Hydrolysis of GTP Associated with the Formation of Tubulin Oligomers Is Involved in Microtubule Nucleation

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ABSTRACT Hydrolysis of GTP is known to accompany microtubule assembly. Here we show that hydrolysis of GTP is also associated with the formation of linear oligomers of tubulin, which are precusors (prenuclei) in microtubule assembly. The hydrolysis of GTP on these linear oligomers inhibits the lateral association of GTP-tubulin that leads to the formation of a bidimensional lattice. Therefore GTP hydrolysis interferes with the nucleation of microtubules. Linear oligomers are also formed in mixtures of GTP-tubulin and GDP-tubulin. The hydrolysis of GTP associated with heterologous interactions between GTP-tubulin and GDP-tubulin in the cooligomer takes place at a threefold faster rate than upon homologous interactions between GTP-tubulins. The implication of these results in a model of vectorial GTP hydrolysis in microtubule assembly is discussed.

INTRODUCTION

It is well known that the hydrolysis of GTP bound to tubulin is associated with microtubule assembly (Weisenberg et al., 1976; David-Pfeuty et al., 1977). After GTP hydrolysis, the interactions between GDP subunits are weakened. Microtubules assembled from GTP-tubulin rapidly depolymerize spontaneously in the absence of GTP (Carlier and Pantaloni, 1978). Hydrolysis of GTP occurs in two consecutive steps, cleavage of the γ-phosphoester bond, followed by the release of P_i (Melki et al., 1990, 1996). Only the latter step is linked to the destabilization of tubulin-tubulin interactions in the microtubule (Carlier et al., 1988). The detailed nature of the tubulin-tubulin contacts involved in GTP hydrolysis is still unknown, and available evidence indicates that lateral tubulin-tubulin contacts between adjacent protofilaments are destabilized after GTP hydrolysis (Howard and Timasheff, 1986; Melki et al., 1989). Because hydrolysis of GTP is not mechanistically coupled to the assembly process (Carlier et al., 1984; Melki et al., 1990), the stochastic loss and gain of a small "cap" of GDP-P_i terminal subunits is thought to account for the functional property of dynamic instability of microtubules (Mitchison and Kirschner, 1984; Horio and Hotani, 1986; Carlier, 1989). The hydrolysis of GTP in tubulin polymerization has been proposed (Carlier et al., 1984; Caplow et al., 1985) and experimentally demonstrated (Carlier et al., 1987; Burns, 1991) to occur essentially in a directional fashion, at the interface between the GDP core and the GTP cap. Evidence has also been obtained for the creation of multiple GTP-GDP-P_i boundaries on the microtubule body in regimes of rapid polymerization (Carlier et al., 1988), which limits the size of the GTP/GDP-P_i cap on rapidly growing microtubules to ~200 subunits, and probably much less (10 subunits on average) at steady state (Melki et al., 1990; Carlier et al., 1987; Carlier, 1991; Drechsel and Kirschner, 1994). Recent cryoelectron microscopy studies (Hyman et al., 1995) have shown evidence for long, curved, open microtubular sheets at the ends of rapidly growing microtubules. This structure was proposed by the authors to represent the GTP cap; however, its evident low structural stability is the opposite of that expected for a thermodynamically stable GTP cap.

The GTP/GDP P_i cap is a dynamic structure. Theoretically, the thermodynamic stability of microtubules (i.e., the concentration of GTP-tubulin at which the rate of growth is zero) is expected to depend on the topology of the GTP cap: however, a quantitative correlation between the structure of the GTP/GDP P_i cap and its dynamic properties is lacking. Analyses of microtubule dynamics in video microscopy have led to the hypothesis that fluctuations in the geometry of the GTP cap could account for the observed broad distribution of the rates of microtubule elongation and rapid shortening (Gildersleeve et al., 1992). Years ago, Monte Carlo simulations (Chen and Hill, 1985) of the large fluctuations in the length of microtubules at steady state, using the GTP cap model, have shown that a putative exponential dependence of the frequencies of loss and gain of a GTP cap on tubulin concentration could well account for experimental observations. However, microscopic rate constants for GTP hydrolysis in different environments in the GTP cap are not known, nor are the different assembly kinetic parameters of microtubule ends with GTP caps of different geometries. The facts that 1) GTP is hydrolyzed in a vectorial fashion, i.e., hydrolysis is favored upon interaction of GTP-tubulin with GDP-tubulin in the microtubule; 2) GDP-tubulin can block microtubule ends (Margolis, 1981; Zeeberg and Caplow, 1981; Engelborghs and Van Houtte, 1981; Carlier et al., 1987); and 3) dimeric GDP-tubulin can be incorporated into the microtubule in the presence of P_i analogs (Carlier et al., 1989) all indicate that different stages

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of GTP hydrolysis on a microtubule subunit are linked to different energies of interaction with neighboring subunits. Heterologous interaction between GTP-tubulin and GDP-tubulin is also one of the features of the "lateral cap" model (Bayley et al., 1989, 1990). Heterologous interactions between molecules of GTP-tubulin and GDP-tubulin are characterized here in further detail. We show that under ionic conditions suitable for microtubule assembly, GTP-tubulin and GDP-tubulin can form prenuclei homo- or heterooligomers in which interaction of a GTP-tubulin subunit with a neighboring subunit, carrying either GTP or GDP, triggers GTP hydrolysis. The significance of the results regarding the regulation of microtubule dynamics and the role of GTP hydrolysis in the nucleation of microtubules is discussed.

MATERIALS AND METHODS

Chemicals

GTP and GDP came from Boehringer, MES from Calbiochem, EGTA from Sigma, beryllium sulfate (Gold Label) from Aldrich, and [γ-^{32}P]-GTP was from Amersham. All other reagents were Merck analytical grade.

Tubulin was purified from pig brain by three cycles of polymerization (Shelanski et al., 1973) followed by phosphocellulose chromatrography (Weingarten et al., 1975) and stored at −80°C at a concentration of 40–80 μM in 50 mM morpholinoethanesulfonic acid (MES), pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 0.1 mM GTP, 3.4 M glycerol (buffer A). The 1:1 [γ-^{32}P]GTP-tubulin complex was prepared by exchanging radioactive GTP for unlabeled bound GTP for 1 h at 0°C, followed by Sephadex G25 chromatography to remove free nucleotide. That this material contained 2 GTPs bound per tubulin, and no GDP, was checked by high-performance liquid chromatography as described (Melki and Carlier, 1993).

