Dynamic Instability of Microtubules Assembled from Microtubule-Associated Protein-Free Tubulin: Neither Variability of Growth and Shortening Rates nor "Rescue" Requires Microtubule-Associated Proteins†

Martin A. Billger,‡ Gourab Bhatacharjee, and Robley C. Williams, Jr.*

Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235

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ABSTRACT: The growth and shortening of microtubules in dynamic instability is known to be modulated by microtubule-associated proteins (MAPs). A full understanding of the mechanism of dynamic instability requires that one distinguish which of its aspects are mediated by microtubule-associated proteins (even in small residual concentrations) and which are intrinsic properties of the tubulin lattice itself. This paper addresses two of those aspects: whether MAPs cause the rescue events of dynamic instability (*i.e.*, the transitions from shortening to growth) and whether MAPs are responsible for the marked variability of the rates at which microtubules grow and shorten. Very pure tubulin was prepared by sequential chromatographies on phosphocellulose and DEAE-Sephadex. Analysis by electrophoresis and immunoblotting showed it to be essentially MAP-free; it contained fewer than one MAP molecule per 10 000 tubulin dimers. When its dynamic instability was studied by video-DIC microscopy, rescues were found to occur at a mean frequency of one per 4 μm of shortening. Variability of rates of growth and shortening, which is observed on the length scale of a few micrometers, was not changed by removal of MAPs. Because the mean distance between bound MAP molecules was calculated to be greater than 14 μm in these experiments, it is concluded that they cannot cause either rescue or variability of rates.

When individual microtubules are observed by light microscopy, both *in vivo* and *in vitro*, they display alternating phases of growth and shortening, collectively termed dynamic instability [Mitchison & Kirschner, 1984; Horio & Hotani, 1986; Walker *et al.*, 1988; Gildersleeve *et al.*, 1992; reviewed by Erickson and O'Brien (1992), by Cassimeris (1993), and by Bayley *et al.* (1994)]. This phenomenon appears to be important in the regulation of such cellular functions as intracellular organelle transport, formation of the mitotic spindle, and maintenance of cellular shape [review, Cassimeris (1993)]. The characteristic large and rapid excursions in length succeed each other in a seemingly stochastic manner. A particular microtubule may grow (by addition of subunits to its end) for some seconds or minutes and then shorten (by loss of subunits) again for some seconds or minutes. A group of microtubules can be statistically characterized in terms of the average values of the rates of growth and shortening of the individual microtubules that compose it and by the average frequencies with which they convert between growth and shortening phases (Walker *et al.*, 1988; Gildersleeve *et al.*, 1992; Gliksman *et al.*, 1993; Martin *et al.*, 1993).

The current understanding of the mechanism of dynamic instability rests on the properties of the hypothetical stabilizing cap at the end of the microtubule, which is composed of subunits bearing either GTP or GDP and P_i[1] [reviews, Erickson and O'Brien (1992) and Bayley *et al.* (1994)]. When the cap is present, the rate of association of subunits with the end is believed to be large and their rate of dissociation slow; when the cap is absent, the association rate is small and the dissociation rate large. The transition between growth and shortening (called *catastrophe*) is thought to occur when a critical number (some or all) of the GTP-containing subunits are lost from the end; the transition from shortening to growth (called *rescue*) must occur through a recapping process.

Rescue is perhaps the least well understood aspect of this dynamic instability mechanism. Rates of shortening of microtubules can be quite rapid, often higher than 10 000 subunits/s (O'Brien *et al.*, 1990; Gildersleeve *et al.*, 1992). It is likely that shortening occurs not by the orderly loss of single tubulin dimers from the microtubule end but by the loss of fairly large pieces of protofilaments (Simon & Salmon, 1990; Mandelkow & Mandelkow, 1991; Mandelkow *et al.*, 1991). Rescue, which must occur by recapping, must somehow involve a halting of this rapid "peeling away" of protofilaments and the restoration of GTP-containing subunits to the microtubular end. The presence of microtubule-associated proteins (MAPs) is known to increase the frequency of rescue (Pryer *et al.*, 1992) and to do so in a concentration-dependent fashion (Drechsel *et al.*, 1992; Kowalski & Williams, 1993a; Vasques *et al.*, 1994; Itoh &

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* Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235. Telephone: 615-322-2072. Fax: 615-343-6707. E-mail: williarc@ctrvax.vanderbilt.edu.

‡ Current address: Department of Zoophysiology, Goteborgs Universitet, Medicinaregatan 18, 413 90 Goteborg, Sweden.

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[1] Abbreviations: DEAE-PC-tubulin, tubulin purified from microtubule protein by successive chromatography on phosphocellulose and DEAE-Sephadex; MAPs, microtubule-associated proteins; 3X-MTP, microtubule protein prepared by three cycles of assembly/disassembly purification; P_i, inorganic phosphate; PC-tubulin, tubulin purified from 3X-MTP by phosphocellulose chromatography; PMEG buffer, 100 mM Pipes (pH 6.8), 1 mM $MgSO_4$, 1 mM EGTA, and 1 mM GTP; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Hotani, 1994; Ainsztein & Purich, 1994; Gamblin *et al.*, 1996). MAPs might do this by bridging protofilaments and holding them together at particular places, thus raising the energetic cost of removal of more pieces of protofilament and thereby allowing recapping to occur. Indeed, as one might expect, several studies have observed the mean length of a shortening event to increase as MAP concentration is decreased, but rescues were still seen at the smallest concentrations of MAPs studied (Drechsel *et al.*, 1992; Kowalski & Williams, 1993; Itoh & Hotani, 1994). Tubulin preparations contain residual MAPs, the concentrations of which were not characterized in detail in the studies cited.

