Stathmin: A Tubulin-Sequestering Protein Which Forms a Ternary T_2S Complex with Two Tubulin Molecules†

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ABSTRACT: Stathmin is an important regulatory protein thought to control the dynamics of microtubules through the cell cycle in a phosphorylation-dependent manner. Here we show that stathmin interacts with two molecules of dimeric $\alpha\beta$−tubulin to form a tight ternary T_2S complex, sedimenting at 7.7 S. This complex appears in slow association−dissociation equilibrium in the analytical ultracentrifuge. The T_2S complex is formed under a variety of ionic conditions, either from GTP− or GDP−tubulin or from the tubulin−colchicine complex. The S16/25/38/63E mutated stathmin in contrast is in rapid equilibrium with tubulin in the T_2S complex. The T_2S complex cannot polymerize in microtubules nor in ring oligomers. Stathmin acts as a pure tubulin-sequestering protein via formation of the T_2S complex. It does not act directly on microtubule ends to promote catastrophe nor enhance microtubule dynamics.

Stathmin plays a pivotal role in signal transduction (1). This ubiquitous, conserved, 17 kDa protein is phosphorylated in multiple and complex ways in response to a variety of extracellular stimuli on four serines (S16, S25, S38, and S63; *2*−*5*). A large body of evidence indicates that stathmin has an essential function in neuronal differentiation and cell division and is suject to cell cycle-regulated phosphorylation (*6*−*11*). Recently, in a search for microtubule destabilizing factors in Xenopus egg extracts, stathmin was identified as a protein regulating the dynamic instability of mitotic microtubules (*12*), by enhancing microtubule catastrophe, i.e., the frequency at which they switch from a regime of slow growth to a regime of rapid depolymerization (*13*, *14*). Stathmin was also demonstrated to inhibit tubulin polymerization in a highly substoichiometric manner (*12*). More recently, however, stoichiometric amounts of stathmin were shown to inhibit tubulin polymerization *in vitro*, and phosphorylation was shown to control its microtubule-destabilizing activity *in vivo*, but not *in vitro* (*15*). Another neuronal, stathmin-related protein, SCG-10, which is abundant in growth cones, has also been reported to cause microtubule disassembly (*16*).

To understand the mechanism of action of stathmin and the molecular basis for its cellular function, we have analyzed the interaction of stathmin with tubulin and microtubules *in vitro* using analytical ultracentrifugation, turbidimetric measurements of microtubule assembly and colchicine binding.

MATERIALS AND METHODS

Proteins. Tubulin was purified from pig brain by two cycles of polymerization (*17*) and phosphocellulose chromatography (*18*) and stored at −80 °C in 50 mM MES-KOH, pH 6.8, 3.4 M glycerol, 0.5 mM EGTA, 0.5 mM $MgCl_2$, and 0.1 mM GTP (M buffer). Before use, tubulin was submitted to one cycle of assembly at 37 °C in MMg buffer (M buffer containing 0.5 mM GTP and 6 mM $MgCl_2$). Sedimented microtubules were resuspended in a buffer containing GTP or GDP, centrifuged at 4 °C, 400000*g* for 10 min, and chromatographed through Sephadex G-25 (PD10, Pharmacia) equilibrated in the chosen buffer. Tubulin concentration was determined spectrophotometrically using an extinction coefficient of 1.2 mg^{-1} cm^2 at 278 nm (*19*).

Recombinant Stathmin was expressed in *Escherichia coli* and purified as described (*20*). Stathmin concentration was determined by amino acid analysis. In Bradford assays, 1 μM stathmin corresponded to 0.21 μM tubulin.

Analytical Ultracentrifugation. Sedimentation velocity was measured at 20 °C in a Beckman Optima XL-A analytical ultracentrifuge. Samples containing tubulin or tubulin−colchicine complex (7−20 μM) equilibrated in GTP- or GDP-containing P buffer (80 mM PIPES-KOH, pH 6.8, 0.5 mM EGTA, and 0.5 mM $MgCl_2$) and different amounts of stathmin were centrifuged at 50 000 rpm. Radial scans were taken at 290 nm at 5 min intervals. Stathmin is not visible at 290 nm due to its lack of aromatic residues. To estimate the concentration of tubulin in the different sedimenting species, the instrument was calibrated by measuring the absorbance of tubulin solutions of known concentrations at low speed (3000 rpm). Scans were analyzed (*21*) to derive the distribution of sedimentation coefficients.

