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GTP Hydrolysis during Microtubule Assembly†

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ABSTRACT: The GTP cap model of dynamic instability [Mitchison, T., & Kirschner, M. W. (1984) *Nature (London) 312*, 237] postulates that a GTP cap at the end of most microtubules stabilizes the polymer and allows continuing assembly of GTP-tubulin subunits while microtubules without a cap rapidly disassemble. This attractive explanation for observed microtubule behavior is based on the suggestion that hydrolysis of GTP is not coupled to assembly but rather takes place as a first-order reaction after a subunit is assembled onto a polymer end. Carlier and Pantaloni [Carlier, M., & Pantaloni, D. (1981) *Biochemistry 20*, 1918] reported a lag of hydrolysis behind microtubule assembly and a first-order rate constant for hydrolysis (k_h) of 0.25/min. A lag has not been demonstrated by other investigators, and a k_h value that specifies such a slow rate of hydrolysis is difficult to reconcile with reported steady-state microtubule growth rates and frequencies of disassembly. We have looked for a lag using tubulin free of microtubule-associated protein at concentrations of 18.5-74 μM, assembly with and without glycerol, and two independent assays of GTP hydrolysis. No lag was observed under any of the conditions employed, with initial rates of hydrolysis increasing in proportion to rates of assembly. If hydrolysis is uncoupled from assembly, we estimate that k_h must be at least 2.5/min and could be much greater, a result that we argue may be advantageous to the GTP cap model. We also describe a preliminary model of assembly coupled to hydrolysis that specifies formation and loss of a GTP cap, thus allowing dynamic instability.

Under defined conditions, microtubules contain approximately one GTP and one GDP molecule per dimeric tubulin subunit, while each free tubulin subunit in solution binds two molecules of GTP with high affinity (Weisenberg et al., 1968, 1976; Gaskin et al., 1974; Kobayashi, 1974; Hamel et al., 1986). Although it has been established that GTP is hydrolyzed during or soon after a tubulin subunit is incorporated into the microtubule polymer (Kobayashi, 1975; David-Pfeuty et al., 1977; McNeal & Purich, 1978; Carlier & Pantaloni, 1981; Hamel et al., 1982; Caplow et al., 1985), hydrolysis is not necessary for assembly. Assembly can take place in the presence of nonhydrolyzable GTP analogues (Weisenberg et al., 1976; Arai & Kaziro, 1976; Purich & MacNeal, 1978) but cannot be initiated from soluble subunits if only GDP is present (Gaskin et al., 1974; Olmstead & Borisy, 1975; Carlier & Pantaloni, 1978). Microtubules formed with nonhydrolyzable GTP analogues are generally much more stable than those formed in the presence of GTP, and this observation led to the suggestion that hydrolysis is not necessary for successful binding of a tubulin subunit to the end of a polymer but is necessary to allow normal disassembly (Weisenberg et al., 1976; Weisenberg & Deery, 1976; Arai & Kaziro, 1976).

To understand the role that hydrolysis of GTP might play in the dynamics of microtubule assembly and disassembly, it is important to know when GTP hydrolysis takes place during the assembly reaction. The idea that hydrolysis of GTP might be concomitant with tubulin assembly has been tested by a number of investigators. Before 1981, most results were consistent with the assumption that hydrolysis was tightly

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coupled to assembly, although it could be argued that none of these studies could distinguish whether hydrolysis took place simultaneously or slightly after the assembly occurred (Kobayashi, 1975; Weisenberg et al., 1976; David-Pfeuty et al., 1977; McNeal & Purich, 1978). Carlier and Pantaloni (1981) were the first to argue strongly that GTP hydrolysis might be uncoupled from assembly, with hydrolysis taking place some time after GTP–tubulin was incorporated into a microtubule. They modeled hydrolysis as first order and calculated a rate constant for hydrolysis (k_h)[1] of 0.25/min from their data. The uncoupled model predicts that newly added GTP–tubulin subunits would constitute a "GTP cap" at the ends of each microtubule, with GDP–tubulin in the interior.

This model was later adapted to explain the steady-state behavior of microtubules assembled from purified tubulin. At the same time most microtubules in a population are slowly growing, a small number are rapidly disassembling ("dynamic instability"; Mitchison & Kirschner, 1984). Steady-state growth rates and free subunit concentrations are thus maintained by the constant disassembly of part of the microtubule population. In a population composed of identical subunits and relatively little contaminating protein, how could these dramatically different behaviors be taking place at the same time? Mitchison and Kirschner (1984) proposed that relatively stable GTP–tubulin caps at the ends of most microtubules prevented catastrophic disassembly until the cap was lost, exposing the labile GDP–tubulin core. The GTP cap model incorporated the idea that the GDP–tubulin core was relatively unstable, conferring lability to the polymer as a whole, as well as the idea that a lag between microtubule assembly and hydrolysis produced GTP caps. However, the proposal that hydrolysis must be uncoupled from assembly to create GTP caps may have been an unnecessary constraint on the model, and one can envision alternative mechanisms that will generate a GTP cap with assembly coupled to hydrolysis (see Discussion).

There are a few difficulties with the uncoupled hydrolysis model as it has been presented. The value reported for k_h by Carlier and Pantaloni (1981) is difficult to reconcile with data on steady-state growth rates. For uncoupled hydrolysis to sustain dynamic instability at steady state, hydrolysis rates must become approximately equal to rates of assembly in order to sustain a stable distribution of cap sizes. With the steady-state growth rates observed by Mitchison and Kirschner (1984) of 1.9 μm/min, a GTP cap of 12 350 subunits would have to be present to generate hydrolysis rates equivalent to assembly, given a k_h of 0.25/min. This is equivalent to 7.5 μm of polymer. It is very unlikely that a GTP cap of this magnitude could be lost by a stochastic process. Further, since the original report by Carlier and Pantaloni (1981), there has been little agreement on whether a lag between assembly and hydrolysis exists. Caplow et al. (1985), although not demonstrating a lag between assembly and hydrolysis, concluded that their data could be explained if a significant amount of GTP–tubulin existed in rapidly assembling polymers, shorter GTP–tubulin caps were present at steady state, and hydrolysis took place primarily at each interface between GTP cap and GDP–tubulin core. Alternatively, Hamel et al. (1982) have presented data showing hydrolysis concurrent with assembly under a wide range of conditions. However, the assembly conditions and tubulin preparations used by Hamel et al. (1982) produced a long nucleation time before rapid assembly began, making a direct comparison with the Carlier and Pantaloni results difficult. Also, while Caplow et al. (1985) and Carlier and Pantaloni (1981) measured hydrolysis using the [^{32}P]phosphomolybdate extraction assay (PM assay) adapted from Nielsen and Lehninger (1958) to measure the evolution of free phosphate during assembly, Hamel et al. (1982) used thin-layer chromatography to evaluate GDP evolved. These differences make it diifficult to compare the conclusions reached by these investigators and leave the presence of a significant lag unresolved.