The first question we address here is whether eliminating *all* MAPs also eliminates rescue. One might imagine that rescue is not directly a property of the fundamental tubulin lattice but rather depends on the presence of at least a few MAP molecules bound to the microtubule lattice. If that were the case, sufficiently pure tubulin would display dynamic instability behavior that lacked rescues; all shortening events would continue until the nucleating structure was reached.

Rates of assembly and disassembly of microtubules made from phosphocellulose-purified tubulin have been observed to be highly variable (Drubin & Kirschner, 1986; Gildersleeve *et al.*, 1992; Drechsel *et al.*, 1992; Chrétien *et al.*, 1995). The cause of the variability is not known, but one hypothesis to be considered is that it is caused by trace amounts of MAPs, which might cluster together at irregular intervals, altering rates of assembly and disassembly by their presence. The second question we address, therefore, is whether sufficiently pure tubulin would display uniform rates of assembly and disassembly.

To address these two questions, we purified tubulin from MAP-containing microtubule protein by sequential chromatography on phosphocellulose and DEAE-Sephadex. By gel electrophoresis and immunoblotting, it was found to have extremely small amounts of contaminating MAPs, less than 1 mol of MAP per 10 000 mol of tubulin dimer. This ratio can be calculated to produce one bound MAP molecule per 13.7 μm of assembled microtubule at the concentrations examined. In spite of this wide spacing between bound MAPs, normal dynamic instability was observed. Extended light-microscopic observation of microtubule growth and shortening revealed a substantial frequency of rescues, thus demonstrating that rescues are a property of the tubulin lattice and not the result of the presence of MAPs. Growth and shortening rates were highly variable, indicating that variability of these properties is also not caused by MAPs.

MATERIALS AND METHODS

Initial Protein Purification

Microtubule proteins (called 3X-MTP) were isolated from bovine brain by three cycles of temperature-induced assembly/disassembly in PMEG buffer [100 mM Pipes (pH 6.8), 1 mM $MgSO_4$, 1 mM EGTA, and 1 mM GTP]. Tubulin (called PC-tubulin) was isolated by phosphocellulose chromatography, and proteins were frozen as drops in liquid N_2 and stored at −70 °C, all as previously described [Williams and Lee (1982) as modified in Correia *et al.* (1987)].

Further Protein Purification

The traces of MAPs present in PC-tubulin were removed in two ways. By a method adapted from Vallee (1986), PC-tubulin (50 mg) was applied to a 1.5 × 10 cm column of DEAE-Sephadex (Pharmacia), pre-equilibrated with PMEGD buffer (PMEG buffer with 1 mM DTE). The column was then washed with 2 volumes of PMEGD followed by 2 volumes of PMEGD with 0.15 M NaCl, at a flow rate of 0.8 mL/min. Tubulin was eluted isocratically in PMEGD with 0.4 M NaCl, concentrated by membrane ultrafiltration (Centricell 20, Amicon Corp., 30 kDa cutoff), gel-filtered into PMEG (NAP-5 columns, Pharmacia), and finally drop-frozen in liquid N_2 and stored at −70 °C. This material is called DEAE-PC-tubulin. The yield of these purification steps was approximately 65%.

PC-tubulin was also subjected to cycles of assembly/disassembly in buffer containing 1 M sodium glutamate [modified from Voter and Erickson (1984)]. The protein was first gel-filtered into 0.025 M Mes, 1 mM EGTA, 0.5 mM $MgSO_4$, and 0.5 mM GTP. Monosodium glutamate was added as a solid to 1 M, and the GTP concentration was adjusted to 1 mM. The protein was incubated for 20 min at 37 °C to allow assembly. Microtubules were pelleted through a cushion of 5% sucrose in the same buffer (60000*g* for 30 min at 37 °C), resuspended in 0.5 mL of PMEG, gel-filtered into PMEG, centrifuged for 20 min at 100000*g* to remove traces of denatured protein, drop-frozen, and stored at −70 °C until use. This material, obtained in 45% yield, is called glutamate-PC-tubulin.

Analysis of Proteins

SDS Gels. Protein concentrations were determined according to Bradford (1976). Protein preparations were analyzed by SDS electrophoresis (Laemmli, 1970), on 8% gels with a thickness of 0.75 mm. Samples were dissolved in freshly made standard sample buffer approximately 1 h before application to the gel, but were not boiled, in order to prevent generation of small peptides that evidently arise from degradation of tubulin at high temperatures (Kowit & Maloney, 1982; Detrich *et al.*, 1983; Kubo, 1995). Detection of tubulin and MAPs was by staining with Coomassie blue (B/T Blu, BT Scientific Technologies, Carlsbad, CA) or by silver staining ("Silver Stain Plus", Bio-Rad, Richmond, CA).

Western Blots. Proteins were transferred to poly(divinylbenzene) membranes for 1 h at 30 V in 0.025 M Tris/glycine (pH 8.6) and 20% methanol (Lobert & Correia, 1994). The efficacy of transfer was assessed by staining the gels recovered from the blotting procedure. Because the intention of the detection method was to reveal as many MAPs as possible with the greatest sensitivity, a mixture of primary antibodies was employed and blots were developed by the amplified alkaline phosphatase method (kit 170-6411, Bio-Rad, Hercules, CA). Rabbit polyclonal anti-MAPs (M-7273, Sigma, St. Louis, MO) was mixed at 1:200 with polyclonal rabbit anti-τ (T-6402, Sigma) at 1:100. In order to obtain an assessment of the upper limits of possible MAP contamination, a calibration series of known amounts of a preparation of PC-MAPs (the entire MAP fraction eluted from phosphocellulose after tubulin) was subjected to electrophoresis in the same gel. The upper limits to the quantity of MAPs remaining in the DEAE-PC-tubulin preparations were then estimated by a comparison technique. To compensate for

possible variations in efficiency of transfer and in staining between one blot and the next, all quantitative comparisons were made between lanes in the same blot, never between blots. Because no MAPs could be detected in DEAE-PC-tubulin, even in the most heavily loaded lanes, the fractional concentrations of MAPs present in those samples were deemed to be less than the minimum detectable in the calibration series. In considering the results below, we therefore deal with upper limits.

