The Conversion of Tubulin Carboxyl Groups to Amides Has a Stabilizing Effect on Microtubules†

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ABSTRACT: In a recent study, we demonstrated that the conversion of carboxyl residues in the C-termini of tubulin to neutral amides with glycine ethyl ester enhanced the ability of the protein to assemble into microtubules and decreased its interaction with microtubule-associated proteins (MAPs). In this work, we investigated the effects of carboxyl modification on the dynamic behavior of microtubules at polymer mass steady state. After steady state, microtubules assembled from unmodified tubulin were sheared, and the mean polymer lengths decreased to 5 μm and then increased to 29 μm within 130 min. In contrast, lengths of sheared microtubules polymerized from tubulin containing 23 modified carboxyl groups increased by only 2-fold. Stabilization of polymer lengths was also observed directly by video-enhanced light microscopy of microtubules grown off of axonemes. Rapid shortening was seen in microtubules composed of unmodified but not modified tubulin. Further evidence for the less dynamic behavior of microtubules as a result of carboxyl modification was obtained from kinetic studies of the elongation phase during assembly which showed a 3-fold lower off-rate constant, k_-, for modified microtubules. Another effect of the modification was a 12-fold reduction in the steady-state rate constant for GTP hydrolysis (165 s^{-1} for unmodified and 14 s^{-1} for modified). These results suggest that reduction of the negative charges in the C-termini by modification of the acidic residues stabilizes microtubules against depolymerization. MAPs may stabilize microtubules in an analogous manner.

Microtubules are dynamic structures that undergo assembly and disassembly to mediate some of their cellular functions. In a population of microtubules at steady state, individual microtubules can behave differently, some growing while others are disassembling rapidly (Mitchison & Kirschner, 1984a). The proposed molecular basis of this phenomenon is that microtubules maintain a cap of tubulin–guanosine 5′-triphosphate (GTP)[1] subunits that stabilizes the polymer ends and that this cap is lost in a stochastic fashion, exposing less stable tubulin–GDP ends (Carlier & Pantaloni, 1981). Evidence for the presence of a GTP cap was provided by kinetic data which showed that GTP hydrolysis lagged behind subunit addition during assembly (Carlier & Pantaloni, 1981; Carlier et al., 1984; Caplow et al., 1985; Burns, 1991). The GTP cap hypothesis postulates that when a labile tubulin–GDP core becomes exposed, disassembly occurs until the ends are recapped by tubulin–GTP. As a result, there is an observed increase in the mean length of steady-state microtubule populations with some microtubules shrinking and others growing. Substantial experimental evidence has accumulated that this so-called dynamic instability behavior exists both in vitro (Mitchison & Kirschner, 1984a,b; Horio & Hotani, 1986; Farrell et al., 1987; Walker et al., 1988) and in cells in culture (Salmon et al., 1984; Cassimeris et al., 1986; Schulze & Kirschner, 1986, 1987, 1988; Sammak et al., 1987; Sammak & Borisy, 1988). However, experiments designed to show the presence of the GTP cap indicate that if it does exist, it is limited to a single layer of GTP subunits (O'Brien et al., 1987; Walker et al., 1988; Stewart et al., 1990; Bayley et al., 1990; Voter et al., 1991).

The dynamic behavior of microtubules is governed by the proportion of growing and shrinking polymers and the rates of tubulin–GTP addition and tubulin–GDP loss. Conditions that stabilize microtubules and decrease the disassembly rates such as microtubule-associated proteins (MAPs) (Kristofferson & Purich, 1981; Horio & Hotani, 1986; Farrell et al., 1987; Keates & Hallett, 1988) and glycerol (Kristofferson et al., 1986) have been found to suppress or greatly reduce the frequency of transitions between growth and depolymerization. Microtubule dynamics in vivo may be influenced by a wide range of cytoplasmic factors, and variations in microtubule stability may be the result of MAP binding or posttranslational modifications (Kirschner & Mitchison, 1986; Schulze & Kirschner, 1988).

