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Report

Straight GDP-Tubulin Protofilaments Form in the Presence of Taxol

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Summary

Microtubules exist in dynamic equilibrium, growing and shrinking by the addition or loss of tubulin dimers from the ends of protofilaments. The hydrolysis of GTP in β-tubulin destabilizes the microtubule lattice by increasing the curvature of protofilaments in the microtubule and putting strain on the lattice. The observation that protofilament curvature depends on GTP hydrolysis suggests that microtubule destabilizers and stabilizers work by modulating the curvature of the microtubule lattice itself. Indeed, the microtubule destabilizer MCAK has been shown to increase the curvature of protofilaments during depolymerization. Here, we show that the atomic force microscopy (AFM) of individual tubulin protofilaments provides sufficient resolution to allow the imaging of single protofilaments in their native environment. By using this assay, we confirm previous results for the effects of GTP hydrolysis and MCAK on the conformation of protofilaments. We go on to show that taxol stabilizes microtubules by straightening the GDP protofilament and slowing down the transition of protofilaments from straight to a curved configuration.

Results and Discussion

The Curvature of Individual Protofilaments Can Be Assayed by AFM

To look at the structure of individual protofilaments by atomic force microscopy (AFM) [1, 2], we used calcium to depolymerize microtubules polymerized with GMPCPP [3]. The depolymerization products of GMPCPP microtubules were adsorbed to mica and imaged with oscillation-mode AFM in buffer (Figure 1A). Numerous curled single protofilaments were observed with a curvature of 31 ± 5 nm (mean ± standard deviation [SD]), close to the curvature of 36 ± 10 nm measured previously with vitreous ice [3]. These previous experiments showed that GMPCPP is not hydrolyzed under these conditions [3]; they also showed that the depolymerization of microtubules either by cooling or by calcium addition generated protofilaments with the same curvature. Therefore, GMPCPP protofilaments depolymerized with calcium are thought to represent the structure of GTP protofilaments [3, 4]. We compared the curvature of GMPCPP protofilaments to those of GDP protofilaments (Figure 1B). Protofilaments present during normal polymerization have radii of 21 ± 4 nm (n = 45), and protofilaments from calcium-induced depolymerization have radii of 21 ± 2 nm (n = 56) (Table 1), again confirming the results of cryo-electron microscopy (EM) (21.1 ± 4.3 nm) [3]. Therefore, we conclude that AFM allows the analysis of the conformation of protofilaments under native conditions.

Taxol Slows the Straight-to-Curved Transition of GDP Protofilaments

We next examined the effect of taxol on the configuration of protofilaments. Taxol does not inhibit the hydrolysis of microtubule-bound GTP [5], but it slows down microtubule depolymerization [6]. Cryo-EM observations show that as with GMPCPP, taxol increases the length of tubulin monomers in microtubules from 4.01 to 4.17 nm [7]. This suggests that taxol could stabilize microtubules by preventing the protofilaments from curving and putting mechanical strain on the tubulin lattice. The small change in the size of the subunit would correspond to the decreased length associated with bending of the subunit.

By using AFM, we showed that microtubules polymerized with taxol contained a significant number of individual protofilaments (Figure 1C). To exclude the possibility that the supporting surface induced the formation of protofilaments, we showed that AFM topographs of glutaraldehyde-fixed microtubules contained similar amounts and appearances of protofilaments compared to unfixed ones (data not shown). Thus, our data are consistent with previous observations that individual protofilaments coexist with microtubules in freshly prepared taxol-stabilized microtubule samples [8].

