Linkages between the Dissociation of $\alpha\beta$ Tubulin into Subunits and Ligand Binding: The Ground State of Tubulin Is the GDP Conformation†

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ABSTRACT: The effects of ligands on the dissociation of the $\alpha\beta$ tubulin dimer into the two subunits were investigated using calf brain tubulin. Sedimentation equilibrium studies showed a number of linkages. In the absence of magnesium in the medium, tubulin-GTP, tubulin-GDP, and tubulin with the exchangeable site unoccupied associate with essentially the same strength ($K_{\alpha\beta} = 1 \times 10^7$ M^{-1}). This indicates that the ground state of tubulin (i.e., in the absence of magnesium) is not affected by occupancy of the exchangeable nucleotide binding site (E site). The $\alpha\beta$ association is enhanced by magnesium ions. The association of tubulin with GDP in the E site is linked to the uptake of twice as many magnesium ions as that of tubulin with GTP in the E site. This suggests that magnesium binding is linked to an E-site-related conformational change. Consideration of the linkages between the binding of magnesium ions, E-site occupancy, and tubulin conformation in terms of the model [Howard, W. D., & Timasheff, S. N. (1986) *Biochemistry 25,* 8292–8300] in which the tubulin $\alpha\beta$ dimer exists in an equilibrium between two conformations, a microtubule-forming ("straight") state favored by GTP and a double-ring-forming ("curved") state favored by GDP, leads to the conclusion that the ground state of tubulin is the ring-forming or "curved" conformation. Thus, in the absence of magnesium, the tubulin heterodimer exists in the ring-forming conformation, whether the E site is occupied by GTP or GDP. It is the strong binding ($K_b \sim 8 \times 10^5$ M^{-1}) of one magnesium ion to tubulin-GTP in the straight conformation that displaces the equilibrium from the ring-forming state toward the microtubule-forming conformation. It is proposed that it is this linkage between a very unfavorable equilibrium ($K \sim 0.01$) from the ring-forming, or storage, form of tubulin to the microtubule assembly-competent state with the binding of a single magnesium ion which is the control mechanism for the activation of microtubule assembly and that this magnesium ion is likely the one complexed to the γ-phosphate of GTP in the E site of tubulin. It is also this coupling of the strong binding of the magnesium to the very weak conformational equilibrium that explains the requirement of a high Mg^{2+} concentration in the microtubule assembly reaction. Dissociation of the $\alpha\beta$ dimer was used to probe the nature of the nonexchangeable nucleotide site (N site). Incubation of tubulin, diluted to a level where α–β dissociation occurs, with a 5000-fold excess of GDP showed no evidence of nucleotide exchange at the N site, leading to the conclusion that GTP must occupy the N site with an affinity 10^6–10^7 times greater than that for the E site.

Tubulin, the basic subunit component of microtubules, is itself composed of two similar but nonidentical subunits, α and β, each of which can bind 1 equiv of guanine nucleotide. The binding site on one subunit, the exchangeable or E site,[1] identified as the β-subunit (Geahlen & Haley, 1977; Hesse et al., 1987), can freely exchange with exogeneous nucleotide, while the site located on the other subunit (nonexchangeable or N site) cannot. That an equilibrium exists between the α and β subunits of tubulin has been well-established by a variety of procedures, including sedimentation equilibrium (Detrich & Williams, 1978; Detrich et al., 1982; Sackett & Lippoldt, 1991), sedimentation velocity (Detrich & Williams, 1978), fluorescence anisotropy (Mejillano & Himes, 1989; Panda et al., 1992) and spectral changes in the fluorescence of Nile Red (Sackett et al., 1990). In the form of the $\alpha\beta$ dimer, tubulin can further polymerize into a diverse array of structures such as microtubules (Weisenberg et al., 1968), double rings (Frigon & Timasheff, 1975), sheets, filaments, and ribbons (Timasheff & Grisham, 1980).

To explain the ability of tubulin to self-assemble into polymers of various geometries, a model has been proposed (Howard & Timasheff, 1986; Melki et al., 1989; Timasheff, 1991) in which the tubulin $\alpha\beta$ dimer exists in an equilibrium between two conformational states, a microtubule-forming or "straight" conformation and a ring-forming or "curved" state, the equilibrium between the two forms being allosterically controlled by the nature of the nucleotide that occupies the exchangeable site. GTP favors the straight form, while GDP favors the curved form. The structure of the final polymer has been shown to be determined by the nature of the γ-phosphate of the guanine nucleotide and its proper coordination with a magnesium ion (Shearwin & Timasheff, 1992). Magnesium ions have been demonstrated to have significant effects on many properties of tubulin, including the assembly of microtubules (Lee & Timasheff, 1974), the formation of 42S double rings (Frigon & Timasheff, 1975; Howard & Timasheff, 1986; Shearwin & Timasheff, 1992), and the binding of vinblastine (Na & Timasheff, 1986).

Given the wide range of effects of these ligands on the conformation of the $\alpha\beta$ dimer and on the nature of any resultant polymer, it was of interest to investigate the relations between

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[1] Abbreviations: E site, exchangeable nucleotide binding site; EDTA, ethylenediaminetetraaceticacid; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; N site, nonexchangeable nucleotide binding site; PEG buffer, 0.01 M sodium phosphate, 0.1 mM EDTA, and 10 μM nucleotide.

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their binding and the association of the α and β subunits. As a result, the dissociation of $\alpha\beta$ tubulin into its subunits has been examined in the presence of GTP and GDP and its linkage to interactions with magnesium ions has been probed in detail. The results are the subject of this paper.

MATERIALS AND METHODS

Tubulin. Fresh calf brains were obtained from a local slaughterhouse, transported on ice, and used within 1 h of slaughter. GTP and GDP were from Sigma Chemical Co. Calf brain tubulin was purified by the method of Weisenberg (1968), as modified by Na and Timasheff (1980). The protein was stored in liquid nitrogen in a buffer containing 0.01 M sodium phosphate, 0.5 mM $MgCl_2$, 0.1 mM GTP, and 1 M sucrose, pH 7.0. Aliquots of tubulin were prepared for use by passing over a small column (1 × 7 cm) of Sephadex G25 from which excess buffer had been removed by gentle centrifugation. Samples were then spun at 35000*g* for 30 min in order to remove any large aggregates and, finally, passed over a column (0.9 × 12 cm) of Sephadex G25 equilibrated with the buffer of interest. Since the GTP used (Sigma, Type III, sodium salt) contained 1–2% GDP, and in the absence of magnesium tubulin in the E site has a higher affinity for GDP than GTP while the reverse is true in the presence of magnesium (Correia et al., 1987), tubulin-GTP samples in magnesium-free medium were prepared by a modified procedure. The thawed tubulin sample which contains magnesium was made 10–20 mM in GTP, incubated 10–15 min, and passed over a dry and then a wet column equilibrated with 10 mM sodium phosphate, 0.1 mM EDTA, and 10 μM nucleotide. HPLC analysis of the resulting protein showed reproducibly that it contained >85% GTP at the E site. Tubulin with GDP occupying the exchangeable site was prepared by incubating tubulin with an excess of GDP 10–20 mM) in the absence of magnesium (30 min, 20 °C), followed by equilibration with the desired buffer via passage over a small dry column and a wet column of Sephadex G25 (Howard & Timasheff, 1986). Tubulin with no nucleotide bound to the exchangeable site (empty tubulin) was prepared by treatment with alkaline phosphatase as described previously (Shearwin & Timasheff, 1992). In experiments designed to examine exchangeability at the nonexchangeable site in dissociated tubulin, tubulin-GDP was diluted to 0.67 μM (total volume 100 mL), supplemented with GDP to a final concentration of 20 μM or 5 mM, and kept at 4 °C for 2 h. The protein was then reconcentrated to approximately 4 mg/mL using an Amicon Centriprep concentrating device and passed over a column of Sephadex G-25 to remove unbound nucleotide. Bound nucleotides were extracted for analysis by HPLC.

