Rigidity of Microtubules Is Increased by Stabilizing Agents

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Abstract. Microtubules are rigid polymers that contribute to the static mechanical properties of cells. Because microtubules are dynamic structures whose polymerization is regulated during changes in cell shape, we have asked whether the mechanical properties of microtubules might also be modulated. We measured the flexural rigidity, or bending stiffness, of individual microtubules under a number of different conditions that affect the stability of microtubules against depolymerization. The flexural rigidity of microtubules polymerized with the slowly hydrolyzable nucleotide analogue guanylyl-(α,β)-methylene-diphosphonate was $62 \pm 9 \times 10^{-24}$ Nm^2 (weighted mean ± SEM); that of microtubules stabilized with tau protein was $34 \pm 3 \times 10^{-24}$ Nm^2; and that of microtubules stabilized with the antimitotic drug taxol was $32 \pm 2 \times 10^{-24}$ Nm^2. For comparison, microtubules that were capped to prevent depolymerization, but were not otherwise stabilized, had a flexural rigidity of $26 \pm 2 \times 10^{-24}$ Nm^2. Decreasing the temperature from 37°C to ~25°C, a condition that makes microtubules less stable, decreased the stiffness of taxol-stabilized microtubules by one-third. We thus find that the more stable a microtubule, the higher its flexural rigidity. This raises the possibility that microtubule rigidity may be regulated in vivo. In addition, the high rigidity of an unstabilized, GDP-containing microtubule suggests that a large amount of energy could be stored as mechanical strain energy in the protein lattice for subsequent force generation during microtubule depolymerization.

Microtubules are long protein polymers that form one component of the eukaryotic cytoskeleton. They are composed typically of 13 protofilaments whose axes run roughly parallel to that of the microtubule; the protofilaments, in turn, are formed from the head-to-tail association of tubulin dimers. Microtubules are thought to have structural, organizational, and motile functions. As structural elements, microtubules help to define and maintain cell shape, and are found in specialized mechanical structures such as cilia and flagella. As cell organizers, microtubules serve as tracks along which organelles are transported. As motile structures, microtubules can generate force on their own; for example, the depolymerization of kinetichore microtubules may drive chromosome segregation during mitosis (Koshland et al., 1988; Coue et al., 1991). The mechanical properties of microtubules are crucial to all of these functions.

The most important large-scale mechanical property of a microtubule is its flexural rigidity, a measure of the microtubule's ability to resist bending forces. The resistance to bending originates in the resistance of the individual protofilaments to the tensile and compressive forces that arise when a microtubule is bent. The flexural rigidity is determined by the bonds between the atoms within each tubulin dimer, as well as the bonds that hold the dimers together in the polymer. Gittes et al. (1993) showed that the flexural rigidity of microtubules polymerized in vitro and stabilized with the antimitotic diterpenoid taxol is sufficiently high to account for the rigidity of living cilia and flagella, organelles whose motility relies on their rigid microtubule-containing cores.

There are circumstances, however, in which the high rigidity of the microtubule cytoskeleton might be a hindrance to cellular function. For example, to permit changes in cell shape, it might be simpler or more economical to make cytoskeletal microtubules more flexible than to remove them altogether by depolymerization. Conversely, an increase in flexural rigidity that straightens a bent microtubule could be used to change a cell's shape. It is, therefore, interesting to ask whether the flexural rigidity of microtubules can be modulated. For example, do microtubule-stabilizing proteins that bind to the surface of a microtubule, like tau and MAP-2, influence the flexural rigidity? Alternatively, do the GTP and GDP forms of a microtubule have different rigidities, so that GTPase regulators could affect the polymer's mechanical properties? Factors that determine microtubule rigidity also determine the capacity of microtubules to do mechanical work. Is the rigidity sufficiently high that a substantial fraction of the energy derived from GTP hydrolysis could be directly stored as potential energy of deformation of the microtubule lattice?

In the present study, we have probed the relationship between the mechanical rigidity of microtubules and their polymerization dynamics. We found that even in the ab-

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sence of stabilizing agents, microtubules are rigid enough to serve their structural and force-generating functions. Microtubule-stabilizing agents increased this rigidity by up to a factor of two. Thus, stabilizing agents induce important structural changes within or between the tubulin subunits, and may regulate microtubule rigidity in vivo.

Materials and Methods

Proteins and Reagents

Tubulin was purified to >95% purity as follows. Two fresh calf brains were stripped of meninges, homogenized in a blender (Waring, New Hartford, CT) in 0.5 ml homogenization buffer (100 mM K-MES, pH 6.5, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 1 mM ATP, 0.25 mM GTP, 0.1% β-mercaptoethanol (βME)[1], 4 mM DTT, and protease inhibitors) per gram of tissue, and centrifuged (Sorvall GSA, 12,000 rpm, 4°C, 50 min). The supernatant was supplemented with 33% glycerol, 6 mM $MgCl_2$, and 2 mM GTP, incubated at 37°C for 60 min, and centrifuged (F28/36; Sorvall Instruments Division, DuPont Co., Newton, CT, 28,000 rpm, 35°C, 70 min). The pellets were resuspended in 100 ml of Pipes buffer (100 mM K-Pipes, pH 6.8, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 0.1 mM GTP, 4 mM DTT, 0.1% βME, and protease inhibitors), incubated on ice for 40 min, and centrifuged (28,000 rpm, 4°C, 40 min). The supernatant was supplemented with 33% glycerol, 3 mM $MgCl_2$, and 1 mM GTP, incubated at 37°C for 40 min, and centrifuged (28,000 rpm, 35°C, 60 min). The pellets were frozen immediately in liquid nitrogen and stored at −80°C; they were later thawed, resuspended in 30 ml of column buffer (50 mM K-Pipes, pH 6.8, 1 mM EGTA, 0.2 mM $MgCl_2$, 10 μM GTP), incubated on ice for 30 min, and centrifuged (28,000 rpm, 4°C, 30 min). The supernatant was rid of microtubule-associated proteins by phosphocellulose chromatography (P11; Whatman Inc., Clifton, NJ); the tubulin-rich eluate was augmented with 30 mM K-Pipes, pH 6.8, 0.8 mM $MgCl_2$, and 0.1 mM GTP, frozen in liquid nitrogen, and stored at −80°C. This tubulin was later thawed, and either cycled once more to remove inactive protein or labeled with one to two tetramethylrhodamines per tubulin dimer, as described by Hyman et al. (1991).

