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Evidence that a Single Monolayer Tubulin-GTP Cap Is Both Necessary and Sufficient to Stabilize Microtubules

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Evidence that 13 or 14 contiguous tubulin-GTP subunits are sufficient to cap and stabilize a microtubule end and that loss of only one of these subunits results in the transition to rapid disassembly (catastrophe) was obtained using the slowly hydrolyzable GTP analogue guanylyl-(a,b)-methylene-diphosphonate (GMPCPP). The minus end of microtubules assembled with GTP was transiently stabilized against dilution-induced disassembly by reaction with tubulin-GMPCPP subunits for a time sufficient to cap the end with an average of 40 subunits. The minimum size of a tubulin-GMPCPP cap sufficient to prevent disassembly was estimated from an observed 25- to 2000-s lifetime of the GMPCPP-stabilized microtubules following dilution with buffer and from the time required for loss of a single tubulin-GMPCPP subunit from the microtubule end (found to be 15 s). Rather than assuming that the 25- to 2000-s dispersion in cap lifetime results from an unlikely 80-fold range in the number of tubulin-GMPCPP subunits added in the 25-s incubation, it is proposed that this results because the minimum stable cap contains 13 or 14 tubulin-GMPCPP subunits. As a consequence, a microtubule capped with 13–14 tubulin-GMPCPP subunits switches to disassembly after only one dissociation event (in about 15 s), whereas the time required for catastrophe of a microtubule with only six times as many subunits (84 subunits) corresponds to 71 dissociation events (84–13). The minimum size of a tubulin-GMPCPP cap sufficient to prevent disassembly was also estimated with microtubules in which a GMPCPP-cap was formed by allowing chance to result in the accumulation of multiple contiguous tubulin-GMPCPP subunits at the end, during the disassembly of microtubules containing both GDP and GMPCPP. Our observation that the disassembly rate was inhibited in proportion to the 13–14th power of the fraction of subunits containing GMPCPP again suggests that a minimum cap contains 13–14 tubulin-GMPCPP subunits. A remeasurement of the rate constant for dissociation of a tubulin-GMPCPP subunit from the plus-end of GMPCPP microtubules, now found to be 0.118 s^{-1}, has allowed a better estimate of the standard free energy for hydrolysis of GMPCPP in a microtubule and release of Pi: this is +0.7 kcal/mol, rather than −0.9 kcal/mol, as previously reported.

INTRODUCTION

Dynamic instability is the property by which microtubules undergo periodic transitions between relatively long periods of growth and short periods of rapid disassembly (Mitchison and Kirschner, 1984). It is believed to be important in generating a functional microtubule network in cells. There is special interest in the mechanism for the transition from growth to rapid disassembly, termed catastrophe, and the reverse transition from rapid disassembly to growth, termed rescue, because the rates for these processes are apparently regulated during the cell cycle (Belmont *et al.*, 1990; Verde *et al.*, 1990, 1992; Glicksman *et al.*, 1992).

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Understanding of these processes will be enhanced by better characterization of the GTP cap that is presumed to transiently stabilize microtubule ends.

Little is known about the GTP cap, except that estimates of its size have progressively decreased as workers have been frustrated in obtaining direct evidence for its presence (Caplow, 1992). The current model for the cap is one in which a monolayer of tubulin-GTP subunits covers the microtubule's 13 or 14 protofilaments (Bayley *et al.*, 1990; Erickson and O'Brien, 1992; Martin *et al.*, 1993; Drechsel and Kirschner, 1994). Longer caps are presumably precluded because GTP hydrolysis is induced in cap-subunits when these become internal by subunit addition. This mechanism for limiting the depth of the GTP cap does require, however, existence of two different routes for inducing GTP hydrolysis at the two microtubule ends (Erickson and O'Brien, 1992). Because the GTP-containing β subunit is at the solvent interface at one end and already interfacing another subunit at the other end, the impact of subunit addition is not expected to be the same at the two ends. Although ockham's razor speaks against a duality of mechanisms for inducing hydrolysis, the induced-hydrolysis model cannot be ruled out.

Evidence against this model was presumably obtained in a study of the GTP hydrolysis that accompanies reaction of tubulin-GTP subunits with covalently cross-linked microtubule seeds (Caplow and Shanks, 1990). If hydrolysis is induced in tubulin-GTP subunits in a monolayer cap when these are the site for tubulin-GTP subunit addition, the GTPase rate is expected to be proportional to the square of the concentration of tubulin-GTP subunits. The rate would be second-order with respect to tubulin-GTP subunits because increases in this concentration would increase both the fraction of seeds that are capped with tubulin-GTP subunits and the rate of reaction of tubulin-GTP subunits with the cap. Although the observed first-order dependence of the GTPase rate on the tubulin-GTP subunit concentration would appear to rule out the induced-hydrolysis model (Caplow and Shanks, 1990), more recent estimates of the equilibrium constant for addition of tubulin-GTP subunits to microtubule ends (Caplow *et al.*, 1994; Hyman *et al.*, 1992) suggest that at the tubulin-GTP concentrations used the equilibrium was favorable for cap formation on the seeds. With the fraction of seed ends having a cap near 1, the GTPase rate would be expected to have the observed first-order dependence on the tubulin-GTP subunit concentration.

The size of the GTP cap sufficient to stabilize microtubules was recently estimated by measuring the number of tubulin-GMPCPP subunits sufficient to stabilize microtubules against dilution-induced disassembly (Drechsel and Kirschner, 1994). Under conditions where an average of 53 subunits was added to the plus-ends of microtubule seeds, only 10% of microtubules were stabilized against dilution-induced disassembly. Although the small fraction of microtubules that were stabilized by addition of 53 subunits suggests that the minimum stabilizing cap must be very large, we present evidence here that the majority of microtubules that were analyzed (Drechsel and Kirschner, 1994) were at the more slowly growing minus-end of the microtubule seed. Therefore, fewer than 53 subunits were added under conditions where 10% of the microtubule population was stabilized. The size of the minimum GTP cap was also estimated using fluorescent tubulin-GMPCPP subunits (Drechsel and Kirschner, 1994), comparing the very weak fluorescence from microtubules that were stable to dilution, with that from microtubules containing 1500–6000 fluorescent subunits. From this it was estimated that the minimum cap consisted of a single tubulin-GTP subunit at the end of each of the microtubule's 13 or 14 protofilaments.

We were especially interested in the proposal (Drechsel and Kirschner, 1994) that stabilization provided by a 13–14 subunit cap might be lost by reduction of the cap length by a single or very few subunits. This model parallels one previously considered by Martin *et al.* (1993), who proposed that exaggerated cooperativity in loss of the GTP cap results from the greater affinity of tubulin-GTP subunits for sites with a greater proportion of surrounding tubulin-GTP subunits.

We report here studies of the minimum size of the tubulin-GMPCPP cap sufficient to stabilize microtubule ends. GTP-microtubules were briefly treated with tubulin-GMPCPP subunits to generate a cap at the microtubule end, and video microscopy was used to determine the delay before onset of rapid disassembly after a subsequent dilution into buffer. The dispersion in the delay time was consistent with a model in which catastrophe is induced in a stable 13–14 tubulin-GMPCPP subunit cap when this loses a single subunit. A similar mechanism is suggested by our finding that the rate of disassembly of the plus-end of microtubules containing both tubulin-GDP and tubulin-GMPCPP subunits is inhibited in proportion to the 13–14th power of the fraction of subunits containing GMPCPP.