Protein concentrations were determined spectrophotometrically at 276 nm, either in 6 M guanidine hydrochloride or in dilute buffer. The extinction coefficients used were 1.03 $mL \cdot mg^{-1} \cdot cm^{-1}$ for tubulin in guanidine hydrochloride and 1.09 $mL \cdot mg^{-1} \cdot cm^{-1}$ for tubulin in buffer (Andreu & Timasheff, 1982).

Ultracentrifugation. Sedimentation equilibrium experiments were carried out at 10 °C with a Beckman Model E analytical ultracentrifuge fitted with RTIC temperature control, xenon arc light source, and photoelectric scanner. In each experiment, three samples were run in a four-place An-F rotor, using 12-mm filled Epon double-sector centerpieces and quartz windows. Because tubulin tends to aggregate when left in solution (Prakash & Timasheff, 1982) it was important that sedimentation equilibrium be attained in the minimum possible time. To this end, a combination of short columns and overspeeding was used such that the experiment was completed within 3–4 h (Sackett et al., 1989a; Sackett & Lippoldt, 1991). Sample volumes of 50 μL, equivalent to a 1-mm column, were used, and the samples were run at 32 000 rpm for 1 h, followed by 1 h at 26 000 rpm. This was sufficient to establish sedimentation equilibrium. Rotor temperature was controlled by the RTIC unit and runs were performed at 10 °C. All samples contained 5 mM sucrose in order to stabilize the gradient. The concentration gradient in the cells, each of which contained a different initial concentration in a given run, was determined by UV absorbance at 276 nm using the photoelectric scanner and the standard Beckman multiplexer to separate the signals. The input voltage from the scanner chart recorder was digitized and saved to a spreadsheet file using a Data Translation (Marlboro, MA) data acquisition board and custom-written software. The program calculates radial distances from the known distance between the counterbalance reference marks (1.61 cm), the number of data points collected within this region, and the frequency of data collection (20 points collected/s). The program also uses the scanner stair steps to calibrate the photomultiplier voltages with the corresponding OD values prior to the start of each scan. Absorbances were converted to concentrations via an empirical extinction coefficient found to be 1.0 $mL \cdot mg^{-1} \cdot cm^{-1}$. During scanning, the photomultiplier carriage was set on the slow speed, giving approximately 4500 points across the entire cell and 250 points across the 1-mm solution column. Multiple scans (usually three) of each cell were taken. Data files were edited to establish the inner and outer reference marks (and hence to calculate radial distances) and to choose the analysis region. This data was imported into a commercial graphics/curve-fitting program (Sigmaplot 4.1, Jandel Scientific, Corte Madera, CA) for data analysis. All data acquisition/curve fitting was done on an Everex 286-based personal computer.

Dissociation Data Analysis. At sedimentation equilibrium, the total concentration (c_T) of a reversibly self-associating species at radial distance r can be expressed as

$$c_{T,r} = \sum c_{i,r_0} \exp\left[A_i M_i \left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] \quad (1)$$

where c_{i,r_0} is the concentration of species i at reference position r_0, M_i is the molecular weight of species i, and A is defined as $(1 - \bar{v}\rho)\omega^2/RT$, where $\bar{v}$ is the partial specific volume, ρ is the solution density, ω is the angular velocity, R is the gas constant, and T is the Kelvin temperature.

For tubulin, which exists as the equilibrium mixture

$$\alpha\beta \rightleftharpoons \alpha + \beta$$

the association constant $K_{\alpha\beta}$ can be defined as $K_{\alpha\beta} = c_{\alpha\beta}/c_\alpha c_\beta$. Equation 1 then becomes

$$c_{T,r} = c_{\alpha,r_0} \exp\left[A_\alpha M_\alpha \left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] + c_{\beta,r_0} \exp\left[A_\beta M_\beta \left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] + c_{\alpha\beta,r_0} \exp\left[A_{\alpha\beta} M_{\alpha\beta} \left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] \quad (2a)$$

Since $c_\alpha = c_\beta$, and assuming that $M_\alpha = M_\beta$, eq 2a simplifies to

$$c_{T,r} = 2c_{\beta,r_0} \exp\left[A_\beta M_\beta \left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] + c_{\alpha\beta,r_0} \exp\left[A_{\alpha\beta} M_{\alpha\beta} \left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] \quad (2b)$$

The natural logarithms of the association constant and the

monomer concentrations were used, thus removing any need to constrain these values to be positive (Sackett & Lippoldt, 1991; Shire et al., 1991). The final equation used for fitting was

$$c_{T,r} = \left[2\exp\left[\ln c_{\beta,r_0} + AM_\beta\left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] + \exp\left(\ln k + 2\ln c_{\beta,r_0} + 2AM_\beta\left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right)\right] + e \quad (2c)$$

where e is a baseline error term. Thermodynamic ideality was assumed, and it was also assumed that $\bar{v}_\alpha = \bar{v}_\beta = \bar{v}_{\alpha\beta}$, $M_\alpha = M_\beta$, and $\epsilon_\alpha = \epsilon_\beta$, where ϵ_i is the extinction coefficient of species i. A value of 0.736 was used for the partial specific volume (Lee & Timasheff, 1974), M_β was taken as 55 000 (Lee et al., 1973),[2] ω was determined by measuring the number of rotations in a given time interval, and buffer densities were measured using an Anton–Paar precision density meter. For each set of scans (~750 data points), a total of seven parameters were fitted; a value of ln c_β and e for each individual scan, and a single value of ln k fitted simultaneously to all three scans. Concentrations were maintained on a mass per volume scale during the fitting procedure, and the resulting association constant, k, was converted to a molar scale via

$$K = kM_1/2 \quad (3)$$

The reference points chosen were approximately one-third of the distance from the cell bottom but it should be noted that eq 2 is completely general and that any point in the solution column could be chosen.