Recombinant full-length bovine tau protein (clone pBT43-12; Himmler et al., 1989) was generously donated by David Drechsel (University College, London, UK). Tubulin dimer and tau protein concentrations were determined using absorption coefficients of 0.10 μM^{-1} cm^{-1} and 0.15 μM^{-1} cm^{-1} at 280 nm, respectively. Guanylyl-(α,β)-methylene-diphosphonate (GMPCPP) was kindly provided by Tim Mitchison (University of California, San Francisco, CA). Taxol was obtained from the National Cancer Institute (Bethesda, MD). DMSO and 2-vinylpyridine were obtained from Aldrich Chemical Co. (Milwaukee, WI); ATP, GTP, and NADPH from Boehringer Mannheim Biochemicals (Indianapolis, IN); and carboxytetramethylrhodamine from Molecular Probes (Eugene, OR). Most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Microtubules

Microtubules were prepared in vitro from purified tubulin in four different ways (Fig. 1). Microtubules were polymerized at 37°C in 80 mM K-Pipes, pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$, and either (*a*) 1 mM GTP, 10 μM unlabeled tubulin, 10 μM labeled tubulin, and 5% DMSO for 50–60 min or (*b*) 1 mM GMPCPP, 0.25 μM unlabeled tubulin, and 0.25 μM labeled tubulin for 9–11 h. To obtain GMPCPP microtubules of lengths greater than a few micrometers, the polymerization solution was replenished with 0.20 μM unlabeled tubulin and 0.20 μM labeled tubulin every half hour. GMPCPP microtubules were diluted 100-fold into observation buffer (80 mM K-Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 0.5–5 mg/ml casein, 4–40 mM D-glucose, 40–200 μg/ml glucose oxidase, and 8–40 μg/ml catalase) at 37°C. The very low rate of hydrolysis of GMPCPP (4×10^{-7} s^{-1}, Hyman et al., 1992) ensured that only ~1% of the GMPCPP in these microtubules was hydrolyzed over the course of our experiments. Microtubules polymerized with GTP were observed after stabilizing them in one of three ways: (*1*) microtubules were diluted 20-fold into observation buffer at 37°C augmented with 10 μM tau protein; (*2*) microtubules were diluted 200-fold into observation buffer at 37°C augmented with 10 μM taxol; (*3*) stable caps were added onto the ends of the microtubules by 20-fold dilution into a solution at 37°C containing 80 mM K-Pipes, pH 6.8, 1 mM EGTA, 1–2 mM $MgCl_2$, 0.5 mM GMPCPP, and 5 μM unlabeled tubulin, followed by incubation for 10–20 min. These cap-stabilized microtubules were then diluted fourfold into a solution at 37°C with final concentrations of observation buffer, or alternatively, 10-fold into redox buffer (see below) at 37°C. Some microtubules were prepared in a similar way, but were stabilized and observed at room temperature (22–26°C); some microtubules were observed in the presence of βME (see below). Recordings were made within a few hours after stabilization. Each polymerization and stabilization of microtubules is referred to as an experiment.

We expect our microtubules to be primarily 14-protofilament microtubules (14-mers), because of two studies that examined protofilament number distributions by electron microscopy. Our GDP microtubule polymerization conditions were nearly identical to the Pipes-DMSO conditions of Ray et al. (1993), who found 14% 13-mers, 72% 14-mers, 11% 15-mers, and 3% 16-mers. Our GMPCPP microtubule polymerization conditions were nearly identical to those of Hyman et al. (1995), who found 0.2% 12-mers, 3.1% 13-mers, 96% 14-mers, and 0.7% 15-mers. From geometrical considerations, the flexural rigidity of a microtubule should depend on the third power of its number of protofilaments (Gittes et al., 1993), so we expect the mean rigidity of GDP and GMPCPP microtubules to be in the ratio

$$\sum_i i^3 p_D(i) \Big/ \sum_i i^3 p_T(i) = 1.018,$$

where $p_D(i)$ ($p_T(i)$) is the probability of there being i protofilaments for the GDP (GMPCPP) microtubules.

Redox Buffer

We constructed a solution, which we call redox buffer, that had ionic concentrations (Patton et al., 1989) and redox potential (Gilbert, 1990) similar to that of cytoplasm. Redox buffer consisted of 130 mM monopotassium glutamate, 8.5 mM monosodium glutamate, 10 mM Hepes, 7 mM glutathione, 140 μM glutathione disulfide, 2 mM $MgCl_2$, 1 mM Na_2ATP, 1 mM EGTA, 0.5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 70 μg/ml thioredoxin, 0.7 mg/ml casein, 4–20 mM D-glucose, 40 μg/ml glucose oxidase, 8 μg/ml catalase, pH 7.2, at 37°C with KOH using ΔpH/°C = −0.014 for Hepes. These concentrations of glutathione and glutathione disulfide correspond to a redox potential of −0.246 V. Glutathione and glutathione disulfide were added and the pH adjusted immediately before use to minimize oxidation of glutathione; observations were made within 60 min. We confirmed that glutathione did not rapidly become oxidized; after 60 min under experimental conditions, the amount of glutathione disulfide in redox buffer only increased from 140 to 490 μM, corresponding to a change in redox potential from −0.246 to −0.226 V. Glutathione disulfide concentration was measured enzymatically by derivatization of glutathione with 2-vinylpyridine followed by glutathione reductase–catalyzed oxidation of NADPH by glutathione disulfide and measurement of the change in absorbance at 340 nm relative to a standard curve (Anderson, 1985). Rhodamine photobleaching was notably slower in redox buffer than in observation buffer due to the presence of glutathione.

Some microtubules were observed in observation buffer in the presence of βME. We determined that our stocks of βME were 97.3–99.4% in the reduced form, depending on the time since the bottle was first opened. Solutions of 1 and 0.25% βME within this range of oxidation have redox

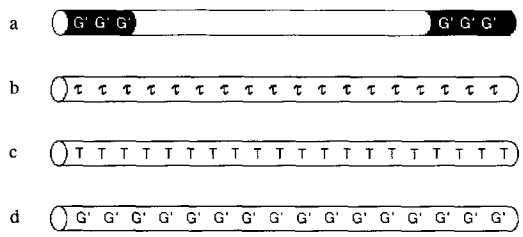

Figure 1. Microtubules assembled and stabilized in vitro. Microtubules were polymerized in vitro from purified tubulin and stabilized in different ways. After polymerization with GTP, microtubules were (*a*) capped with GMPCPP-tubulin, (*b*) stabilized with 10 μM tau, or (*c*) stabilized with 10 μM taxol. (*d*) Other microtubules were polymerized with GMPCPP along their entire lengths. *G'*, GMPCPP-tubulin; τ, tau protein; *T*, taxol.