MATERIALS AND METHODS

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 contained 1.08 mol exchangeable guanine nucleotide/mol tubulin, determined by an isotope dilution procedure (Caplow *et al.*, 1994). The 0.08 mol of excess free nucleotide was probably derived from tubulin that denatured during freezing, storage, and thawing; this denatured protein was removed by centrifugation (13,000 × *g*, 10 min, 4°C)

immediately before kinetic experiments. Reactions were in BRB80 buffer (80 mM Na Pipes, 1 mm $MgCl_2$, 1 mm EGTA, pH 6.8), at 37°. Hela cell kinesin and sea urchin axonemes were, respectively, gifts from V. Lombillo and J.R. McIntosh, University of Colorado, Boulder, CO, and from E.D. Salmon, University of North Carolina, Chapel Hill, NC.

Guanylyl-(a,b)-methylene-diphosphonate (GMPCPP) and gamma-[^{32}P]GMPCPP were synthesized as described (Hyman *et al.*, 1992). [^{3}H]GMPCPP was synthesized by catalytic tritiation of the 8-bromo derivative, which had been synthesized from 8-bromoguanosine isopropylidene, using the procedure described for guanosine isopropylidene.

Methods

The polarity of microtubules formed at axoneme ends with tubulin-GMPCPP subunits could be determined by the pattern for elongation of the two ends. About twice as many microtubules were nucleated from one end and these grew more slowly so that the end had a paintbrush-like appearance. The paintbrush-like end was shown to be the minus-end from the direction of movement of kinesin-linked latex beads (Kowalski and Williams, 1993) on axonemes that had been elongated with tubulin-GTP subunits. After dilution-induced disassembly of the GTP-microtubules, the polarity-identified axonemes were elongated with tubulin-GMPCPP and the paintbrush pattern was formed at the minus-end. The numerous short microtubules at the minus-end made it difficult to measure rates, and in studies of the disassembly kinetics for microtubules assembled with mixtures of GMPCPP and GTP, the rate was only measured for the plus-end.

Microtubule dynamics were analyzed using a microscope enclosed in a plastic box that was maintained at 37° by a flow of heated air. Microtubules were formed at the ends of axonemes that adhered to the surface of a coverslip, by a brief incubation with a tubulin-nucleotide mixture. Reaction mixtures (approximately 3.5 μl) were in a flow cell (Vale, 1991) that allowed rapid replacement of the mixture used for microtubule assembly by flow of 15–20 μl of solution; this was wicked into the cell at about 2 μl/s, measured with a dye/buffer mixture. Thus, less than 1 s elapsed between the time microtubules were seen to move as a result of the flow of the added solution, which was taken as zero-time in kinetic studies, and the time when the microtubules were exposed to the added solution. The flow cell was sealed with Vaseline for reactions that were incubated for more than 50 min.

Images from a Hamamatsu 2400 Newvicon camera were processed with an Argus 10 (Hamamatsu, Bridgewater, NJ), recorded on Super VHS tape, and analyzed with the computer-based RTM system (Glicksman *et al.*, 1992). The disassembly rates were too slow to use this method with microtubules formed with GMPCPP in the absence of GTP. Therefore, images were recorded by making a trace on a sheet of transparent plastic of three to nine viewing areas immediately after dilution with buffer and again after 30–240 min. Decreases in microtubule length could be measured because relatively few microtubules were lost by physical breakage during the incubation and about 75% of the microtubules had a unique orientation and/or length so that they could be identified in traces made at intervals. The fraction of microtubules that showed no change in length during the incubation is reported; an upper limit for disassembly was obtained for these by assuming that a length change equal to 0.5 mm in the video image (190 subunits) would have gone undetected.

The microtubules used to determine the lifetime of a tubulin-GMPCPP cap on GTP-microtubules were assembled with 22 μM tubulin and 100 μM GTP. These were sequentially reacted with a mixture of tubulin at varying concentrations and 1 mM GMPCPP, followed by buffer containing 100 μM GMPCPP. It was difficult to time precisely the flow of buffer/GMPCPP so that the duration of the reaction with tubulin-GMPCPP subunits was for 20–30 s; it was assumed that elongation with tubulin-GMPCPP subunits was for 25 s in estimating the number of tubulin-GMPCPP subunits added.

We were interested in the effect of very low concentrations of tubulin-GDP subunits on the stability of microtubules assembled with GMPCPP in the absence of added GTP. GDP comes from the pool of nucleotide in tubulin subunits and to measure the extent of this incorporation it was necessary to radiolabel both GDP and GTP in the pool to known specific activity before the assembly reaction. As described above, the tubulin contained 1.08 mol guanine nucleotide/mol tubulin and the fraction of this that was GDP and GTP was determined by thin layer chromatography analysis on PEI cellulose of an aliquot from a mixture containing a trace amount of [α-^{32}P]GTP, 10 μM tubulin, and nucleoside diphosphate kinase (Sigma, St. Louis, MO; beef liver enzyme, 3 U/ml), after this had been incubated for 10 min. The distribution of radiolabel was 63% in GDP and 37% in GTP. Thus when 10 μM tubulin was assembled with 1 mM GMPCPP the mixture contained about 6.8 μM GDP and 4.0 μM GTP. The amount of this GDP and GTP that was incorporated into microtubules during assembly with 1 mM GMPCPP was determined with tubulin that had been mixed with a trace amount of [α-^{32}P]GTP, or a mixture of [α-^{32}P]GTP and [α-^{32}P]GDP. The latter mixture was obtained as a filtrate (Microcon 10, Amicon, Beverly, CA) from a mixture of a trace amount of [α-^{32}P]GTP, 10 μM tubulin, and nucleoside diphosphate kinase (see above), after a 10- or 30-min incubation. The amount of radiolabeled GDP and GTP incorporated into microtubules was calculated by assembling microtubules with radioactive GTP and GDP at two different known specific activities to solve for the two unknowns in equation 1:

$$\text{cpm in pellet} = (\text{moles GTP incorporated into microtubules})(\text{specific activity GTP}) + (\text{moles GDP incorporated in microtubules})(\text{specific activity GDP}) \quad (1)$$

The microtubule assembly was done in two cycles, an initial 30-min incubation at 25° with 1 mM GMPCPP, isolating the product by centrifugation (Airfuge, 10 min, 30 psi, 37°), followed by disassembly for 20 min in ice-cold buffer. After a trace of insoluble protein was removed by centrifugation (13,000 × *g*, 2 min, 4°) and the protein concentration was diluted to 10 μM, an aliquot was assayed for radioactivity. A second assembly reaction was performed with either 50 μM or 1 mM GMPCPP (30 min, 25°), and the protein and radioactivity was determined after the pellet from centrifugation (Airfuge, 10 min, 30 psi, 37°) was dissolved in 0.1 M NaOH. An 83% yield of protein was obtained in the second assembly reaction.

Determining the amount of GTP and GMPCPP incorporated into microtubules assembled with a mixture of GMPCPP and GTP (in excess of the tubulin concentration) was simpler, because endogenous GTP does not significantly influence the specific activity of added radioactive GTP. In this case, microtubules were assembled with [α-^{32}P]GTP and [^{3}H]GMPCPP in separate, otherwise identical assembly reactions. Microtubules were isolated by centrifugation (Airfuge, 10 min, 30 psi, 37°) and the protein and radioactivity were measured in the pellet. Control experiments in which [^{3}H]CTP was added to the assembled microtubules before centrifugation indicated that about 0.5% of the nucleotide in a reaction is nonspecifically entrapped in pelleted microtubules.

RESULTS

Kinetic Properties of GMPCPP Microtubules Containing Minimal GDP

We measured the kinetic properties of tubulin-GMPCPP microtubules to estimate the stability provided by tubulin-GTP subunits that presumably cap microtubules assembled with GTP. These studies required measurement of the rate constant for dissociation of tubulin-GMPCPP subunits from microtubules

in which the highest possible fraction of subunits had this analogue bound rather than GDP. We had previously found (Table 3 in Caplow *et al.*, 1994) that a low concentration of tubulin-GDP subunits in GMPCPP microtubules can significantly alter the subunit dissociation rate. Tubulin-GMPCPP subunits obtained from an assembly/disassembly cycle with 1 mM GMPCPP were additionally assembled with GMPCPP/tubulin ratios of 5/1 and 100/1. Although the tubulin-GDP content of the microtubules formed in the two reactions were similar (23% with the 5/1 ratio and <8% with a 100/1 ratio), the disassembly rate was fivefold higher at the plus-end with the former microtubules. The minus-end rates were apparently comparable, but this may have resulted because the rates were so slow that only an upper limit could be estimated. We have extended these studies, making a greater effort to determine the very slow minus-end rates and have determined that the rate is influenced by reducing the microtubules's GDP content to even lower levels.

