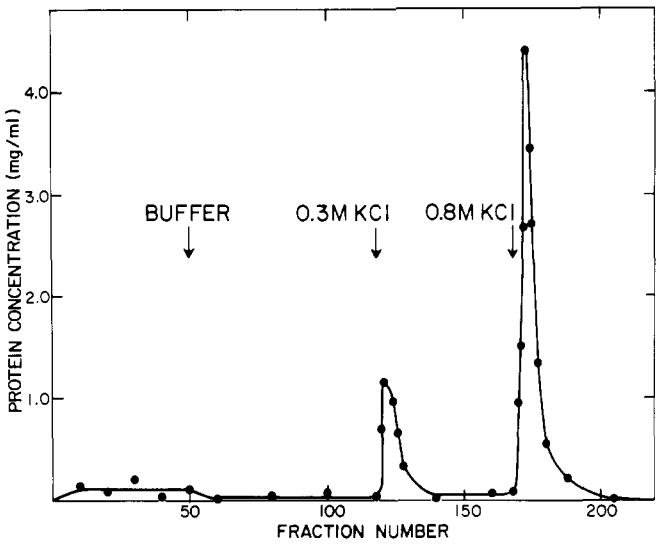

FIGURE 3: Separation of HMW from tubulin by chromatography on DEAE-Sephadex. Microtubule protein (47 mg) purified by two cycles of in vitro assembly was prepared at 0.9 mg/mL in PM containing 0.1 mM GTP and applied to a column containing DEAE-Sephadex. The column was washed with equilibration buffer to remove unbound protein and HMW and tubulin removed by stepwise elution with 0.3 and 0.8 M KCl, respectively, in the same solution. The column fractions (1.0 mL) were assayed for protein content by the Lowry procedure and their composition was determined by gel electrophoresis.

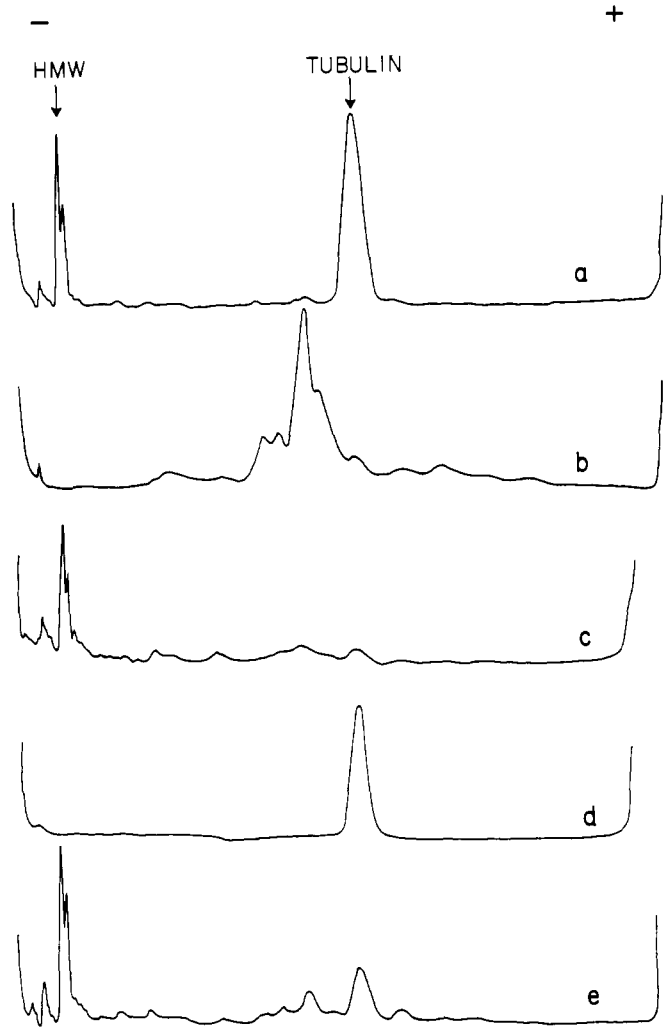

FIGURE 4: Gel electrophoretic analysis of column fractions from chromatography on DEAE-Sephadex. Densitometer tracings of 5% acrylamide gels containing 4-μg loadings of unfractionated microtubule protein (a), unbound fraction (b), HMW fraction (c), tubulin fraction (d), and total nontubulin fraction (e).

