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Biochemistry

Participation of guanine nucleotides in nucleation and elongation steps of microtubule assembly

(brain tubulin/GTP hydrolysis/elongation by GDP/nucleotide analog effects)

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ABSTRACT **Critical concentrations for formation of microtubules from subunits with GTP and its β,γ-imido and β,γ-methylene analogs are similar when adequate time is given for equilibration. Dilution of microtubules into GTP and GDP yielded values of 0.1 and 0.19 mg/ml for the critical concentration, results similar to those reported by Carlier and Pantaloni [Carlier, M. & Pantaloni, D. (1978) *Biochemistry* 17, 1908–1915]. GDP is capable of supporting elongation of preformed microtubules, but it efficiently poisons the nucleation events. Reported experiments also demonstrate that the critical tubulin concentration of the tubulin·GDP complex can be accurately measured in both the assembly and disassembly directions. Evidence is presented that GTP is involved in early nucleation events but that microtubules are stabilized in the presence of either GTP or GDP. These results are discussed in terms of a condensation-equilibrium model in which tubulin subunits equilibrate rapidly with microtubule ends, and their affinity for the ends is governed by the nucleotide ligand at the exchangeable site.**

GTP is hydrolyzed during microtubule assembly, and there is now general agreement that the exchangeable guanine nucleotide site is involved (1–3). Nonetheless, the role of nucleotide hydrolysis in terms of promoting essential protein–protein interactions during nucleation,* elongation, or stabilization of microtubules is only poorly understood. Part of the problem in assigning a role to GTP hydrolysis arises from the observation that the analogs guanosine 5′-[β,γ-imido]triphosphate (Guo*PP*[NH]*P*) and guanosine 5′-[β,γ-methylene]triphosphate (Guo*PP*[CH_2]*P*) also support assembly, suggesting that GTP hydrolysis is not necessary for assembly (4–6). The analogs and GTP were recently shown to have similar interactions with tubulin, on the basis of fluorescence quenching measurements (7). The finding that tubules formed with Guo*PP*[NH]*P* resist subsequent depolymerization upon dilution led Weisenberg and Deery (6) to propose that hydrolysis is important in processing microtubules for eventual disassembly. Weisenberg *et al.* (8) reported that, whereas GDP was a potent inhibitor of microtubule assembly, it did not depolymerize preformed microtubules. Carlier and Pantaloni (9) have more recently examined nucleotide interactions with tubulin (Tb) purified by ion-exchange chromatography and assembled in the presence of glycerol and high magnesium ion concentrations. These authors were able to demonstrate microtubule stability in the presence of GDP added after condensation equilibrium had been reached. More recently, our finding that the mole fraction of phosphorylated exchangeable nucleotide sites, [Tb·GTP]/([Tb·GTP] + [Tb·GDP]), may be as low as 0.6 without marked inhibition of the rate or extent of polymerization suggested that hydrolysis and assembly may not be strictly coupled (3).

To further probe the role of nucleotides in assembly, we have employed measurements of the critical concentration. It has been found that the redistribution of tubulin between unpolymerized and polymerized forms after dilution of Guo*PP*[CH_2]*P* and Guo*PP*[NH]*P* tubules parallels the behavior of GTP tubules. We have also observed that Tb·GDP and Tb·GTP have different critical concentrations and that Tb·GDP can participate in the elongation of microtubules. These findings suggest that GTP hydrolysis during elongation may be a consequence of an induced hydrolysis required during nucleation but not in elongation. The data also suggest that fluctuations in the GDP and GTP concentrations of the cell will have little effect on assembled microtubules.

MATERIALS AND METHODS

Materials. GDP, GTP, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 2-(*N*-morpholino)ethanesulfonic acid (Mes), and Guo*PP*[CH_2]*P* were products of Sigma. Guo*PP*[NH]*P* was obtained from ICN. The buffer used in this study contained 0.1 M Mes, 1 mM EGTA, 1 mM $MgSO_4$, and KOH to adjust the pH to 6.8. The protein used throughout this work was obtained from bovine brains by using an experimental protocol developed in this laboratory (10). Immediately prior to use, protein that had been stored in pellet form at −80°C after three cycles of polymerization/depolymerization was dissolved in Mes/EGTA/$MgSO_4$ buffer and either GTP or GDP was added, depending on the experiment. After a 30-min incubation at 4°C, the sample was spun at 48,000 × *g* for 15 min to remove any large aggregates.