Ligand Concentrations. Magnesium chloride was prepared as a concentrated stock solution (~0.5 M) in water and the exact concentration was determined by titrating with EDTA of known concentration, with eriochrome black T as an indicator (Vogel, 1961). Free concentrations of magnesium were controlled by including in buffer solutions a known concentration (0.1 mM) of EDTA. The free magnesium concentration was then obtained by solving the equation

$$[Mg]_{total} = [Mg]_{free}\left(1 + \frac{K_1[EDTA]_{total}}{1 + K_1[Mg]_{free}}\right) \quad (4)$$

where K_1 is the apparent stability constant of the EDTA–metal complex (2.5×10^5 M^{-1} at pH 7.0) (Dawson et al., 1989). Magnesium binding to nucleotides was also taken into account by adding further terms to eq 4. Mean ionic activities were calculated according to Robinson and Stokes (1959).

HPLC. Nucleotides were extracted from tubulin samples and standards by the procedure of Seckler et al. (1990) and separated by isocratic reversed-phase ion-pair HPLC (Perrone & Brown, 1984) on a 250- × 4.3-mm C18 column (Supelco). Twenty-microliter samples were injected and the column was run at 1 mL/min, using a mobile phase consisting of 0.2 M K_2HPO_4, 0.1 M acetic acid, and 4 mM tetrabutylammonium phosphate. Detection was at 256 nm.

RESULTS

Tubulin Subunit Association. The stability of the tubulin $\alpha\beta$ heterodimer, which is the active state of the protein, and its dissociation into the α and β subunits were examined by sedimentation equilibrium in PEG buffer (0.01 M sodium phosphate, 0.1 mM EDTA, and 10 μM nucleotide)[3] at pH 7.0 and 10 °C. The data from a typical run are shown in Figure 1. The dots (~250/scan) are the experimental points and the lines represent the best fit to the data based on eq 2, where the equilibrium constant was fit to all three scans simultaneously. The residuals (Figure 1B) are small and distributed about zero. The values of the fitted α–β association constant, $K_{\alpha\beta}$, for tubulin-GTP and tubulin-GDP were found to be essentially identical. An unavoidable complication in experiments carried out in the absence of magnesium is the differential affinity of the nucleotides GTP and GDP for the tubulin E site. The affinity of the exchangeable site for GTP is approximately 1100-fold lower than for GDP in the absence of magnesium (Correia et al., 1988). Given the relatively weak binding of GTP in the absence of magnesium and the length of time required to complete the sedimentation equilibrium experiments, the extent of E-site occupancy was measured as a function of time. HPLC analysis of the tubulin nucleotide contents showed in the absence of magnesium a slow depletion of GTP from the E site, from approximately 78% occupancy at time 0 to 45% at 2 h and <10% after 3 h. Thus, there was a gradual decrease in the GTP contents of the E site over the course of the sedimentation experiments,

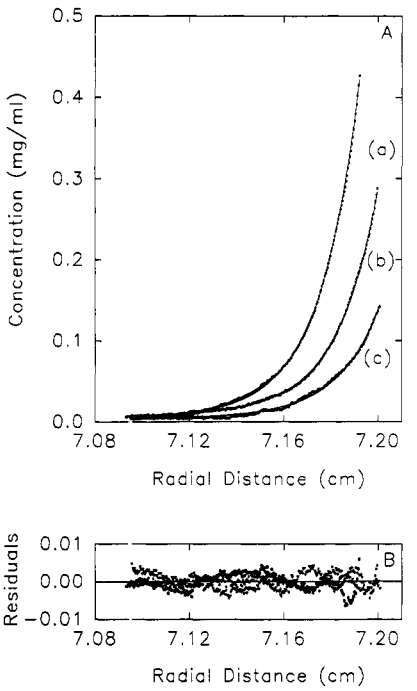


FIGURE 1: Sedimentation equilibrium of tubulin-GTP in 0.01M sodium phosphate buffer, pH 6.5, containing 0.1 mM EDTA, 10 μM GTP, and 5 mM sucrose. Samples (50 μL) were run in double-sector cells in an An-F rotor for 1 h at 32 000 rpm, followed by 1 h at 26 000 rpm at 10.0 °C. (A) Following attainment of equilibrium, the concentration distribution across the solution column was recorded as described in Materials and Methods. Initial concentrations were (a) 0.25 mg/mL, (b) 0.13 mg/mL, and (c) 0.09 mg/mL. The dots represent the experimental data (~250 points across the 1-mm column), while the solid lines show the best fit to the data obtained by simultaneously fitting the three scans to eq 2. (B) Residuals; the differences between the experimental data and the data calculated from the fitted values are shown.

[2] The molecular weight of each tubulin subunit is the value measured by five totally independent physicochemical methods which gave 55 000 ± 1000. It is unclear why the value calculated from sequence data (Ponstingl et al., 1981; Valenzuela et al., 1981) is lower by 5000 M_r units.

[3] Since tubulin preparations are known to carry weakly bound magnesium ions, it is expected that, in the absence of magnesium in the equilibrating buffer, the medium would contain an infinitesimally small concentration of free magnesium ions due to dissociation from tubulin of residually bound ions. This level is below our measuring capacity and certainly ≪0.046 mM, the lowest magnesium ion concentration used in these studies.

Table I: Effect of Mg^{2+} on the $\alpha\beta$ Tubulin Subunit Interaction for Tubulin-GTP and Tubulin-GDP at pH 7.0, 10 °C

GTP			GDP		
free Mg^{2+} concn (mM)	$K_{\alpha\beta}$ (M^{-1})	$\Delta G°$ (kcal/mol)	free Mg^{2+} concn (mM)	$K_{\alpha\beta}$ (M^{-1})	$\Delta G°$ (kcal/mol)
0[a]	9.2×10^6	−9.0	0[a]	1.2×10^7	−9.2
0.046	1.1×10^7	−9.1	0.05	9.2×10^6	−9.0
0.094	1.7×10^7	−9.4	0.099	1.7×10^7	−9.4
0.39	3.7×10^7	−9.8	0.149	4.3×10^7	−9.9
0.89	7.0×10^7	−10.2	0.199	3.2×10^7	−9.7
			0.398	9.6×10^7	−10.3
			0.896	4.8×10^8	−11.3

[a] In the equilibrating buffer system.

from approximately 45% to essentially 0 during the time period in which the equilibrium distributions were recorded. It was observed, however, that over this same time period the distributions were invariant, suggesting that tubulin $\alpha\beta$ dissociation does not change as GTP is lost. To verify this, tubulin with an empty E site was prepared by treatment with immobilized alkaline phosphatase (Shearwin & Timasheff, 1992) and subjected to sedimentation equilibrium. The fitted $\alpha\beta$ association constant, $K_{\alpha\beta}$, for empty tubulin (8.3×10^6 M^{-1}; $\Delta G = -9.0$ kcal/mol) was found to be identical to that for tubulin-GTP (9.2×10^6 M^{-1}; $\Delta G = -9.0$ kcal/mol) and tubulin-GDP (1.2×10^7 M^{-1}; $\Delta G = -9.2$ kcal/mol). Hence, despite the variation in E-site occupancy as a result of low GTP affinity in the absence of magnesium, the values quoted for tubulin-GTP are indeed appropriate to tubulin-GTP, which, in fact, behaves identically to tubulin-GDP and empty tubulin.