Colchicine Binding. The kinetics of colchicine binding to tubulin was monitored by the increase in fluorescence of colchicine (*22*) using a Spex spectrofluorimeter thermostated at 30 °C. Excitation and emission wavelengths were 365 and 425 nm, respectively. Precycled tubulin (10 μM), equilibrated in P buffer containing 50 μM GTP, was reacted with 150 μM colchicine. Data were analyzed in terms of a double exponential (*22*).

Polymerization Studies. Tubulin polymerization was monitored turbidimetrically at 350 nm (*23*) in a Kontron-Uvikon spectrophotometer thermostated at 37 °C, using cuvettes of 150 μL and 1 cm light path (Hellma). Experi-

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ments were carried out either in MMg or PMg buffers containing 0.5 mM GTP. To start the reaction, $MgCl_2$ at a concentration of 6 mM was added to the tubulin solution in M or P buffer that had been preheated in the cuvette for 1 min. The increase in turbidity was recorded.

Critical concentration plots, representing the amount of polymerized tubulin versus the total concentration of tubulin, were obtained by polymerizing a stock solution of tubulin in a water bath at 37 °C, followed by addition of stathmin and serial dilution with warm polymerization buffer containing the same amount of stathmin. The absorbance of each sample was measured at 37 °C, then after 15 min incubation at 0 °C to ensure complete depolymerization. The difference between the two values was plotted versus the total concentration of tubulin, yielding a linear curve in the tubulin concentration range investigated. The abscissa intercept represented the concentration of unassembled tubulin at steady state. The values of absorbance read at 0 °C increased slightly with tubulin concentration (see Figure 4a, open circles, as an example), but at a given concentration of tubulin, the values were independent of the concentration of stathmin present in the sample.

To validate the use of turbidimetric measurements of microtubule assembly, the amount of protein present in the supernatants of microtubules sedimented for 8 min at 300000*g*, 37 °C, was assayed. The concentration of unassembled tubulin was calculated as the difference between the measured amount of protein and the concentration of stathmin. The values were identical to those derived from turbidimetric data. SDS−PAGE demonstrated the absence of stathmin in the microtubule pellets.

Polymerization of tubulin−colchicine complex was monitored at 420 nm (*24*) in MMg buffer.

GTP Hydrolysis Measurements. Tubulin was polymerized at 45 μM in PMg buffer containing 200 μM γ-^{32}P-labeled GTP. When steady state was reached (from absorbance measurements), the solution was split into different tubes using a truncated tip and was supplemented with different concentrations of stathmin. Hydrolysis of GTP at steady state was measured as described (*25*).

RESULTS

Stathmin Interacts with Two αβ-Tubulin Heterodimers in a Ternary T_2S Complex. The sedimentation velocity of tubulin was examined in the presence of different amounts of stathmin. In P buffer, tubulin sedimented as a single species of 5.6 S. In the presence of an excess of stathmin, tubulin sedimented as a single species at 7.7 S. At substoichiometric amounts of stathmin, the 5.6 and 7.7 S species sedimented as two independent noninteracting components (Figure 1a), which indicated that the tubulin−stathmin complex is in slow association−dissociation equilibrium. The proportion of tubulin in the 5.6 and 7.7 S species at each concentration of stathmin was derived from the distribution of sedimentation coefficients. Figure 1b shows that the mass fraction of the 7.7 S species increased with the amount of stathmin added and reached a maximum of 1 as soon as 0.5 M equiv stathmin per tubulin was present in the sample. No other species was observed upon increasing stathmin up to a 2:1 molar ratio to tubulin. These results demonstrate that stathmin (S) interacts with two molecules of tubulin (T) in a ternary T_2S complex sedimenting at 7.7 S at 20 °C. No intermediate complex was observed. This result was obtained with GTP−tubulin, GDP−tubulin, and tubulin−colchicine. The complex appeared slightly weaker with GTP−tubulin. Upon addition of increasing amounts of the S16/25/38/63E mutated stathmin to tubulin, the 7.7 S complex was eventually formed, but the distribution of sedimentation coefficients always displayed a single gaussian curve, with a maximum S value increasing from 5.6 to 7.7 S, which indicated that, in contrast to stathmin, the mutated stathmin was in rapid equilibrium with tubulin (*26*).

