Linkage between Ligand Binding and Control of Tubulin Conformation†,‡

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ABSTRACT: The effect of both antimitotic drugs and nucleotide analogues on the magnesium-induced self-association of purified tubulin into 42S double rings has been examined by sedimentation velocity. In the absence of magnesium, all complexes sedimented as the 5.8S species. The binding of colchicine to tubulin led to a small but consistent (–0.1 to –0.2 kcal/mol) enhancement in the self-association of tubulin α–β dimers. In the absence of nucleotide at the exchangeable site, tubulin retained a weak ability (K_2 = 7.5 × 10^3 M^{-1}) to self-associate, which was unchanged by the addition of guanosine or GMP. Analogues with altered P–O–P bonds (GMPPCP, GMPPNP) did not support ring formation at the protein concentrations examined, although GMPPCP supported microtubule assembly. When the exchangeable site was occupied by nucleotides altered on the γ-phosphate (GTPγS, GTPγF), rings were formed; tubulin–GTPγF formed rings to an extent slightly greater than did tubulin–GTP, and tubulin–GTPγS to about the same extent as tubulin–GDP. Both of these analogues are inhibitors of microtubule assembly. These results are consistent with a model [Melki, R., Carlier, M.-F., Pantaloni, D., & Timasheff, S. N. (1989) *Biochemistry 28,* 9143–9152] in which an equilibrium exists between straight (microtubule-forming) and curved (ring-forming) conformations of tubulin. Furthermore, the present results indicate that the "switch" which controls the nature of the the final polymeric product via free energy linkages is the occupancy of the γ-phosphate binding locus of the exchangeable site by a properly coordinated metal–nucleotide complex.

Tubulin is able to assemble into a remarkably diverse array of structures, including microtubules (Weisenberg et al., 1968), double rings (Frigon & Timasheff, 1975a,b), sheets, ribbons, and filaments (Timasheff & Grisham, 1980). Microtubules and rings are formed under similar solution conditions and in fact have been observed simultaneously (Lee & Timasheff, 1974) in tubulin reconstitution experiments. While rings were once proposed to be involved in the assembly process, statistical thermodynamic analysis has shown that this is not the case (Erickson & Pantaloni, 1981). However, electron micrographs of depolymerizing microtubule-associated protein (MAP)[1]-containing microtubules (Erickson, 1974; Kirschner et al., 1974) suggested that ring formation may be part of the disassembly process. Subsequent kinetic measurements using labeled tubulin (Melki et al., 1989) have shown that rings are indeed formed during the depolymerization of microtubules.

Tubulin consists of two nonidentical subunits, each of which can bind 1 equiv of GTP. One binding site, believed to be on the β-subunit (Geahlen & Haley, 1977), can freely exchange with exogenous nucleotide, while the other (on the α-subunit) is unable to exchange (Weisenberg et al., 1968). The exchangeable site (E-site) GTP can be replaced by GDP and a variety of nucleotide analogues [e.g., see Kirsch and Yarbrough (1981), Monasterio and Timasheff (1987), and Seckler et al. (1990)]. The nature of the nucleotide occupying the exchangeable nucleotide binding site is intimately involved in determining the structure of the final polymer formed. Assembly into microtubules requires either GTP, a triphosphate analogue, the GDP·P_i complex, or a structural analogue (e.g., GDP·BeF_3) (Melki et al., 1990). Examination by sedimentation velocity of tubulin double-ring formation, however, has shown that, in this process, the strength of the polymerization is greater when the E-site is occupied by GDP than GTP (Frigon & Timasheff, 1975a; Howard & Timasheff, 1986). These results have given rise to a model in which an equilibrium exists between two conformational states of tubulin: a microtubule-forming "straight" form and a double-ring-forming "curved" form,[2] the equilibrium between these two states being thermodynamically linked (Wyman, 1964; Wyman & Gill, 1990) to the occupancy of the exchangeable nucleotide binding site, i.e., allosterically controlled by it (Melki et al., 1989).

Nucleotides are not the only ligands capable of inducing a conformational change in the tubulin molecule. The antimitotic drug colchicine binds with high affinity to tubulin. Several properties of the protein–colchicine complex, including quenched protein fluorescence, perturbed circular dichroism spectrum, induction of a GTPase activity, and altered geometry of polymerization products, are indicative of a conformational change upon ligand binding (Andreu & Timasheff, 1982). Similar effects have been observed with structural analogues of colchicine (Medrano et al., 1991; Andreu et al., 1991).

It is well established that the binding of the antimitotic drug colchicine to tubulin results in an assembled structure morphologically different from that obtained with pure tubulin (Saltarelli & Pantaloni, 1982; Andreu et al., 1983). Protofilaments, amorphous aggregates, and spirals are formed, rather than microtubules. The thermodynamics of the two processes, however, are very similar (Andreu & Timasheff, 1982). The differences between the resulting structures are

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‡ This paper is dedicated to Professor Jeffries Wyman on the occasion of his 90th birthday.

[1] Abbreviations: GMP, guanosine 5′-monophosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GMPPCP, guanosine 5′-(β,γ-methylenetriphosphate); GMPPNP, guanosine 5′-(β,γ-imidotriphosphate); GTPγF, guanosine 5′-*O*-(3-fluorotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HPLC, high-performance liquid chromatography; MAP, microtubule-associated protein.

[2] The terms "straight" and "curved" refer to the mutual orientation of consecutive longitudinal bonds between tubulins inside a protofilament assembled into microtubules (180°) and double rings (158°). No inference is made on particular bends within the tubulin molecule.

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thought to reflect a dislocation of the inter-tubulin bonds in the tubulin–colchicine polymer (Andreu et al., 1983) arising from a weakening in the lateral interactions (Saltarelli & Pantaloni, 1982). In addition, a conformational change in the tubulin molecule is known to occur upon binding of the tropolone portion of the colchicine molecule (Garland, 1978; Andreu & Timasheff, 1982), with a resulting stabilization of the α–β dimer (Detrich et al., 1982).

A study has been carried out, therefore, in an effort to gain further insight into the relationship between ligand binding and the control of tubulin conformation. The effects of colchicine-related antimitotic drugs and various nucleotide analogues on the self-assembly of tubulin into rings and microtubules were investigated, and the results are discussed in terms of a model an essential feature of which is the proper coordination of a nucleotide–metal complex with the exchangeable site.

MATERIALS AND METHODS

Fresh calf brains were obtained at a local slaughterhouse, transported on ice, and used within 1 h of slaughter. GTP, GDP, GMP, and guanosine were purchased from Sigma Chemical Co. Nucleotide analogues (GTPγS, GMPPCP, GMPPNP) and calf intestinal alkaline phosphatase (enzyme immunoassay grade) were obtained from Boehringer. Ultrapure sucrose and ammonium sulfate were from ICN/Schwarz Mann, while guanidine hydrochloride was from Heico. Colchicine was supplied by Aldrich Chemical Co. All other chemicals were of reagent grade or better.

Tubulin. Calf brain tubulin was prepared by the method of Weisenberg (1968), as modified by Na and Timasheff (1980). Tubulin was stored in liquid nitrogen in a buffer consisting of 0.01 M sodium phosphate, 0.5 mM $MgCl_2$, 0.1 mM GTP, and 1 M sucrose, pH 7.0. Each preparation was routinely checked both for its ability to assemble and by its behavior in the ultracentrifuge. Aliquots of protein were prepared for use by centrifuging over a small column (1 × 7 cm) of Sephadex G-25 from which excess buffer had been removed by gentle centrifugation. Samples were then subjected to high-speed centrifugation (35000*g*, 20 min) in order to remove any large aggregates, followed by passage over a larger (0.9 × 12 cm) column of Sephadex G-25 equilibrated with the buffer of interest (Na & Timasheff, 1982). The tubulin–colchicine complex was prepared by incubation of tubulin with an excess of colchicine for 10 min at 20 °C (Andreu & Timasheff, 1982). Unbound colchicine was removed by passage over a column of Sephadex G-25.