Effect of Ions. Previous investigations of the strength of association between the α and β tubulin subunits in the $\alpha\beta$ tubulin dimer (Detrich & Williams, 1978; Detrich et al., 1982; Mejillano & Himes, 1989; Sackett et al., 1989b; 1990; Sackett & Lippoldt, 1991) have reported association constants that differed by a factor of 6. These studies, however, had been carried out under a variety of solution conditions. It is known that the strengths of associations undergone by tubulin are modulated by the ionic composition of the buffer. Therefore, the linkages between the interactions with magnesium and hydrogen ions and the strength of α–β tubulin subunit association were examined.

The results obtained as a function of magnesium concentration are presented in Table I. Figure 2 shows the effect of magnesium ions (added as $MgCl_2$) on $K_{\alpha\beta}$ at pH 7.0, in terms of the Wyman linkage relation (Wyman, 1964; Wyman & Gill, 1990)

$$\frac{d \log K}{d \log a_x} = \Delta\nu_{app} \qquad (5)$$

where $\Delta\nu_{app}$ is the difference between the bindings of the ligand to the product and to the reactant in an equilibrium system, expressed as moles of ligand bound per mole of protein, and a_x is the activity of the ligand. Occupancy of the E site had a major effect on the dependence of α–β subunit interaction on magnesium ion concentration. In both states of E-site occupancy, magnesium ions strengthened the association. Taking the least-squares fit of the linear increase of log $K_{\alpha\beta}$ with log of magnesium activity gave slopes ($\Delta\nu_{app}$) of 0.60 ± 0.03 for tubulin-GTP and 1.3 ± 0.1 for tubulin-GDP, which indicates a much stronger effect of magnesium ions for the latter. When magnesium concentration was increased above 1 mM, the best fit of the data for both tubulin-GTP and tubulin-GDP was no longer to the $\alpha + \beta \rightleftharpoons \alpha\beta$ equilibrium but rather to the dimerization of the $\alpha\beta$ subunits (not shown),

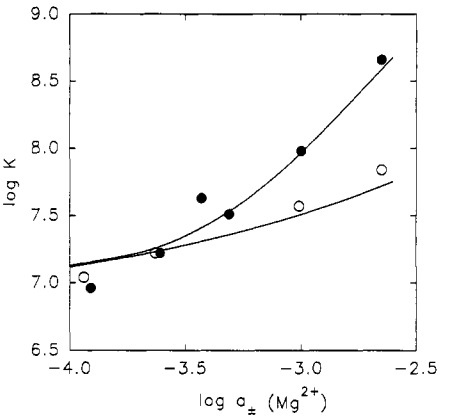


FIGURE 2: Wyman plot of the effect of magnesium ions on tubulin $\alpha\beta$ association. Mean ionic activities were calculated according to Robinson and Stokes (1959). Open circles show the results obtained with tubulin-GTP, and solid circles are the results found with tubulin-GDP. The lines represent the best fit to the data according to eq 7. See Discussion for details.

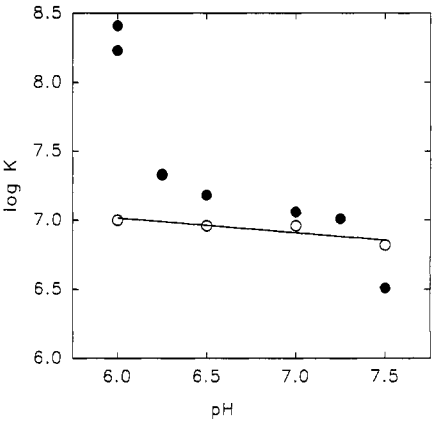


FIGURE 3: Wyman plot of the effect of pH on tubulin $\alpha\beta$ association. Open circles represent the results obtained for tubulin-GTP, and solid circles show the results obtained for tubulin-GDP.

which is consistent with the self-association characteristics of the early stages of double-ring formation (Frigon & Timasheff, 1975). At low magnesium concentrations (50 μM), the association constants for the two states of tubulin were almost identical. As the magnesium ion concentration was increased, however, for tubulin-GDP the association became much stronger than for GTP-tubulin. For example, at 0.9 mM $MgCl_2$ (free concentration) the association constant was 4.8×10^8 M^{-1} ($\Delta G° = -11.3$ kcal/mol) for tubulin-GDP, compared to 7.0×10^7 M^{-1} ($\Delta G° = -10.2$ kcal/mol) for tubulin-GTP. The apparent slope of the Wyman plot (1.3 ± 0.1) indicates that approximately twice as many magnesium ions are taken up during the association of tubulin-GDP as during the association of tubulin-GTP from the subunits. It should be cautioned, however, that these are effective values and do not mean a priori that the ions participate directly in the association process.

The effect of pH on the associations of tubulin-GTP and tubulin-GDP is shown in Figure 3 in the form of a Wyman plot. These experiments were performed in PEG buffer between pH 6.0 and 7.5, which for tubulin-GTP are the limits of stability of tubulin (Lee & Timasheff, 1977; Croom et al., 1986). As seen in the figure, a difference is apparent between the effect of pH on α–β tubulin dissociation whether the E site is occupied by GTP or GDP. For tubulin-GTP, the association constant varied from 1.0 x 10^7 M^{-1} ($\Delta G° = -9.1$ kcal/mol) at pH 6.0 to 6.7×10^6 M^{-1} ($\Delta G° = -8.8$ kcal/mol)

Table II: Effect of Buffer Composition on $\alpha\beta$ Association of Tubulin-GTP

buffer[a]	$K_{\alpha\beta}$ (M^{-1})
0.1 M MES and 0.1 mM EDTA	7.4×10^6 (−8.9)[b]
0.01 M sodium phosphate and 0.01 M EDTA	9.6×10^6 (−9.0)
0.1 M MES, 1 mM EGTA, 1 mM DTT, and 1 mM $MgCl_2$[c]	3×10^8 (−11.0)
0.01 M sodium phosphate, 0.1 mM EDTA, and 1 mM $MgCl_2$[d]	7.0×10^7 (−10.2)

[a] All buffers contained 10 μM GTP; the presence of 1 mM dithiothreitol had no significant effect on $\alpha\beta$ dissociation. [b] Numbers in parentheses are the corresponding free energy values in kilocalories per mole. [c] Buffer used by Sackett & Lippoldt. Free $Mg^{2+} \approx 1$ mM. [d] Free $Mg^{2+} = 0.89$ mM.

at pH 7.5. The slope of the Wyman plot, 0.1 ± 0.04, indicates that, within the pH range studied, essentially no protons are taken up or released upon formation of the $\alpha\beta$ dimer. With GDP occupying the exchangeable site, the pH dependence of the association became more complex. Between pH 6.5 and 7.25, the slope was similar to that of tubulin-GTP. As the pH was lowered from 6.25 to 6.00, the association constant increased sharply, with a greater uptake of protons, while at pH 7.5, the effect was opposite. This may indicate that the pH zone of conformational stability for tubulin-GDP is even narrower than that of tubulin-GTP. It should be noted that all our other experiments were carried out at pH 7.0, i.e., within the zone in which the association constants of tubulin in the two states of E-site occupancy are essentially the same.