The carboxyl-terminal domains of α- and β-tubulin appear to be involved in modulating microtubule dynamics since MAPs bind to these regions (Serrano et al., 1984; Littauer et al., 1986; Maccioni et al., 1988; Vera et al., 1988; Joly et al., 1989; Cross et al., 1991) and stabilize microtubules. In a recent study, we prepared tubulin with chemically modified carboxyl side chains, primarily in the C-termini, using glycine ethyl ester or methylamine in a carbodiimide-catalyzed reaction (Mejillano & Himes, 1991). Conversion of these negatively charged groups to neutral amides resulted in a protein with an increased propensity to assemble into micro-

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; AMPPNP, 5′-adenylyl imidodiphosphate; MAPs, microtubule-associated proteins; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, ethyl glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide.

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tubules (lower critical protein concentration, C_c), and a decreased ability to interact with MAPs and other positively charged ligands. To determine whether carboxyl group modification of tubulin changes the dynamic behavior of microtubules at polymer mass steady state, we investigated properties such as the polymer length redistribution, the kinetics of GTP hydrolysis, and the rates of tubulin dimer addition to and dissociation from microtubules. In these studies, we demonstrate that the chemical modification of carboxyl groups in the C-termini of tubulin reduces the dynamic instability behavior of the polymers at steady state.

Experimental Procedures

Materials. Pipes was obtained from Research Organics, GTP from Boehringer Mannheim, [^{14}C]glycine ethyl ester hydrochloride from DuPont, glycine ethyl ester hydrochloride from Eastman, and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) from Pierce Chemical Co.

Purification of Tubulin. Tubulin was purified from bovine brain by three cycles of assembly and disassembly followed by chromatography on a phosphocellulose–Biogel P10 piggyback column (Algaier & Himes, 1988).

Preparations of Chemically Modified Tubulin. The carboxyl residues of tubulin were modified by incubating microtubules assembled in vitro with 0.3 M glycine ethyl ester and 3 mM EDC at 37 °C for 60 min, as described previously (Mejillano & Himes, 1991). The extent of modification of tubulin was determined from the amount of [^{14}C]glycine ethyl ester incorporated into the protein. In the experiments reported in these studies, the extent of modification ranged from 20 to 23 glycine ethyl ester groups incorporated per tubulin dimer.

Microtubule Assembly Reactions. Assembly of unmodified or modified tubulin was generally carried out at 37 °C in the presence of 0.5 mM GTP in PEM buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$). In some cases, 10% DMSO was present in the assembly mixture. Polymerization was monitored by the increase in the apparent absorbance at 350 nm.

The critical protein concentration for assembly was determined from the amount of pelleted polymerized tubulin or from the protein concentration in the supernatant (Algaier & Himes, 1988). Unmodified or modified tubulin was assembled in a 0.4-mL volume at different protein concentrations and centrifuged at 200000g and 37 °C for 4 min in a Beckman TL-100 ultracentrifuge, and the concentration of unpolymerized tubulin in the supernatant was determined. The pelleted protein was resuspended in cold buffer and centrifuged for 10 min at 27000g and 4 °C, and the protein concentration in the supernatant was determined.

Microtubule Length Measurements. Samples from the assembly reaction mixtures were taken for polymer length measurements by electron microscopy according to the procedure of Mejillano and Himes (1990). For each time period, the lengths of 150–200 microtubules were measured, and the mean microtubule length was used to calculate the microtubule number concentration, $[m] = C_P/X1690$ where C_P is the concentration of tubulin in the polymer form and 1690 is the number of dimers per unit of polymer length (Amos & Klug, 1974).

Steady-State Microtubule Length Dynamics. Steady-state length distributions of microtubules assembled from modified or control tubulin were compared under unperturbed or sheared conditions. Microtubules were mechanically sheared shortly after reaching polymer mass steady state by passing the solution several times through a 25-gauge needle. Aliquots were then taken at various time periods for measurements of microtubule lengths and amount of protein polymerized.