Isoelectric Focusing. Tubulin samples were subjected to isoelectric focusing in 3.9% acrylamide/0.21% bis-acrylamide gels (16 × 14 × 0.15 cm) in the presence of 9.16 M urea, 2% NP-40, 2.5% Servalyte 5-6, 1.25% Servalyte 4-6, and 1.25% Servalyte 5-7 (Serva Biochemicals, Paramus, NJ). Following prefocusing at 8 W and 16 °C for 1 h, tubulin samples prepared with urea, detergent, and Servalytes as above and supplemented with 4.5% β-mercaptoethanol were applied (12.5 μg per lane) and focused at 10 W and 16 °C for 5 h, with the use of 0.05 M H_3PO_4 as the anolyte and 0.05 M NaOH (degassed) as the catholyte. Gels were fixed for 1 h in 5% trichloroacetic acid/5% HAc, stained for 2 h with 0.2% Coomassie Brilliant Blue in 45% methanol/20% HAc, and destained in 10% HAc.

Videomicroscopy of Microtubules. Microtubules assembled on sea urchin axonemal pieces were observed at 37 °C by video-enhanced DIC microscopy, as described earlier (Gildersleeve *et al.*, 1992; Williams, 1992). A solution of axonemes (2−4 μL) was placed on a 22 × 22 mm coverslip (Fisher, #1.5, 0.16−0.19 mm thick), and sufficient tubulin (diluted in PMEG buffer and 1 mM GTP) was added to make a final volume of 12 μL. The coverslip was then sealed to a glass slide with VaLaP (a 1:1:1 mixture of Vaseline, lanolin, and paraffin) and mounted in the microscope. A useful concentration of DEAE-PC-tubulin was found to be 1.8 mg/mL (final concentration) and for glutamate-PC-tubulin 1.5 mg/mL. Below these values, which were used in all the experiments, nucleation frequency quickly became negligible. We suspect that the difference between the two useful levels resulted from the presence of small amounts of inactive tubulin. Selected microscopic fields, generally containing only a few axonemes, were recorded. To allow establishment of a constant temperature and to avoid analysis of aged proteins, measurement included, but did not extend beyond, a 60 min time period starting 15 min after the onset of assembly.

Analysis of Dynamics Data. Analysis was carried out by means of a computer program similar to that described by Kowalski and Williams (1993), making use of the approach described by Gildersleeve *et al.* (1992). The lengths of individual microtubules were measured over time with a computer-generated video-overlaid mouse cursor during playback of recorded sequences in real time (Walker *et al.*, 1988; Williams, 1992). Periods of shortening were additionally measured in a frame-by-frame mode by advancing the tape several frames between measurements, manually entering the time recorded by the image processor. Rates of growth were calculated by an 11−point running second-order curve fit of the data. A rate that differed from zero at the 95% confidence level (*i.e.*, by more than 1.64 times its standard deviation) was classified as a significant growth or shortening rate. Those periods of time when the rate of change of length could not be distinguished from zero were termed "inactive". This category certainly includes microtubules that are growing or shortening at very small rates and may also include some that are not changing length at all [the "pause" referred to by Walker *et al.* (1988) or the "attenuated" state of Toso *et al.* (1993)]. A catastrophe was defined as a range of significant rates where the sign of the slope changed from positive to negative, while rescues were defined similarly when the sign of the slope changed from negative to positive. For consistency with past analyses, changes to and from inactive periods were not included in the evaluation of catastrophes and rescues. Frequencies for catastrophes (or rescues) were calculated as the number of catastrophes (or rescues) divided by the total time spent in growth (or shortening) (min^{-1}) or the total length covered by growth (or shortening) (mm^{-1}) (Kowalski & Williams, 1993). Following rate analysis, data from several individual microtubule measurements were pooled to obtain population statistics. No attempt was made to differentiate (+)-ends from (−)-ends in the gathering or analysis of data.

RESULTS

Purity of DEAE-PC-Tubulin and Glutamate-PC-Tubulin. When examined by Coomassie blue-stained SDS−PAGE, ordinarily prepared PC-tubulin was seen to contain traces of polypeptides with molecular weights greater than that of tubulin [not shown, see Williams and Lee (1982)]. Because of their apparent molecular weights and their copurification with tubulin during assembly/disassembly, these polypeptides probably corresponded to MAP1, MAP2, τ, and other minor proteins. Further purification by DEAE chromatography produced tubulin (DEAE-PC-tubulin) which did not reveal these polypeptides. Figure 1 shows characterization, by SDS electrophoresis and by immunoblotting, of the purified tubulin used for measurement of dynamics. Figure 1A shows that non-tubulin components could not be detected on heavily overloaded Coomassie-stained gels. The smallest total amount of MAPs that could be visualized by Coomassie blue staining was somewhat greater than 0.1 μg per lane (lane 5) and by silver stain about 0.03 μg (not shown). To improve the sensitivity of detection of MAPs, and to make it possible to reveal MAPs that might be obscured by the large tubulin bands in overloaded gels, immunoblots were performed. Figure 1B shows a blot probed with a mixture of antibodies intended to yield broad specificity in detection of MAPs. Lanes 3 and 4 of this blot contained DEAE-PC-tubulin. Lane 3 is the most heavily loaded, with 194 mg of protein. Lane 5 of this blot contained 0.1 μg of PC-MAPs, and it is clear from direct observation that most MAPs are easily visible in a lane loaded with this amount of material. Because no MAP bands at all are visible in lane 3, although MAPs are easily visible in lane 5, one can infer that the weight fraction of all MAPs together in the tubulin sample is less than (0.1 μg)/(194 μg), or 0.00052. To extend the limit further, another blot was probed with a higher concentration of primary antibodies, as shown in Figure 1C. Greater sensitivity was obtained under these conditions, but at the cost of noticeable cross-reactivity; tubulin was labeled as well as several of the molecular weight marker proteins. A contaminating band is also seen at an apparent molecular weight of 68000. Because of its apparent molecular weight, because of the fact that it is not accompanied by the "ladder"of related isoforms characteristic of τ proteins, and because it was not systematically present on all blots probed with these antibodies (not shown), we infer that this band is