GDP found in GMPCPP microtubules comes from the GDP and GTP contained in the exchangeable site (E-site) of purified tubulin subunits. To determine the extent of this incorporation during two cycles of assembly with GMPCPP, before the first assembly reaction the tubulin nucleotide pool was labeled with a mixture of [α-^{32}P]GTP and [α-^{32}P]GDP (reactions 1–3, Table 1). Because nucleotide can be incorporated from both endogenous GTP and GDP, it required assembly with nucleotides at two different specific activities to determine the uptake of nucleotide (see equation 1). A typical reaction (Table 1, reaction 1) contained 27,680 cpm/μl (5.6 μCi total) in the 4 μM GTP derived from the 10 μM tubulin in the reaction mixture (see above) and 4,150 cpm/μl in the 6.8 μM GDP. Thus, the specific activities were 6,920 cpm/μl/μM for GTP and 610 cpm/μl/μM for GDP. The resuspended pellet contained 10 μM tubulin and 3195 cpm/μl; this was reduced to 2760 cpm/μl after correcting for a 0.5% entrapment from free nucleotide (see above). From this value and results from reaction 2 (Table 1), solution of equation 1 indicated that the 10 μM tubulin in the pelleted microtubules contained the equivalent of 0.473 μM GTP (from the 4 μM endogenous GTP, see above) and 0.06 μM GDP (from the 6.8 μM endogenous GDP, see above), giving a total of 0.53 μM GDP/10 μM tubulin in microtubules (0.053 μM/μM tubulin). Because the stoichiometry for guanine nucleotide incorporation in assembly with excess GMPCPP is about 0.71 (Caplow *et al.*, 1994), GDP constituted about 7.5% of the nucleotide in the microtubules. Results from a third reaction (Table 1, reaction 3) were in agreement with the stoichiometries calculated from reactions 1 and 2.

A second assembly was done with subunits obtained by cold-induced disassembly of the microtubules formed with 1 mM GMPCPP (reaction 1 in Table 1). When assembly was with a fivefold excess of GMPCPP (reaction 4), 12% of the GDP from the prior assembly was retained; this corresponds to a stoichiometry of about 0.0064 mol of GDP/mol tubulin and because the GMPCPP content is 0.71 − .0064 = 0.70, the GDP is 1/110 total guanine nucleotide. The fraction of the microtubule with tubulin-GDP subunits was lower when the second assembly was performed with a 100-fold excess of GMPCPP (Table 1, reaction 4). Here (Table 1, reaction 5) only about 5% of the GDP that remained after a prior assembly with 1 mM GMPCPP was in the microtubules, corresponding to an overall retention of about 0.0026 mol of GDP/mole tubulin in microtubules; therefore, GDP constitutes about 1/270 total guanine nucleotide.

Table 1. Incorporation of endogenous GDP and GTP into GMPCPP-microtubules[a]

Reaction no.	Assembly	GTP S.A.[b]	GDP S.A.[b]	cpm pellet[c]	calc cpm pellet[d]
1.	First assembly	6920	610	2760	3308
2.	with 1 mM	3310	2190	1700	1700
3.	GMPCPP	2230	2240	1370	1190
4.	Second assembly with 50 μM GMPCPP		2760	348 (12%)	
5.	Second assembly with 1 mM GMPCPP		2760	144 (5%)	

[a] 10 μM tubulin was assembled with [alpha-^{32}P]GTP and [alpha-^{32}P]GDP at three different specific activities (reactions 1–3) with 1 mM GMPCPP. The microtubules from reaction 1 were disassembled by treatment with ice-cold BRB80 buffer, the tubulin was diluted to 10 μM, and the mixture was assembled with either 50 μM GMPCPP (reaction 4) or 1 mM GMPCPP (reaction 5). Results from one of four experiments are presented.

[b] cpm/μl/μM.

[c] cpm/μl, after the pellet was taken up in sufficient buffer to give 10 μM tubulin.

[d] Calculated by multiplying the 0.473 μM GTP and 0.06 μM GDP/10 μM assembled tubulin by the nucleotide specific activity.

We were surprised to find that the dilution-induced disassembly rate was decreased by reducing the tubulin-GDP content of GMPCPP microtubules from 1/110 to 1/270 (cf. reactions 1 and 3 in Table 2). This difference did not result from incomplete removal by the diluent of the GMPCPP that had been used at different concentrations for assembling the microtubules in reactions 1 and 3. Evidence that it is the small difference in tubulin-GDP that accounts for the results was obtained by comparing the rates in buffer containing 100 μM GMPCPP (cf. reactions 2 and 4 in Table 2). The slower disassembly rates with 100 μM GMPCPP in the buffer (cf. reactions 1 and 2, and 3 and 4 in Table 2)) probably resulted because this prevents formation of nucleotide-free (apotubulin) subunits at microtubule ends. We have found (Caplow and Shanks, 1995) that reducing the concentration of free GTP (at fixed concentrations of tubulin-GTP subunits) induces catastrophe at the plus-end of microtubules, presumably because the tubulin-GTP cap is destabilized by dissociation of GTP to form apotubulin from a fraction of the subunits. Additional evidence that the rate of disassembly of GMPCPP microtubules can be influenced by apotubulin subunit formation is provided by the greater disassembly rate in the presence of alkaline phosphatase (cf. Reactions 5 and 4 in Table 2), which would promote formation of apotubulin subunits at microtubule ends by reducing the concentration of free GMPCPP in the dilution buffer.

Because the rate of disassembly of the minus end of GMPCPP microtubules is extremely slow, video studies were only able to provide an upper limit for the rate constant (Table 2, reaction 4); even with a 240-min incubation only an upper limit for the rate could be measured for 44% of the microtubules. The rate constant could be determined from the equilibrium constant and the rate of elongation at the minus-end. Because GMPCPP is not hydrolyzed when tubulin-GMPCPP subunits are added to microtubules, subunit addition is a true equilibrium and because the equilibrium constant is identical at the two ends (Wegner, 1976), equation 2 holds:

$$K_{eq} = [k_- (+ \text{ end})/k_+ (+ \text{ end}) = [k_- (- \text{ end})/k_+ (- \text{ end})] \quad (2)$$

with k_+ and k_- equal to the rate constants for subunit addition and loss.

The rate constant for addition to the plus and minus-end were found to be 2.9 (SD 0.8, n = 9) and 1.6 (SD 0.2, n = 8) × 10^6 M^{-1} s^{-1}, respectively, measured with 4.5 μM tubulin. From these constants and that for dissociation at the plus-end (Table 2, reaction 4), the rate constant for loss of tubulin-GMPCPP at the minus-end is calculated to be 0.065 s^{-1}; this is in reasonable agreement with 0.037 s^{-1} determined with the video assay.

