doi:10.1006/jmbi.2000.3554 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2000) 297, 211–220

JMB

A Reassessment of the Factors Affecting Microtubule Assembly and Disassembly *in Vitro*

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Current models of microtubule assembly from pure tubulin involve a nucleation phase followed by microtubule elongation at a constant polymer number. Both the rate of microtubule nucleation and elongation are thought to be tightly influenced by the free GTP-tubulin concentration, in a law of mass action-dependent manner. However, these basic hypotheses have remained largely untested due to a lack of data reporting actual measurements of the microtubule length and number concentration during microtubule assembly.

Here, we performed simultaneous measurements of the polymeric tubulin concentration, of the free GTP-tubulin concentration, and of the microtubule length and number concentration in both polymerizing and depolymerizing conditions. In agreement with previous work we find that the microtubule nucleation rate is strongly dependent on the initial GTP-tubulin concentration. But we find that microtubule nucleation persists during microtubule elongation. At any given initial tubulin-GTP concentration, the microtubule nucleation rate remains constant during polymer assembly, despite the wide variation in free GTP-tubulin concentration. We also find a remarkable constancy of the rate of microtubule elongation during assembly. Apparently, the rate of microtubule elongation is intrinsic to the polymers, insensitive to large variations of the free GTP-tubulin concentration. Finally we observe that when, following assembly, microtubules depolymerize below the free GTP-tubulin critical concentration, the rate-limiting factor for disassembly is the frequency of microtubule catastrophe. At all time-points during disassembly, the microtubule catastrophe frequency is independent of the free GTP-tubulin concentration but, as the microtubule nucleation rate, is strongly dependent on the initial free GTP-tubulin concentration. We conclude that the dynamics of both microtubule assembly and disassembly depend largely on factors other than the free GTP-tubulin concentration. We propose that intrinsic structural factors and endogenous regulators, whose concentration varies with the initial conditions, are also major determinants of these dynamics.

Keywords: microtubules; dynamics; nucleation; elongation; disassembly

Introduction

Microtubules are vital cell structures that can be assembled from pure GTP-tubulin *in vitro* (Dustin, 1984). Tubulin assembly into microtubules is a reversible process which involves a continuous exchange between the soluble and the polymeric tubulin pools. For many years, the kinetics of microtubule assembly have been interpreted according to simple law of mass action-based models (Oosawa & Kasai, 1962; Oosawa & Higashi, 1967; Johnson & Borisy, 1977). Exchanges between the soluble and the polymeric tubulin pools were supposed to be rapid, with a continuous and direct effect of the free GTP-tubulin concentration on microtubule behavior. In this view, the rates of microtubule nucleation and of microtubule elongation are both supposed to be dependent

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0022-2836/00/010211–10 $35.00/0

at all time-points on the free GTP-tubulin concentration. When the free GTP-tubulin concentration decreases during microtubule polymerization, the rate of microtubule nucleation decreases as a function of a high power of this concentration, whereas the rate of microtubule elongation varies linearly with the free GTP concentration (Carlier & Pantaloni, 1978; Voter & Erickson, 1984; Fygenson *et al*., 1994, 1995; Flyvbjerg *et al*., 1996). Hence, microtubule assembly seems to proceed in two phases, a phase of microtubule nucleation followed by a phase of microtubule elongation at constant microtubule number concentration. Microtubule disassembly, caused for instance by a decrease of the free GTP-tubulin concentration, is viewed as mechanistically identical with microtubule elongation, with a negative net assembly rate.

The discovery of microtubule dynamic instability has modified these views, suggesting that microtubule dynamics are strongly influenced by structural transitions occurring at microtubule ends (Mitchison, 1984a,b; Horio & Hotani, 1986; Walker *et al*., 1988; Mandelkow *et al*., 1991). It is still thought that microtubule nucleation and elongation are dependent at all time-points on the free GTP-tubulin concentration. But a central hypothesis of all dynamic instability models is that microtubule disassembly and microtubule assembly are different processes. According to these models, disassembling and assembling microtubules react in different ways with their molecular environment, including free GTP-tubulin dimers (Mitchison & Kirschner, 1984b; Chen & Hill, 1987; Mandelkow *et al*., 1991; Bailey *et al*., 1990; Chrétien *et al*., 1995).

To our knowledge, these basic hypotheses concerning microtubule assembly dynamics have remained largely untested. For instance, the hypothesis that the microtubule number concentration remains constant during microtubule elongation has been central to the interpretation of microtubule assembly curves, but complete sets of data including measurements of the microtubule length and number concentration over a wide range of tubulin concentrations are lacking. In fact, the description of the first reliable method for microtubule length measurements (at least in the case of pure tubulin polymers) appeared in 1984 (Michison, 1984a,b). The same papers reported the discovery of dynamic instability. Following this discovery, the main interest of scientists in the field shifted to the video-microscopy examination of microtubule dynamic instability close to the critical GTP-tubulin concentration.

Here, we examine the kinetic behavior of both assembling and disassembling microtubules over a wide range of GTP-tubulin concentrations, using simultaneous measurements of the free-GTP tubulin concentration, of the mass of polymeric tubulin and of the microtubule length and number concentration. We find that at most time-points during assembly, microtubule nucleation and elongation proceed at constant rates, independent of the free GTP-tubulin concentration. We also provide evidence that, as predicted by dynamic instability models, the dynamics of assembling and disassembling microtubules depend on distinct factors. Finally, we propose that microtubule assembly and disassembly dynamics depend to a large extent on structural factors and on the effect of tubulin oligomers, whose concentration varies with the initial conditions.