1. *Abbreviations used in this paper*: βME, β-mercaptoethanol; GMPCCP, guanylyl-(α,β)-methylene-diphosphonate.

potentials in the range of −0.291 to −0.311 V, and −0.272 to −0.293 V, respectively. Oxidized βME was assayed by equilibration of a 1% solution with 10 mM DTT in 50 mM KH_2PO_4 (pH 7.2 with NaOH) and measurement of the change in absorption at 290 nm using an absorption coefficient for oxidized dithiothreitol of 273 $M^{-1}cm^{-1}$ (Cleland, 1964). Concentrations of βME are given as percent volume per volume; a 1% solution is equivalent to 143 mM.

Redox potentials for glutathione- and βME-containing solutions were calculated using the Nernst equation: $E = E_0 + (kT/ne)\ln(c_o/c_r^2)$. Here, E is the redox potential relative to the standard hydrogen electrode, E_0 is the standard redox potential at pH 7.0 which equals −0.26 V for both glutathione (Gilbert, 1990) and βME (Lees and Whitesides, 1993), k is the Boltzmann constant, T is the absolute temperature, n is the number of electrons transferred which equals 2 in a thiol–disulfide exchange, e is the elementary charge, and c_o and c_r are concentrations of the oxidized and reduced species.

Microscopy

A microliter of solution containing stable microtubules was pipetted onto a glass microscope slide, a cover glass was placed on top, and the edges of the cover glass were sealed with immersion oil to prevent fluid flow. Microtubules in the resulting shallow chamber (<3 μm) were observed with an epifluorescence microscope equipped with a ×100, 1.25 NA oil immersion objective (Diastar, Leica Inc., Buffalo, NY). Images were detected with a silicon-intensified target camera (Hamamatsu C2400-8; Bartels and Stout, Bellevue, WA) and recorded with a 1/2-inch video cassette recorder (Panasonic AG-7350, Proline, Seattle, WA). For microtubules examined at 37°C, the solution was maintained at ~37°C throughout all manipulations, and at 37 ± 1°C during recordings. The slide and cover glass were prewarmed on a heated aluminum block, and the microscope objective, which contacted the cover glass through a layer of immersion oil, was warmed by circulating warm water through copper tubing wrapped around the objective. Some microtubules were recorded at room temperature (22–26°C).

Measurement of Flexural Rigidity

The flexural rigidity of individual microtubules was measured from thermal fluctuations in microtubule shape (Fig. 2), as previously described by Gittes et al. (1993). Briefly, microtubules were digitized, and their shapes were parametrized by tangent angle, θ, as a function of arc length, s. Each shape was decomposed into Fourier modes with amplitudes, a_n, given by

$$a_n = \sqrt{\frac{2}{L}}\int_0^L ds\ \theta(s)\cos\left(\frac{n\pi s}{L}\right),$$

where L is the microtubule's length and $n = 1, 2, \ldots$ is the mode number. The mode amplitudes were estimated numerically and the flexural rigidity, EI, was calculated from the variance of the mode amplitudes:

$$EI = \left(\frac{L}{n\pi}\right)^2 \frac{kT}{\mathrm{var}(a_n)},$$

where $\mathrm{var}(a_n) = \langle(a_n - a_n^o)^2\rangle$ and a_n^o are the variance and mean of the nth mode's amplitude. In general, a_n^o was nonzero due to a microtubule's intrinsic shape (the shape in the absence of thermal or applied forces). One advantage of our method over other methods is that it permits subtraction of the intrinsic curvature, which can be large compared to the fluctuations. Failure to subtract the mean curvature would artifactually increase the variance, and lead to an underestimation of the flexural rigidity. A second advantage of this method is that different bending modes of a single microtubule can provide independent estimates of flexural rigidity; this is a consequence of the orthogonality of the components of a Fourier expansion and the assignment of equal energy to each of these components according to the equipartition theorem (Reif, 1965).

Microtubules of lengths 24–68 μm were analyzed. 25–84 video images of each microtubule were digitized by taking 6–12 points along its length. This was repeated every 10 s (or, in a few cases, every 5 s). An illustration of the method is given in Fig. 2 for a tau-stabilized microtubule.

When it was possible to obtain rigidity estimates from more than one mode of a particular microtubule, a χ^2 test was performed to determine whether the estimates were consistent with each other. For 38 microtubules, rigidity estimates from different modes were consistent with each other ($P > 0.05$), and were therefore combined into a weighted mean rigidity for each of these microtubules. Two microtubules, whose different modes yielded significantly different rigidities ($P < 0.05$), were excluded