Kinetic Properties of Microtubules Containing GMPCPP and GDP

Because the lifetime of tubulin-GMPCPP subunits is about 8 s at the plus-end and 15 s at the minus-end, compared with about 1 ms for tubulin-GDP subunits (Walker *et al.*, 1988; Caplow *et al.*, 1989), it might be expected that microtubules would be dramatically stabilized when tubulin-GMPCPP subunits constitute even a minor fraction of the microtubule. To test this, microtubules with varying fractions of tubulin-GMPCPP subunits were generated by assembling with increasing concentrations of GMPCPP, with a fixed (100 μM) concentration of GTP. Even though

Table 2. Kinetics for disassembly of GMPCPP-microtubules[a]

Reaction no.	Fraction MT with Tu-GDP	Disassembly conditions	k (plus-end) (s^{-1}) (n, S.D., %)	k (minus-end) (s^{-1}) (n, S.D., %)[b]	Duration (min)[c]
1.	1/110	Buffer	0.59 (52, .59, 10)	0.34 (53, .34, 11)	94
2.	1/110	0.1 mM GMPCPP	0.34 (108, .26, 19)	0.056 (157, .009, 64)	90
3.	1/270	Buffer	0.24 (76, .1, 3)	0.064 (31, .05, 23)	207
4.	1/270	0.1 mM GMPCPP	0.118 (108, .08, 13)	0.037 (134, .034, 44)	240
5.[d]	1/270	Alkaline phosphatase	0.61 (49, 0.24, 0)	0.18 (29, .14, 28)	100
5A.			0.69 (100, .46, 5)	0.29 (44, .27, 30)	38

[a] Tubulin-GMPCPP subunits, obtained by cold-induced disassembly of microtubules that had been assembled from 10 μM tubulin and 1 mM GMPCPP were reassembled with either 50 μM or 1 mM GMPCPP. The GDP content of the resulting microtubules was determined as described in Table 1. The rate of disassembly was determined after these were diluted with buffer, with and without 0.1 mM GMPCPP, or in the presence of alkaline phosphatase (5.6 U/ml).

[b] Values in parentheses are number of microtubules measured, standard deviation, and percent of microtubules showing no measurable change in length during the incubation. It was assumed that these microtubules shortened by 0.5 mm on the video screen (190 subunits), but this shortening could not be detected.

[c] Time for study of the disassembly reaction.

[d] 5 and 5A are duplicate reactions, varying the duration of the disassembly only.

tubulin-GTP and tubulin-GMPCPP subunits assemble at about the same rate (Hyman *et al.*, 1992) high GMPCPP concentrations were required for incorporation (Table 3 and Figure 1), apparently because GTP binds 30-times tighter than GMPCPP to tubulin subunits (Caplow *et al.*, 1994).

It was surprising that despite the approximately 10000-fold lower rate for dissociation of tubulin-GMPCPP subunits compared with tubulin-GDP, only an eightfold reduction in the disassembly rate was seen when tubulin-GMPCPP constituted about 62% of the microtubule (Figure 2). The rate decrease induced by tubulin-GMPCPP subunits was markedly dependent on the fraction of the microtubule containing these subunits (Table 4 and Figure 3).

Size of the Minimum Tubulin-GMPCPP Cap for Stabilizing a Microtubule End

To obtain information about the minimum size of a cap for stabilizing a microtubule end, GTP-microtubules assembled on axonemes were first briefly treated with a dilute solution of tubulin-GMPCPP subunits and then with subunit-free buffer. Those GTP-microtubules that did not disassemble in the dilute mixture of tubulin-GMPCPP had apparently been capped by tubulin-GMPCPP subunits, so that they were transiently stable in buffer. The size of the stabilizing GMPCPP-cap could be estimated from the delay before initiation of disassembly, because this is expected to be equal to the product of the number of tubulin-GMPCPP subunits in the cap and the time for dissociation of a single tubulin-GMPCPP subunit. According to this scheme, because the lifetime of a single tubulin-GMPCPP subunit is about 15 s ($1/0.065\ s^{-1}$; see above) a microtubule that initiates disassembly after 500 s must be capped by 32 tubulin-GMPCPP subunits.

Because we wished to determine the size of the cap just sufficient to prevent catastrophe, the treatment with tubulin-GMPCPP subunits was brief (about 25 s), with only 1 μM tubulin-GMPCPP. Although the measured second-order rate constants for reaction of tubulin-GMPCPP subunits predict that an average of 73 subunits would be added to the plus-end and 40 subunits at the minus-end during the 25-s incubation, only a fraction of microtubules were stabilized by addition of tubulin-GMPCPP and most of these had a free minus-end (Table 5). Stabilization was much less efficient at the plus-end, presumably because the much higher catastrophe frequency at this end (Walker *et al.* 1988) more than offset the 1.8-fold faster addition of tubulin-GMPCPP subunits.

GTP-microtubules that had not disassembled in a dilute solution of tubulin-GMPCPP, apparently because they were capped with tubulin-GMPCPP subunits, did not start to disassemble for 25–2000 s after exposure to buffer (Figure 4A). In contrast, GTP-mi-

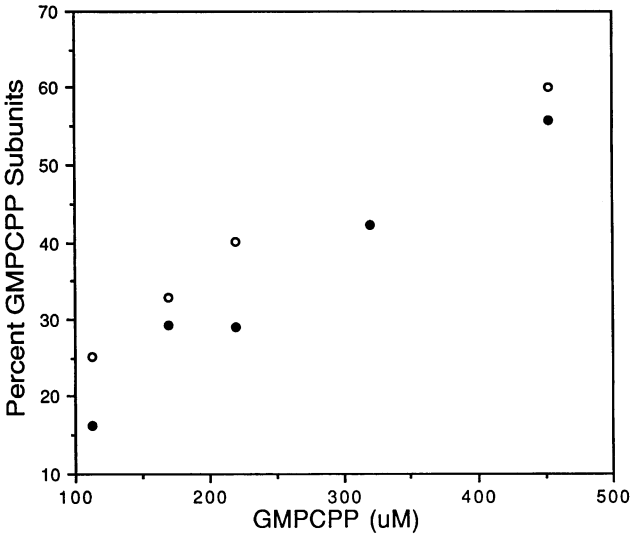

Figure 1. Composition of microtubules assembled with 100 μM GTP and varying concentrations of GMPCPP. Results are given from two experiments. Reliable results could not be obtained from assembly with concentrations of GMPCPP greater than 450 μM because when the mole fraction of the microtubule with tubulin-GMPCPP is >0.5 an increase in concentration reduces the specific activity of the [^{3}H]GMPCPP more than it can increase the amount of radioactive GMPCPP incorporated into microtubules.

Table 3. Composition of microtubules assembled with GTP and GMPCPP[a]

GMPCPP (μM)	Tu in MT[b] (μM)	GXP/Tu in MT	GDP/MT	GMPCPP/MT	%GMPCPP
113	10.04	.527	3.93	1.342	25
170	10.79	.603	4.35	2.154	33
227	13.13	.664	5.21	3.507	40
453	15.90	.727	4.60	6.958	60

[a] Microtubules were assembled with 30 μM tubulin, 100 μM [alpha-^{32}P]GTP, and the indicated concentrations of [^{3}H]GMPCPP. Results from this and an identical experiment are given in Figure 1.

[b] Concentration of tubulin after pelleted microtubules were resuspended in a volume of BRB80 equal to that used for assembly.