Results

We wanted to investigate the kinetics of both microtubule polymerization and depolymerization, in a chemically simple and defined system. For this, we assembled pure GTP-tubulin in the absence of excess free GTP. In such conditions, a net microtubule assembly phase and a net microtubule disassembly phase occur sequentially (Carlier & Pantaloni, 1978; O'Brien & Erickson, 1989; Carlier *et al*., 1997). Furthermore the GTP-tubulin concentration can be determined directly and accurately. Since tubulin forms a 1:1 complex with GTP (Carlier & Pantaloni, 1978; O'Brien & Erickson, 1989), the GTP-tubulin concentration can be assayed simply by measuring the remaining GTP-tubulin concentration at each time-point (see Materials and Methods). This is not the case in other experimental systems using either excess GTP, or GTP-regenerating systems. In such systems, the GTP-tubulin concentration cannot be measured directly and depends on a complex set of reactions through which GDP-tubulin is converted back to GTP-tubulin (Pirollet *et al*., 1987; Mandelkow *et al*., 1988; Carlier *et al*., 1997). Starting with four different GTP-tubulin concentrations, we measured the concentration of polymeric tubulin and the free GTP-tubulin concentration at various time-points. The microtubule number concentration, defined as the number of microtubules per unit of volume (expressed in pM), was determined in parallel, when technically possible (microtubule mean length <60 μm, see Materials and Methods). Results showed that the maximum concentration of polymeric tubulin increased with increasing initial GTP-tubulin concentration (Figure 1(a)-(d)). The transition between the assembly and the disassembly phases occurred at a GTP-tubulin concentration of 30(±5) μM (Figure 1(e)), independent of the initial GTP-tubulin concentration and corresponding to the tubulin critical concentration in the buffer conditions used (Mitchison & Kirschner, 1984b). Both the rates of tubulin polymerization and of tubulin depolymerization increased sharply with increasing initial GTP-tubulin concentrations (Figure 1(a)-(d)).

Microtubule nucleation

In current kinetic models of microtubule assembly, the microtubule number concentration is thought to to be constant during microtubule polymerization. Here, we determined the microtubule

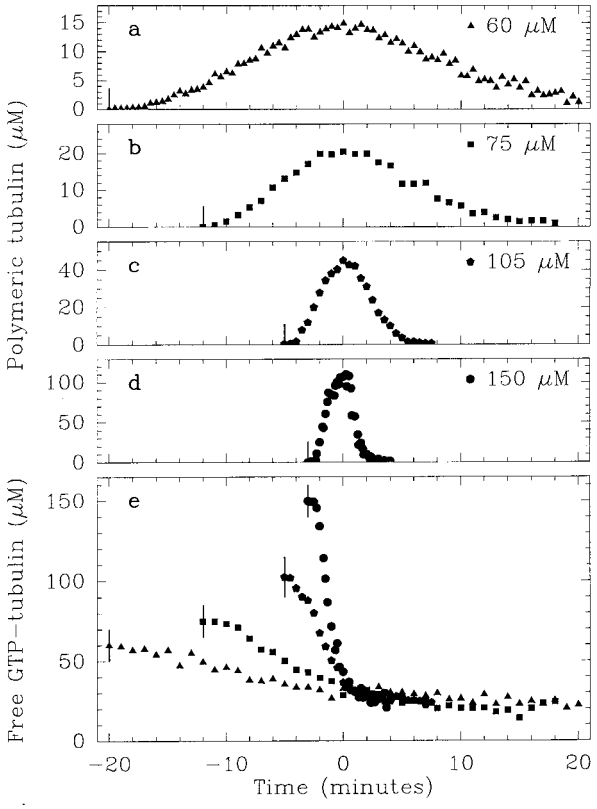

Figure 1. Assembly of GTP-tubulin. GTP-tubulin was assembled in the absence of free GTP, at different initial tubulin concentrations, as indicated. (a)-(d) Concentration of tubulin in polymers. (e) GTP-tubulin concentration, measured at the indicated time-points. Time-zero corresponds to maximum tubulin assembly. Vertical bars indicate the beginning of experiments. Note that the ordinate scale is different in (a), (b), (c) and (d).