Comparison with Earlier Studies. The absolute values of the association constants obtained in the present study are higher than those of previous studies (Mejillano & Himes, 1989; Sackett et al., 1990; Panda et al., 1992). For example, at ~1 mM magnesium there is a 14-fold difference in the values of the association constant for tubulin-GTP: 4.9×10^6 M^{-1} ($\Delta G° = -8.5$ kcal/mol) obtained by Sackett and Lippoldt (1991) at 5 °C for rat brain tubulin and 7.0×10^7 M^{-1} ($\Delta G°$ = −9.2 kcal/mol) determined in the present study. The possibility that the difference is due to the differences in buffer composition was examined in control experiments employing the buffer conditions of Sackett and Lippoldt (1991). The results are presented in Table II. In the absence of magnesium in the medium, the association constants were found to be essentially identical, whether the buffer was phosphate or MES (rows 1 and 2). Addition of magnesium raised $K_{\alpha\beta}$, regardless of the buffer used (rows 3 and 4). Thus, the linkage to magnesium concentration is observed in both phosphate and MES buffers. In MES buffer, the $\alpha\beta$ association appears to be linked more strongly to magnesium binding than in phosphate buffer, which is similar to the effect of MES buffer on microtubule assembly (Himes et al., 1977; Lee & Timasheff, 1977). One may conjecture that the differences observed between the various studies reside in the method of tubulin preparation. The present study employed tubulin purified by the method of Weisenberg (Weisenberg et al., 1968), while all previous studies used tubulin prepared by cycles of assembly and disassembly (Shelanski et al., 1973). It is possible to speculate, for example, that the two procedures extract nonidentical fractions of the many tubulin isotypes found in brain tissue.

Probing the N Site. One of the two nucleotide binding sites of the tubulin heterodimer, the exchangeable or E site, can freely exchange its ligand with free nucleotide in solution. When tubulin is assembled into higher polymers, however, the E site can no longer exchange (Weisenberg et al., 1976). It might be conjectured, therefore, that, in an analogous manner, the nonexchangeability of the N site is a result of blockage of the site by intermolecular contacts in the intact heterodimer and that upon dissociation into the α and β subunits the N site nucleotide may acquire the ability to exchange. This was tested by nucleotide exchange experiments, as described in Materials and Methods. Exchange of the N-site nucleotide upon dissociation should have increased the GDP contents of the protein, with a corresponding decrease in the GTP contents. To test this, the protein was diluted to a concentration at which it dissociates into the subunits. It was then exposed to free GDP in the medium at a level of 20 μM, i.e., twice that in the sedimentation equilibrium experiments, or 5 mM, a concentration sufficient to exchange completely the E site. Analysis of the reconcentrated GDP-tubulin showed no change in the proportions of bound GTP and GDP (Table III). A similar result had been observed by Correia et al. (1988). It is evident, therefore, that the nonexchangeable site remained so whether the tubulin existed as the $\alpha\beta$ dimer or as the dissociated subunits.

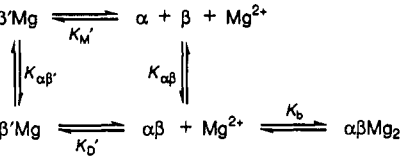
Table III: Nucleotide Contents of Tubulin-GDP before and after Dilution and Reconcentration (mol/mol) in an Excess of GDP

[GDP] during incubation	nucleotide	before	after
20 μM	GDP	1.04	0.98
	GTP	0.96	1.02
5 mM	GDP	0.88	0.94
	GTP	1.12	1.06

DISCUSSION

The Stability of $\alpha\beta$ Tubulin Is Identical in the GTP and GDP States. The present study has described the linkages that exist between the binding of ligands to tubulin and its dissociation into its constituent (α and β) subunits. The association constants for tubulin-GTP, $K_{\alpha\beta} = 9.2 \times 10^6$ M^{-1}, and tubulin-GDP, 1.2×10^7 M^{-1}, at pH 7.0 indicate that $\alpha\beta$ dissociation is not linked to nucleotide binding and suggest that, in the absence of magnesium, the two nucleotide states of tubulin exist in solution in the same or similar conformation(s). This is supported by the observation that the $\alpha\beta$ dissociation of tubulin with no nucleotide bound to the exchangeable site was identical to those of tubulin-GTP and tubulin-GDP. In addition, in the absence of magnesium in the medium, the binding affinity of the colchicine analogue allocolchicine to the tubulin heterodimer is the same whether the E site is occupied by GTP or GDP [Shearwin & Timasheff (1994) (following paper in this issue)]. In contrast, Sackett and Lippoldt (1991) had reported for rat brain tubulin a 3-fold difference between the association constants for tubulin-GTP and tubulin-GDP, the association of the GDP form being stronger. What is the source of the apparent contradiction on the strengths of association for tubulin-GTP and tubulin-GDP? Previous measurements of tubulin $\alpha\beta$ association/dissociation (Detrich & Williams, 1978; Sackett & Lippoldt, 1991) were performed in buffer systems that contained magnesium ions (~1 mM), while in the present study the comparison was made essentially in the absence of magnesium. As shown in Figure 2 and Table I, however, magnesium ions affect the association of the α and β subunits of tubulin-GTP and tubulin-GDP to different extents. In the absence of magnesium in the medium, the association constants for the two nucleotide states of tubulin are almost identical, but as the free magnesium ion concentration is raised, the strength of the association increases more rapidly for tubulin-GDP than for tubulin-GTP. Therefore, the differences in the association constants reported for the two nucleotide states of

Scheme I[a]



[a] For tubulin-GTP: $K_{\alpha\beta} = 9.2 \times 10^6$ M^{-1}; $K_D' \approx 8 \times 10^3$ M^{-1}; $K_M' \approx 3 \times 10^3$ M^{-1}; $K_b \approx 5 \times 10^6$ M^{-2}. For tubulin-GDP: $K_{\alpha\beta} = 1.2 \times 10^7$ M^{-1}; $K_D' \approx 1 \times 10^3$ M^{-1}; $K_M' \approx 0$; $K_b \approx 6 \times 10^6$ M^{-2}.

tubulin in the various studies can be ascribed to differences in the magnesium ion concentrations.

