GDP State of Tubulin: Stabilization of Double Rings†

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ABSTRACT: Purified tubulin, with GDP occupying the exchangeable nucleotide binding site, has been examined conformationally and for its ability to self-associate into double rings. The circular dichroism spectrum increased by ca. 10% in negative amplitude between 205 and 225 nm over the spectrum of tubulin in the GTP state, but there were no significant shape changes. This indicates that replacement of GTP by GDP induces tubulin to adopt a more ordered conformation. The sedimentation coefficients of tubulin α–β dimers in the GDP and GTP states were identical, with $s_{20,w}$ = 5.8 S. A sedimentation velocity study of tubulin in the GDP state showed that, in the presence of magnesium ions, this protein undergoes a reversible Gilbert-type self-association. The end product of this reaction was found to be 26 subunit double rings identical with those described by Frigon and Timasheff [(1975) *Biochemistry 14*, 4567–4599] for a similar polymerization of tubulin in the GTP state. Analysis of the data showed that Tu–GDP has a much stronger propensity for the formation of double rings than Tu–GTP, the corresponding equilibrium constants for the $26Tu \rightleftharpoons Tu_{26}$ being 4.2×10^{119} M^{-25} and 2.27×10^{109} M^{-25} for Tu–GDP and Tu–GTP, respectively. This leads to Tu–GTP being predominantly in the form of α–β dimers and Tu–GDP in the form of double rings under normal experimental conditions used in the study of microtubule assembly. Comparison of these results with literature data on the microtubule assembly–disassembly process has led to the proposal that double rings of tubulin in the GDP state can be stable products of microtubule disassembly.

The guanosine nucleotides guanosine 5′-triphosphate (GTP)[1] and guanosine 5′-diphosphate (GDP) are known to play important roles in the modulation of the assembly and disassembly of tubulin into microtubules (for review, see: Purich & Kristofferson, 1984; Correia & Williams, 1983; Timasheff & Grisham, 1980). The α–β tubulin heterodimer contains two guanosine nucleotide binding sites; one (the E site) is exchangeable with free nucleotide and the other (the N site) is not (Weisenberg et al., 1968). Tubulin with GTP in the E site (Tu–GTP) is effective in promoting the initiation of microtubule assembly, while Tu–GDP is not (Carlier & Pantaloni, 1978). Once incorporated into the microtubule, the GTP at the E site is hydrolyzed to GDP and inorganic phosphate, the GDP remaining tightly bound (Kobayashi, 1975; Kobayashi & Simizu, 1976; Weisenberg et al., 1976). The hydrolysis step, however, is not mandatory for assembly, since nonhydrolyzable GTP analogues, such as GMP-PCP and GMP-PNP, are effective in supporting microtubule assembly (Weisenberg et al., 1976; Penningroth et al., 1976; Arai & Kaziro, 1976).

The role of GDP in microtubule function has been the subject of much discussion with no definite conclusion, the experimental observations on the solution physicochemical behavior of Tu–GDP being seemingly contradictory. Thus, Carlier and Pantaloni (1978) concluded that Tu–GDP could elongate preformed microtubules, although they demonstrated that the affinity of Tu–GDP for microtubules was significantly weaker than that of Tu–GTP. On the other hand, Zackroff et al. (1980) and Jameson and Caplow (1980) came to the opposite conclusion that Tu–GDP did not elongate previously formed microtubules. While the role of Tu–GDP appears to be complex, it does destabilize microtubules, facilitating the depolymerization process (Weisenberg & Deery, 1976). Furthermore, tubulin is known to assemble spontaneously into nonmicrotubule structures, yet little is known about the influence of nucleotides on these self-association processes. Some of the structures identified in the electron microscope were single and double rings (Frigon & Timasheff, 1975a,b; Kirschner et al., 1974), protofilaments (Borisy et al., 1972), and flat or helical sheets of protofilaments (Borisy et al. 1975; Erickson, 1974). The early observation by Borisy and Olmsted (1972) that removal of particulate structures, namely, rings, by high-speed centrifugation resulted in the inhibition of microtubule assembly raised the question of the possible roles that these structures might play in assembly. The roles proposed for rings have been (1) that rings serve as direct intermediates of microtubule assembly, i.e., rings stack together to form microtubules (Borisy et al., 1975); (2) that rings serve as templates, controlling the initial steps of microtubule assembly (Jacobs et al., 1975); (3) that rings break open, forming protofilaments that initiate assembly (Kirschner et al., 1974); (4) that rings are a storage form of tubulin, serving no direct function in the assembly process (Weisenberg et al., 1976).

In an attempt to understand better the role of GDP in the self-assembly of tubulin into various structures, a systematic biophysical characterization of Tu–GDP has been initiated. In this paper, we report on the differences in conformation and ability to form double rings between Tu–GDP and Tu–GTP and the implications that these differences have for the microtubule assembly–disassembly process.

Materials and Methods

Calf brains were obtained from freshly slaughtered animals, kept on ice, and used within 1 h of slaughter. The nucleotides,

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP-PCP, guanosine 5′-(β,γ-methylenetriphosphate); GMP-PNP, guanosine 5′-(β,γ-imidotriphosphate); Tu, tubulin; PMG buffer, 10 mM sodium phosphate, 16 mM $MgCl_2$, and 1.0 mM GDP or GTP, pH 7.0; PG buffer, 10 mM sodium phosphate and 1.0 mM GDP, pH 7.0; CD, circular dichroism; EM, electron microscope.

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GTP Type II-S and GDP Type I were purchased from Sigma. [γ-^{3}H]GDP, trisodium salt, 10 Ci/mmol, and [γ-^{3}H]GTP, tetrasodium salt, 10 Ci/mmol, both stored in 1:1 ethanol:water were from New England Nuclear. Ultrapure ammonium sulfate and ultrapure sucrose were from Schwarz/Mann. Ultrapure guanidine hydrochloride, from Heico Co., was filtered through a scintered glass funnel prior to use. Sephadex G-25 and DEAE–Sephadex were from Pharmacia. PEI-Cellulose F chromatography plates were from EM-Reagents. All other chemicals were of reagent grade or better.

Preparation of Calf-Brain Tubulin. Calf-brain tubulin was prepared by a modified Weisenberg procedure as previously described (Na & Timasheff, 1980). Protein aliquots (20 mg at 60–80 mg/mL) were stored in liquid nitrogen in a buffer consisting of 10 mM sodium phosphate, 0.1 mM GTP, 0.5 mM $MgCl_2$, 1 M sucrose, pH 7.0. Prior to each experiment the bulk of the sucrose was removed from the tubulin solution by a Sephadex G-25 batch procedure (Na & Timasheff, 1980). The resulting protein solution was cleared of aggregates by centrifugation at 35000 *g* for 30 min. The final equilibration of the protein with the desired buffer was by gel chromatography on a Sephadex G-25 column. All steps were performed at 4 °C. Protein concentrations were determined in 6 M guanidine hydrochloride at 275 nm with an absorptivity value of 1.03 mL mg^{-1} cm^{-1}.

