Induction of Microtubule Catastrophe by Formation of Tubulin−GDP and Apotubulin Subunits at Microtubule Ends†

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ABSTRACT: The recent discovery that GTP linked to latex beads binds to microtubule ends suggested that nucleotide interactions at this site may play a role in regulating microtubule (MT) dynamics. Evidence for this was sought using DIC microscopy to analyze effects of the free GTP and GDP concentration on the rates of MT elongation and phase transition to rapid shortening (catastrophe, k_c). That nucleotide can dissociate and thereby destabilize the plus end by forming nucleotide-free (apotubulin) subunits was indicated by an increase in k_c from 0.001 to 0.05 s^{-1}, when the free GTP concentration was reduced from 100 to 0.5 μM, during assembly with 15 μM tubulin−GTP subunits (TuT). That nucleotide can bind to the minus end was indicated by a nearly 5-fold decrease in the rate of elongation when the free GDP concentration was increased from 1.6 to 175 μM, during assembly with a mixture of 36 μM TuT and 54 μM TuD. Further evidence that nucleotide can bind to both ends was provided by the observation that with a mixture of 36 μM TuT and 54 μM TuD, k_c was increased from 0.0036 to 0.05 s^{-1} at the plus end, and from 0.0005 to 0.005 s^{-1} at the minus end, when the free GDP concentration was increased from 1.6 to 175 μM. Our evidence for destabilization of microtubules by formation of apotubulin and by nucleotide exchange to form terminal TuD subunits suggests that microtubule dynamics can be regulated in cells by an exchange factor that generates apotubulin subunits, or by a GTPase activating protein that forms TuD subunits at microtubule ends.

Despite the early discovery that GTP is required (Weisenberg, 1972) and apparently plays a regulatory role in microtubule assembly (Mitchison & Kirschner, 1984), the detailed mechanism for this function has not been fully elucidated. The currently accepted model is one in which tubulin−GTP (TuT)[1] subunits make stable additions to microtubule ends without GTP hydrolysis, which subsequently (Mitchison & Kirschner, 1986; Caplow, 1992) occurs within the core of the microtubule. There is a near-zero change in free energy, apparently because the energy from hydrolysis is almost entirely converted into strain in the microtubule lattice (Caplow et al., 1994), with the result that dissociation of tubulin−GDP subunits (TuD) from microtubule ends is both thermodynamically and kinetically very favorable.

Tubulin subunits contain 2 mol of guanine nucleotide. Little is known about the nucleotide at a "nonexchangeable" site (N-site), except that this is GTP which does not hydrolyze, and dissociates with a rate of $<1 \times 10^{-5}$ s^{-1} (Zeeberg & Caplow, 1978). The K_d for binding to the exchangeable site (E-site) is equal to about 22 nM for GTP and about 60 nM for GDP (Zeeberg & Caplow, 1979; Fishback & Yarbrough, 1984; Hamel et al., 1986; Correia et al., 1987) with half-times for dissociation equal to 5 s for GDP (Brylawski & Caplow, 1983) and 25 s for GTP (Caplow et al., 1984). Nucleotide dissociation from tubulin oligomers is a two-step reaction in which TuD subunits first dissociate ($k = 0.11$ s^{-1}), followed by GDP dissociation from subunits ($k = 0.139$ s^{-1}; Zeeberg et al., 1980; Caplow & Shanks, 1990). Guanine nucleotide in tubulin subunits in microtubules does not exchange with nucleotide in solution (Jacobs et al., 1974). The E-site of subunits at microtubule ends is accessible, however, to GTP bound to fluorescent latex beads (Mitchison, 1994); binding was about 25 times better at the plus end.

Our goal was to determine whether nucleotide binding to the E-site of terminal subunits can influence microtubule dynamics and in particular to test whether GTP binding may be significantly weaker to tubulin subunits at microtubule ends than with subunits in solution. This would predict that conditions exist where unpolymerized tubulin subunits are largely saturated with GTP while a significant fraction of subunits at microtubule ends are in the nucleotide-free (apotubulin) state. These conditions would correspond to when the GTP concentrations is equal to the tubulin concentration with these concentrations greater than the K_d for GTP dissociation. For example, with 20 μM tubulin and 20 μM GTP, the equilibrium concentrations of free GTP (and Tu) and TuT are 0.65 and 19.35 μM, respectively. Thus, if the K_d for nucleotide dissociation from terminal subunits is >0.65 μM, the microtubule dynamics would be different for tubulin subunits with a low equilibrium concentration of GTP than with excess GTP, provided that the formation of terminal apotubulin subunits affects the microtubule stability.

We also hoped to determine the effect of GDP exchange into terminal subunits. Although there have been no

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[1] Abbreviations: AP, alkaline phosphatase; BRB80 buffer, 80 mM Na-Pipes, 1 mM $MgCl_2$ and 1 mM EGTA, pH 6.8; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; E-site, exchangeable guanine nucleotide binding site on tubulin; GMPCPP, the GTP analogue guanylyl α,β-methylenediphosphonate; Tu, tubulin free of E-site nucleotide; P_i, inorganic phosphate; TuT, tubulin−GTP subunits; TuD, tubulin−GDP subunits; DIC, differential interference contrast.

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measurements of nucleotide affinity to terminal subunits, the relative affinity of GTP and GDP for this site can be estimated from comparison of the equilibrium constants for addition of TuT and TuD to microtubule ends. This estimation is possible since an identical product is expected to be formed by TuT addition and GTP exchange, and by TuD addition and GDP exchange. Since the thermodynamics for a reaction are independent of path, the relative K_d's for GTP and GDP are determined by the relative affinities of TuT and TuD for microtubule ends. Although the equilibrium constants for TuT and TuD addition to microtubule ends have not been determined, the equilibrium for addition of tubulin−GMPCPP was found to be 132-fold more favorable than for tubulin−GMPCP (Caplow et al., 1994). The terminal subunits therefore appear to have a higher preference for GTP than dimeric tubulin, which binds GTP only about 2.8-fold more tightly than GDP (Zeeberg & Caplow, 1978; Correia et al., 1987). Because terminal subunits better discriminate against GDP than do tubulin subunits, the likelihood of observing an effect of GDP exchange into MT-terminal subunits was dependent on GDP having a appreciable effect on microtubule dynamics, even at relatively low levels of occupancy in terminal subunits.

These predictions have been realized. We have been able to obtain evidence for the formation of apotubulin subunits at the microtubule plus end, and exchange of GDP into terminal subunits at both microtubule ends increases the frequency for the transition from microtubule growth to rapid disassembly (i.e., catastrophe). Furthermore, predictions (Martin et al., 1993) that TuD subunits will differently influence the rate of elongation and the catastrophe frequency at the microtubule's plus and minus ends have been confirmed. There are no prior examples where microtubule dynamics have been found to be altered by systematic modification of the nucleotide composition of microtubule ends.