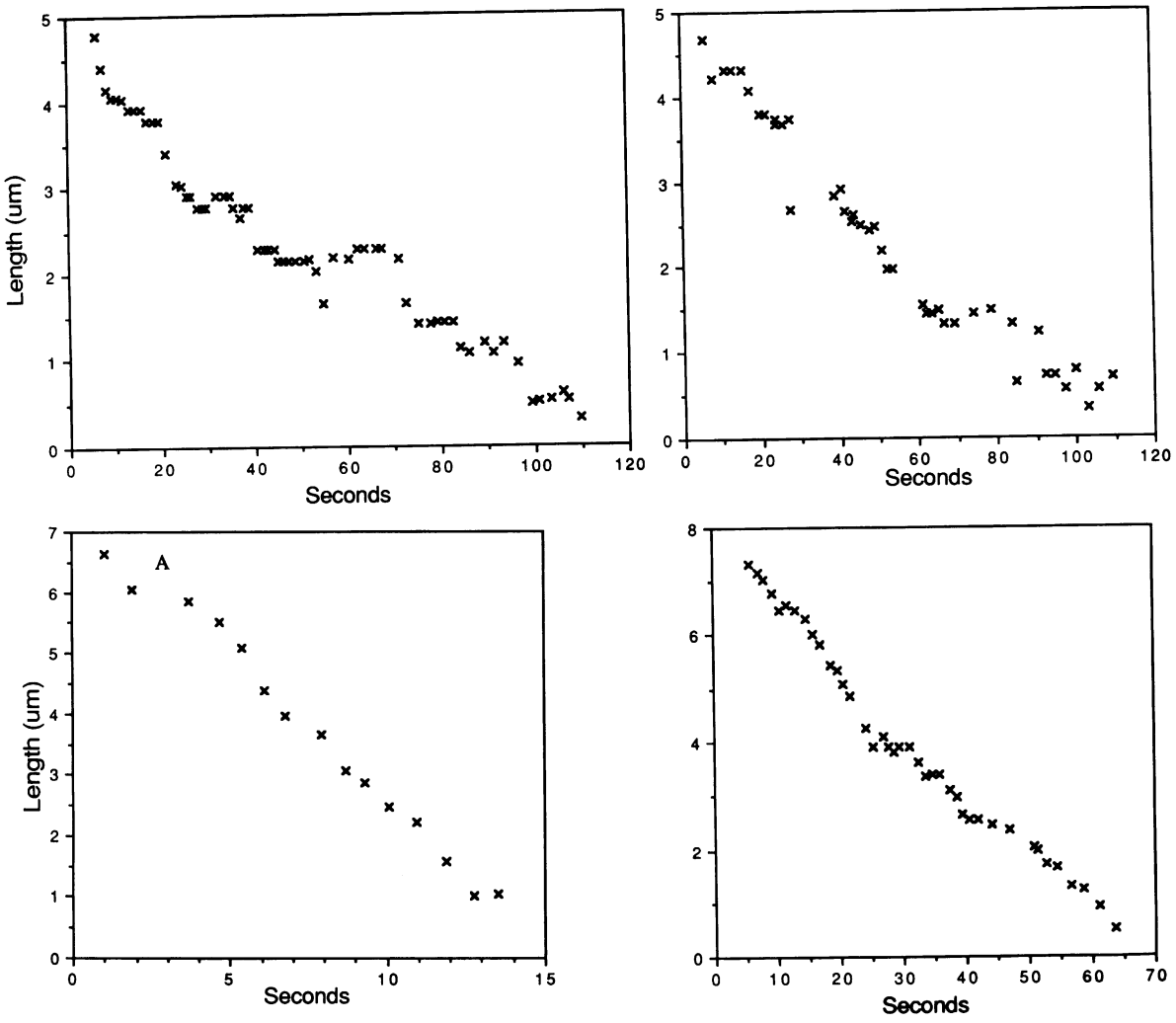

Figure 2. Kinetics for disassembly of microtubules containing tubulin-GDP (lower left panel) and tubulin-GMPCPP subunits (other three panels). Microtubules were assembled with 100 μM GTP, 100 μM GTP, and 520 μM GMPCPP. The irregularities in the disassembly rate were reproducible in multiple measurements from a video recording.

crotubules that had not been reacted with tubulin-GMPCPP subunits started to disassemble 5–8 s after dilution (our unpublished results).

DISCUSSION

Rate of Dissociation of Tubulin-GMPCPP Subunits

The rate of dissociation of tubulin-GTP is important for deciding how growing microtubules lose their GTP cap (Caplow *et al.*, 1988; Voter *et al.*, 1991; Walker *et al.*, 1991). The rate with MAP-free microtubules has been estimated at 0.1 to 44 s^{-1} by extrapolating to zero concentration a plot of the microtubule assembly rate versus the concentration of tubulin-GTP. The very wide range may result because the lowest concentration of tubulin-GTP used for assembly was 7–10 μM (Walker *et al.*, 1988; O'Brien *et al.*, 1990; Trinczek *et al.*, 1993, 1995) or 23 μM (Trinczek *et al.*, 1993) so that small errors in the rates generate a large error in the value obtained by extrapolation to zero tubulin. This error is likely to have been smaller in studies in which the lowest subunit concentration was only 2–3 μM, which gave rates equal to 0.1 (Drechsel *et al.*, 1992) and 0.37 s^{-1} (Mitchison and Kirschner, 1984). Evidence in support of these lower rates was obtained with microtubules containing GMPCPP, which had a subunit dissociation rate equal to 0.1 s^{-1} at the plus end (Hyman *et al.*, 1992).

Because our analysis of the minimum size of a cap sufficient to stabilize a microtubule end required knowing the rate of dissociation of tubulin-GMPCPP subunits from the plus and from the minus end, we

Table 4. Rate of disassembly of microtubules containing GDP and GMPCPP[a]

μM GMPCPP	% Tu-GMPCPP[b]	No.	Rate (s^{-1})	SD	Calc. Rate[c]
0	0	12	740	178	
200	36	9	516	193	732 (721)
320	47	33	368	103	462 (340)
400	56	43	257	75	73 (46)
520	62	31	95	31	16 (11)

[a] The rate of dilution-induced disassembly of the plus-end of microtubules was determined with microtubules that had been assembled with 15 μM tubulin, 100 μM GTP, and the indicated concentration of GMPCPP.

[b] Estimated from results in Figure 1.

[c] The rate was calculated for the mechanism described in Figure 5, from equation 6,

$$1/k_{obs} = 1 - (f_{GMPCPP}{}^{n}/1 - f_{GMPCPP})/k_{GDP} + (f_{GMPCPP})^{n}/k_{GMPCPP} + (f_{GMPCPP})^{n+1}/k_{GMPCPP}/n + 1) + (f_{GMPCPP})^{n+2}/(k_{GMPCPP}/n + 2) + (f_{GMPCPP})^{n+3}/(k_{GMPCPP}/n + 3) \quad (6)$$

with k_{GDP} and k_{GMPCPP} equal to 740 s^{-1} (see above) and 0.118 s^{-1} (Table 2, reaction 4), respectively, and with n equal to the minimum number of tubulin-GMPCPP subunits to form a stable cap. Equation 6 is similar to equation 3, except that the numerator of the term with k_{GDP} describes the fraction of the time that ends contain tubulin-GDP or less than n tubulin-GMPCPP subunits. The numerator for the terms with k_{GMPCPP} describes the probability for arrival of n uniquely arranged tubulin-GMPCPP subunits; the denominator is as in equation 3, except that k_{GMPCPP} is corrected (by division by n + 1, n + 2, etc.) for the reduced dissociation rate when more than one tubuline-GMPCPP subunit must be lost to destabilize the end when more than n uniquely arranged tubulin-GMPCPP subunits arrive at the disassembling end. The rates calculated for n equals 14 and 13 (in parenthesis) are given in the right-hand columns.

determined these values. Our results are consistent with rates equal to 0.118 and 0.065 s^{-1}, respectively, for the plus and minus ends of near pure GMPCPP microtubules (tubulin-GDP subunits constituted only about one part per 270 parts). We are unable to provide a decisive interpretation for the decreased stability of GMPCPP-microtubules in which tubulin-GDP constituted one part per 110 parts (Table 2). Tubulin-GDP will truncate runs of contiguous tubulin-GMPCPP, but this will be infrequent and, therefore, expected to have only a small effect on the disassembly kinetics. However, if discovery of a tubulin-GDP subunit at a microtubule end were to result in an unraveling of the microtubule end that persists for a period after loss of the tubulin-GDP subunit, then a significant effect on the disassembly rate would result. Although this mechanism predicts that disassembly would occur in pulses corresponding to arrival of a tubulin-GDP subunit at an end, the limited resolution of the measurements would not allow detection of such pulses, if they were to exist.