Sedimentation Velocity. Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with an RTIC temperature control, electronic speed control, and a UV absorbance scanner. Filled Epon centerpieces with quartz windows were used throughout. Schlieren profiles were recorded on Kodak metallographic plates. Data analysis of the Schlieren profile was performed with a Nikon Model 6C microcomparator equipped with a Mitutoyo digimatic micrometer to record radial values and an IKL, Inc., Model 9200 precision digital positioner interfaced to a modified Kaypro II computer for recording heights of the sedimentation boundary and for the calculation of the weight-average sedimentation coefficients, $\bar{s}_w$.

The value of $\bar{s}_w$ was calculated from the measured rate of movement of the second moment of the boundary

$$\bar{r}^2 = \frac{\sum_{r_m}^{r_p} r^2(\partial c/\partial r)}{\sum_{r_m}^{r_p} (\partial c/\partial r)} \quad (1)$$

where $\bar{r}^2$ is the square of the value of the radius at the second moment of the boundary, r_m is the radius at the meniscus, r_p is the radius at some point in the plateau region, and r^2 is the square of the radial value at a point within the boundary where the concentration gradient, $(\partial c/\partial r)$, is evaluated. A plot of log r vs. time yields $\bar{s}_w$

$$\bar{s}_w = \frac{2.303}{60\omega^2} \frac{\mathrm{d} \log \bar{r}}{\mathrm{d}t} \quad (2)$$

where ω is the angular velocity of the rotor in radians/s.

Electron Microscopy. Samples for electron microscopy were prepared essentially according to the previously described procedure (Frigon & Timasheff, 1975a). Protein samples (0.5 mg/mL) were applied to individual carbon-coated Formvar films cast on copper grids and blotted dry with adsorbant paper after 1–2 min. The grids were stained with 15 drops of a 1% uranyl acetate solution applied over a period of 15 s, followed by blotting and air-drying of the grids. The protein samples were examined with a Phillips Model 300 electron microscope at either 60- or 80-kV acceleration potential. The field of view was recorded on 35-mm film and then enlarged to 8 × 10 inch prints.

Analysis of Nucleotide Contents. The fraction of E sites exchangeable with free GDP was estimated by incubating radiolabeled GDP with tubulin, followed by a desalting step to remove the free nucleotide from that bound. An assumption inherent in this procedure is that the exchanged GDP remains bound to tubulin during the desalting step. Thus, within this assumption, the fraction of readily exchangeable sites was measured by removal of free nucleotide by Sephadex G-25 chromatography, followed by quantitation of the amount of radiolabeled nucleotide remaining in the protein fractions. The experimental conditions used to estimate the exchangeability of GTP for GDP were as follows: [γ-^{3}H]GDP (3.76×10^9 cpm/mmol), at a total concentration of 1 mM, was incubated with tubulin (8.0 mg/mL in 10 mM sodium phosphate, 6 mM $MgCl_2$, pH 7.0) at 20 °C for 30 min. These experimental conditions were identical with those used for the sedimentation velocity experiments, except for the altered Mg^{2+} ion concentrations, where indicated. Following the incubation period, free GDP was separated from the exchangeably bound GDP by applying the protein–nucleotide mixture to a Sephadex G-25 column (0.9 × 25 cm). Elution was carried out at a flow rate of 4 mL/min. The procedure required about 5 min for separation. The column fractions containing tubulin were measured in a Beckman LS 7500 liquid scintillation counter giving the moles of GDP that had exchanged in per mole of tubulin. No correction was made for the inhibitory effect of the released nucleotide, since the GDP was at all times 100 times in excess of the GTP.

Circular Dichroism. Circular dichroism (CD) spectra were obtained with a Jobin Yvon Auto-Dichrograph Mark V. Spectra were routinely recorded from 200 to 290 nm for the far-UV measurements and from 250 to 350 nm for the near-UV measurements. A band-pass of 2 nm with a sensitivity of 1.25×10^{-3} differential absorbance units for a full-scale deflection was used for all experiments. The far-UV measurements were performed with a 0.01-cm jacketed cell, while the near-UV measurements utilized a 1.0-cm jacketed cell. Each spectrum represents an average from three scans. A value of 109 was used for the mean residue weight of tubulin in the calculation of ellipticities (Lee et al., 1978). All runs were performed at 20 °C, the temperature being regulated by a Brinkman Instruments Lauda K-2/R water bath.

RESULTS

Exchange of Free GDP with GTP–Tubulin. Tubulin purified by our procedure contains principally GTP bound at the E site. Therefore, in order to carry out experiments with the Tu–GDP complex, it was necessary to replace stoichiometrically GTP at the E site with GDP. Although several procedures described in the literature have met with varying degrees of success (Correia & Williams, 1983), an incubation of tubulin with 1.0 mM GDP at 20 °C for 30 min in PM buffer (10 mM sodium phosphate, 6 mM $MgCl_2$, pH 7.0) was sufficient to exchange the majority of the E-site GTP for GDP in our protein. Figure 1 shows a typical experiment for [^{3}H]GDP incorporation into tubulin after incubation of free GDP with tubulin, followed by removal of free nucleotide by Sephadex G-25 chromatography. It is evident that complete separation occurs between bound and free nucleotide. An average of 83% (n = 3) of the theoretical number of exchangeable sites (based on M_r 108000 for tubulin α–β dimer) were found to be occupied by GDP. This result should be considered as a minimal estimate, since some bound GDP

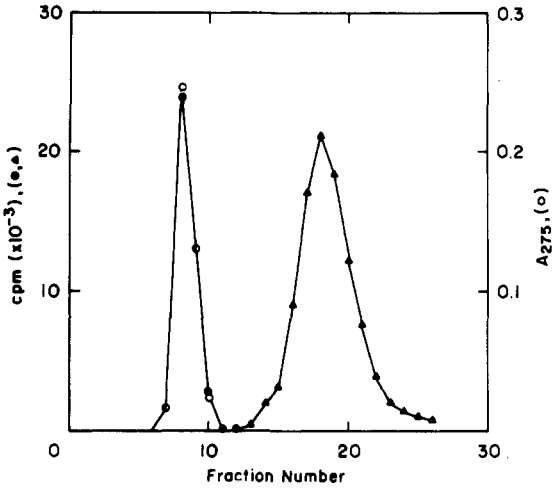

FIGURE 1: Column elution profiles of tubulin (8.0 mg/mL) after incubation with 1.0 mM [^{3}H]GDP (3.8 × 10^9 cpm/mmol) at 20 °C for 30 min. The initial tubulin concentration was 10 mg/mL. (●), cpm in protein fractions; (○), absorbance at 275 nm in 6 M GuHCl; (▲), cpm in nucleotide fractions × 10^{-1}.