Turbidity Assay. The increase in optical density at 350 nm was used in these experiments to monitor the weight of polymeric protein in solution (11). In an earlier study (3) it was determined that the OD_{350} was a linear function of polymeric protein concentration up to 2.5 mg/ml. All turbidimetric assays were performed with a Cary 118 spectrophotometer equipped with a thermostated sample chamber.

Dilution of Microtubules into GDP or GTP. A concentrated protein solution containing 0.1 mM GTP was diluted with cold Mes/EGTA/$MgSO_4$ buffer containing 0.1 mM GTP to give a stock solution of 1.75 mg/ml. Appropriate aliquots of this solution were delivered to test tubes and incubated for 60 min at 30°C. Another series of protein aliquots from this 1.75 mg/ml stock were delivered to test tubes and immediately diluted to various amounts with buffer containing 0.1 mM GTP and warmed to 30°C, and the initial stages of the polymerization reaction were monitored. This gives an accurate measurement for the initial OD_{350} for the individual reaction aliquots. After

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Abbreviations: Guo*PP*[NH]*P*, guanosine 5′-[β,γ-imido]triphosphate; Guo*PP*[CH_2]*P*, guanosine 5′-[β,γ-methylene]triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tb, tubulin.

* The term "nucleation" is used here to refer to the initial steps in self-assembly, and no detailed assembly mechanism is implied.

the 60-min incubation, the samples were diluted with warm buffer containing either 0.1 mM GTP or 2.0 mM GDP (containing 2.33 mM $MgSO_4$ to ensure that the level of free magnesium remained constant). After a 25-min incubation at 30°C, the final OD_{350} was determined. The final OD_{350} was recorded continuously for 5 min after the incubation period to ensure that equilibration had been reached.

Dilution of Microtubules in Buffer Containing GTP, Guo*PP*[NH]*P*, or Guo*PP*[CH_2]*P*. These experiments were performed analogously to those above except the protein samples were made 1.5 mM in their respective nucleotides and were incubated an additional hour with alkaline phosphatase to evacuate the exchangeable nucleotide site (12). The data presented in the *inset* to Fig. 1 were obtained by diluting 0.4 ml of a 6.75 mg/ml solution of Guo*PP*[NH]*P*-formed microtubules approximately 1:32 in a specially fabricated rapid mixing cuvette (13) and monitoring the decrease in turbidity with time in the spectrophotometer.

Elongation of Microtubules with Tb·GDP Complex. To determine the critical concentration in the assembly direction for Tb·GDP complex, it was possible to utilize microtubules assembled with GTP as seeds for further elongation. Protein was prepared as described above with 2 mM GDP, and a separate sample was prepared with 3.15 mM GTP. A 60-μl sample of the latter (15.7 mg/ml in microtubule protein) was polymerized for 30 min at 30°C. Simultaneously, solutions containing GDP and a variable concentration of microtubule protein were thermally equilibrated at 30°C to await addition of the polymerization seeds. Immediately prior to initiation of the elongation reaction, polymerized microtubules were sheared by three rapid passages through a 50-μl Hamilton syringe, and a 35-μl aliquot was added to each sample of Tb·GDP complex to give a final concentration of 0.11 mM GTP and 2 mM GDP and the desired quantity of microtubule protein in each 1-ml sample. The progress of the elongation reaction was monitored at 350 nm, and sufficient time was provided for equilibration to occur before the final optical density was taken. The increased turbidity was then plotted versus Tb·GDP complex, and the extrapolated value at zero optical density was defined as the critical concentration. In control experiments involving turbidity measurements and electron microscopy, we verified that Tb·GDP complex was incapable of assembly.