binding proteins (tau factor) could be isolated in distinct fractions. Accordingly, the HMW components and tau factor were separated from tubulin on DEAE-Sephadex using a stepwise elution procedure as shown in Figure 3. A small portion of the sample did not bind to the column and contained proteins of lower molecular weight than HMW. HMW components were eluted with 0.3 M KCl; a fraction containing nearly pure tubulin was eluted with 0.8 M KCl. The highest yield and also the highest purity of both the 0.3 and 0.8 M KCl fractions occurred when the sample was applied at concentrations below 1.0 mg/mL. Above this value an increasing proportion of the sample did not bind to the column and there was a corresponding decrease in the purity of the 0.3 M KCl fraction and in the yield of the 0.8 M KCl fraction. In addition, it was determined that the yield of the 0.8 M KCl fraction was linear over the range explored (data not shown), up to 6 mg of protein sample per mL Sephadex. Densitometer tracings of gels of the protein fractions obtained from this column are shown in Figure 4, and the compositional analysis of these fractions as determined by gel electrophoresis is shown in Table I.

Microtubule protein prepared by two cycles of in vitro assembly-disassembly contained 75% tubulin and 25% microtubule-associated protein, of which 60% was composed of HMW and 40% consisted of other trace components (Figure 4a). The unbound fraction (Figure 4b) (5% of the sample) eluted with the column equilibration buffer and contained four major proteins, including two components of approximately 70 000 MW (P26, 27) and two components near 80 000 MW (P24, 25) as well as several other components, but contained only a small amount of tubulin (7%) and a trace amount of HMW. The 0.3 M KCl fraction (Figure 4c) consisted primarily of HMW components (71%), some tubulin (4%), and other components (25%). The 0.8 M KCl fraction consisted of greater than 99% tubulin with no HMW detectable, and only traces of other components were detected on overloaded gels containing 100 μg of protein (Figure 4d). When the 0.8 M KCl fraction was examined on stacking gels, only the α and β tubulin bands were present, confirming that all of the nontubulin components were indeed separated from tubulin by the salt-fractionation procedure (data not shown). For some experiments a fraction containing all of the nontubulin components (Figure 4e) was prepared (see Materials and Methods). This fraction was generally contaminated somewhat more by tubulin (12%) than the unbound fraction or the HMW fraction but otherwise essentially had the composition of the unbound and HMW fractions combined, namely, 51% HMW, 12% tubulin, and 37% other species.

Stability of Tubulin and the Stimulatory Activity of the Microtubule-Associated Proteins. Before examining the activity of the components in the nontubulin fraction in more detail, it was first necessary to examine the stability of these components after separation from tubulin. Previous results showed that, as determined by sedimentation, tubulin reconstituted with HMW 2 h after fractionation by ion-exchange chromatography formed 78% as much polymer as the unfractionated material. For longer term storage, separated tubulin and nontubulin components were frozen in liquid nitrogen and kept at −80 °C and then thawed immediately before use in a polymerization experiment. We found that the separated components when recombined after a month of storage