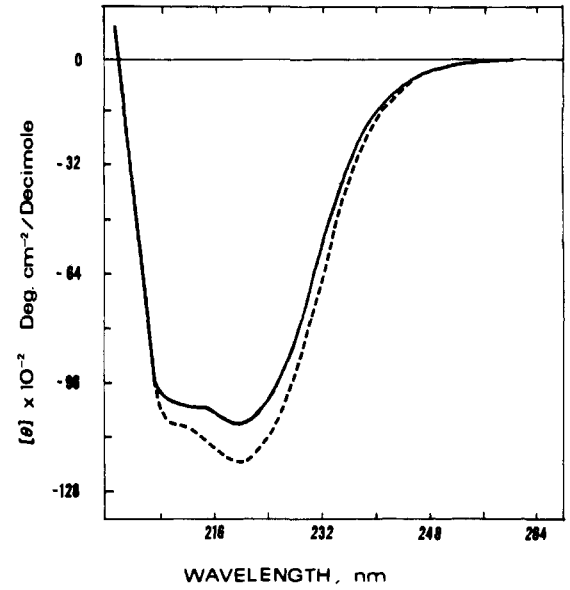

FIGURE 2: Far-UV CD spectrum of tubulin (1.0 mg/mL) in PMG buffer, pH 7.0, with either 0.1 mM GDP (---) or 0.1 mM GTP (—). The temperature was 20 °C. Each spectrum represents an average of three scans.

should dissociate during the column chromatography, once the free nucleotide has been separated from the Tu–GDP complex. This assumption is supported by the correlation between the amount of GDP bound and the fraction number, since increasing fraction numbers were found to contain higher measured apparent binding stoichiometries. For example, fraction 8 had 77% of the theoretical E sites occupied with radiolabeled nucleotide, fraction 9 had 80%, and fraction 10 had 91%. That protein fractions that have been sequestered from free GDP for longer periods of time (lower fraction numbers) contain less bound [^{3}H]GDP suggests that a small amount of dissociation of bound nucleotide is occurring during the desalting step. If this were not the case, all fractions should have contained the same ratio of moles of bound GDP to mole tubulin.

Conformation of Tu–GDP. The CD spectra of tubulin (1.0 mg/mL) in PMG buffer (10 mM sodium phosphate, 16 mM $MgCl_2$ and 0.1 mM GDP or GTP, pH 7.0) at 20 °C are shown in Figure 2. A difference between the spectra of Tu–GDP and Tu–GTP is clearly evident in the far-UV region. While there are no major shape changes in the CD spectrum, the negative signal amplitude between 205 and 225 nm is increased by ca. 10% in the presence of GDP. This indicates that the replacement of GTP by GDP induces tubulin to adopt a more ordered conformation, either increasing the amount of α-helix or β-sheet, or both. The near-UV spectra under the same experimental conditions displayed little difference between the tubulins in the two states of liganding, indicating the absence of significant differences in the environment of the aromatic chromophores.

The conformational difference between Tu–GDP and Tu–GTP was further probed in sedimentation velocity experiments, since the sedimentation coefficients of these two forms of tubulin could be different if the increase in order in the secondary structure led to differences in shape. The sedimentation coefficient of Tu–GDP was measured in PG buffer (10 mM NaPi, 1.0 mM GDP, pH 7.0). Analysis in terms of the standard equation (Schachman, 1959)

$$s = s^0(1 - gC_T) \quad (3)$$

gave a value of $s^0_{20,w}$ = 5.8 S, i.e., identical with that of Tu–GTP, assuming g = 0.018 mL/mg (Frigon & Timasheff, 1975a). Therefore, conformational differences between tubulin in the two states are not reflected in any gross differences in the hydrodynamic properties of these two forms of the protein.

Effect of GDP on the Mg^{2+}-Induced Self-Association of Tubulin. Among the causes of the lower effectiveness of Tu–GDP in promoting microtubule elongation the two most likely are (1) Tu–GDP has a weaker affinity than Tu–GTP for the microtubule end; i.e., it has a weaker tendency to self-associate; (2) the conformational difference between the two states of tubulin leads to a shift in the mutual geometric arrangement of the two self-assembly sites on the tubulin α–β dimer. This would permit tubulin to bind to the end of the growing microtubule with a high affinity but would preclude further elongation, in a manner similar to that proposed for the tubulin–colchicine complex (Andreu et al., 1983). To test these possibilities, the tendency of Tu–GDP to self-associate was examined in sedimentation velocity experiments. Figure 3A shows a comparison of the sedimentation boundaries of Tu–GDP and Tu–GTP obtained at identical Mg^{2+} and protein concentrations. In both cases, bimodal sedimentation boundaries were obtained. In the presence of GDP, the fraction of the protein under the rapidly sedimenting "peak" is strikingly greater than with Tu–GTP, indicating a considerably stronger degree of self-association. Furthermore, both patterns exhibit characteristics of a modified Gilbert system (Gilbert, 1955, 1959), i.e., of a polymerizing protein system in rapid dynamic equilibrium leading to a definite terminal polymer.

In Figure 3B are shown sedimentation patterns of Tu–GDP at several protein concentrations in the double ring forming buffer. All patterns are bimodal, with clear characteristics of a reaction boundary behaving as a Gilbert system. First, the two peaks in the boundary never fully separated as the pattern never reached the base line between them. Second, once bimodality had set in, all the new area was added to the rapid peak, the area under the slow peak remaining essentially constant. The slight increase in area under the slow peak as a function of protein concentration can be attributed to the small amount of tubulin that, as shown above, had not exchanged with free GDP. This residual Tu–GTP can be expected to behave as nonreacting protein, since the tendency of Tu–GTP to self-associate is weaker than that of Tu–GDP, as shown in Figure 3A. Its sedimentation at 5.8 S as α–β