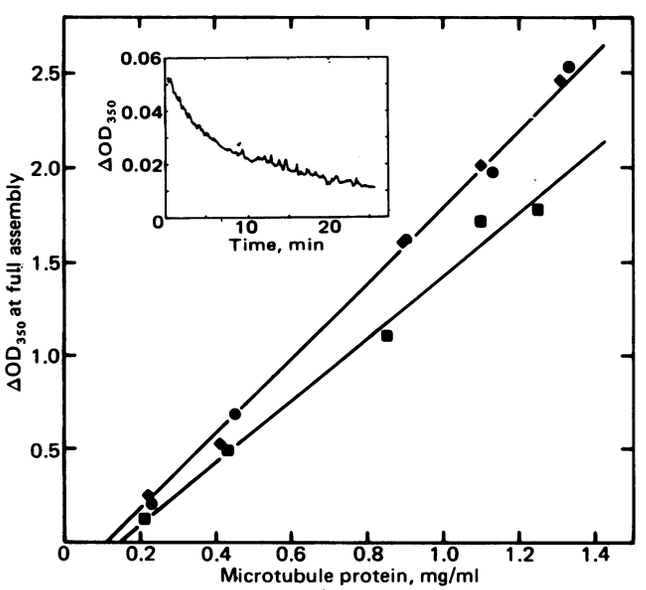

FIG. 1. Stability of microtubules formed in the presence of 0.5 mM Guo*PP*[NH]*P* (◆), Guo*PP*[CH_2]*P* (■), or GTP (●) after dilution into buffer containing 0.5 mM of their respective nucleotide or nucleotide analog. (*Inset*) Time course for the depolymerization of microtubules formed in the presence of 0.5 mM Guo*PP*[NH]*P* after dilution into a rapid mixing cell.

Negative stain electron microscopy was used to confirm the presence of microtubules in all experiments performed. Samples were routinely fixed on grids by standard procedures using cytochrome *c* and uranyl acetate and were inspected with a Siemens Elmiskop I. At the conclusion of experiments, samples were routinely subjected to cooling to 4°C for 30 min and in all cases the turbidity decreased to baseline levels.

"GTP–GDP Shift" Experiments. Protein prepared as described above containing 0.1 mM GTP was diluted to 1.15 mg/ml with Mes/EGTA/$MgSO_4$ buffer containing 0.2 mM GTP. A 1-ml aliquot was extracted and warmed from 4°C to 30°C in the spectrophotometer. A 40-μl aliquot of 50 mM GDP solution and a 13-μl aliquot of a 100 mM $MgSO_4$ solution were added to the protein solution at the indicated times (dotted lines of Fig. 4, curves A–E). Curve F of Fig. 4 represents addition of GDP followed by warming to 30°C for 5 min and subsequent recooling to 4°C. After incubation at 4°C for 25 min the sample was warmed to 30°C. After 80 min at 30°C, the sample was made 1.5 mM in GTP.

THEORY

The condensation-equilibrium model of Oosawa and Kasai (14) may be used to define the critical microtubule protein concentration. Consider the equilibrium between unpolymerized microtubule protein X and the polymerized form MT,

$$X + MT_n \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MT_{n+1}, \qquad [1]$$

in which n and $n + 1$ indicate the number of assembled units of X. The kinetics of this process may be described by

$$\frac{dX}{dt} = -k_1[X]\sum_{n=l}^{m-1}[MT_n] + k_2\sum_{n=l-1}^{m-1}[MT_{n+1}], \qquad [2]$$

in which the summation is made over all polymer lengths from l to a maximal length m and represents the weight concentration. We may assume that the polymers are long and that the binding of additional monomeric units does not alter the reactivity of the polymer end. Thus we may regard the two summations as alternate expressions of [MT], the approximate concentration of polymer ends. At equilibrium $dX/dt = 0$ and the dissociation constant K_d for Eq. 1 may be reduced to [X], the equilibrium concentration of unpolymerized protein. Oosawa and Kasai (14) use the term "critical concentration" to indicate this threshold concentration required for the formation and maintenance of polymerization nuclei. In this study, we apply the concept of the critical concentration to examine the stability of microtubules in the presence of various nucleotide ligands. It is also useful to note that X_∞ may be evaluated by diluting preformed microtubules into an appropriate buffer and extrapolating to zero polymer (actually zero optical density) in plots of optical density change versus the total weight concentration of X. As shown earlier (11), the optical density change at 350 nm may be used as a measure of the weight concentration of polymer.