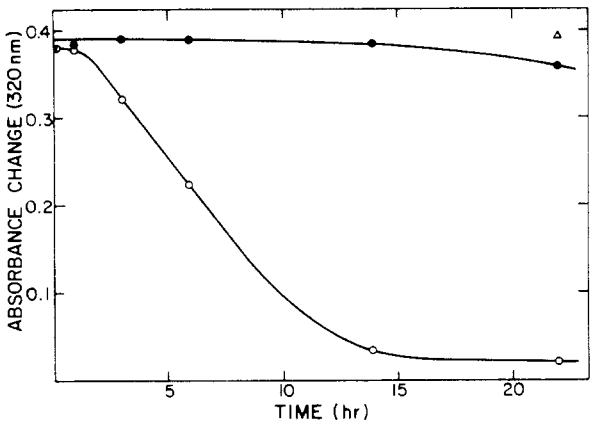

FIGURE 5: Stability of the isolated tubulin and nontubulin fractions. The tubulin fraction (○) was assayed for its competency to form microtubules, and the nontubulin fraction (●) was examined for its ability to stimulate tubulin assembly at various times after isolation. In both cases, the fraction whose activity was to be examined was combined 1:1 (v/v) with the complementary fraction whose activity was kept constant by storage in liquid N_2. The mixtures were placed at 37 °C and polymerization was monitored by observing the change in turbidity at 320 nm (see Materials and Methods). The open triangle gives the extent of polymerization when the frozen tubulin and nontubulin fractions were thawed and mixed 1:1. The concentrations of tubulin and nontubulin protein in all mixtures were 1.5 and 0.5 mg/mL, respectively.

TABLE I: Compositional Analysis of Microtubule Protein and Column Fractions Obtained by DEAE-Sephadex Chromatography.[a]

Fraction	Volume (mL)	Concn (mg/mL)	Protein (mg)	Tubulin (%)	HMW[b] (%)	Other[c] (%)
Microtubule protein	55	0.95	52.3	75	15	10
Unbound protein	115	0.03	2.6	7	0	93
0.3 M KCl fraction	65	0.20	13.0	4	71	25
0.8 M KCl fraction	72	0.35	24.9	>99	0	<1

[a] Microtubule protein purified by two cycles of assembly (52.3 mg of protein in PM containing 0.1 mM GTP) was applied to 16 mL of DEAE-Sephadex. Unbound protein was eluted with buffer and the HMW and tubulin fractions were collected by stepwise elution with 0.3 and 0.8 M KCl in the same buffer. The yields represent data from a typical column isolation. A portion (20%) of the applied sample was not recovered from the column. The percent composition of tubulin and microtubule-binding proteins was determined by gel electrophoresis as described in Materials and Methods. [b] HMW refers to the total HMW material as defined in Results. [c] "Other" refers to all of the microtubule-binding proteins except the HMW components.

in the frozen state retained greater than 80% of their polymerizing ability. However, it was also necessary to examine the stability of the tubulin and the stimulatory activity while in solution and over the periods of time required to carry out a fractionation experiment. For these experiments, both tubulin and nontubulin fractions were separately prepared and divided into two aliquots. One aliquot of protein, whose stability was to be tested, was maintained at 0 °C. The other aliquot was subdivided, frozen, and stored in liquid nitrogen. After various times, aliquots of the frozen HMW and tubulin fractions were each combined 1:1 (v/v) with an aliquot of the complementary unfrozen fraction, and the extent of polymerization was determined by monitoring the change in turbidity at 320 nm following polymerization at 37 °C. As seen in Figure 5, the competency of pure tubulin to polymerize decayed rapidly at 0 °C with a half-life of 6.8 h, whereas 92% of the activity of the nontubulin fraction remained after 22 h. Thus, both the tubulin and nontubulin fractions could be prepared as solutions, and frozen and stored with no detectable loss in activity when returned to polymerizing temperatures. However, in solution, whereas the stimulatory activity was moderately stable, the tubulin itself was very labile.