EXPERIMENTAL PROCEDURES

Materials. Beef brain tubulin was purified by two cycles of thermal-induced assembly and disassembly, followed by chromatography on phosphocellulose (Weingarten et al., 1975). The protein in the void volume fraction from this column was concentrated with an Amicon YM10 membrane. Immediately before use, any protein that denatured during the several hours required for the concentration step or during freezing, storage, and thawing was removed by centrifugation at 13000*g* for 10 min at 4 °C. Concentrations were determined from a molar extinction coefficient equal to 132 000, at 278 nm. Although all of the experiments reported here were obtained with a single batch of tubulin, similar results were obtained for representative experiments with other batches of tubulin. Axonemes were obtained by treating *Tetrahymena* with dibucaine (Thompson et al., 1974; Whitman et al., 1978). HeLa cell kinesin, a gift from V. Lombillo and J. R. McIntosh, was linked to latex beads using the method described previously (Lombillo et al., 1995). All reactions were in BRB80 buffer.

Guanine Nucleotides in Purified Tubulin. It was necessary to identify the amount and composition of the exchangeable (E-site) guanine nucleotides associated with purified tubulin, since these influence the TuT/TuD ratio in reaction mixtures. For these determinations, the GDP moiety in endogenous GDP and GTP (and added GTP, see below) was brought to isotopic equilibrium with a trace amount of added [α-^{32}P]-GTP by reaction with nucleoside diphosphate kinase (Sigma, N2635, 0.017 unit/mL, 20 °C, 30 min). The amount of guanine nucleotide in the purified tubulin was then determined by isotope dilution. In this procedure, a 50 μL aliquot of 40 μM tubulin was chromatographed, with or without the addition of 40 μM GTP, on a 1 mL Sephadex G-25 column, and the amount of bound and free nucleotide was determined from measurements of radiolabel in the void and included volumes. The void peak contained 56.6% of the label in the reaction without added GTP, and 37.5% in the reaction with 40 μM added GTP. Since the amount of label bound to tubulin subunits is proportional to the specific activity of the nucleotide, and the specific activity is inversely proportional to the nucleotide concentration, the endogenous guanine nucleotide contained in purified tubulin (E) is described by the relationship (eq 1):

$$\frac{\text{cpm in void peak (GTP added} = 0)}{\text{cpm in void peak (GTP added} = A)} = \frac{E + A}{E} \quad (1)$$

In this equation, "*A*" corresponds to the concentration of added GTP. Based on the observation that 40 μM added GTP decreased the bound radioactive nucleotide from 56.6 to 37.5% of the total, it is calculated that the 40 μM tubulin was associated with 78.5 μM guanine nucleotide. The 38.5 μM excess nucleotide is presumably derived from the denatured tubulin removed before the protein was used. The remaining soluble tubulin was almost fully active since the stoichiometry for nucleotide binding to subunits was 0.71 (Caplow et al., 1994). Also, as described below, the fraction of nucleotide in unpolymerized tubulin subunits that was resistant to hydrolysis by AP corresponded to a unitary stoichiometry for nucleotide binding.

The composition of the endogenous nucleotide and the relative fraction of endogenous GTP and GDP bound to subunits were similarly determined by adding [α-^{32}P]GTP and nucleoside diphosphate kinase. Aliquots of the unchromatographed protein and the void volume peak were deproteinized with perchloric acid and then chromatographed on a poly(ethylenimine)−cellulose plate. The chromatogram was developed with 1 M P_i, pH 3.4, and the reaction products were quantitatively identified by scanning the plate with an Ambis Radioanalytic Imaging System. The guanine nucleotide was 56% GDP and 44% GTP for a stock solution of tubulin at 100 μM before it had been chromatographed on Sephadex G-25, and 69% GTP and 31% GDP for the tubulin−nucleotide complex obtained in the void volume.

To compare the latter values with theory, it is noted that the concentrations of bound GTP and GDP depend on the dissociation constants and nucleotide concentrations according to eq 2:

$$0 = [Tu_f^3] + (K_T + K_D + [GTP_T] + [GDP_T] - [Tu_T])[Tu_f^2] + (K_T K_D + K_D[GTP_T] + K_T[GDP_T] - [Tu_T]K_D - [Tu_T]K_T)[Tu_f] - [Tu_T]K_T K_D \quad (2)$$

where $[Tu_f]$ is the concentration of nucleotide-free dimeric tubulin; $[Tu_T]$ is the total tubulin subunit concentration, equal to the sum of [TuT], [TuD], and $[Tu_f]$; $[GTP_T]$ is the sum of [TuT] and free [GTP]; $[GDP_T]$ is the sum of [TuD] and free [GDP]; K_T and K_d are the dissociation constants for GTP and GDP (equal to 22 and 62 nM, respectively, for GTP and GDP) (Zeeberg & Caplow, 1978). Equation 2 was

derived from the three conservation equations and the two dissociation constants. The concentrations of GDP and GTP were calculated from the amount of added and endogenous dissociable nucleotide; the latter was calculated based on our observation that the tubulin contained 1.96 mol of nucleotide/mol of tubulin consisting of 56% GDP and 44% GTP. Thus, a stock solution of tubulin at 100 μM contains 86 μM GTP and 110 μM GDP. Based on eq 2, it is expected that this tubulin solution would be 56% TuT and 44% TuD; this is in reasonable agreement with the observed values (69% TuT, 31% TuD).

Some of our studies focused on the effect of TuD and of excess GDP on microtubule dynamics, and it was therefore necessary to identify the bound nucleotide in tubulin that had been supplemented with excess GDP. The Sephadex G-25 chromatography procedure was used to determine the TuT/TuD ratio in a mixture of 100 μM tubulin to which 144 μM GDP was added. Adding 144 μM GDP to the 100 μM tubulin solution would be predicted to contain 254 μM GDP and 86.4 μM GTP, while eq 2 predicts 40 μM TuT and 60 μM TuD. Indeed, chromatography on Sephadex G-25 showed that the protein in the void peak was 42% TuT and 58% TuD. The 100 μM tubulin/144 μM added-GDP mixture was also analyzed by measuring the nucleotides that were resistant to hydrolysis by added AP and which are presumably bound to tubulin. In this case, 29.5% of the nucleotide, which was 50% GTP and 50% GDP, was resistant to hydrolysis by AP (5.6 units/mL, 10 min, 20 °C). This agrees with the predictions since the 100 μM tubulin/144 μM GDP mixture contains 254 μM GDP and 86 μM GTP, and if the 100 μM tubulin binds 1 mol of nucleotide, then 100/(254 + 86) = 29.4% of the nucleotide would be resistant to AP treatment.

In studies to determine the effect of excess free GDP and GTP on microtubule dynamics, the concentration of free nucleotide associated with tubulin subunits was reduced to the minimum equilibrium concentration by reaction with AP (5.6 units/mL, 10 min, 20 °C). This was done with tubulin in which the appropriate TuT/TuD ratio had been generated by a 5 min incubation at 20 °C following the addition of 100 μM GTP to 50 μM tubulin, or 144 μM GDP to 100 μM tubulin. For kinetic studies, the tubulin/GTP mixture was generally diluted to 20 μM total tubulin, and the tubulin/GDP mixture was diluted to 90 μM. The composition of these diluted mixtures, with and without AP treatment, is given in Table 1.