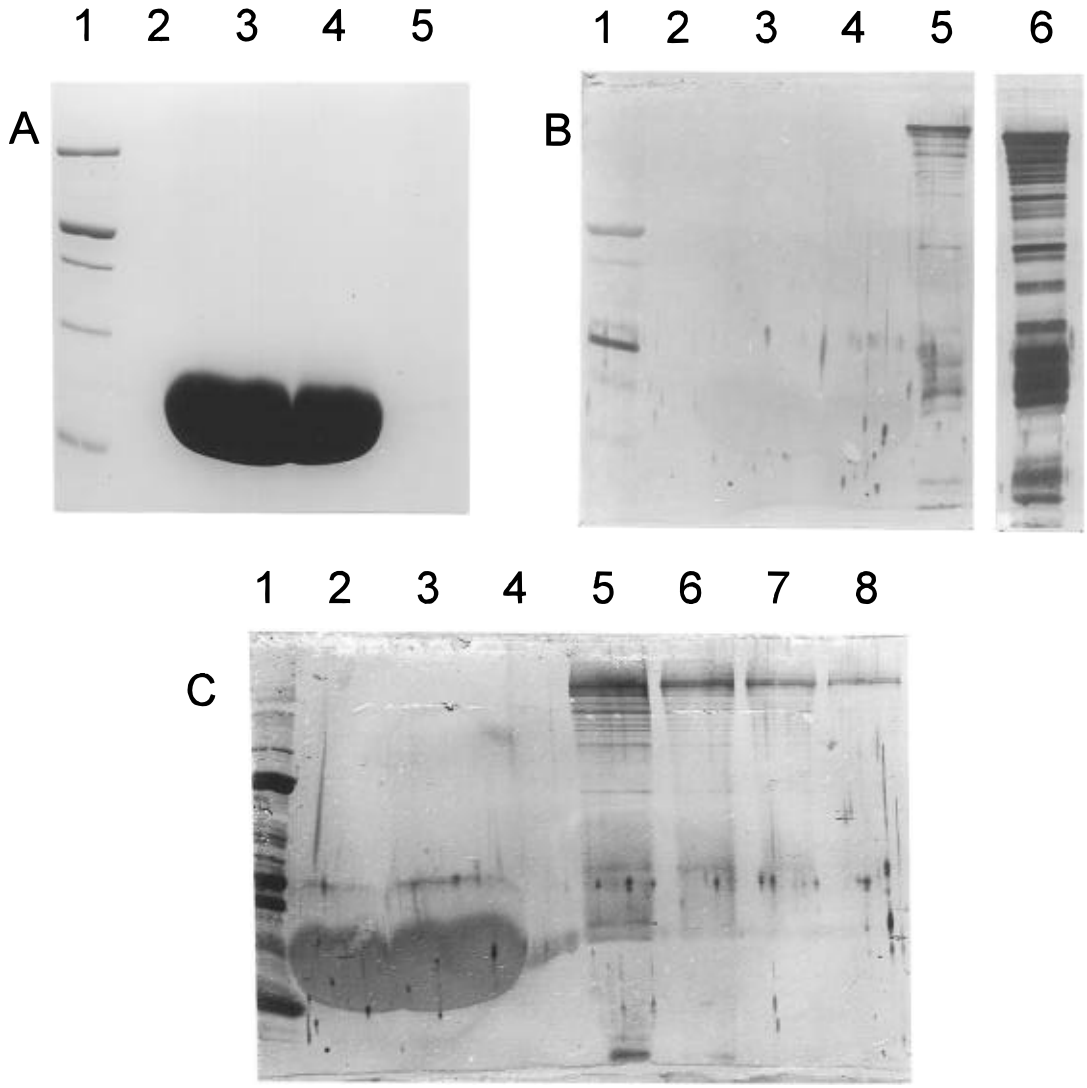

FIGURE 1: Electrophoretic analysis of tubulin purified by phosphocellulose and DEAE chromatography (DEAE-PC-tubulin). Calibration standards were run in the same gels and blots as the experimental samples. (A) Coomassie Brilliant Blue-stained gel. Lanes: 1, molecular weight markers; 2, empty; 3, DEAE-PC-tubulin (197 μg); 4, DEAE-PC-tubulin (94 μg); and 5, MAPs (0.1 μg) (no bands are visible). (B) Lanes 1−5, identically numbered immunoblot of an identical gel, run simultaneously and probed with a mixture of anti-τ and a broad-specificity anti-MAP antibody (see Materials and Methods). Lane 6, lane from the same blot, 0.37 μg of PC-MAPs. (C) Immunoblot probed with twice the concentration of primary antibodies as in panel B. Lanes: 1, molecular weight standards; 2, DEAE-PC-tubulin (97 μg); 3, DEAE-PC-tubulin, (197 μg); 4, empty; and 5−8, MAPs (0.1, 0.06, 0.04, and 0.02 μg, respectively). The band just above tubulin (seen to be a doublet on close examination) appears to result from contamination during the preparation of the gel. Because of its molecular weight and idiosyncratic distribution, it seems likely that it represents keratin from the investigator's fingers, most probably basic keratin type II (Moll *et al.*, 1982; Sun *et al.*, 1985). A similar band can be seen in lane 4 of panel B, but not in lane 3, where the quantity of tubulin was twice as great.

almost certainly adventitious human keratin. By comparing the pattern presented by the 0.02 μg of MAPs present in lane 8 with the MAP-free pattern presented by 194 μg of tubulin in lane 3, one may infer that the fraction of high-molecular weight MAPs present in DEAE-PC-tubulin (chiefly MAP2 in our preparations) is less than (0.02 μg)/(194 μg), or 0.00010.