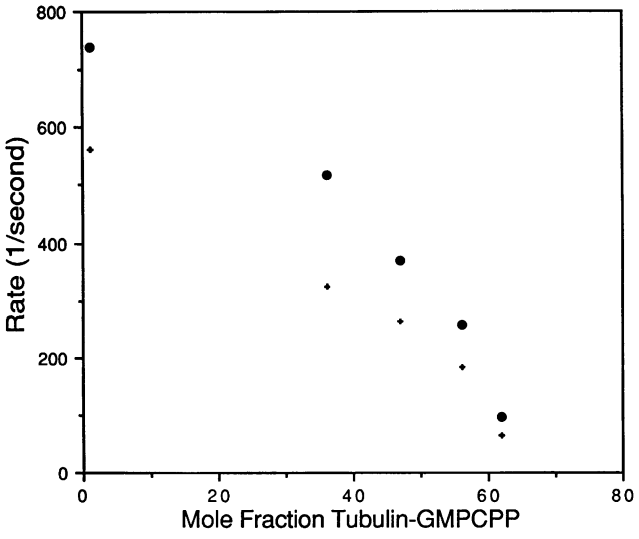

Figure 3. Dependence of the rate of disassembly of the microtubule plus-end on the mole fraction of subunits with GMPCPP. The observed rate (●) and 1 SD (+) are shown.

Table 5. Stabilization of GTP-microtubules by tubulin-GMPCPP

μM Tubulin	% Plus-end stabilized (n)	% Minus-end stabilized (n)
0.25	0 (34)	0 (37)
0.5	0 (89)	30 (34)
1.0	10 (44)	57 (39)
2.0	18 (67)	93 (62)
3.0	40 (40)	93 (35)

Microtubules assembled with 22 μM tubulin and 100 μM GTP were exposed for 25 s to 1 mM GMPCPP containing tubulin at the indicated concentrations, before they were treated with BRB80 buffer. Stabilized microtubules were defined as microtubules that were not disassembling when the buffer flow was complete.

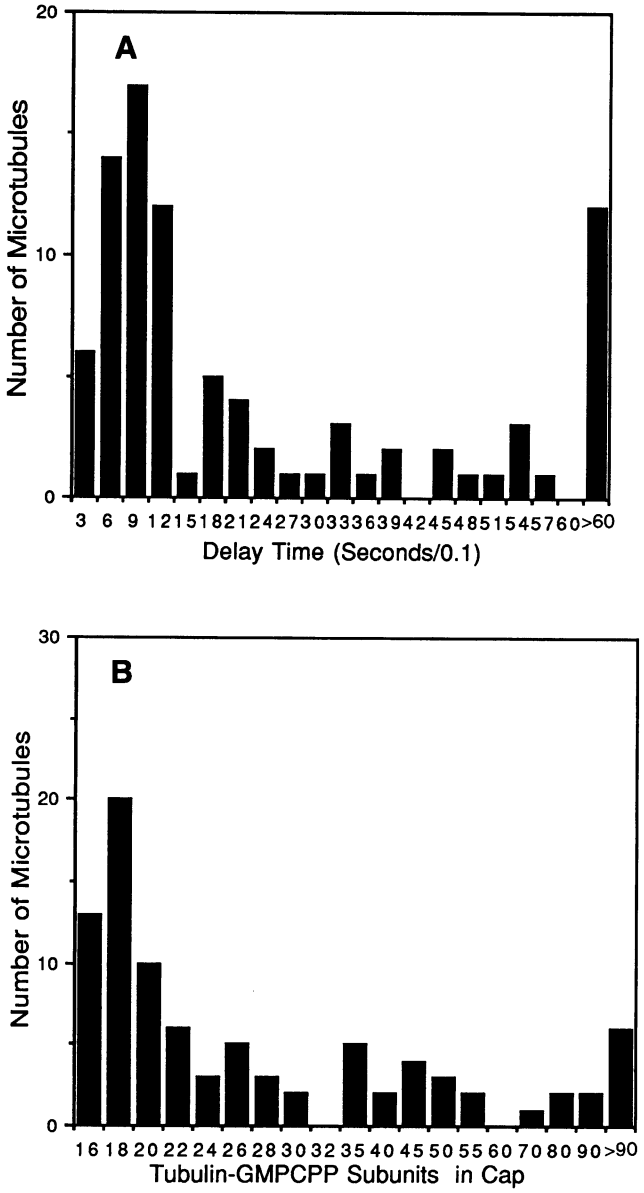

Figure 4. (A) Lifetime of GTP-microtubules capped with GMPCPP subunits. GTP-microtubules at the minus end of axonemes were reacted for about 25 s with 0.5 μM tubulin-GMPCPP and then diluted with buffer. Microtubules that had not started to disassemble 10 s after the dilution with buffer were presumed to have been capped with tubulin-GMPCPP subunits, and the time before disassembly started is given. (B) Estimated length of a tubulin-GMPCPP cap at microtubule ends, assuming that rapid disassembly follows when the number of tubulin-GMPCPP subunits at the microtubule end is reduced to less that 14 subunits. The size of the tubulin-GMPCPP cap was calculated by adding 13 subunits to the value obtained by dividing the time that elapsed between dilution and initiation of disassembly (panel A), by the lifetime of a tubulin-GMPCPP subunit at the microtubule's minus-end (i.e., 15 s).

Influence of Tubulin-GMPCPP Subunits on GDP Microtubules

The data above show that the lifetime of a tubulin-GMPCPP subunit in a near pure GMPCPP lattice is about 8 s at the plus end (i.e., $1/0.118\ s^{-1}$), which is about 6300-fold slower than with GDP. This vast difference in rates is expected to have a dramatic effect on the kinetic behavior of microtubules containing a mixture of subunits with GDP and GMPCPP. Because rapidly dissociating tubulin-GDP subunits will be trapped in the microtubule until overlying tubulin-GMPCPP subunits are lost from ends, the rate is expected to be primarily influenced by that for the slowly dissociating tubulin-GMPCPP subunits. This behavior is apparent from equation 3, which describes disassembly of a mixed lattice microtubule containing tubulin-GDP and tubulin-GMPCPP subunits:

$$1/k_{obs} = f_{GDP}/k_{GDP} + f_{GMPCPP}/k_{GMPCPP} \qquad (3)$$

In this equation f_{GXP} represents the mole fraction of subunits with each nucleotide, and k is the rate constant for dissociation of the indicated subunit from a homopolymer. According to equation 3 with k_{GMPCPP} at the plus-end equal to only 0.118^{-1}, compared with $740\ s^{-1}$ for k_{GDP}, the rate is predicted to be reduced by a factor of two when tubulin-GMPCPP subunits constitute one subunit in 6300. It was therefore unexpected that microtubules assembled with 200 μM GMPCPP and 100 μM GTP, in which tubulin-GMPCPP subunits constituted about 36% of the subunits, disassembled at a rate equal to $516\ s^{-1}$; this is 1600-times faster than predicted from equation 3. The fact that microtubules containing GMPCPP and GDP subunits are less stable than pure GMPCPP microtubules provides evidence that tubulin-GMPCPP subunits are randomly distributed within the microtubule lattice. Further evidence for formation of a random copolymer is the effect of GMPCPP subunits on microtubule stability is proportional to a high power of the mole fraction of the microtubule with GMPCPP (see next paragraph). A first-order dependence is expected if tubulin-GMPCPP subunits were preferentially assembled together in microtubules; i.e., the probability of neighboring tubulin-GMPCPP subunits is influenced by something other than chance.