Microtubule Dynamics by Light Microscopy. The dynamic instability of microtubules assembled from modified and control tubulin was also assayed directly by video-enhanced light microscopy (Schnapp, 1986; Walker et al., 1988; Cross & Williams, 1991; Williams, 1991). Sperm-tail axonemes, prepared as described by Cross and Williams (1991), were employed as nucleating structures, and assembly was carried out at 37 °C in PEM buffer in the presence of 1 mM GTP. Microtubule lengths were measured in videotaped images.

Measurement of GTP Hydrolysis Rates. At different time periods during assembly of modified or unmodified tubulin in the presence of 0.5 mM GTP, aliquots (100 μL) of the reaction mixture were removed and precipitated with 5% perchloric acid, and after centrifugation, the supernatant was neutralized with a 4 M potassium acetate/10 M KOH solution. The sample was centrifuged, and supernatants were analyzed for GDP formation by anion-exchange HPLC as described previously (Mejillano et al., 1990). The rate of GTP hydrolysis was determined from the concentration of GDP measured at various time periods up to 35 min after assembly was initiated. Values were corrected for GDP present at zero time, which resulted from that which occupied the E-site of tubulin as isolated, and the contaminating GDP in the GTP. By linear regression analysis, these plots gave R values of 0.983–0.998. The hydrolysis rate constants were calculated from the observed rates of GDP formation and microtubule number concentration calculated from the mean polymer length as described above.

Determination of Polymerization and Depolymerization Rates. Chemically modified or control tubulin at 35 μM each was polymerized at 37 °C in the presence of 0.5 mM GTP. The observed elongation rate constant, k_{app} was measured from the slope of the plot of ln $(A_\infty - A_t)$ vs time where A_∞ is the maximum or final absorbance value and A_t is the absorbance at any given time between 30 and 70% of the completion of the assembly reaction. R values of 0.992–0.999 were obtained from these plots by linear regression analysis. The rate constant for tubulin dimer addition to microtubules, k_+, was determined from the observed elongation rate constant and the microtubule number concentration ($k_{app} = k_+[m]$). The rate constant for dimer release from the microtubules, k_-, was calculated using the relationship $C_c = k_-/k_+$ where C_c is the critical protein concentration, determined as described above.

Results

Microtubule Length Dynamics Measured by Electron Microscopy. To investigate the changes in the steady-state dynamics of microtubules assembled from tubulin with modified carboxyl residues, length redistributions of populations of unsheared and sheared microtubules were examined at different time periods. As shown in Figure 1, when microtubules assembled from unmodified tubulin (in the absence of DMSO) were sheared shortly after reaching polymer mass steady state, there was a rapid decrease in absorbance to about 60% of the initial value followed by an increase back to the steady-state value. This change in absorbance is caused by transient disassembly upon shearing followed by reassembly and reestablishment of polymer mass steady state (Mitchison & Kirschner, 1984b). In the case of microtubules assembled from modified tubulin (23 modified carboxyl groups) or unmodified tubulin in the presence of 10% DMSO, shearing caused a similar drop in absorbance after which no further change was observed. In these latter two samples, depolymerization did not occur upon shearing as determined by a lack of decrease in polymerized tubulin. We do not understand

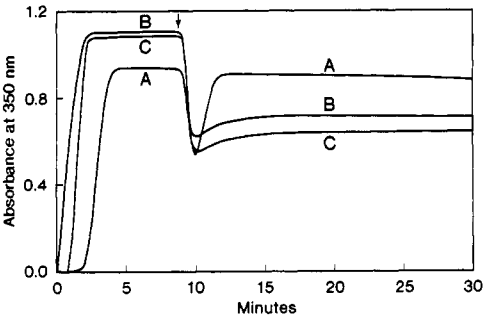

FIGURE 1: Effect of shearing microtubules assembled from modified and unmodified tubulin. Microtubules were assembled in PEM buffer with 0.5 mM GTP at 37 °C using (A) 35 μM unmodified tubulin, (B) 35 μM modified tubulin containing 23 glycine ethyl ester groups, and (C) 20 μM unmodified tubulin in the presence of 10% DMSO. Microtubules were sheared shortly after reaching steady state (at the arrow).

why a drop in turbidity occurs in these cases, but we have consistently observed this phenomenon.