A less extensive characterization of glutamate-PC-tubulin in Coomassie blue-stained gels yielded an upper limit for fractional MAP contamination of 0.001. The purity of both DEAE-PC-tubulin and glutamate-PC-tubulin was further examined by perchloric acid extraction of τ (Lindwall & Cole, 1984). No detectable amount of τ was seen on the resulting SDS gels (data not shown).

Isoelectric focusing was employed to assess whether the extensive purification of tubulin might have caused the inadvertent removal of one or more particular tubulin isoforms. Figure 2 shows the patterns produced by PC-tubulin and the two tubulins purified from it. The number of tubulin isoforms is large in each case, but there were no obvious differences between the three preparations. The combined use of phosphocellulose chromatography and either DEAE chromatography or cycling in 1 M glutamate therefore appears to remove non-tubulin polypeptides without perturbing the isoform distribution of tubulin itself.

Calculation of the Extent of Binding. From the knowledge of the upper limit to the fraction of MAPs present in a solution of tubulin, it is possible to estimate the upper limit to the extent of binding of MAPs to microtubules. From that number, one can then obtain the *lower* limit to the mean length of shortening that would be observed, under the extreme assumption that a single bound MAP molecule is able to stop a shortening excursion. That lower limit is equivalent to the mean distance between bound MAP

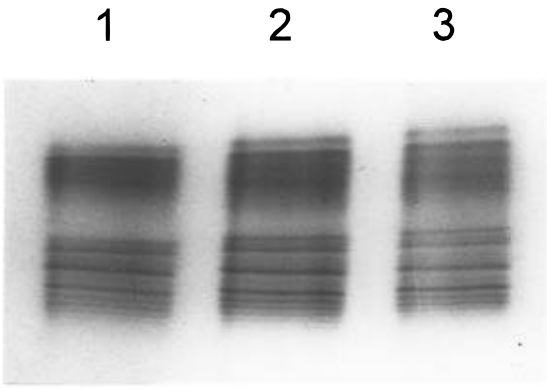

FIGURE 2: Isoelectric focusing gel of tubulins: lane 1, DEAE-PC-tubulin; lane 2, PC-tubulin; and lane 3, glutamate-PC-tubulin. Each lane contained 12.5 μg of protein. Gel was stained with Coomassie Brilliant Blue.

molecules or clusters of molecules. Because MAP binding can be cooperative, MAPs may tend to bind in clusters. At the extremely small concentrations of MAPs present in our preparations, the extent of binding could not be measured directly, but it can be estimated from measurements made at higher concentrations. The most fully applicable are those of Wallis *et al.* (1993), who analyzed binding of MAPs to taxol-stabilized microtubules under conditions closely similar to those used here. They were able to fit their data, which related the concentration of bound MAPs to the total concentration of MAPs, by an equation of McGhee and von Hippel (1974):

$$\nu/L = K(1 - n\nu)[[(2\omega + 1)(1 - n\nu) + \nu - R]/[2(\omega - 1)(1 - n\nu)]]^{n-1}[[1 - (n + 1)\,\nu + R]/[2(1 - n\nu)]]^2 \quad (1)$$

where ν represents the number of MAPs bound per tubulin dimer (much smaller than 1 in the present circumstances), L is the concentration of free MAPs, n is the number of tubulin dimers covered by a MAP when it binds, and ω is the factor by which the association constant, K, is increased when a MAP has a nearest neighbor. The quantity R is given by

$$R = [[1 - (n + 1)\nu]^2 + 4\omega\nu(1 - n\nu)]^{1/2} \quad (2)$$

With the use of the binding parameters given in Figure 4 of Wallis *et al.* (1993) (K = 0.04 mM, n = 75, and ω = 1500), one can calculate the approximate occupancy of sites on the microtubule. The parameters may not bear the precise physical significance assigned them in the theory, but they serve to describe well the binding behavior within the low-concentration range. τ protein can be disregarded because it represents a very small fraction of the MAPs in our preparations. It binds noncooperatively and much less strongly than does MAP2 (see Discussion).

The tubulin dimer concentration in the experimental solutions was 18 μM. The primary constituents of the MAPs are the high-molecular weight MAPs, MAP1 and MAP2, leading one to assign a rough average molecular weight of 163 000 to the mixture (Kurz & Williams, 1995). Because the approximate upper limit to the molar fraction of MAPs is about 1.0×10^{-4}, the upper limit of the MAP concentration is 18 μM $\times$ 0.00010 $\times$ (100000/163000) = 0.0011 μM. At that concentration, a value of ν of 4.8×10^{-5} MAP/(tubulin dimer), or 1 MAP per 20 800 tubulin dimers, can be calculated from eqs 1 and 2 and from the concentration of tubulin present in microtubules.[2] If MAPs bound with uniform spacing, this would correspond to 1 MAP per 12.8 μm. Because of cooperativity, MAPs tend to cluster, increasing somewhat their mean spacing. The mean spacing in the McGhee−von Hippel model is given by Wegner (1979) by the quantity g, where

$$g = \frac{2(n\nu - 1)(\omega - 1)}{1625(1 + \nu - n\nu - R)} \quad (3)$$

Under these circumstances, one estimates a minimum distance between small clusters of MAPs (mean cluster size is 1.07 molecules) equal to 13.7 mm. This distance is large when compared to the mean length of a shortening event (see below).