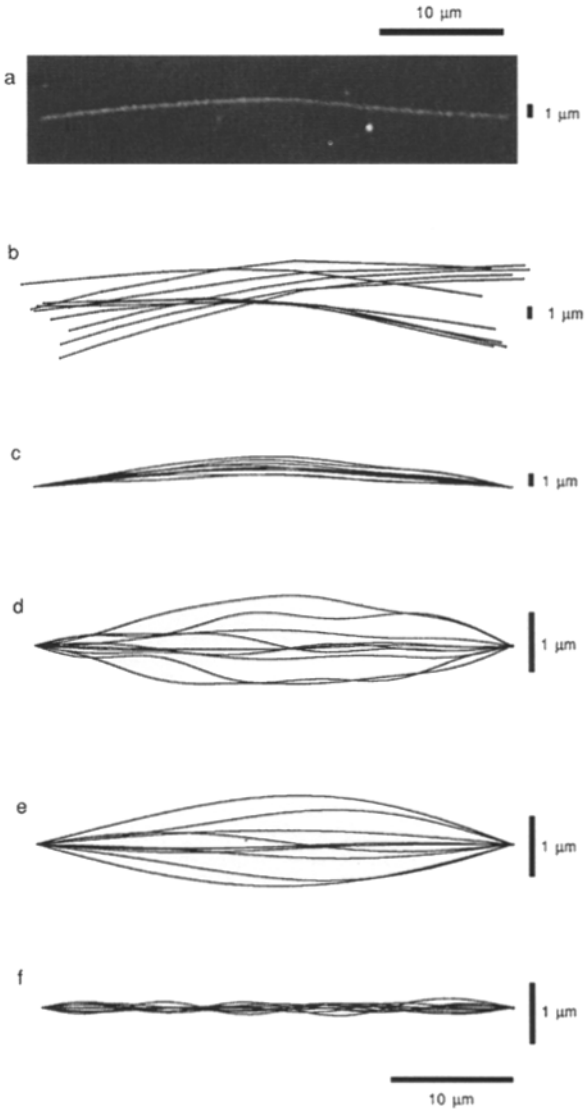

Figure 2. Shape analysis of a tau-stabilized microtubule. Shapes were analyzed from fluorescence images of the microtubule, such as the image in *a*. Every 10 s, a set of eight points was digitized along the microtubule; 10 such sets of digitized points are shown in *b*. Note that the microtubule diffuses laterally and rotationally (about an axis perpendicular to the focal plane), showing that the microtubule is free to move, and is not immobilized in the 2–3-μm depth of the chamber. The shape of the microtubule, parametrized by the tangential angle versus arc length, was decomposed into independent Fourier bending modes, and the amplitudes of these modes were calculated for each set of points. In principle, a complete set of mode amplitudes completely describes the shape. A reconstruction of the microtubule shapes from all of the modes is shown in *c*. The intrinsic shape (determined from mean mode amplitudes) is subtracted out in *d*, leaving only the fluctuating portion of the shape; for clarity, the vertical scale has been expanded by a factor of three. While variance in the lowest order modes (fluctuations of the lowest spatial frequencies) reflected thermal bending, variance in the higher order modes (fluctuations of higher spatial frequencies) were dominated by measurement error. A reconstruction using only the first two modes, each of which provided an independent estimate of the microtubule's rigidity, is shown in *e*. The sum of the four remaining higher order modes, shown in *f*, indicates the magnitude of the noise.

from further analysis. For each condition, a χ^2 test was performed on the weighted mean rigidities (or first mode rigidities, if higher modes were not available) of the microtubules to test the hypothesis that the population of microtubules was homogeneous. For 12 conditions, this hypothesis was substantiated ($P > 0.05$). For two conditions, the populations were heterogeneous ($P < 0.05$), but removal of one outlier in each case left populations that were homogeneous; these cases are described in Results. Two to four different experiments were performed for each condition described. Statistics were performed on the natural logarithms of the data (see Gittes et al., 1993). Flexural rigidities are reported as a weighted mean ± standard error of the population with the outlier (if any) removed.

Rotational Diffusion of Microtubules

The rotational diffusion coefficient, D, (about an axis perpendicular to the microtubule) was measured, and the perpendicular drag coefficient per unit length, $c_\perp$, was calculated for each of six microtubules as they diffused in our experimental chambers according to $c_\perp = 24kT/L^3D$ (Hunt et al., 1994). The expected perpendicular drag coefficient per unit length was calculated as $c_\perp \sim 2 \cdot 4\pi\eta/\cosh^{-1}(h/r)$ (Hunt et al., 1994), where η is the viscosity (0.7 kg m^{-1} s^{-1} at 37°C), h is the distance of the microtubule from each surface (taken to be 1 μm), r is the radius of the microtubule (15 nm), and the prefactor of two is included to roughly account for both surfaces of the chamber. First-mode relaxation time constants, τ, were estimated as described by Gittes et al. (1993):

$$\tau \cong \frac{c_\perp}{EI}\left(\frac{2L}{3\pi}\right)^4.$$

Clamped Microtubules

To check the accuracy of our results, we measured the flexural rigidity of microtubules using an independent method. Rhodamine-labeled microtubules grown from short biotinylated microtubule seeds were "clamped" via a streptavidin linkage to biotinylated BSA adsorbed to the glass surface of a flow cell (Gittes, F., E. Meyhöfer, S. Baek, and J. Howard, manuscript in preparation). Microtubules of lengths 8–17 μm were recorded on videotape at room temperature and digitized for analysis. The spring constant (stiffness) of a slender rod clamped at one end and deflected at the other is $\kappa = 3EI/L^3$ (Feynman et al., 1964). The potential energy associated with a deflection through distance x is $^1/_2\kappa x^2$. According to the equipartition theorem (Reif, 1965), the mean potential energy is equal to $^1/_2kT$, so $\kappa = kT/\langle x^2\rangle$ where $\langle x^2\rangle$ is the mean square deflection (in one dimension). Substituting this into the earlier equation for κ gives $EI = {}^1/_3kTL^3/\langle x^2\rangle$.

While this method is relatively simple, it is prone to systematic errors. For example, we found that it was necessary to keep the flow rate very low when changing solutions, because rapid flow irreversibly increased the mean-squared displacement of the free end up to 10-fold, and thus led to an apparent decrease in a microtubule's flexural rigidity to as little as 10% of its original value. This may have been due to weakening of the free portion of the microtubule adjacent to the clamp. It was also crucial to eliminate fluid flow during measurements, since such external forces generally dominate the fluctuations, making microtubules appear flexible. Other methodological problems include difficulties in estimating the digitization noise and in determining the length of the free polymer.

Microtubule Elasticity and Energetics

If we model a 14-protofilament microtubule as a cylinder whose cross-section has an inner radius of 11.48 nm and a wall thickness of 2.7 nm (which gives the correct mass per unit length), then its second moment of area is $I = 1.8 \times 10^{-32}$ m^4 (Landau and Lifshitz, 1986; Gittes et al., 1993). If we model a protofilament as having a rectangular cross-section of thickness $a = 2.7$ nm and width $b = 5.15$ nm ($= 11.48$ nm $\times\ 2\pi/14$) then its second moment is $I_p = a^3b/12 = 8.4 \times 10^{-36}$ m^4 (Landau and Lifshitz, 1986). If we assume that the microtubule is isotropic, then a flexural rigidity $EI = 26 \times 10^{-24}$ Nm^2 (see Table I, *caps*) corresponds to a Young's modulus $E = 1.4$ GPa. The flexural rigidity of a protofilament is then expected to be $EI_p = 1.2 \times 10^{-26}$ Nm^2. The work per dimer required to bend a protofilament into an arc of radius R is $W = {}^1/_2EI_pd/R^2$, where d is the dimer length (Landau and Lifshitz, 1986). For example, for $E = 1.4$ GPa, $d = 8$ nm, and $R = 20$ nm, $W = 1.2 \times 10^{-19}$ J. Assuming $E = 3.4$ GPa (using the flexural rigidity of 62×10^{-24} Nm^2 measured for GMPCPP microtubules) and a radius of curvature $R = 70$ nm then $W = 0.23 \times 10^{-19}$ J.