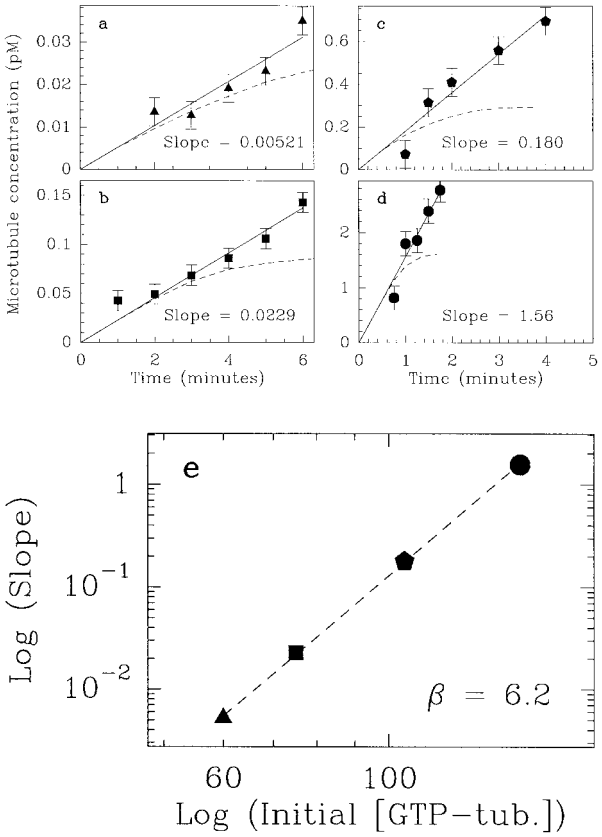

Figure 2. Plots of microtubule number concentration *versus* time. The microtubule number concentration was determined at different time-points and at different initial GTP-tubulin concentrations: (a) 60 μM; (b) 75 μM; (c) 105 μM; (d) 150 μM. Time-zero corresponds to the beginning of experiments. After six minutes of assembly, the microtubule mean length exceeded 60 μm in (a) and (b), and this precluded further determination of the microtubule number concentration. The data shown in (c) and (d) concern the first 80 % of the microtubule polymerization phase, excluding the transition between assembly and disassembly regimes. Continuous lines, best linear fit to experimental data. Corresponding slopes are indicated. Confidence intervals are displayed in the form of error bars on measured points. (e) Log-log plot of these slopes *versus* the corresponding initial GTP-tubulin concentrations and linear regression fit (broken line). The slope of the regression line equals 6.2. Broken lines in (a)-(d), expected plots of microtubule number concentration over time, assuming a microtubule nucleation rate proportional at all time-points to [free GTP-tubulin]$^{6.2}$ (see the text), as predicted by current nucleation models. Note that the deviation between the observed and the expected data is minimal at early times of microtubule assembly ((a)) and becomes evident only when enough free tubulin has been incorporated into polymers, so that the current tubulin concentration is markedly different from the initial tubulin concentration ((b), (c) and (d)).

number concentration during assembly, starting at four different tubulin-GTP concentrations, ranging from 60 to 150 μM (Figure 2(a)-(d)). In apparent contrast with expectation, our data showed persistent microtubule nucleation during most of the polymerization phase, despite the decreasing free GTP-tubulin concentration. At all tested initial GTP-tubulin concentrations, we observed a linear increase in microtubule number concentration during tubulin polymerization, indicating a constant rate of microtubule nucleation. The rate of microtubule nucleation was strongly influenced by the initial GTP-tubulin concentration. The nucleation rate at 150 μM initial GTP-tubulin concentration (Figure 2(d)) was 300 times higher than the nucleation rate at 60 μM initial GTP-tubulin concentration (Figure 2(a)). Previous measurements made at initial time-points of microtubule assembly (Voter & Erickson, 1984) showed that the rate of microtubule nucleation and free tubulin concentration are linearly related on a log-log plot. A similar relationship is shown in Figure 2(e). We find that the rate of microtubule nucleation was proportional to [initial-GTP-tubulin]$^{6.2}$ (Figure 2(e)).

Thus, in agreement with previous work, we find that the free GTP-tubulin concentration strongly

influences microtubule nucleation at initial time-points. But current models of microtubule nucleation are based on the hypothesis that the relationship between the free GTP-tubulin concentration and the rate of microtubule nucleation persists unchanged at subsequent time-points of microtubule assembly. In contrast, we find that the nucleation rate remains apparently constant during microtubule assembly, independent of the free GTP-tubulin concentration.

Are our data truly incompatible with a persistent influence of the free GTP-tubulin concentration on the microtubule nucleation rate? If the influence of the free GTP-tubulin concentration on the rate of microtubule nucleation was conserved during microtubule polymerization, the nucleation rate would be proportional to [free GTP-tubulin]$^{6.2}$ at all time-points. To compare our data with the data expected according to such proportionality, we calculated at all time-points the expected microtubule number concentration using the observed values of the free GTP-tubulin concentration (Figure 1(d)) and this proportionality (see Materials and Methods). The resulting curves are shown in Figure 2(a)-(d) (broken lines). In contrast to the observed linear plots of the microtubule number concentration over time, the calculated curves plateaued. This reflected the expected effect of decreasing free GTP-tubulin concentration on the calculated microtubule nucleation rate during assembly. At the highest initial GTP-tubulin concentrations and at the latest time-points during assembly, the difference between the observed and calculated curves was massive, with a two- to threefold difference between the calculated and observed values of the microtubule number concentrations (Figure 2(c) and (d)). At initial time-points during microtubule assembly, the expected and the experimental curves remained close because the free GTP-tubulin concentration remained near to its initial value. The difference between the observed curves and the calculated curves was significant, but relatively small, at 60 μM initial tubulin concentration (Figure 2(a)). In this condition, the microtubule length measurements were technically feasible only at initial time-points during assembly. Within this phase of assembly, the variation of the free GTP-tubulin concentration was small.

