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Role of GTP Hydrolysis in Microtubule Polymerization: Evidence for a Coupled Hydrolysis Mechanism†

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ABSTRACT: The relationship between GTP hydrolysis and microtubule assembly has been investigated by using a rapid filtration method. Microtubules assembled from phosphocellulose-purified tubulin, double-labeled with [γ-^{32}P]- and [^{3}H]GTP, were trapped and washed free of unbound nucleotide on glass fiber filters. The transient accumulation of microtubule-bound GTP predicted by uncoupled GTP hydrolysis models [Carlier & Pantaloni (1981) *Biochemistry 20*, 1918–1924; Carlier et al. (1987) *Biochemistry 26*, 4428–4437] during the rapid assembly of microtubules was not detectable under our experimental conditions. By calculating hypothetical time courses for the transient accumulation of microtubule-bound GTP, we demonstrate that microtubule-bound GTP would have been detectable even if the first-order rate constant for GTP hydrolysis were 4–5 times greater than the pseudo-first-order rate constant for tubulin subunit addition to microtubules. In a similar manner, we demonstrate that if GTP hydrolysis were uncoupled from microtubule assembly but were limited to the interface between GTP subunits and GDP subunits (uncoupled vectorial hydrolysis), then microtubule-bound GTP would have been detectable if GTP hydrolysis became uncoupled from microtubule assembly at less than 50 μM free tubulin, 5 times the steady-state tubulin concentration of our experimental conditions. In addition, during rapid microtubule assembly, we have not detected any microtubule-bound P_i, which has been proposed to form a stabilizing cap at the ends of microtubules [Carlier et al. (1988) *Biochemistry 27*, 3555–3559]. Also, several conditions that could be expected to increase the degree of potential uncoupling between GTP hydrolysis and microtubule assembly were examined, and no evidence of uncoupling was found. Our results are consistent with models that propose cooperative mechanisms that limit GTP hydrolysis to the terminal ring of tubulin subunits [e.g., O'Brien et al. (1987) *Biochemistry 26*, 4148–4156]. The results are also consistent with the hypothesis that a slow conformational change in tubulin subunits after GTP hydrolysis and P_i release occurs that results in destabilized microtubule ends when such subunits become exposed at the ends.

Microtubules assembled in vitro, in the absence of stabilizing agents such as microtubule-associated proteins (MAPs)[1] or glycerol, can be highly dynamic (Horio & Hotani, 1986; Walker et al., 1988). At steady state under appropriate conditions, a microtubule can be either growing relatively slowly or disassembling rapidly at either end, and the ends may alternate between the growing and shortening phases frequently relative to the lifetime of the microtubule. The sug-

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[1] Abbreviations: MAP(s), microtubule-associated protein(s); GXP, guanine nucleotide in microtubules, in the form either of GDP or of GTP; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; column buffer, 50 mM Pipes, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8; polymerization buffer, 100 mM Pipes, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8; stabilizing buffer, 30% glycerol (v/v) and 10% dimethyl sulfoxide (v/v) in polymerization buffer; PBS, phosphate-buffered saline solution; BSA, bovine serum albumin.

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gestion that the presence or absence of GTP subunit "caps" at the ends of microtubules may be the mechanistic basis for such behavior (Mitchison & Kirschner, 1984a) has focused considerable attention on the involvement of GTP hydrolysis in microtubule dynamics. Despite much effort, the existence of a GTP cap remains controversial.

The presence of a sizable GTP subunit cap was first inferred from simultaneous measurements of microtubule assembly and GTP hydrolysis which indicated that the release of stoichiometric amounts of inorganic phosphate into the medium and the attainment of steady-state rates of phosphate release lagged several minutes behind the time that polymer mass steady state was reached (Carlier & Pantaloni, 1981). GTP hydrolysis was proposed to occur as a random first-order reaction on subunits within the microtubule. In this model, the ends of microtubules would be capped by a gradient of GTP-liganded tubulin subunits with an average length proportional to the assembly rate. During the initial moments of microtubule polymerization, virtually the entire polymer could consist of GTP-liganded subunits. For the cap to be lost at steady state required that the hydrolysis rate be approximately equal to the steady-state microtubule assembly rate. Then stochastic fluctuations in the size of the caps could result in the loss of some caps, exposing the unstable GDP-liganded tubulin core of the microtubule.

Earlier experiments had suggested that GTP hydrolysis and tubulin assembly were concurrent events (MacNeal & Purich, 1978), and more recent studies from several laboratories have not supported the hypothesis that GTP hydrolysis is substantially uncoupled kinetically from subunit addition to the ends of microtubules (Hamel et al., 1982, 1986a; Caplow et al., 1985; O'Brien et al., 1987; Schilstra et al., 1987). The inability to detect a lag in GTP hydrolysis led O'Brien et al. (1987) to propose that addition of GTP-liganded subunits at the ends of a microtubule "forces" the hydrolysis of the molecules of GTP on the previously terminal subunits. In this model, GTP hydrolysis is kinetically coupled to assembly, yet a GTP subunit cap of only a few terminal subunits is maintained to distinguish growing from shortening microtubules. The small cap could be lost stochastically by dissociation of the terminal GTP subunits or by the slow spontaneous hydrolysis of GTP liganded to the terminal subunits.

A vectorial model for GTP hydrolysis that involved the existence of a GTP cap was proposed by Carlier et al. based upon their more recent experiments (Carlier et al., 1987). They found that assembly and hydrolysis were coupled at low assembly rates. However, as assembly rates were increased by increasing the tubulin concentration, GTP hydrolysis reached a maximum rate at a tubulin concentration designated c'. In their model, at tubulin concentrations greater than c' (high assembly rates), GTP hydrolysis would become uncoupled from tubulin addition, and microtubules would contain large regions of GTP-liganded subunits at their ends. GTP hydrolysis was proposed to occur only at the interface between the GTP-liganded and the GDP-liganded subunits. Hydrolysis would then proceed vectorially from the internal boundary of the GTP cap toward the ends of the microtubule. At tubulin concentrations below c', when the rates of GTP hydrolysis are greater than the rates of assembly (e.g., at steady state), the GTP cap was proposed to be maintained by the requirement that GTP could only be hydrolyzed when GTP-liganded tubulin subunits became buried within the microtubule. In this latter respect, the "vectorial" hydrolysis model is similar to the "forced" hydrolysis model (O'Brien et al., 1987). Models of GTP hydrolysis involving cooperative interactions at the interface between GTP-liganded and GDP-liganded subunits have also been discussed by other investigators (Karr et al., 1979; Caplow et al., 1985).

Much of the controversy concerning the coupling of GTP hydrolysis and microtubule assembly may be due to the experimental difficulties inherent in comparing simultaneous but separate measurements of assembly and GTP hydrolysis [discussed by Carlier (1989)]. Definitive evidence for the existence of a GTP cap, which would circumvent problems associated with superimposing separate kinetic measurements, would be a direct detection of E-site GTP incorporated in microtubules. In fact, Carlier and Pantaloni (1986) used a rapid filtration–trapping method to demonstrate directly the transient accumulation of F-actin-bound ATP and P_i during actin assembly. However, efforts to measure microtubule-bound GTP or P_i by filtration–trapping methods have not been successful (Hamel et al., 1986b; Carlier, 1988).