In the present study, we have investigated GTP hydrolysis during microtubule assembly under conditions chosen to maximize our ability to observe a lag. We have assembled a wide range of tubulin concentrations in the buffer used by Carlier and Pantaloni (1981), as well as a glycerol-free buffer known to allow dynamic instability (Mitchison & Kirschner, 1984). We studied the assembly of two preparations of MAP-free tubulin that differed somewhat in purity and assembly activity. Also, in addition to the PM assay, we used high-pressure liquid chromatography (HPLC) to measure the accumulation of GDP, a method similar in principal to that of Hamel et al. (1982). This allowed an evaluation of the PM assay, as well as a direct comparison of our results with previous investigations. Finally, we graphed the reactions in a manner that should have made visualization of a lag obvious, plotting both assembly and hydrolysis on the same micromolar scale.

Materials and Methods

Tubulin Purification. Porcine brain tubulin was purified by two cycles of assembly and disassembly in a buffer of 100 mM Mes, 1 mM EGTA, 0.5 mM $MgSO_4$, and 3.4 M glycerol, pH 6.6, followed by passage over phosphocellulose and a third cycle of assembly in 1 M sodium glutamate as described previously (Voter & Erickson, 1984). Alternatively, tubulin was concentrated after phosphocellulose chromatography by Amicon filtration followed by a cycle of assembly and disassembly. Both preparations of tubulin were then stored in 3.4 M glycerol at −80 °C. On the day of an experiment, tubulin purified with glutamate underwent a final warm/cold centrifugation cycle to remove aggregates and tubulin inactivated during storage. This preparation, designated "precycled", was essentially free of MAPs as judged by silver-stained SDS–PAGE and had very little inactive tubulin present. Tubulin purified without glutamate underwent a cold centrifugation step to remove aggregates but did not undergo a warm cycle before use. This preparation was less pure than the precycled tubulin preparation and contained 20–30% inactive tubulin. It is designated "nonprecycled" in the results presented. The nonprecycled tubulin was found by HPLC to contain a small amount of nucleoside diphosphokinase activity, which was not present in the tubulin prepared with glutamate.

For seeded assembly, precycled tubulin was placed into PB, a glycerol-free Pipes buffer (80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM GTP; Mitchison & Kirschner, 1984), by passage through a Sephadex G-25 column (Pharmacia PD-10) immediately before use. Without seeds, this tubulin did not assemble appreciably for 2–5 min after being warmed to 37 °C. We therefore prewarmed the tubulin for only 45–60 s before addition of seeds. Seeds were prepared by warming

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HPLC, high-pressure liquid chromatography; k_h, first-order rate constant for GTP hydrolysis; MEM buffer, 50 mM Mes, 1 mM EGTA, 5 mM $MgSO_4$, and 3.4 M glycerol, pH 6.6; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PB buffer, 80 mM Pipes, 1 mM EGTA, and 1 mM $MgCl_2$, pH 6.8; PM assay, phosphomolybdate extraction assay; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); MAP, microtubule-associated protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCA, perchloric acid; Me_2SO, dimethyl sulfoxide.

precycled tubulin in a Mg/glycerol PB buffer (containing 10 mM $MgCl_2$ and 3.4 M glycerol) to 37 °C for 10 min and then shearing with two passes through a 28-gauge needle immediately before use. Alternatively, a stoichiometric amount of taxol was added to tubulin in PB without glycerol and the same procedure followed as with the Mg–glycerol seeds. The seeds were always made at the same tubulin concentration as that of the tubulin to be assembled.

Experimental Procedures. Microtubule assembly without seeds was carried out in a buffer of 50 mM Mes, 1 mM EGTA, 5 mM $MgSO_4$, and 3.4 M glycerol, pH 6.6 (MEM buffer), with 100–500 μM GTP. When appropriate, [γ-^{32}P]GTP (Amersahm Inc.; 20 Ci/mM) was added to the tubulin solution at least 1 h before the final assembly reaction was begun to ensure equilibration with E-site GTP already present on the tubulin monomers. If a GTP regeneration system was used, it also was added at least 1 h before assembly was begun. The regenerating system (McNeal et al., 1977) included an increased concentration of acetate kinase (2 units/mL, Sigma, from *Escherichia coli*) along with 20 mM acetyl phosphate, which at 37 °C was sufficient to remove approximately 200 μM GDP/min from solution, and at 4 °C removed >500 μM GDP in 30 min.

Immediately before assembly was begun, aliquots of the tubulin solution were taken for following the reaction turbidimetrically, and "time zero" samples were taken for analysis of starting guanine nucleotide and free phosphate concentrations. These samples were pipetted into perchloric acid (PCA) to a final concentration of 0.3 M PCA and then rapidly cooled in an ice/water slurry. Assembly was then initiated by pipetting tubulin solutions into prewarmed glass test tubes and then swirling each aliquot for 8–12 s (depending on the volume of solution) in 40 °C water. Timing was begun as soon as swirling began. Assembly without glycerol was initiated by the addition of microtubule seeds (1% v/v) to prewarmed tubulin in PB. The solutions were then either placed in a 37 °C water bath or pipetted into a 37 °C semi-micro quartz cuvette (1-cm path length × 0.2 cm wide) in the temperature-controlled cuvette holder of a Shimadzu UV 240 recording spectophotometer set to monitor turbidity at a wavelength of 350 nm. At various times after assembly was initiated, 100-μL samples were taken and pipetted into PCA as described above. During the peak of optical density, a 100-μL sample was taken and centifuged in a prewarmed Beckman type 70.1Ti rotor (10 min at 35 000 rpm). The supernatant and pellet were immediately separated for later evaluation of tubulin concentration to determine the amount of polymer assembled during the reaction.