The free energy associated with the hydrolysis of GTP is $\Delta G = \Delta G^0 + kT\ln([GDP][P_i]/[GTP])$. Using $\Delta G^0 = -6.04 \times 10^{-20}$ J per molecule $\equiv -36.4$ kJ/mol (assumed equal to that of ATP at pH 7.1, 2 mM Mg^{2+}, 25°C, 0.2 ionic strength; Daniels and Alberty, 1975), [GDP] = 0.01 mM, [P_i] = 1 mM, and [GTP] = 1 mM, this gives $\Delta G = 1.1 \times 10^{-19}$ J per molecule.

Results

Microtubules were assembled in vitro, stabilized in different ways (Fig. 1), and visualized by fluorescence microscopy. The flexural rigidities of individual microtubules were measured from their thermally driven fluctuations in shape as previously described (Gittes et al., 1993). Fig. 2 graphically depicts the analysis, using a tau-stabilized microtubule as an example. The principal results are summarized in Table I.

Caps of GMPCPP Tubulin Stabilize Microtubules

To examine the effects of nucleotide hydrolysis and stabilizing agents on microtubule rigidity, it was necessary to study microtubules that contain exclusively GDP (so-called GDP microtubules) in the absence of such agents. But this posed a problem: unstabilized microtubules are either growing or shrinking, and therefore fluctuate in length over just a few minutes (Mitchison and Kirschner, 1984); measurements of flexural rigidity, which require several minutes, are thus precluded. To circumvent this problem we used GMPCPP, a slowly hydrolyzable analogue of GTP (Hyman et al., 1992), to polymerize stable tubulin caps onto microtubules originally grown in the presence of GTP. By fluorescently labeling the caps to a lesser extent, it was possible to restrict rigidity measurements to the more brightly labeled interior portion of a microtubule. Virtually all the tubulin in this interior region is in the GDP form, since the lag between subunit addition and GTP hydrolysis is less than ~5 s (Erickson and O'Brien, 1992).

This interior GDP-containing portion of the microtubule is expected to be stabilized only by the GMPCPP-

Table I. Microtubule Rigidity Depends on Nucleotide, Tau Protein, Taxol, and Temperature

Polymerization nucleotide	Stabilization method	Temperature	Flexural rigidity*	Critical concentration‡
		°C	10^{-24} Nm^2	μM
GMPCPP	–	37	62 ± 9	0.02
GTP	Tau	37	34 ± 3	1.3
GTP	Taxol	37	32 ± 2	0.15
GTP	Caps	37	26 ± 2	≥90
GTP	Taxol	25	21 ± 1§	–

*Weighted mean ± standard error.

‡Critical concentrations of GMPCPP, cap-stabilized (GDP), and tau-stabilized microtubules were calculated from the ratio of the rate of dilution-induced shrinkage to the rate of growth of the microtubule's plus end. For GMPCPP microtubules, the critical concentration derived from microtubules polymerized with 1 mM GMPCPP that had a shrinkage rate of 0.0038 μm min^{-1}, and a growth rate of 0.19 μm min^{-1} μM^{-1} at 37°C (Hyman et al., 1992). For GDP microtubules, the critical concentration derived from a shrinkage rate of 11 μm min^{-1} for GDP microtubules, and a growth rate of 0.12 μm min^{-1} μM^{-1} for GTP microtubules at 34°C (Drechsel et al., 1992). For tau-stabilized microtubules, the critical concentration in the presence of 10 μM tau derived from a shrinkage rate of 5 μm min^{-1}, and a growth rate of 0.4 μm min^{-1} μM^{-1} measured in 1 μM tau at 34°C (Drechsel et al., 1992). For taxol-stabilized microtubules, we use a critical concentration of 0.15 μM for microtubules assembled in the presence of 10 μM taxol at 37°C (Schiff and Horwitz, 1981).

§From Gittes et al. (1993).

containing caps at both ends. A fortuitous observation allowed us to confirm this prediction. A small fraction (<1%) of cap-stabilized microtubules at room temperature broke in the interior and each interior end rapidly depolymerized toward its respective capped end. The depolymerization rates were ~1 μm/s, about 10,000 times faster than the rates for GMPCPP microtubules (Hyman et al., 1992). Thus we can rule out the possibility that stabilization was due to the incorporation of GMPCPP into the interior section of the microtubule. Furthermore, it is unlikely that the caps exerted a long-range influence on the interior of the microtubule, since mixed-nucleotide microtubules such as these possess clear structural boundaries (Hyman et al., 1995).

Six measurements from six cap-stabilized microtubules at 37°C gave a flexural rigidity of $26.9 \pm 3.0 \times 10^{-24}$ Nm^2, and five measurements from four cap-stabilized microtubules at 37°C that had been polymerized in the presence of 70 mM Pipes and 10 mM MES, rather than 80 mM Pipes, gave a flexural rigidity of $23.9 \pm 3.4 \times 10^{-24}$ Nm^2. Because the ionic composition of our observation buffer differs considerably from mammalian cytoplasm, and because differences in buffers might explain the differences in microtubule rigidity reported by different laboratories (see Discussion), cap-stabilized microtubules were also observed in a very different, more physiological solution, which we call redox buffer. This solution closely approximated the cytoplasm with respect to pH, ion concentrations, ionic strength, and redox potential (see Materials and Methods). Seven measurements from six cap-stabilized microtubules in redox buffer at 37°C gave a flexural rigidity of $25.8 \pm 2.6 \times 10^{-24}$ Nm^2. (When an outlier, as defined in Materials and Methods, was included, the mean increased by 12%.) Since the above three estimates are not significantly different from each other, we combined them to yield a flexural rigidity of $25.7 \pm 1.7 \times 10^{-24}$ Nm^2 for cap-stabilized microtubules at 37°C.

βME Decreases the Rigidity of Cap-stabilized Microtubules

The flexural rigidity of cap-stabilized microtubules described above is almost seven times higher than the value we initially reported (Mickey et al., 1993). The reason for this revision is that experiments subsequent to our original report showed that the flexural rigidity of cap-stabilized microtubules is markedly reduced by high concentrations of βME, a disulfide-reducing reagent commonly used in conjunction with an antioxidant enzyme system to slow photobleaching of fluorophores (Kishino and Yanagida, 1988). That βME had such a dramatic effect on cap-stabilized microtubules was surprising, since we had shown earlier that it has no effect on taxol-stabilized microtubules (Gittes et al., 1993).