In the present work, we describe efforts to detect microtubule-bound GTP and P_i using a modified rapid filtration–trapping method. The major drawback of this approach previously had been the length of time required to process samples. We have reduced the sample processing time of the method to 15–20 s, which has allowed us to assign lower limits to the rate of GTP hydrolysis during assembly of microtubules. The results place important limitations on models of GTP hydrolysis that predict substantial GTP subunit caps at the ends of microtubules, and support the idea that the hydrolysis of GTP is closely coupled to addition of tubulin at microtubule ends.

Materials and Methods

Preparation of Microtubule-Associated Protein (MAP)-Free Bovine Brain Microtubules. MAP-free bovine brain tubulin, prepared from MAP-rich microtubules essentially as described by Mitchison and Kirschner (1984b), was used in all experiments described in this work. Bovine brain microtubule protein, consisting of approximately 70% tubulin and 30% MAPs, was first isolated by a modification of the procedure of Asnes and Wilson (1979) as described by Farrell and Wilson (1984). Pellets of three-cycle MAP-rich microtubules were resuspended by Dounce homogenization in column buffer (50 mM Pipes, 0.5 mM EGTA, and 0.5 mM $MgSO_4$, pH 6.8) plus 0.1 mM GTP. After 20 min of incubation at 0 °C, solutions were clarified by centrifugation (150000*g*, 30 min, 4 °C). Supernatants (60–70 mg total protein) were applied to 70-mL phosphocellulose columns (P11, Whatman Chemical Separation, Ltd., U.K.) equilibrated in column buffer at 4 °C. The flow rate was 1 mL/min. Peak flow-through fractions were pooled, the buffer was adjusted to the composition required for polymerization (polymerization buffer: 100 mM Pipes, 1 mM EGTA, and 1 mM $MgSO_4$) with a stock solution of 10× polymerization buffer, and tubulin solutions were drop-frozen in liquid nitrogen and stored at −70 °C. The tubulin was essentially free of MAPs as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and Coomassie Blue stain.

Immediately before assembly, the tubulin solution was thawed and clarified by centrifugation (29900*g*, 20 min, 4 °C). If necessary, the supernatant was concentrated by centrifugation using a Centricon 30 tube (Amicon Corp., Danvers, MA). Microtubule seeds were prepared by assembling tubulin at 37 °C in PEM buffer plus 30% glycerol (v/v). Before use, seeds were sheared by three passes through a 1-in., 23-gauge hypodermic needle. Microtubule assembly without further glycerol was initiated by adding 2–3% (v/v) of the seed suspension to the tubulin solution prewarmed to 37 °C in a cir-

culating water bath (maximum glycerol content, 0.9% v/v). Polymerization was monitored by turbidimetry at 350 nm in a Response II spectrophotometer (Gilford Inc.) fitted with a circulating water bath to maintain constant temperature. A GTP-regenerating system consisting of 10 mM acetyl phosphate and 1 unit/mL acetate kinase (final concentrations) was used in some experiments to ensure constant GTP concentration or to remove free GDP from solution.

Collection of Microtubules by Centrifugation. Microtubules were sedimented with a Beckman Airfuge (Beckman Instruments, Inc.). The driving air jet was prewarmed by passage through a large coil of copper tubing submerged in a circulating water bath at 65 °C which maintained the rotor inside the Airfuge at approximately 35 °C. Microtubules were quantitatively sedimented for 4 min at top speed (28 psi, ca. 90000*g*). To limit contamination by free nucleotides, either microtubules were sedimented through 30–50% sucrose cushions or microtubule pellets after sedimentation were washed 3 times with stabilizing buffer (30% glycerol v/v and 10% dimethyl sulfoxide v/v, in polymerization buffer) at 37 °C.

Rapid Collection and Analysis of Double-Radiolabeled Microtubules on Glass Fiber Filters. The basic filtration assay for determining radiolabeled GTP incorporation into microtubules during assembly has been described in detail by Wilson et al. (1982). A modified fixation–stabilization buffer was developed for use with MAP-free microtubules, which are not stable in the stabilizing buffer used with MAP-rich microtubules. Aliquots (20 μL) of microtubule suspensions assembled from tubulin, double-labeled with [^{3}H]- and [γ-^{32}P]GTP (final specific activity for both 200–300 Ci/mol), were diluted 100-fold into a fixation solution consisting of 0.75% glutaraldehyde and 50% sucrose (w/v) in polymerization buffer at 37 °C. The suspensions of fixed microtubules were filtered within 5 s through Whatman GF/F 2.4-cm glass fiber filters supported on a 10-place filter holder manifold (Hoefer Scientific Instruments). Filters were immediately washed at 37 °C with two 3-mL volumes of polymerization buffer containing 30% glycerol (v/v) and 0.75% glutaraldehyde. The entire filtration and washing procedure was carried out by two individuals working together for maximum speed, and as a rule was accomplished within 15 s and never more than 20 s. Finally, filters were transferred to scintillation vials to which 10 mL of Ready Protein (Beckman Instruments) was added for determination of radioactivity. Spillover correction factors were determined by counting samples of [^{3}H]- and [γ-^{32}P]GTP separately.

Two methods were used to determine the background radioactivity bound to the filters that was not bound to microtubules. In one method, 20-μL aliquots of the radiolabeled PC tubulin solutions, prewarmed to 37 °C, were filtered and washed in the absence of microtubules by not adding any glycerol-assembled microtubule seeds. In the second method, aliquots of microtubule suspensions at steady-state were centrifuged in an airfuge (90000*g*, 4 min, 35 °C) to remove the microtubules quantitatively. Aliquots (20 μL) of the resulting supernatants were filtered and washed as described above. The two methods of determining background amounts of filter-bound radioactivity in the absence of microtubules gave identical results. The radioactivity values shown in the figures include the background radioactivity, which was not subtracted. The background levels of radioactivity are indicated on the figures.

In all experiments, the steady-state quantities of [^{3}H]GXP trapped in microtubules on filters corresponded to a molar ratio with tubulin in the microtubule in a narrow range between 0.45 and 0.50 mol of [^{3}H]GXP/mol of tubulin (data not shown), in agreement with results of previous studies (Maccioni & Seeds, 1982; Wilson et al., 1985; Croom et al., 1985). For comparison to the stoichiometries obtained with the filter assay, the stoichiometry of nucleotide incorporation was also determined by sedimenting microtubules polymerized in the presence of [^{3}H]GTP. At steady state in three replicate experiments, the amount of [^{3}H]GXP bound to washed and resuspended microtubule pellets corresponded to a stoichiometry of 0.51 mol of [^{3}H]GXP/mol of tubulin. Thus, [^{3}H]GXP exchange and incorporation into microtubules as determined by filter trapping quantitatively reflected the incorporation of GXP into microtubules and the mass of polymer assembled at any moment in time. For convenience, the filter assay incorporation data were normalized (multiplied by a factor of approximately 2) to a stoichiometry of 1 mol of radiolabeled GXP per mole of assembled tubulin at steady state, so that radiolabeled nucleotide incorporation on a molar basis correlated 1:1 with the incorporation of tubulin into the microtubules. We assumed that the amount of GTP incorporated per mole of tubulin remained constant during polymerization.