The rate of AP-catalyzed hydrolysis of free GTP was determined by adding a trace amount of [α-^{32}P] GTP to a mixture of the enzyme (5.6 units/mL) and 50 μM tubulin. Aliquots were quenched in perchloric acid and analyzed as described above. The rate of the AP hydrolysis of nucleotide in TuT and TuD was determined by adding AP to 50 μM tubulin containing a trace amount of freshly prepared [α-^{32}P]-TuT and [α-^{32}P]TuD. The latter materials were prepared by a 5 min incubation of a trace amount of labeled GDP and GTP with 50 μM tubulin, followed by chromatography on Sephadex G-25. The radioactive GDP, GTP, and GMP in aliquots from reaction mixtures was determined by thin-layer chromatography.

Measuring Microtubule Dynamics. Microtubule dynamics were analyzed using video-enhanced DIC microscopy, with the microscope enclosed in a chamber maintained at 37 °C. Microtubules were assembled at the ends of *Tetrahymena* axonemes that bound to a coverslip in a flow cell (Vale, 1991) with an approximately 2.5 μL capacity. This small volume was replaced in about 10 s using filter paper to induce rapid flow of 20 μL of a displacing solution containing tubulin/nucleotide mixtures. Microtubule lengths were measured with the computer-based RTM system (Glicksman et al., 1992), with a catastrophe scored when the video image of a microtubule was seen to decrease by 3 mm (0.4 μm microtubule length) or more.

RESULTS

We wished to obtain evidence for formation of nucleotide-free (apotubulin) subunits from a fraction of the TuT subunits in the cap at microtubule ends and determine whether this influences microtubule dynamics. This required a method for preparing TuT subunits that are associated with a minimum concentration of free GTP.

Preparing TuT and TuD Subunits. With a given amount of tubulin, the minimum concentration of free GTP and/or GDP corresponds to the equilibrium concentration in an equimolar mixture of tubulin and nucleotide. This equilibrium-free nucleotide concentration depends on K_d and the concentrations of TuD and TuT. For example, based on a K_d equal to 22 nM for GTP (Zeeberg & Caplow, 1979), the calculated equilibrium concentration of free GTP equals 0.46 and 1.47 μM for 10 and 100 μM TuT, respectively. With GDP, K_d is equal to 61 nM (Zeeberg & Caplow, 1979), so that the minimum (equilibrium) concentrations of free GDP are 0.75 and 2.44 μM for 10 and 100 μM TuD, respectively.

Purified tubulin contains both GTP and GDP (see above), so that it was necessary to add excess GTP or GDP to the protein to generate a solution containing predominantly TuT or TuD subunits. This excess nucleotide had to be removed to obtain subunits with a minimum equilibrium concentration of free nucleotide. An attempt to remove excess GTP or GDP from tubulin by Sephadex G-25 chromatography was not successful, since it was not possible to use a sufficiently large column to get the requisite resolution of free and bound nucleotide without diluting the tubulin below the concentration required for our studies of microtubule dynamics. We were able to obtain TuT and TuD subunits associated with a minimum equilibrium concentration of free nucleotide by reacting mixtures of tubulin and excess GTP and/or GDP with AP. The rationale for this approach is based on our observation (Brylawski & Caplow, 1983; Caplow et al., 1984) that enzymes that react rapidly with free GTP or GDP react very slowly with tubulin-bound nucleotides. This slow rate results because at modest concentrations of nucleotide-consuming enzyme the free GDP or GTP formed by dissociation from tubulin is more likely to rebind to the approximately micromolar equilibrium concentration of nucleotide-free tubulin (equal to the free nucleotide concentration), than react with the nucleotide-consuming enzyme, i.e.:

$$\mathrm{TuT} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{apotubulin} + \mathrm{GTP} \xrightarrow{k_2(\mathrm{AP})} \mathrm{P_i} + \mathrm{GDP} \quad (3)$$

while the hydrolysis rate is described by

$$\text{rate} = k_2(\mathrm{AP})[\mathrm{TuT}]k_1/[k_{-1}(\text{apotubulin}) + k_2(\mathrm{AP})] \quad (4)$$

At concentrations of AP where k_{-1}(apotubulin) > k_2(AP):

$$\text{rate} = k_2(\mathrm{AP})[\mathrm{TuT}]k_1/k_{-1}(\text{apotubulin}) \quad (5)$$

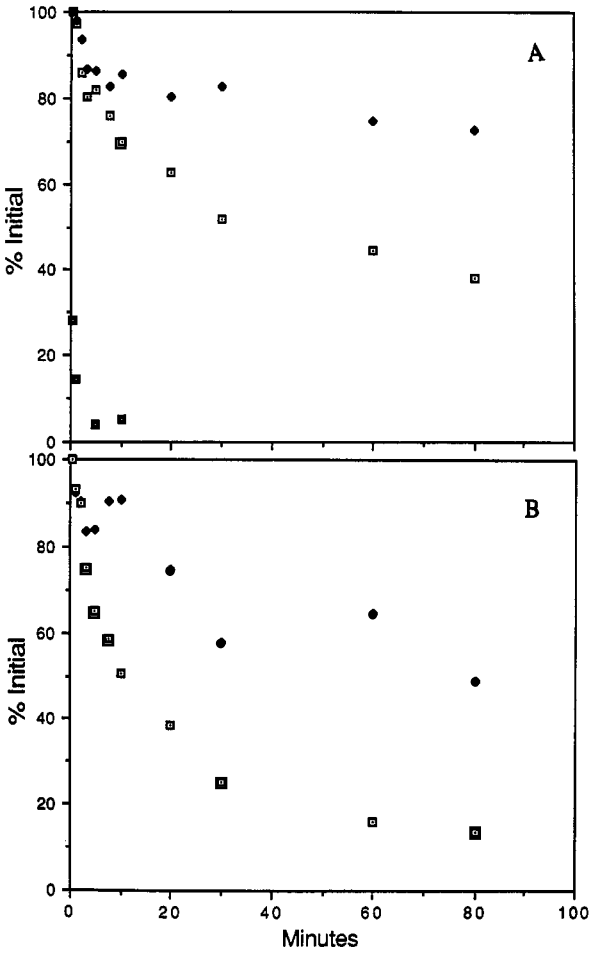

FIGURE 1: Alkaline phosphatase hydrolysis of free and tubulin-bound nucleotides. In panel A, the concentration of AP was 5.6 units/mL, and the hydrolysis rate was determined after a trace amount of [α-^{32}P]GTP was added to a mixture of 50 μM tubulin and AP (□), a mixture of AP and [^{32}P]TuT (♦), and a mixture of AP and [^{32}P]TuD (⊡). The reaction of AP at 22.4 units/mL with [^{32}P]TuT (♦) and [^{32}P]TuD (⊡) is shown in panel B.

The rate is slow because the k_1/k_{-1}(apotubulin) term in eq 5 [equal to K_d/[apotubulin]] is <1 under conditions where GTP binding to tubulin is favorable; for example, K_d/[apotubulin] = 22 nM/1038 nM with 50 μM TuT.