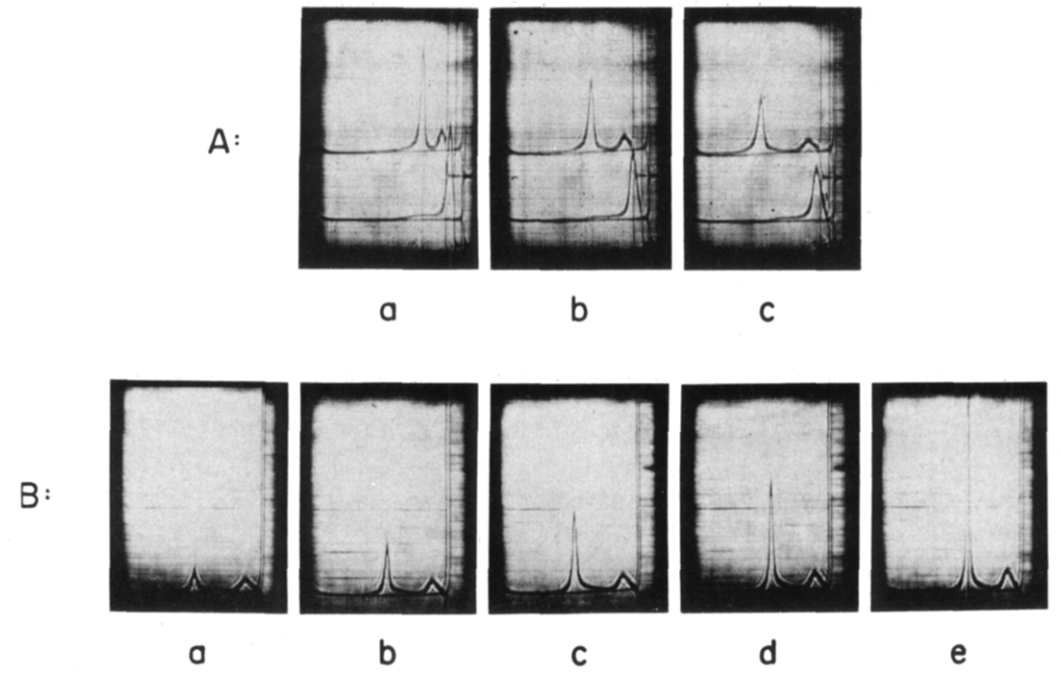

FIGURE 3: (A) Sedimentation velocity profiles of tubulin in PMG buffer (16 mM $MgCl_2$, pH 7.0) with 1.0 mM GDP (upper pattern) or GTP (lower pattern). The direction of sedimentation is from right to left. The times after reaching full speed are (a) 0 min, (b) 4 min, and (c) 8 min. The rotor speed was 48 000 rpm. The protein concentration was 5.0 mg/mL in both cells. (B) Sedimentation patterns of tubulin in PMG buffer (12 mM $MgCl_2$, 1.0 mM GDP) at pH 7 and 20 °C. The direction of sedimentation is from right to left; the rotor speed was 48 000 rpm. The protein concentrations were (a) 2.5 mg/mL; (b) 3.5 mg/mL; (c) 5.0 mg/mL; (d) 6.0 mg/mL; (e) 8.5 mg/mL. The photographs were taken 8 min after reaching full speed.

tubulin dimer should increase the area of the slow peak as a function of protein concentration, resulting in a situation similar to that treated theoretically for β-lactoglobulin (Timasheff & Townend, 1961). The primary diagnostic criterion of a Gilbert-type system is that, once bimodality appears, the area under the slow peak remains constant with increasing protein concentration, even though the total area of the boundary increases indefinitely. It is evident that this criterion has been met by Tu–GDP. The Gilbert-type sedimentation pattern means that the polymerization of this protein in the presence of Mg^{2+} ions involves a single highly favored terminal step, which effectively truncates the polymerization with the formation of a polymer of definite size, and that the stoichiometry of this polymer from protein monomers is greater than 2. Frigon and Timasheff (1975a,b) have shown rigorously that the magnesium-induced self-association of Tu-GTP satisfies these criteria. Furthermore, these investigators had found that, prior to the appearance of bimodality, the sedimentation rate of the slow peak did not remain constant at some value between that of monomer and polymer, as predicted by simple Gilbert theory (Gilbert, 1955) but rather that the $\bar{s}_w$ of the slow peak increased gradually with protein concentration until the appearance of bimodality and remained constant thereafter. This behavior led to the conclusion that polymerization of Tu–GTP proceeds first as an isodesmic linear polymerization that is truncated by a favorable intermolecular bond-forming step in which double rings are formed (Frigon & Timasheff, 1975a,b). This mechanism can be represented as

$$\begin{aligned} A_1 + A_1 &\rightleftharpoons A_2; \quad K_2 \\ A_2 + A_1 &\rightleftharpoons A_3; \quad K_3 \\ &\vdots \\ A_{i-1} + A_1 &\rightleftharpoons A_i; \quad K_i \\ &\vdots \\ A_{n-1} + A_1 &\rightleftharpoons A_n; \quad K_n \\ K_2 &= K_3 = K_i \ll K_n \end{aligned} \tag{4}$$

where K_j is the equilibrium constant for step j in the polymerization, expressed in M^{-1} units. The results obtained with Tu–GDP indicate that it behaves in the same way. Figure 4 shows the weight-average sedimentation coefficients obtained as a function of protein and Mg^{2+} concentrations. At low magnesium concentration (4 mM), where the boundary shape

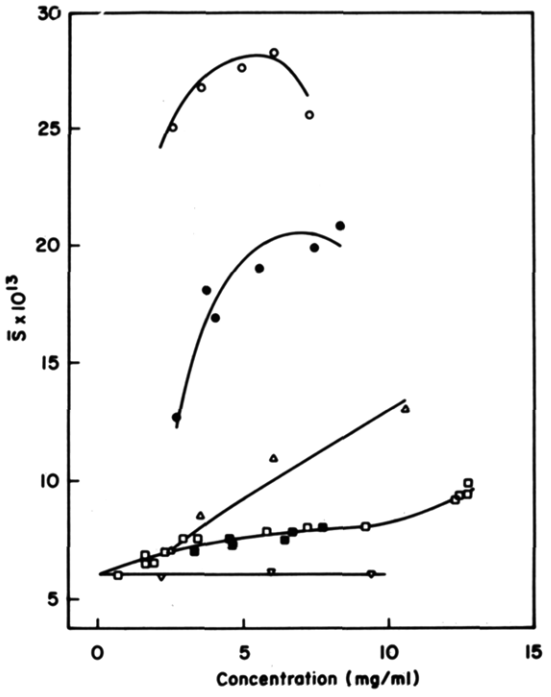

FIGURE 4: Protein concentration dependence of weight-average sedimentation coefficients determined experimentally at various $MgCl_2$ concentrations. All experiments were in PMG buffer (1.0 mM GDP) at pH 7 and 20 °C with the following magnesium ion concentrations: (▽), 2 mM; (□), 4 mM; (△), 6 mM; (●), 8 mM; (○), 12 mM. (□) denotes data from GTP in PMG under identical conditions, at 8 mM Mg^{2+}, taken from Frigon and Timasheff (1975a). The lines through the data represent the calculated best fits to the model described in the text.