Identification of the Stimulatory Activity. To identify the molecular species that promoted tubulin assembly, we first examined the stimulatory activity of three different fractions of microtubule-associated proteins. The HMW fraction, the unbound fraction (tau factor), and a fraction containing all of the nontubulin components were prepared in PM containing 1.0 mM GTP, added to the tubulin fraction, and monitored for microtubule formation by viscometry at 37 °C. A unit of polymerization was defined as an increase of 0.1 in specific viscosity after 30 min incubation at 37 °C per mg of protein added to 1 mL of a fixed concentration of pure tubulin (1.8 mg/mL). The specific stimulatory activity was found to be 0.9 unit for the total nontubulin fraction, 1.3 units for the HMW fraction, and 1.4 units for the unbound fraction, respectively. However, as shown by acrylamide gel analysis (see Figure 4), these same fractions differed extensively in composition. The content of HMW in the nontubulin, HMW, and unbound fractions was 51, 71, and 0%, respectively; whereas the major 70 000 MW species present in the unbound fraction was present at only trace levels in the HMW fraction. Therefore, all three fractions stimulated polymerization with comparable specific activities but no *single* component appeared to be present in each of the fractions and responsible for the stimulatory activity. These data suggested that stimulatory activity was not restricted to a single component and that microtubule-associated proteins had approximately equal activity when compared on a mass basis.

To examine the stimulatory activity of the nontubulin components in more detail, a total nontubulin protein fraction was prepared and components were fractionated by molecular sieve chromatography on a column containing 4% agarose and assayed for stimulatory activity using frozen aliquots of pure tubulin as a source of competent subunits as described in Materials and Methods. Sodium dodecyl sulfate gels of fractions resulting from this procedure are shown in Figure 6, and the elution profile is shown in Figure 7a. The composition of these fractions, as determined from densitometer tracings of the gels, is indicated in Figure 7b for the HMW components (solid triangles) and for other nontubulin material (open triangles). When these two components were summed and added to the contribution due to tubulin, a profile of total protein was obtained (closed circles) which was very similar to that obtained by the Lowry reaction for protein (Figure 7a). The elution profile shows a large peak of voided material (peak 1) followed by a large secondary peak (peak 2) with a trailing edge. Peak 1 was turbid and contained all of the residual tubulin and 345 000 MW component present in the sample. Peak 1 also contained bands corresponding to HMW 1 and 2, a component of 70 000 MW plus some other trace species. The other fractions of the column were clear. Peak 2 contained primarily HMW 1 and 2, and the trailing edge of peak 2 contained the 70 000 MW component and several components of lower molecular weight. Thus, HMW 1 and 2 were completely

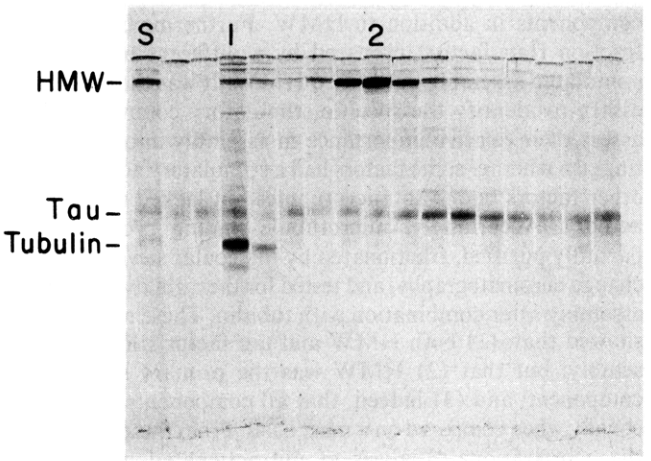

FIGURE 6: Gel electrophoretic analysis of column fractions after chromatography of the nontubulin protein fraction on 4% agarose. The gel labeled S indicates the sample applied to the column; the gels labeled 1 and 2 correspond to peak 1 and 2 as shown in the elution profile in Figure 7a.