The 1:1 GDP-tubulin complex was prepared as follows. Microtubules were assembled at 37°C in buffer A supplemented with 6 mM $MgCl_2$, sedimented through a 30% sucrose cushion made in buffer A containing no GTP and 6 mM $MgCl_2$. The pellet was resuspended in buffer A without GTP. After depolymerization at 0°C for 15 min, the solution (routinely 60–80 μM GDP-tubulin) was clarified by sedimentation at 4°C, 150,000 × g for 30 min before use.

The nucleotide content of the perchloric extract of GTP- and GDP-tubulin was checked by anion exchange high-performance liquid chromatography (Synchropak, Synchrom) with isocratic elution by 0.35 M KH_2PO_4, 1.3 M NaCl, pH 3.5.

Tubulin polymerization was carried out at 37°C in buffer A containing 6 mM $MgCl_2$ and the indicated amount of GTP and GDP, and was monitored turbidimetrically at 350 nm in a Kontron Uvikon 860 spectrophotometer as described previously (Carlier and Pantaloni, 1978). Above 20 μM tubulin, the change in turbidity was no longer correlated with the mass amount of polymerized tubulin, because of the nonideality of the polymer solution (Carlier and Pantaloni, 1978). The second viriel coefficient had to be taken into account, as described by the following equation:

$$\frac{Hc_w}{\tau} = \frac{1}{M} + 2Bc_w + \dots \quad (1)$$

where the constant H can be measured separately, c_w is the mass amount of polymerized tubulin, τ is the turbidity, M is the molecular mass of tubulin, and B is the second viriel coefficient.

According to Eq. 1, the linear double-reciprocal plot of $1/\Delta A$ versus $1/c_w$ was used to convert the absorbance change in molar units of polymerized tubulin (Fig. 1, *inset*). The value of c_w was derived from a sedimentation assay; c_w was equal to the difference between the total concentration of tubulin and the concentration of tubulin in the supernatants of microtubules sedimented at 300,000 × g for 8 min at 37°C.

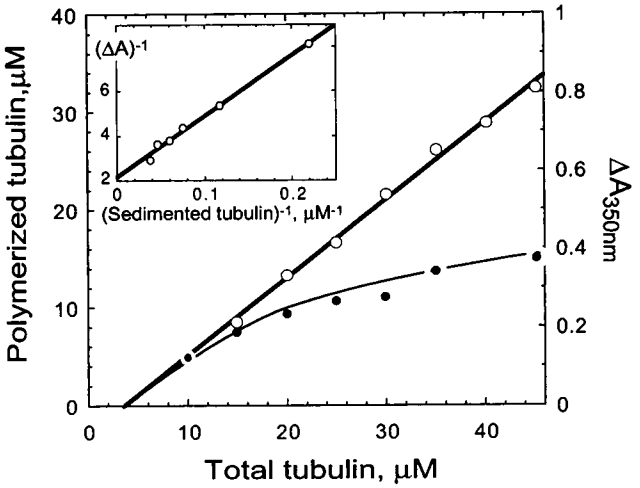

FIGURE 1 Correlation between the extent of turbidity change upon microtubule assembly and the mass amount of polymerized tubulin. Tubulin was polymerized at the indicated concentration. The extent of turbidity increase ΔA at 350 nm, light path 0.5 cm (●) was measured. The mass amount C_w of assembled tubulin (○) was measured in pararallel in sedimentation assay. The turbidity data were convert into molar amounts of polymerized tubulin by using the double-reciprocal plots shown in the inset.

Hydrolysis of [γ-^{32}P]GTP was assayed by extraction of the ^{32}P-labeled phosphomolybdate formed in 1 N HCl, as previously described (Carlier et al., 1987). The accuracy and sensitivity of this method in measuring acid-labile P_i are well known (Carlier, 1989).

GTP hydrolysis and microtubule assembly were monitored simultaneously by removing 50-μl aliquots of the polymerizing tubulin solution at different time intervals and rapidly delivering them into the ice-cold ammonium molybdate/HCl quench solution. The incident light beam was interrupted, to make a mark on the turbidity recording, at the time the aliquot of the polymerizing solution was acid quenched. The uncertainty in the GTP hydrolysis time points was less than 2 s. The error in the measurements of the GTPase rate is typically 15%, taking into account the cumulated pipetting errors involved in the processing of aliquots at each time point and the radioactivity measurements involved in the GTPase measurement assays.

RESULTS

Hydrolysis of GTP by tubulin in microtubules and in prenuclei oligomers

The kinetic correlation between spontaneous microtubule assembly and associated GTP hydrolysis has been studied in the past (Carlier and Pantaloni, 1981; Hamel et al., 1984; O'Brien et al., 1987; Burns, 1991) under conditions in which a large amount of free GTP was present in solution. Under these conditions, an appreciable steady-state GTPase activity follows the burst of hydrolyzed GTP associated with microtubule assembly, as a result of the extensive monomer-polymer exchange reactions occurring at steady state. During the polymerization process itself, and more markedly in the late stages of assembly, the dynamic instability behavior of microtubules inevitably leads to a larger amount of hydrolyzed GTP than of apparently assembled tubulin at the same time. Indeed, in the following sequence of reactions—dissociation of GDP-tubulin from the micro-

tubule, GTP exchange for bound GDP on dimeric tubulin, reincorporation of GTP-tubulin in the microtubule, and hydrolysis of GTP—the average mass of polymer remains constant while one GTP per recycled tubulin has been hydrolyzed. This complexity, inherent in the microtubule system, increases the difficulty in appreciating the temporal correlation between the individual steps of tubulin polymerization and GTP hydrolysis, and provides a plausible explanation for the discrepancy in the data coming from different groups, with GTP hydrolysis either delayed relative to polymerization (Carlier and Pantaloni, 1981; Carlier et al., 1987; Burns, 1991; Melki et al., 1996) or proceeding faster than polymerization in other works (Hamel et al., 1984; O'Brien et al., 1987). This complexity was decreased here in two ways. First the use of a glycerol-containing polymerization buffer reduces (but does not totally abolish) dynamic instability. Second, the absence of free GTP rules out the possibility of "turnover" outlined above. In other terms, GTP-tubulin polymerizes and hydrolyzes its bound GTP only once; in the case of dissociation of GDP-tubulin from microtubules in the late stages of assembly, no reassembly can occur, and net depolymerization of microtubules should be observed.