Measurements of the mean polymer lengths revealed that for the control microtubules, the mean length shortened from 25 to 5.0 μm upon shearing but increased again shortly thereafter (Figure 2A). With time, the fraction of shorter microtubules decreased, and the length distribution spread toward longer microtubules. After 130 min, the mean length had increased by 474% to 29 μm. In the case of microtubule populations from modified tubulin, the polymer lengths were initially much shorter, and shearing induced a smaller and less extensive redistribution (Figure 2B). During a 130-min period, the mean length merely doubled from 3.6 to 7.1 μm. Similarly, shearing of unmodified polymers assembled in the presence of DMSO generated only a 70% increase in the mean length (Figure 2C). In the latter two cases, the length increases stopped after 70 min, but in the case of unmodified tubulin in the absence of DMSO, length redistribution was still occurring after 130 min. As shown in Figure 3A, when the microtubules from the control samples were left undisturbed, only a small increase (43%) in mean length was detected. An even smaller increase (27%) in lengths of polymers assembled from chemically modified tubulin was observed in undisturbed samples (Figure 3B). In all cases, under both unperturbed and sheared conditions, the concentration of tubulin in the polymerized form remained constant during the time period that the length measurements were made.

Microtubule Length Dynamics Observed in the Light Microscope. Assembly of modified tubulin was observed by video-enhanced light microscopy at a series of concentrations. No microtubules were observed when the initial modified tubulin concentration was 4 μM. At 16 μM, the field of view rapidly became saturated with microtubules, making observation of individual structures difficult. At 7.1 and 9.5 μM, both free and axoneme-attached microtubules were observed. The free microtubules ranged in length from a few micrometers to 15 μm and appeared somewhat uneven in cross section, as if they were partly formed by side-to-side association. The axoneme-attached microtubules were an order of magnitude shorter (typically 1–4 μm in length) than those formed from unmodified tubulin. This was undoubtedly caused by the lowering of the free tubulin concentration due to the formation of large numbers of non-axoneme-attached microtubules, consistent with the fact that modified tubulin nucleates microtubules more readily than does the unmodified protein. At 7.1 μM tubulin, the axoneme-attached microtubules, could be clearly observed and measured. Figure 4A shows the lengths of two typical modified microtubules measured over times up to 30 min after the initiation of assembly. There was no indication of the large (micrometers) and frequent (every few minutes) excursions in length as exhibited by microtubules composed of unmodified tubulin under identical conditions