AP-catalyzed hydrolysis of GTP and GDP in TuT and TuD was found to be slow, compared to free GTP, for which the rate is equal to k_2(AP) (Figure 1A). The validity of the assignment k_{-1}(apotubulin) > k_2(AP) was tested by measuring the rate of hydrolysis of GTP in TuT, as a function of the AP concentration (Figure 1A,B). The observed dependence of the rate on the AP concentration is predicted by eq 4 for the case where k_{-1}(apotubulin) > k_2(AP); the rate will be independent of the AP concentration if the reverse holds.

Although the half-time for hydrolysis of excess free GTP was less than 1 min (Figure 1), there was relatively little hydrolysis of tubulin-bound GTP in 80 min. The observed more rapid hydrolysis of GDP bound in TuD is expected since k_1 is about 5 times greater for TuD than for TuT (Brylawski & Caplow, 1983; Caplow et al., 1984) and since eq 4 predicts a dependence of the hydrolysis rate on k_1. The important point from Figure 1 is that it is possible to remove rapidly excess free GTP and GDP with relatively slow concomitant hydrolysis of nucleotide bound to tubulin. Thus, during the few minutes required for each of the kinetic studies described below, the TuD and TuT concentrations remained approximately constant while the free nucleotide concentration remained low.

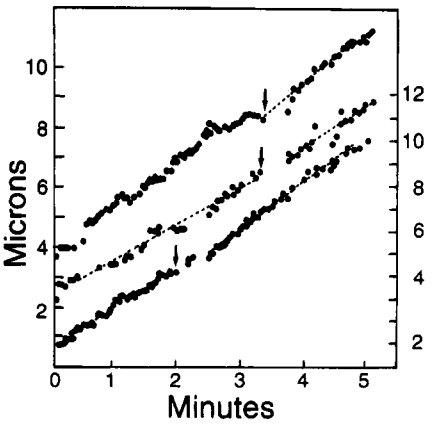

FIGURE 2: Effect of AP treatment on assembly properties of tubulin. Axonemes were elongated with 20 μM tubulin, to which 50 mM P_i and 100 μM GTP were added after the protein had been treated with AP. The length of the same microtubules was measured before and after AP-treated tubulin was replaced (indicated by arrow) with untreated 20 μM tubulin and 40 μM GTP. The lowest curve is represented by the right-hand ordinate. The ratio of the rates with enzyme-treated and untreated tubulin was 0.99 (n = 10, SD 0.34).

Table 1: Effects of Excess Nucleotide and TuD on Microtubule Dynamics[a]

reaction mixture	AP treated	[TuT] (μM)	[free GTP] (μM)	[TuD] (μM)	[free GDP] (μM)
A	−	17.1	40.2	2.9	19.1
B[b]	+	16.6	0.5	2.7	0.22
C	−	36.2	41.8	53.8	175.2
D[b,c]	+	35.8	0.39	52.2	1.61

[a] Concentrations were calculated from eq 2, using the nominal tubulin concentration, without correction for the observed stoichiometry (equal to 0.71) for bound nucleotide. This convention, which allows comparison with work from other labs, is without effect on the conclusions derived, since these were from experiments under the same conditions, except for the concentration of excess of free nucleotide. [b] For mixtures B and D, it was assumed that AP rapidly hydrolyzes excess free GDP and GTP, and an equilibrium is then established between free and bound GTP and GDP. The concentration of free nucleotide probably strays a bit below the equilibrium level as a result of the AP digestion, since the k_1 reaction in eq 3 may not be rapid enough to maintain the equilibrium concentration. Although the AP will eventually hydrolyze all nucleotide, these concentrations hold during the very brief period during which microtubule dynamics were studied. [c] The sum of [TuT] and [TuD] is equal to 90 μM in mixture C and only 88 μM in mixture D because the concentration of nucleotide-free subunits is only 0.019 μM in mixture C and 2 μM in mixture D.

Effect of a Low Concentration of Free GTP on Microtubule Dynamics. AP, which was used to remove excess nucleotide from tubulin, does not induce irreversible effects on its assembly properties, since the elongation rate was 1.48 μm/min (n = 10, SD 0.35) with tubulin in which the action of AP was stopped by addition of 50 mM P_i and excess GTP, compared with 1.32 μm/min (n =10, SD 0.39) with tubulin that had not been treated with AP (Figure 2). In this comparison, the elongation rate of the same microtubules was determined for enzyme-treated and untreated tubulin. Such a comparison was important, since there is considerable heterogeneity in the rate of elongation of individual microtubules (O'Brien et al., 1990; Gildersleeve et al., 1992).

We were surprised to find (Figure 3A) that only one axoneme end was elongated by 16.6 μM TuT containing approximately 0.5 μM free GTP (mixture B, Table 1). In contrast, a significant fraction of axonemes was elongated