Protein concentrations were determined by dilution of the protein into 6 M guanidine hydrochloride and measurement of the absorbance at 275 nm. Extinction coefficients used were 1.03 $mL \cdot mg^{-1} \cdot cm^{-1}$ for tubulin and 1.16 $mL \cdot mg^{-1} \cdot cm^{-1}$ for the tubulin–colchicine complex (Andreu & Timasheff, 1982). Alternatively, the protein assay of Bradford (1976) was employed, using purified tubulin as a standard. For sedimentation experiments involving empty tubulin, protein concentration was determined via a calibration plot relating the area under the schlieren peak to protein concentration, obtained at a constant phase plate angle and using GTP–tubulin or GDP–tubulin.

Nucleotides, Drugs. The concentrations of nucleotide solutions were measured by ultraviolet absorption, using an extinction coefficient at 256 nm of 13 700 $M^{-1} \cdot cm^{-1}$ (Dawson et al., 1969). Both GTPγS and GMPPNP were found to contain up to 20% of breakdown products (mainly GDP) and so were repurified by DEAE-Sephadex A25 chromatography before use. The nucleotide solutions were applied to an 0.9 × 30 cm column of DEAE-Sephadex A25 and eluted with a 0.01–1 M gradient of triethylammonium bicarbonate, pH 8.0. GTPγF was synthesized by the procedure of Monasterio and Timasheff (1987). Colchicine analogues were prepared as described previously (Medrano et al., 1991).

Nucleotides were extracted from tubulin samples, standards, and buffers by the procedure of Seckler et al. (1990), and separated by isocratic reversed-phase ion-pair HPLC (Perrone & Brown, 1984) on a column (250 × 4.3 mm) of octadecylsilica (Supelco). The mobile phase consisted of 0.2 M K_2HPO_4, 0.1 M acetic acid, and 4 mM tetrabutylammonium phosphate. Twenty-microliter samples were injected, the column was run at 1 mL/min, and nucleotides were detected by the absorbance at 254 nm.

Nucleotide Replacement. Tubulin, with GDP occupying the exchangeable site, was prepared by the method of Howard and Timasheff (1986). Briefly, tubulin was thawed and the majority of the sucrose and free nucleotide removed by centrifuging over a drained column of Sephadex G-25. GDP was added to a final concentration of 10–20 mM and the protein incubated at 20 °C for 30 min, followed by passage over a second drained column and a wet column of G-25, both equilibrated with the required buffer. Complete removal of E-site nucleotide was accomplished by treatment of tubulin–GDP with alkaline phosphatase (Purich & MacNeal, 1978; Seckler et al., 1990). However, because some of the nucleotides to be studied were also subject to hydrolysis by this enzyme, soluble alkaline phosphatase could not be used. In order to overcome this difficulty, immobilized alkaline phosphatase was employed. This procedure has been used previously to remove nucleotide from tubulin (Purich & MacNeal, 1978; Jemiolo & Grisham, 1982), as well as from other nucleotide binding proteins [e.g., see John et al. (1990)]. Complete and efficient removal of nucleotide is best performed on tubulin–GDP in the presence of high (millimolar) concentrations of the nonhydrolyzable analogue GMPPCP, which is then removed, along with the hydrolysis products, by gel filtration (Seckler et al., 1990).

The enzyme was covalently coupled to glutaraldehyde-activated Ultrogel Ac-A22 (Weston & Avrameas, 1971) and the gel thoroughly washed to remove unbound protein. Tubulin–GDP (with or without colchicine) was prepared in 20 mM imidazole, pH 7.0 (phosphate being an inhibitor of alkaline phosphatase), freed of excess GDP and spun (35000*g*, 30 min) to remove aggregates. The nonhydrolyzable analogue GMPPCP was added (1 mM) and the solution applied to the alkaline phosphatase column (1-mL volume). The column outlet was blocked and the sample allowed to remain in place for 1 h at room temperature. Following this incubation, the tubulin was eluted and equilibrated with experimental buffer as described earlier. It should be noted that GMPPCP binds sufficiently weakly to allow complete removal during the final chromatography step. Samples were supplemented with the appropriate nucleotide and used within 3 h. Tubulin prepared in this manner (empty tubulin) was routinely examined by HPLC to ensure complete removal of exchangeable nucleotide. Control experiments performed on E-site-depleted tubulin prepared in this manner showed that, upon readdition of GTP or GDP, the protein exhibited the same weight average sedimentation coefficient (at 12 mM $MgCl_2$) as untreated tubulin–GTP or tubulin–GDP.

Assembly. Assembly of tubulin was followed turbidimetrically by monitoring the increase in absorbance at 350 nm. Empty tubulin, equilibrated with nucleotide-free assembly

buffer (0.01 M sodium phosphate, 12 mM $MgCl_2$, and 3.4 M glycerol, pH 7.0), was supplemented with the appropriate nucleotide and incubated on ice for 20 min prior to assembly. The protein was then placed in a thermostated cuvette maintained at 5 °C, and assembly was initiated by rapidly switching the water supply to a second water bath maintained at 37 °C.

Ultracentrifugation. Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and RTIC temperature control. Samples were run in an An-D rotor in 12-mm double-sector cells with filled Epon centerpieces and quartz windows. Experiments were performed at 20 °C, employing a phase plate angle of 65° and a speed of 48 000 rpm, the precise speed for each run being determined by recording the number of revolutions in a given time interval. Sedimentation profiles were recorded on Kodak OMC diagnostic film. Measurements of schlieren patterns were carried out on a Nikon Model 6C microcomparator equipped with a Mitutoyo digital micrometer to record radial values, and interfaced to a modified Kaypro computer for calculation of weight-average sedimentation coefficients. These values were calculated from the rate of movement of the second moment of the boundary (Goldberg, 1953) and corrected to water at 20 °C, using a value of 0.736 for the partial specific volume (Lee & Timasheff, 1974). The concentration dependence of the sedimentation coefficients was considered in terms of the standard equation:

$$s = s^0(1 - gc_T) \tag{1}$$

where g is the hydrodynamic coefficient, c_T is the total concentration of protein (milligrams per milliliter), and s^0 is the sedimentation coefficient at zero protein concentration.

Binding Studies. The binding of nucleotide analogues to empty tubulin was measured using the Amicon MPS1 micropartition system (Seckler et al., 1990; Mejillano et al., 1990). For GMPPNP binding, nucleotide-depleted tubulin (15 μM) in 0.01 M sodium phosphate, 1 mM $MgCl_2$, and 3.4 M glycerol, pH 7.0, and 0–100 μM total nucleotide were incubated for 20 min at 20 °C, followed by centrifugation (3000g, 10 min) of the MPS1 units. Aliquots of retentate (total nucleotide) and filtrate (free nucleotide) were extracted, and the nucleotide concentrations were determined by HPLC. GTPγS binding was determined using [^{35}S]GTPγS (New England Nuclear). Empty tubulin (14 μM) in 10 mM sodium phosphate/1 mM $MgCl_2$, pH 7.0, was incubated with 0–125 μM [^{35}S]GTPγS ($\sim 10^{13}$ cpm/μmol). Total and free nucleotides were separated by centrifugation, and the free concentration of ligand was determined by liquid scintillation counting.