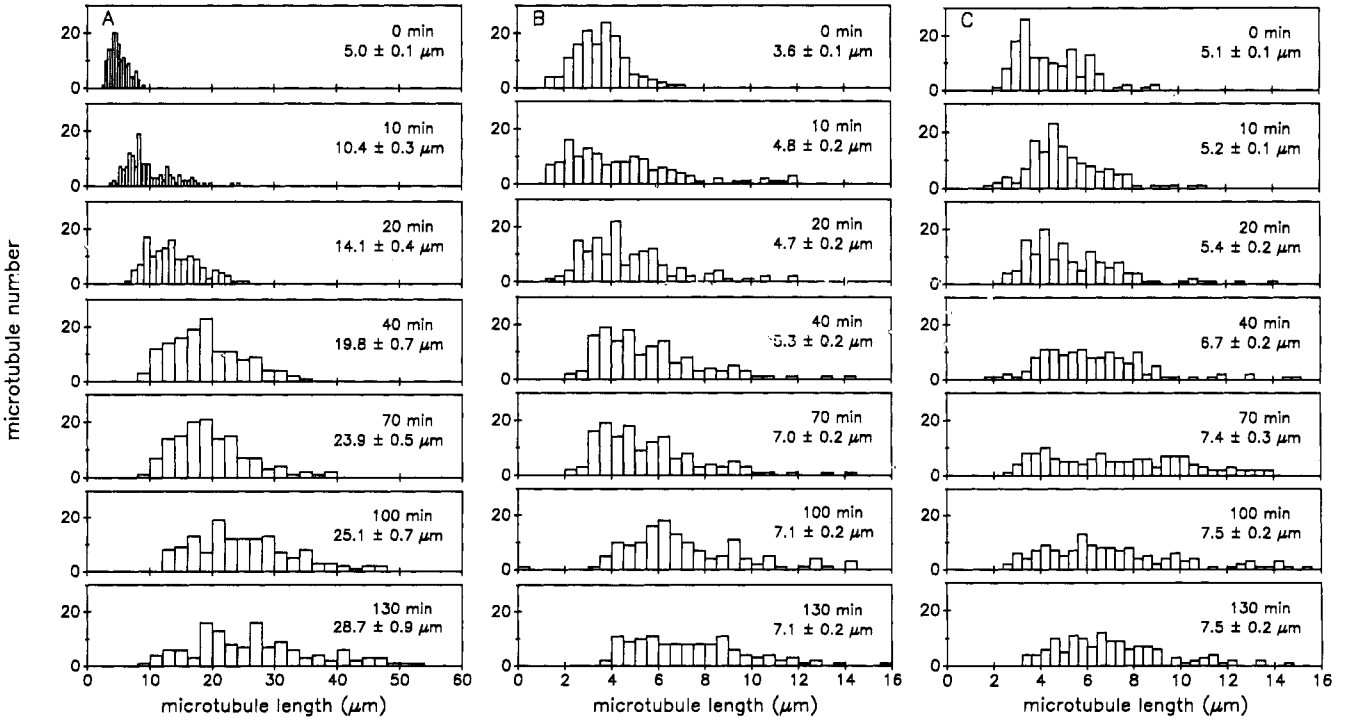

FIGURE 2: Length distributions of sheared microtubules assembled from tubulin which was (A) unmodified, (B) modified (23 glycine ethyl ester groups), or (C) unmodified in the presence of 10% DMSO. (Note the differences in horizontal scales). After shearing of the microtubules formed in the experiment described in Figure 1, aliquots of the assembly mixtures were taken at the indicated time periods during steady state and prepared for length measurements. Zero time refers to immediately after shearing or about 8 min after the initiation of assembly. Values of the mean polymer lengths are given with standard errors. The length distributions of (A) and (B) before being sheared were similar to those reported in Figure 3A,B, respectively.