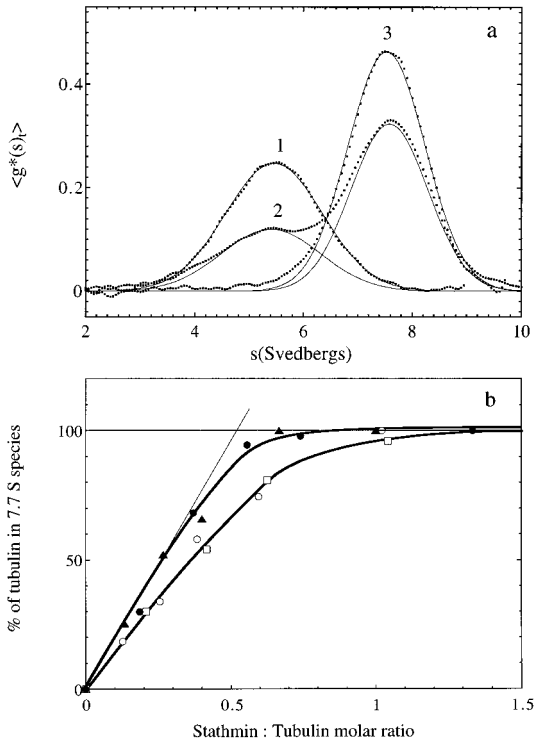

FIGURE 1: Stathmin forms a ternary T_2S complex with two tubulin $\alpha\beta$ heterodimers. (a) Distribution of the sedimentation coefficients of GDP−tubulin (13.5 μM) in the absence (1) and in the presence of 5 μM (2) and 7.5 μM (3) stathmin. Dots are the data and thin lines the deconvolution in one (curves 1, 3) or two (curve 2) gaussian curves. (b) Sedimentation velocity of tubulin in P buffer in the presence of stathmin. The percent of tubulin in the 7.8 S complex is plotted versus the stathmin:tubulin molar ratio. ●, GDP−tubulin (13.5 μM); ▲, tubulin−colchicine (15 μM); □, GTP−tubulin (7.5 μM); ○, GTP−tubulin (12 μM).

Independent evidence for the interaction of stathmin with two molecules of tubulin was provided by the effect of stathmin on the rate of colchicine binding to tubulin, displayed in Figure 2. Colchicine binding was slowed down by stathmin, consistent with the formation of a ternary complex of two tubulins and one stathmin, to which colchicine bound with a 3-fold slower rate. The change in fluorescence of colchicine upon binding to tubulin is known to be biphasic (*27*), the first phase accounting for 70% of the overall fluorescence change. Only the rate constant of the first phase was decreased by stathmin, the slow phase remained unaffected. The overall increase in fluorescence also was unaffected by stathmin.

Mechanism for Destabilization of Microtubules by Stathmin. Spontaneous polymerization of tubulin into microtubules was inhibited by stathmin. Figure 3 shows that in the presence of stathmin, the lag time preceding microtubule assembly was longer and the extent of turbidity increase was

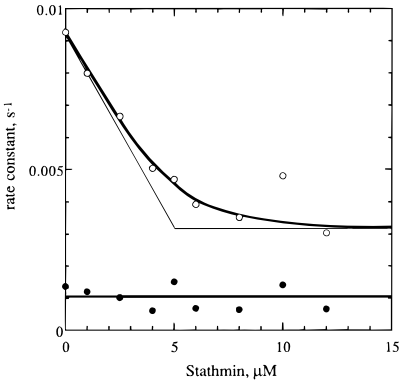

FIGURE 2: Stathmin slows down colchicine binding to tubulin with a 1:2 molar ratio. The time course of colchicine binding to tubulin (10 μM in P buffer) was examined in the presence of the indicated concentrations of stathmin. The values of the first order rate constant for the rapid phase (○) and the slow phase (●) of the fluorescence change are plotted versus stathmin concentration. The lower limit value of the rate constant of the rapid phase was confirmed by other data (not shown) up to 25 μM stathmin.

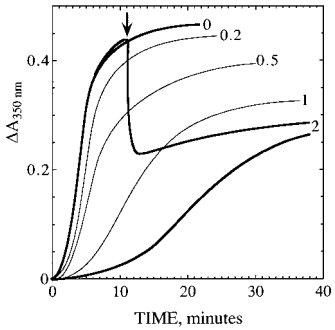

FIGURE 3: Stathmin inhibits spontaneous polymerization of pure tubulin and causes microtubule disassembly. Tubulin was polymerized at 15 μM in MMg buffer in the absence (0) or in the presence of stathmin at the indicated concentrations (in micromolarity). Arrow indicates the addition of 2 μM stathmin to preassembled microtubules.