Data Analysis. Sedimentation velocity results were analyzed in terms of the model outlined by Frigon and Timasheff (1975a). The mechanism can be stated in terms of two phases: first, a linear polymerization (growth), followed by a final ring-closing step:

$$\begin{array}{lll} A_1 + A_1 \rightleftharpoons A_2 & & K_2' = m_2/m_1^2 \\ A_2 + A_1 \rightleftharpoons A_3 & & K_3' = m_3/m_2m_1 \\ \vdots \quad \vdots \quad \vdots & & \\ A_{n-1} + A_1 \rightleftharpoons A_n & & K_n' = m_n/m_{n-1}m_1 \\ K_2' = K_3' = K_i' & \ll & K_n' \end{array} \tag{2}$$

where K_i' are the equilibrium constants for each successive bond formation between monomer and $(i-1)$-mer expressed on a molar scale. On a milligram per milliliter concentration scale

$$K_i = i(K_2'/2)^{i-1} \tag{3}$$

and

$$K_n = n(K_2'/2)^{n-1}\gamma \tag{4}$$

where K_2' is the equilibrium constant for i monomers forming an i-mer, n is the stoichiometry of the final polymer, and $-RT$ ln γ is the contribution of the additional free energy of the favorable ring-closing step.

For a system of n species in rapid, dynamic equilibrium, the rate of movement of the reaction boundary can be rigorously described by the weight-average sedimentation coefficient:

$$\bar{s}_w = \sum s_i c_i / \sum c_i \tag{5}$$

(Schachman, 1959) where s_i is the sedimentation coefficient of the ith aggregate. The total weight concentration of the protein, c_T, is

$$c_T = \sum c_i = \sum K_i c_1^{\,i} \tag{6}$$

The experimentally observed $\bar{s}_w$ therefore becomes

$$\bar{s}_w = \frac{\sum s_i(1 - g_i c_T)K_i c_1^{\,i}}{\sum K_i c_1^{\,i}} \tag{7}$$

In fitting this function to experimental results, it was assumed that (i) the sedimentation coefficients of all aggregates except double rings could be calculated using spherical symmetry (Nichol et al., 1964):

$$s_i = s_1 i^{2/3} \tag{8}$$

(ii) the values of g_i for all species other than g_n are identical to g_1 (0.018 mL/g), and (iii) the concentration of aggregates greater than hexamer ($i = 6$), except for polymer, was negligible.

Monomer concentrations (c_1) were calculated from the positive real root of eq 6, and the unknown parameters, K_2 and K_n, were obtained by minimizing the standard root mean square deviation of the observed data from the calculated curve, as described previously (Frigon & Timasheff, 1975a; Howard & Timasheff, 1986).

It should be noted that, because the system is in rapid equilibrium, the velocity of neither the fast nor the slow peak represents a unique protein species. However, when the association constant is large and the stoichiometry of the reaction high, the sedimentation of the fast peak closely approaches that of the largest species (Josephs & Harrington, 1968).

RESULTS

Colchicine and Double-Ring Formation. As the primary effect of colchicine binding on tubulin self-assembly is an alteration in the interaction between tubulin molecules, the effect of the drug on another mode of tubulin self-assembly, the magnesium-induced self-association into double rings, was examined in some detail. Initially, the sedimentation coefficient of the tubulin–colchicine complex was determined in the absence of magnesium, in order to detect whether any conformational change induced by the drug is reflected in altered hydrodynamic properties of the protein. Extrapolation of the results to standard conditions (Schachman, 1959) gave a value of $s^0_{20,w} = 5.8$ S for both GTP– and GDP–tubulin–colchicine, identical to that of the drug-free protein (Frigon & Timasheff, 1975a; Howard & Timasheff, 1986), indicating

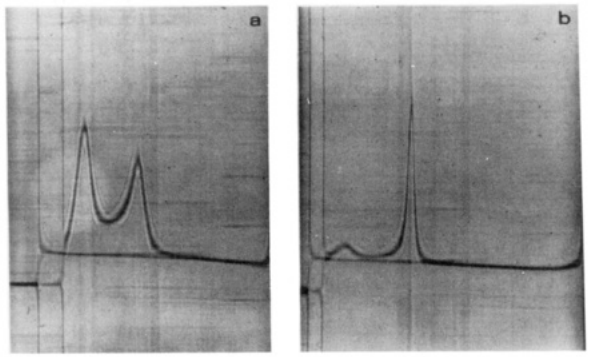

FIGURE 1: Sedimentation velocity profiles of (a) tubulin–colchicine–GTP and (b) tubulin–colchicine–GDP. Runs were performed at 48 000 rpm and 20 °C in a buffer containing 0.01 M sodium phosphate, 12 mM $MgCl_2$, and 1 mM nucleotide, pH 7.0. Sedimentation is from left to right. The protein concentration was (a) 14.0 and (b) 5.7 mg/mL, and the photographs were taken 8 min after reaching speed.

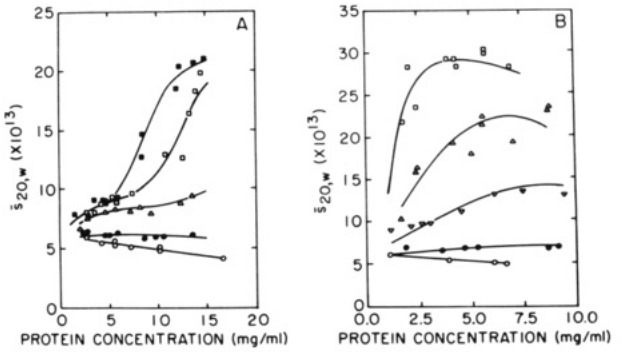

FIGURE 2: Protein concentration dependence of the weight-average sedimentation coefficient of (A) tubulin–colchicine–GTP and (B) tubulin–colchicine–GDP at various magnesium concentrations. Sedimentation velocity experiments were performed at 20 °C and 48 000 rpm in 0.01 M sodium phosphate/1 mM nucleotide, pH 7.0, containing (A) 0 (○), 2 (●), 8 (△), 12 (□), or 16 mM (●) $MgCl_2$ and (B) 0 (○), 2 (●), 6 (▽), 8 (△), or 12 mM (□) $MgCl_2$. The solid lines represent the best fit to the data calculated according to the model described in the text.

the subtle nature of the shape change. In the presence of magnesium, sedimentation patterns of the tubulin–colchicine complex exhibited all the characteristics of a modified Gilbert system (Gilbert, 1955, 1959), that is, a progressive self-association leading to a definite higher polymer. As was the case for tubulin–GTP and tubulin–GDP, the patterns became bimodal above a certain protein concentration, the region between the peaks never reaching the base line (Figure 1). As the protein concentration was increased, all the new area was added to the rapid peak. The proportion of the protein under the rapidly sedimenting peak was much greater for tubulin–colchicine–GDP than for tubulin–colchicine–GTP (Figure 1a,b), indicative of a stronger association, just as had been found with the drug-free tubulin. In order to examine whether the nature of the final polymer was altered by the binding of the drug, the sedimentation coefficient of the polymer was obtained by plotting the velocity of the fast peak as a function of protein concentration and extrapolating to zero protein concentration. Analysis of the tubulin–GDP data in terms of eq 1 yielded values of $s^0_{20,w} = 42 \pm 1$ S and $g_n = 0.037$ mL/mg. The sedimentation coefficient is identical to those obtained for GTP- and GDP–tubulin. The value of the hydrodynamic coefficient is slightly higher (cf. 0.019 and 0.028 mL/mg), perhaps indicating a minor change in the frictional properties of the final polymer upon binding of the drug. That the overall structure was basically unchanged was confirmed by electron microscopy, which revealed double-ring structures indistinguishable from those obtained in the absence of colchicine (Frigon & Timasheff, 1975a; Howard & Timasheff, 1986). On the basis of these results, the stoichiometry (n) of the polymer was again taken as 26.