Linkages between Mg^{2+} Binding and $\alpha\beta$ Stability. The difference in the linkages of the association of tubulin to the binding of magnesium ions, whether tubulin is in the GTP or GDP state, can be explained by a conformational transition of tubulin that is differentially linked to the binding of magnesium ions for the two occupancies of the E site. To probe this, let us explore the free energy (linkage) relationships between $\alpha\beta$ dissociation, ligand binding, and the conformational states of tubulin and then relate these to linkages known to exist in the tubulin self-assembly processes.

As shown above, the uptake of magnesium ions coupled to the association of the α and β subunits for tubulin-GDP is twice that found for tubulin-GTP. This suggests a close linkage between an E-site-related conformational transition and the binding of magnesium ions. Furthermore, the observation that the value of $\Delta\nu$ for tubulin-GDP is greater than 1 points to the involvement of at least two weakly bound Mg^{2+} ions in the α–β association process. A simple reaction mechanism which takes into account these observations is shown in Scheme I. In this scheme the ground state is denoted as $\alpha\beta$ tubulin. It can bind magnesium ions in two modes: in the first (K_b), 2 Mg^{2+} bind and strengthen the dimer association without any conformational change. In the second mode (K_D'), the binding of one Mg^{2+} induces a conformational change. The experimentally measured dependence of the α–β association on magnesium ion concentration represents then the combination of the two reactions as defined by the equilibrium constants of the scheme. As a consequence, the association constant measured in the presence of magnesium is only an apparent quantity, $K_{\alpha\beta}^{app}$, defined by

$$K_{\alpha\beta}^{app} = \frac{[\alpha\beta] + [\alpha\beta'Mg^{2+}] + [\alpha\beta Mg_2^{2+}]}{[\alpha]([\beta] + [\beta'Mg^{2+}])} \quad (6)$$

Substitution of the appropriate equilibrium constants and rearrangement gives

$$K_{\alpha\beta}^{app} = \frac{K_{\alpha\beta}(1 + K_b a_{Mg^{2+}}^2 + K_D' a_{Mg^{2+}})}{1 + K_M' a_{Mg^{2+}}} \quad (7)$$

where $a_{Mg^{2+}}$, is the activity of magnesium ions and $K_{\alpha\beta}$ is the association constant in the absence of magnesium. From experiments carried out in the absence of magnesium, $K_{\alpha\beta}$ is known to be identical for the two states of tubulin; its value is given in Table I. Application of equation 7 to the magnesium dependence data of Table I resulted in the approximate values of the various equilibrium constants listed under Scheme I. From these, the expected Wyman plots were constructed. They are shown as the solid lines of Figure 2. Their upward curvature reflects the predominance of K_b over K_D', much more so for tubulin-GDP than tubulin-GTP. The relative values of K_D', which is ca. 1 order of magnitude greater for tubulin-GTP than tubulin-GDP, indicate a stronger ability of the former to undergo the conformational transition and to compete with the dimer-stabilizing binding reaction, K_b.[4] Similarly, the relative values of K_M', which for tubulin-GTP is $\sim[Mg^{2+}]^{-1}$ and for tubulin-GDP is $\ll[Mg^{2+}]^{-1}$, show that dissociated tubulin in the GTP state undergoes the isomerization reaction at the magnesium concentrations used, while the GDP state practically does not. On the other hand, $K_{\alpha\beta}$ and K_b are essentially identical for the two nucleotide states of tubulin. Therefore, the ground state of tubulin (in the absence of Mg^{2+}), $\alpha\beta$, is affected by the nature of the E-site occupancy with regard neither to dissociation ($K_{\alpha\beta}$) nor to the $\alpha\beta$-stabilizing magnesium binding (K_b). The whole difference between the two states resides in their abilities to undergo a magnesium-dependent conformational change ($\alpha\beta \rightleftharpoons \alpha\beta'$) that destabilizes the $\alpha\beta$ dimer. For tubulin-GTP this transition is much stronger than for tubulin-GDP. Hence the difference in the Wyman plots of Figure 2.

The T or Curved Conformation Is the Ground State of Tubulin.* Let us now consider these conclusions in the light of the model in which the $\alpha\beta$ dimer exists in a state of equilibrium between two conformations (Croom et al., 1985; Howard & Timasheff, 1986; Melki et al., 1989; Timasheff, 1991; Shearwin & Timasheff, 1992), T and T*, the self-association of one of which (T*, curved) leads to double rings, while that of the other (T, straight) leads to microtubules. Since rings are the only polymers that are observed to form from tubulin-GDP,[5] which cannot form microtubules, the equilibrium must be overwhelmingly favorable to T*. Therefore, for the analysis one may assume that tubulin-GDP exists only in the T* form. The GTP state, however, can self-associate into either microtubules or double rings, the simultaneous presence of which has, in fact, been observed and identified as the end products of two simultaneous competitive reactions (Lee & Timasheff, 1977). This means that, under assembly conditions, tubulin-GTP exists as a mixture of the two conformational forms. How is the equilibrium between these two conformations linked to the binding of magnesium ions? This information is contained in the effect of magnesium ions on double-ring formation in the two states of E-site occupancy. The equilibrium between the two states of tubulin for tubulin-GTP, defined by $K_c = [T^*]/[T]$ (Howard & Timsasheff, 1986), is related to the difference between the free energies of double ring formation, ΔG°_{ring}, from $\alpha\beta$ tubulin in the GDP and GTP states by

$$\Delta G^{\circ GDP}_{ring} - \Delta G^{\circ GTP}_{ring} = -2nRT \ln\left(1 + \frac{1}{K_c}\right) \quad (8)$$

where $n = 26$ is the number of $\alpha\beta$ tubulin protomers per double ring. Detailed studies of the polymerization of tubulin into the 42S double rings have generated free energy values for the overall formation of the double-ring structure (ΔG°_{ring}) for both tubulin-GTP and tubulin-GDP as a function of Mg^{2+} ion concentration (Frigon & Timasheff, 1975; Howard & Timasheff, 1986; Shearwin & Timasheff, 1992). A plot of these values against magnesium ion activity is shown in Figure 4. The striking result is that extrapolation to zero magnesium concentration leads, within experimental error, to identical values of the free energy of formation of double rings whether tubulin is in the GTP state or the GDP state, $\Delta G^{\circ intr}_{ring} \approx$

[4] It must be noted that K_b is the binding constant for two Mg^{2+} ions. As such it is an Adair constant and not the intrinsic equilibrium constant per bound ion.

[5] It is interesting to note that the state of the tubulin polymerized within the zinc-induced sheets conforms to the curved conformation (Melki & Carlier, 1993).