Microtubule Length Determinations. An indirect immunofluorescence method for measuring microtubule lengths was adapted from that described by Mitchison and Kirschner (1984b). Microtubules were fixed at 37 °C by slow injection into 0.5% glutaraldehyde in PEM buffer with cut-off Pipetman tips to minimize breakage. Solutions were gently mixed and diluted to a final protein concentration of 5 μg/mL. Aliquots (300–500 μL) of fixed microtubule suspensions were placed as large drops on acid-washed, polylysine-coated coverslips. Microtubules were allowed to settle onto coverslips for 10–15 min; then excess suspension was removed with a pipet. Coverslips were placed in 100% methanol at −20 °C for 10 min to dehydrate the samples and then transferred to a 0.1% solution of bovine serum albumin in PBS, pH 7.4, at room temperature for rehydration (PBS–BSA).

Normal goat serum (50–75 μL), diluted 1:3 with PBS–BSA, was applied to the surface of the coverslips which were contained in a covered petri dish humidified with wet filter paper. After 10–15 min at 37 °C, coverslips were washed 3 times for 5 min in PBS–BSA and then incubated for 15–30 min at 37 °C with 50–75 μL of mouse anti-β-tubulin antibody (kindly supplied by Dr. M. Klymkowski, University of Colorado, Boulder, CO) diluted 1:500 with PBS–BSA. After three 5-min washes in PBS–BSA, 50–75-μL aliquots of fluorescein isothiocyanate (FITC)-labeled secondary antibody (Cappel Inc.; goat anti-mouse), diluted 1:300 with PBS–BSA, were applied. Coverslips were incubated in the dark for 15–30 min at 37 °C and then thoroughly washed with PBS–BSA. Coverslips were inverted onto a small drop (5 μL) of mounting medium (0.1% *p*-phenylenediamine in 95% glycerol) on glass slides. Coverslips were secured with nail polish and stored in the dark until examined.

Microtubule fields were selected that contained an appropriate density of microtubules with random orientations (rather than aligned by flow or other shear forces) and were photographed at a magnification of 100× on a Zeiss Axioplan microscope. Microtubule lengths were measured with a Zeiss MOP-3 digitizer either by projecting the negatives onto the back of the digitizing pad or by printing the negatives at 7.5× enlargment. The microtubule number concentration was calculated by using the mean length and a value of 1680 dimers/μm of microtubule; i.e., [microtubule] = [tubulin]/[mean length (μm) × 1680].

Reagents and Miscellaneous Procedures. EGTA, GTP (type III), and glutaraldehyde (25%, grade II) were obtained from Sigma Chemical Co., St. Louis, MO. GTPγS was obtained from Boehringer Mannheim GmbH, West Germany. Pipes (free acid) was obtained from Research Organics Inc., Cleveland, OH. [^{3}H]GTP, [γ-^{32}P]GTP, and [γ-^{35}S]GTP were obtained from New England Nuclear, Claremont, CA. Radiolabeled nucleotides were routinely analyzed for contaminants by HPLC. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Results

The absence of a detectable lag of GTP hydrolysis relative to microtubule assembly in experiments in which the rates of assembly and GTP hydrolysis were determined from simultaneous but separate measurements (MacNeal & Purich, 1978; Hamel et al., 1986; O'Brien et al., 1987; Schilstra et al., 1987; our own unpublished work) led us to look for a more sensitive and more direct method to measure GTP hydrolysis during microtubule assembly. For this purpose, a glass fiber filter assay (Wilson et al., 1982) was modified (see Materials and Methods) to allow microtubule assembly and GTP hydrolysis to be measured simultaneously during assembly of phosphocellulose-purified tubulin double-labeled at the E site with [^{3}H]- and [γ-^{32}P]GTP. Filter-trapped ^{3}H, which represents both GDP and any GTP bound in the microtubules (noted as GXP in the figures), is a measure of microtubule assembly, while filter-trapped ^{32}P, which represents any GTP or P_i bound in the microtubules, permits determination of the extent of GTP hydrolysis and P_i release from tubulin subunits incorporated into the microtubules.

Models of uncoupled GTP hydrolysis predict that hydrolysis should become increasingly uncoupled from addition of tubulin to microtubules as the rate of addition increases (Carlier & Pantaloni, 1981; Carlier et al., 1987). Therefore, we have used a high concentration of tubulin to obtain high initial rates of polymerization. Seeded assembly using 79 μM tubulin double-labeled with [^{3}H]- and [γ-^{32}P]GTP is shown in Figure 1. The tubulin solution was warmed to 37 °C for 2 min, and then glycerol-assembled seeds (3% v/v) were added (zero time). Incorporation of [^{3}H]GXP into the microtubules obeyed the first-order rate equation:

$$[GXP]_{microtubule}(t) = [GTP]_{tubulin}(t_0)(1 - e^{-k_1 t}) \quad (1)$$

where k_1 is the pseudo-first-order rate constant for microtubule assembly. As is clear from the data in the figure, even during the most rapid period of assembly there was no detectable ^{32}P and, therefore, no GTP or P_i trapped in the microtubules on the filters above background levels. The steady-state level of microtubule-bound [^{3}H]GXP in Figure 1 corresponded to a stoichiometry of 0.45 mol of radiolabeled GXP incorporated per mole of tubulin (28 μM [^{3}H]GXP and 61 μM assembled tubulin). After the stoichiometry was normalized (see Materials and Methods), an initial rate of assembly of 70 μM min^{-1} was calculated from the initial rate of [^{3}H]GXP incorporation. The number concentration of microtubules was 1.8 nM, as determined from length measurements at steady state (see Materials and Methods). If we assume that the rate of hydrolysis continued in unquenched fashion during the 20 s it took to process the samples, the minimum rate of GTP hydrolysis must have been 53 μM min^{-1} (70 μM/1.33 min), or approximately 490 microtubules^{-1} s^{-1}. This is more than 10 times the maximum rate of GTP hydrolysis, 35–40 microtubules^{-1} s^{-1}, reported by Carlier et al. (1987).

Hypothetical Transient Accumulation of Microtubule-Bound GTP Predicted by the Uncoupled Stochastic Hydrolysis Model. Precise determination of microtubule-bound GTP with a filter trapping assay requires either that GTP hydrolysis be quenched during the sample processing procedure or that the processing procedure be carried out essentially instantaneously. The processing procedure we have used requires a maximum of 20 s. We cannot determine whether hydrolysis is quenched in the glutaraldehyde fixation–stabilization buffer during the 20-s processing delay time because we cannot detect any incorporated ^{32}P in the microtubules. Thus, in order to estimate a minimum rate of hydrolysis, we assume that quenching of GTP hydrolysis does not occur. This is a worst case assumption and, indeed, glutaraldehyde may quench ATP hydrolysis during actin assembly (Carlier & Pantaloni, 1986).