The time courses of polymerization of the 1:1 GTP-tubulin complex (30 μM, [γ-^{32}P]GTP-labeled) and associated GTP hydrolysis are shown in Fig. 2 (*set of curves a*). As previously observed (Carlier and Pantaloni, 1978), microtubules spontaneously assembled, then disassembled. Hydrolysis of GTP accompanied microtubule assembly. The lag in hydrolysis of GTP after microtubule assembly was 3–4 s. This delay is in agreement with a recent study using rapid kinetics to monitor P_i release continuously in microtubule assembly (Melki et al., 1996). The hydrolysis process had to be included as a kinetic step before P_i release to account for the data. In addition, because the short microtubules and open microtubular sheets that are formed in the early stages of assembly (10% of the overall turbidity change) scatter light less than longer microtubules assembled in the later stages of assembly (Berne, 1974), the relative mass amount of polymer formed in the first stages of assembly is likely to be higher than estimated from the turbidity data; hence the actual uncoupling between GTP hydrolysis and polymerization is probably slightly greater than conveyed by inspection of the raw data in Fig. 2 *a*. After the maximum turbidity was reached, GTP hydrolysis continued while a portion of the polymers on which hydrolysis of GTP had occurred started to depolymerize.

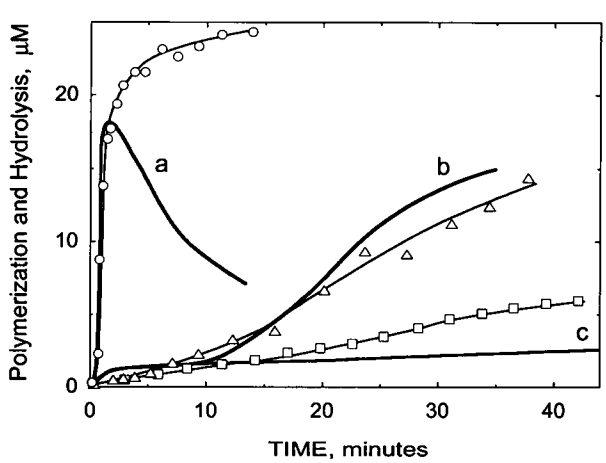

FIGURE 2 Polymerization of the GTP-tubulin 1:1 complex and accompanying GTP hydrolysis, in the presence of different amounts of GDP. The 1:1 γ-labeled GTP-tubulin complex (29 μM) was incubated at 0°C in the presence of GDP at the following concentrations in μM: *curve a*, 0; *curve b*, 200; *curve c*, 300. Polymerization was followed by turdibidy (*solid line*) and GTP hydrolysis (○ in *a*, △ in *b*, □ in *c*) as described in Materials and Methods. The turbidity data were converted into micromolar units of polymerized tubulin by using the correspondence diagram between sedimented microtubules and turbidity change, which was established in Fig. 1, *inset*.

In the presence of GDP (*sets of curves b and c*), nucleation of microtubules was inhibited, as demonstrated by the increase in lag time preceding the slow polymerization of GTP-tubulin. An appreciable linear hydrolysis of GTP developed during the lag period, in the absence of microtubule assembly. Although the rate of GTP hydrolysis increased as microtubules slowly assembled, no clear correlation could be established, in the presence of GDP, between the time courses of GTP hydrolysis and tubulin polymerization, because the two curves crossed each other. These results suggest that during the lag period, although no appreciable turbidity developed, interactions take place between GTP-tubulin and GDP-tubulin, with associated hydrolysis of GTP.

In the experiment displayed in Fig. 3, [γ-^{32}P]GTP-tubulin 1:1 complex (30 μM) was polymerized in the presence of 400 μM GDP and in either the presence or absence of 200 μM (BeF_3^-, H_2O), which has been shown to bind tightly to the site of the γ-phosphate of GTP on GDP-microtubules (Carlier et al., 1988) and mimic the stable GDP-P_i state of microtubules. In the absence of BeF_3^-, no polymerization was observed for at least 30 min, and hydrolysis of ~4 μM GTP proceeded linearly with time during this period. In the presence of BeF_3^-, polymerization of 30 μM tubulin developed after a 12-min lag period, during which the hydrolysis of GTP proceeded at the same rate as in the control; additional hydrolysis of 10 μM GTP accompanied the delayed polymerization process, which demonstrated, in agreement with previous results (Carlier et al., 1989), that 70% of the microtubules had assembled from GDP-tubulin units, the incorporation of which into the polymer was driven by BeF_3^-. in the presence of BeF_3^-, practically all of the tubulin polymerized in microtubules, consistent with the large decrease in the critical concentration (Carlier et al., 1988) promoted by this phosphate analog.

Homologous (T-GTP–T-GTP) and heterologous (T-GTP–T-GDP) interactions during prenucleation events

The interaction between either GTP-tubulin molecules or between GTP-tubulin and GDP-tubulin with associated GTP hydrolysis and the role of these reactions in nucleation were examined in greater detail in the following experi-

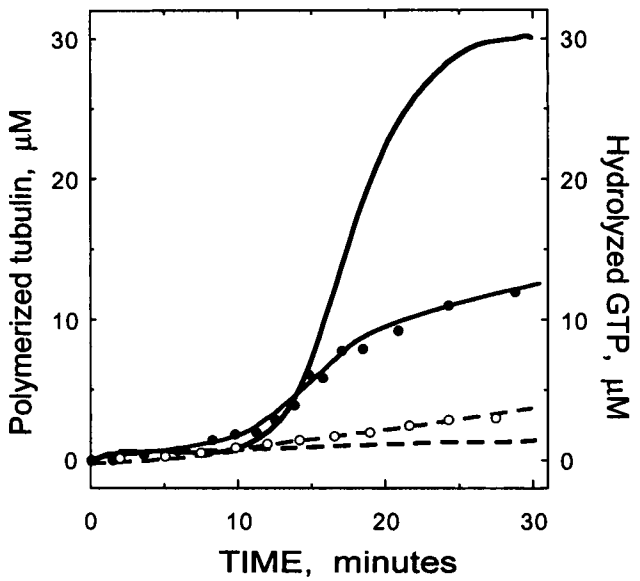

FIGURE 3 Effect of BeF_3^- in the polymerization of tubulin and accompanying GTP hydrolysis in the presence of GDP. The 1:1 γ-^{32}P-labeled GTP-tubulin complex (30 μM) was polymerized in the presence of 400 μM GDP, 200 μM Be^{2+} and either 5 mM NaF (——, ●) or 5 mM NaCl (– – –, ○). Thick lines represent polymer formation derived from turbidimetric recordings. Symbols represent hydrolyzed GTP.