Taxol-stabilized protofilaments were mainly straight or slightly curved, but occasionally formed rings (Figure 1C). The radius of curvature of slightly curved protofilaments was 245 ± 105 nm (n = 51), (compared to 31 ± 5 nm for GMPCPP protofilaments; Table 1). The radius of closed rings (cf. Figure 1D) was 21 ± 2 nm (n = 48), similar to the radius of GDP protofilament rings. To test whether the slightly curved taxol protofilaments represented transition states between straight

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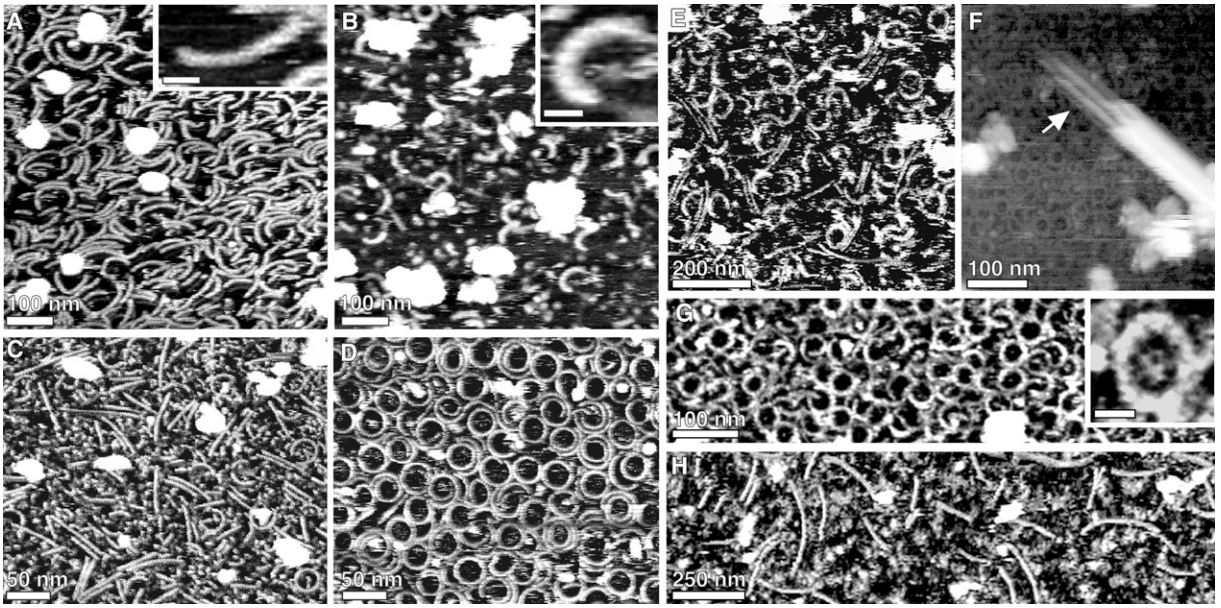

Figure 1. Conformations of Individual Tubulin Protofilaments Imaged by AFM

(A) Products of calcium- (40 mM) induced GMPCPP microtubules depolymerization.

(B) Unstabilized GDP protofilaments imaged quickly after deposition on mica.

(C) Individual protofilaments are present in the sample of taxol-stabilized microtubules and are present either in straight, curved, and closed conformations or in open ring-like conformations.

(D) After 2 days of aging, the taxol-stabilized protofilaments present a ring-like conformation, closed (1), open (2), or even double ring (3).

(E–G) The addition of MCAK/AMPPNP induced ring-like protofilaments from GMPCPP-stabilized microtubules (E) and taxol-stabilized microtubules (F and G). Nevertheless, ends of taxol-stabilized microtubules maintain a straight (blunt) conformation (F). (G) shows ring-like protofilaments obtained after MCAK addition to taxol-stabilized microtubules (the inset shows a higher-resolution image).

(H) MCAK-ATP, unlike MCAK-AMPPNP, does not induce the accumulation of ring-like protofilaments.