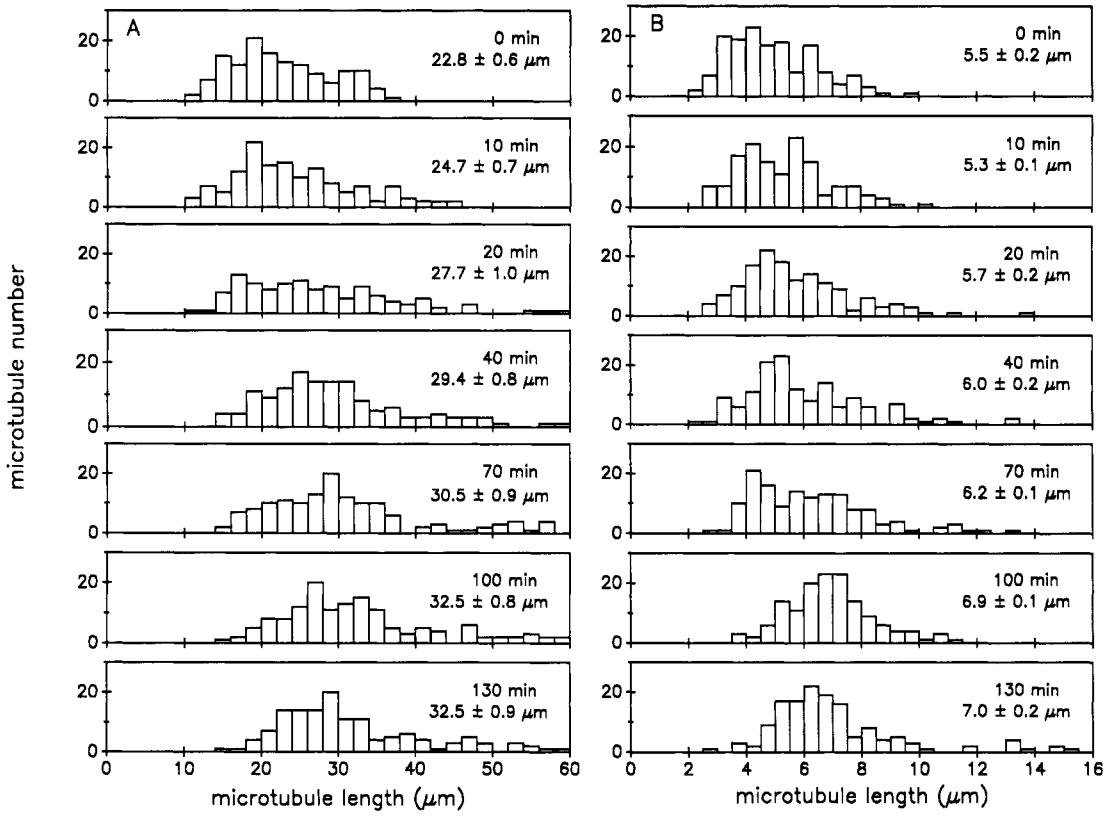

FIGURE 3: Length distributions of unperturbed microtubules assembled from (A) unmodified (in the absence of DMSO) or (B) tubulin containing 21 modified carboxyl groups. (Note the differences in horizontal scales.) The assembly conditions were the same as in Figure 1 except that the microtubules were not sheared. Length measurements were made at different time periods during steady state. Zero time refers to immediately after reaching polymer mass steady state (about 8 min after initiation of assembly) as judged by the apparent absorbance at 350 nm.

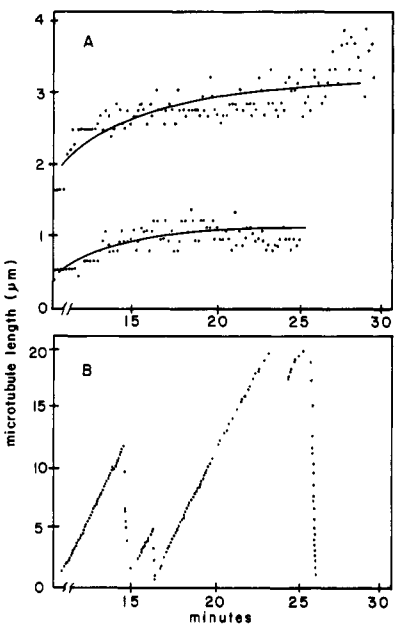

FIGURE 4: Microtubule lengths measured by light microscopy. (A) Modified tubulin at 7.1 μM. (B) Unmodified tubulin at 13 μM. Note the 5-fold difference between the scales of the vertical axes. Each point represents a single measurement of length. Elapsed time after the initiation of assembly is indicated. The interrupted horizontal axes represent the fact that approximately 10 min was required to assemble the microscope chamber and to begin observations.

(Figure 4B). Instead, the pattern was one of steady growth to an essentially constant length. In observations of approximately 100 microtubules, only 1 event with any characteristic of rapid shortening was observed, and that one may have been due to breakage of the microtubule. In contrast, rapid

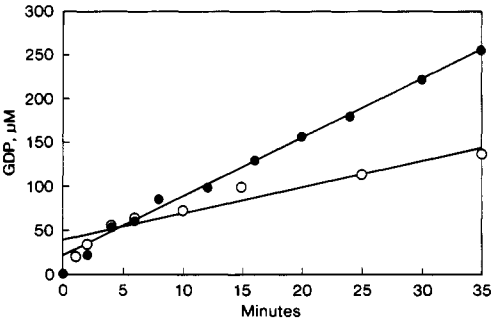

FIGURE 5: Time course of GTP hydrolysis during assembly of unmodified tubulin (●) and tubulin containing 20 modified carboxyl groups (○). Tubulin (35 μM) was polymerized at 37 °C in PEM in the presence of 0.5 mM GTP. At different time periods, aliquots of the assembly mixture were taken and analyzed for GDP formation by anion-exchange HPLC and for length measurements. The microtubule number concentration, [m], was 0.66 nM for the unmodified sample and 3.04 nM for the modified sample.

shortening of microtubules composed of unmodified tubulin was observed for every microtubule examined.