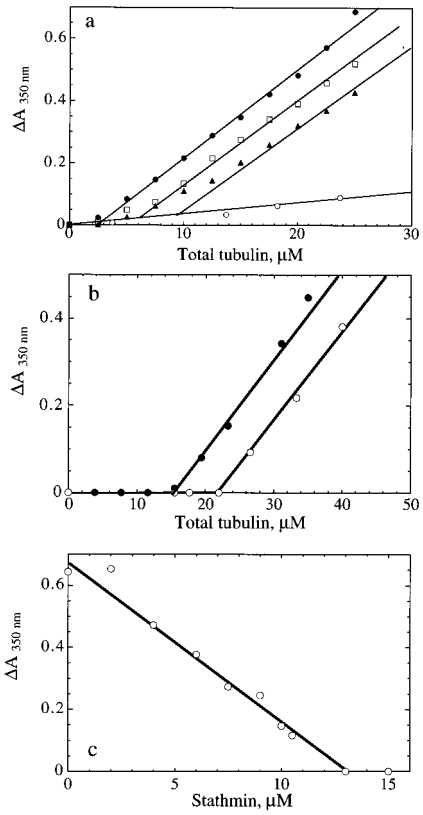

FIGURE 4: Stathmin depolymerizes microtubules into the T2S complex. (a) Critical concentration plots for microtubule assembly in MMg buffer in the absence (●) and in the presence of 1.5 μM (□) and 3 μM (▲) stathmin. Open circles: absorbance at 0 °C following depolymerization of microtubules. (b) Critical concentration plots in PMg buffer, in the absence (●) and in the presence (○) of 3 μM stathmin. The values of absorbance at 0 °C have been subtracted. (c) Stathmin sequesters tubulin in a 1:2 molar ratio. Tubulin was polymerized at 45 μM in PMg buffer, and stathmin was added as indicated at steady state. ΔA represents the difference in absorbances measured at 37 and 4 °C.

lower, indicating that a lower amount of microtubules were assembled at steady state. The same final turbidity level was reached at late times whether stathmin was added to preassembled microtubules or to tubulin before polymerization was started. This result indicates that a defined steady state exists in the presence of stathmin.

The effect of stathmin on the amount of tubulin polymerized at steady state provides a means to quantitatively assess the function of stathmin in microtubule assembly. Critical concentration plots were derived from turbidimetric measurements and validated by sedimentation assays (see Materials and Methods).

In MMg buffer (Figure 4a), the critical concentration for microtubule assembly was 2.5 μM. In the presence of stathmin, the critical concentration plots were shifted while remaining parallel to the control straight line observed in the absence of stathmin. This result indicated that stathmin caused depolymerization of the same amount of microtubules at all tubulin concentrations. The amount of unassembled tubulin in complex with stathmin can be derived from the shift of the plot. In the presence of 1.5 μM stathmin, the critical concentration shifted from 2.5 to 5.5 μM, indicating that 3 μM tubulin had depolymerized. In the presence of 3 μM stathmin, the concentration of unassembled tubulin was 9.5 μM, indicating that 7 μM tubulin had depolymerized. Therefore 2 mol of tubulin are depolymerized per mole of sathmin added, consistent with the formation of a ternary T_2S complex that does not participate in microtubule assembly. The remaining microtubules coexist at steady state with dimeric tubulin at an unmodified critical concentration. The curvature of the critical concentration plots at low tubulin concentration is consistent with the relatively facile nucleation of microtubules in glycerol-containing MMg buffer. Similar curved plots are indeed observed for actin assembly in the presence of nucleating agents (*28*).

Sedimentation velocity measurements of microtubules assembled in MMg buffer in the presence of stathmin were carried out at 37 °C. Only tubulin and the T_2S species, sedimenting at 3.2 S in this buffer, were observed following the rapid sedimentation of microtubules. Once corrected for viscosity and temperature, the 3.2 S value corresponded to 8 S at 20 °C in water. Analysis of the absorbance scans showed that 4.2 and 9.4 μM tubulin was present in the 3.2 S species following addition of 2 and 5 μM stathmin respectively to polymerized tubulin.