Dynamic Instability Measurements. Microtubules formed by DEAE-PC-tubulin and by glutamate-PC-tubulin displayed all the qualitative characteristics of dynamic instability, including catastrophes, rescues of shortening microtubules, and variable rates of growth and shortening. Figure 3A shows several representative examples of length *vs* time plots of microtubules measured in real time. Catastrophes and rescues were frequent. Growth rates varied, both within individual microtubules and between neighboring microtubules in the same microscopic field. Similar characteristics were observed in measurements of glutamate-PC-tubulin. Shortening events were measured separately in a frame-by-frame mode because their rapidity allowed only a few data points to be collected in real time, in spite of frequent sampling. Examples appear in Figure 3B. Shortening rates were found to vary, both within and between microtubules.

To expand the characterization of dynamic instability in these essentially MAP-free microtubules, a full set of measurements of length *vs* time was performed. Panels A and B of Figure 4 show frequency histograms of these measurements of rates of growth and shortening of DEAE-PC-tubulin. They reveal a broad distribution of growth rates and of shortening rates, similar to those that appear in measurements of microtubules prepared by ordinary purification. Figure 4C shows the distribution of growth rates obtained for glutamate-PC-tubulin. This distribution is, again, broad.

Table 1 presents the numerical values of dynamic instability parameters for DEAE-PC-tubulin and for glutamate-PC-tubulin. The first point that emerges is that rescues are observed in highly purified tubulin with about the same frequency (0.25 rescues per micrometer of shortening) as in ordinarily purified tubulin (0.24 rescues per micrometer of shortening). The observed mean length of shortening (4.0 μm) is about $^1/_3$ of the minimum spacing between bound MAPs calculated above. A second point is that the catastrophe and rescue frequencies, the mean rates of growth and

[2] In these saturation calculations, it is necessary to estimate the fraction of the total tubulin actually present in microtubules. The total molar concentration of MAPs, c_{tot}, is composed of bound MAPs and free MAPs. Therefore, $c_{tot} = c_{free} + nc_{mt}$, where c_{free} is the molar concentration of free MAPs, n is the fraction of tubulin dimers bearing a bound MAP, and c_{mt} is the molar concentration of tubulin dimers present in microtubules. In the conservative spirit of this estimate, it was assumed that the concentration of tubulin in microtubules was 2 μM. Larger assumed values of c_{mt} produce slightly larger calculated mean spacings between MAPs, although the calculated spacing is quite insensitive to the assumed value of c_{mt}.

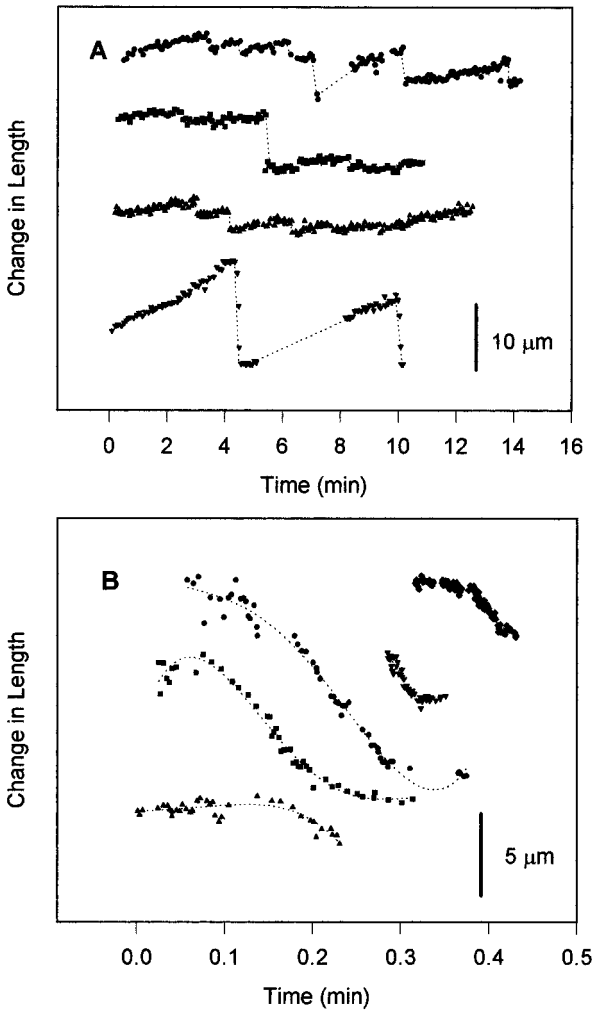

FIGURE 3: Length *vs* time plots of microtubules assembled from DEAE-PC-tubulin. Microtubules consisting of DEAE-PC-tubulin were nucleated on sea urchin axonemes, and their lengths were measured over time. The polarities of the four ends shown were not identified but probably represent two (−)-ends and two (+)-ends. Plots are offset from each other, to allow easier comparison: (A) lengths measured in real time and (B) lengths measured in frame-by-frame mode for shortening events. Points of different shapes represent measurements of different microtubules. The curves are offset vertically for clarity of representation. Dashed lines indicate the trend of the data. Bars represent scales of length.