After completion of the assembly reaction, aliquots were reserved for determination of total protein and total guanine nucleotide present. Each time point sample (in PCA) was then centrifuged 5 min at 4 °C in an Eppendorf desktop centrifuge (15600*g*) to remove precipitated protein. Fifty microliters from each sample was then pipetted into 1 mL of a 5% ammonium molybdate/2 M sulfuric acid solution (PM assay) and vortexed thoroughly. Extraction was essentially as described by Carlier and Pantaloni (1981), except that cyclohexane was substituted for benzene and 5 mM H_3PO_4 was used as "carrier" during extraction. Aliquots of the organic phase were pipetted into scintillation vials for counting directly with no fluor.

For analysis of GDP and GTP concentrations by HPLC, 16.7 μL of 4 M potassium acetate was added to the 150 μL of each remaining sample, followed by Eppendorf centrifugation to remove the precipitated potassium perchlorate. Samples were stored in ice water until processing by HPLC. Separation of nucleotides was accomplished in 1 M potassium phosphate, pH 5.3, using a SynChropak AX300 ion-exchange column (Synchrom, Inc.) and an LKB 2150 HPLC pump. Samples were loaded by using a 100-μL injection loop. The absorbance of the outflow as monitored and recorded at a 256-nm wavelength using an ISCO 1840 absorbance monitor, and the areas under each peak were determined by using a Numonics 1224 digitizing planimeter. The concentration of total guanine nucleotides present in the tubulin solution was determined after precipitation of the protein with PCA, Eppendorf centrifugation, and determination of the absorbance of the supernatant at 256 nm, using a molar extinction coefficient of 12 400. This value was then used to calculate absolute GTP and GDP concentrations using HPLC data for the relative amounts of each nucleotide. Nucleoside diphosphokinase activity seen with nonprecycled tubulin was demonstrated by the presence of a GMP peak, which increased in size during an assembly reaction. Calculations of GDP evolved were corrected for this activity by adding back twice the increase in GMP concentrations at each time point measured. Determination of the starting GTP concentration was also used to calculate a specific activity of [γ-^{32}P]GTP in the PM assay. Protein concentrations were determined by using the Bradford protein assay (Bio-Rad Laboratories) using purified tubulin as a standard [A_{278} for a 1 mg/mL solution = 1.19 (Voter & Erickson, 1984)].

Results

Two independent assays were used to measure GTP hydrolysis. The evolution of free phosphate was measured with the phosphomolybdate extraction assay (PM assay) Neumann, 1902; Nielsen & Lehninger, 1958; Penningroth & Kirschner, 1977; Carlier & Pantaloni, 1981; Pollard & Weeds, 1984; Carlier et al., 1984; Caplow et al., 1985) using [γ-^{32}P]GTP. We also measured the production of GDP using HPLC to separate GDP from GDP, and UV absorption spectroscopy to detect and quantitate the concentration of total guanine nucleotides.

In our early attempts to correlate hydrolysis with assembly, it was apparent that hydrolysis, as measured with the PM assay, closely followed the general time course of assembly. At the time that assembly reached a plateau value, GTP hydrolysis had reached a steady-state rate. However, the calculated concentration of phosphate released was 20–50% lower than that of tubulin assembled. We therefore investigated the possibility that the relatively low value for the amount of GTP hydrolyzed was caused not by a lag in GTP hydrolysis behind assembly but by a problem with the extraction assay. After extraction, all free phosphate is expected to be in the organic phase (as the molybdate salt), while GTP stays in the aqueous phase. We found that extraction of free phosphate was very efficient when little or no protein was present. However, as the amount of protein in the aqueous phase was increased, fewer counts were present in the organic phase after extraction.

We therefore examined the sulfuric acid solution for unextracted ^{32}P counts, without addition of the organic phase. Centrifugation of the sulfuric acid solution after addition of protein (bovine serum albumin was used for this test to conserve tubulin) and free [^{32}P]orthophosphate showed that a significant fraction of added counts had been pelleted. The fraction of added free phosphate that pelleted was seen to increase significantly at protein concentrations of 50 μg/mL or above in the sulfuric acid. With a 25-μL sample volume, this corresponded to a concentration of 2 mg/mL in the starting sample. At 75 μg/mL in the acid, slightly more than 50% of added counts were pelletable. Without centrifugation,

the counts were found to be present in a whitish precipitate that collected near the aqueous/organic interface during extraction. Pelletable counts did not seem to be extractable into the organic phase.

We were able to improve the assay significantly by removing protein from solution before extraction was begun. Free [^{32}P]phosphate, in the presence of various concentrations of protein, was added to small volumes of 0.3 M perchloric acid (PCA) and placed on ice. The solutions were then centrifuged, the supernatants were removed, and the process was repeated with fresh PCA to wash trapped counts from the pellet. The supernatants were then added to sulfuric acid extraction solutions, while the pellet was counted directly by Cerenkov scintillation counting. Both the aqueous and organic phase solutions were then similarly analyzed for radioactivity. Only a background fraction of added free phosphate counts was found in the PCA pellet (2–3%) at all protein concentrations tested, while the remainder were found in the organic phase after extraction. Thus, the sulfuric acid/ammonium molybdate solution appears to trap phosphate in a protein precipitate, while PCA precipitates the protein cleanly. We therefore routinely used PCA to remove tubulin from solution before extraction. Even with this improvement, which has been incorporated into the data presented, the PM assay gave values for GTP hydrolysis that were usually somewhat lower than the concentration of tubulin assembled when compared at the earliest stage of the turbidity plateau. However, the discrepancies no longer increased with increasing tubulin concentration, and the shapes of the hydrolysis curves more closely followed those of assembly. Although not mentioned in most recent applications of the PM assay, and not known by the present authors until this study had been completed, the importance of removing protein before adding ammonium molybdate had been recognized quite early in the development of the assay (Fiske & Subbarow, 1925).