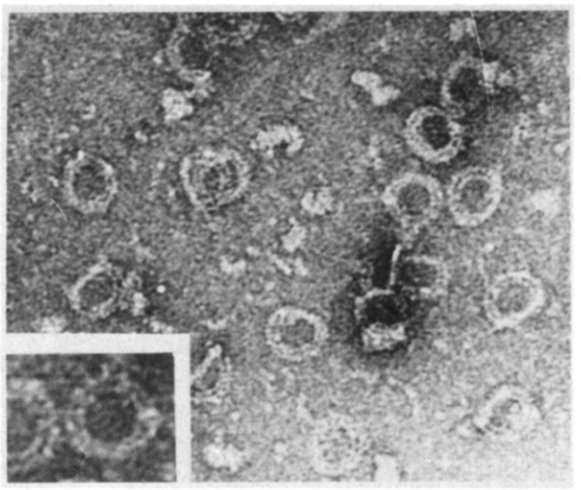

FIGURE 5: Electron micrograph of structures formed by tubulin (0.5 mg/mL) in pH 7.0 sodium phosphate (10 mM) buffer, containing 16 mM $MgCl_2$ and 1.0 mM GDP. Inset shows double ring structure at higher magnification.

is a single forward skewed peak, $\bar{s}_w$ increased as a function of protein concentration. This means that, like Tu–GTP, Tu–GDP is self-associating according to an indefinite linear polymerization model (Frigon & Timasheff, 1975a,b). In both cases, an increase in Mg^{2+} ion stimulates tubulin self-association. The onset of a Gilbert-type bimodality at higher Mg^{2+} concentrations shows the formation of an end polymer.

That this end product is the same for both Tu–GDP and Tu–GTP was confirmed with EM photographs, which showed the formation of double ring structures from Tu–GDP, shown in Figure 5, indistinguishable from those formed with Tu–GTP (Frigon & Timasheff, 1975a). The formation of the same end product suggested that the mechanism of double ring formation should be identical for the two nucleotide systems. The significantly decreased area under the slow peak for Tu–GDP relative to Tu–GTP (Figure 3A) under identical conditions indicated that Tu–GDP has a stronger propensity for forming double rings than Tu–GTP.

A quantitative comparison of the self-association properties of these two forms of tubulin was carried out in terms of the model developed by Frigon and Timasheff (1975a). With protein concentration expressed in mass units (mg/mL), the scheme of eq 4 was analyzed in terms of equilibrium constants K_2', K_i', and K_n' and the stoichiometry n, where K_2', is the equilibrium constant for the first step, formation of a dimer from two monomers, K_i is the equilibrium constant for i monomers forming an aggregate of size i, and

$$K_n' = n(K_2'/2)^{n-1}\gamma \tag{5}$$

where γ is the contribution of the favorable additional free energy of the formation of the end polymer, i.e., $-RT \ln \gamma$ (Gilbert, 1955, 1959). In the analysis of Frigon and Timasheff (1975b), γ was defined as

$$\Delta G_\gamma^\circ = \Delta G_2^\circ + \Delta G_{\text{cratic}} + RT \ln 4 + \Delta G_R \tag{6}$$

where ΔG_R represents the contribution of all other entropic effects. The strong similarity to the GTP system—namely, a measured $s_{20,w}$ value of 41 S for the terminal polymer and the appearance of the structures detected by electron microscopy, led to the assignment of a stoichiometry of ring formation of 26 tubulin monomers (n = 26). This model contains two basic assumptions: (i) that the free energies of bond formation between each successive aggregate and the monomeric unit (tubulin heterodimer) are identical, and (ii) that the final step in the self-association is favored.

Table I: Fitted Values of Equilibrium Constants for Self-Association of Tubulin as a Function of Mg^{2+} Ion Concentration

	$MgCl_2$[a] (mM)			
	4	6 (5)	8	12 (16)
K_2' (mL/mg)	0.213	0.241 (0.14)	0.263 (0.224)	0.321 (0.332)
K_n' $(mL/mg)^{-25}$		1.0×10^{-11}	1.0×10^{-5} (5.5×10^{-16})	990 (4.2×10^{-10})

[a] The numbers in parentheses represent data taken from Frigon and Timasheff (1975a) for the GTP system. All other values are for the GDP system.

In the analysis of the sedimentation of a polydisperse reacting system in dynamic equilibrium, the rate of movement of the boundary is rigorously described by the velocity of the square root of the second moment of this boundary, i.e., the weight-average sedimentation coefficient, $\bar{s}_w$ (Schachman, 1959)

$$\bar{s}_w = \sum_i s_i C_i / \sum_i C_i \tag{7}$$

where s_i is the sedimentation coefficient of the ith aggregate and C_i is its concentration given by the term $K_i' C_1^i$. The total weight concentration is

$$C = \sum_i C_i = \sum_i K_i' C_1^i \tag{8}$$

With the equilibria expressed as above, the experimentally measured $\bar{s}_w$ becomes

$$\bar{s}_w = \sum_i s_i (1 - g_i C) K_i' C_1^i / (\sum_i K_i' C_1^i) \tag{9}$$

where g_i expresses the hydrodynamic nonideality affecting the observed rate of sedimentation. The sedimentation coefficients of all aggregates except double rings were calculated by assuming spherical symmetry (Cann, 1970; Nichol et al., 1964)

$$s_i = s_1 i^{2/3} \tag{10}$$

While the assumption of spherical symmetry is not strictly true, a more detailed analysis would only serve to complicate the process without adding any additional insight. The values of g_i for all polymers, other than double rings, were assumed to be identical with g_1 for monomer (0.018 mg/mL). The values of g_n and s_n for double rings were determined by measuring the protein concentration dependence of the rate of transport of the rapid peak, according to eq 3. Therefore, analysis of the experimentally determined $\bar{s}_w$ in terms of the above assumptions reduces itself to the problem of determining two unknowns, namely, K_n and K_2, the monomer concentration, C_1, being defined by the positive real root of eq 9. The experimentally obtained values for the GDP system of g_n = 0.028 and s_n = 41 S for 8 mM $MgCl_2$ were used in the reduction of the data.