The gray levels of the AFM topographs correspond to vertical scales of 6 nm (A), 10 nm (B and D), 22 nm (C), 20 nm (E and H), 8 nm (G), and 30 nm (F). Scale bars of insets represent 20 nm.

and protofilaments rings, we examined the structure of protofilaments with time after polymerization. We prepared taxol-stabilized microtubules and imaged them by AFM 1, 4, 6, 7, 24, and 50 hr after polymerization (Figure 2). These topographs show that straight protofilaments are very stable—up to 4 hr, most of taxol protofilaments are straight. The most likely explanation for these data is that taxol initially stabilizes protofilaments in a straight conformation. Because GTP is hydrolyzed in taxol-stabilized microtubules [9], these data suggest that taxol actively straightens protofilaments. The majority of the protofilaments are in a straight configuration for up to 6 hr after preparation. Given the time scale of microtubule lifetime during polymerization (minutes), it is likely that the protofilaments remain straight when taxol stabilizes microtubules. However, at longer time scales, the protofilaments slowly convert to the curved conformation, and by 24 hr they eventually close up into rings, reminiscent of the structure of GDP protofilaments. How does taxol slow down the straight-to-curved transition of GDP protofilaments? One likely possibility is that taxol slows down the intrinsic structural transition from straight to curved that takes place in GDP protofilaments. However we cannot rule out that taxol itself is slowly inactivated in an aqueous environment.

Table 1. Radius of Curvature of Tubulin Protofilaments

Sample	Protofilament Radius of Curvature (nm ± Standard Deviation)
GDP protofilaments	21 ± 4 (n = 45)
GDP protofilaments + 40 mM Ca^{2+}	21 ± 2 (n = 56)
GMPCPP protofilaments + 40 mM Ca^{2+}	31 ± 5 (n = 35)
GMPCPP protofilaments + MCAK-AMPPNP	27 ± 4 (n = 43)
Taxol-stabilized protofilaments (fresh)	245 ± 105 (n = 51)
Taxol-stabilized protofilaments + 40 mM Ca^{2+}	21 ± 2 (n = 57)
Taxol-stabilized protofilaments (2 days old)	21 ± 2 (n = 48)
Taxol-stabilized protofilaments + MCAK-AMPPNP	19 ± 2 (n = 37)

Van Buren et al. [10] proposed that taxol might stabilize microtubules by making the protofilaments less stiff rather than by making them less curved: In both cases, the mechanical strain energy penalty for tubulin in the microtubule lattice is reduced. We have no evidence indicating that taxol softens protofilaments because our AFM images provide no information about protofilament rigidity; however, we note that it is not established that taxol even has a softening effect on microtubules—see, e.g., B. Mickey and J. Howard [11], who found that taxol actually increased slightly the flexural rigidity of GDP microtubules.

GTP Hydrolysis and Kink between Tubulin Monomers

Previous experiments suggest that protofilament curvature increases after GTP hydrolysis because tubulin