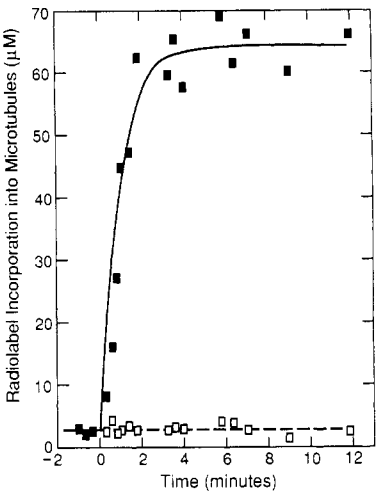

FIGURE 1: Polymerization of microtubules with 79 μM double-radiolabeled ([γ-^{32}P]-[^{3}H]GTP) tubulin. Glycerol-assembled microtubule seeds (3% v/v) were added at time zero, and microtubules were collected by rapid filtration at the times indicated as described under Materials and Methods. The time course of microtubule polymerization was calculated (solid line) by using eq 1 with the pseudo-first-order rate constant 1.06 min^{-1} determined from the ^{3}H incorporation data (solid squares). The background level of filter-trapped ^{32}P (broken line) was the average of the measurements made in the absence of microtubules before addition of seeds. Filter-trapped ^{32}P (open squares) was within the background range throughout the polymerization reaction.

The time course for accumulation of microtubule-bound GTP that theoretically should have been detected if a stochastic uncoupled hydrolysis mechanism were operative was calculated by using an equation for the accumulation of the intermediate products of two consecutive first-order reactions as described by Carlier and Pantaloni (1981):

$$[GTP]_{microtubule}(t) = [GTP]_{tubulin}(t_0)[k_1/(k_1 - k_2)](e^{-k_1 t} - e^{-k_2 t}) \quad (2)$$

where k_1 is the pseduo-first-order rate constant for microtubule assembly and k_2 is the first-order rate constant for GTP hydrolysis within the microtubule. With a first-order rate constant for GTP hydrolysis of 0.25 min^{-1} (Carlier & Pantaloni, 1981) and the pseudo-first-order rate constant for microtubule polymerization calculated from the data in Figure 1 (1.06 min^{-1}), there would have been more than 35 μM microtubule-bound GTP at 2 min of assembly, despite the 20-s sample processing delay [Figure 2A (alternating short and long dashes)], a quantity that would have been detected readily by the assay.

Assuming a stochastic uncoupled GTP hydrolysis mechanism for the data of the experiment in Figure 1, we wanted to visualize what the hypothetical transients for accumulation

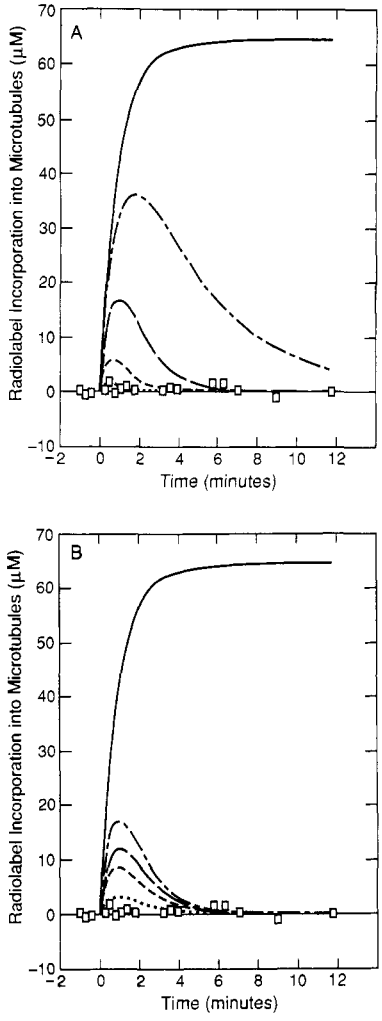

FIGURE 2: Hypothetical transients of GTP bound to filter-trapped microtubules predicted by the stochastic uncoupled GTP hydrolysis model during rapid microtubule assembly. The data are from the experiment shown in Figure 1. The time course of assembly (solid line) was calculated as described in Figure 1, and the filter-bound ^{32}P data, corrected for subtraction of background (open squares), are shown for comparison. (A) Effect of different rate constants for GTP hydrolysis. The time course for accumulation of GTP in microtubules during assembly was calculated by using first-order rate constants for GTP hydrolysis of 0.25 min^{-1} (alternating long- and short-dash line), 1.0 min^{-1} (long-dash line), 3.0 min^{-1} (short-dash line), and 5.0 min^{-1} (dotted line). (B) Effect of sample processing delay times. The time course for accumulation of GTP in microtubules was calculated by using a first-order rate constant for GTP hydrolysis equal to the pseudo-first-order microtubule assembly rate constant (1.0 min^{-1}) and sample processing delays of 20 s (alternating long-dash and short-dash line), 40 s (long-dash line), 60 s (short-dash line), and 120 s (dotted line).

of microtubule-bound GTP might be at GTP hydrolysis rates greater than 0.25 min^{-1}, and we also wanted to determine what the minimum rate of GTP hydrolysis of microtubule-bound GTP would have to have been in order not to be able to detect any GTP bound in the microtubules. Thus, we calculated hypothetical time courses for accumulation of microtubule-bound GTP using multiples of the measured pseudo-first-order microtubule assembly rate constant (1.06 min^{-1}) for GTP hydrolysis rate constants. From this analysis (Figure 2A), it appears that microtubule-bound GTP could have been detected and measured reliably during the most rapid period of assembly even if the first-order rate constant for GTP hydrolysis were 4–5 times higher than the pseudo-first-order rate constant for microtubule assembly. This calculation included a 20-s filter processing time delay. This would be a first-order rate

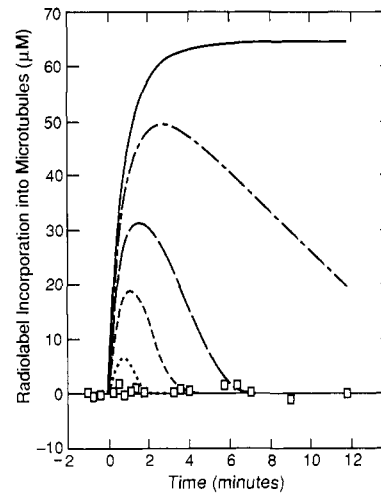

FIGURE 3: Hypothetical transients of GTP bound to microtubules predicted by the uncoupled vectorial GTP hydrolysis model during rapid microtubule assembly. The data are from the experiment of Figure 1. The time course for assembly (solid line) was calculated as in Figure 1, and the filter-bound ^{32}P data from the experiment of Figure 1, corrected for subtraction of background radioactivity, are shown for comparison (open squares). The time course for accumulation of GTP in microtubules during assembly was calculated by using linear rates of GTP hydrolysis of 35 s^{-1} (alternating short-dash and long-dash line), 105 s^{-1} (long-dash line), 175 s^{-1} (short-dash line), and 263 s^{-1} (dotted line). A 20-s sample processing delay time was included in the calculations.

constant for GTP hydrolysis 20 times higher than the rate constant originally reported for GTP hydrolysis by Carlier and Pantaloni (0.25 min^{-1}, 1981) and twice as high as the minimum value estimated by O'Brien et al. (1987).