Figure 2 shows the dependence of the weight-average sedimentation coefficients of tubulin–colchicine–GTP and tubulin–colchicine–GDP on protein and magnesium concentrations. A quantitative analysis of these results was performed in terms of the model outlined under Materials and Methods. The results of this analysis are summarized in Table I. Data previously obtained in the absence of drug (Frigon & Timasheff, 1975a; Howard & Timasheff, 1986) are shown for comparison. It can be seen that the effects of colchicine binding on double-ring formation are small but consistent. The drug appears to enhance slightly (–0.1 to –0.2 kcal/mol) the strength of the inter-tubulin bonds in the self-association of tubulin monomers into higher aggregates in the presence of magnesium, in agreement with the preliminary results of Andreu et al. (1983). Both GTP- and GDP–tubulin interactions are affected to the same extent.

The quantity $-RT \ln \gamma(\Delta G^\circ_\gamma)$ represents the favorable additional free energy of formation of the polymer and is equal to

$$\Delta G^\circ_\gamma = \Delta G^\circ_b + \Delta G_{cratic} + RT \ln 4 + \Delta G^\circ_R \qquad (9)$$

(Frigon & Timasheff, 1975b) where ΔG°_b is the free energy of forming the bond and ΔG°_R is the additional contribution of other entropic effects. The value of ΔG°_γ is more negative for tubulin–GDP than for tubulin–GTP. Given the similarity of ΔG°_2 for the GTP and GDP forms of tubulin, the observed differences must lie in the term ΔG°_R, indicating that the conformation of tubulin–GDP–colchicine is more favorable for ring closure than that of tubulin–GTP–colchicine, as is the case for pure tubulin (Timasheff, 1991). It appears that the final ring-closing step is slightly less favorable in the presence of colchicine than in its absence by between 1 and 3 kcal/mol.

The data in Table I were subjected to analysis in terms of the Wyman linkage function (Wyman, 1964; Wyman & Gill, 1990):

$$\frac{d \ln K}{d \ln a_x} = \Delta\nu_{app} \qquad (10)$$

where $\Delta\nu_{app}$ is the difference between the binding of the ligands to the product and reactant in an equilibrium system, expressed as moles of ligand bound per mole of tubulin at the given ligand concentration. Plots of $\ln K_2$ versus mean ionic activity of $MgCl_2$ (a_x) (Figure 3) yielded slopes close to 1 for both tubulin–colchicine–GTP and tubulin–colchicine–GDP, indicating the apparent binding of one magnesium ion per α–β-tubulin dimer upon self-association during the growth stage of the polymerization. Wyman plots of $\ln K_n$, on the other hand, gave different values of $\Delta\nu_{app}$ for the two nucleotide states of tubulin. The ring-closing step of tubulin–colchicine–GTP was linked to the apparent binding of 12 magnesium ions, while that of tubulin–colchicine–GDP was linked to the binding of approximately 32 magnesium ions.

Nucleotides and Double-Ring Formation. Because of the striking difference in propensity for double-ring formation between tubulin–GTP and tubulin–GDP ($\Delta\Delta G^\circ_\gamma \approx 10$ kcal/mol at 12 mM $MgCl_2$) (Howard & Timasheff, 1986), it has been proposed that occupancy of the γ-phosphate binding locus in tubulin is a critical factor in the control of the interconversion between two conformational states of tubulin (Melki et al., 1989). In an effort to learn more about the

Table I: Fitted Values for Association Constants as a Function of Magnesium Ion Concentration[a]

$[Mg^{2+}]$ (M)	nucleotide/drug[b]	K_2^{fitted} (M^{-1})	$\Delta G°_2$ (kcal/mol)	K_n^{fitted} (M^{-25})	$\Delta G°_n$ (kcal/mol)	γ (M^{-1})	$\Delta G°_\gamma$ (kcal/mol)
0.006	GDP/+	1.65×10^4	−5.65	2.50×10^{115}	−155	9.14×10^9	−13.4
0.006	GDP/−	1.33×10^4	−5.50	4.22×10^{113}	−153	3.6×10^{10}	−14.2
0.008	GDP/+	2.20×10^4	−5.82	5.0×10^{122}	−165	1.38×10^{14}	−19.0
0.008	GDP/−	1.45×10^4	−5.60	4.2×10^{119}	−161	4.1×10^{15}	−20.9
0.012	GDP/+	2.40×10^4	−5.88	3.81×10^{129}	−174	5.52×10^{19}	−26.5
0.012	GDP/−	1.77×10^4	−5.70	4.12×10^{127}	−172	2.80×10^{21}	−29.5
0.008	GTP/+	1.69×10^4	−5.67	7.08×10^{110}	−149	1.34×10^5	−6.9
0.008	GTP/−	1.23×10^4	−5.50	2.27×10^{109}	−147	1.3×10^7	−9.5
0.012	GTP/+	2.4×10^4	−5.88	7.7×10^{115}	−155	1.39×10^6	−8.2
0.016	GTP/+	2.5×10^4	−5.90	1.50×10^{117}	−157	1.56×10^7	−9.6
0.016	GTP/−	1.83×10^4	−5.70	1.76×10^{115}	−155	4.8×10^8	−11.6

[a] Data in the absence of colchicine are taken from Howard and Timasheff (1986) (GDP) and from Frigon and Timasheff (1975a) (GTP). [b] +, with colchicine, −, without colchicine.

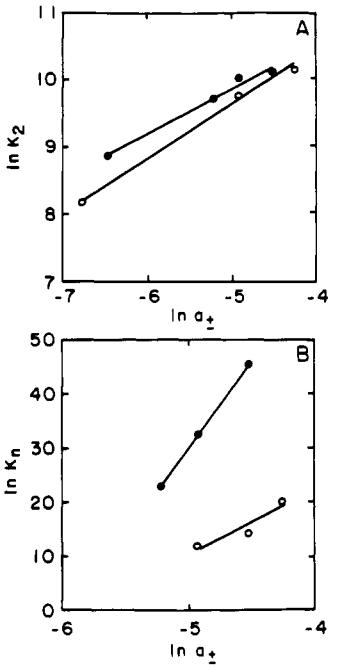

FIGURE 3: Wyman plot of fitted values for (A) K_2 and (B) K_n as a function of the mean ionic activity of magnesium chloride. Open circles represent the results obtained with GTP–tubulin and filled circles results obtained with GDP–tubulin.