RESULTS

Stability of Microtubules Assembled with GTP, Guo*PP*[NH]*P*, or Guo*PP*[CH_2]*P*. Weisenberg and Deery (6) relied upon dilution experiments to draw the conclusion that GTP hydrolysis may play a role in preparing microtubules for

disassembly. They observed that GTP tubules and Guo*PP*[NH]*P* tubules behave differently upon dilution into their respective nucleotide. Whereas GTP tubules gave a typical critical concentration of 0.2 mg/ml, the Guo*PP*[NH]*P* tubules yielded a negative value. These authors speculated that GTP hydrolysis leads to a tubule state that is susceptible to disassembly, but Guo*PP*[NH]*P*-supported assembly does not. To further test this hypothesis, we repeated these experiments with GTP, Guo*PP*[NH]*P*, and Guo*PP*[CH_2]*P*. The methylenediphosphonate analog was included because it should behave like the imidodiphosphonate derivative if GTP hydrolysis is a necessary feature. In these experiments (data not shown), we duplicated the results of Weisenberg and Deery (6), but the Guo*PP*[CH_2]*P* tubules gave a positive intercept slightly higher than the GTP intercept. We recognized the possibility that the Guo*PP*[NH]*P* tubules may require a greater time for disassembly. To explore this possibility, we assembled Guo*PP*[NH]*P* tubules and subsequently diluted them 1:32 into a 5-cm pathlength mixing cell. Earlier experiments demonstrated that GTP tubules depolymerize in an apparently first-order fashion with a rate constant of 0.02 sec^{-1}, and the entire process is over in 3–5 min (13). On the other hand, Guo*PP*[NH]*P* tubules disassemble much more slowly (Fig. 1 *inset*), and the 5-min value used in the Weisenberg and Deery (6) experiments may have been insufficient to permit adequate equilibration. As shown in Fig. 1, the dilution experiments may be repeated by allowing 45 min for disassembly after dilution, and all three types of tubules have similar behavior. Most significantly, GTP and Guo*PP*[NH]*P* tubules behave almost identically, yielding a critical concentration of 0.1 mg/ml. The Guo*PP*[CH_2]*P* tubules also have a positive critical concentration, 0.13 mg/ml. These results serve to reopen the question concerning the role of GTP hydrolysis in microtubule assembly and disassembly.

Critical Concentration of Microtubules in Buffers Containing GDP and GTP. It was possible to use the concept of the critical concentration to further probe microtubule assembly/disassembly behavior. MacNeal and Purich (3) have reported that the mole fraction, [Tb·GTP]/([Tb·GTP] + [Tb·GDP]), prior to assembly can be as low as 0.6 without significantly affecting the extent of assembly. This indicates that Tb·GDP complex may be incorporated into microtubules, and it was of interest to determine whether GDP and GTP stabilize microtubules to the same extent. Microtubules formed in the presence of 0.1 mM GTP were diluted to various extents into Mes/EGTA/$MgSO_4$ buffer containing 2 mM of either GDP or GTP. Under these conditions, the unpolymerized tubulin will fully exchange its nucleotide within a period of 4–5 min (unpublished findings). Because Tb·GDP and Tb·GTP complexes have similar dissociation constants (15), this exchange leads to a condition in which the unpolymerized tubulin is largely present as Tb·GDP or Tb·GTP. The results shown in Fig. 2 demonstrate that the critical concentration with GDP is about 2-fold greater than with GTP. The finding that depolymerization is incomplete with GDP substantiates the earlier conclusion (3) that Tb·GDP may be incorporated into microtubules. To add further credence to this statement, we examined the critical concentration for Tb·GDP addition to microtubules that had been assembled with GTP. Microtubular protein containing 2 mM GDP (which by itself would not assemble) was added to preformed microtubules as described under *Materials and Methods*, and the increased turbidity was monitored until a new equilibrium plateau had been reached. The increased OD_{350} was then plotted versus added Tb·GDP and extrapolated to zero optical density as shown in Fig. 3. Comparison of Fig. 3 with Fig. 2 reveals that the intercepts for "GDP perturbed tubules" are identical for the assembly and disassembly routes