The contribution of different sample processing delay times with the filtration method was explored by calculating hypothetical time courses for accumulation of microtubule-bound GTP, assuming a number of different sample processing delay times and using the data obtained in the experiment of Figure 1 (Figure 2B). For the examples shown, a first-order rate constant for GTP hydrolysis equal to the pseudo-first-order rate constant of microtubule assembly (1.06 min^{-1}) was used. It was evident that even with a 120-s delay, during which it was assumed that hydrolysis of microtubule-bound GTP continues at the same rate as before microtubule fixation, there would still have been measurable microtubule-bound GTP. Thus, the filter processing delay time of 20 s does not seriously limit the ability to detect microtubule-bound GTP at the previously reported rates of GTP hydrolysis.

Transient Accumulation of Microtubule-Bound GTP Predicted by the Uncoupled Vectorial Hydrolysis Model. The uncoupled vectorial hydrolysis model requires that GTP hydrolysis reach a maximum rate at a tubulin dimer concentration designated c' (Carlier et al., 1987). Further, the model predicts that during the initial moments of microtubule polymerization when the free tubulin dimer concentration is above c', large transient GTP subunit caps would exist at the microtubule ends. For the experiment shown in Figure 1, the initial tubulin dimer concentration was 10 times higher than the c' of 7 μM reported by Carlier et al. (1987).

A minimum estimate of c' for our experimental conditions was determined by calculating the hypothetical time course for the transient accumulation of microtubule-bound GTP predicted by the vectorial hydrolysis model using several multiples of the reported maximum rate of GTP hydrolysis (Figure 3). A 20-s sample processing delay time was included in the calculations. From this analysis, it appears that microtubule-bound GTP could have been measured readily

during the most rapid period of assembly even if the maximum rate of GTP hydrolysis were 7–8 times the maximum rate previously reported (Carlier et al., 1987). This implies that the minimum value of c' for the experimental conditions of the experiment used to calculate the data of Figure 3 would be greater than 50 μM, or 5 times the measured tubulin concentration of 10 μM in a microtubule suspension at polymer-mass steady state.

Microtubule-Bound GXP during Glycerol-Induced Assembly of Tubulin at Room Temperature. The inability to detect GTP bound to microtubules that is predicted to be bound according to uncoupled GTP hydrolysis models led us to search for conditions that might increase the extent of uncoupling between GTP hydrolysis and microtubule assembly. We reasoned that if GTP hydrolysis and tubulin subunit addition were independent reactions as postulated, then it might be possible to enhance or retard one reaction relative to the other.

Glycerol is thought to promote tubulin polymerization by affecting the structure of the solvent rather than by interacting directly with the tubulin dimer (Lee & Timasheff, 1977). Therefore, we reasoned that glycerol might enhance the rate of addition of tubulin dimer to microtubules relative to the GTP hydrolysis rate and, thereby, increase any kinetic uncoupling that may exist between microtubule polymerization and GTP hydrolysis. An additional reason for studying microtubule polymerization in glycerol was that the earlier data which had indicated that GTP hydrolysis was uncoupled from microtubule polymerization were obtained with microtubules assembled in the presence of glycerol (Carlier & Pantaloni, 1981; Carlier et al., 1987).

Assembly of microtubules in the presence of glycerol also allowed us to examine the relative effects of temperature on polymerization and GTP hydrolysis. Lowering the temperature of polymerization might decrease the intrinsic GTP hydrolysis rate, while glycerol might counterbalance the effects of lower temperature on the polymerization rate, resulting in an increase in the extent to which GTP hydrolysis and polymerization might become uncoupled. Therefore, high concentrations of tubulin were polymerized in the presence of glycerol at a number of different temperatures.

In one such experiment, tubulin (64 μM), double-radiolabeled with [^{3}H]- and [γ-^{32}P]GTP, was polymerized in PEM buffer plus 3.4 M glycerol at 23 °C, the lowest temperature studied (Figure 4). Background measurements were made by centrifuging aliquots of microtubule suspensions at steady state to remove the microtubules and filtering the resulting supernatant fractions. Except for three early data points during the lag before assembly began in this experiment, there was no filter-bound ^{32}P above background and, therefore, no microtubule-bound GTP or P_i. Similar results were obtained at higher temperatures (27 °C, and 37 °C, data not shown), with no filter-bound ^{32}P higher than background at any time during the experiment. These data indicate that neither polymerization in the presence of glycerol nor assembly at low temperatures enhances or causes the uncoupling of microtubule assembly and GTP hydrolysis within the limits of our ability to detect such uncoupling in the form of microtubule-bound GTP or P_i. Further, the data also indicate that the presence of glycerol does not account for the discrepancy between the data reported here and those previously reported by Carlier et al. (1987).

Microtubule-Bound Nucleotides during Assembly of Tubulin in the Presence of GTPγS. In their vectorial GTP hydrolysis model, Carlier et al. (1987) proposed that at tubulin concentrations below c' (e.g., at steady state) a one-subunit-deep GTP cap is maintained at the ends of microtubules because cooperative interactions between GDP-liganded subunits and GTP-liganded subunits at the interface between a GTP cap and a GDP core are required for GTP hydrolysis. If this were true, incorporation of tubulin dimers containing a nonhydrolyzable GTP analogue into microtubules might disrupt the cooperative interactions at such an interface. Then, hydrolysis of GTP might be inhibited on the next and perhaps on many subsequent GTP-liganded subunits that assemble onto the microtubule ends, resulting in a measurable accumulation of microtubule-bound GTP. On the other hand, if GTP hydrolysis and subunit addition to the microtubule were strictly coupled, nonhydrolyzable GTP analogues should inhibit assembly.

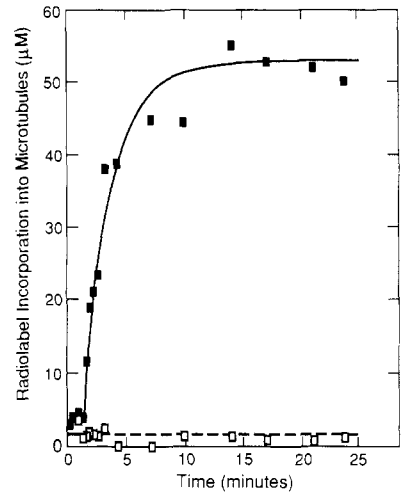

FIGURE 4: Polymerization of microtubules in glycerol at 23 °C with 64 μM double-radiolabeled ([γ-^{32}P]- and [^{3}H]GTP tubulin. The time course for microtubule polymerization was calculated (solid line) with eq 1 using a pseudo-first-order rate constant calculated from the initial ^{3}H incorporation data (solid squares). The background amount of filter-trapped label (broken line) was determined by filtering aliquots of the supernatant obtained by sedimenting microtubules from aliquots of the microtubules at steady state. The amount of the filter-bound ^{32}P (open squares) was at or below the background level throughout the polymerization reaction.