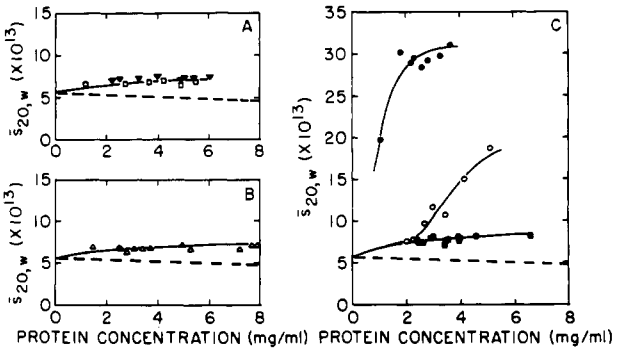

FIGURE 4: Dependence upon protein concentration of the weight-average sedimentation coefficient of tubulin–colchicine, with various nucleotides occupying the exchangeable site. Tubulin was depleted of exchangeable nucleotide as described under Materials and Methods and supplemented with the following: (A) (□) no addition, (▼) 2 mM guanosine; (B) (△) 5 mM GMP, (▲) 1 mM GMPPNP; (C) (■) 1 mM GMPPCP, (○) 1 mM GTPγF, (●) 1 mM GTPγS. All runs were performed at 20 °C, 48 000 rpm in 10 mM sodium phosphate/12 mM $MgCl_2$, pH 7.0. The solid lines represent the best fit to the data calculated via eq 7 and the data in Table II, while the dashed lines represent the result which would be expected in the absence of any self-association.

linkages that exist between nucleotide binding and tubulin conformation, i.e., about this "allosteric control" (Wyman, 1964; Wyman & Gill, 1990), a sedimentation velocity study of tubulin containing various nucleotides and analogues at the exchangeable nucleotide binding site was performed.

Figure 4 shows the results of the sedimentation velocity study of tubulin prepared in various states of nucleotide occupancy of the E-site, carried out over a range of protein concentrations and at a fixed concentration (12 mM) of $MgCl_2$. For every nucleotide examined, runs were carried out on both tubulin and the tubulin–colchicine complex. Only minor differences in K_2 and K_n were observed between the two states of tubulin liganding, similar in magnitude to those listed in Table I. For the sake of clarity, the results shown are those obtained with tubulin–colchicine. In the absence of magnesium, tubulin sedimented as a single 5.8S species, irrespective of the nucleotide occupying the exchangeable site.

Analysis of the protein concentration dependence of sedimentation coefficients in terms of eq 7 reveals (Table II) that, even in the absence of exchangeable nucleotide, tubulin retains a weak ability to self-associate in the presence of magnesium ions. The strength of the association is such that only lower order structures can form and no evidence of ring formation was observed over the concentration range examined. Guanosine (2 mM) and guanosine 5′-monophosphate (5 mM) had little or no effect on the equilibrium constant, K_2. Nucleotide analogues with modified P–O–P bonds, GMPPNP and GMPPCP (each 1 mM), were also unable to support double-ring formation, though again a weak ability to self-associate was retained. For each of these nucleotide ligands, only single schlieren peaks were ever observed in the concentration range examined (Figure 5). The association constant for tubulin–GMPPCP was significantly higher than those of the other nucleotides. Attempts to observe ring formation by extending the study to higher protein concentrations were hampered by technical limitations of the nucleotide depletion procedure and the relatively short lifetime of tubulin in solution.

Tubulin with γ-phosphate-modified nucleotides (GTPγS, GTPγF) occupying the exchangeable site gave bimodal sedimentation patterns that exhibited all the characteristics of the modified Gilbert system described above (Figure 5). The area under the fast peak was much greater, at a given protein concentration, for tubulin–GTPγS than tubulin–GTPγF. That 26 ± 4 subunit double rings were again the structure of the final polymer was confirmed by electron microscopy. Quantitative analysis of these results (Table II) revealed that the tendency of tubulin–GTPγS to form double rings was very similar to that of tubulin–GDP, the most favorable step again being the formation of the final ring-closing bond. Tubulin–GTPγF, on the other hand, assembled

Table II: Free Energy of Tubulin Double-Ring Formation as a Function of Exchangeable Nucleotide[a]

nucleotide	K_2^{fitted} (mL/mg)	K_2 (M^{-1})	ΔG_2 (kcal/mol)	K_n^{fitted} [$(mL/mg)^{-25}$]	K_n (M^{-25})	$\Delta G°_n$ (kcal/mol)	γ (M^{-1})	$\Delta G°_\gamma$ (kcal/mol)
GTP	0.45	2.40×10^4	−5.88	2.3×10^{-9}	7.70×10^{115}	−155.0	1.39×10^6	−8.2
GDP	0.45[b]	2.40×10^4	−5.88	9.12×10^4	3.81×10^{129}	−173.7	5.52×10^{19}	−26.5
GTPγS	0.45[b]	2.40×10^4	−5.88	5.1×10^7	2.13×10^{132}	−177.4	3.08×10^{22}	−30.2
GTPγF	0.45[b]	2.40×10^4	−5.88	1.11×10^{-4}	4.63×10^{120}	−161.8	6.70×10^{10}	−14.5
GMPPCP	0.321[c]	1.77×10^4	−5.69	0				
GMPPNP	0.180	9.90×10^3	−5.36	0				
GMP	0.120	6.60×10^3	−5.12	0				
guanosine	0.195	1.07×10^4	−5.40	0				
empty	0.136	7.50×10^3	−5.19	0				

[a] Results shown were obtained on the tubulin–colchicine complex. [b] The value for GTP–colchicine was used. [c] RMS deviations for the non-ring-forming species were in the range 0.07 (GMP) to 0.11 (empty).

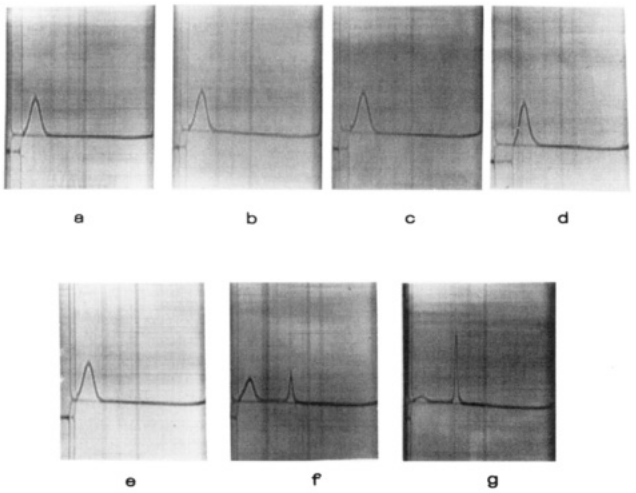

FIGURE 5: Schlieren profiles of tubulin–colchicine supplemented with nucleotide analogues. Runs were carried out at 20 °C, 48 000 rpm in 0.01 M sodium phosphate/12 mM $MgCl_2$, pH 7.0. The concentrations of added nucleotide, the protein concentration, and the time after reaching speed are as follows: (a) none, 4.61 mg/mL, 8 min; (b) 2 mM guanosine, 5.07 mg/mL, 8 min; (c) 5 mM GMP, 5.27 mg/mL, 8 min; (d) 1 mM GMPPNP, 4.95 mg/mL, 8 min; (e) 1 mM GMPPCP, 4.59 mg/mL, 8 min; (f) 1 mM GTPγF, 4.15 mg/mL, 6 min; (g) 1 mM GTPγS, 3.28 mg/mL, 6 min. Sedimentation was from left to right.