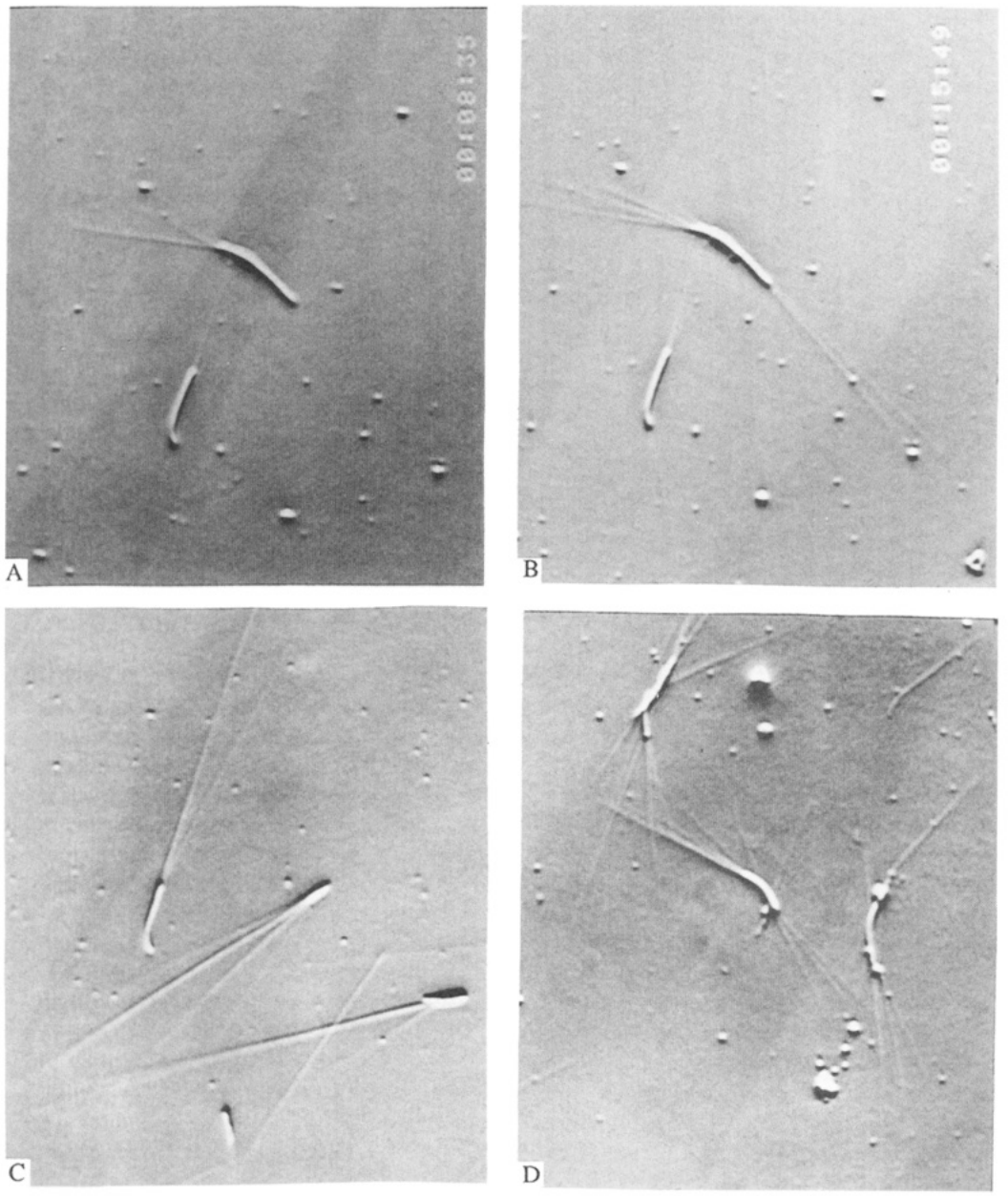

FIGURE 3: Effect of low free GTP concentration on the elongation of microtubules. Axonemes were elongated with 20 (mixture B), 30, or 40 μM AP-treated tubulin containing 16.6 (A), 24.9 (C), or 33.2 μM TuT (D). (B) shows the same axoneme as in (A), except after a 9 min reaction with mixture B (Table 1) the axoneme was reacted for an additional 7 min with a mixture of 20 μM AP-treated tubulin, 50 mM P_i, and 100 μM GTP.

at both ends with 10 μM tubulin and 1 mM GTP (data not shown). This agrees with published results showing that both microtubule ends can be elongated by tubulin at concentrations greater than 7 μM in the presence of excess GTP (Walker et al., 1988). The assembly, however, was only at one end with 20 μM tubulin (mixture B) (Figure 3A) or with 30 μM tubulin (Figure 3C), while both ends could be elongated at 40 μM tubulin (Figure 3D). As described below, this is an important observation since it suggests the existence of a kinetic barrier for formation of apotubulin at microtubule ends.

The lack of assembly at one end in mixture B apparently resulted from the low free GTP concentration since both axoneme ends were elongated when P_i, to stop the action of AP, and excess GTP were added (Figure 3B). We could show that the microtubules that are growing from one axoneme end are unstable rather than slow-growing by exposing axonemes which had been elongated at both ends with 20 μM TuT/40 μM GTP to mixture B. Microtubules at only one end underwent catastrophe, and this occurred within about 19 s (k_c = 0.05 s^{-1}, Table 2). This lifetime before catastrophe may be overestimated because of the following: (1) The time did not include the approximately 10 s required to refocus after adding the new tubulin mixture. (2) A microtubule seen to be shortening when the field was first observed was assumed to have undergone catastrophe when the microtubule was first seen, rather than when the flow was initiated. (3) Results were ignored where microtubules were entirely lost from the end before the axoneme could be viewed, since it is possible that this resulted from breakage of the microtubules from the end, rather than by progressive disassembly.

To determine further the impact of the low concentration of GTP in mixture B, a control reaction run with the same batch of tubulin (at 15 μM) with excess GTP (100 μM) and without AP treatment was used to challenge axonemes that had been elongated at both ends with 20 μM TuT/40 μM GTP. The microtubules at both ends were relatively stable

Table 2: Effect of Nucleotide Concentration on the Elongation and Catastrophe Rate

conditions	mixture[a]	end elongation rate (μm/min) (n, SD)	catastrophe events/s k_c[b] (s^{-1})	range[c]
20 μM TuT/100 μM GTP		− 1.1 (12, 0.3)		
		+ 1.9 (9, 0.4)		
15 μM TuT/40 μM GTP	A	+	6/4500, 0.0013	0.0005/0.0029
16 μM TuT/0.5 μM GTP	B	+	15/277, 0.05	0.03/0.09
90 μM TuT + TuD/excess GDP	C	+ not observed	0.05[d]	
		− 0.72 (16, 0.18)	40/7847, 0.005	0.004/0.007
90 μM TuT + TuD/low GDP[e]	D	+ 1.0 (7, 0.2)	7/3343, 0.0036	0.0008/0.0043
		− 3.5 (12, 0.4)	1/2100, 0.0005	0.0001/0.0026

[a] The composition of reaction mixtures is given in Table 1. [b] Calculated from the ratio catastrophe events/cumulative seconds of microtubule elongation. [c] The lower/upper mean calculated for a confidence limit of 95%, assuming a Poisson distribution of rates (Johnson & Kotz, 1969). [d] Because elongation at this end was not seen, the rate was determined by treating axonemes that had been elongated with TuT and excess GTP with mixture C. Approximately 50 catastrophes were observed, and these were concomitant with or shortly after flow. [e] Rates were determined as described in the legend for Figure 5. A comparable rate for the minus end was determined in a reaction in which axonemes were first elongated with mixture C and then with mixture D (Figure 6).

and had a catastrophe frequency of only about 0.0013 s^{-1} (Table 2) at the more rapidly growing (presumably plus) end, which contrasts with the plus end stability with mixture B.

To identify which microtubule end is unstable in the presence of a low free GTP concentration, studies were done with axonemes whose polarity had been determined by observation of the direction of movement of kinesin-linked latex beads. Microtubules at the axoneme's plus end rapidly disassembled in the mixture containing 16.6 μM TuT and 0.5 μM GTP (Figure 4).

Effect of Excess Free GDP on the Plus End Catastrophe Rate. We prepared a mixture of TuT and TuD with (mixture C, Table 1) and without excess free GDP (mixture D, Table 1) to determine whether binding of GDP from solution to subunits at microtubule ends influences microtubule dynamics. Since the TuT and TuD concentrations are virtually identical in mixtures C and D, differences in microtubule dynamics in these two mixture will reflect binding of free nucleotide to terminal subunits.

Excess free GDP and TuD in mixture C had a dramatic effect on microtubule dynamics: axoneme elongation was slow and occurred at only one end. The latter observation might have resulted because there is a kinetic barrier for initiating microtubule growth on axonemes (Walker et al., 1988). We tested this hypothesis by treating axonemes that had been bidirectionally elongated in a reaction with 20 μM TuT/40 μM GTP, with mixture C. Microtubules at one end of the axoneme underwent almost immediate catastrophe, while the other end continued to grow. The catastrophe rate was at least as fast as described for reactions with low free GTP (mixture B; 0.05 s^{-1}), but as described above, it is difficult to estimate the catastrophe rate when this event is almost concomitant with the flow of the new tubulin solution into the observation chamber.