The nonlinear regression routine developed by Marquardt (1963) was used to determine the unknown parameters from the data of Figure 4. Once a stable solution was found, the initial guesses were varied to ensure that the solution did not represent a local minimum. The results of this analysis are summarized in Table I, where they are compared with values obtained previously for Tu–GTP. Clearly, both equilibrium constants are increased for tubulin–GDP for all magnesium concentrations examined. The dramatic effect for Tu–GDP, however, residues in the term γ, which is 8 orders of magnitude higher (Mg^{2+} = 8 mM) than γ determined for GTP. The calculated thermodynamic parameters for the Mg^{2+}-induced tubulin self-association at 20 °C are presented in Table II,

Table II: Free Energy of Tubulin Self-Association into Double Rings at 20 °C

$C_{Mg^{2+}}$ (M)	nucleotide[a]	K_2^{fitted} (M^{-1})	ΔG_2 (kcal/mol)	K_p^{fitted} (M^{-25})	$\Delta G_p°$ (kcal/mol)	γ (M^{-1})	$\Delta G_\gamma°$ (kcal/mol)
0.004	GDP	1.17×10^4	−5.47				
0.005	GTP	0.75×10^4	−5.2				
0.006	GDP	1.33×10^4	−5.5	4.2×10^{113}	−153	3.6×10^{10}	−15.5
0.008	GDP	1.45×10^4	−5.6	4.2×10^{119}	−161	4.1×10^{15}	−20
0.008	GTP	1.23×10^4	−5.5	2.27×10^{109}	−147	1.3×10^7	−9.5
0.012	GDP	1.77×10^4	−5.7	4.12×10^{127}	−172	2.8×10^{21}	−29.5
0.016	GTP	1.83×10^4	−5.7	1.76×10^{115}	−155	4.8×10^8	−11.6

[a] Data for GTP taken from Frigon and Timasheff (1975a,b).

where they are compared with the values obtained in the GTP system. In these calculations, the equilibrium constants expressed in mass units, K_i', were converted to molar units, K_i, as follows:

$$K_i = K_i' M_1^{i-1} / i \quad (11)$$

Then $\Delta G_p°$, the standard free energy change for ring formation from tubulin monomers, is given by

$$\Delta G_p° = -RT \ln (K_2^{n-1} \gamma) \quad (12)$$

Both $\Delta G_2°$ and $\Delta G_p°$ are consistently more negative for the GDP system. The most striking difference, however, lies in the term $\Delta G_\gamma°$, which was calculated by setting $n = 26$ and introducing the values of $\Delta G_2°$ and $\Delta G_p°$ from Table II into eq 9. This term is equal to

$$\Delta G_\gamma° = \Delta G_b° + \Delta G_R'° + \Delta G_{cratic} + RT \ln 4 \quad (13)$$

where $\Delta G_b°$ is the standard free energy change of forming the bond and $\Delta G_R'°$ is the additional standard free energy change associated with the overall effect of ring closure on the polymer. Since, once the final closed structure is formed, the intersubunit bonds become indistinguishable, the intrinsic standard free energy of formation of the last bond can be regarded as identical with those of the other 25 intersubunit bonds (Frigon & Timasheff, 1975b). Thus, the difference between the $\Delta G_2°$ values of Tu–GDP and Tu–GTP does not account for the observed differences in $\Delta G_\gamma°$, and the primary source of this difference must reside in the term $\Delta G_R°$, leading to the hypothesis that Tu–GDP is in a more favorable conformation for ring closure than is Tu–GTP.

The results presented in this paper are summarized in Figure 6, where the equilibrium distribution of protein species are shown as a function of total protein concentration at identical magnesium concentration (8 mM). It is quite clear that the GDP system already forms double rings at protein concentrations that are commonly used in the laboratory for microtubule assembly. To the contrary, the GTP system has a much lower tendency to form double rings, and significant ring formation occurs only at protein concentrations well above those used for microtubule assembly. This result indicates that, were double rings important in microtubule assembly, GDP, rather than GTP, should have stimulated microtubule formation, since its presence should direct assembly toward double rings. Its impediment of microtubule formation can then be taken as evidence that double rings are not an intermediate on the microtubule assembly pathway but, in fact, are the products of a parallel competing polymerization reaction.

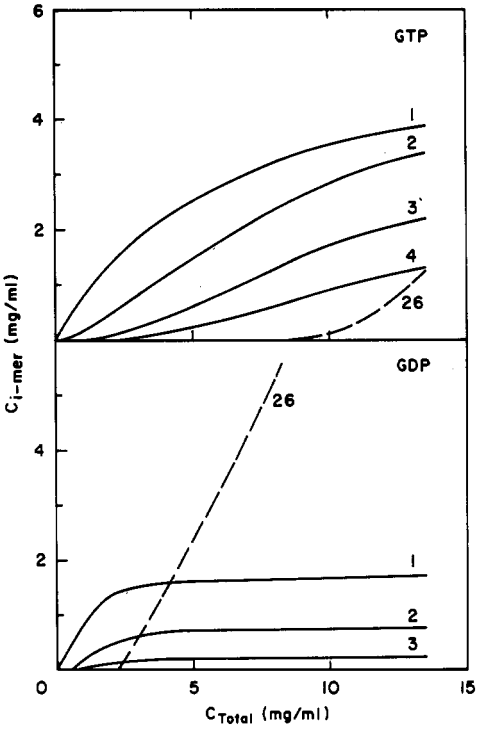

FIGURE 6: Dependence of the concentration of individual associated protein species on total protein concentration in the self-association of tubulin in the presence of 8 mM $MgCl_2$. The lines for GDP (1.0 mM) were calculated by using the parameters in Table I. For comparison, the lines for GTP were taken from Frigon and Timasheff (1975a). The numbers refer to the size of the aggregates in units of α–β tubulin monomers.

Discussion

The lack of agreement that exists currently on the exact role of nucleotides in the microtubule assembly process (see Introduction) may be due in great part to the diversity of tubulin purification procedures and compositions of buffers utilized in the various studies. The differences, and even apparent contradictions, may then well reflect differences in degree rather than all or none specific effects. The results reported in the present study can serve as a good illustration of this point; namely, the formation of double rings (hereafter referred to as rings) in the presence of Mg^{2+} ions can take place whether GTP or GDP is bound at the E site of tubulin. This self-association, however, is much stronger in the presence of GDP and could easily be undetected when working with Tu–GTP at the usual concentrations. As will be shown below, the strong enhancement of double ring formation in the presence of GDP has important implications in defining the relative roles of GTP, GDP, and rings in the microtubule assembly–disassembly cycle.