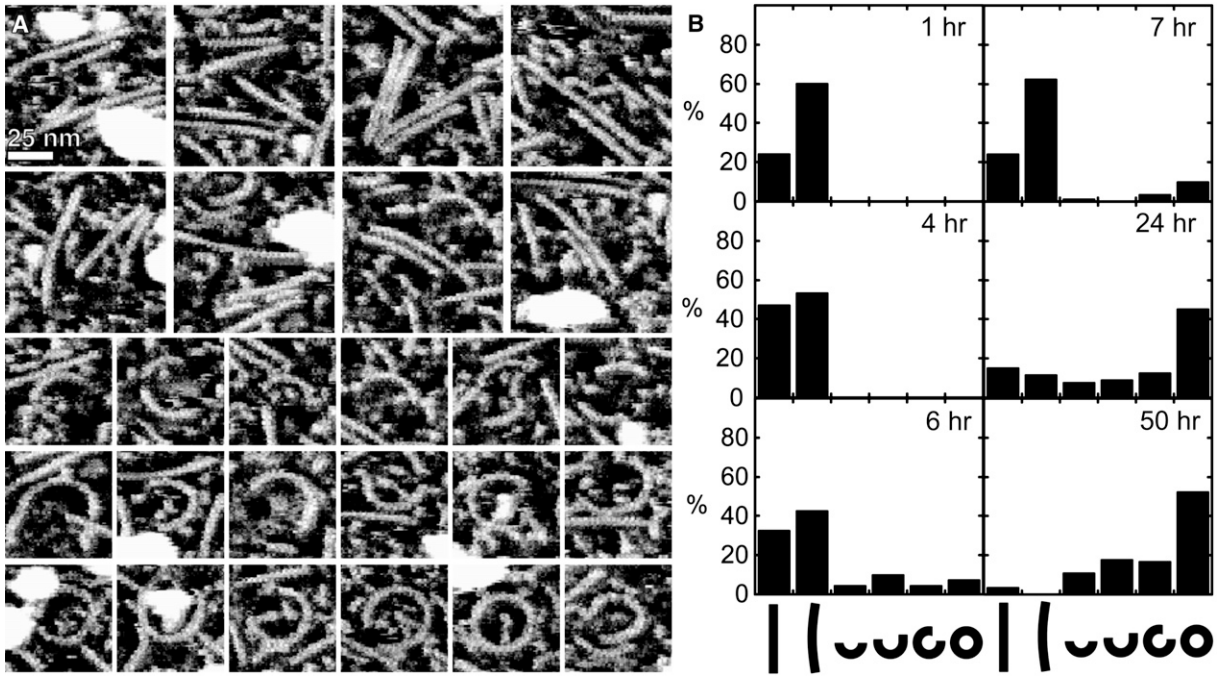

Figure 2. Straight-to-Ring Transition of Tubulin Protofilaments

(A) Gallery presenting the different protofilaments conformations observed from taxol-stabilized microtubules. The gray levels of all AFM topographs correspond to vertical scales of 6 nm.

(B) Occurrence of the different structural intermediates depends on sample aging. For each condition imaged by AFM, we analyzed about 100 single protofilaments to determine their conformational statistics. Cartoons at the bottom relate the structural intermediates occurring during the straight-to-ring transition of protofilaments: the straight, slightly curved, highly curved, half-ring, almost-closed, and closed ring conformations.

subunits in the lattice shorten, changing the repeat length of the tubulin dimers from 4.20 to 4.05 nm [12]. This lead to the conclusion that a conformational change within tubulin subunits induced by GTP hydrolysis induces protofilament curvature. Recent structural analysis of the two alternative tubulin structures, both straight and curved protofilaments, at atomic resolution have shown that, indeed, GTP hydrolysis induces a 12° kink between the tubulin subunits [13]. The kink angle was obtained by the reconstruction of cryo-EM images recorded from double-layered tubes of GDP-bound tubulin [4]. Thus, it was not possible to directly compare these tubulin polymer conformations with those of protofilaments induced by various stabilizing and destabilizing factors and conditions. By using high-resolution AFM topographs, we were able to distinguish individual tubulin subunits in the protofilaments (Figure 3). By averaging the tubulin distances contoured in the height profiles of ten straight taxol-stabilized protofilaments such as shown in Figure 3A, we obtained a periodicity of 4.2 ± 1.6 nm (n = 104) (Figure 3B), corresponding well to the known size of monomeric tubulin, 4 nm [14]. Second, combining tubulin periodicity and protofilament radius of curvature allowed us to calculate the kink angle established between the tubulin subunits (see the Experimental Procedures).

We calculated the kink in GMPCPP-stabilized protofilaments and GDP protofilaments and compared this to the published data from other methods. GMPCPP protofilaments form curled protofilaments with an average radius of curvature of 31 ± 5 nm (n = 35) in presence of 40 mM Ca^{2+}. This gives a calculated value of 7.8 ± 1.2° (n = 63, 40 mM Ca^{2+}) for the kink angle between subunits. This differs from the 5° kink found between subunits in tubulin sheets [13]. This difference might be due to the kink of subunits within tubulin sheets is constrained [15]. The radius of GDP protofilament rings was 21 ± 2 nm (n = 56) independently of whether 40 mM Ca^{2+} or MCAK was present in the sample (Figure 3F). With 30 subunits to a ring (Figures 3D and 3E), the tubulin subunits within these protofilaments had a kink of 12°, exactly that found between tubulin monomers of a GDP-tubulin dimer in the relaxed, curved conformation [13]. Thus, these data confirm that a change in the kink between tubulin subunits can account for the change in radius of curvature of the protofilaments.