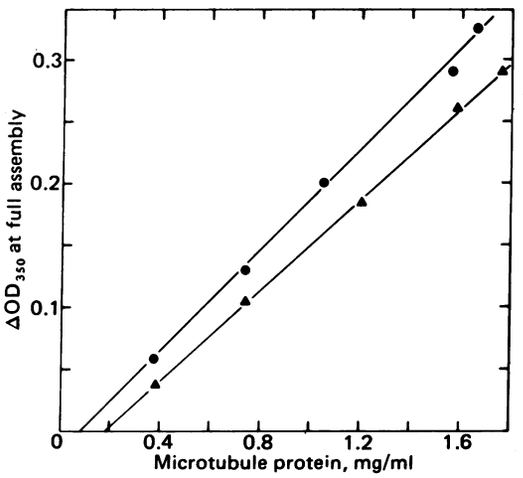

FIG. 2. Dilution of microtubules into buffer containing either 2.0 mM GTP (●) or 2.0 mM GDP (▲). Microtubular protein (1.75 mg/ml) was assembled at 30°C in the presence of 0.1 mM GTP. After 60 min the samples were diluted with buffer containing the appropriate nucleotide and the final OD_{350} was determined at 25 min.

to equilibrium. It should be noted that Tb·GDP required longer periods of time to reach equilibrium than Tb·GTP, but electron microscopy revealed no differences in morphology or relative abundance of the GDP tubules. In addition, both samples consistently showed identical sensitivity to cold-induced disassembly.

Effect of Added GDP During GTP-Supported Assembly. We have routinely observed that GDP does not support microtubule assembly over a 90-min period, and a number of workers (3, 8, 9) have demonstrated that GPD is an effective inhibitor of polymerization. In view of our present finding that addition of Tb·GDP leads to elongation of preexisting microtubules, we carried out a series of experiments in which the predominant nucleotide was shifted from GTP to GDP at various times after assembly of the microtubule protein was initiated with 0.2 mM GTP. As shown in Fig. 4, curve A, the addition of GDP to microtubules in the steady state leads to a drop to a new steady state that is consistent with the increased critical concentration in the presence of GDP and the slight

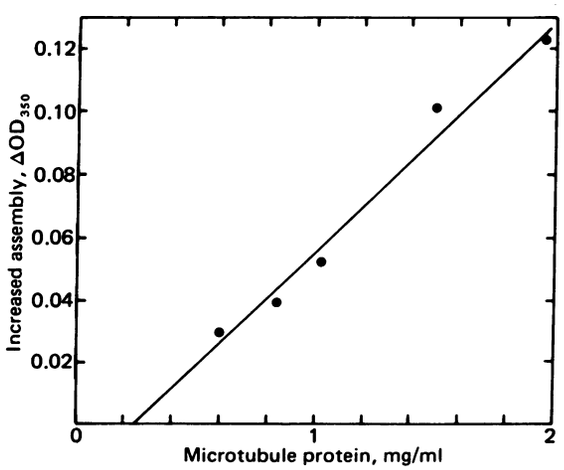

FIG. 3. Increased extent of assembly after addition of Tb·GDP complex. Each data point represents the increase in OD_{350} after a plateau value was reached for assembly subsequent to the addition of sheared microtubules (15.7 mg/ml) to the specified concentrations of Tb·GDP complex.

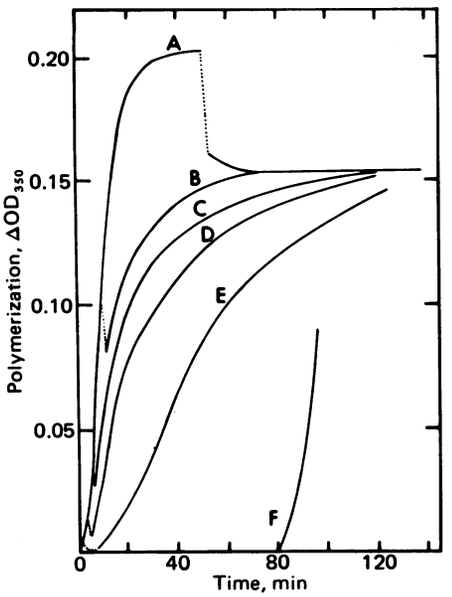

FIG. 4. Effect of added GDP during GTP-supported assembly. Identical samples containing microtubular protein at 1.15 mg/ml and 0.2 mM GTP were assembled at 30°C. At the indicated times (dotted lines) the samples were made 2.0 mM GDP and 2.33 mM $MgSO_4$ (curves A–E). Curve F represents the polymerization profile in which the GDP was added to the protein sample at 4°C for 2 min, warmed to 30°C for 5 min, and recooled to 4°C for 25 min, followed by warming to 30°C. At 80 min the protein was made 1.5 mM in GTP.