These predictions were examined by polymerizing tubulin in the presence of different ratios of GTP and GTPγS, a nonhydrolyzable analogue of GTP (Kirsch & Yarbrough, 1981; Hamel & Lin, 1984; Roychowdhury & Gaskin, 1986). Turbidimetric analysis of tubulin polymerization in the presence of GTPγS and GTP indicated that GTPγS inhibited the initial rate of microtubule polymerization in a concentration-dependent manner but that it did not appear to inhibit the final extent of polymerization (Figure 5). Radiolabeled nucleotides incorporated into microtubules in the presence of GTPγS were determined in two separate but identical polymerization experiments (Figure 6). In the first experiment, 45 μM tubulin was polymerized in the presence of 50 μM unlabeled GTPγS and a 100 μM mixture of [^{3}H]- and [γ-^{32}P]GTP. The second identical assembly reaction contained 50 μM [γ-^{35}S]GTP and 100 μM unlabeled GTP. Backgrounds for both reactions were determined by centrifuging aliquots of the steady-state microtubule suspensions and filtering the supernatant solutions through glass fiber filters. As shown in Figure 6, GTPγS became incorporated into the microtubules to a small extent (0.8 μM), in agreement with the results of Roychowdhury and Gaskin (1986), but the incorporated GTPγS did not cause any detectable inhibition of GTP hydrolysis within the microtubules as evidenced by the absence of filter-bound ^{32}P above background measurements. The fact that some incorporation of a nonhydrolyzable GTP analogue

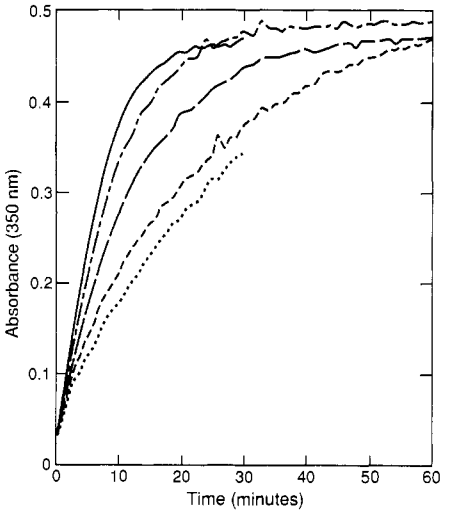

FIGURE 5: Effect of GTPγS on the polymerization of tubulin. Polymerization at 37 °C was monitored by turbidimetry at 350 nm. Glycerol-assembled microtubule seeds were added at zero time to aliquots of 28 μM tubulin in PEM buffer plus 100 μM GTP and 0 μM GTPγS (solid line), 25 μM GTPγS (alternating long-dash and short-dash line), 50 μM GTPγS (long-dash line), 100 μM GTPγS (short-dash line), and 200 μM GTPγS (small squares).

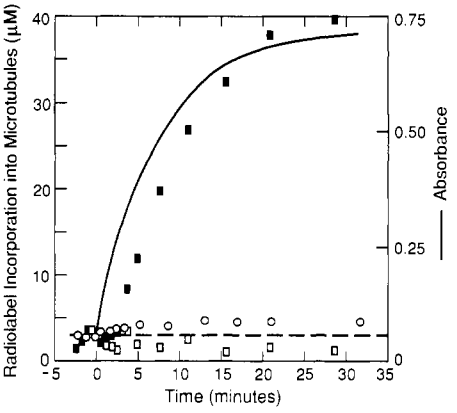

FIGURE 6: Effect of GTPγS on GTP hydrolysis during microtubule assembly. Two suspensions of 45 μM tubulin in PEM buffer were assembled at 37 °C by the addition of glycerol-assembled microtubule seeds at zero time. The first suspension contained a mixture of 100 μM [γ-^{32}P]- and [^{3}H]GTP and 50 μM unlabeled GTPγS. The second contained 100 μM unlabeled GTP and 50 μM [γ-^{35}S]GTP. The label incorporation data from both solutions were plotted together. The solid line represents polymerization determined by turbidimetry at 350 nm in a separate aliquot of the same microtubule suspension. The background amount of filter-bound radiolabel (broken line) was plotted as the average of the measurements made before the addition of microtubule seeds. Filter-bound ^{32}P (open squares) is below the background level throughout the assembly reaction. Filter-bound ^{35}S (open circles) reached 0.8 μM above background at steady state.

into microtubules occurred might be taken to indicate that GTP hydrolysis is not obligately coupled to tubulin addition to microtubules. However, the extent of incorporation was small and may have been insufficient to block polymerization. Further, although tubulin aggregates were not readily detected in the microtubule suspensions, it is also possible that the filter-bound ^{35}S was bound to tubulin aggregates rather than to bona fide microtubules, because the extent of incorporation was so small.

Other Assembly Conditions with Potential Effects on GTP Hydrolysis. O'Brien and Erickson (1989) and Gal et al. (1988) found that the rate of microtubule disassembly is dependent upon the Mg^{2+} concentration, with microtubules being more stable at low concentrations than at high concentrations of Mg^{2+}. In agreement with these results, we found that when tubulin was polymerized in the presence of a stoichiometric or a slightly greater than stoichiometric concentration of GTP, the rate at which the microtubules disassembled after depletion of the GTP decreased as the free Mg^{2+} concentration was decreased (data not shown). This permitted us to examine the possibility that the stabilizing effects of very low Mg^{2+} concentrations were due to a decrease in the intrinsic GTP hydrolysis rate. Tubulin (65 μM), freed of all unbound Mg^{2+} by gel filtration, was polymerized in the presence of a mixture of [^{3}H]- and [γ-^{32}P]GTP as described for previous experiments in this work. Similar to the results of the other experiments, microtubule-bound ^{32}P did not rise above the background measurements at any time during the polymerization reaction (data not shown).

Al^{3+} has been reported to inhibit GTP hydrolysis by tubulin while not affecting its assembly into normal microtubules (MacDonald et al., 1987). This property would make Al^{3+} a useful tool for studying the properties of GTP-liganded microtubules and for testing the GTP cap hypothesis. In experiments designed to confirm that Al^{3+} inhibits the hydrolysis of GTP, PC tubulin was assembled in PEM buffer plus 50 μM Al^{3+} and [^{3}H]- and [γ-^{32}P]GTP. As with all the other assembly conditions examined in this work, filter-bound ^{32}P was within the range of the background measurements at all times during the assembly reaction (data not shown). Thus, in disagreement with the work of MacDonald et al. (1987), at the conditions used in this work, Al^{3+} did not inhibit GTP hydrolysis during polymerization.

Discussion

To understand how the spatial distribution of microtubules in the cell is established and controlled, it will be necessary to understand the detailed mechanisms responsible for microtubule assembly and disassembly dynamics. Toward this end, it is important to determine if GTP hydrolysis and subunit addition to the microtubule occur as a concerted reaction, or if GTP hydrolysis is mechanistically and kinetically uncoupled from microtubule assembly.

Comparisons of GTP Hydrolysis and Microtubule Assembly Rates. Comparison of the rates determined simultaneously for GTP hydrolysis and microtubule assembly (Hamel et al., 1982, 1986a; Caplow et al., 1985; O'Brien et al., 1987; Schilstra et al., 1987) has failed to confirm the existence of a kinetic lag between GTP hydrolysis and microtubule assembly (Carlier & Pantaloni, 1981; Carlier et al., 1987; Carlier, 1989). The continuing controversy regarding the existence or nonexistence of a GTP cap is due to several difficulties associated with superimposing separate kinetic measurements of GTP hydrolysis and microtubule assembly, as was done in all of the above studies (Carlier, 1989).