ments. The [γ-^{32}P]GTP-tubulin (1:1 complex, freed of GTP) was polymerized at 37°C at different concentrations ($[C_o]$) and in the presence, at each tubulin concentration, of varying amounts of GDP. The rate of GTP hydrolysis was measured, for each sample, during the lag time preceding microtubule assembly. In the absence of GDP, concentrations of GTP-tubulin higher than 14 μM could not be assayed because the lag time preceding microtubule assembly becomes too short (i.e., shorter than 2 min) for a linear hydrolysis to be conveniently measured with sufficient accuracy before polymerization starts. Time points at intervals of 15–100 s were taken for periods of 2–20 min. Typically, eight data points were collected over a period of time during which less than 20% of the bound GTP was hydrolyzed linearly with time under initial rate conditions, and the intervals fulfilling this condition were adjusted at each tubulin concentration. The partial concentration of GTP-tubulin (T-GTP) in each sample before oligomerization started (i.e., if only dimeric GTP-tubulin and GDP-tubulin species were present in solution) was derived from the concentrations of total tubulin ($[C_o]$), total GTP (equal to the total tubulin concentration, $[C_o]$) and total GDP ($[X_o]$), and from the ratio (α) of the equilibrium dissociation constants for binding of GDP and GTP, respectively, to tubulin, using the following equation:

$$[T-GTP] = \frac{[C_o](2\alpha-1)+[X_o]}{2(\alpha-1)} \times \frac{\pm\sqrt{([C_o](2\alpha-1)+[X_o])^2-4\alpha[C_o]^2(\alpha-1)}}{2(\alpha-1)} \quad (2)$$

A value of $\alpha = 3$ (Caplow et al., 1985) was used for the calculation of [T-GTP]. However, we determined that using other values of α, in the range 2–10, affected the results only quantitatively, but did not change the general shape of the curves displayed in Fig. 4, or the essential conclusion that in the presence of GDP, the data points fall above the curve obtained in the absence of GDP.

In each series of measurements carried out by increasing $[X_o]$ at a given $[C_o]$, the concentration of T-GTP decreased from $[C_o]$ (in the absence of GDP) to 0 (at infinite GDP), whereas conversely the concentration of T-GDP increased from 0 to $[C_o]$, with [T-GTP] + [T-GDP] = $[C_o]$. In Fig. 4, the rate of GTP hydrolysis was plotted versus the concentration of T-GTP, calculated using Eq. 2. This representation provides the immediate comparison of the rates of GTP hydrolysis that were measured, in different series, at the same concentration of T-GTP, but in the presence of different concentrations of T-GDP. The following observations were made.

In the absence of GDP, the rate of GTP hydrolysis during the lag period preceding microtubule assembly increased cooperatively with the concentration of T-GTP, consistent with hydrolysis of GTP occurring upon interaction between

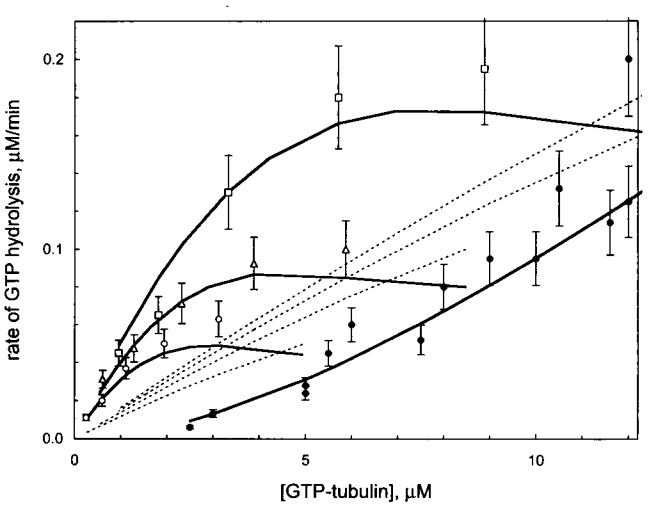

FIGURE 4 GTP hydrolysis linked to interactions between GTP-tubulin and GDP-tubulin. ●, The 1:1 [γ-^{32}P]-GTP-tubulin complex at different concentrations was brought to 37°C in polymerization buffer, and the initial rate of GTP hydrolysis during the lag time preceding microtubule assembly was measured. ○, □, △, The 1:1 [γ-^{32}P]-GTP-tubulin complex was isolated at the following concentrations (C_o, in μM): □, 20; △, 12; ○, 9. It was incubated in the presence of GDP at different concentrations (X_o), resulting in amounts of GTP-tubulin and GDP-tubulin that can be calculated according to Eq. 1. The solution was brought to 37°C in polymerization buffer, and the initial rate of GTP hydrolysis was measured as described under Fig. 2. Lines represent calculated curves assuming random GTP hydrolysis in oligomers. Dashed lines were calculated within a model in which GTP is hydrolyzed at the same rate upon interaction with either a GTP- or a GDP-bound subunit. Solid lines were calculated within a model in which GTP is hydrolyzed at a 3.5-fold higher rate when interacting with a GDP-bound subunit than with a GTP-bound subunit. The following values were used for equilibrium and rate parameters: $K = 0.04$ μM^{-1}; $K' = 0.08$ μM^{-1}; $H_{TT} = 0.023$ min^{-1}; $H_{TD} = 0.08$ min^{-1}.

molecules of T-GTP. When GDP was added, the presence of GDP-tubulin resulted in a higher GTPase rate, at any given concentration of T-GTP, than the one measured at the same concentration of T-GTP but in the absence of T-GDP. At large enough concentrations of T-GDP, data obtained at different values of $[C_o]$ all led to the same linear dependence of the rate of GTP hydrolysis on the partial concentration of T-GTP. These results indicate that GTP hydrolysis occurs upon interaction of T-GTP with T-GDP as well as with T-GTP; the fact that the rate of GTP hydrolysis increases linearly with [T-GTP] in the presence of saturating amounts of T-GDP suggests that under these conditions each molecule of T-GTP interacts with T-GDP only.