First, let us address the question of the exchangeability of guanosine nucleotides with tubulin. Croom et al. (1985), using phosphocellulose purified tubulin, have shown clearly that only a fraction of tubulin dimers are readily exchangeable with nucleotide (~80%), the other 20% exchanging very slowly. These results are in very good agreement with those presented here, namely, that only 83% of the theoretical number of exchangeable sites were exchanged with GDP. To account for the lack of full exchangeability, Croom et al. (1985) postulated that tubulin is present in solution in the form of two conformers: Tb, which exchanges readily, and Tb', which

does not, the equilibrium between the two forms being slow. This model accounts for reports in the literature that only 60–80% of all the E sites are readily exchangeable (for a review, see: Correia & Williams, 1983). In the present study it has been found that the gel filtration step utilized to separate free from bound nucleotide results in the dissociation of some of the bound nucleotide. With the assumption that the dissociation rate constant for GDP is similar to that for GTP, this observation is consistent with the finding of Brylawski and Caplow (1983) that the half-life for GTP dissociation is on the order of 5 min. Thus, in such a gel filtration experiment, some dissociated nucleotide around the trailing edge of the protein peak should separate from the protein. The correlation between fraction number and fraction of E sites exchanged supports this conclusion. Even though these considerations would increase the value of the extent of exchange on the E sites, it is probably safe to conclude that, in our system as well, a small fraction of tubulin (Tb′) does not exchange readily with free nucleotide. This does not detract significantly from the results described in this paper, since at least 80% of the tubulin exists in the Tu–GDP complex. The only consequence is that the values of the polymerization equilibrium constants of Tu–GDP reported here are somewhat underestimated.

The results presented in this paper show that GDP is considerably more effective than GTP in promoting double ring formation from purified tubulin. In order to understand the possible mechanisms of this action, the effect of nucleotides on the equilibrium constants of ring formation must be examined. It has been found that the measured apparent dimerization constant, K_2^{GDP}, is consistently greater than K_2^{GTP}. This need not mean a priori that, in fact, dimerization of tubulin in the GDP state is stronger than that in the GTP state. The reason for this resides in the nature of the data analysis in which only aggregates with one to six tubulin dimers were considered significant in calculating theoretical $\bar{s}_w$ values in the model of ring formation (eq 4). Therefore, the calculated $\bar{s}_w$ should be much less sensitive to changes in K_2 than to those in K_n, due to the r^2 weighting in determining weight-average sedimentation coefficients. Furthermore, it is possible that the small oligomers derived from the initial steps of the self-association of Tu–GDP may have more compact shapes than those derived from Tu–GTP. This would result in the experimentally observed $\bar{s}_w$ values for the same size distribution of aggregates being higher for Tu–GDP. The constraint of spherical symmetry put on these oligomers to simplify the analysis would, then, necessarily lead to higher apparent values of K_2 to account for the higher values of $\bar{s}_w$. As a result, a much more reliable parameter for comparison of ring formation from Tu–GDP and Tu–GTP at higher Mg^{2+} concentrations (>4 mM) is K_n, the overall ring formation constant.

The observation of the effect of GDP on ring formation, when taken together with the observed conformational difference between Tu–GTP and Tu–GDP, suggests a possible simple model for the various equilibria involved in tubulin self-assembly to rings. In this model, tubulin exists in a state of equilibrium between two conformations, T and T*, only one of which is capable of undergoing self-association into rings. Let form T* be the ring-forming conformer. This leads to the reaction scheme

$$\begin{aligned} T &\rightleftharpoons T^*; \quad K_c \\ T^* + T^* &\rightleftharpoons T_2^*; \quad K_2 \\ &\vdots \\ T_{25}^* + T^* &\rightleftharpoons T_{26}; \quad K_{26} \end{aligned} \tag{14}$$

where K_c is the equilibrium constant for the transconformation reaction. Because GDP enhances this polymerization, T* would correspond to the "GDP form", and T to the "GTP form". This does not mean, however, that tubulin must be liganded to GDP to form rings, since in that case rings could not have been observed previously (Frigon & Timasheff, 1975a,b). By the Wyman (1964) linkage theory, in this model GDP must promote the conformational equilibrium to T*. That equilibrium, however, exists also in Tu–GTP but to a weaker extent. The observed apparent equilibrium constant, $K_{2,app}$, which distinguishes only between monomers and aggregates, without being cognizant of conformational difference, is then given by

$$K_{2,app} = [T_2^*]/([T^*] + [T])^2 = \frac{K_2}{[1 + (1/K_c)]^2} \tag{15}$$

The more general model in which both forms of tubulin are capable of self-association but with only T* forming rings gives

$$T^* + T^* \rightleftharpoons T_2^*; \quad K_2 \tag{16}$$

and

$$T + T \rightleftharpoons T_2; \quad K_2^* \tag{16a}$$

Then

$$K_{2,app} = ([T_2] + [T_2^*])/([T] + [T^*])^2 = \frac{K_2^* + K_2K_c^2}{(1 + K_c)^2} \tag{15a}$$

Since this mode of self-association leads to an intractable solution for determining K_c, a few simplifying assumptions were introduced. First, as in the model of eq 14, let us assume that only T* can form aggregates; second, let us assume that, in the GDP state, all of the tubulin is in the T* form. Then, by eq 5 and 6, we have

$$\Delta G_p'^{GDP-GTP} = \Delta G_p^{GDP} - \Delta G_p^{GTP} = -RT[\ln (K_2^n\gamma') - \ln (K_{2,app,T}^n\gamma')] \tag{17}$$

where $\Delta G_p'^{GDP-GTP}$ is the difference between the free energies of intertubulin bond formation (26 bonds) in the formation of double rings from 26 Tu–GDP and 26 Tu–GTP α–β heterodimers. $K_{2,app,T}$ is the apparent growth constant of Tu–GTP and $\gamma' = (\gamma - \Delta G_2^\circ)$. $\Delta G_2^\circ = -RT \ln K_2$, in fact, is the free energy change of bond formation in the assembly of the final structure, equally apportioned between all bonds.