Evidence that free GDP is responsible for the high catastrophe frequency in mixture C was obtained by measuring this rate with mixture D, in which the free GDP concentration had been reduced to 1.6 μM. Under these conditions, the catastrophe frequency was only 0.0036 s^{-1} (Table 2) at the less stable, slower-growing plus end. Evidence that it is the plus end that is unstable in the presence of excess GDP (mixture C) was obtained in experiments with axonemes whose polarity had been determined with kinesin-linked latex beads (data not shown). The lower catastrophe frequency at the plus end with mixture D allowed bidirectional elongation of about 15% of axonemes in 10 min; a smaller fraction of axonemes was elongated at both ends when mixture D was diluted to 50 μM total tubulin, and only unidirectional elongation was seen with 37.5 μM total tubulin.

Effect of TuD and Excess GDP on Minus End Elongation and Catastrophe Rates and Effect of TuD on the Plus End Elongation Rate. Mixture C contains TuD and excess free GDP, both of which can be expected to inhibit elongation by the TuT in this mixture. The combined inhibitory effect of TuD and GDP was determined by comparing the elongation rate with mixture C with that with a mixture containing TuT and excess free GTP. The separate inhibitory effect of excess free GDP was determined by comparing the rate with mixture C with that with mixture D in which excess GDP had been removed by treatment with AP.

Minus End: Although the plus end is unstable in mixture C, the minus end was slowly elongated in this mix. The combined inhibitory effect of TuD and excess free GDP resulted in a rate of only 0.72 μm/min (Table 2). It was not possible to measure the rate at other dilutions of mixture C and therefore not possible to determine the second-order rate constant for elongation. Even a 2-fold dilution to 50 μM total tubulin was not possible since the elongation rate was reduced while the mean microtubule lifetime of only about 120 s before catastrophe precluded accurate measurements. The inhibitory effect of TuD and excess free GDP in mixture C was apparent by comparing the elongation rate (0.72 μm/min) with that for a mixture of TuT and excess GTP: the minus and plus end rates were 1.1 and 1.9 μm/min, respectively, with 20 μm tubulin and 100 μm GTP (Table 2). Assuming a linear dependence of the rate on the TuT subunit concentration, the predicted rate for elongation of the minus end with the 36 μM TuT in mixture C is 2.0 μm/min, which is almost 3 times the value observed.

As described above, the separate inhibitory effects of GDP in mixture C could be determined by comparing dynamics in mixtures C and D. Excess free GDP in mixture C has a major effect on the rate of elongation of the microtubule minus end: the rate of elongation in mixture D was 3.5 μm/min (Table 2, Figure 6), compared to only 0.72 μm/min in mixture C. In addition to the almost 5-fold slower rate in mixture C, elongation of the minus end with this mixture was punctuated by frequent catastrophe (Figure 7), apparently because of the excess free GDP. The participation of excess free GDP in inducing catastrophe was indicated by the reduction of k_c from 0.005 s^{-1} with 175 μM excess GDP (mixture C; Table 2) to about 0.0005 s^{-1} with 1.6 μM excess

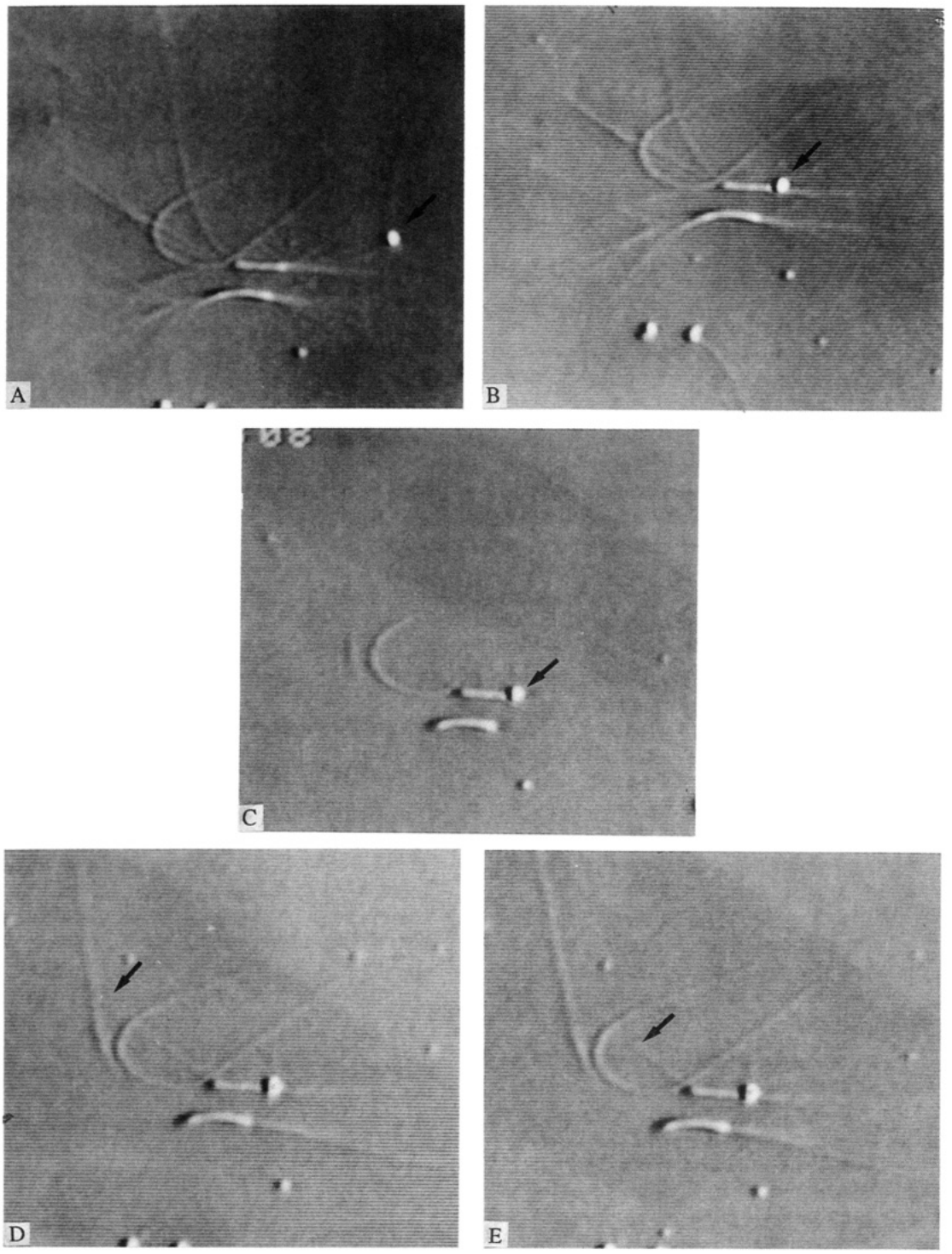

FIGURE 4: Assignment of the polarity of the microtubule end that is unstable in the presence of a low free GTP concentration. A kinesin-coated microsphere (arrow) moved on a microtubule (A) and stopped at the axoneme (B), so that the right-hand side of the axoneme is the minus end. Microtubules and excess beads were removed by treatment with buffer (C), and after microtubules had been regrown with 20 μM TuT and 40 μM excess GTP, they were reacted with 16.6 μM TuT with 0.5 μM free GTP. Photos taken after 156 (D) and 164 (E) s are shown; the microtubule whose end is identified with an arrow disassembled to completion shortly after the second photograph was made. Note that the curved microtubule on the left of the axoneme is irrelevant. Microtubules were also seen to disassemble from only one end of the axoneme below the one analyzed here, but since we had not observed the movement of the bead at the end of the axoneme, the polarity was not established.