The fact that hydrolysis of GTP accompanies the interaction of T-GTP with T-GDP was verified in the following experiment. Increasing amounts of T-GDP 1:1 complex, prepared as described in Materials and Methods, were added to 6 μM [γ-^{32}P]GTP-tubulin (1:1 complex), and the initial rate of GTP hydrolysis was measured. Fig. 5 shows that the GTPase rate increased cooperatively with the concentration of T-GDP and reached a maximum value of 4–5 $\times$ 10^{-4} s^{-1} at saturation by T-GDP, consistent with the data displayed in Fig. 4.

Sedimentation of heterologous oligomers containing T-GTP and T-GDP

All results thus far are consistent with the formation of linear oligomers of T-GTP or T-GDP, or both, in polymerization buffer at 37°C. These oligomers are likely to be precursors in microtubule nucleation (Erickson and Pantaloni, 1981). Similar oligomers of ~12 tubulin dimers have recently been invoked in kinetic studies of microtubule nucleation (Kuchnir-Fygenson et al., 1995) and have been frequently observed as open rings or short, curved linear oligomers in electron microscopy. Isodesmic polymerization of pure tubulin leading to 42S rings has been extensively studied by analytical ultracentrifugation at 4°C (Frigon and Timasheff, 1975; Howard and Timasheff, 1986). The formation of rings was favored when GDP rather than GTP was bound to tubulin (Howard and Timasheff, 1986), essentially because the isomerization constant leading to closure of the 26-tubulin oligomer into a ring is higher for GDP-tubulin, whereas the propagation constants for oligomer formation are identical for GTP-tubulin and GDP-tubulin. To verify that the same type of isodesmic oligomerization is taking place in the experiments described above, GDP-tubulin (30 μM) in polymerization buffer was supplemented with GTP and GDP at different concentrations, the total nucleotide concentration being 1 mM, so that each sample contained known amounts of T-GTP and T-GDP. The samples were centrifuged for 40 min at 400,000 $\times$ g, 13°C, in the Beckman TL100 Tabletop ultracentrifuge, using the TLA100.1 rotor (k factor = 12). Under these conditions, oligomers containing at least four tubulin subunits sediment to the bottom of the tubes. The amount of protein in the top half of the supernatants was assayed. Data displayed in Fig. 6 show that the concentration of tubulin in the top half of the supernatant increased from 12.5 μM to 17

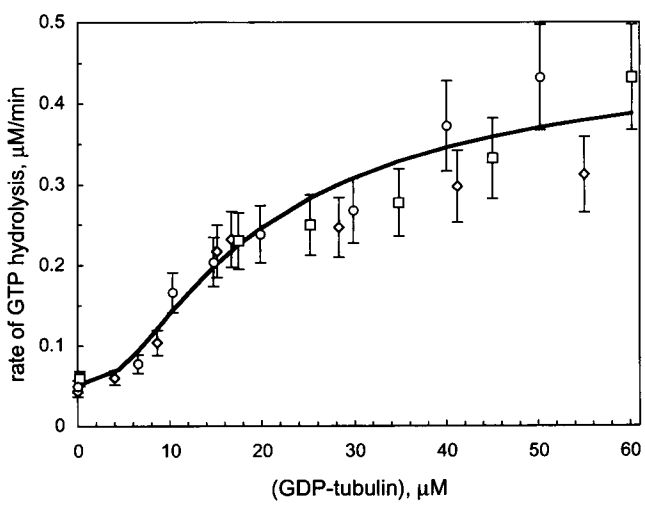

FIGURE 5 Cooperative interactions between GTP-tubulin and GDP-tubulin are accompanied by hydrolysis of GTP. Mixtures of [γ-^{32}P]-GTP-tubulin 1:1 complex (6 μM) and GDP-tubulin 1:1 complex at the indicated concentrations were brought to 37°C in polymerization buffer. The initial rate of GTP hydrolysis during the lag time was measured. Different symbols refer to independent experiments. The solid curve was calculated using the same values of the parameters as in Fig. 4 (*solid lines*).

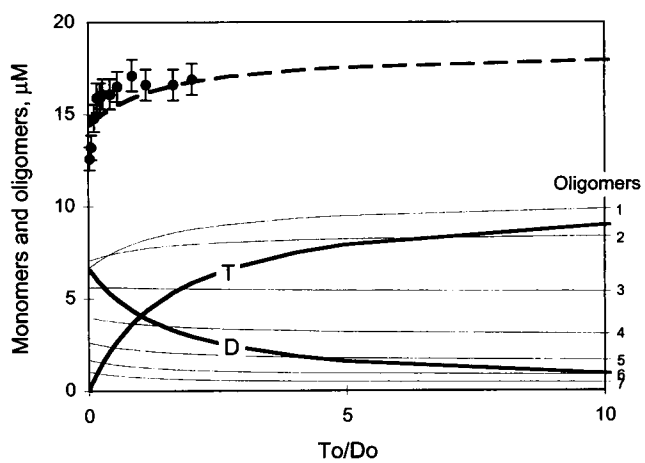

FIGURE 6 Partial dissociation of GDP-tubulin oligomers is induced by GTP. GDP-tubulin 1:1 complex (30 μM) in polymerization buffer was supplemented with known amounts of GTP and GDP (with GTP + GDP = 1 mM). The ratio [T-GTP]/[T-GDP] (T_o/D_o) was calculated for each sample. Oligomers containing at least four tubulin subunits were sedimented to the bottom of the tubes at 13°C, 400,000 $\times$ g for 40 min. The amount of tubulin in the upper half of the supernatant was assayed in each sample. Closed circles represent experimental data. Solid lines represent calculated values of the mass amount of tubulin in different species at equilibrium as follows. Curves T and D represent monomeric GTP-tubulin and GDP-tubulin, respectively. Curve 1 represents total monomers (T + D). Curves 2, 3, . . . , 7 represent tubulin in dimers, trimers, . . . , heptamers, respectively. The concentration of each species is calculated using K = 0.04 μM^{-1} and K'' = 0.08 μM^{-1}. The dashed curve is the sum of curves 1 and 2 (monomers and dimers), which reasonably fits the data.