First, determining the rate of microtubule assembly by turbidimetry requires that the specific turbidity be a linear function of microtubule mass. This may not be true at all times during the assembly reaction. Initially, microtubule mass could be underestimated because of the presence of large numbers of microtubules less than 1 μm long that scatter light to a lesser extent than longer microtubules (Berne, 1974). At steady state, the relationship between turbidity and microtubule mass has also been shown to deviate from linearity at high tubulin concentrations; turbidity underestimated microtubule mass determined by sedimentation (Carlier & Pantaloni, 1978). Aggregation of inactive tubulin and formation of nonmicrotubule polymers which do not hydrolyze GTP can also contribute to turbidity. Any of these situations would lead to misinterpretation when turbidity data were superimposed on GTP hydrolysis data.

Second, the interpretation of GTP hydrolysis data gathered during polymerization requires that assumptions be made regarding the assembly mechanism. In the original study that led to the GTP cap hypothesis, Carlier and Pantaloni (1981) assumed that the reaction that gave rise to the steady-state rate of GTP hydrolysis (i.e., subunit turnover) also operated throughout assembly. Therefore, the steady-state rate of GTP hydrolysis was subtracted from the entire time course of the hydrolysis data, which accentuated the apparent lag in GTP hydrolysis. In later studies (O'Brien et al., 1987), the assumption was made, on the basis of the dynamic instability model, that no subunit turnover occurred during assembly before steady state was reached. Probably neither assumption was correct in light of recent results (Walker et al., 1988) which have demonstrated that microtubules undergo phase changes and therefore subunit turnover even during assembly, but at a lower frequency than at steady state. This would result in a superstoichiometric release of P_i that could mask a slight lag in GTP hydrolysis.

Additional problems include the possibility of the incorporation of a small proportion of GDP-liganded subunits into the microtubules (Manser & Bayley, 1985; Hamel et al., 1986a), which could lead to an underestimation of the GTP hydrolysis rate. Also, there is lack of agreement on the most accurate method of extracting [^{32}P]P_i from a solution of microtubules (Carlier, 1989; O'Brien et al., 1987).

Direct Determination of Microtubule-Bound GTP. An experimental approach designed to demonstrate microtubule-bound GTP directly would circumvent the experimental difficulties described above. In initial efforts, microtubules were separated from free nucleotide by centrifugation and then analyzed for total bound nucleotide. No evidence of an excess of GTP over GDP was found. Centrifugation, though, has the drawback of being a relatively slow method for collecting microtubules. It was likely that GTP hydrolysis within the microtubule would have continued during the sedimentation time and in the pellet. In addition, it was very difficult to control or measure the background contamination of the pellet by free nucleotide.

The glass fiber filter assay as used herein has allowed the rapid separation of microtubules from free nucleotide and the quantitation of microtubule-bound radiolabeled nucleotide. Since any filter-bound ^{32}P would represent microtubule-bound GTP or P_i, the postulation that P_i release from microtubules following GTP hydrolysis may be slow relative to microtubule assembly could be examined, which was an additional advantage of the filter assay method as compared with methods of extracting P_i or GDP from microtubule solutions to determine the rate of GTP hydrolysis. The slow release of P_i could result in the ends of microtubules being capped and stabilized by GDP–P_i subunits in a manner analogous to that proposed for GTP subunit caps (Carlier, 1988). However, evidence against this possibility has recently been obtained by Caplow et al. (1989), who found that the rates of shortening and the frequency of transition from growth to rapid shortening of individual microtubules were not affected by high concentrations of P_i.

In the experiments described in the present work, even at an initial rate of assembly of 70 μM min^{-1}, during which substantial amounts of microtubule-bound GTP or P_i should have been present if GTP hydrolysis or P_i release were kinetically uncoupled from subunit addition to the microtubule, microtubule-bound GTP or P_i was not detected (Figure 1). Microtubule-bound GTP was not detectable during seeded microtubule assembly, or under glycerol-promoted assembly

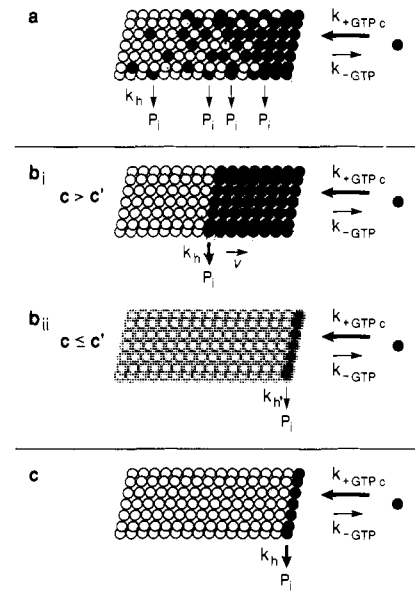

FIGURE 7: Mechanistic models for hydrolysis of GTP at microtubule ends. (a) Uncoupled stochastic GTP hydrolysis. A growing end of a microtubule would be capped by a gradient of GTP-liganded subunits (shaded circles). The length of the cap would be proportional to the polymerization rate $k_{+GTP}c$ where c = the tubulin concentration and k_+ is the association rate constant. During the initial moments of polymerization (high c), virtually the entire microtubule could be composed transiently of GTP-liganded subunits. GTP hydrolysis would occur randomly at a single rate (k_h) on all GTP-liganded subunits, independent of interactions with neighboring subunits, giving rise to GDP-liganded subunits (open circles). ($b_{i,ii}$) Uncoupled vectorial GTP hydrolysis. GTP hydrolysis within the microtubule would be greatly enhanced by cooperative interactions at the interface between GTP-liganded (shaded circles) and GDP-liganded (clear circles) subunits. At a tubulin subunit concentration designated c', GTP hydrolysis would reach a maximum rate (k_h). (b_i) When the tubulin concentration is greater than c' (i.e., $c > c'$), hydrolysis would become uncoupled kinetically from subunit addition to the microtubule end, and a growing microtubule end would become capped by a large region of GTP-liganded subunits. GTP hydrolysis would occur as a vectorial wave progressing at a constant rate (v) toward the tip of the microtubule. (b_{ii}) Below c', the rate of GTP hydrolysis would be greater than the rate of tubulin addition to the microtubule end until the GDP/GTP interface reached the terminal ring of subunits. GTP liganded to the terminal subunits would be hydrolyzed ($k_{h'}$) only as new GTP-liganded tubulin molecules added to the microtubule end. Below c', GTP hydrolysis would be kinetically but not mechanistically coupled to microtubule polymerization (i.e., $k_{h'} = k_{+GTP}$). For this model to be operative, c' would have to be greater than 50 μM (see Results). (c) Forced GTP hydrolysis. At all tubulin concentrations, a growing microtubule end would be capped by a single ring of GTP-liganded subunits. Addition of new GTP-liganded subunits would be kinetically and mechanistically coupled to the "forced" hydrolysis of GTP on previously terminal subunits. Thirteen terminal GTP-liganded subunits are indicated in this and in the previous diagram (b_{ii}). However, we consider that there would be one GTP-liganded subunit per elongating site.

conditions similar to those used in earlier studies that had suggested that GTP hydrolysis was uncoupled from assembly (Carlier & Pantaloni, 1981). In addition, there was no microtubule-bound GTP or P_i detected during rapid assembly at reduced temperature (Figure 4), or in the presence of GTPγS (Figure 6), conditions that theoretically could have enhanced the degree of uncoupling between GTP hydrolysis and tubulin addition to microtubule ends.