GTP Hydrolysis at Steady State. Slower rates of exposure of tubulin–GDP ends and dissociation of tubulin–GDP from the microtubule ends would result in a decreased rate of GTP hydrolysis at steady state. In Figure 5, the time course of GDP formation during the assembly and steady-state phase using untreated and modified tubulin (35 μM) is presented. In both cases, a biphasic reaction was observed which consisted of an initial more rapid GDP formation (assembly) followed by a slower constant rate (steady state). The rate for GTP hydrolysis at the steady-state phase in the case of modified microtubules (2.6 μM/min) was significantly lower than that for control microtubules (6.5 μM/min). The hydrolysis rate constant, calculated using the microtubule number concen-

Table I: Kinetic Constants for Assembly of Modified and Unmodified Tubulin

tubulin sample	C_c (μM)	mean MT length (μm)	[m] (nM)	k_{app} ($\times 10^3$ s^{-1})	k_+ ($\times 10^{-6}$ M^{-1} s^{-1})	k_- (s^{-1})
unmodified	10.5	21.4	0.77	9.8	12.7	133
modified	3.4	5.7	2.49	33.4	13.4	46
unmodified (+10% DMSO)	1.5	4.9	3.04	42.4	13.9	21

tration, was calculated to be 165 s^{-1} for the unmodified microtubules and 12-fold higher than for modified microtubules (14 s^{-1}). The observed hydrolysis rate for unmodified microtubules in the presence of 10% DMSO was similar to that of unmodified microtubules. Addition of 1 mM AMPPNP, which inhibits non-tubulin GTPase activity (Carlier & Pantaloni, 1982), did not change the rate of GTP hydrolysis, indicating that the observed rate was strictly due to tubulin addition to microtubule ends.

Rates of Addition and Loss of Modified Tubulin Subunits. Another approach to the study of the kinetic properties of modified microtubules is to examine the kinetics of the elongation phase of the assembly reaction, i.e., to determine k_+ and k_-. The results of these studies are presented in Table I. The rate constants we obtained for unmodified tubulin were in good agreement with previously reported values using the same approach (Cote & Borisy, 1981; Algaier & Himes, 1988). It is evident that carboxyl modification of tubulin resulted in an increase in k_{app} ($=k_+$[m]) values. This was due solely to an increase in the number concentration, [m], since the values for k_+ were similar for modified and unmodified tubulin. A 3-fold reduction in the off-rate constant, k_-, accounted for a corresponding decrease in the value of C_c, the critical protein concentration required for assembly. For assembly of unmodified tubulin in the presence of 10% DMSO, the values for [m] and k_+ were not significantly different from those of modified tubulin, but the C_c and k_- values were about 2-fold lower.