μM upon increasing the ratio T-GTP/T-GDP from 0 to 5, which indicated that the tubulin association equilibrium is only slightly shifted toward the monomer in the presence of GTP. Therefore the equilibrium association constants for oligomerization of GTP-tubulin and GDP-tubulin are not very different, in fair agreement with Howard and Timasheff's results obtained in the absence of glycerol. The data are reasonably consistent with the calculated curves, using values of 0.04 μM^{-1} and 0.08 μM^{-1} for K and K', respectively (see model below).

Model

The results described in this paper were tentatively analyzed with a model of isodesmic oligomerization of tubulin, in which GTP hydrolysis is associated with the interaction of GTP-tubulin with either GTP-tubulin or GDP-tubulin. These oligomers lead to either microtubule nuclei or to rings, depending on the conditions (temperature, nucleotide, ionic conditions). Nuclei are formed as soon as the probability of association of a GTP-tubulin molecule in a lateral position with a linear oligomer of N subunits generates a bidimensional microtubular sheet (Carlier and Pantaloni, 1978; Erickson and Pantaloni, 1981), which then closes into a microtubule. Linear oligomers therefore are predominant during the prenucleation phase (lag time) preceding the onset of turbidity rise corresponding to microtubule growth. According to this model, formation of homopolymers of T-GTP or of T-GDP as well as of copolymers of T-GTP and T-GDP is described by the following equations, in which T and D represent T-GTP and T-GDP, respectively:

$$T + T \Leftrightarrow T_2$$
$$T_2 + T \Leftrightarrow T_3$$
$$\overline{\qquad\qquad}$$
$$T_i + T_j \Leftrightarrow T_{i+j}$$

The equilibrium association constant for oligomerization of GTP-tubulin is

$$K = \frac{[T_j]}{[T_{j-1}]\cdot[T]} \tag{3}$$

The concentration of oligomers made of j molecules of GTP-tubulin is

$$[T_j] = \frac{(K[T])^j}{K} \tag{4}$$

Similarly, the oligomerization of GDP-tubulin can be represented as follows:

$$D + D \Leftrightarrow D_2$$
$$D_2 + D \Leftrightarrow D_3$$
$$\overline{\qquad\qquad}$$
$$D_i + D_j \Leftrightarrow D_{i+j}$$

The equilibrium association constant for oligomerization of GDP-tubulin is

$$K' = \frac{[D_i]}{[D_{i-1}][D]} \tag{5}$$

The concentration of oligomers made of j molecules of GDP-tubulin is

$$[D_i] = \frac{(K'[D])^i}{K'} \tag{6}$$

The number concentration of homopolymers is (assuming $K[T] < 1$ and $K'[D] < 1$)

$$\sum_{i=1}^{i=\infty} D_i = \frac{D}{1 - K'D} \quad \text{and} \quad \sum_{j=1}^{j=\infty} T_j = \frac{T}{1 - KT}$$

The mass amount of tubulin in homopolymers is

$$\sum_{i=1}^{i=\infty} i \cdot D_i = \frac{D}{(1 - K'D)^2} \quad \text{and} \quad \sum_{j=1}^{j=\infty} j \cdot T_j = \frac{T}{(1 - KT)^2}$$

In a copolymer, T-T, D-D, D-T, and T-D interactions occur with equilibrium association constants K, K', K''_{DT}, and K''_{TD}, respectively. Within a simple statistic copolymerization scheme, it is assumed that $K''_{TD} \cdot K''_{DT} = KK'$ and $K'' = \sqrt{K \cdot K'}$, therefore,

$$K'' = \frac{[D_i\, T_j]}{[D_i][T_j]} \tag{7}$$

which means that the stability of a copolymer only depends on its composition and not on the environment of the D and T subunits.

Nonstatistical copolymerization illustrated by two extreme models is excluded by these assumptions: the fully ordered alternate TDTDTDTD... polymers ($K'' \gg \sqrt{K \cdot K'}$), and the "block" polymers... DDDDDDTTTTTT..., containing only a minimum of DT interface ($K'' \ll \sqrt{K \cdot K'}$).

Then,

$$[D_i\, T_j] = K'' \frac{(K'[D])^i}{K'} \frac{(K[T])^j}{K} \tag{8}$$

Let $[C_o]$ be the total tubulin concentration, and $[T_o]$ and $[D_o]$ be the total concentrations of T-GTP and T-GDP, respectively:

$$[C_o] = [T_o] + [D_o] \tag{9}$$

$$[T_o] = \sum_{j=1}^{j=\infty} j \cdot \frac{(K \cdot [T])^j}{K} + \sum_{i=1}^{i=\infty} \sum_{j=1}^{j=\infty} K'' j \cdot \frac{(K[T])^j}{K} \cdot \frac{(K'[D])^i}{K'} \cdot C^j_{i+j}$$

$$[D_o] = \sum_{i=1}^{i=\infty} i \cdot \frac{(K'[D])^i}{K'} + \sum_{i=1}^{i=\infty} \sum_{j=1}^{j=\infty} K'' i \cdot \frac{(K[T])^j}{K} \cdot \frac{(K'[D])^i}{K'} \cdot C^i_{i+j}$$

which can be written with small aproximations, if $K[T] < 1$ and $K'[D] < 1$,

$$T_o = \frac{[T]}{(1 - (K[T] + K'[D]))^2} \quad (10)$$

$$D_o = \frac{[D]}{(1 - (K[T] + K'[D]))^2} \quad (11)$$

and without approximation, if $K = K'$.
The number concentration of all (2^i) copolymers containing i subunits is

$$C_i = (K^{i-1/i}[T] + K'^{i-1/i}[D])^i$$

The total mass amount of tubulin is

$$[C_o] = \sum_{i=1}^{i=\infty} i \cdot C_i$$

This model was used to quantitatively describe the interactions between tubulin molecules as a function of bound nucleotide and tubulin concentration.

The data shown in Figs. 4–6 have been used to derive the values of the propagation constants K and K' and the rate constants for GTP hydrolysis associated with tubulin-tubulin interactions. Analysis of the sedimentation data in Fig. 6 yields values of 0.04 μM^{-1} and 0.08 μM^{-1} for K and K'. The values of the rate constants H_{TT} and H_{TD} for GTP hydrolysis associated with the interaction of GTP-tubulin with GTP-tubulin or GDP-tubulin could then be derived from adjustment of the model to the data in Fig. 5, using the determined values of K and K'. It was found that the resulting best fit values of K, K', H_{TT}, and H_{TD} could also well account for the experimental data presented in Fig. 4. Hence all theoretical curves in Figs. 4–6 were calculated by using the same values $K = 0.04\ \mu M^{-1}$, $K' = 0.08\ \mu M^{-1}$, $H_{TT} = 0.023\ min^{-1}$, $H_{TD} = 0.08\ min^{-1}$.