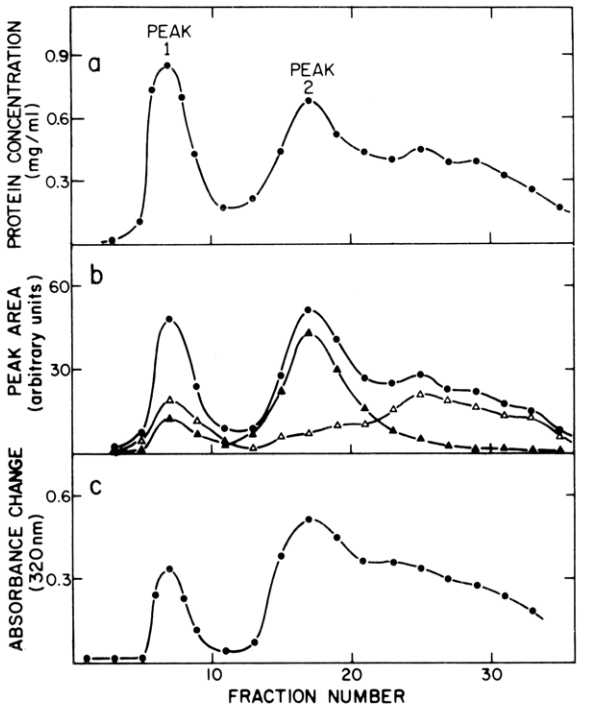

FIGURE 7: The nontubulin proteins fractionated by chromatography on 4% agarose. Determination of stimulatory activity. The nontubulin protein fraction (15 mg) purified by ion-exchange chromatography was prepared at 6.6 mg/mL in PM and applied to a column containing 4% agarose and 0.8-mL fractions were collected. (a) Elution profile showing protein concentration, (b) quantitative analysis of the gels shown in Figure 6 indicating the total amount of Coomassie-staining material (●), and the amount of material due to HMW (▲), and other nontubulin proteins (△), (c) activity profile obtained by combining column fractions 1:1 (v/v) with tubulin (2.4 mg/mL, final concentration) and monitoring the change in turbidity at 320 nm that occurred after warming the mixture to 37 °C.

resolved from tubulin and the 345 000 MW component and were partially separated from the components of 70 000 MW.

The amount of stimulatory activity present in these fractions, determined as the amount of cold-reversible polymer formed at 37 °C (see Materials and Methods), is shown in Figure 7c. The major peak of activity was coincident with the peak position for HMW as determined from the gel analysis. However, the activity profile had the same general shape as the elution profile for total protein. Whereas there was a symmetrical distribution of HMW 1 and 2 in peak 2 as determined from the gels, the distribution of stimulatory activity over this peak had a trailing edge, indicating that the other nontubulin components in our preparations also stimulated assembly. When the activity profile was resolved into portions due to HMW and to other components, approximately 60% of the activity was estimated to be due to HMW and 40% to the other species. These results confirmed the conclusions of the three-fraction analysis that stimulatory activity was approximately proportional to the mass of the nontubulin proteins irrespective of their molecular weight. In addition, the activity assay on the column fractionated material pointed to HMW 1 and 2 as the principal stimulatory species.

Other Stimulatory Factors. The inference that stimulatory activity was a more general property of microtubule-associated proteins suggested that any factor that bound to tubulin might promote the association of tubulin dimers and hence also stimulate polymerization. Since tubulin is an acidic protein and is known to bind to DEAE-resins with high affinity, various positively charged factors such as basic proteins and polycations might be expected to bind to tubulin and display stimulatory activity. To examine this possibility a variety of polycationic and anionic substances were tested for their ability to induce the polymerization of purified tubulin. As seen in Table II, cationic substances, such as DEAE-Dextran and polylysine, and basic proteins, such as protamine and histone, stimulated polymerization; by comparison, anionic substances, such as Dextran sulfate and tRNA, and acidic proteins, including ovalbumin, serum albumin, and urease, did not promote assembly. Polymerization was also stimulated by 4 M glycerol, indicating that, in the presence of this substance, no additional factors were required at all. In all cases where polymerization occurred, electron microscopy showed that the assembly products were microtubules and that aberrant forms such as sheets or twisted ribbons were not present. Thus, numerous factors such as DEAE-Dextran, which associate with tubulin due to their net positive charge, also stimulated microtubule

TABLE II: Stimulation of Tubulin Polymerization by Nonspecific Factors.[a]

Factor	Factor Concn (mg/mL)	Microtubule formation
None		−
DEAE-Dextran	0.025	+
Polylysine	0.05	+
Protamine chloride	0.025	+
Calf thymus histone	0.05	+
Dextran sulfate	0.25	−
tRNA	0.28	−
Chicken ovalbumin	0.05	−
Bovine serum albumin	0.25	−
Urease	0.05	−
Glycerol	4.0 M	+