Since γ' is identical for the polymerizations of Tu–GTP and Tu–GDP into double rings, consisting only of statistical and entropic terms (e.g., changes of entropy of interaction with the solvent on polymerization), we have by eq 14 and 17

$$\frac{\Delta G_p'^{GDP-GTP}}{n} = -RT \ln \left(1 + \frac{1}{K_c}\right)^2 \tag{18}$$

where K_c is explicitly defined as the equilibrium constant for the isomerization of Tu–GTP from the T state to the T* state. Taking the values of ΔG_p° in 8 mM $MgCl_2$ for Tu–GDP and Tu–GTP (Table II), we have $\Delta G_p'^{GDP-GTP} = -14000$ cal/mol, which, by eq 18, gives $K_c = 1.70$. The definition of $K_c = [T^*]/[T]$, set by eq 14, leads to the result that, in the GTP state, 63% of the tubulin is in the T* form. If it is assumed that the interconversion between the T and T* states is kinetically slow, this number takes on significance relative to the fraction of tubulin monomers that are exchangeable with free nucleotide (Croom et al., 1985), namely, that only 60–80% of tubulin in the GTP state has been found to exchange with free GTP. In this model, the T* state, defined as that which can associate to double rings, would be equated to the Tb′ state

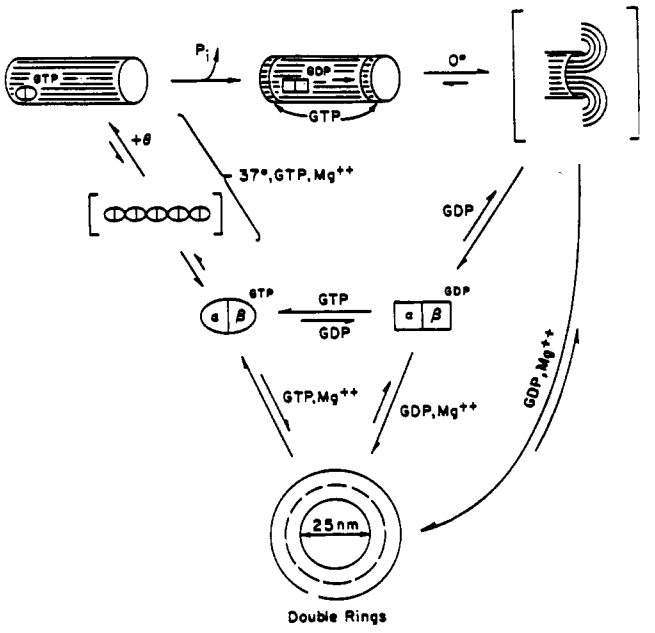

FIGURE 7: Working model for the microtubule assembly–disassembly cycle of purified tubulin.

proposed by Croom et al. (1985) as that which can exchange readily its E-site nucleotide. The hypothesis that the equilibrium between the two conformational states is slow is consistent with the known slowness of the binding process of nucleotides to tubulin (Brylawski & Caplow, 1983; O. Monasterio and S. N. Timasheff, unpublished results; G.-M. Wu and S. N. Timasheff, unpublished results). Furthermore, rates of interconversion between conformational states of proteins are known to be frequently very slow. For example, the interconversion between the α- and γ-states of chymotrypsin takes several weeks, even though a single surface residue (Tyr-146) is involved (Freer et al., 1970).

Comparison of the values of $\Delta G_\gamma°$ for Tu–GDP and Tu–GTP, given in Table II, reveals that this parameter is twice as great for the GDP state of tubulin as for the GTP state. This suggests that the conformation of the Tu–GDP is in a more favorable geometry for ring formation. If the ring forms by the head-to-tail polymerization of tubulin monomers, the curvature required would be on the order of 26° (assuming 14 subunits in the ring). This value is not an unreasonable one, considering the observed plasticity of tubulin in forming a variety of polymorphic structures. In fact, it is the same curvature as in microtubule formation, although the axes of polymerization are perpendicular to each other in the two structures. This suggests that the observed conformational changes may be the result of such an alteration of the geometry of the α–β tubulin dimeric molecule upon binding GDP.

The strong enhancement of tubulin ring formation by GDP may have significant implications for the role of rings and Tu–GDP in microtubule assembly. A number of studies have been published in which rings have been either implicated or discounted in microtubule assembly (Timasheff & Grisham, 1980). For example, Jacobs et al. (1975) have suggested that rings might serve as templates for microtubule nucleation. If this were the case, the number concentration of microtubules should be of the same order as that of rings. Purich and Kristofferson (1984) have estimated that the number concentration of microtubules is 100 times smaller than the initial concentration of rings, rendering this model not very likely. Other investigators have addressed the possibility that rings open and align forming sheets of protofilaments that eventually close into microtubules (Erickson, 1974; Kirschner et al., 1974). Karr and Purich (1980) and Engelborghs et al. (1980), however, have proposed that rings break down to small oligomers prior to assembly into microtubules. Lee and Timasheff (1977) have suggested that rings are a mode of polymerization independent of microtubule formation. Finally, Mandelkow et al. (1980) in an elegant small-angle X-ray scattering study have clearly demonstrated that microtubule assembly is preceded by the breakdown of rings.

The concept of rings supporting directly microtubule initiation is inconsistent with the results described in this paper: rings are favored by the GDP form of tubulin and GDP is ineffective in promoting microtubule initiation. On the other hand, the propensity of Tu–GDP to form rings suggests an explanation for structures seen in electron microscope photographs of depolymerizing microtubules in which the ends of the microtubules have the appearance of curled protofilaments that resemble rings (Erickson, 1974; Kirschner et al., 1975; Mandelkow & Mandelkow, 1985). Tubulin in depolymerizing microtubules should be predominantly in the GDP state (Carlier, 1982; Pantaloni & Carlier, 1986). Furthermore, Kirschner et al. (1974) have shown that the stable products of the cold depolymerization of MAP-containing microtubules are double rings and spirals of similar curvature. These observations, taken with our finding of double ring stabilization by GDP, suggest that double rings composed of Tu–GDP can be direct depolymerization products of microtubules. This hypothesis would be consistent with the reported lower dissociation constant of Tu–GDP from microtubules (Carlier & Pantaloni, 1981), since the monomeric protein would be stabilized in the ringlike structures formed in the ends of depolymerizing microtubules.

These considerations lead to a working scheme of the cycle of microtubule assembly–disassembly, which is shown in Figure 7. In this, α–β tubulin in the GTP state could assemble into microtubules through a series of filamentous intermediates (Erickson & Pantaloni, 1981; Bordas et al., 1983). Following assembly, GTP is hydrolyzed to GDP + P_i, tubulin undergoing a conformational change and the steady-state microtubule being stabilized by two Tu–GTP caps (Carlier, 1982; Pantaloni & Carlier, 1986). Depolymerization, e.g., by cold, would be accompanied by either the curling up of Tu–GDP protofilaments with the formation of double rings or the depolymerization into Tu–GDP α–β dimers. Exchange of GDP to GTP would lead to depolymerization of the double rings and completion of the cycle by both pathways. Depending on the levels of GTP, GDP, and Mg^{2+} ions in the environment, the tubulin disassembled from microtubules could exist in the state of either α–β dimers or of double rings stabilized by GDP and Mg^{2+}. This last possibility would be consistent with the proposal of Weisenberg et al. (1976) that the rings are a storage form of tubulin.

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