Data in Fig. 4 were fitted as follows. The concentration of GTP-tubulin in oligomers is $[T_0] - [T]$. For a given value of [T], the concentration of [D] was derived from the concentration of added GDP ($[X_0]$), and free GTP, using the following equation:

$$D = (T/\alpha) \cdot (GDP/GTP) \quad (12)$$

where GDP and GTP represent the concentrations of free GDP and free GTP, respectively. The total concentration of GTP-tubulin (free GTP-tubulin + oligomerized GTP-tubulin), $[T_o]$, was derived using Eq. 10. An iteration loop was introduced in the calculation to adjust the value of [T], so that the resulting calculated value of $[C_o]$ was identical to the experimental value. The concentration of free GTP was also calculated in the iteration loop, as GTP = $[C_o] - [T_o]$. The experimental values of the rate of GTP hydrolysis were plotted versus the calculated values of $[T_o]$. In the presence of GDP, all data points were found to lie above the curve obtained in the absence of GTP. This feature was observed for all alternative values of K and K' assayed. Therefore the faster rate of GTP hydrolysis is not due solely to the shift of the oligomerization equilibrium toward a higher level of assembled GTP-tubulin triggered by GDP-tubulin. The data indeed show that GTP is hydrolyzed faster when GTP-tubulin in oligomers is interacting with GDP-tubulin. To fit the experimental data, the rate of GTP hydrolysis V_h was calculated as the weighted sum of T-T and T-D interactions in all oligomers $[D_i\ T_j]$:

$$V_h = ([T_o] - [T])[H_{TT} \cdot \beta + H_{TD} \cdot (1 - \beta)] \quad (13)$$

where H_{TT} and H_{TD} are the rate constants for GTP hydrolysis on a polymerized GTP subunit interacting with a GTP-bound or a GDP-bound neighbor, respectively, and $\beta = ([T_o] - T)/([C_o] - (T + D))$. No satisfactory fit could be obtained by using $H_{TT} = H_{TD}$ (*dashed lines* in Fig. 3). The best fit to experimental data points was obtained when the ratio H_{TD}/H_{TT} had a value between 3 and 4, consistent with the fit to data in Fig. 5.

The assumption that GTP hydrolysis is associated with longitudinal interactions between tubulin molecules in linear oligomers, rather than with lateral interactions occurring upon the nucleation of a bidimensional sheet is supported by the observation that GTP hydrolysis also occurs upon heterologous interaction between GTP-tubulin and GDP-tubulin. Lateral interactions are known to be destabilized by GDP; hence a lower rate of GTP hydrolysis would be observed if the hydrolysis reaction were linked to unfavorable lateral contacts. It is generally assumed, in models of microtubule assembly (Erickson and Pantaloni, 1981) and observed, in the electron microscope, that lateral interactions are much weaker than longitudinal interactions; hence linear oligomers are the predominant species, in mass and in number, during the prenucleation period. Although the above model is obviously oversimplified, it has merit, in that it accounts for the data; hence we may reasonably expect that a more complex model would not be, in essence, drastically different.

The experimental data points obtained in the absence of GDP (*filled circles* in Fig. 4) were well fitted by using values of 0.04 μM^{-1} for K and 0.023 min^{-1} for H_{TT}. This value of K is consistent with the one that can be extrapolated at 37°C from the series of determinations of K made at different temperatures between 5°C and 30°C (Howard and Timasheff, 1986). The data obtained in the presence of GDP and at different values of $[C_o]$ (lopen symbols in Fig. 4) were well fitted by using the value of 0.08 μM^{-1} for K' and 0.08 min^{-1} for H_{TD}. These figures correspond to $\alpha = 3$. Other values would be found for $3 < \alpha < 10$. The hydrolysis rate constants are much lower than the value of 40 s^{-1} (Carlier et al., 1987) found for vectorial GTP hydrolysis per microtubule in microtubule assembly. This difference is consistent with the notion that the linear oligomers of tubulin are in rapid equilibrium, and the lifetime of a molecule of GTP-tubulin in oligomers is on the order of 1 ms; hence