As an independent assay of GTP hydrolysis, HPLC was used to determine the concentration of GDP present during the assembly reaction. This assay seemed to reliably follow changes in GDP present, and the shapes of hydrolysis curves generated with this method were very similar to those produced with the PM assay. However, HPLC determination of the moles of GTP hydrolyzed was higher than that found using the PM assay and corresponded much more closely with the moles of tubulin assembled during the early stages of assembly. To compare PM extraction with HPLC more directly, [γ-^{32}P]GTP was added to a tubulin solution before initiation of assembly. The free phosphate peak, as well as the various guanine nucleotide elution peaks (A_{256}) from HPLC, could then be counted directly and the free phosphate and remaining GTP compared with values determined by the extraction procedure. The PM extraction assay consistently showed fewer counts extracted as free phosphate than measured directly by HPLC.

In the figures that follow, we have presented the kinetics of assembly and hydrolysis on the same vertical scale. This presentation is similar to that of Hamel et al. (1982), in that microtubule assembly has been graphed as the concentration of tubulin in polymer. The time course of assembly was followed as the increase in absorbance at a wavelength of 350 nm (A_{350}). The concentration of tubulin actually polymerized was then determined at the time of maximum turbidity (A_{max}) by protein assay of the pellet and supernatant after warm centrifugation. The increase in A_{350} during assembly was then graphed such that A_{max} corresponded to the final concentration of tubulin in polymer. This allowed a direct comparison of

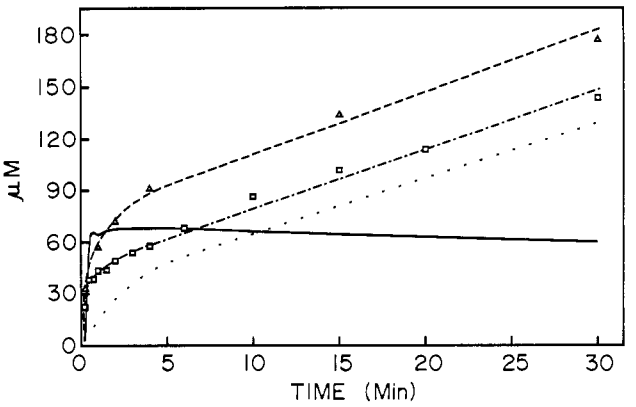

FIGURE 1: Time course of assembly of 74 μM precycled tubulin and accompanying GTP hydrolysis. Precycled tubulin was assembled at 37 °C in MEM buffer. Solid line, microtubule assembly; triangles, concentration of GDP evolved, determined by HPLC; squares, concentration of phosphate released, PM assay. Included in this figure (dotted line) is a graph of the calculated time course of GTP hydrolysis given the pseudo-first-order rate constant for the reaction presented (k_{app} = 10.1/min) and a k_h of 0.25/min.

the kinetics of polymer formation and GTP hydrolysis. We sometimes observed a gradual decline in turbidity after the final plateau was reached. Warm centrifugation of samples taken after the final plateau was reached and again at 30 min showed an equal concentration of tubulin in polymer at both times. We chose the early plateau A_{350} value with which to prorate the concentration of polymer.

If GTP hydrolysis is uncoupled from microtubule assembly, a lag of hydrolysis behind assembly should become more apparent as rates of assembly are increased (Carlier & Pantaloni, 1981). This is true for a comparison between the moles of tubulin assembled and moles of GTP hydrolyzed, a "lag in stoichiometry", at a given time. Carlier and Pantaloni (1981) stated that they observed no lag during assembly of 17.2 μM tubulin, while finding a significant disparity between hydrolysis and assembly with tubulin concentrations of 34.5 μM. To enhance our ability to measure a lag of hydrolysis behind assembly, we assembled highly purified tubulin at concentrations as high as 74 μM.

In Figure 1, a microtubule assembly reaction is presented that includes both PM assay and HPLC measurements of GTP hydrolysis. Assembly of 74 μM precycled tubulin was initiated by rapid warming from 0 to 37 °C. Assembly at this concentration of tubulin was very rapid, and apparent steady state was reached by about 3 min. The PM assay and HPLC curves both showed very rapid initial hydrolysis rates that corresponded almost exactly with assembly, with hydrolysis slowing after 0.5 min and reaching steady state by 2–3 min. As described above, the PM assay generated a lower estimate of the moles of GTP hydrolyzed at the time steady state was reached, although the curves generated by each assay were similar in general shape.

We have included in Figure 1 an estimate of the hydrolysis expected given the equations and first-order rate constant presented by Carlier and Pantaloni (1981) (k_h = 0.25/min). We used a pseudo-first-order rate constant for assembly calculated from the assembly reaction shown in Figure 1 (10.1/min) and added our observed steady-state hydrolysis rate to the result (prorated to our steady-state polymerized tubulin concentration of 68 μM), from 6.18 min on. This was the time that the calculated hydrolysis rate equaled the observed steady-state hydrolysis rate. The time lag predicted from this treatment, calculated at 50% of the plateau value of assembly (50% A_{max}), was 2.83 min; i.e., it should have taken

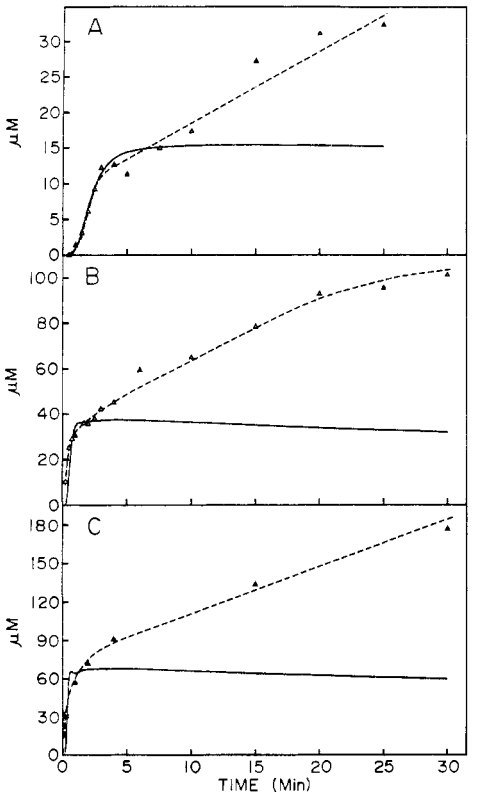

FIGURE 2: Assembly and hydrolysis for three concentrations of precycled tubulin in MEM buffer, HPLC assay only. Symbols as described in the legend to Figure 1. (A) 18.5 μM total tubulin; (B) 41 μM tubulin; (C) 74 μM tubulin.