dilution (5%) during GDP addition. Curves B–E indicate that the GTP-to-GDP shift at any time after warming still leads to microtubule assembly (electron microscopy confirmed that the increase in turbidity in these experiments was due to the formation of microtubules). Curves A–E approach the same final plateau value, but it should be noted that GDP was added to the samples in A–E after warming to 30°C, whereas in curve F the GDP was added at 4°C 2 min prior to warming. GTP was capable of reversing the inhibition by GDP after an 80-min incubation, as indicated in Fig. 4, curve F. It should be clear that elongation with GDP is slower, requiring twice as long to achieve a final plateau condition.

DISCUSSION

This report emphasizes the value of critical concentration measurements in providing information on the energetics of self-assembly processes and in serving as a quantitative basis for interpreting the role of nucleotides in certain assembly reactions. The theory as elaborated by Oosawa and Kasai (14) suggests that the value of the critical concentration is a measure of the monomer–polymer equilibrium and that the length distribution is of no particular importance in judging the critical concentration. Indeed, polymerization is viewed as a sequential process: nucleation → elongation → monomer–polymer equilibrium → length redistribution. Gaskin *et al.* (11) demonstrated that turbidity is a measure of the weight concentration of polymerized tubulin, and thus our measurements reflect the amount of polymer without bias by the length distribution. It is interesting to note that Cooke (16) rigorously applied the concept of the critical concentration to the role of hydrolysis of nucleotides in the polymerization of G-actin·ATP, G-actin·ADP, or G-actin·Ado*PP*[NH]*P* to form filamentous F-actin polymer. One important conclusion of that study was that polymerization of G-actin·ATP may be explained by nucleotide dephosphorylation occurring in a step following the polymerization step. This proposal and the observed polymerization with Ado*PP*[NH]*P* fit with the idea that ATP hydrolysis is not strictly coupled to polymerization. With microtubule assembly, MacNeal and Purich (3) have also obtained kinetic evidence against tight coupling of hydrolysis and assembly, and we are drawn to conclude that the role of GTP hydrolysis in microtubule assembly remains unclear.

The findings presented in this report permit us to make four statements: (*i*) GTP, Guo*PP*[NH]*P*, and Guo*PP*[CH_2]*P* support microtubule assembly in a manner that does not substantially alter microtubule stability. (*ii*) The critical concentration in the presence of GTP is only slightly less than with GDP, suggesting that there are no major differences between Tb·GDP and Tb·GTP interactions with tubules. (*iii*) Shifting the nucleotide bound to tubulin from GTP to GDP does alter the extent of elongation and retards nucleation and the assembly rate. (*iv*) Cooling microtubules to 4°C after the shift from GDP to GTP destroys the ability of microtubule protein to polymerize upon warming. These observations indicate that the role of GTP hydrolysis in microtubule assembly is not simply to surmount a thermodynamic barrier to polymerization. Indeed, the observation that Tb·GDP complex will support elongation suggests that the elongation process will occur spontaneously without the use of the free energy of hydrolysis. The nucleation of assembly by Tb·GTP appears to be a necessary feature, and Tb·GDP complex alone will not support nucleation. In this respect, we incline towards models that utilize GTP hydrolysis to support effective nucleation. Fig. 5 shows schematically the equilibrium processes that appear to be involved. We perceive the microtubule ends to be capable of freely associating with tubulin dimers that contain either GTP or GDP but with a slightly lower affinity for the Tb·GDP complex. This model says nothing about when or how GTP is hydrolyzed upon polymerization, but the experiments presented in this report serve to emphasize that whereas the hydrolytic events are irreversible (i.e., disassembly does not proceed via the mechanism $MT_n \cdot GDP + xP_i \rightarrow MT_{n-x} + GTP$), the assembly/disassembly processes can be reversible. For there to be a difference in the critical concentration with GDP and GTP, we must conclude that Tb·GTP must bind to the ends and occupy the site as MT·Tb·GTP complex for a sufficiently long enough time to establish the equilibrium. It will be of interest to determine the number of Tb·GTP dimers that persist at the ends of microtubules without hydrolysis. This matter relates to the possibility of induced hydrolysis of GTP upon subsequent dimer addition steps.