The insensitivity of the plus-end disassembly rate to the fraction of the microtubule containing tubulin-GMPCPP subunits (Figure 3) can be taken to indicate that the stabilizing effect of GMPCPP requires the presence of multiple neighboring tubulin-GMPCPP subunits. As described in the legend for Figure 5 the number of tubulin-GMPCPP subunits required to stabilize an end transiently can be estimated from the exponent that describes the dependence of the rate on the mole fraction of subunits with GMPCPP. The exponents 13 and 14 fit the observed rate (Figure 6),

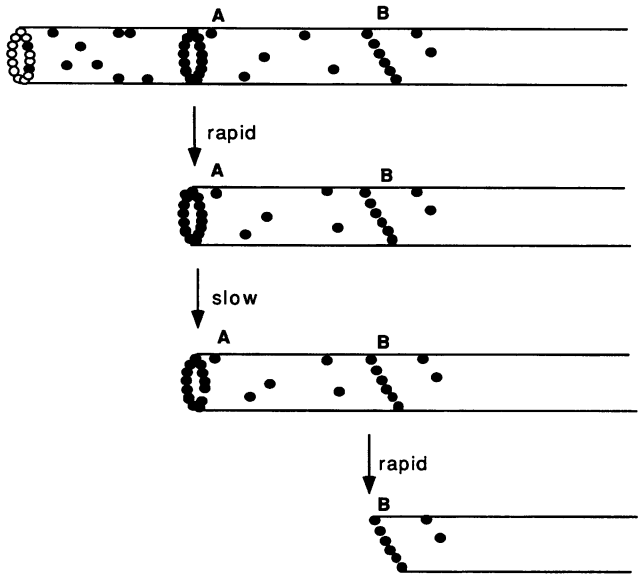

Figure 5. Mechanism of disassembly of mixed lattice microtubules and a model for the tubulin-GTP cap. According to the proposed mechanism dissociation of tubulin-GDP and tubulin-GMPCPP (●) subunits from a mixed lattice microtubule have the same rapid rate until multiple contiguous tubulin-GMPCPP subunits arrive at the end (region A and/or B). The stability provided by the tubulin-GMPCPP cap is lost when this is reduced from 14 to 13 (or from 13 to 12) subunits; subsequent loss of tubulin-GMPCPP subunits from the disrupted cap is rapid. The 14 tubulin-GMPCPP subunits may not be contiguous as in region A; the arrangement in region B may represent a stable cap. The probability for arrival of a number (n) of uniquely arranged tubulin-GMPCPP subunits that can stabilize the end of a disassembling microtubule is equal to:

$$p(n) = (f_{GMPCPP})^n \quad (4)$$

where f_{GMPCPP} is the mole fraction of subunits with GMPCPP. The exponent in equation 4 gives the stoichiometry of the stabilizing tubulin-GMPCPP cap. The exponent in equation 4 does not, however, give the stoichiometry of a stable cap if multiple arrangements of 14 (or 13) tubulin-GMPCPP subunits can stabilize a microtubule end. When multiple (i.e, n') arrangements of n tubulin-GMPCPP subunits can stabilize an end, the probability for forming a stable cap with n subunits is:

$$p(n,n') = (f_{GMPCPP})^{n + \log n'/\log f_{GMPCPP}}. \quad (5)$$

For example, with f_{GMPCPP} equal to 0.62 the probability for arrival at an end of 14 uniquely arranged tubulin-GMPCPP subunits is $f_{GMPCPP}^{14} = 0.00124$; this is equal to $f_{GMPCPP}^{12.55} = 0.00248$ if two arrangements of 14 tubulin-GMPCPP subunits can stabilize an end; if 10 unique arrangements of 14 tubulin-GMPCPP subunits form a stable cap the rate with f_{GMPCPP} equal to 0.62 would be equal to $(f_{GMPCPP})^{9.18}$. Our observation that the stability conferred by tubulin-GMPCPP subunits can be described by a high exponent for f_{GMPCPP} (Figure 6) suggest that there are not many configurations of tubulin-GMPCPP subunits that can form a stable cap.

suggesting that a microtubule end is stable when 13 or 14 tubulin-GMPCPP subunits with a unique orientation arrive at the end of a disassembling microtubule (Figure 5). Because rate measurements can only provide information about the stoichiometric composition of the rate-limiting transition state and not the arrangement of atoms in the transition state, the arrangement of the 13 or 14 tubulin-GMPCPP subunits that generate a transiently stable end is not known (Figure 5). The possibility that numerous configurations of 13 or 14 subunits can stabilize an end is unlikely because this would decrease the requirement for a high mole fraction of subunits with GMPCPP to reduce the rate; i.e., the exponent for f_{GMPCPP} in equation 4 would be less than 13 or 14 (see the legend to Figure 5).

The dependence of the rate on the 13th or 14th power of the mole fraction of subunits with GMPCPP also provides information about the mechanism by which a stable end is lost. Dissociation of a single tubulin-GMPCPP subunit from the cap apparently destabilizes the ring of adjacent tubulin-GMPCPP subunits and allows these to dissociate rapidly. If the cap were still stable after loss of one or two tubulin-GMPCPP subunits from a 13–14 subunit cap the rate would have been proportional to $(f_{GMPCPP})^{11-12}$, which corresponds to the probability of ends having 11–12 contiguous tubulin-GMPCPP subunits.

The proposed mechanism predicts that disassembly occurs in pulses, with a rapid phase (about 740 s^{-1} = 0.45 μm/s) corresponding to dissociation of tubulin-GDP and interspersed tubulin-GMPCPP subunits, punctuated with pauses corresponding to arrival at the end of 13 or 14 tubulin-GMPCPP subunits with an appropriate orientation. Approximately 8-s pauses corresponding to the time for dissociation of a single tubulin-GMPCPP subunit are expected (the large standard error for this rate constant gives considerable latitude for this value); approximately 16- or 24-s pauses for loss of two or three tubulin-GMPCPP subunits are expected less frequently when 15 or 16 appropriately arranged tubulin-GMPCPP subunits arrive at an end. In a typical reaction in which the rate is reduced about eightfold compared with GDP microtubules, equation 4 predicts that 14 contiguous tubulin-GMPCPP subunits arrive at microtubule ends once during disassembly of each 0.5 μm of microtubule length. Although the predicted irregularity of the disassembly rate was observed (Figure 2), the loss of 0.45-μm segments in 1-s steps is very close to the experimental error in the measurements, so that we cannot accept these results as further proof for the proposed mechanism.

Estimate of the Size of the Minimum Tubulin-GMPCPP Cap Necessary to Stabilize Microtubules

When microtubules assembled with GTP were stabilized by a brief reaction with tubulin-GMPCPP subunits, and then diluted to induce disassembly, the time required for onset of disassembly from the minus end was extremely varied (Figure 4A). Sev-

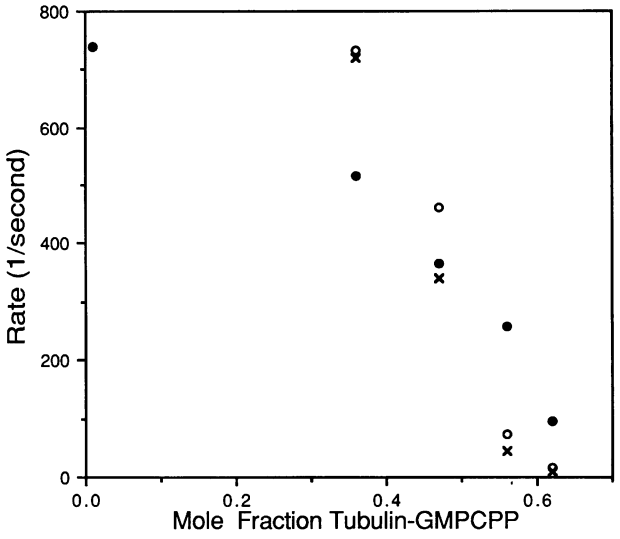

Figure 6. Observed and calculated dependence of the rate of disassembly of mixed lattice microtubules. The observed rate (●) and calculated rate (right column of Table 4), assuming that disassembly is slowed when 13 (×) or 14 (○) contiguous tubulin-GMPCPP subunits are at an end.