Next, we calculated the time-dependent changes in the kink angle between the subunits in taxol-stabilized protofilaments (Figure 3F). One hour after the preparation of taxol-stabilized protofilaments, the tubulin monomers of the protofilaments showed an average kink close to 0°, and after 7 hr, this kink increased slightly to ~1°. However, after 24 hr, all taxol-stabilized protofilaments were in rings, with a calculated kink angle of 12°. These results support previous analysis by Arnal and Wade [7], who looked at the periodicity of tubulin subunits in the lattice and showed that the most likely mechanism by which taxol stabilizes microtubules is by preventing the GDP subunit from adopting a kinked configuration after GTP hydrolysis.

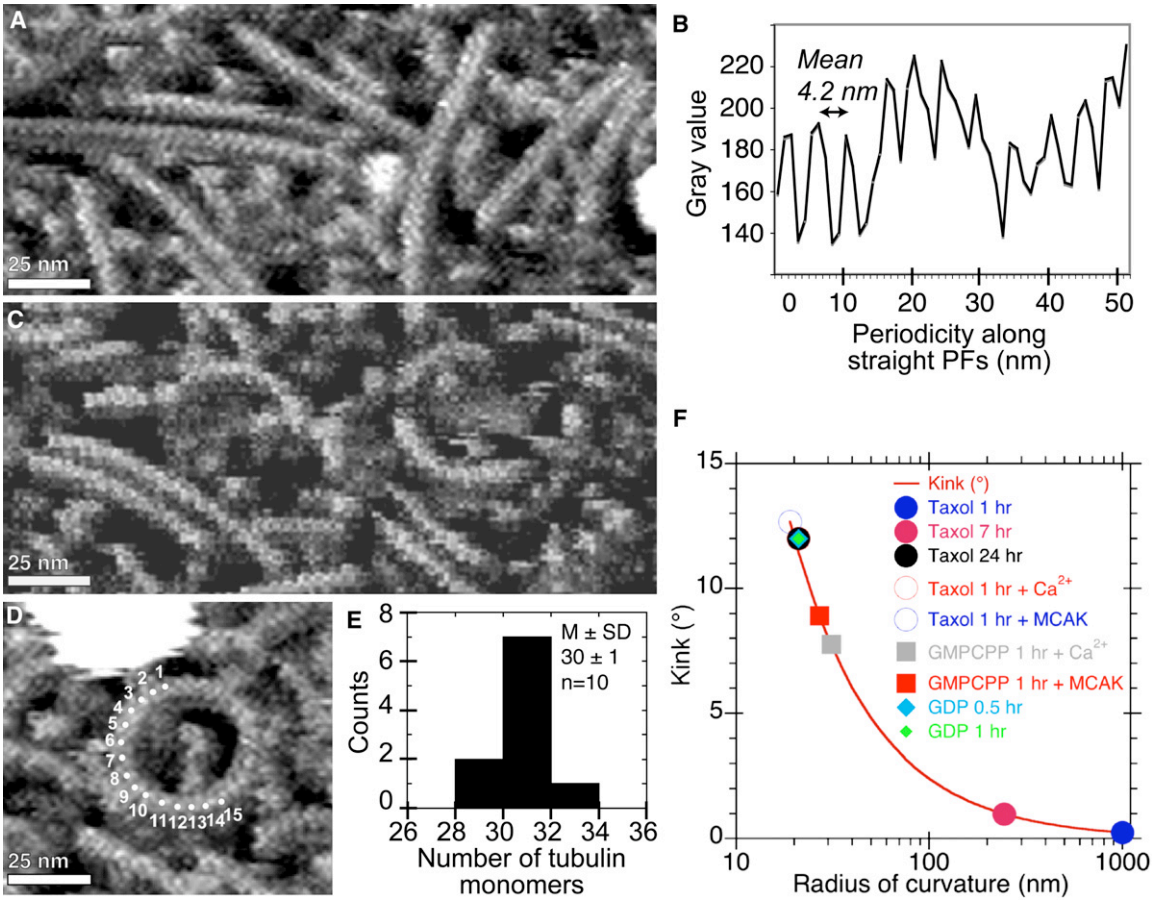

Figure 3. High-Resolution AFM Directly Observes Single Tubulin Molecules Forming Protofilaments

(A) Freshly prepared straight taxol-stabilized protofilaments.