Data obtained in PMg buffer are displayed in Figure 4, panels b and c. Dynamic instability is known to be extensive in this buffer, while it is attenuated by glycerol present in MMg buffer. The critical concentration was 15 μM in PMg buffer. In the presence of 3 μM stathmin, a 6 μM shift in the critical concentration plot was observed, indicating that again stathmin sequestered tubulin in a 1:2 complex (Figure

Table 1: Stathmin Causes Dissociation of Ring Oligomers of Tubulin

	f		
stathmin, μM	5.6 S	7.8 S	32−40 S
0	68.1	0	31.8
5	25	60.7	13.5

[a] Sedimentation velocity patterns of GDP−tubulin (50 μM in 10 mM phosphate buffer pH 7.0 containing 0.1 mM GDP and 10 mM $MgCl_2$) in the presence of stathmin were analyzed in terms of three components, a heavy 32−40 S component, a 7.8 S, and a 5.6 S species. The percent mass of tubulin in each species, *f*, was derived.

4b). When increasing amounts of stathmin were added to a solution of 45 μM tubulin preassembled in microtubules, the amount of polymerized tubulin decreased linearly with stathmin. Total depolymerization was observed with 13.5 μM stathmin (Figure 4c). Since the concentration of polymerized tubulin was initially 45 − 15 = 30 μM, this result reasonably confirms that stathmin causes microtubule disassembly by forming a nonpolymerizable T_2S complex in all buffers. Similar experiments carried out at other concentrations of tubulin, in either MMg or PMg buffer, (not shown) strengthened this conclusion. Sedimentation assays of microtubule solutions containing different amounts of stathmin and protein measurements in the supernatant confirmed that 2 mol of tubulin were depolymerized per mole of stathmin added. Finally, the S16/25/38/63E mutant of stathmin depolymerized microtubules in a manner quantitatively identical to stathmin, in agreement with Horwitz et al. (*15*).

In conclusion, the simple tubulin-sequestering activity of stathmin appears to satisfactorily account for its microtubule-destabilizing activity. Stathmin had first been proposed to increase the rate of catastrophe of microtubules (*12*). The more frequent losses of GDP subunits are expected to lead to an increase in the turnover of microtubules at steady state, which can be estimated by measuring the resulting increase in steady state GTPase activity of microtubules. The addition of 1 or 2 μM stathmin to a solution of 45 μM polymerized tubulin in PMg buffer caused no detectable change in the steady state GTPase rate, consistent with a pure tubulin-sequestering function of stathmin.

Stathmin Prevents Longitudinal Tubulin−Tubulin Interactions. A microtubule is a bidimensional lattice in which tubulin subunits interact via longitudinal bonds (along protofilaments) and lateral bonds (between adjacent protofilaments). Tubulin can also assemble into linear, ring-shaped oligomers. The formation of rings is favored in the presence of GDP and millimolar amounts of Mg^{++} ions and at low temperature (*29*). The effect of stathmin on the formation of rings was examined in the analytical ultracentrifuge. In the absence of stathmin, dimeric tubulin was in equilibrium with a population of oligomers sedimenting as two species at 32 and 40 S. Upon addition of stathmin, the proportion of the rapidly sedimenting species decreased as a 7.7 S species became predominant. Data are summarized in Table 1. Therefore, the T_2S complex cannot assemble into linear oligomers of tubulin.

This conclusion was confirmed by examination of the effect of stathmin on the polymerization of tubulin−colchicine. Under solution conditions similar to those leading to microtubule assembly, tubulin−colchicine complex assembles into ring oligomers that display a high tendency to associate into amorphous aggregates (*24*). Polymerization of tubulin−colchicine (TC) was inhibited by stathmin in a manner consistent with the formation of a nonpolymerizable $(TC)_2S$ complex. Results similar to those shown for microtubules in Figure 4c were obtained upon addition of stathmin to polymerized tubulin−colchicine. Namely, the critical concentration for polymerization of tubulin−colchicine was 10 μM. Increasing amounts of stathmin were added to a solution of 31 μM preassembled tubulin−colchicine. The absorbance at 420 nm decreased linearly with stathmin and reached the value measured at 0 °C at 12 μM stathmin, in reasonable agreement with the expected value of (31 − 10)/2 μM = 10.5 μM corresponding to the formation of the $(TC)_2S$ complex.