an additional 2.83 min to hydrolyze 34 μM GTP than it took to assemble that amount of tubulin. Alternatively (see Discussion), we could have added the observed steady-state rate from the approximate time that nucleation events should have become negligible (0.5 min). This treatment generates a time lag at 50% A_{max} of 1.5 min (data not shown). Our observed hydrolysis curves show no time or stoichiometry lag at 50% A_{max}.

Figure 2 shows tubulin assembly for 18.5, 41, and 74 μM starting tubulin concentrations, with GTP hydrolysis measured by HPLC. Although this was a 4-fold difference in starting tubulin concentration, the curves had many important features in common. In each of the graphs, GTP hydrolysis appeared closely coupled to tubulin assembly for the first two-thirds of the polymerization reaction. At about this point, the curves began to diverge. The rate of hydrolysis slowed before the assembly curve, beginning to decrease to the eventual steady-state rate. The steady-state rate of GTP hydrolysis was established at about the same time maximum turbidity was reached.

At high concentrations of tubulin, we often observed a "bump" in the turbidity curve: turbidity rose rapidly to a peak and then fell slightly before rising to a final plateau. This was particularly evident at high tubulin concentrations (Figures 1 and 2C) and with tubulin that had not been "precycled" before use (see Materials and Methods, Figures 3 and 4). In Figure 2B, the bump appeared as a shoulder reached at about 1 min, where turbidity leveled off before rising more slowly to its actual maximum. We believe that turbidity during the bump period is probably in excess of that due to assembled microtubules. Rearrangements of the forms of polymer present may be taking place, scattering light differently than during rapid growth or steady state. Because the bump phenomenon is evident at the 90% level of A_{max}, and a lag should be clearly

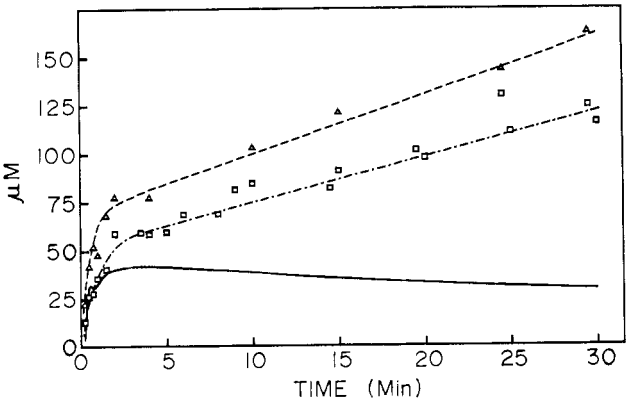

FIGURE 3: Assembly of 63 μM nonprecycled tubulin in MEM buffer and accompanying GTP hydrolysis. Symbols as described in the legend to Figure 1.

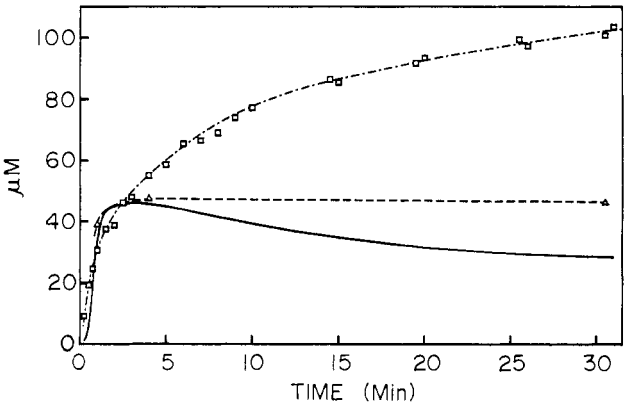

FIGURE 4: Assembly of 61 μM nonprecycled tubulin in MEM buffer and accompanying GTP hydrolysis, with a GTP regenerating system present. Measurements by HPLC should reflect only GDP that had been bound to polymerized tubulin. Symbols as described in the legend to Figure 1.

evident during the most rapid phase of microtubule assembly, we chose to evaluate these and the curves that follow for a lag of hydrolysis behind assembly at 50% of the A_{max}. As presented in Figure 2, no lag was detected at the 50% level of assembly at any tubulin concentration.

To test for a lag under conditions more like those present in the experiments of Carlier and Pantaloni (1981), we modified our procedure for purifying tubulin. The revised procedure eliminated the use of glutamate to assemble tubulin after passage over the phosphocellulose column, a step which normally helps to remove any MAPs that may remain after the column. We also eliminated the cycle of assembly and disassembly normally done on the day of an experiment to reduce the concentration of inactive tubulin. Examples of assembly reactions carried out with nonprecycled tubulin are shown in Figures 3 and 4. The lack of a cycle of assembly and disassembly before use increased the final percentage of inactive tubulin. Only 42 and 46 μM tubulin was assembled from 63 and 61 μM total tubulin, respectively. Given a steady-state free tubulin concentration of our tubulin in this buffer of approximately 3 μM, this implies that inactive tubulin was approximately 29% and 21% of total. This is similar to the values reported in Figure 2 of Carlier and Pantaloni (1981) and compares with 7% (Figure 2A), 2% (Figure 2B), and 4% (Figure 2C) inactive tubulin seen with precycled tubulin as shown in Figure 2 of the present paper. In contrast to our precycled tubulin preparation, as well as that of Carlier and Pantaloni (1981), our nonprecycled tubulin contained a nucleoside diphosphokinase activity which was evident in the presence of a peak of GMP during HPLC separation of nu-

cleotides. The GMP concentration increased linearly during assembly, from approximately 15% to 25% of total guanine nucleotide. Calculation of GDP evolved therefore necessitated adding back twice the increase in GMP seen at a particular time point.