(B) The height profile along protofilament sections reveals an average tubulin periodicity of 4.2 ± 1.9 nm (n = 104).

(C) With increasing time, taxol-stabilized protofilaments transform into slightly curved conformations.

(D) Example of a ring-like taxol-stabilized protofilament observed after 2 days' aging of the sample. Dots and numbers indicate single tubulin monomers.

(E) The average number of tubulin monomers counted per ring-like protofilament corresponds to 30 ± 1 (n = 10).

(F) Kink between tubulin monomers calculated from the radius of curvature of protofilaments observed under different conditions. The red line represents kink over protofilament curvature radius calculated for a subunit size of 4.2 nm. Experimental averages of radii are given for native and for taxol- and GMPCPP-stabilized protofilaments, in the absence and presence of calcium or MCAK. No time given in the legend indicates that the structures were observed roughly 1 hr after protofilament preparation and immediately after MCAK or calcium addition to protofilaments. The gray levels of all AFM topographs correspond to a vertical scale of 6 nm.

MCAK Increases the Curvature of GTP Protofilaments and Kink between Tubulin Subunits

Previous EM results suggested that MCAK induces curvature in GTP-like protofilaments [16]. On the basis of these and other observations [17, 18], it has been suggested that the microtubule-destabilizing factors, such as KLP-7, bend tubulin dimers and thus destabilize microtubules [17, 18]. To further test this idea, MCAK was added to microtubules grown both in the presence of GMPCPP or GTP with taxol. AMPPNP was added so that MCAK could be inhibited and so that it could be tightly bound to microtubules [17]. After 5 min incubation, the resulting structures were imaged by AFM. The AFM topographs showed some intact microtubule ends and numerous ring-like protofilaments (Figures 1F and 1G). In contrast to the effects observed with only the MCAK motor core [17], protofilaments obtained in the presence of the complete MCAK protein were predominantly single rings with a 19 ± 2 nm (n = 37) radius of curvature, whereas no double or triple concentric rings were observed. The protofilament radius from taxol-stabilized microtubules, 19 ± 2 nm (Figure 1G, Table 1), was similar to previously published EM data [17]. The radius of ring-like protofilaments from GMPCPP microtubules was 27 ± 4 nm (n = 43) (Figure 1E, Table 1). From these data, we calculated that MCAK increased the kink between tubulins from 7.8 ± 1.2° (n = 63, 40 mM Ca^{2+}) to 8.9 ± 1.2° (n = 43) (Table 1, Figure 3F). Thus, in agreement with Desai [16] and Moores [17], the AFM data indicate that MCAK bends tubulin protofilaments made of GTP tubulin. Interestingly, the addition of MCAK to the sample of taxol-stabilized microtubules in the presence of ATP instead of AMPPNP did not induce the formation of protofilament rings. We speculate that ATP hydrolysis and MCAK release from protofilaments occur more rapidly than does protofilament bending. An alternative possibility is that the complete ATPase cycling allows the disassembly of the protofilaments

into tubulin dimers, suggesting that MCAK might induce a greater kink between tubulin monomers, increasing protofilament curvature.