In conclusion, our data show that microtubule nucleation persists at a constant rate during most of the microtubule elongation phase, despite wide variation of the free GTP-tubulin concentration. Such insensitivity to the free GTP-tubulin concentration is not compatible with current models, which suppose the formation of microtubule nuclei from free GTP-tubulin dimers. Clearly, this raises new questions with regard to the mechanism of microtubule nucleation (see Discussion).

Microtubule elongation

It is assumed that the rate of individual microtubule growth during polymerization is directly proportional to the free GTP-tubulin concentration. Here, the microtubule mean length was determined during tubulin polymerization (Figure 3(a)). A striking observation was that, during most of the polymerization phase, the microtubule mean length increased linearly over time, at a constant rate, independent of both the initial and the current

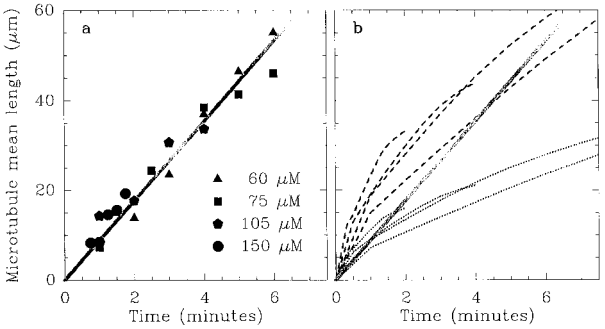

Figure 3. Plots of microtubule mean length *versus* time. (a) The microtubule mean length was measured at different time-points and different initial GTP-tubulin concentrations, as indicated. Limitations in experimental measurements were as described in the legend to Figure 2. Time-zero corresponds to the beginning of experiments. Continuous line, best linear fit of experimental data. As estimated from the slope of the continuous line, the microtubule mean length increased over time at a rate of 8.9 μm/minute. (b) Predicted evolution of the microtubule mean length under two different models of individual microtubule elongation. Broken and dotted curves, individual microtubule elongation proportional to the free GTP-tubulin concentration. In this case, the rate of microtubule elongation depends on the free GTP-tubulin concentration and on an elongation constant, ε (see equation (4) in Materials and Methods) whose value is unknown. Model curves of microtubule mean lengths at the four tested initial free GTP-tubulin concentrations were calculated for a variety of different ε values. The model curves were invariably incompatible with the observed data shown in (a); (b) illustrates this result for two particular values of ε chosen to best fit experimental data observed at 60 μM initial GTP-tubulin and at 150 μM initial GTP-tubulin, respectively. The two different sets of calculated curves are shown with an elongation constant adjusted to best fit the experimental data observed at 60 μM tubulin concentration (broken curves), or an elongation constant adjusted to best fit the experimental data observed at 150 μM tubulin concentration (dotted curves). Whatever the value chosen for the elongation constant, model curves were incompatible with the experimental data. Continuous line, individual microtubule elongation independent of the free GTP-tubulin concentration. The individual microtubule elongation rate was fixed at twice the rate of the microtubule mean length increase (2×8.9 μm/minute $= 17.8$ μm/minute, see equations (5) and (10) in Materials and Methods). The model plot of microtubule mean length is identical with the linear fit of experimental data shown in (a).

free GTP-tubulin concentrations (Figure 3(a)). The microtubule mean length depended only on the time-point at which it was determined, not on the free GTP-tubulin concentration.

Such a linear increase of the microtubule mean length, regardless of the free GTP-tubulin concentration, seemed poorly compatible with proportionality between the individual microtubule elongation rate and the GTP-tubulin concentration. However, the microtubule mean length is a complex parameter that depends both on the rate of individual microtubule growth and on the rate of new microtubule formation (nucleation rate). Therefore we tested whether or not the observed linear increase of the mean microtubule length over time was indeed incompatible with proportionality between the rate of individual microtubule elongation and the free GTP-tubulin concentration.

For this, we calculated values of the mean microtubule length during microtubule polymerization, at the different initial GTP-tubulin concentrations, according to the observed nucleation rates, to the observed free GTP-tubulin concentrations, and to the law of mass action (see Materials and Methods). The calculated values deviated strongly from the observed values (Figure 3(b), broken and dotted lines). Therefore our data were incompatible with the hypothesis of proportionality between the individual microtubule growth rate and the free GTP-tubulin concentration.

The simplest alternative hypothesis was that the individual microtubules themselves elongated at a constant rate, independent of the free GTP-tubulin concentration during the assembly phase. Is this hypothesis compatible with models and data? To answer this question we modeled the evolution of the microtubule mean length over time as above, except that the rate of individual microtubule elongation was assumed to be constant, independent of the free GTP-tubulin concentration. Simple calculations showed that in this case the mean microtubule length increases linearly over time, independent of both the initial and current free GTP-tubulin concentration (equation (10), see Materials and Methods). Furthermore, the rate of the mean microtubule length increase is mathematically equal to half the individual microtubule elongation rate (equation (10)). We observed a rate of microtubule mean length increase of 8.9 μm/minute. We then calculated the values of the microtubule mean length at all time-points and initial tubulin concentrations under the hypothesis of a constant microtubule growth rate of 17.8 μm/minute. The calculated plot was identical with the observed plot (Figure 3(b), continuous line, compared to Figure 3(a), continuous line). We conclude that, in contrast with expectation, individual microtubules elongate at a constant rate, independent of the free GTP-tubulin concentration during most of the polymerization phase.