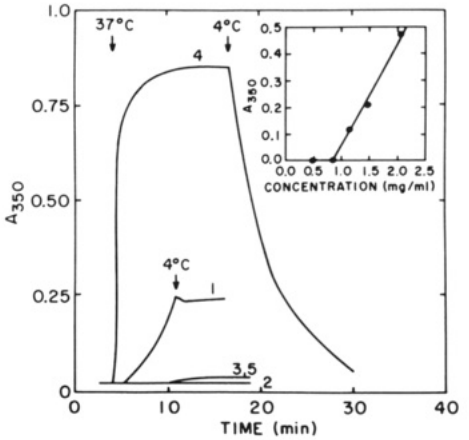

FIGURE 6: Self-assembly of tubulin into microtubules. Exchangeable nucleotide was removed from tubulin by treatment with immobilized alkaline phosphatase. Empty tubulin (2.9 mg/mL) in 0.01 M sodium phosphate, 3.4 M glycerol, and 12 mM $MgCl_2$, pH 7.0, was supplemented with nucleotide and incubated on ice for 20 min prior to assembly. Curve 1, buffer only; curve 2, 0.5 mM GTPγS; curve 3, 1 mM GMPPNP; curve 4, 0.5 mM GMPPCP; curve 5, 0.5 mM GTPγF. Assembly was followed by monitoring the turbidity at 350 nm. Inset: determination of the critical concentration for assembly of tubulin–GMPPCP.

into rings to an extent slightly greater than that of tubulin–GTP, the ring-closing free energy change being more favorable by ≈6 kcal/mol.

Self-Assembly into Microtubules. The correlation between nucleotide structure and the preferred mode of tubulin polymerization was probed further by examining the ability of the nucleotide analogues to support microtubule assembly. E-Site-depleted tubulin (2.9 mg/mL) was prepared in assembly buffer as described under Materials and Methods. In the absence of added nucleotide, the absorbance at 350 nm steadily increased at the assembly temperature (37 °C) (curve 1, Figure 6). Upon cooling to 5 °C, the absorbance remained constant and did not return to the base line, indicating that empty tubulin is rapidly denatured under these conditions. In the presence of GTPγS (0.5 mM), no absorbance increase above the base line was observed (curve 2), implying that this nucleotide binds to tubulin and protects it against denaturation, but is unable to support assembly. This result is in agreement with that of Hamel and Lin (1984) but in contrast to those of Kirsch and Yarbrough (1981). The same situation pertained when GMPPNP (1 mM) was the nucleotide of interest. No increase in A_{350nm} was observed (curve 3), in agreement with the findings of Hamel and Lin (1990), but opposed to those of O'Brien and Erikson (1989) and Mejillano et al. (1990). In contrast, when 1 mM GMPPCP was incubated with empty tubulin, turbidity increased rapidly upon warming, with no noticeable lag phase (curve 4). A true plateau was obtained which, upon cooling, returned almost to the base line. This system was able to undergo multiple cycles of assembly–disassembly (Seckler et al., 1990). GTPγF (0.5 mM) (curve 5) was unable to support assembly, in agreement with previous studies from this laboratory (Monasterio & Timasheff, 1987) which showed that GTPγF is a competitive inhibitor of tubulin assembly.

The inset to Figure 6 shows the evaluation of the critical concentration for assembly of tubulin–GMPPCP in 0.01 M sodium phosphate, 12 mM $MgCl_2$, 3.4 M glycerol, and 0.5 mM GMPPCP, pH 7.0, obtained by monitoring solution turbidity at 350 nm. Extrapolation to zero protein concentration yields a critical concentration of 0.84 mg/mL. This corresponds to a growth constant, $K_g = C_r^{-1}$ (Oosawa & Kasai, 1971; Lee & Timasheff, 1977), of 1.3×10^5 M^{-1}, giving a $\Delta G°_{app}$ for assembly of −7.3 kcal/mol, similar to that of the assembly of tubulin–GTP for which $\Delta G°_{app}$ was −7.0 kcal/mol under identical conditions.

Binding of Nucleotides to Tubulin. As shown in Figure 6, addition of either GTPγS or GMPPNP to empty tubulin protected it against heat denaturation, presumably by binding to the exchangeable site. The ultrafiltration method was used to quantitate these interactions. Since labeled GMPPNP is no longer commercially available, the binding of this analogue was quantitated by HPLC. In the presence of 3.4 M glycerol, GMPPNP bound to a single site with an association constant of 9 (±3) × 10^4 M^{-1}. The presence of high concentrations

Table III: Summary of Results

nucleotide	assembly ΔG° (kcal/mol)	double rings	binding constant (M^{-1})
GTP	−7.1	+	$(6.4–9) \times 10^7$ [b]
GDP	−[a]	++	$(2.5–2.7) \times 10^7$ [b]
GMP	−	−	ND[g]
guanosine	−	−	ND
none	−	−	
GMPPCP	−7.3	−	2.9×10^4 [c,d] $(5.3–7.1) \times 10^4$ [e]
GMPPNP	−	−	9.0×10^4 [d]
GTPγF	−	+	9.1×10^5 [f]
GTPγS	−	++	2.3×10^4

[a] Not observed. [b] Correia et al. (1987). [c] Seckler et al. (1990). [d] In the presence of 3.4 M glycerol. [e] Mejillano et al. (1990). [f] Monasterio & Timasheff (1987); obtained from inhibition of microtubule assembly. [g] ND, not determined.

of glycerol is known to strengthen tubulin–nucleotide interactions by approximately 1 order of magnitude (Seckler et al., 1990). In the absence of glycerol, the interaction was too weak to determine by HPLC. GTPγS binding was measured by radioactivity, in the absence of glycerol. In the presence of 1 mM $MgCl_2$, GTPγS bound to one site with an association constant of 2.3 (±0.2) × 10^4 M^{-1}. A similar value was obtained in the absence of magnesium, indicating that the binding of this nucleotide is not linked to the binding of magnesium ions.

DISCUSSION

The results presented in this paper demonstrate further the complex nature of the tubulin molecule. The antimitotic drug colchicine, while able to stabilize tubulin against denaturation (Prakash & Timasheff, 1992) and to influence the final morphology of the assembly products (Saltarelli & Pantaloni, 1982; Andreu et al., 1983), has only minor effects on the formation of 42S double rings, regardless of the nature of the nucleotide occupying the exchangeable site. This would indicate that the conformational change induced in tubulin by interaction with colchicine (Garland, 1978; Andreu et al., 1983) is distinct from that controlled by occupancy of the nucleotide binding site. Indeed, energy-transfer studies have shown the colchicine and nucleotide binding sites to be separated by approximately 2.4 nm (Ward & Timasheff, 1988).

Studies performed with the reversibly binding colchicine analogues allocolchicine, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB), and 2,3,4,4′-tetramethoxy-1,1′-biphenyl (TMB) gave very similar results, the order of effect on ring formation (on tubulin–GDP at 12 mM $MgCl_2$) being colchicine > allocolchicine > TKB ≈ TMB ≈ no drug. Taken together, these results are consistent with the notion (Garland, 1978; Andreu & Timasheff, 1982) that a small conformational change is induced upon the binding of colchicine to tubulin.

The use of nucleotide analogues to study tubulin self-associations has led to some intriguing findings. A summary of these results is shown in Table III. The nonhydrolyzable GTP analogue GMPPCP will support assembly, while the closely related GMPPNP will not. Neither of these will support double-ring formation to any significant extent. Guanine nucleotide analogues with modified γ-phosphate groups cannot induce assembly into microtubules but will, in the presence of magnesium ions, induce 42S double-ring formation. Quantitative analysis of these results in terms of a modified Gilbert theory shows that tubulin–GTPγS forms rings to an extent similar to that of tubulin–GDP, while the energetics of ring formation from tubulin–GTPγF are closer to those of tubulin–GTP. Guanosine 5′-monophosphate and guanosine have no effect on either mode of tubulin assembly.