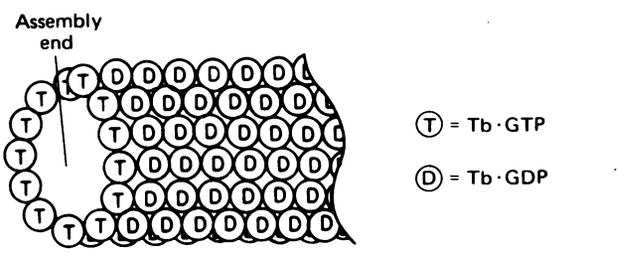

FIG. 5. Equilibrium boundary model for tubulin–nucleotide interactions. Tubulin complexed with either GTP or GDP equilibrates rapidly with microtubule ends. Boundary and interior dimers are distinguished by the number of nearest neighboring tubulin dimers, and the stability largely depends on the dimer–dimer interactions at tubule ends. Association constants are $K_T = 1/[Ⓣ]_{crit}$ and $K_D = 1/[Ⓓ]_{crit}$, in terms of critical concentrations. A basic feature of this model is the existence of Tb·GTP dimers at the assembly end of the tubule even after polymerization is attained, but no stoichiometry is implied by this diagram.

It is interesting to speculate on the metabolic consequences of GTP hydrolysis and the ability of Tb·GDP to participate in the elongation reaction. B. J. Terry and D. L. Purich (unpublished) have examined the initial rate of microtubule assembly in the presence of GDP and ATP via the action of the nucleoside diphosphate kinase. The rate was inversely related to the [ADP]/[ATP] ratio, indicating that the rate of *de novo* formation of microtubules may be metabolically regulated by the phosphorylation state of the adenine nucleotide system. Yet our findings would suggest that existing microtubules would be relatively insensitive to instantaneous fluctuations in the [ADP]/[ATP] ratio because Tb·GDP and Tb·GTP may participate in elongation. A high [ADP]/[ATP] ratio would increase the critical concentration only 2-fold (see Fig. 2). Another consequence of the incorporation of Tb·GDP and GTP hydrolysis during Tb·GTP incorporation is that P_i is not sequestered in the microtubule. Indeed, Weisenberg *et al.* (8) and MacNeal and Purich (3) have shown that P_i is stoichiometrically released from microtubules assembled from Tb·GTP complex. Because the total tubulin concentration in brain is 50–75 μM, this amount will be freed upon assembly. A third possibility is that the so-called "treadmilling" process, in which dimers add to a primary assembly site at one end and dimer release at a disassembly site at the other end (17), depends upon nucleotide hydrolysis. Whether the treadmilling process plays a role in cytokinetic events is unclear, but Margolis *et al.* (18) have considered an elaborate and intriguing model for chromosome movement based upon the unidirectional flow of dimers by multiple addition and release steps at opposite ends of the tubule. It will be of interest to determine whether Guo*PP*[NH]*P* tubules or Guo*PP*[CH_2]*P* tubules are capable of sustaining the treadmilling process. Finally, one might argue that the hydrolysis of GTP during elongation is merely the extended use of the same hydrolytic process essential for nucleation, and this would obviate the need for two classes of tubulin—one for nucleation and one for elongation. In any event, the addition of Tb·GDP during elongation will require considerably more study for us to understand the intricate behavior of microtubules *in vitro*, and it is true that the *in vivo* behavior may be greatly modified and regulated by microtubule organizing centers in the cell.

Lastly, we should note here that our experiments were carried out with bovine brain tubulin, which is devoid of GDPase (unpublished findings). With porcine brain microtubule protein, there is a contaminating GDPase activity (9, 19) which might affect the GDP "shift" and critical concentration measurements. In this regard, there is yet much to be learned about the enzymatic activities associated with microtubule protein samples obtained from different tissues and preparations.

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