We made 21 measurements of nine cap-stabilized microtubules at room temperature in the presence of 1 or 2% (143 or 286 mM) βME, but in the absence of taxol. The flexural rigidity did not depend on the time that the microtubules were exposed to βME before measurement (15 min to 2 h), and was the same at the two βME concentrations. Combining the results gave a value of $3.77 \pm 0.18 \times 10^{-24}$ Nm^2. This large decrease in rigidity accompanying addition of βME could be unrelated to the reducing power of βME, but the fact that βME is a strong reducing agent raised the possibility that the rigidity of microtubules could be regulated by the redox state of the protein. Indeed, a precedent for the redox control of a protein has been reported (Benezra, 1994) and strong reducing conditions are known to partially inhibit microtubule assembly in vitro (Khan and Ludueña, 1991). But this effect of βME may not be physiologically relevant because a buffer containing 1% βME is more reducing than the cytoplasm: a 1% solution of βME has a redox potential between −0.29 and −0.32 V (see Materials and Methods), significantly lower than the redox potential of healthy heart and liver cells (−0.24 to −0.275 V; Gilbert, 1990). To determine whether microtubule rigidity is low at more physiological redox potentials, we repeated the measurements in two less reducing buffers. First, we made 11 measurements from seven cap-stabilized microtubules at 37°C in the presence of 0.25% βME (redox potential between −0.27 and −0.30 V). The flexural rigidity was $21.7 \pm 2.0 \times 10^{-24}$ Nm^2, not significantly different from that of cap-stabilized microtubules at 37°C in the absence of βME ($P = 0.14$, χ^2 with $d = 1$). Second, we examined cap-stabilized microtubules in a different solution, redox buffer, in which the peptides glutathione and glutathione disulfide kept the redox potential between −0.23 and −0.25 V. As described above, microtubules in redox buffer were quite rigid at 37°C, and very similar to those measured in our standard observation buffer in the absence of βME. Thus, only the highest concentrations of βME, corresponding to very negative redox potentials, modify the rigidity of non–taxol-stabilized microtubules. Whether these modifications can occur under physiological conditions is not known.

Tau, Taxol, and Raised Temperature Moderately Increase Microtubule Rigidity

Microtubules stabilized with the microtubule-associated protein tau are more rigid than microtubules stabilized with caps. Nine measurements from eight microtubules in a solution containing 10 μM tau at 37°C gave a flexural rigidity of $33.9 \pm 2.8 \times 10^{-24}$ Nm^2, significantly higher than that of cap-stabilized microtubules at 37°C (χ^2, $P < 0.01$). Since the dissociation constant of tau is ~0.1 μM (Butner and Kirschner, 1991), we expect that each microtubule's surface was saturated with tau (~1 tau per five tubulin dimers; Cleveland et al., 1977). It was clear from the stability of our microtubules that tau was bound and active: the rate of depolymerization of tau-stabilized microtubules was <~0.1 μm/min (compared with a rate greater than ~10 μm/min without tau).

Taxol also increased the flexural rigidity of microtubules. Seven measurements from six microtubules in buffer containing 10 μM taxol at 37°C gave a flexural rigidity of $32.0 \pm 3.2 \times 10^{-24}$ Nm^2. 10 measurements from 7 such taxol-stabilized microtubules at 37°C in the presence of 0.25% βME gave a flexural rigidity of $32.1 \pm 2.6 \times 10^{-24}$ Nm^2. Combining these two values yielded a flexural rigidity of $32.1 \pm 2.0 \times 10^{-24}$ Nm^2, significantly higher than that of cap-stabilized microtubules at 37°C (χ^2, $P < 0.025$).

Taxol-stabilized microtubules were more rigid at 37°C than at room temperature. As described above, we found

that taxol-stabilized microtubules at 37°C have a flexural rigidity of 32.1 ± 2.0 × 10^{-24} Nm^2. This value is 1.5 times the flexural rigidity of taxol-stabilized microtubules at room temperature, which we measured previously (Table I; and Gittes et al., 1993).

GMPCPP Microtubules Are About Twice as Rigid as Other Stabilized Microtubules

To examine the effect of GTP hydrolysis on the flexural rigidity, we polymerized tubulin in the presence of the slowly hydrolyzable GTP analogue GMPCPP. Because the rates at which tubulin associates with the microtubule during polymerization are similar in the presence of either nucleotide, it is thought that the GMPCPP-containing dimer mimics the GTP-bound state (Hyman et al., 1992). The slow rate of hydrolysis and slow off-rate of GMPCPP-tubulin ensured that the lifetime of GMPCPP microtubules was sufficiently long to perform flexural rigidity measurements; we estimate that only ~1% of the GMPCPP is hydrolyzed over the course of the experiments (see Materials and Methods). Six measurements from six GMPCPP microtubules at 37°C gave a flexural rigidity of 62.2 ± 8.8 × 10^{-24} Nm^2. The flexural rigidity of these GMPCPP microtubules is roughly twice that of cap-stabilized microtubules at 37°C. Further experimental support for this increase in flexural rigidity induced by GMPCPP comes from 10 measurements from seven GMPCPP microtubules at room temperature in the presence of 2% βME; the flexural rigidity of 36.4 ± 2.9 × 10^{-24} Nm^2 was 1.7 times greater than that of taxol-stabilized microtubules at room temperature in the presence of 1% βME. Taxol does not further increase the flexural rigidity of GMPCPP microtubules. Six measurements of four GMPCPP microtubules derived from a single experiment gave a flexural rigidity of 30.1 ± 3.2 × 10^{-24} Nm^2 at room temperature in the presence of 10 μM taxol and 2% βME. (Inclusion of an outlier decreased the mean by 26%.)

Evaluation of Systematic Errors

Gittes et al. (1993) considered many potential sources of error associated with our method of measuring microtubule rigidity and described two internal checks for systematic errors. The first is a comparison of rigidity estimates from different bending modes of a particular microtubule and the second is a comparison of rigidity estimates from microtubules of different lengths. These comparisons check for artifacts such as convective flow that are expected to affect different modes and different length microtubules to different extents. As described in Materials and Methods, we performed χ^2 tests to determine if estimates from different modes were consistent with each other, and found a statistically significant difference in only 5% of cases. There was no dependence of flexural rigidity on length.