The idea that microtubule stabilizers and destabilizers work through the curvature of protofilaments is a fascinating link between the global structural change of a microtubule and the small chemical change from GTP to GDP, within its tubulin dimers. By straightening the GDP protofilaments, taxol apparently prevents GTP hydrolysis from putting strain on the microtubule lattice. In this context, it is interesting that taxol-stabilized protofilaments had radii either greater than 200 nm or around 20 nm, and both populations were found in the same samples. This indicates that kinking occurs simultaneously in the 20–30 subunits of a protofilament, suggesting a long-distance cooperative interaction between the tubulins of a protofilament. Cooperative kinking might act as a growth-to-shrinkage switch and explain why microtubules undergo catastrophic disassembly. It will be fascinating to see how other modulators of microtubule stability alter the conformation of the protofilaments.

Taxol is used as an anticancer agent [19]. Its action on microtubules suggests a mechanism by which arrest in mitosis is due to changes in microtubule stability that induce apoptosis of cancerous cells. Our data on the effect of taxol on the microtubule lattice suggest a potentially more complex explanation for the efficiency of taxol. By straightening the protofilaments, taxol induces a more GTP-like configuration in the protofilament. Because proteins can distinguish the GTP from the GDP configuration microtubule lattice [20, 21], perhaps putting the protofilaments into a GTP-like configuration triggers physiological changes in the apoptosis machinery that are different from those induced by simple microtubule depolymerization.

Experimental Procedures

Preparation of Taxol-Stabilized Microtubule Sample

Taxol-stabilized microtubules were prepared by the incubation of tubulin (30 μM final concentration) for 20 min in BRB80 buffer containing 1 mM GTP at 37°C and then the addition of 170 μl of 10 μM taxol in BRB80. Then microtubules were pelleted by ultracentrifugation at 109,000 g (TLA 100 rotor, OptimaTM MAX Ultracentrifuge, Beckman) for 5 min and resuspended in 50 μl BRB80 containing 10 μM taxol (BRB80-taxol). Taxol-stabilized microtubules are always diluted in a taxol-containing buffer.

Preparation of GMPCPP Microtubule Sample

GMPCPP microtubules were polymerized as described [22] at a tubulin concentration of 10 μM instead of 4 μM.

Calcium and MCAK-AMPPNP-Induced Microtubule Depolymerization

Microtubule depolymerization was induced by the addition of calcium (40 mM final concentration) or MCAK-AMPPNP (0.2 μM MCAK, 0.5 mM AMPPNP) to the microtubule sample in BRB80 as previously described [3]. Recombinant MCAK was obtained as described [23]. The mixture was deposited on freshly cleaved mica immediately and left at least 1 min on the surface before being extensively washed with BRB80 buffer.

Determining Protofilament Radius, Tubulin Distance, and Kink between Tubulin Monomers

We used Image J software to fit a circle of a given radius onto bent or ring-like protofilaments. Then we measured the periodicity of the height profile along the protofilament, revealing the average distance between tubulin monomers. Both protofilament radius, r, and average tubulin distance, l, were used to calculate the kink according to $kink(^\circ) = 2\pi r/(360 \bullet l)$. By determining the average radius of protofilaments at a certain time after preparing the sample, we could then directly relate this time to the progression of the kink.

Sample Adsorption to Mica and AFM Imaging

Samples were allowed to adsorb to freshly cleaved mica for at least 1 min before being rinsed (at least 3 times) with buffer (50 mM HEPES [pH 7.4] adjusted with KOH, 200 mM KCl 2 mM $MgCl_2$, or BRB80). Mixtures of microtubules + 40 mM Ca^{2+} or microtubules + MCAK were deposited on freshly cleaved mica immediately after preparation and left 1 min for adsorption before being extensively washed with BRB80 or HEPES buffer. AFM (Nanoscope III, Veeco) imaging was performed in buffer solution with oscillation mode. The AFM cantilevers (NPS-oxide-sharpened silicon nitride probes [Veeco] exhibiting spring constants of 0.32 or 0.58 N/m) were activated at their resonance frequencies, ranging from 8.5 to 9.5 kHz. For the feedback controls, typical values of setpoint for imaging were between 0.5 and 1.5 V, depending on the drive amplitude. The oscillation amplitude was maintained at 5–10 nm for protofilament imaging.

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