[a] Purified tubulin was prepared in PM containing 1.0 mM GTP as described in Methods. Microtubule formation in samples of tubulin supplemented with various factors was monitored at 37 °C by viscometry or by turbidity at 320 nm and by electron microscopy. The concentration of tubulin ranged from 1.9 to 2.5 mg/mL; figures for the concentration of factor indicate the final concentration in the sample.

formation, demonstrating that the stimulatory effect is not necessarily specific for the proteins that associate and copurify with microtubules during in vitro assembly. While this work was in progress a similar stimulation of tubulin polymerization by polycationic substances (Erickson and Voter, 1976) and glycerol (Lee and Timasheff, 1975) was reported.

Discussion

Recently a possible mechanism for the regulation of microtubule assembly has been suggested by the demonstration of a requirement for accessory proteins for polymerization of tubulin in vitro (Murphy and Borisy, 1975; Weingarten et al., 1975). However, these studies have reported different stimulatory species referred to as HMW and tau factor, respectively. In an attempt to resolve these differences we have investigated in a more detailed way the identity and relative stimulatory activity of the nontubulin proteins associated with microtubules.

It now seems clear that the amount and kind of nontubulin proteins present in preparations of microtubules depend on the method of purification. Microtubule protein prepared under optimal conditions for in vitro assembly according to the procedure of Borisy et al. (1975) contains as the major nontubulin species a doublet band of 286 000 and 271 000 MW referred to as HMW 1 and 2, plus other minor species.

In contrast, brain microtubule protein prepared in a different buffer system in the presence of glycerol has been reported not to contain HMW but instead to contain trace amounts (5%) of other nontubulin material designated tau factor (Weingarten et al., 1974, 1975). However, other workers, also using a glycerol assembly procedure to purify microtubule protein, have reported the presence of varying levels of high-molecular-weight material (Gaskin et al., 1974; Erickson, 1974; Keates and Hall, 1975; Kuriyama, 1975; Sloboda et al., 1975). A comparative study (Scheele and Borisy, 1976) of microtubule protein isolated by the procedures of Borisy et al. (1975) and Weingarten et al. (1974) showed that the glycerol isolated protein indeed contained some high-molecular-weight species but at lower levels than protein isolated in the absence of glycerol. Thus, both the amount of protein bound to tubulin and the kind of protein detected appear to depend on the conditions used for the purification of microtubule protein.

The amount and kind of nontubulin proteins also depended upon the number of cycles of assembly–disassembly used to purify the microtubule protein. Preparations of microtubules polymerized from extracts of porcine brain tissue in 0.1 M Pipes buffer contained 35 detectable nontubulin proteins that associated with tubulin through two cycles of assembly–disassembly. After six cycles of purification, most of these components were no longer detected and the HMW components were the only factors present in significant amounts. Species of 345 000, 180 000, and 70 000 were detectable but only at low levels. Of greater significance is the fact that HMW 1 and 2 and the species of 345 000 and 180 000 were maintained at constant stoichiometry to tubulin through all six cycles. The material at 70 000 was depleted in the first four cycles but persisted at constant stoichiometry thereafter. Thus, although many cellular proteins bound to microtubules, only a few proteins, namely, the above described species, showed a high affinity for tubulin. Of these high affinity species, HMW 1 and 2 were the primary components.