GDP (mixture D; Table 2). It is noted that the 0.005 s^{-1} catastrophe frequency with excess GDP corresponds to growth periods with a mean length 1/0.005 = 200 s, which would suggest that there would be very little microtubule elongation under these conditions. However, the minus end rescue frequency was very high (33 rescues/52 s rapid disassembly = 0.6 s^{-1}), so that the mean duration for rapid shortening was only 1.6 s. As a result, despite the high catastrophe frequency, it was possible to generate long microtubules at the minus end, even though this end grew relatively slowly under these conditions (see the early time points in Figure 6).

Plus End. Elongation of the plus end was more sensitive than the minus end to inhibition by TuD subunits. As seen in the representative results in Figure 5, the minus end elongated faster (3.5 μm/min) than the plus end (1.0 μm/

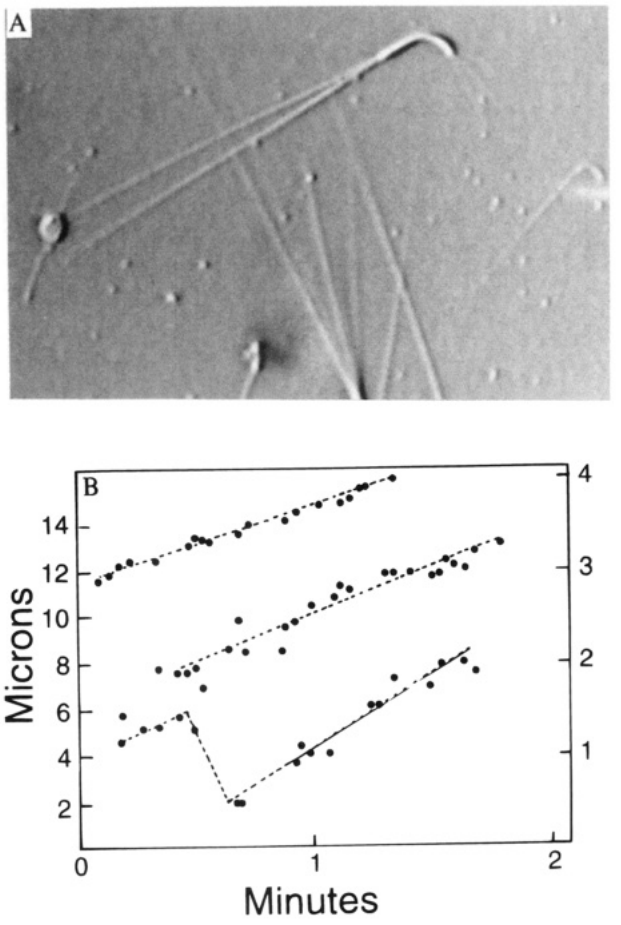

FIGURE 5: Effect of TuD on the rate of elongation of the microtubule plus and minus ends. Axonemes were elongated with mixture D, which contains TuT and TuD, with a minimum concentration of free GDP. Under conditions where virtually all axonemes were elongated at one end, about 15% of the axonemes were elongated at both ends (see A). The growth rate of these bidirectionally elongated axonemes was measured (see B). The minus end was identified by the near-identity of its elongation rate (3.5 μm/min) with that for the minus end in the experiment described in Figure 6 (2.5 μm/min). Also, in a limited number of reactions the axoneme plus end was identified by the much higher catastrophe frequency for microtubules grown at this end with 15 μM TuT and 30 μM GTP. The opposite end was elongated at a rate equal to 2.8 um/min in a reaction with mixture D. The right-hand ordinate describes the kinetics for elongation of two minus end microtubules (lowest and middle curve), and the left-hand ordinate is for the plus end microtubule described in the top curve.

min, Table 2) in mixture D. The plus end ordinarily elongates faster than the minus end (Walker et al., 1988).

DISCUSSION

We have determined the effects of GDP and GTP on microtubule dynamics that result from binding of nucleotide to microtubule ends, rather than to dimeric tubulin subunits. This was made possible by our observation that free GTP and GDP can be reduced to very low concentrations by reaction with AP, with only slow changes in the concentration of subunit-bound nucleotide. Since the free nucleotide concentration could be decreased independent of any significant changes in the concentration of TuT or TuD subunits, any observed effects of free nucleotide concentration on microtubule dynamics must result from effects on microtubule ends.

It was important in these studies to demonstrate that effects of AP-induced depletion of free nucleotide did not result from

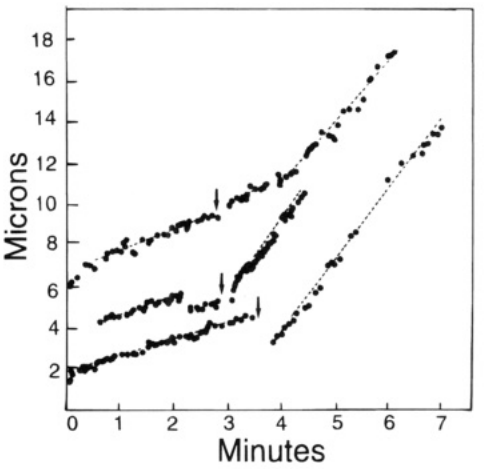

FIGURE 6: Effect of excess free GDP on the rate of elongation of the microtubule minus end. Microtubules were first elongated with mixture C and after the time indicated by the arrow, by mixture D. The rate was 0.67 μm/min (n = 8, SD 0.27) in mixture C and 2.5 μm/min (n = 8, SD 1.0) with mixture D.

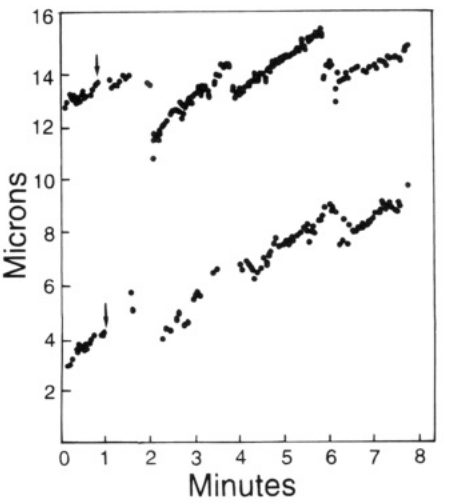

FIGURE 7: Microtubule elongation with a mixture of TuT and TuD and excess GDP is associated with a high catastrophe and rescue frequency. The rate of elongation of individual microtubules was measured in the presence of 20 μM TuT and 40 μM GTP (mixture A) and after addition (arrow) of mixture C. That the rate is for the minus end is indicated since only this end is stable in mixture C.

effects of the enzyme on tubulin subunits. AP treatment does not induce irreversible changes in tubulin since the microtubule elongation rates were indistinguishable for untreated tubulin subunits and for AP-treated tubulin subunits, where the action of AP was inhibited by P_i and excess GTP was added (Figure 2). Further evidence that free nucleotide and not tubulin is the target for AP was also provided by the observation that AP treatment could both inhibit and promote microtubule assembly, by reducing the concentration of free GTP (Figure 3) and by reducing the concentration of free GDP (Figure 6). If tubulin were the target, then either inhibition or promotion would probably be observed.