Analysis of microtubule disassembly

What determines the kinetics of microtubule disassembly? This question could be addressed in the case of experiments run at 105 and 150 μM initial GTP-tubulin concentration. In these experiments, the microtubule lengths could be measured during the disassembly phase. During the phase of rapid microtubule disassembly (corresponding to a drop in the polymeric tubulin concentration from 90 % to 0 % of its maximal value), the GTP-tubulin incorporation into microtubules was negligible. Such incorporation represented less than 2 % of the maximal mass of polymeric tubulin (Figure 1(e)). Thus, in our experimental conditions, sustained microtubule growth and/or microtubule rescues during the net microtubule disassembly phase were apparently rare events.

In the absence of significant assembly, the rate-limiting factor for bulk microtubule disassembly could either be the rate of individual microtubule subunit loss, or the frequency of microtubule catastrophe, or both. These different possible situations would be completely different with regard to the evolution of the microtubule mean length over time. The rate of individual microtubule subunit loss could be the limiting factor if all polymers underwent rapid end transition toward a disassembly configuration at the onset of the disassembly phase. Subsequently, all polymers shrink and, as a result, the microtubule mean length would decrease over time. Alternatively the frequency of microtubule catastrophe could be rate-limiting during bulk microtubule disassembly. This would mean that the slow step during the microtubule disassembly reaction is the transition of microtubule ends toward a disassembly configuration, whereas the subsequent depolymerization step is, comparatively, quasi-instantaneous. In this case, microtubules would disappear from suspensions at random, independently of their length, and the microtubule length distribution would remain constant during the disassembly phase.

To test which of these possibilities was true, we determined the microtubule length distribution at various time-points during disassembly. We observed that the microtubule mean length and the microtubule length distribution remained essentially constant during the microtubule disassembly phase (Figure 4(a)-(c)). As discussed above, such constancy means that the rate of bulk microtubule disassembly depends on the frequency of microtubule catastrophe, not on the rate of individual microtubule subunit loss.

What is the relationship of the microtubule catastrophe frequency with the GTP-tubulin concentration? To address this issue we examined the half microtubule disassembly time ($t_{1/2}$) at the various initial GTP-tubulin concentrations. These $t_{1/2}$ values are equal to the average microtubule half-life and are therefore inversely proportional to the catastrophe frequency. The observed $t_{1/2}$ values are shown in Figure 4(d), as estimated from data pre-

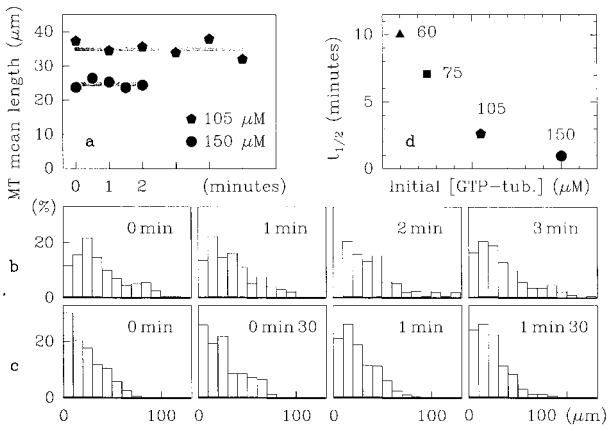

Figure 4. Analysis of microtubule disassembly: influence of the initial GTP-tubulin concentration. (a) Plots of microtubule mean length *versus* time measured at two initial GTP-tubulin concentrations as indicated. Time-zero corresponds to the maximum of assembly curves. (b) and (c) Microtubule length distribution at indicated time-points. Ordinates, percentage of total microtubules. Abcissas, microtubule length in μm. The initial GTP-tubulin concentration was either (b) 105 μM or (c) 150 μM. (d) Plot of the microtubule half-disassembly time ($t_{1/2}$) *versus* the initial GTP-tubulin concentration. With the hypothesis that microtubule disassembly occurs through random polymer catastrophe (see the text), $t_{1/2}$ corresponds to the average microtubule half-life.

sented in Figure 1(a)-(d). Results showed that $t_{1/2}$ was strongly dependent on the initial GTP-tubulin concentration, being 20 times shorter at 150 μM initial tubulin concentration than at 60 μM initial tubulin concentration (Figure 4(d)). This intriguing effect of the initial conditions could not be explained by differences in the free GTP-tubulin concentrations during disassembly, since these concentrations were similar, whatever the initial tubulin concentration (Figure 1(e)). Thus, the frequency of microtubule catastrophe was strongly influenced by the initial GTP-tubulin concentration, regardless of the current free GTP-tubulin concentration.

Discussion

We find that, in assembling tubulin solutions, the rate of microtubule nucleation is strongly dependent on the initial concentration of free GTP-tubulin, but remains constant during most of the assembly phase, being completely insensitive to wide drops in this concentration during assembly. Apparently, the rate of microtubule elongation is also constant during most of the assembly phase and is also insensitive to wide variations in the free GTP-tubulin concentration. Thus, there is strong evidence that, in assembly conditions, the limiting factor for both microtubule nucleation and elongation is not the free GTP-tubulin concentration. Our results deviate from current models of microtubule assembly (Oosawa & Kasai, 1962; Oosawa & Higashi, 1967; Johnson & Borisy, 1977).