Discussion

As has been shown previously by others (Mitchison & Kirschner, 1984; Farrell et al., 1987; Kristofferson et al., 1986; Keates & Hallett, 1988), a population of MAP-free microtubules at steady state undergoes rapid depolymerization upon exposure of tubulin–GDP ends, which is followed by regrowth. This behavior can be explained by the disassembly of uncapped and unstable microtubules, exchange of bound GDP for GTP on the released tubulin dimer, and re-addition of tubulin–GTP to the ends of other microtubules. Experimental evidence for this phenomenon is a considerable increase in the mean polymer length and change in length distribution. In contrast, the instability behavior was drastically reduced in sheared microtubules assembled from tubulin with modified carboxyl groups as demonstrated by a distinctly smaller increase in microtubule mean length at steady state. Although the length increases were less prominent in the unperturbed samples, a greater extent of length redistribution still occurred in the microtubules assembled from unmodified tubulin. The decreased extent of steady-state length redistribution implies that modification of the acidic residues in the C-termini stabilizes microtubules. This was evident from the kinetic studies which showed a 3-fold lower k_- value for modified microtubules. At least part of the small redistribution of lengths found with microtubules formed from modified tubulin may be due not to microtubule growth and shortening processes, but to end-to-end joining (annealing), a process which has been shown to yield quite similar length redistributions in the case of total taxol-stabilized microtubules (Williams & Rone, 1989). Stabilization was also evident by direct observation of dynamic instability as the solution approached polymer mass steady state (Figure 4). The large length excursions typical of unmodified microtubules were absent in the microtubules composed of chemically modified tubulin, which simply grew steadily to a nearly unchanging plateau of length. Another notable difference between microtubules formed from unmodified and modified protein was a 12-fold lower rate constant for steady-state GTP hydrolysis. We also observed a significant decrease in the mean length and an increase in the number concentration, [m], of microtubule populations formed from modified protein when compared to microtubules formed from identical or higher concentrations of unmodified tubulin, indicating that carboxyl modification enhanced the nucleation step of the assembly reaction. This was also evident from a shorter lag phase during the assembly reaction (Figure 1).

The stability of microtubules assembled from modified tubulin resembles that of microtubules assembled in the presence of MAPs (Kristofferson & Purich, 1981; Horio & Hotani, 1986; Farrell et al., 1987; Keates & Hallett, 1988). Horio and Hotani (1986) observed that MAPs suppressed conversions between growing and shrinking microtubules and stabilized the polymers in the growing phase while others (Farrell et al., 1987; Keates & Hallett, 1988) demonstrated that in MAP-rich microtubule preparations increases in polymer length distribution were invariably small. MAPs stabilize the microtubules throughout the polymer length and decrease the depolymerization rate by lowering the rate for tubulin dimer dissociation (k_-). Johnson and Borisy (1977) reported a value of 7 s^{-1} for k_- measured by seed-induced assembly in the presence of MAPs. This value is considerably lower than what we and others obtained (93–130 s^{-1}) for MAP-free tubulin (Cote & Borisy, 1981; Algaier & Himes, 1988). Since we demonstrated in a previous work that in the absence of MAPs tubulin with modified carboxyl residues polymerizes with a lower C_c than unmodified tubulin (Mejillano & Himes, 1991), it is not surprising that the modification caused similar effects on microtubule dynamics as those produced by MAPs. Conversion of the acidic groups in the C-termini into neutral amides appears to mimic the binding of MAPs to this region and regulate the dynamic behavior of microtubules in an analogous manner. It can be envisioned that removal of some of the negative charges at the C-termini by carboxyl modification or MAPs binding reduces the charge–charge repulsion between neighboring subunits in the polymer and, as a result, may stabilize the microtubules against depolymerization.

Little is known about the regulation of microtubule assembly and disassembly in vivo. MAP binding or posttranslational modifications may play important roles in modulating microtubule dynamics. One type of posttranslational modification of tubulin, polyglutamylation of Glu-445 of the α-subunit and Glu-438 of the βIII-subunit (Edde et al., 1990; Alexander et al., 1991), adds a variable number of negative charges to the C-termini. It is conceivable that such modification will result in more dynamic polymers since we have shown in this study that reduction of the negatively charged groups in the C-termini decreases the dynamic instability behavior of microtubules.

We have also shown that control microtubules assembled in the presence of DMSO, a known microtubule stabilizing

agent, are less dynamic and exhibit less extensive length changes. In addition, GTP hydrolysis at steady state occurred at a slower rate compared to that in the absence of DMSO. These results can also be explained by a slower dissociation of tubulin–GDP from the microtubule ends which is consistent with an earlier report (Algaier & Himes, 1988). Similarly, length fluctuations in the presence of another stabilizer, glycerol, have been found to be less pronounced (Kristofferson et al., 1986).

Registry No. GTP, 86-01-1; GTPase, 9059-32-9.

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