The dynamic properties of both microtubule ends were found to be sensitive to the solution nucleotide composition, presumably because nucleotide is able to exchange into the β subunit at both ends. However, while high concentrations of free GDP inhibited elongation at both ends (Table 2), only the plus end was sensitive to low concentrations of GTP. The rate of elongation at the plus end was also more sensitive to inhibition by TuD. Results are summarized in Table 2 and discussed next.

(1) Formation of Apotubulin. The instability of the microtubule plus end in the presence of a low free GTP concentration can be accounted for if (a) nucleotide-free (apotubulin) subunits form at this end and (b) apotubulin has similar low affinity for ends as terminal TuD subunits. With regard to (a), formation of apotubulin subunits at microtubule ends is expected, since this is an intermediate in binding of GTP linked to latex beads (Mitchison, 1993). Our observation that the plus end is unstable in the presence of the 0.5 μM GTP that accompanies the 16.6 μM TuT (mixture B) is consistent with a K_d for terminal subunits that is in the micromolar range, since this would allow accumulation of apotubulin subunits in mixture B. With regard to (b), that apotubulin subunits have similar low affinity for ends as TuD subunits, this is consistent with a mechanism in which the γ-phosphate moiety of GTP makes an important contribution to the stability of TuT subunits in microtubules; this moiety is absent in both TuD and apotubulin subunits. It is not known what fraction of terminal subunits at the plus end must be in the form of apotubulin to induce rapid disassembly; this also applies for TuD subunits in a terminal TuT cap. Also, as with TuD formation at ends from TuT, there is a kinetic barrier for formation of apotubulin within the TuT cap. If there were no kinetic barrier, so that the nucleotide composition of ends remained constant irrespective of the growth rate, then no elongation of the plus end would have been observed when the GTP concentration was constant at the minimum equilibrium level while the concentration of TuT was increased to 40 μM (see Figure 3D).

Our conclusion that apotubulin subunits destabilize microtubules is of interest, since it suggests a route for regulating microtubule stability. Ends could be destabilized by a protein that binds to and stabilizes the transition state for forming apotubulin from TuT, and also binds relatively tightly to the resultant apotubulin product. The resultant accumulation at ends of catalyst-bound apotubulin subunits could be expected to induce the phase transition to rapid disassembly.

(2) Exchange of GDP into Terminal Subunits. Our finding that the microtubule plus end is not stable when the free GDP concentration is high (mixture C) is consistent with a mechanism in which GDP exchange into terminal subunits destabilizes the end. The mechanism for this destabilization would require, however, that the terminal subunits not be in equilibrium with free GDP and GTP. If rates of nucleotide equilibration were rapid so that kinetic factors did not influence the composition of ends, then microtubule dynamics would be identical with tubulin mixtures C and D since both have a free GDP/free GTP ratio of 4.2; this is not the case. The factors that apparently influence the composition and behavior of ends are the relative concentrations of TuT and free GDP. How these concentrations may influence microtubule dynamics is described next.

The lower stability of the plus end at high GDP concentrations (at a fixed free GDP/free GTP ratio) can be accounted for by assuming that under the reaction conditions used here three things may happen when GTP dissociates from terminal subunits to generate apotubulin subunits: (1) catastrophe; (2) GDP binding, which is presumed to either increase the likelihood for catastrophe and/or decrease the rate of addition of TuT subunits (rescue); (3) TuT subunit addition (rescue). Evidence for reaction 3 comes from our observation that high concentrations of TuT subunits prevent catastrophe under conditions where microtubules are apparently destabilized by formation of apotubulin subunit formation (compare Figure 3A and Figure 3D). We propose that microtubule ends are stabilized by reducing the GDP concentration to micromolar levels (mixture D) because this allows the rate for reaction 3 with micromolar concentrations of TuT to compete with reaction 2. This scheme requires that addition of free GDP to the microtubule by reaction 2 reduces the stability of the end compared to an end with apotubulin, because formation of TuD at ends increases catastrophe and/or decreases rescue.

Our observation that excess free GDP increases the catastrophe frequency at both microtubule ends (Table 2) does not conflict with the fact that GTP linked to latex beads binds 25 times better to the microtubule plus end (Mitchison, 1993). This better binding was presumed to result because the plus end has the E-site-containing β subunit at the microtubule's interface with solvent (Mitchison, 1993), so that there is little steric hindrance for binding. Recent work has suggested that the α subunit lies at the interface with solvent at the plus end (Song & Mandelkow, 1995). Irrespective of where the β subunit is located, the steric requirements for binding free GTP are expected to be less than for binding GTP linked to a microsphere, so that the two ends are likely to show less discrimination for binding of free nucleotide than for GTP linked to a bead.

Because the GTP/GDP ratio in cells is ordinarily very high, it is unlikely that GDP exchange into terminal subunits is important in regulating microtubule stability in cells. However, our finding that microtubule ends are destabilized when these contain GDP suggests that a GTPase activating protein (GAP) directed at terminal TuT subunits could be expected to induce the phase transition from growth to rapid disassembly.

(3) Inhibition by TuD. The effectiveness of TuD as an inhibitor for elongation of the microtubule plus end is indicated by our observation that the rate of growth at this end in mixture D is less than at the ordinarily slower-growing minus end (Table 2). However, we cannot rule out the possibility that this may actually result from the 1.6 μM residual free GDP present after AP treatment, since it is not possible to study reactions of TuD in the total absence of free GDP.

TuD presumably inhibits microtubule elongation by TuT subunits by reacting with the microtubule end (Caplow & Reid, 1985). Although the position of the equilibrium is expected to be unfavorable for net elongation of microtubule ends with the 53.8 μM TuD in mixture D, TuD may inhibit by transiently adding to a cap of TuT subunits at MT ends. Evidence for inhibition resulting from TuD subunit binding to TuT subunits at microtubule ends was recently obtained in studies in which the total tubulin subunit concentration was kept constant at 14 or 17 μM, and the fraction of subunits as TuD was increased from 0 to 55% (Vandecandelaere et al., 1995). The rate was decreased by increasing the fraction of subunits as TuD, presumably by an effect other than that resulting from a decrease in the concentration of TuT. It is especially interesting that both our work and this previous study show that the inhibition by TuD is greater at the faster growing end, and that this behavior had been predicted from the lateral cap model (Martin et al., 1993). In this model, the affinity of TuD for microtubule ends was predicted to be 20% that of TuT at the β subunit-out end and only about 1% that of TuT at the α subunit-out end. Addition of TuD

to ends would both inhibit subsequent addition on TuT, thus reducing the elongation rate, and induce hydrolysis of GTP in subjacent TuT subunits in the microtubule cap, thereby reducing the lifetime of the cap. Evidence for the predicted increase in microtubule dynamics in the presence of TuD was obtained (Vandecandelaere et al., 1995), but the effect of TuD on the catastrophe frequency could not be measured. Our observation that TuD increases the plus end catastrophe frequency is therefore in accord with the predictions of the lateral cap model (Martin et al., 1993).

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