These results support our earlier conclusion (Murphy and Borisy, 1975) that the HMW species was specifically bound to microtubules. However, the HMW fraction used in the previous study to stimulate assembly contained several trace components in addition to HMW. Furthermore, a protein fraction (tau factor) prepared by a different method also stimulated assembly but lacked HMW. It was therefore necessary to identify the specific stimulatory components and assess their relative importance in assembly in order to distinguish whether some factors had a stimulatory activity while other factors bound to microtubules but lacked stimulatory activity. Accordingly, microtubule-binding proteins were partially purified, fractionated by molecular sieve or ion-exchange chromatography, and tested for their ability to promote assembly after combination with tubulin. These experiments showed that: (1) both HMW and tau factor stimulated assembly; but that (2) HMW was the primary stimulatory component; and (3) indeed, that all components stimulated equally when compared on a mass basis. From these results and the compositional analysis of unfractionated microtubule protein, we conclude that approximately 60% of the stimulatory activity in our preparations of twice cycled microtubule protein is due to HMW. The percentage of stimulatory activity ascribable to HMW would be higher in preparations purified by additional cycles of assembly–disassembly in which tau and other nontubulin proteins would be depleted; however, the percentage would be lower for protein isolated in the presence of glycerol which contains lower levels of HMW.

Since many factors appear to stimulate assembly in these preparations, the question arises whether some of these proteins are related to one another. By using controlled digestion with trypsin, Vallee and Borisy (1977) have demonstrated that HMW can be cleaved into a large (255 000 MW) nonbinding and nonstimulatory fragment and a small (~35 000 MW) species that both binds and stimulates microtubule assembly. Sloboda et al. (1976) have also shown that high-molecular-weight components break down spontaneously during storage over long periods of time to form smaller fragments, including one of 70 000 MW. By using immunological techniques, these workers have also presented evidence that several of these proteins may in fact be related to each other. Together these results suggest the possibility that some of the lower molecular weight stimulatory peptides are derived from species of higher molecular weight. However, in these studies proteins corresponding to tau were not specifically identified as fragments of HMW capable of stimulating polymerization, and it remains possible that tau factor and HMW are of independent origin.

It is important to note that, when compared on a molar basis, the HMW components account for a smaller proportion of the nontubulin material, but have a greater specific activity than the other components in promoting tubulin assembly. In addition, the findings of Vallee and Borisy (1977) indicate that only a small portion (approximately 10%) of the HMW molecule is involved in the stimulation of assembly, whereas the other 90% is nonstimulatory and probably represents that portion of the molecule that projects away from the microtubule surface. Thus on a molar basis, the HMW component may be more active than the other nontubulin peptides, perhaps reflecting its biological specificity.

An additional point is that the stimulatory effect was not an exclusive property of the microtubule-binding proteins since numerous polycationic substances such as DEAE-Dextran and basic proteins also promoted tubulin assembly. A similar observation has been reported by Erickson and Voter (1976) who have suggested that these observations argue against a specific protein factor being involved in the assembly reaction. Since anionic substances were ineffective, the stimulatory effect was presumed to be due to the enhancement of dimer interactions

that resulted from ionic associations between the polycations and the negatively charged tubulin dimers. Here our interpretation is in agreement with that of Erickson (Erickson and Voter, 1976) who proposed that the facilitation of assembly may be based on the coacervation of polyelectrolytes. In addition, at least one solvent, glycerol, also stimulated polymerization, suggesting that this solvent can substitute for either the polycations or the microtubule-binding proteins (see also Lee and Timasheff, 1975). Thus, the assembly of purified tubulin does not absolutely require microtubule-binding proteins and may be stimulated by nonspecific factors. This is not to say, however, that HMW or some other factor does not perform this function in the cell. Indeed, the observations of Vallee and Borisy (1977) point strongly to the conclusion that only a small, specific part of the HMW molecule possesses stimulatory activity.

What then may we conclude concerning the identity of accessory proteins essential for the assembly of microtubules in vitro?

(1) Activity for stimulating polymerization appears to be a generalized property of molecules that bind to tubulin.

(2) Microtubule-associated proteins, including HMW, tau factor, and perhaps other nontubulin species, are all stimulatory factors in vitro.

(3) HMW 1 and 2 are the principal stimulatory species in our preparations of microtubule protein.

(4) The relative abundance of HMW and tau factor depend on the method used for isolating microtubule protein.

Clearly, the problem of interest is now to identify which of these protein species, if any, regulate microtubule assembly in the cell.

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