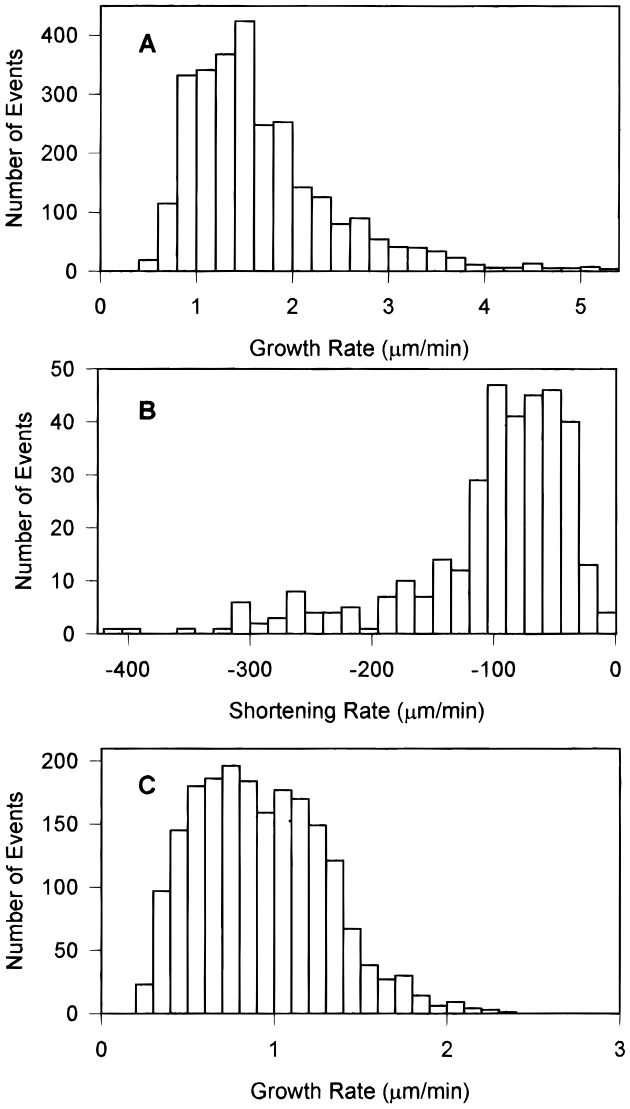

FIGURE 4: Histograms of rates of growth and shortening of microtubules assembled from highly purified tubulins. The data include both (+)-ends and (−)-ends, which were not differentiated in the analysis. (A and B) Growth and shortening rates, respectively, for microtubules of DEAE-PC-tubulin. Growth rates were measured in real time; shortening rates were measured in frame-by-frame mode (see Materials and Methods). Note that the scale for shortening rates increases in absolute value from right to left. (C) Growth rates for microtubules of glutamate-PC-tubulin.

shortening, and the variabilities of these rates are similar to those previously observed for ordinary PC-tubulin under similar conditions. The observed mean shortening rate is notably greater, by a factor of 1.5−2, than that previously observed under similar circumstances (Walker *et al.*, 1988; Gildersleeve *et al.*, 1992; Kowalski & Williams, 1993b; Gamblin & Williams, 1995). The reasons for this result are not clear. It can nevertheless be concluded that the qualitative causes of catastrophe and rescue as well as the cause for rate variability are contained in tubulin itself.

DISCUSSION

Purity of Tubulin. The purification of tubulin by successive chromatographies on negatively and positively charged media yielded material with concentrations of MAPs too small to measure. The comparison standards employed to estimate concentration were the mixtures of MAPs eluted after tubulin in phosphocellulose chromatography. The composition of this "whole MAP" preparation, as assessed by SDS gel electrophoresis, is very similar to that of the solution of microtubule protein from which it is purified (Kurz & Williams, 1995) and probably represents a faithful sample of the presumed residual MAPs whose concentrations are assayed. MAP2a/b is the chief constituent of this fraction, along with much smaller amounts of MAP1a/b/c, τ, tubulin, and neurofilament proteins. The possibility that, in the tubulin purification, one or more particular MAPs are selectively enriched over the others during the chromatographies cannot be definitely excluded. Such a selectively enriched protein, though, would produce a denser band on electrophoresis and would be easier to detect than if no enrichment had taken place. Thus, for the purposes of establishing an upper limit to the fractions of contaminating MAPs, the assumption that no fractionation takes place is the conservative one.[3]

The comparative technique yields an estimate of the upper limit of MAP concentrations which applies to the *sum* of

Table 1: Dynamic Instability Data for DEAE-PC-Tubulin and Glutamate-PC-Tubulin[a]

material	mean length of shortening event (μm)	mean growth rate (μm min^{-1})	mean shortening rate (μm min^{-1})	catastrophe frequency		rescue frequency	
				(min^{-1})	(μm^{-1})	(min^{-1})	(μm^{-1})
DEAE-PC-tubulin	4.0	1.5 ± 0.8	103 ± 71	0.75	0.18	2.0	0.25
glutamate-PC-tubulin	2.9	0.94 ± 0.59	nd	0.35	0.21	0.83	0.34
PC-tubulin, (+)-ends[b]	8.2	2.28 ± 0.84	58 ± 31	0.42	0.16	3.0	0.24
PC-tubulin, (−)-ends[b]	3.9	1.34 ± 0.64	82 ± 53	0.24	0.17	6.0	0.21

[a] Statistical summary of data shown in Figure 4. Mean rates are shown together with their standard deviations. Data from both (+)-ends and (−)-ends are included in the analysis. Note that the mean length of a shortening event is the reciprocal of the rescue frequency when it is expressed on a per-micrometer basis. The number of catastrophes and rescues observed was too small to specify their uncertainties. Growth events were observed in 99 microtubules and shortening events in 13 for DEAE-PC-tubulin. The total time of observation was 511 microtubule minutes for DEAE-PC-tubulin and 481 microtubule minutes for glutamate-PC-tubulin (1 microtubule minute constitutes observation of one microtubule for 1 min). [b] Data for (+)-ends and (−)-ends of microtubules, taken under similar conditions with tubulin purified by phosphocellulose chromatography with the use of standard methods [from Figure 4 and Table 2 of Kowalski and Williams (1993b)].