DISCUSSION

We have shown that, under several ionic conditions, stathmin interacts with two $\alpha\beta$−tubulin heterodimers to form a ternary (T_2S) complex of 217 kDa sedimenting at 7.7 S. This value of the sedimentation coefficient is somewhat lower than the 9 S value expected for a globular protein of the same molecular mass, which suggests that the T_2S complex has an elongated shape. The sedimentation data (Figure 1) demonstrate that the T_2S complex has a high stability ($> 10^6$ M^{-1}) and is in slow association−dissociation equilibrium, suggesting that tubulin must be the physiological target of stathmin. At the tubulin concentrations used in the experiments (10^{-5} M), stathmin bound apparently almost as well to GTP−tubulin as to GDP−tubulin. Only binding assays carried out at tubulin concentrations lower than the equilibrium dissociation constant would allow to measure the difference in affinity of stathmin for GTP− and GDP−tubulin with accuracy.

Neither TS nor TS_2 complexes have been observed in the analytical ultracentrifuge. Hence, the two molecules of tubulin in the T_2S complex must interact with each other, thus contributing to the overall stability of the ternary complex.

Quantitative analysis of the interference of stathmin with microtubule assembly demonstrates that stathmin acts as a tubulin-sequestering protein. The effect of stathmin on tubulin polymerization can be described by the following scheme.

$$T \Leftrightarrow MTs$$

$$2T + S \Leftrightarrow T_2S$$

The critical concentration for microtubule assembly is not affected by stathmin, indicating that stathmin does not interact with microtubule ends appreciably. One can imagine that stathmin establishes a reservoir of unassembled tubulin that can be used, upon appropriate stimulation, to assemble new microtubules, a process which actively operates in neurite outgrowth and in mitosis.

The difference in the regulation of sequestration of actin and tubulin is striking. The major G-actin sequestering agent is thymosinβ4, which forms a 1:1 complex with G-actin (*30*). The equilibrium dissociation constant for the Tβ4−actin complex is 10^{-6} M, in the range of variation of the critical concentration for actin assembly. Hence, the concentration of sequestered actin (Tβ4−actin complex, TA) is controlled

by changes in critical concentration elicited by capping proteins and profilin (*31*, *32*), as described by the following equation (*32*).

$$[TA] = [T_0]\frac{C_C}{C_C + K_T}$$

where T_0 is the total concentration of Tβ4, C_C the critical concentration for actin assembly, and K_T the equilibrium dissociation constant of the TA complex.

In the case of tubulin, the stathmin(tubulin)$_2$ complex is very tight. The concentration of T_2S is insensitive to changes in critical concentration, because $K_T << C_C$, which results in $[T_2S] = [S_0]$ as soon as tubulin is present in sufficient amounts. The regulation of the amount of T_2S has to be elicited by another mechanism, most likely phosphorylation of stathmin. The S16/25/38/63E mutant surprisingly depolymerizes microtubules like stathmin *in vitro* (*15*; and the present work), while it does not *in vivo* (*15*). The fact that the mutated stathmin is in rapid equilibrium with tubulin suggests that it has a lower affinity for tubulin than stathmin. While this difference in affinity does not show up *in vitro* at micromolar concentrations of the proteins, it may be greatly enhanced *in vivo* due to macromolecular crowding which buffers the concentrations of free proteins to a low value.

The fact that 1 mol of stathmin depolymerizes 2 mol of tubulin seems somewhat at variance with the highly substoichiometric effect of stathmin reported earlier (*12*). The discrepancy is only apparent. The stathmin:tubulin stoichiometry had been expressed (*12*) as the molar ratio of total stathmin:total tubulin. The present work shows that the functionally relevant stoichiometry is stathmin:(unpolymerized tubulin $- C_C$). To be specific, when the critical concentration for microtubule assembly is 15 μM, at a total concentration of 17 μM tubulin, 2 μM tubulin is polymerized and only 1 μM stathmin causes total disassembly (in this particular circumstance this represents a 1:17 molar ratio).

For the same reason, stathmin looked like a catastrophin in the *in vitro* assay used (*12*) because, in decreasing the concentration of free tubulin available for microtubule growth to concentrations close to the critical concentration, it increased the rate of catastrophe. In other words, the simple sequestering activity of stathmin reveals a functionally important aspect of microtubule dynamic instability, i.e., the steep dependence of the frequency of rapid depolymerization on tubulin concentration (*13*, *14*).

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