The main novelty of our study compared to previous work concerns the introduction of systematic microtubule length measurements. These measurements present technical difficulties; breakage of very long microtubules cannot be excluded. Here microtubule breakage would introduce an underestimation of the microtubule mean length, especially at low initial tubulin concentration. However, such bias cannot explain the observed deviation of our results from previous models. Thus, microtubule nucleation deviates maximally from expected values at the highest initial GTP-tubulin concentrations, where the microtubule mean length is small. With regard to microtubule elongation, a putative underestimation of the microtubule mean length at low initial tubulin concentration compared to high initial tubulin concentration would tend to minimize deviations from previous models. Finally, the apparent invariance of the microtubule mean length during disassembly can hardly be related to bias in the microtubule length measurements.

How can the rate of microtubule nucleation be determined by the initial conditions and then remain constant during assembly? In descriptive terms, this means both that microtubule nuclei form from precursors which are present at a concentration determined by the initial tubulin concentration, and that the tubulin molecules used for microtubule nucleation are distinct from the free GTP-tubulin dimers used for microtubule elongation.

The simplest explanation for these puzzling features of microtubule nucleation is probably that at the beginning of assembly, tubulin solutions contain stable nucleation-competent tubulin oligomers whose concentration is proportional to the initial free GTP-tubulin concentration. There is probably a bifurcation at the beginning of tubulin assembly through which tubulin dimers segregate in different pools, a large pool of assembly-competent dimers, and a small pool composed of nucleation-competent oligomers. Previous nucleation models have involved tubulin oligomers in the formation of microtubule nuclei (Palmer *et al.*, 1982; Spann *et al.*, 1987; Carlier *et al.*, 1997). However, these oligomers were thought to be at rapid equilibrium with tubulin dimers, whose concentration was thought to drive the nucleation reaction. In contrast, our data strongly suggest that persistent tubulin oligomers, not free GTP-tubulin molecules, are the basic building blocks of microtubule nuclei. Apparently, given the nucleation exponent that we observe (6.2), about six oligomers combine to form microtubule nuclei. However, the mechanism of microtubule nuclei formation from oligomers could be flexible, and different values of the apparent nucleation exponent have been observed in different experimental conditions (Voter & Erickson, 1984; Carlier & Pantaloni, 1978; Flyvbjerg *et al.*, 1996; Fygenson *et al.*, 1994, 1995).

It will be of great interest to elucidate the structure of the putative nucleation competent oligomers. They may or may not contain non-tubulin proteins present in contaminating amounts in tubulin solutions. The main difficulty is that the concentration of these oligomers could be low. The identification of the nucleation-competent oligomers will require the design of methods able to stabilize, assay and purify such oligomers. This work is now in progress in the laboratory.

In our experimental conditions, the rate of microtubule elongation is apparently determined by intrinsic properties of the microtubule system, independently of the free GTP-tubulin concentration. These data agree with previous evidence that the rate of microtubule elongation is limited by the probability that tubulin dimers stick to the microtubules, and not the rate at which they diffuse to the microtubule end (Fygenson *et al*., 1994). Our data fit well with structural data suggesting that microtubule assembly proceeds through the formation of 2D tubulin sheets that progressively close into tubes (Chrétien *et al*., 1995). During microtubule elongation, sheet extensions are present at microtubule ends as long as the GTP-tubulin concentration is above its critical value. In these conditions, the rate-limiting step for microtubule elongation should be the intrinsic rate of tubulin sheet closure. Close to the critical tubulin concentration, the rate of sheet closure into microtubules apparently becomes higher than the rate of formation of sheet extensions (Chrétien *et al*., 1995). In these conditions, the free GTP concentration can become rate-limiting.

We find that the frequency of microtubule catastrophe, not the rate of microtubule disassembly, is rate-limiting during the microtubule disassembly phase. This is compatible with previous observations that also suggest that microtubule catastrophe *in vitro* is a rare event, followed by rapid polymer disassembly (Mitchison & Kirschner, 1984b; Horio & Hotani, 1986). The microtubule structure can be affected by the polymer elongation rate (Chrétien *et al*., 1992) but in our conditions the microtubule elongation rate was independent of the initial tubulin concentration. What is puzzling is the strong influence of the initial GTP-tubulin concentration on the rate of polymer catastrophe. This influence must be as strong as in the case of nucleation to account for the remarkable symmetry of the microtubule assembly-disassembly curves. To explain such influence one has to invoke catastrophe factors present in tubulin solutions in increasing concentrations at increasing initial GTP-tubulin concentrations. An obvious possibility was that the GDP-tubulin molecules produced during microtubule depolymerization functioned as catastrophe factors. However, addition of GDP-tubulin to tubulin solutions induced a decrease in the microtubule catastrophe rate (data not shown). These catastrophe factors may comprise non-tubulin proteins. However, in this case, one would expect tubulin kinetics to vary widely with the degree of tubulin purity and this has not been our experience: we have further purified tubulin on affinity columns without observing drastic changes in microtubule kinetics (Paturle *et al*., 1989). We believe that the catastrophe factor will turn out to be tubulin oligomers, as in the case of the nucleation competent complexes (see above). In fact, the ability of tubulin molecules to form various types of oligomers has long been recognized, and these oligomers have been proposed as important regulators of microtubule dynamics (Palmer *et al*., 1982; Spann *et al*., 1987; Lange *et al*., 1988; Mandelkow *et al*., 1988; Marx & Mandelkow, 1994; Carlier *et al*., 1997). It has been suggested that endogenous catastrophe factors, possibly consisting of tubulin oligomers, were needed to account for synchronized microtubule oscillations (Marx & Mandelkow, 1994). Further work should reveal the structure of the putative catastrophe oligomers and their relationship with the nucleation-competent complexes.