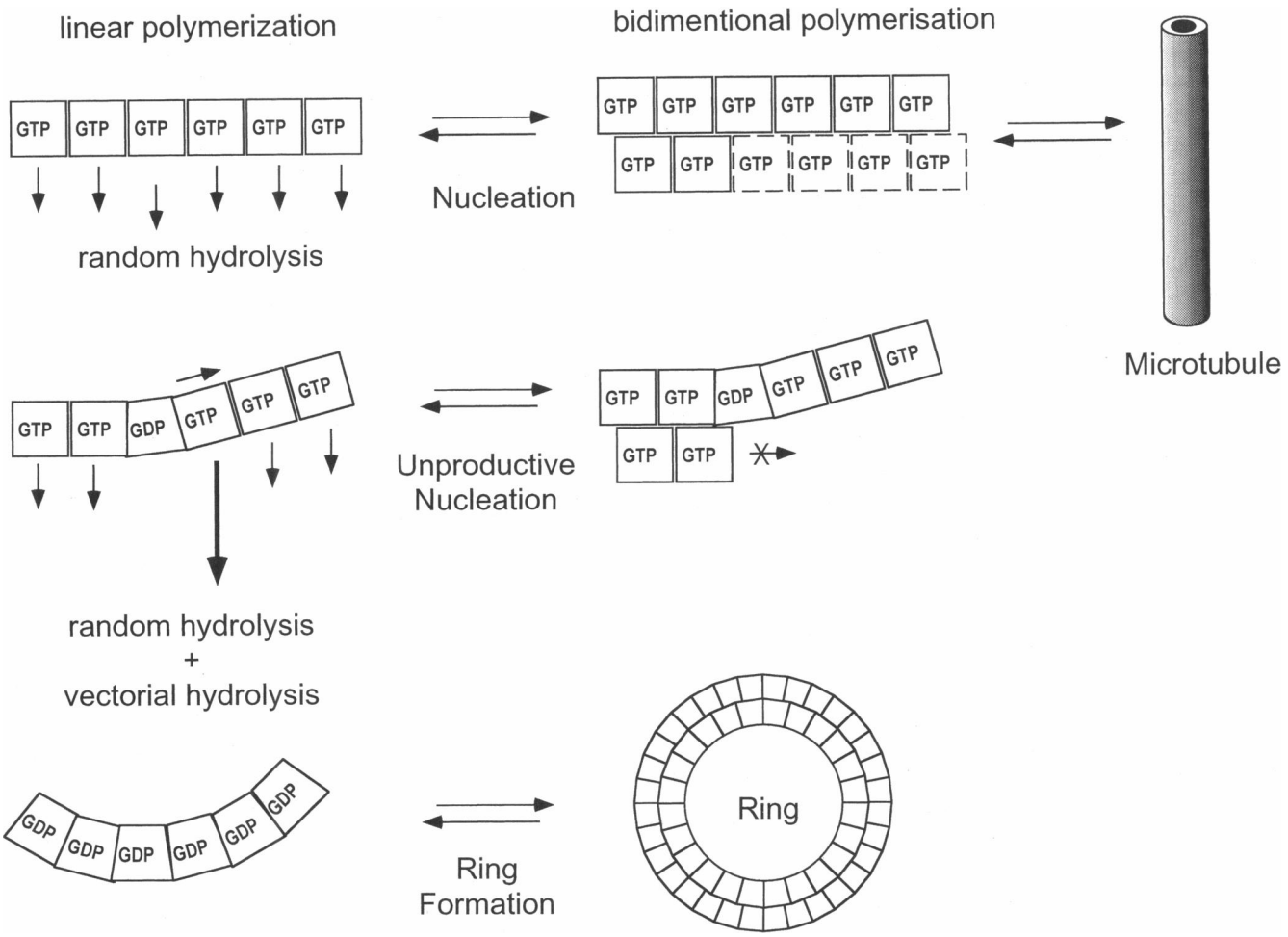

FIGURE 7 Model for inhibition of nucleation by GTP hydrolysis occurring on linear oligomers of GTP-tubulin. For simplicity, only the relevant reactions that take place on an oligomer the size of a prenucleus are displayed. In the present example, GTP-tubulin may associate laterally to a linear oligomer of GTP-tubulin, making a T-GTP/T-GTP lateral bond that initiates the formation of a bidimensional lattice. GTP is hydrolyzed on the linear oligomer. The probability of a lateral T-GTP/T-GTP contact decreases as GTP hydrolysis proceeds in the oligomer. The dependence of the probability of lateral bond formation on the proportion of GTP-tubulin in the oligomer is not known.

the probability of hydrolysis of GTP at a rate on the order of 1 s^{-1} becomes very small. The situation is different in growing microtubules, where the GTP subunits are immobilized in a bidimensional lattice.

DISCUSSION

The results presented here show that GTP hydrolysis is associated with the interactions between molecules of GTP-tubulin that lead to the formation of microtubule nuclei. In mixtures of GDP and GTP, heterologous interactions between GTP-tubulin and GDP-tubulin occur and are associated with GTP hydrolysis. Heterologous interactions between T-GTP and T-GDP are of the same nature as the homologous functional interactions between molecules of GTP-tubulin leading to oligomers involved in microtubule assembly. Evidence for this conclusion is provided by the experiment described in Fig. 3, carried out under conditions where GTP-tubulin interacts principally with GDP-tubulin. In the presence of [BeF_3^-, H_2O], the oligomers resulting from such interactions are stabilized and can work as functional nuclei supporting the elongation of microtubules.

A simple model of isodesmic polymerization of T-GTP and T-GDP could well account for both the sedimentation and GTP hydrolysis data (Figs. 4–6). According to this model, which is consistent with previous sedimentation studies of tubulin oligomers (Howard and Timasheff, 1986), GTP-tubulin and GDP-tubulin undergo linear isodesmic oligomerization with very similar values for the propagation constants. The straight shape of the GTP-tubulin molecule would allow easy building of a bidimensional sheet, whereas the curved conformation of GDP-tubulin would prohibit it. The vision of GTP-tubulin and GDP-tubulin as "straight" and "curved" molecules is useful for illustrating the fact that lateral interactions are not possible in the GDP bound state, whereas longitudinal interactions are not affected. Hence GTP hydrolysis on prenuclei leads to unproductive GDP oligomers. These results convey the conclu-

sion that nucleation of microtubules must develop in two steps: 1) linear oligomers are formed by sequential association of GTP-tubulin subunits; 2) these prenuclei are stabilized by lateral association of GTP-tubulins. Lateral association of GTP-tubulin must proceed at a rate high enough to prevail over the generation of unproductive GDP oligomers via GTP hydrolysis. The hydrolysis of GTP therefore may represent a kinetic barrier accounting for the second nucleation step in the heterogeneous nucleation model previously proposed (Voter and Erickson, 1984) to describe the spontaneous polymerization of tubulin and for the recently reported different nucleation pathway (Kuchnir-Fygenson et al., 1995). A scheme describing this mechanism is provided in Fig. 7. The nucleation-counteracting function of GTP hydrolysis will, of course, generate a more cooperative dependence of the polymerization rate on tubulin concentration than a simpler scheme in which GTP hydrolysis is not involved. The easy nucleation of microtubules observed with the nonhydrolyzable analog of GTP, GMPCPP (Sandoval et al., 1977), supports this conclusion. It is expected that putative regulatory proteins that would inhibit GTP hydrolysis associated with nuclei formation would favor microtubule nucleation.

The fact that GTP is hydrolyzed upon interaction of GTP-tubulin with GDP-tubulin may introduce a bias in experiments aimed at measuring the uncoupling between microtubule assembly and GTP hydrolysis when the tubulin used may not have consisted of 100% GTP-tubulin, because a thorough equilibration by Sephadex G25 chromatography in a GTP-containing buffer had not been performed. The full exchangeability of the nucleotide bound to the E-site, which is known to depend on ionic conditions (Croom et al., 1985), is not invariably observed in different reports (Hyman et al., 1995). Our data (Fig. 2) show that in mixtures of GTP- and GDP-tubulin, hydrolysis of GTP occurs before microtubule assembly and does not appear to be tightly coupled to the assembly process; the GTP hydrolysis curve actually crosses the polymerization curve. The same behavior can be observed in previous works (Hamel et al., 1984; O'Brien et al., 1987, figures 1–3), suggesting that incomplete nucleotide exchange might account for these observations.

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