Implications for GTP Cap Models. Although the data do not absolutely rule out an uncoupled GTP hydrolysis mechanism, they make it extremely unlikely. The data allow us to assign lower limits for the rate constants that would be involved. For the case of stochastic uncoupled GTP hydrolysis (see Figure 7a), the first-order rate constant for GTP hy-

drolysis and P_i release (K_h) would have to be at least 5 times higher than the calculated pseudo-first-order rate constant for microtubule polymerization in order to account for the absence of detectable microtubule-bound GTP or P_i (Figure 1). While still a theoretical possibility, a first-order rate constant for GTP hydrolysis this high would preclude the existence of a GTP subunit cap in microtubule suspensions at steady state, in which the tubulin concentration is only approximately 10 μM. This simple model, then, cannot reasonably account for the observed data.

Similarly, an uncoupled vectorial GTP hydrolysis mechanism (Figure 7b) cannot absolutely be eliminated. However, the data demonstrate that, under the experimental conditions of this study, for this mechanism to be operative the minimum rate of GTP hydrolysis must be 6–7 times greater than originally reported (35–40 s^{-1}; Carlier et al., 1987), and the lowest tubulin concentration at which the rate of GTP hydrolysis could become slower than tubulin addition to microtubule ends would be more than 50 μM (Figure 3). While it remains possible that GTP hydrolysis at the interface of a potential GTP cap and the GDP core could become slower than microtubule assembly at some very high tubulin concentration, the functional relevance in vitro and in the cell becomes questionable. Since microtubules would be capped by at most a single ring of GTP subunits at all times during polymerization of less than 50 μM tubulin and at steady state at any tubulin concentration, the question of whether GTP hydrolysis is uncoupled from microtubule assembly becomes unimportant in seeking to understand the behavior of microtubules in cells.

Of the models of GTP hydrolysis that predict a GTP subunit cap, the results are most consistent with a "forced" hydrolysis mechanism as first proposed by O'Brien et al. (1987). This mechanism (Figure 7c) can account for hydrolysis of GTP concomitant with addition of tubulin at microtubule ends while a cap of GTP-liganded tubulin is maintained at the microtubule ends to distinguish growing from shortening microtubules at steady state. A cap of five to eight GTP-liganded subunits would be below the resolution of the filter assay method and, at present, any other known method for measuring microtubule-bound GTP. For example, the microtubule number concentration for the experiment shown in Figure 1 was 1.8 nM. If both ends of the microtubules were capped with [^{32}P]GTP or P_i, then the solution would have contained 20–40 nM microtubule-bound ^{32}P (not considering a 20-s filter processing delay time). The background measurement of ^{32}P was on the order of 200 nM.

Case for a Large Stretch of Conformationally Stable Tubulin at Microtubule Ends (a "Conformational Cap"). It has been firmly established by direct microscopic observation (Horio & Hotani, 1986; Walker et al., 1988) that under certain conditions two distinct populations of steady-state microtubules exist in vitro, one growing and one shortening, and that individual microtubules can transition from one population to the other infrequently relative to the rates of subunit addition to and loss from the microtubule. Further, there is good evidence that it is the ends of the microtubules that somehow differentiate these populations. First, shearing of bulk steady-state MAP-free microtubules results in a sudden, rapid, and extensive loss of polymer mass, which is then followed by recovery (Mitchison & Kirschner, 1984a; Farrell et al., 1987). This phenomenon is consistent with the idea that the majority of microtubules at polymer-mass steady state exist in a metastable state with the stability controlled at the microtubule ends. Shearing creates new ends by exposing the unstable microtubule core, and results in rapid depolymerization. Second, at least one end of individual microtubules has been observed directly to depolymerize rapidly when the end is severed by UV microbeam irradiation in vitro (Walker et al., 1989).

Although the existence of a cap of GTP-liganded tubulin at the ends of microtubules provides an aesthetic explanation for the observed dynamics of steady-state microtubules, and despite its widespread acceptance, there is little experimental evidence to support the existence of such a cap. Other explanations are possible. For example, one model that could account for most of the experimental observations would be that a relatively slow first-order transition in tubulin from a GTP-liganded conformation to a GDP-liganded conformation might occur after GTP hydrolysis and release of the P_i. GTP hydrolysis and P_i release may be mechanistically as well as kinetically coupled to microtubule assembly. This model differs from the original GTP cap model (Carlier & Pantaloni, 1981) only in that the intrinsic rate-determining step in the tubulin subunit assembly/disassembly cycle is the occurrence of a conformational change in the tubulin subunits following hydrolysis of the GTP, rather than hydrolysis of the GTP itself. When tubulin in the GTP-liganded conformation is at the microtubule end, the end is postulated to be stable relative to the presence of tubulin at the microtubule end in the GDP-liganded conformation. Rate-determining protein conformational changes are known. As one example, the rate-limiting step for actin-activated ATPase of myosin has been demonstrated to be a slow conformational change that occurs after hydrolysis of the ATP, either before or after the relatively rapid release of P_i from the enzyme–product complex (Trentham et al., 1972; Bagshaw & Trentham, 1974).

In addition to the kinetic studies which indicate that GTP hydrolysis and microtubule assembly are closely coupled (MacNeal & Purich, 1978; Hamel et al., 1982, 1986a; Caplow et al., 1985; O'Brien et al., 1987; Schilstra et al., 1987; this study) other experimental evidence is available to support the idea that conformational changes in tubulin at microtubule ends may be responsible for controlling microtubule assembly dynamics. Results of studies in which there was a lag in microtubule depolymerization following a sudden temperature decrease are inconsistent with the idea that a terminal ring of GTP-liganded subunits at the ends of microtubules forms a stabilizing cap (Caplow et al., 1988). The delay in microtubule disassembly following a temperature decrease is consistent with the existence of a large region of stabilized tubulin at microtubule ends. Although the results were interpreted in terms of a large cap of GTP-liganded subunits, no GTP measurements were made, and the results are equally interpretable in terms a large conformational cap.

In summary, the minimum rates of GTP hydrolysis that have been determined from the data of the present study preclude mechanisms that involve the existence of GTP within microtubules at steady state other than mechanisms that limit hydrolysis of GTP to the extreme ends of the microtubules. Such mechanisms must involve cooperative interactions between GTP- and GDP-liganded subunits as suggested in the vectorial and forced hydrolysis models (see Figure 7). The existence of a steady-state GTP cap any larger than a single turn of subunits at the microtubule end would require the mechanism to operate by long-range cooperative interactions between subunits.

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