Measurements of GTP hydrolysis presented in Figure 3 show that, by the time steady state had been reached, a superstoichiometric amount of GTP had been hydrolyzed relative to the amount of tubulin assembled into polymer. This was evident by both HPLC and PM assays, although the measurements by PM assay were lower. The relatively greater amount of hydrolysis seen with nonprecycled tubulin was reproducible. Apparent steady state was reached at about the same time in both hydrolysis curves and correlated well with the time the plateau of turbidity was reached. As with precycled tubulin, a bump in the turbidity curve was evident before the A_{max} was reached.

Assembly with a GTP regenerating system present allowed calculation of the GDP–tubulin in polymer in an interesting way (Figure 4). We used nonprecycled tubulin and a regenerating system adequate to remove all accessible GDP from solution very quickly [approximately 40 times the concentration of actetyl kinase coommonly used (MacNeal et al., 1977; Margolis & Wilson, 1978)]. The rapid precipitation with PCA, used to quench the assembly and hydrolysis reactions, also inactivated acetate kinase, liberating bound GDP and preventing its rephosphorylation to GTP. The only GDP present in these samples should therefore have been that which was formed by hydrolysis in polymer and which remained in polymer. The PM assay was unaffected by the presence of the regenerating system since acetate kinase does not use free phosphate as its substrate, except that the specific activity of the [^{32}P]GTP is gradually diluted by newly formed unlabeled GTP. This dilution was not corrected for in Figure 4 and may explain the lack of linearity in the steady-state hydrolysis measured by PM extraction.

Samples taken before the start of assembly showed no GDP detectable by HPLC. During assembly, the concentration of GDP measured by HPLC was equal to or slightly greater than the concentration of assembled polymer at all time points measured. This is consistent with one GDP being bound per tubulin subunit in polymer, with no evidence of a significant component of E site GTP present even at the earliest time point measured. The PM assay showed hydrolysis had achieved approximate stoichiometry with assembly by the time maximum turbidity was reached. Given that this assay provides estimates that are low relative to HPLC, it is probable that a superstoichiometric amount of GTP had been hydrolyzed by this time (3 min). Interestingly, the presence of the regenerating system abolished the increase in GMP during assembly normally seen with this tubulin, presumably by limiting the availability of GDP to the diphosphokinase. This strengthens the argument that the regenerating system removes free GDP from solution very quickly and that the GDP we measure by HPLC must be almost all from assembled polymer.

We also evaluated GTP hydrolysis during assembly in the glycerol-free buffer used to demonstrate dynamic instability of microtubule growth (Mitchison & Kirschner, 1984). We used two different types of seeds to initiate assembly of prewarmed tubulin (Figure 5). One type of microtubule seed was assembled in 10 mM $MgSO_4$ and 3.4 M glycerol (Figure 5A,B), while the other was assembled with a stoichiometric amount of taxol in the non-glycerol buffer (Figure 5C,D). Both preparations of seeds were sheared before adding them

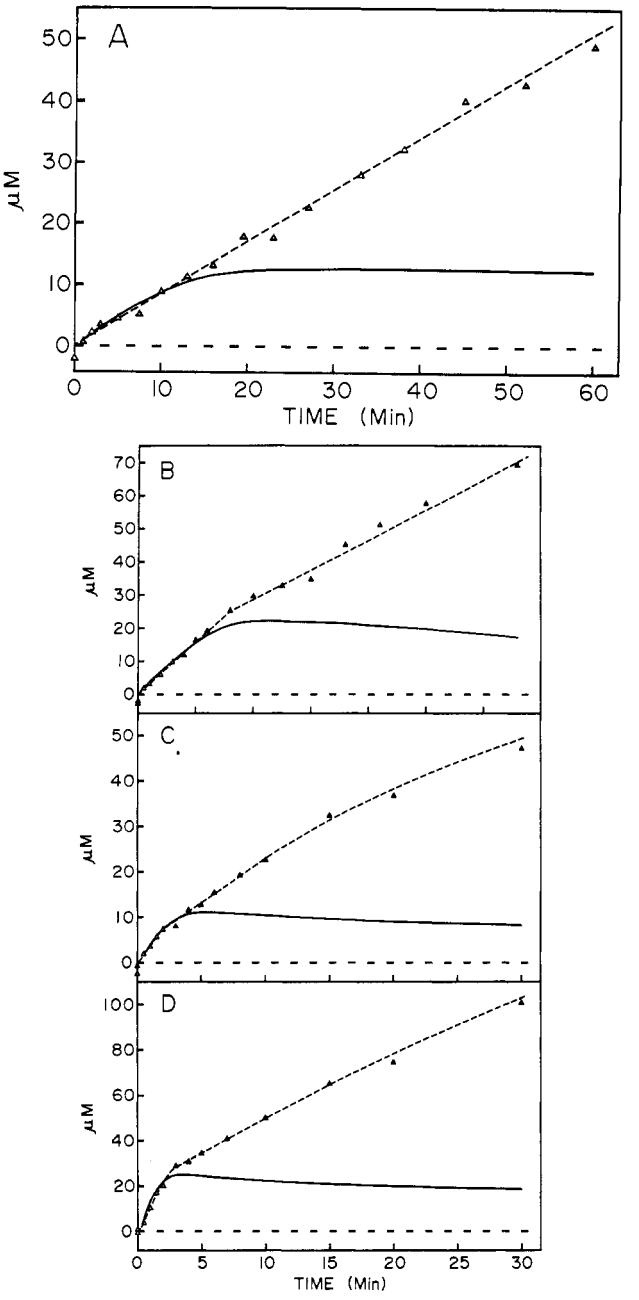

FIGURE 5: Microtubule assembly in PB using two different kinds of seeds. Assembly of prewarmed tubulin in PB was initiated by the addition of a 1% volume of a solution of microtubule seeds. Seeds were assembled with either 10 mM magnesium and 3.4 M glycerol (Mg–glycerol) (A and B) or a stoichiometric concentration of taxol (C and D), and both were sheared just before use. Zero time is when seeds were added. Preassembly (zero time) GDP concentrations are preented as less than zero to reflect the small but measurable amount of GDP generated by the seeds before they were added to the non-glycerol tubulin solution. Symbols as described in the legend to Figure 1. Tubulin concentrations were (A) 30, (B) 41, (C) 24.5, and (D) 38 μM.