Some comment should perhaps be made at this stage on the many conflicting reports in the literature regarding the ability of nucleotide analogues to support assembly. As noted by Seckler et al. (1990), most of these discrepancies may be explained by differences in experimental technique. Common factors involved include variations in tubulin preparations (presence and absence of MAPs), incomplete replacement of nucleotides, and contamination of commercial nucleotide preparations. As discussed by Hamel and Lin (1990), this last factor especially can lead to spurious and irreproducible results. In light of this, care was taken in the present study to use pure calf brain tubulin, to ensure complete removal of E-site nucleotide, and when necessary to purify nucleotide analogues before use.

The reference state for these studies must be tubulin with the exchangeable site unoccupied. A weak self-association (K_2 = 7.5 × 10^3 M^{-1}) of empty tubulin is observed under ring-forming conditions. By analogy, assembly of empty tubulin into microtubules was not observed at protein concentrations obtainable in the laboratory. However, this does not mean that it cannot occur. As is the case with all nucleotide states of tubulin, provided that sufficient added linkage free energy is supplied by the binding of a ligand [such as taxol or the nucleotide itself (Carlier & Pantaloni, 1983; Howard & Timasheff, 1988)], the overall strength of the polymerization can be shifted to a level observable at practical protein concentrations. This principle of linkage applies whether the final product is microtubules or 42S double rings.

By what mechanism do nucleotides control the nature of tubulin assembly? Microtubule formation under laboratory conditions requires occupancy of the γ-phosphate binding locus in the proper conformation: for example, by GTP, GDP·P_i, GDP·BeF_3 (Carlier et al., 1988, 1989; Melki et al., 1990), or GMPPCP (Seckler et al., 1990; present results). Occupancy of this site is thought to yield a "straight" conformation able to form linear polymers, i.e., microtubules, while with an empty γ-phosphate binding locus, the preferred conformational state is "curved", giving rise to double rings (Howard & Timasheff, 1986; Monasterio & Timasheff, 1987; Melki et al., 1989; Timasheff, 1991).[2] The present results allow us to scrutinize further the structural features that engender this allosteric control of protein conformation.[3]

A result seemingly inconsistent with the proposed model was obtained with the nonhydrolyzable analogue GMPPNP. No microtubule formation was observed with this triphosphate compound, although it binds to tubulin. The closely related compound GMPPCP, however, readily induced microtubule polymerization. NMR studies have demonstrated that, in the presence of magnesium, the pK's for all the nucleotide analogues are sufficiently low to ensure that the fully deprotonated form is the predominant species at neutral pH (Vogel & Bridger, 1982). This would seem to exclude differences in charge as an explanation of the observed results. While a crystallographic comparison of the bond angles and bond lengths of inorganic pyrophosphate, imidodiphosphate (Larsen et al., 1969), and methylene diphosphonate (Yount et al., 1971) reveals that in all three compounds P–P distances

[3] The only direct evidence for a conformational difference between tubulin in the GTP and GDP states is that seen by Raman scattering (Audenaert et al., 1989). The circular dichroism difference between GTP– and GDP–tubulins, reported by one of us (S.N.T.) (Howard & Timasheff, 1986), could not be reproduced and has been traced to a systematic drift in the instrument used at the time. It is hereby retracted.

are essentially the same, it should be noted that under physiological conditions nucleotides almost always exist as metal ion–nucleotide complexes. Hence, the solution structure determines nucleotide binding properties. In this connection, ^{17}O NMR studies of adenine nucleotide analogues (Reynolds et al., 1983) show strong evidence for the existence of imido tautomers of the P–N–P bridge; i.e., the proton exists on the oxygen rather than on the nitrogen. Furthermore, direct coordination of the magnesium with the P–N–P bridge has been suggested on the basis of ^{31}P NMR studies (Tran-Dinh & Roux, 1977). The methylene analogue, on the other hand, resembles more closely the structure of the native triphosphate compound (Gerlt et al., 1983). It appears, then, that the solution structures of the GMPPNP– and GMPPCP–metal ion complexes may be markedly different and that these differences can manifest themselves in altered interactions with the exchangeable nucleotide binding site. In the presence of GMPPCP, which closely resembles GTP, the straight $\rightleftharpoons$ curved equilibrium is shifted in favor of the straight conformation, and microtubule assembly can proceed. Since the present experiments were performed under conditions where the nucleotide binding site is fully occupied, the inability of GMPPCP to form double rings at the protein concentrations examined can be explained by the strengthening of the "straight" conformation in comparison to tubulin–GTP. Thus, at low protein concentrations, the remaining free energy is insufficient to overcome the unfavorable steric constraint imposed by the straight conformation.[4]

Data obtained with nucleotides modified on the terminal phosphate (sulfur or fluorine) can be interpreted in the same way. Stability constants for nucleotide–magnesium complexes decrease when sulfur replaces oxygen due to (a) an alteration in the charge distribution between oxygen and sulfur (Frey & Sammons, 1985) and (b) destabilization of the coordination structure by loss of an internal hydrogen bond between an oxygen of coordinated phosphate and metal-bound water (Pecoraro et al., 1984). As a result, GTPγS is unable to coordinate properly a magnesium ion on the γ-phosphate and so behaves essentially like GDP: it forms rings strongly and fails to support assembly into microtubules. Similarly, when the terminal titratable –OH group of the nucleotide is replaced by a fluorine atom, a negative charge is lost, and the affinity constant for the metal–nucleotide complex is decreased by approximately 36-fold as shown by Haley and Yount (1972) for ATPγF. NMR studies have shown that a high-affinity metal binding site still exists at the same locus as that of the exchangeable nucleotide when GTPγF is the nucleotide of interest (Monasterio, 1987). However, the metal may be complexed with the α- and β-phosphates rather than the γ-phosphate (Yount et al., 1971; Monasterio, 1987), with the consequence that tubulin–GTPγF complexation is similar to that of tubulin–GDP. The protein then should exist in the curved conformation. It is unable to assemble into microtubules, but is able to form double rings.

That the proper coordination of the γ-phosphate with a metal ion is essential for the straight microtubule-forming conformation can be inferred also from studies in which the metal ion, rather than the nucleotide, had been altered. Manganese (Buttlaire et al., 1980; Jemiolo & Grisham, 1982) and chromium (Carlier et al., 1991) can substitute for magnesium and support assembly. Moreover, it has been determined that, in the absence of magnesium, the affinity of tubulin for GTP is at least 1100-fold weaker than for GDP (Correia et al., 1987), indicative of the integral part played by the metal ion in the binding interaction. It should be noted that, in addition to the tightly bound metal, the presence of weakly bound metal ions appear to be required for the formation of both rings and microtubules (Frigon & Timasheff, 1975b). Wyman linkage analysis has shown that the effective binding of one additional magnesium ion is linked to the self-association of tubulin into microtubules (Lee & Timasheff, 1977) and its addition to rings during their growth phase (present results). A larger number of magnesium ions is linked to the final ring-closing step. The apparent uptake of 12 (tubulin–GTP) or 32 (tubulin–GDP) magnesium ions, though, does not imply direct participation of the ions in the ring-closing process, but rather may reflect altered surface electrostatic properties of the tubulin molecules upon formation of the final bond.