Another potential systematic error, which can also be ruled out, is that a microtubule might interact so strongly with the surfaces of the observation chamber that it only samples a small subset of its unconstrained bending conformations. For example, if the microtubule were stuck to a surface or pinned between two surfaces, it would be prevented from bending and its rigidity would be overestimated. To rule out this potential artifact, rigidity measurements were made only on microtubules that diffused translationally and rotationally (about an axis perpendicular to the focal plane) as shown in Fig. 2. Since the microtubules were not irreversibly stuck, there must have been times during which they were free to undergo fluctuations in shape. The issue, then, is how long a microtubule takes to sample adequately its available bending conformations, i.e., how long one must wait to observe a shape that is uncorrelated with the previous shape. To address this question, we divided the measured rotational diffusion coefficients of six microtubules (under six different experimental conditions) by their corresponding predicted values. The mean ratio was 0.29 (range 0.09–0.65), indicating that on average the microtubules spent at least 29% of their time freely diffusing. From the diffusion measurements, the relaxation time for the thermal bending can be estimated (see Materials and Methods). The mean relaxation time for the first mode was 1.4 s (range 0.6–3.2 s). Since this time is much less than the duration of the experiment (several minutes), the microtubules had time to explore the full range of bending conformations and so the mean rigidity should not have been overestimated. And since this time is less than our sampling interval (10 s), sequentially digitized shapes should have been uncorrelated as assumed in our error analysis.

We also checked the accuracy of our Fourier method for measuring microtubule rigidity by using an independent method, in which taxol-stabilized microtubules were fixed at one end (clamped) to a glass surface and fluctuations of the free end were observed at room temperature. Seven clamped microtubules had a weighted mean flexural rigidity of 15.2 ± 2.1 × 10^{-24} Nm^2. This value is similar to 21.5 ± 0.8 × 10^{-24} Nm^2, which was obtained using our curvature fluctuation method (Gittes et al., 1993).

Discussion

We measured the flexural rigidity of naked GDP microtubules to be 26 ± 2 × 10^{-24} Nm^2 at 37°C. The similarity of this value to that measured for taxol-stabilized microtubules at 25°C (Gittes et al., 1993) implies that the conclusions drawn in the earlier work remain valid. In particular, the rigidity of microtubules polymerized with purified tubulin in vitro is sufficiently high to account for the rigidity of flagellar microtubules measured in vivo. Thus, the microtubule-associated proteins found in the flagellum are not essential for maintaining the microtubule's rigidity. Consistent with this conclusion is our present finding that the neuronal microtubule-associated protein tau only slightly increased the rigidity of microtubules in vitro.

One of our hypotheses was that cells might regulate the flexural rigidity of their microtubules during changes in cell shape or organization. That tau increases microtubule rigidity by only 30% implies that it does not play a major role in the regulation of rigidity. Similarly, microtubule-associated proteins like MAP-2 that contain microtubule-binding domains homologous to tau's (Lewis et al., 1988) are not likely to strongly regulate microtubule rigidity. On the other hand, the twofold difference in rigidity between a GDP microtubule and a GMPCPP microtubule indicates that regulation of tubulin's GTPase activity could appre-

ciably modulate microtubule rigidity in vivo. The finding that the phosphate analogues aluminum fluoride and beryllium fluoride increase the rigidity of microtubules (Venier et al., 1994) suggests that the phosphate-release step, rather than the hydrolysis step, is associated with a change in rigidity. The high flexural rigidity of GDP microtubules, even in the absence of microtubule-associated proteins, and the lack of a known physiological factor that decreases this rigidity, together suggest that a cell depends on dismantling (rather than deforming) its microtubule scaffold during major changes in shape.

Mechanical Energy Storage within the Microtubule Lattice

The rigidity of GDP microtubules is sufficiently high that much of the free energy available from GTP hydrolysis could be stored as a mechanical deformation of the tubulin dimers within the microtubule. As shown in Fig. 3, the protofilaments at the ends of a growing, GTP microtubule are fairly straight (Chrétien et al., 1995) while those at the ends of a shrinking, GDP microtubule curve outward (Mandelkow et al., 1991). The simplest interpretation is that while the GTP dimer is fairly straight, the GDP dimer is curved. The corollary of this structural difference is that since the reaction GTP → GDP occurs within the straight wall of the microtubule, the resulting GDP dimer must be in a strained conformation. Release of this strain would then drive depolymerization and could be harnessed to generate force as the microtubule depolymerizes (Koshland et al., 1988; Coue et al., 1991).

The amount of mechanical energy stored as internal strain depends on the mechanical properties of the microtubule. If we make the strong assumption that the microtubule is mechanically isotropic, our measurements imply that the flexural rigidity of an individual GDP protofilament is $\sim 0.013 \times 10^{-24}$ Nm^2; to straighten such a protofilament that has curved into an arc with a radius of 20 nm (Mandelkow et al., 1991) requires $\sim 1.2 \times 10^{-19}$ J of work per 8-nm-long dimer (Materials and Methods). To constrain the curved GDP protofilament in the straight wall of the microtubule therefore requires this amount of energy. On the other hand, because the GTP protofilament is straighter, a smaller amount of energy, perhaps $\sim 0.2 \times 10^{-19}$ J per dimer, is required to constrain it in the wall of the microtubule, even if the rigidity for GTP is twice that for GDP as suggested by our experiments with GMPCPP (we assume a radius of curvature of 70 nm; see Materials and Methods). Thus the difference in mechanical energy associated with the reaction GTP → GDP occurring within the straight wall of the microtubule is $\sim 10^{-19}$ J per dimer. Since this energy is comparable to the free energy available from GTP hydrolysis (Materials and Methods), we propose that the storage of energy as mechanical strain of the GDP dimer takes place and is the structural basis for the thermodynamic results of Caplow et al. (1994), who found that much of the free energy of GTP hydrolysis is coupled to a change in the internal energy of the microtubule.

To a first approximation, the amount of mechanical energy stored in the microtubule lattice depends only on the rigidity of the GDP microtubule since the GTP protofilaments are nearly straight, and are, therefore, little strained.