the concentrations of all the MAPs in the mixture. It is inherently limited because it treats all MAPs as if they were the same. The subsequent analysis attempts to mitigate this difficulty by making conservative assumptions where definitive information is lacking. In the estimation of the mean distance between bound MAPs, the characteristics (dissociation constant and cooperativity parameters) of MAP2 were chosen to represent the binding of all MAPs. This assumption is certainly conservative in regard to τ, which binds to microtubules more weakly than MAP2. Dissociation constants for the noncooperative binding of unphosphorylated τ species are approximately 1 μM (Gustke *et al.*, 1994), reflecting a binding about 25 times weaker than that of MAP2, and this quantity is weakened approximately another 10-fold by the phosphorylation that is present in protein isolated from brain (Drechsel *et al.*, 1992). For the purposes of calculation of upper limits, we may therefore neglect the difference between τ and MAP2. In order to convert the observed weight-based fractions to the required molar fractions, the analysis again presumed that all MAPs are alike and used a conservative effective weight-average molecular weight in the calculation (Kurz & Williams, 1995).

The estimate of levels of MAPs detectable rests partly on the presumed broad specificity of the mixture of antibodies employed. In particular, the mixture may react only weakly with MAP1a/b/c. These proteins constitute, at most, 10% of the MAP fraction, do not appear on silver-stained gels, and are likely to have been removed with approximately the same efficiency as the other MAPs, justifying the neglect of the difference between them and MAP2.

Taking the above considerations into account, it appears that the upper limit of 0.00010 to the mole fraction of MAPs is therefore a sound approximation. The actual concentration of MAPs is likely to be even less than this small number.

Dynamic Instability

Comparison with Other Preparations. The data in Table 1 show that the behavior of DEAE-PC-tubulin and glutamate-PC-tubulin does not differ qualitatively from that of tubulin prepared by other methods (Walker *et al.*, 1988; Gildersleeve *et al.*, 1992; Kowalski & Williams, 1993a,b; Trinczek *et al.*, 1993). Data obtained from PC-tubulin under closely similar conditions (Kowalski & Williams, 1993b) are included for comparison. These data were obtained under conditions that allowed differentiation of (+)-ends from (−)-ends, a fact which must be kept in mind in making the comparison. The conclusions drawn in this paper, though, are independent of the polarity of the microtubules. Microtubules made of tubulin virtually free of MAPs display all the characteristics associated with dynamic instability, including rescue and variable rates of growth and shortening. Therefore, neither the extensive chromatography nor the complete removal of MAPs makes a qualitative change in this phenomenon.

Rescue. The data show that rescue, the transition from shortening to growth, is not qualitatively inhibited by the reduction of MAP concentrations to very low levels. Table 1 shows that rescue frequencies remain in the same range in the MAP-depleted tubulin preparations as in the controls. There is no doubt, of course, that MAPs at moderate concentrations modulate dynamic instability (Pryer *et al.*, 1992; Kowalski & Williams, 1993a; Vasquez *et al.*, 1994; Ainsztein & Purich, 1994; Olesen, 1994; Panda *et al.*, 1995), increasing rescue frequencies, among other effects. But the data definitely show that MAPs probably do not *cause* rescues.

The data allow the interpretation to be extended to the semiquantitative level. The upper limit to the mean distance between MAP molecules under the conditions of measurement was estimated to be about 14 μm. That distance can be compared to the mean length of a shortening event, which is about 4 μm for DEAE-PC-tubulin and about 3 μm for glutamate-PC-tubulin. The extreme case of influence of MAPs on rescue would occur if every bound MAP molecule caused a rescue when the microtubule shortened to its location. At that extreme, the mean length of shortening events (*i.e.*, the number of micrometers lost between a catastrophe and a rescue) would be comparable to the distance between MAP molecules, equal to $^1/_2$ that distance in the limit of a large number of events. Because the free energy of binding of a single MAP is comparable to the free energy of binding of a tubulin molecule (Robinson & Engelborghs, 1982), it is quite unlikely that only one could stop the depolymerization process, which involves the cooperative peeling away of whole protofilaments (Simon & Salmon, 1990; Mandelkow *et al.*, 1991; Mandelkow & Mandelkow, 1991). Furthermore, if only one bound MAP were capable of halting a shortening event, they would become immeasurably short at moderate MAP concentrations, contrary to observations (Pryer *et al.*, 1992; Kowalski

[3] The phrase *conservative assumption* here denotes one which tends to negate the conclusion being arrived at. In the case of an estimation of an upper limit, a conservative assumption is one which tends to raise the apparent value of the quantity being calculated. The assumption that MAPs were not fractionated is the conservative one in this sense because it leads to a higher estimate of possible contamination than does the assumption that fractionation takes place.

& Williams, 1993a; Itoh & Hotani, 1994; Dhamodharan & Wadsworth, 1995; Panda *et al.*, 1995). Therefore, one can conclude quite firmly that MAPs are not the cause of rescues, which must be an intrinsic property of the tubulin lattice.

Variability of Rates. Gildersleeve *et al.* (1992) had speculated that the presence of trace amounts of MAPs might be the cause of the variability of rates observed in microtubule assembly and disassembly. Because the variability occurs on a length scale of several micrometers and involves large sections of the microtubule, it also appears impossible that one MAP molecule every 14 μm could be responsible. Variability must be a property of the tubulin lattice alone, possibly caused by fluctuations in the structure of the growing and shortening ends (Gildersleeve *et al.*, 1992; Chrétien *et al.*, 1995).

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