All dynamic instability models suppose that assembling and disassembling microtubules react differently with GTP-tubulin dimers. This hypothesis has gained very strong support from structural data showing different conformations of microtubule ends during assembly and disassembly. However, this hypothesis has remained formally unproved (Chrétien *et al*., 1995). Our data provide strong evidence that assembling and disassembling microtubules are indeed sensitive to different factors.

The nucleation-competent oligomers and the endogenous catastrophe factors, whose existence is suggested by our data, may turn out to be truly important to the regulation of microtubule dynamics *in vivo*. In this case one would expect to find physiological effectors of oligomer formation and stability and such effectors could be involved in the regulation of microtubule dynamics in cells.

As they are, our results indicate a built-in capacity of the microtubule system to undergo transition from a few long and persistent microtubules to numerous short and dynamically unstable polymers, simply by increasing the concentration of assembly-competent tubulin dimers. This apparent intrinsic capacity of the microtubule system to shift from an interphasic to a mitotic configuration (Belmont *et al*., 1990; Verde *et al*., 1990), may be important for the cell cycle-dependent microtubule re-organization in cells.

Materials and Methods

Preparation of GTP-tubulin

Tubulin was purified from fresh bovine brain by phosphocellulose chromatography as described previously (Paturle *et al*., 1991). Purified tubulin (60-150 μM) was incubated in PEM buffer (100 mM Pipes (pH 6.65), 1 mM EGTA, 1 mM $MgCl_2$), for ten minutes at 4 °C, in the presence of 0.5 mM GTP, 100 μCi/μM [^{3}H]GTP and 100 μCi/μM [γ-^{32}P]GTP. Free nucleotides were removed using Biogel P30 chromatography. The GTP-tubulin

concentration was adjusted in PEM buffer and aliquots were stored in liquid nitrogen.

Microtubule assembly from GTP-tubulin

We needed simultaneous measurements of the concentration of polymeric tubulin, of the GTP-tubulin concentration and of the microtubule mean length. GTP-tubulin was incubated in identical conditions whatever the parameter to be measured. GTP-tubulin was thawed at 4 °C and aliquoted (20 µl) on ice in 5 ml plastic tubes. Tubulin assembly was initiated by immersing tubes in a water bath equilibrated at 35 °C. Temperature equilibration in samples was achieved within 30 seconds following immersion, as determined using thermocouple measurements. At selected time-points, reactions were stopped. Stop procedures and further processing of the samples were adapted to the parameter to be measured, as described below. All measurements were done in triplicate. For each experiment, the control values at time-zero were determined in quadruplicate. Several different preparations of tubulin were used with identical results.

Measurement of polymeric tubulin concentration

Tubulin assembly was stopped by adding to samples 1 ml of MEM buffer (100 mM Mes (pH 6.65), 1 mM EGTA, 1 mM $MgCl_2$) containing 0.75 % (v/v) glutaraldehyde and 50 % (w/v) sucrose as previously described (Pirollet *et al*., 1987). Samples were filtered through GF/F filters to trap ^{3}H-labeled cross-linked microtubules. Further processing of the filters and radioactivity counting was as described (Pirollet *et al*., 1987).

Assay of GTP-tubulin concentration

GTP hydrolysis was stopped by sample immersion in liquid nitrogen. Samples were thawed at 4 °C and diluted with 80 µl of cold PEM buffer. GTP-tubulin complexes were then separated from free ^{32}P using gel filtration: an 80 µl aliquot of the mix was immediately loaded into a 1 ml spin column containing Biogel P30 equilibrated in PEM buffer, at 4 °C. The column was prespun at 450 *g* for five minutes prior to sample application and [γ-^{32}P]GTP-tubulin complexes were eluted by spinning the column at 600 *g* for five minutes. Then, ^{32}P radioactivity was counted. The GTP-tubulin concentration present in the tubulin solution at the time of sample freezing was then calculated as (^{32}P radioactivity at the time of freezing divided by ^{32}P radioactivity at time zero) multiplied by the initial GTP-tubulin concentration. These measurements require that the β phosphate group of GDP is not used for GTP generation by a contaminating regenerating system. Such a reaction would generate GMP. We have checked using HPLC, that no detectable amounts of GMP appeared in solution during experiments.