to unassembled tubulin in a 1:100 (v/v) ratio. Assembly with both types of seeds was approximately linear during the intial phase of the reaction. Hydrolysis was approximately equal to assembly until the assembly curves began to approach steady state. In each case, by the time a plateau of turbidity was reached, more GTP had been hydrolyzed than tubulin assembled. Interestingly, assembly of lower concentrations of tubulin with either kind of seed produced hydrolysis rates that did not change appreciably once steady state was reached (Figure 5A,C). In taxol-seeded reactions, steady state was reached much more quickly than with Mg/glycerol seeds,

perhaps implying relatively shorter seeds produced by this method. No lag of hydrolysis behind assembly was evident under either of these conditions.

Discussion

Dynamic instability of microtubules is a well-established empirical phenomenon, and the GTP cap model explaining this behavior is attractive. Evidence for a GTP–tubulin cap at the ends of microtubules is based primarily on the observation of a lag between GTP hydrolysis and microtubule assembly during the early, rapid phase of polymerization (Carlier & Pantaloni, 1981). Because of the theoretical importance of the GTP cap and the uncoupled GTP hydrolysis model explaining its existence, we have carefully looked for a lag under conditions similar to those of the original study, as well as conditions that have been shown to allow dynamic instability. In contrast to the conclusions of the earlier work, we found no lag of hydrolysis behind assembly under any of the conditions employed. We therefore must address two possibilities: either the uncoupled model is correct but the lag between assembly and hydrolysis is too small to be measured in our system, or hydrolysis of GTP is tightly coupled to assembly.

If the uncoupled hydrolysis model is correct, how small would k_h have to be to produce a visible lag in our experiments? In the uncoupled model, the lag of hydrolysis behind assembly is a result of both the sequential nature of the reactions (first a subunit is incorporated into the polymer; then it can hydrolyze) and also the size of k_h [see Carlier and Pantaloni, (1981)]. A lag should be evident in the difference in the intial rate of formation of polymer and liberation of GDP or free phosphate, becoming easily measurable by the 50% level of assembly. For example, if k_h is 0.25/min, the minimum expected time lag at the 50% assembly point, defined as the extra time needed for hydrolysis to produce as much product (GDP or phosphate) as tubulin assembled, is 2.8 min. We believe we could have discerned a time lag of 10–15 s during the rapid phase of assembly, corresponding to k_h values of about 5.6–2.8/min, respectively. Since we saw no lag, k_h for an uncoupled model is probably at least 2.8/min and could be much greater.

In fact, rather than a lag of hydrolysis behind assembly, our most rapid assembly reactions indicated an apparent "negative lag": at the earliest time points, hydrolysis preceded the rise in A_{350} (Figures 1, 2B, 2C, 3, and 4). We considered the possibility that changes in absorbance were not accurately reflecting the concentration of microtubules during rapid assembly. Turbidity is a good indicator of polymer mass when microtubules are significantly longer than the incident wavelength of light (Gaskin et al., 1974). However, at early stages of rapid, self-nucleated microtubule assembly, large numbers of short microtubules are produced. Under these conditions, turbidity might underestimate the mass of polymer present. To check this possibility, we fixed solutions of microtubules in 1% glutaraldehyde at various times during assembly and determined the quantity of pelletable polymer for each time point. This assay showed excellent agreement with turbidity for all time points in slow assembly reactions (90% of the final plateau reached in 3–5 min), and at all times past 1–1.5 min in fast reactions (90% of the final plateau reached in 1–3 min). However, during the first minute of a fast assembly, turbidity appeared to lag 15–20 s behind assembly as quantitated by fixation and centrifugation. This is similar to the lag of turbidity behind hydrolysis noted in our fast assembly reactions and is the probable origin of the negative lag seen during fast assembly.

It may be argued that no one has convincingly demonstrated a lag of hydrolysis behind assembly. For example, in the original data presented by Carlier and Pantaloni (Carlier & Pantaloni, 1981; Figure 1), if the nonnormalized data for assembly in the figure are redrawn such that the final plateau of assembly corresponds to the 30 μM level discussed in the figure legend, little if any time or stoichiometry lag is seen. If a further correction is made for the fraction of total tubulin that characteristically assembled into polymer, as stated in the caption to Figure 2 of the same paper, a lag is even less apparent. The normalized curves presented in the inset to this figure depend critically upon when steady-state hydrolysis is estimated to begin, which depends on the judgement of the investigator. Also, as discussed below, there are problems with an interpretation of the uncoupled model that specifies subtracting a steady-state hydrolysis rate from the "burst" of hydrolysis throughout assembly. It is therefore more straightforward to consider the unmodified data, graphed on the same micromolar scale. Further, since the PM assay has been found to significantly underestimate the amount of GTP hydrolyzed, particularly at high protein concentrations, data generated with the unmodified assay should be thought of as a minimum estimate of hydrolysis. Even with a minimum estimate of hydrolysis, however, no convincing lag was demonstrated.

Similarly, Caplow et al. (1985), although circumventing the problems associated with the PM assay by pipetting very small volumes of reaction mixture into the PM solution, also do not demonstrate a lag when their data for both hydrolysis and assembly are graphed together on the same scale. Instead, their conclusion that GTP hydrolysis is not coupled to assembly depends on the hypothesis that their hydrolysis curve would have been linear throughout the reaction except that assembly was inhibited by the buildup of free GDP. Further, although a recent experiment by Hamel and co-workers showed a lag of hydrolysis behind microtubule assembly at a relatively high pH, they could find no evidence of GTP in assembled polymer and found no lag at the pH range normally used to promote assembly (Hamel & Lin, 1981; Hamel et al., 1982, 1986). Our result with the GTP regenerating system (Figure 4) also corroborates the findings of Weisenberg et al., (1968, 1976), Kobayashi (1974), and also Hamel et al. (1986), in the observation that the fraction of microtubule polymer that was GTP–tubulin was not measurable. Although indirect, our result seems especially important at the 1-min time point, since this is when almost none of the assembled polymer should have been GDP–tubulin, given the uncoupled model and a k_h of 0.25/min.