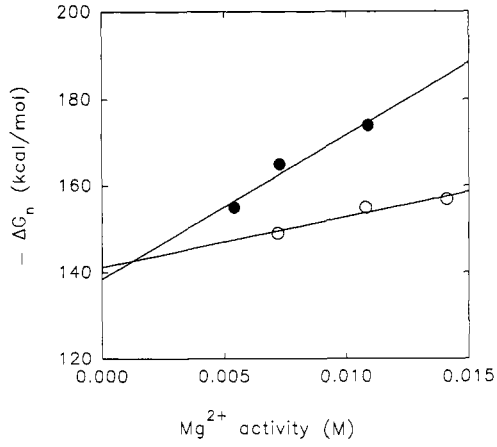
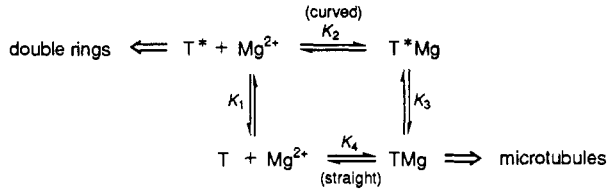


FIGURE 4: Free energy of double ring formation as a function of magnesium ion activity. The data points are taken from Shearwin and Timasheff (1992). Open circles show the data for tubulin-GTP, while solid circles represent the results for tubulin-GDP. Extrapolation to zero magnesium activity gives the same free energy value for the formation of double rings from both tubulin-GTP and tubulin-GDP.

Scheme II[a]



[a] For tubulin-GTP: $K_1 = 0.01$; $K_3 \approx 16$; $K_4 \gg K_2$. For tubulin-GDP: K_1 and $K_3 = 0$.

-140 ± 3 kcal/mol.[6] Thus, intrinsically, ring formation is thermodynamically identical for the two nucleotide states of tubulin, i.e., $\delta\Delta G_{ring}^{GDP-GTP} = 0$ in the absence of magnesium. Given that tubulin-GDP exists overwhelmingly in the T* form, it follows that, for tubulin-GTP, $K_c \rightarrow \infty$ as $[Mg]^{2+} \rightarrow 0$. Therefore, in the absence of magnesium, by the criterion of this transition as well, tubulin-GTP and tubulin-GDP both exist in the same conformation, and that conformation is the T* or curved form. The existence of this conformation in the absence of ligands other than the nucleotides permits us to assign to it the status of the ground, or relaxed, state of tubulin. This assignment is fully consistent with Weisenberg's (Weisenberg et al., 1976) identification of double rings as the storage form of tubulin.

Following this conclusion, let us extend the linkage analysis to double-ring formation in the two states of E-site occupancy. A straight-line analysis of the Wyman plots of the dependence of double-ring formation on magnesium activity gave slopes of 0.8 and 1.8 per tubulin-GTP and tubulin-GDP protomer incorporated, respectively. The uptake of twice as many additional magnesium ions per $\alpha\beta$ tubulin incorporated into rings in the GDP state as in the GTP state and the fact that in the GDP state this uptake is greater than one suggest that the T $\rightleftharpoons$ T* transition, which tubulin-GTP must undergo, is accompanied by the loss of one magnesium ion. If T* is taken as the ground state, these considerations may be expressed by the mechanism of Scheme II. K_c then becomes

$$K_c = \frac{([T^*] + [T^*Mg^{2+}])}{([T] + [TMg^{2+}])} \quad (9)$$

[6] With this free energy value, double ring formation would be detectable only at tubulin concentrations above 45 mg/mL, which is unattainable in the laboratory.

or, upon substituting the equilibrium constants

$$K_c = \frac{1}{K_1}\frac{(1 + K_2 a_{Mg^{2+}})}{(1 + K_4 a_{Mg^{2+}})} = \frac{1 + K_2 a_{Mg^{2+}}}{K_1 + K_3 K_2 a_{Mg^{2+}}} \quad (10)$$

Since, in the absence of magnesium, the protein exists predominantly in the T* state for both E-site occupancies, the value of K_1, the isomerization constant, must be very small. For tubulin-GDP, both K_1 and K_3 must be essentially equal to zero. For tubulin-GTP, if K_1 is assigned a value of 0.01, the limited data (Frigon & Timasheff, 1975; Howard & Timasheff, 1986; Shearwin & Timasheff, 1992) give, by eq 10, a value of $K_3 \approx 16$. Therefore, under conditions of saturating magnesium concentrations, >90% of tubulin-GTP would exist in the T form. It follows that $K_4 \gg K_2$ ($K_4/K_2 \approx 1.6 \times 10^3$); i.e., the magnesium ion that induces the conformational change binds much more strongly to the T state than to the T* state. By the Wyman linkage relationship, therefore, magnesium ions, by binding to the T state, must displace the equilibrium from the T* or ring-forming state to the T or microtubule-forming state; hence the simultaneous observation of microtubules and double rings in the case of tubulin-GTP (Lee & Timasheff, 1977).

The Microtubule Assembly Switch. How are the processes expressed by Scheme I ($\alpha\beta$ association) and Scheme II (curved $\rightleftharpoons$ straight equilibrium) related? Comparison of the two schemes suggests that species $\alpha\beta$ of Scheme I is, in fact, T* of Scheme II and that $\alpha\beta'$Mg of Scheme I is TMg of Scheme II. This renders $K_D' = K_1K_4$ and leads, within the assignment of the value of K_1, to $K_4 \approx 8 \times 10^5$ M^{-1} and $K_2 \approx 5 \times 10^2$ M^{-1} for tubulin-GTP. The stabilization of the microtubule-forming conformation of tubulin is effected then by the strong binding of one magnesium ion to a conformation-switching specific site. This magnesium should be identifiable now with the single magnesium taken up during the addition of each tubulin subunit to a growing microtubule (Olmsted & Borisy, 1975; Lee & Timasheff, 1977). This last observation, however, was made by means of a Wyman plot of the variation of the microtubule growth constant, K_g, over a magnesium concentration range of 1–16 mM, i.e., much higher than K_4^{-1}. This apparent disagreement is simply a reflection of the linkage of the magnesium effect on microtubule growth to the binding and conformational transition processes described by Scheme II. Specifically, the high magnesium ion concentration is needed to overcome the coupling of K_4 to the very weak constant K_1 and the competition with double-ring formation from T*. The relative proportions of microtubules and double rings observed then are a complex function of magnesium ion concentration. The available data on the magnesium dependence of microtubule assembly and of double-ring formation, however, could not be analyzed further, since the data had been obtained under nonidentical solution conditions.

Of the two processes discussed, $\alpha\beta$ dissociation is probably biologically not significant, since dissociation takes place at tubulin concentrations much lower than the cellular levels of free tubulin. The other process, that of the T* $\rightleftharpoons$ T transition, does account for much of the behavior of tubulin and the assembly of tubulin into microtubules (O'Brien et al., 1990). It is tempting, therefore, to suggest that the set of linked reactions expressed by the four equilibria of Scheme II is, in fact, a control mechanism for the activation of microtubule assembly through the shifting of the tubulin equilibrium from the ground or storage state, T*, to the activated, assembly-competent state, T. In such a case, the switching mechanism between storage and assembly would be an influx of magnesium

ions and the ensuing binding of one magnesium to a key site on the T form of the protein.