eral puzzling inferences are generated by assuming that the delay preceding rapid disassembly results from the stepwise loss of tubulin-GMCPP subunits with a mean lifetime of about 15 s: the brief delay (30–120 s) for many microtubules suggests that microtubules can be stabilized by as few as two to eight tubulin-GMPCPP subunits. If this were true then microtubules in which tubulin-GMPCPP constitutes 35–60% of the subunits would be extremely stable, which was not observed (Table 4). Also, according to the measured second-order rate constant for elongation with tubulin-GMPCPP, reaction of the microtubule minus-end with 1 μM tubulin-GMPCPP for 25 s would add about 40 tubulin-GMPCPP subunits so that addition of only two to eight subunits to a significant fraction of the microtubules is unlikely. Finally, the dispersion in the lifetime of the stabilized microtubules is unreasonably large; lifetimes from 25–2000 s would correspond to an 80-fold variation in the number of tubulin-GMPCPP subunits added during the 25-s incubation.

To account for these discrepancies, the size of the tubulin-GMPCPP cap at the end of stabilized microtubules was estimated by dividing the delay time by the 15-s lifetime of a terminal tubulin-GMPCPP subunit, further assuming that 14 tubulin-GMPCPP subunits, one at the end of each of the microtubule's 14 protofilaments, are required for the cap to be stable. Loss of a single tubulin-GMPCPP subunit is presumed to destabilize the cap and allow catastrophe. According to this model a microtubule with 14 tubulin-GMPCPP subunits has a lifetime of only 15 s, whereas a sixfold longer cap with 84 subunits would have a 71-fold longer lifetime, corresponding to dissociation of 71 tubulin-GMPCPP subunits. Unlike the distribution of microtubule lifetimes described in Figure 4A, the calculated length distribution (Figure 4B) has a realistic dispersion and the cap lengths are in reasonable accord with those expected for a reaction in which about 40 GMPCPP subunits are added to a microtubule end. The peak of the histogram at 18 rather than 40 subunits, calculated from the rate determined with a 4.5-fold higher concentration of tubulin-GMPCPP subunits, might result because the elongation rate is not perfectly proportional to the subunit concentration. Also, there may be a short lag for addition of tubulin-GMPCPP subunits on a GTP cap, and microtubules that are stabilized by tubulin-GMPCPP subunits may grow more slowly than the average population. Based on these results we propose that the presence of a single tubulin-GMPCPP subunit at the end of each of the microtubule's 13 or 14 protofilaments is both sufficient and necessary to stabilize a microtubule against disassembly. As described above, this mechanism can also account for the dependence of the disassembly rate on the fraction of subunits containing GMPCPP, in microtubules containing both tubulin-GDP and tubulin-GMPCPP subunits (Figure 6).

Earlier Results

Our estimate of the size of the minimum stabilizing tubulin-GMPCPP cap is in agreement with the 22-subunit value determined from measurements of the intensity of the fluorescence of caps (Drechsel and Kirschner, 1994). However, this 22-subunit value was not in agreement with the observation that only 10% of microtubules had a stable cap when an average of 53 tubulin-GMPCPP subunits had been added to the end (Drechsel and Kirschner, 1994). This discrepancy is believed to result from a mistaken assumption that it was the more rapidly growing plus-end that was capped by added tubulin-GMPCPP subunits; the addition of 53 subunits was calculated from the higher rate constant for elongation of this end. Our results (Table 5) suggest that it was the slower-growing minus-end that was capped, so that fewer than 53 subunits were added to the end of stabilized microtubules. In support of this, in the earlier study only 50% of the GTP-microtubules at the ends of stable seeds were stabilized when these were diluted into 1 μM tubulin containing 1 mM GMPCPP and only about 10% of microtubule seeds were stable at both ends. Although it was assumed that the reaction occurred randomly at the two ends, we found that despite the more facile addition of tubulin-GMPCPP subunit at

the plus-end, the higher catastrophe rate at this end apparently makes stabilization less frequent (Table 5).

It was previously suggested that the microtubule cap contains about 23–44 tubulin-GTP subunits, because microtubules start to disassemble within 1 second after dilution (Voter *et al.*, 1991) and the rate constants are equal to 23–44 s^{-1} (Walker *et al.*, 1988) for dissociation of tubulin-GTP subunits from the ends of growing microtubules. However, as described above, the rate constant for tubulin-GTP subunit dissociation has also been reported to be 0.37 and 1.1 s^{-1} (Mitchison and Kirschner, 1984), so that the onset of disassembly within 1 second would correspond to destabilization of a cap by loss of only one tubulin-GTP subunit from a 14-subunit cap, in agreement with the model proposed here.

In summary, results in Figures 3 and 4A are consistent with the model that had been proposed by Bayley *et al.* (1990) and Drechsel and Kirschner (1994), in which loss of a single tubulin-GTP subunit from a 14-subunit cap results in destabilization of the cap and the phase transition to rapid disassembly. The important element in the model is that the phase transition to rapid disassembly is precipitous, depending on a single molecular event, hydrolysis of a single GTP molecule within a monolayer of tubulin-GTP subunits at the microtubule end.

Recalculation of the Equilibrium Constant for Hydrolysis of Nucleotide Triphosphate in Microtubules

Results given here show that our previous determination of the equilibrium constant for hydrolysis of GMPCPP in microtubules was based on an incorrect rate constant for dissociation of tubulin-GMPCPP subunits. The measured rate was too high because tubulin-GDP subunits, even at very low fractional concentrations, have a dramatic effect on the rate of dissociation of tubulin-GMPCPP subunits. Our study of this phenomenon showed that tubulin-GDP alters the disassembly rate, even when present at one part per 110 parts.

Using data obtained with GMPCPP microtubules in which tubulin-GDP subunits constitute only one part per 270 parts, the equilibrium constant for the reaction:

$$\text{Tubulin-GMPCPP} + \text{MT} \rightleftharpoons \text{Tubulin-GMPCPP-MT} \quad (1)$$

derived from the ratio of the rate constants for assembly and disassembly at the microtubule's plus-end is equal to $4 \times 10^6\ M^{-1}\ s^{-1}/0.118 = 3.39 \times 10^7$, corresponding to a change in free energy equal to −10.24 kcal/mol. Based on the earlier measurements of the related equilibria (Caplow *et al.*, 1994), a standard free energy equal to +0.7 kcal/mol is calculated for hydrolysis of microtubule-bound nucleotide triphosphate and dissociation of Pi.

$$\text{Tubulin-GMPCPP-MT} \rightleftharpoons \text{Tubulin-GMPCP-MT} + \text{Pi} \quad (2)$$

Assuming a K_d equal to 250 mM for Pi dissociation the standard free energy is −0.10 kcal for the reaction:

$$\text{Tubulin-GMPCPP-MT} \rightleftharpoons \text{Tubulin-GMPCP-MT-Pi} \quad (3)$$

The high K_d for Pi is supported by studies of the effect of Pi on microtubule dynamics (Caplow *et al.*, 1989; Trinczek *et al.*, 1993). It is important to note that although the positive free energy for reaction 2 means hydrolysis is thermodynamically unfavorable, because Pi is buffered in cells at approximately 1 mM, if the K_d for Pi is <1 the change in free energy for nucleotide triphosphate hydrolysis in the microtubule will ultimately be favorable when Pi is released from the microtubule. Assuming a $K_d < 1$, and Pi in cells at 1 mM, the change in free energy for reaction 2 is −3.38 kcal, so that 1.7 kcal of the 5.08 kcal potentially available from GMCPP hydrolysis would be stored in the microtubule lattice.

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