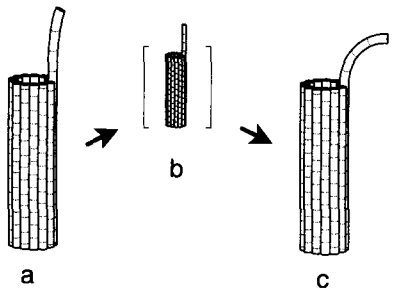

Figure 3. Model of protofilament force production. Assembling microtubules tend to have fairly straight protruding protofilaments (*a*; *GTP*), while disassembling microtubules tend to have protruding protofilaments that curve away from the microtubule axis (*c*; *GDP*). If the conformational change from the initial to the final state occurs after incorporation of the protofilament into the closed wall of the microtubule, mechanical potential energy will be stored as strain. The amount of energy, which ultimately derives from GTP hydrolysis, is proportional to the flexural rigidity of the protofilament.

However, the small curvature of the GTP protofilaments may be very important. One of the difficulties of the GTP-cap model of the dynamic instability of microtubules is that the cap, if it exists, is very small (for review see Erickson and O'Brien, 1992). Furthermore, the cap does not get longer as the rate of polymerization increases, as expected if there is a fixed lag time between attachment of a GTP dimer to the end of the microtubule and hydrolysis. Instead, it appears that hydrolysis is coupled to polymerization. How could this be? There is a very simple mechanical model that could explain this coupling. If the transition state for the hydrolysis reaction, perhaps corresponding to GDP-P_i, is straighter than the initial GTP state (Fig. 3 *b*), then the closure of the microtubule would catalyze the hydrolysis and the associated conformational change! The reason is that the straightening of the protofilament attending closure of the microtubule provides some of the energy required to enter the transition state. For example, if fully straightening the GTP dimer (a → b in Fig. 3) costs 0.2×10^{-19} J, then the hydrolysis rate might be accelerated $\sim$100-fold ($= \exp[0.2 \times 10^{-19}\,\text{J}/kT]$) as a result of incorporation into the straight wall of the microtubule. This might explain why GTP (Table I; GMPCPP) and GDP-P_i (Venier et al., 1994) microtubules have high rigidities, since the greater the rigidity, the greater is the energy difference between states a and b in Fig. 3, and thus the slower the hydrolysis before microtubule closure.

Structural Implications

We found that several ligands that increase microtubule stability also increase microtubule rigidity. Table I shows critical concentrations that correspond to our measured rigidities. The correlation between rigidity and stability could be due to the more stable dimers having internally more rigid conformations. Indeed, Hyman et al. (1995) have detected a structural difference between the GMPCPP and GDP forms of the tubulin dimer using cryoelectron microscopy. It is equally plausible that a significant portion of a microtubule's elasticity resides in the same bonds that determine stability, namely those bonds between tu-

bulin dimers along a protofilament. If doubling the interdimer bond stiffness also doubled the bond energy, then the twofold range of rigidities we observe could readily account for the 5,000-fold range in stabilities (Erickson, 1989).

The greater rigidity of the GMPCPP microtubule may explain why the distribution of protofilament numbers is more uniform when microtubules are polymerized, in GMPCPP (96% 14-mers) compared to GTP (72% 14-mers) (Ray et al., 1993; Hyman et al., 1995). The nucleation structures of GMPCPP microtubules may "breathe" less and so be less prone to closure with an inappropriate number of protofilaments.

Comparison to Other Work

Our measurements of flexural rigidity are not in quantitative agreement with four recent studies (Dye et al., 1993; Vale et al., 1994; Venier et al., 1994; Kurachi et al., 1995). While this disagreement could reflect genuine structural differences between the microtubules due to different sources and preparations of the protein, or to details of the polymerization procedures, we believe that most of the disagreement arises from large experimental uncertainties and theoretical errors in these other studies.[2] Nevertheless, there remain some baffling, though minor, discrepancies. For example, while we found that taxol slightly increased the flexural rigidity, Dye et al., Venier et al., and Kurachi et al. found that taxol decreased it. This discrepancy might be explained by our taxol-stabilized microtubules having a structure different from theirs, as our microtubules did not exhibit the wavy appearance noted by Dye et al. or the helicoidal superstructure that Venier et al. observed. Our finding that taxol, like GMPCPP, increased the rigidity of microtubules, while not in agreement with these laboratories, is consistent with a recent cryoelectron microscopy study showing that stabilization of microtubules with taxol induces upon the tubulin dimer a similar conformational change as does stabilization with GMPCPP (Arnal and Wade, 1995).

[2]The Dye et al. (1993) estimate of the rigidity of unstabilized microtubules is about one-twentieth of ours. (Their flexibility parameter λ equals $kT/2EI$.) However, their theory does not take the intrinsic curvature of microtubules into account, and is therefore not applicable. Furthermore, it is likely that their measurements are dominated by digitization errors since, for a 15-μm-long microtubule, their inferred flexibility corresponds to a root-mean-square fluctuation of the end-to-end distance of only 56 nm—well below the resolution of the microscope. The Venier et al. (1994) estimate of the rigidity of unstabilized microtubules is about one-third of ours. The agreement of their two methods appears to lend credence to their results. But their first (hydrodynamic) method is not a solution to the hydrodynamical equations and does not appear to fully account for the nearby surface (Brennen and Winet, 1977). We believe this leads to an underestimation of the flexural rigidity. Their second method (which is similar to our clamped method) contains an error—the constant in their equation 12 should be $^1/_3$ rather than $^2/_3$ since, like us, they measure the end fluctuation in just one dimension (see Materials and Methods of the present work); so they overestimated the flexural rigidity by a factor of two. Because the two errors are in opposite directions, the two methods are inconsistent with each other. The Vale et al. (1994) measurements correspond to a rigidity ~300-fold smaller than ours. (If their histogram of curvatures (ρ) is approximated by an exponential distribution with mean ρ_o, then the flexural rigidity may be estimated as $EI = kT/2\rho_o^2\Delta s$, where Δs is the arc length over which the curvature is measured and $2\rho_o^2 = \langle\rho^2\rangle$ is the mean square curvature of the distribution.) This low apparent rigidity is roughly that of an actin filament, and is certainly wrong. It is likely that intrinsic bends or large external forces during the freezing process made their microtubules appear artificially flexible. Kurachi et al. (1995) used optical tweezers to measure microtubule rigidity. Their measurements fall within a wide range ($1–200 \times 10^{-24}$ Nm^2) due to an unexpected dependence on length that we have not observed. This apparent length dependence may be due to uncertainty in the boundary conditions in their buckling assay.

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