Determination of microtubule length and concentration

Tubulin assembly was stopped by adding to samples 1 ml of MEM buffer containing 0.75 % glutaraldehyde and 50 % sucrose. Mixtures were gently mixed, then the cross-linked microtubules were centrifuged on coverslips and processed for immunofluorescence analysis as previously described (Pirollet *et al*., 1987). The microtubule length was measured by means of image analysis. The fluorescence images were captured with a chilled CCD camera. The masks of the microtubules were obtained by adaptative thresholding followed by a skeletonization and pruning step. The microtubule length was obtained by counting the pixels of the pruned skeleton. At least 200 microtubules were analyzed on each coverslip. Microtubule length measurements become irreproducible when polymers are too long, presumably to increased incidence of polymer breakage (Mitchison, 1984a,b). In our hands, measurements were reproducible for microtubule mean lengths below 60 µm. To determine the microtubule number concentration, we derived the number of tubulin dimers in polymers per unit of volume from the molar concentration of polymeric tubulin and the Avogadro number. The microtubule number concentration was calculated as the ratio of this number of tubulin molecules per unit of volume divided by the average number of tubulin molecules per microtubule. This average number was calculated from the microtubule mean length, assuming 1625 tubulin dimers per micrometer of microtubule length (Bayley *et al*., 1994).

Modeling the evolution of microtubule number concentrations during tubulin polymerization

This modeling was based on experimental data showing that:

(i) At each initial free GTP-tubulin concentration, new microtubules were nucleated at a constant rate α_C (cf. indicated slopes in Figure 2(a)-(d)):

$$\frac{dN(t)}{dt} = \alpha_C \qquad (1)$$

where $N(t)$ is the number of microtubules at time t.

(ii) When the observed values of α_C are plotted *versus* the corresponding initial free GTP-tubulin concentrations on a log-log scale, a linear plot is observed (Figure 2(e)). Therefore:

$$\frac{dN(t)}{dt} = \alpha_C = K \times [\text{initial} - \text{GTP} - \text{tubulin}]^{\beta} \qquad (2)$$

where β is equal to the slope of the log-log plot (β = 6.2).

Equation (2) reflects the observation that, during polymerization, microtubules were nucleated at a constant rate, determined by the initial free GTP-tubulin concentration. Current models would involve the current free GTP-tubulin concentration instead of the initial free GTP-tubulin concentration in equation (2). To test whether such models were compatible with our data, we calculated the corresponding microtubule number concentrations by numerical integration using the experimentally determined free GTP-tubulin concentrations (Figure 1(e)) and the experimentally determined value of β:

$$N(T) = \int_0^T K \times [\text{GTP} - \text{tubulin}(t)]^{\beta} \times dt \qquad (3)$$

The results of such integration are shown in Figure 2(a)-(d), broken lines.

Modeling the evolution of microtubule mean length during tubulin polymerization

Expected values of the microtubule mean length were calculated under two different hypotheses: (a) the rate of individual microtubule growth is proportional to the free

GTP-tubulin concentration; (b) individual microtubules grow at a constant rate.

As judged from GDP-tubulin *versus* time plots, microtubule depolymerization was negligible at the time of microtubule mean length measurements (data not shown). Therefore, disassembly events did not need to be taken into account in models.

Rate of individual microtubule elongation proportional to the free GTP-concentration

In this case, the length of a microtubule created at time t and observed at time T is:

$$l(t, T) = \int_t^T \varepsilon \times [\mathrm{GTP} - \mathrm{tubulin}(t)] \times \mathrm{d}t \qquad (4)$$

where ε is the elongation constant in $\mu m\ \mu M^{-1}\ min^{-1}$ and $[GTP - tubulin(t)]$ the GTP-tubulin concentration. For any given ε value and any (t,T) set, $l(t,T)$ can be determined by numerical integration using the free GTP-tubulin concentration data presented in Figure 1(e). The microtubule mean length at time T, $\bar{L}(T)$, can be subsequently deduced by numerical integration of equation (9) (see below).

Constant rate of individual microtubule growth

$$\frac{\mathrm{d}l}{\mathrm{d}t} = C \qquad (5)$$

l is the individual microtubule length, and C the elongation constant in $\mu m\ min^{-1}$. The length of a microtubule created at time t and observed at time T is:

$$l(t, T) = C \times (T - t) \qquad (6)$$

Therefore, the total length of microtubules ($L_{\mathrm{Tot}}(T)$) at time T is:

$$L_{\mathrm{Tot}}(T) = \int_0^T \alpha_C \times l(t, T) \times \mathrm{d}t \qquad (7)$$

and the total number of microtubules is obtained by integrating equation (1):

$$N(T) = \int_0^T \alpha_C \times \mathrm{d}t \qquad (8)$$

The microtubule mean length $\bar{L}(T)$ at time T is equal to the ratio of equations (7) and (8), and simplifies to:

$$\bar{L}(T) = \frac{1}{T}\int_0^T l(t, T) \times \mathrm{d}t \qquad (9)$$

Taking $l(t,T)$ from equation (6) and performing a straightforward analytical integration, one obtains:

$$\bar{L}(T) = \frac{1}{2} \times C \times T \qquad (10)$$

Thus, the expected microtubule mean lengths increase linearly with time, at a rate equal to half the rate of individual microtubule elongation.

Acknowledgments

We thank Dr Yasmina Saoudi for useful advice during this work, and Drs A. Andrieux, C. Bosc, E. Denarier, A. Fourest and R.H. Wade for review of our manuscript. This work was supported by grants from the Association pour la Recherche sur le Cancer and from La Ligue.

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Edited by A. Klug

(Received 23 October 1999; received in revised form 21 January 2000; accepted 25 January 2000)