If the uncoupled model is correct but a lag of hydrolysis behind microtubule assembly is not measurable with the techniques currently used, the average size of the GTP cap must be much smaller than previously thought. What are the implications of much larger values of k_h for dynamic instability? In our opinion, a large k_h is advantageous to the model. As pointed out in the introduction, a k_h of only 0.25/min would produce a very large average cap size at the rates of steady-state growth recently reported (Mitchison & Kirchner, 1984; Horio & Hotani, 1986), and it would be very unlikely for caps of this size to be lost by a stochastic process, allowing disassembly. It is our belief, therefore, that a minimum value for k_h of 2.5/min for uncoupled hydrolysis is closer to values that could explain the observed behavior of microtubules at steady state. In fact, starting from the assumption that both addition and hydrolysis are stochastic events per unit time, we found the best estimate for k_h that could explain both the observed

rates of growth and frequencies of catastrophe sited above was 100–300/min. This yields an average cap size per growth site of about 8–12 GTP subunits (Voter et al., 1987).

The advantage of considering hydrolysis as uncoupled from assembly is that the uncoupled model provides a rationale for the existence of a GTP cap at the ends of microtubules. As described in the introduction, the GTP cap model nicely incorporates known properties of GTP and GDP–tubulin polymers, and can explain rapid disassembly under dilution conditions as well as growth of some polymers at the steady-state free tubulin concentration and below. In the original paper describing the possibility of a GTP cap, Carlier and Pantaloni (1981) stated that a coupled model precludes a GTP cap at the growing points of a microtubule. This conclusion was derived from the assumption that coupled hydrolysis necessitates hydrolysis of GTP on the subunit being added to the growth site:

$$T_{T(sol)} + T_{D(MT)n} \rightarrow T_{D(MT)n+1}$$

where $T_{T(sol)}$ is a GTP–tubulin subunit free in solution and $T_{D(MT)n}$ is a linear polymer of n GDP–tubulin subunits. However, as pointed out by Caplow et al. (1985), coupled assembly does not necessarily demand hydrolysis of the subunit being added to the polymer but could entail hydrolysis of GTP on the end subunit:

$$T_{T(sol)} + T_{T(MT)}T_{D(MT)n} \rightarrow T_{T(MT)}T_{D(MT)n+1}$$

This reaction model constantly maintains a one-subunit-deep cap per growth site.

Can a one-subunit-deep cap allow dynamic instability? The assumptions required for dynamic instability would be that free GTP–tubulin bind preferentially but not exclusively to GTP–tubulin growth sites, maintaining the cap. The cap could be lost by occasional disassembly of the terminal GTP–tubulin subunit from a microtubule end. Alternatively, a low rate of spontaneous hydrolysis of GTP in the polymer could provide an equivalent loss of cap. Cap loss would allow rapid disassembly of the GDP–tubulin polymer, as before. Once lost, the GTP cap could be restored (microtubule rescue) by addition of GTP–tubulin to a GDP end. Cap loss by dissociation of the terminal GTP subunit is attractive as the reverse of rescue, both involving the interaction of a GTP subunit with a GDP–tubulin microtubule end. An equilibrium constant and critical concentration can thus be postulated for this reaction, leading to predictions for the frequency of both catastrophe and rescue under various conditions. Thus, the rate of growth as well as the frequency of rescue would depend on the concentration of free GTP–tubulin in solution, while the frequency of catastrophe would depend only on a first-order rate constant for cap loss. These parameters could eventually be extended from a simple linear polymer model to incorporate multiple growth sites per microtubule end, with cooperativity between growth sites with respect to growth, rescue, and catastrophe. Although this model holds some promise in explaining observed behavior, it is presented at this time primarily to illustrate that a coupled hydrolysis model that could explain our results is not necessarily at odds with observations of dynamic instability and rapid microtubule disassembly rates. Our data cannot distinguish between these models, but studies of the properties of individual microtubules, both during rapid growth and at apparent steady state, should be able to offer further insight.

At steady state, all assembly must be balanced by an equal amount of disassembly. As observed in this and previous studies, hydrolysis of GTP persists at a substantial and approximately constant rate after turbidity has reached a plateau. Under conditions that promote dynamic instability, steady-state hydrolysis probably reflects the continued growth of most microtubules. However, steady-state hydrolysis is also significant in buffers such as the Mg/glycerol buffer that apparently suppresses the dynamic behavior of microtubules. In this case, steady-state hydrolysis also may be due to continued assembly events, but with shorter or less extensive episodes of disassembly. If hydrolysis takes place only as a consequence of the addition of a subunit to a microtubule, the steady-state rate of GTP hydrolysis must also equal the rate of steady-state disassembly. Carlier and Pantaloni (1981) suggested that the same rate of disassembly should obtain during the rapid elongation phase of assembly, and they therefore subtracted out the steady-state hydrolysis rate from early hydrolysis in order to compare hydrolysis and net assembly. However, the GTP cap model of dynamic instability predicts that rapid assembly, with consequent buildup of the GTP cap, would virtually eliminate early disassembly events. Similarly, the coupled model we have described above predicts that a constant rate of spontaneous cap loss would be masked during rapid assembly by much more frequent rescue events, reducing disassembly to well below that at steady state. Under these models, therefore, subtraction of steady-state hydrolysis from the "burst" of hydrolysis during rapid assembly, in order to compare the stoichiometry of net assembly and hydrolysis, is not justified.

Since the correct model for GTP hydrolysis during microtubule assembly has not yet been determined, we chose not to compensate for a possible constant rate of disassembly during rapid assembly. If we had included this correction in the calculations presented, our values for predicted time lags based on the equations presented by Carlier and Pantaloni (1981) and our estimate of a minimum k_h would have been changed slightly. However, our conclusion that a lag of hydrolysis behind assembly is not measurable using current assay methods would not have been affected. It is clear that determining the mechanism of steady-state assembly and hydrolysis in various buffer systems will be an important next step in refining our understanding of microtubule assembly, as well as the dynamic behavior of microtubules.

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