Can the switching magnesium ion be identified with the very high affinity divalent cation coordinated to the γ-phosphate of GTP? While the present results cannot answer this question, comparison with the literature might give some insight. The current value of the binding constant of the magnesium to the T form of tubulin-GTP ($K_4 \sim 8 \times 10^5$ M^{-1}) is strikingly similar to the affinity of magnesium for GTP on the E site ($\sim 1 \times 10^6$ M^{-1}) (Correia et al., 1988). Furthermore, in metal exchange experiments, the E-site magnesium could be replaced by manganese or cobalt in column equilibration against a buffer that contained a large excess of the competing metal (Himes et al., 1982; Jemiolo & Grisham, 1982; Monasterio, 1987; Ward & Timasheff, 1988; Correia et al., 1988). On the other hand, replacement of E-site GTP by GDP in a magnesium-free buffer requires a large excess of GDP (20 mM) in the medium (Seckler et al., 1990). Yet, the affinity of GTP, noncoordinated to magnesium, is very low ($<1.4 \times 10^4$ M^{-1}) (Correia et al., 1987) and it should be expected to leave once the magnesium ion had been lost. The affinity of Mg^{2+}-GTP for the E site, however, is very high ($\sim 10^8$ M^{-1}) (Correia et al., 1987). An observation that is inconsistent with the identity of the E-site GTP-bound magnesium and the ion identified in the current study is the observation that, in the presence of taxol, tubulin polymerizes into microtubules whether the E site is occupied by GTP or GDP (Schiff & Horowitz, 1981; Carlier & Pantaloni, 1983), yet magnesium is required (Howard & Timasheff, 1988; Andreu et al., 1992).

It is evident that these observations lead to ambiguous conclusions and cannot answer the question. Aesthetically, however, it is appealing to identify the T* → T switching magnesium ion with that tightly complexed to the γ-phosphate of GTP when bound to the tubulin E site. This obviates the necessity of invoking the existence of one further controlling site. This hypothesis engenders certain implications dictated by the linkages of Scheme II. These can be tested in terms of existing knowledge. First, the GTP bound to the E site of tubulin in the T*, curved, conformation would not be complexed with magnesium, nor would that incorporated into double rings. Double rings, however, are strongly favored by GDP, which does not have a γ-phosphate but does bind a magnesium ion with a low affinity ($K = 2 \times 10^3$ M^{-1}) (Correia et al., 1988). Interestingly, our analysis results in the binding of magnesium to the T* form of tubulin with a similarly weak affinity ($K_2 = 5 \times 10^2$ M^{-1}). The similarity of these values to the affinity of one of the αβ-stabilizing magnesium ions (see Scheme I) ($K_b^{1/2} \approx 2.2 \times 10^3$ M^{-1}) suggests a further possible identity. In this scheme, the T*-GTP-Mg species would be very rare since it would be either converted to the T-Mg, microtubule-forming state by the large value of K_3 or incorporated into double rings, a possibility suggested by the above-mentioned observation that magnesium concentrations above 1 mM led to a molecular weight equilibrium distribution consistent with the first steps of ring growth in both tubulin-GTP and tubulin-GDP. The weak binding of magnesium to T*-GTP, while it is very strong for T-GTP, is consistent with the knowledge that the latter state possesses a strong γ-phosphate binding subsite, while the T* state of tubulin does not, as shown by the lack of incorporation of BeF_3 into tubulin-GDP, except when it is within a microtubule (Carlier et al., 1989). Mechanistically, these considerations imply that, with tubulin in the T* conformation, the magnesium-free γ-phospate of GTP would be incapable of forming productive contacts with tubulin, hence the weak binding ($K < 1 \times 10^4$ M^{-1}) (Correia et al., 1988). Furthermore, the weakness of magnesium binding to T*-GTP relative to that of T-GTP suggests that the magnesium ion complexed to the γ-phosphate of T-GTP makes with the protein a binding subsite-stabilizing contact. It is this linkage that shifts the equilibrium toward the microtubule-forming conformation of tubulin, in agreement with the earlier conclusion (Shearwin & Timasheff, 1992) that the straight or T conformation requires the complexing of a magnesium ion (or other metal) in the proper coordination to the γ-phosphate of the E-site-occupying nucleotide. The difference between the affinities of magnesium for T and T* ($\delta\Delta G^\circ \approx -4.5$ kcal/mol), then, is a measure of the energetic contribution of the linkage to the microtubule assembly enabling transformation. Structurally, since the chemical nature of the longitudinal bonds appears to be identical in microtubules and double rings (Melki et al., 1989), the whole activation must be concentrated in the creation of a tubulin geometry, such that the lateral bonds could form properly between tubulin molecules when protofilaments are aligned in a straight mode, i.e., in microtubules.

Nature of the Nucleotide Binding Sites. What information does the present study convey on the nature of the nucleotide binding sites? When tubulin dimers form longitudinal contacts, such as during assembly into microtubules, the contact between the amino terminus of the β subunit of one protomer and the carboxy terminus of the α subunit of the other (Kirchner & Mandelkow, 1985) blocks the E site and the nucleotide (which in a microtubule is GDP) is unable to exchange, even though occupation of the E site by GDP inside a microtubule is thermodynamically unfavorable (Melki et al., 1989). Within an αβ dimer, the amino-terminal region of the α subunit is in contact with the carboxy-terminal domain of the β subunit (Kirchner & Mandelkow, 1985). If it is assumed that the nucleotide binding site on the α subunit (the N site) is similarly located in the amino-terminal region, the dimer forming contact could block the N site (α) nucleotide, but leave the E site (β) nucleotide free to exchange. In such a case, dissociation of the dimer would open the N site to exchange. As shown in the present study, however, the N site (α) nucleotide remained nonexchangeable on dissociation of the dimer. The nonexchangeability of the N-site nucleotide, therefore, must be the result of an intrinsic difference between the natures of the nucleotide binding sites rather than the result of steric factors. Incubation of tubulin diluted to a level at which α–β dissociation occurs with a 5000-fold molar excess of GDP did not promote nucleotide exchange. Since these experiments were carried out in the absence of magnesium, where the affinity of the E site for GDP is 1100-fold higher than that for GTP (Correia et al., 1987), a simple calculation shows that GTP must occupy the N site with an affinity 10^6–10^7 times greater than it does the E site. There is some evidence in the literature of a difference in the nature of the two sites. Sternlicht et al. (1987) found that α tubulin lacks the guanine-specific binding loop found in β tubulin and proposed that the GTP binding domains in the two subunits are conformationally different. Correia et al. (1988) studied metal ion exchange at the nucleotide binding sites and found that the N-site ion was able to exchange slowly divalent cations, indicating that the exchange at this site occurs by a mechanism that involves protein breathing. This leads to the conclusion that the exchangeability of the E site is modulated by steric factors, while the nucleotide–protein interaction at the N site is invariant.

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