In summary, occupancy of the γ-phosphate binding locus is a necessary, but not sufficient, condition for microtubule formation at protein concentrations observable in the laboratory. Correct coordination of the terminal (γ) phosphate of the nucleotide with a metal ion is required for maintaining tubulin in the "straight" microtubule-forming conformation. Under these conditions, tubulin–GTP maintains enough linkage free energy to overcome unfavorable steric constraints and allows the formation of double rings. Similarly, a correctly coordinated tubulin–GMPPCP–Mg^{2+} complex permits the formation of microtubules, but provides less linkage free energy for polymerization into double rings. When the full chelation structure is unavailable, such as exchangeable-site occupancy either by a nucleoside triphosphate with an altered coordination structure (GTPγF, GTPγS) or by a nucleoside diphosphate (GDP), tubulin assumes the "curved" or ring-forming conformation. In the absence of nucleotide, insufficient free energy is available, at protein concentrations obtainable in the laboratory, to proceed past lower order polymers. In this regard, it is of interest to examine for the various nucleotide states the additional free energy made available per α–β dimer as a result of ring closure. On the basis of a 26-subunit structure, –0.4 kcal/mol is available to tubulin–GTP, –1.0 kcal/mol to tubulin–GDP, –1.2 kcal/mol to tubulin–GTPγS, and –0.6 kcal/mol to tubulin–GTPγF. Hence, the extra free energy available to each subunit is relatively small, but taken together over the whole structure, it becomes significant, reflecting the cooperativity of the process.

An analogy may be drawn between tubulin and other GTP binding proteins such as transducin, elongation factor Tu, and the *ras* oncogene product (p21). Each of these proteins exists in an active state when complexed with GTP and in an inactive state when GDP is bound. X-ray crystallographic studies of *ras* protein (Schlichting et al., 1990; Milburn et al., 1990) reveal significant structural differences between the two states of the protein, the trigger for the change being an altered coordination of the active-site magnesium as a result of the loss of the γ-phosphate of GTP. The metal ion is proposed to play a pivotal role in the mechanism of GTP hydrolysis by polarizing the P–O bond and assisting the nucleophilic attack on the γ-phosphate.

Let us consider now the factors that control the degree of polymerization in the "straight" and "curved" conformational modes. Microtubules have been observed to assume different lengths depending on the medium (Regula et al., 1981; Krist-

[4] For example, using a value of –10 kcal/mol for $\Delta G^{\circ}_{\gamma}$, ΔG°_{n} was calculated to be –151 kcal/mol for GMPPCP–tubulin, –141 kcal/mol for GMPPNP–tubulin, and –137 kcal/mol for empty tubulin. The protein concentrations at which bimodality would be expected to be seen in the ultracentrifuge would then be 18 mg/mL for GMPPCP–tubulin, 32 mg/mL for GMPPNP–tubulin, and 42 mg/mL for empty tubulin.

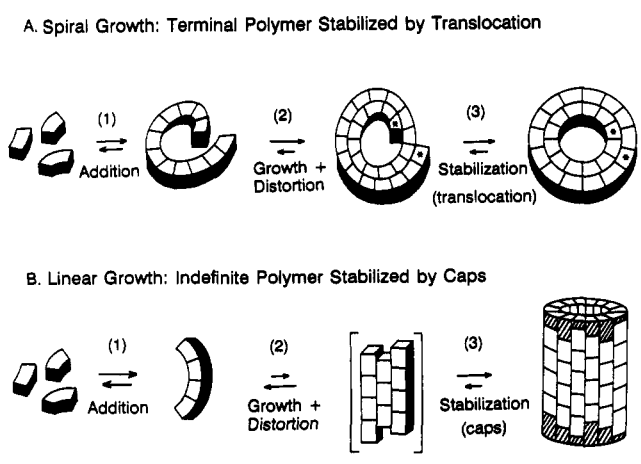

FIGURE 7: Energetics of tubulin polymerization. (A) Double-ring formation from tubulin–GDP. The polymerization of tubulin proceeds by curvilinear growth. The formed spiral structure results in increasing distortion of the inter-tubulin bond angle. Growth stops when the distortion free energy becomes equal to the bonding free energy. For tubulin, this occurs at two turns of the spiral. The asterisks indicate the two unsatisified terminal bonds in the spiral, which, in a thermodynamically favorable step, undergoes a translocation that permits closure of the final bond and yields the stable double-ring structure. (B) Hypothetical polymerization of tubulin–GDP into microtubules. Significant linear polymerization of "curved" tubulin is not possible because $\Delta G_{distortion}$ is always greater than $\Delta G_{addition}$. Only when stabilizing terminal caps (such as tubulin–GTP, represented by hatching) provide sufficient additional free energy can a linear structure be maintained.

offerson et al., 1986). Ring polymers appear to favor mostly the geometry of two concentric rings. Let us examine the thermodynamic factors that control these degrees of assembly. Polymer growth can be described in terms of a balance between the bonding free energy of subunit addition and the distortion free energy arising from departure of the protein conformation from its optimal state (Oosawa & Asakura, 1975). For each step in the polymerization, the net free energy of growth ($\Delta G_{stability}$) is given by

$$\Delta G_{stability} = \Delta G_{addition} + \Delta G_{distortion} \quad (11)$$

Infinite growth of the polymer is possible when the bonding free energy remains greater than the distortion free energy. When the distortion free energy becomes greater than the bonding free energy, polymer growth must stop. This is shown schematically in Figure 7. In the case of "curved" tubulin, polymerization must lead to the formation of spirals with a continuously increasing angle between consecutive longitudinal bonds (Figure 7A), once the lateral bonds start forming with the second turn. This entails a continuously increasing distortion free energy with each step in the polymerization until it balances the bonding free energy ($\Delta G_{addition}$), when polymer growth stops. This cessation of growth leaves two unsatisified bonds at the ends of the spiral. To relieve this thermodynamically unfavorable situation, a dislocation takes place, transforming the spiral into concentric rings that become stabilized by the thermodynamically favorable closure of the final longitudinal bond (Timasheff, 1991). In the case of tubulin, the distortion free energy seems to attain a value equal and opposite to $\Delta G_{addition}$ at the level of two turns of the spiral. Hence, the self-association terminates at the level of double rings.

When the same concepts are applied to microtubule formation, direct polymerization of tubulin–GDP ("curved" tubulin) into linear polymers (microtubules) is not possible (Figure 7B) because the distortion free energy required to maintain a "straight" orientation of the molecules is always greater than the free energy of subunit addition. Microtubule formation, therefore, can arise only from the polymerization of "straight" GTP–tubulin subunits, a state in which the distortion free energy is greatly reduced and the bonding free energy is the dominant factor. Upon incorporation in the microtubule, however, GTP is hydrolyzed to the GDP·P_i complex, followed by leaving of the inorganic phosphate. Trapped within the microtubule, the resulting tubulin–GDP is constrained into the straight conformation by the lateral and the two longitudinal inter-tubulin bonds per protein subunit. If present at the ends of the assembled structure, the constrained GDP–tubulin molecules possess unsatisfied bonding sites (one longitudinal bond per end subunit and probably some lateral bonds). This reduces their $\Delta G_{addition}$ relative to molecules inside the structure to a level at which it cannot overcome $\Delta G_{distortion}$, hence the requirement of the thermodynamic linkage to the stable GTP (or GDP·P_i)-containing subunits (caps) at the ends of the microtubule (Melki et al., 1990). Loss of the caps shifts the balance in favor of the distortion free energy. As a consequence, the GDP subunits become free to return to the favorable curved conformation, and the structure falls apart rapidly (catastrophic disassembly). As the number of tubulin subunits incorporated into a given microtubule is increased, so too is the free energy required to constrain the GDP subunits in the unfavorable straight conformation. Consequently, the free energy (and probably also the size) of the cap must also increase to maintain the integrity of the microtubular structure. Thus, the phenomenon of dynamic instability, consisting of alternating phases of elongation and disassembly, can be considered in terms of changes in the balance between the distortion free energy and the free energy of the stabilizing cap.

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