Mg\(^{2+}\) Dependence of Guanine Nucleotide Binding to Tubulin*

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The relationship between the concentration of Mg\(^{2+}\) and the binding of GDP and GTP to tubulin dimers was investigated by measuring the displacement of the nucleotide bound at the exchangeable site (E-site) by radio-labeled GDP and GTP. A wide range of concentrations of GTP, GDP, and Mg\(^{2+}\) was explored. In the absence of GDP, the affinity of tubulin for GDP was found to be much greater than its affinity for GTP. In the presence of 1.0 mm Mg\(^{2+}\), however, its affinity for GDP was slightly less than for GTP. The results could be quantitatively described in terms of a small number of reversible equilibria. Equilibrium constants, pertaining to measurements at 0 °C, in 0.1 M piperazine-N,N'-bis(2-ethanesulfonic acid), 0.2 mM dithioerythritol, 2 mM EGTA, pH 6.9, were obtained by nonlinear least squares fitting of the data. When the association constant of tubulin for GDP uncomplicated with Mg\(^{2+}\) was taken to be 1.6 \(\times\) 10\(^{-7}\) M\(^{-1}\), that for the GDP-Mg\(^{2+}\) complex was found to be 2.5-2.7 \(\times\) 10\(^{-7}\) M\(^{-1}\), while that for the GTP-Mg\(^{2+}\) complex is 6.4-9.0 \(\times\) 10\(^{-7}\) M\(^{-1}\).

Tubulin binds two molecules of guanine nucleotide per \(\alpha\beta\) heterodimer (1, 2); a molecule of GTP at the "nonexchangeable" or N-site that can apparently be removed only by denaturation of the protein (3) and a molecule of either GDP or GTP at the "exchangeable" or E-site that is thought to undergo rapid exchange with free nucleotide (for reviews see Refs. 4 and 5). During or subsequent to the polymerization of tubulin to form microtubules, GTP at the E-site is hydrolyzed to GDP (6, 7). Upon subsequent disassembly of microtubules in the presence of excess GTP, the resulting tubulin dimers are thought to exchange the GDP at the E-site for a GTP from the solvent, so that the next cycle of polymerization takes place with GTP-bearing protein. The nucleotide exchange process undergone by the tubulin dimer is thus an important part of the cyclic assembly/disassembly of microtubules.

Mg\(^{2+}\) is required for assembly of microtubules (8-10) and for stability of tubulin (1, 11, 12, 26). Tubulin binds one Mg\(^{2+}\)/dimer in the course of microtubule assembly (13). For these reasons most studies of microtubule assembly have been carried out in Mg\(^{2+}\)-containing buffers. Both GTP and GDP bind Mg\(^{2+}\), and there is reason to expect that the binding of GTP and GDP to tubulin is linked to the binding, and hence to the concentration, of Mg\(^{2+}\) (14, 15).

The affinities of GTP and GDP for the E-site of tubulin have been measured by Zeeberg and Caplow (16), who employed a combination of the Hummel-Dreyer approach and competitive displacement methods and obtained apparent association constants of 4.5 \(\times\) 10\(^{-7}\) M\(^{-1}\) for GTP and 1.6 \(\times\) 10\(^{-7}\) M\(^{-1}\) for GDP, and by Fishback and Yarbrough (17) who employed fluorescence methods and competitive binding to obtain values of 5.9 \(\times\) 10\(^{-7}\) M\(^{-1}\) for GTP and 1.2 \(\times\) 10\(^{-7}\) M\(^{-1}\) for GDP. Partly because of these large affinities and partly because of unknown factors, it has been difficult to obtain complete exchange of nucleotides at the E-site. Ordinarily, if tubulin is incubated in a saturating concentration of a radio-labeled nucleotide and the free nucleotide is then removed, one finds that only 0.5-0.8 mol of label have become attached to a mol of tubulin dimer (6, 14, 16-26). Croom et al. (26) addressed this problem and showed that exchange of guanine nucleotide is strongly influenced by the presence of Mg\(^{2+}\). They found that incubation of tubulin in a buffer free of GTP and Mg\(^{2+}\) resulted in the rapid release of a fraction of the nucleotide bound to the E-site. In this paper, we show that the binding and exchange of GTP and GDP are strongly linked to the concentration of Mg\(^{2+}\) and that the binding and exchange can be quantitatively explained in terms of equilibria only. In the complete absence of Mg\(^{2+}\), the affinity of GTP for tubulin is at least 1100-fold weaker than that of GDP.

**MATERIALS AND METHODS**

Reagents—[8,5-'\(^{3}\)H]GTP (31.4 Ci/mmol), [\(^{3}\)H]GDP (19 Ci/mmol), and \([\gamma-^{3}\)P]ATP (2500 Ci/mmol) were purchased from Du Pont-New England Nuclear. [8-'\(^{3}\)H]GTP (16 Ci/mmol) purchased from ICN was used in some experiments. ATP was purchased from Pharmacia LKB Biotechnology Inc. Pipes, EGTA, dithioerythritol, GDP (Type I), and GTP (Type II-S) were from Sigma. MgSO\(_4\) was ACS reagent grade from Fisher. PMD buffer (0.1 M Pipes-NaOH (pH 6.9), 2 mM EGTA, 1 mM MgSO\(_4\), and 0.2 mM dithioerythritol) or PD buffer (0.1 M Pipes-NaOH (pH 6.9), 2 mM EGTA, and 0.2 mM dithioerythritol) were used, with additions as noted, in all experiments. N\(_{2}\)/SO\(_2\) was Schwabe/Marietta.

Preparation of Tubulin—Microtubule protein was purified from bovine brain by three assembly/disassembly cycles according to the method of Shelanski et al. (27), with modifications described by Williams and Lee (28). ATP (to 2.5 mm) was added to the supernatant of the first high speed centrifugation (29) to increase the yield.
Exchange of Guanine Nucleotides by Tubulin.

Tubulin was separated from microtubule-associated proteins (MAPs) by chromatography on Whatman P11 phosphocellulose in PDM buffer (0.1 mM GTP, 30 mM, and MgSO₄, to 1 mM) was added to fractions of the eluate that contained tubulin. (31). In some preparations, tubulin was eluted in a buffer containing 0.1 mM GDP to take advantage of the strong binding of this nucleotide in the absence of Mg²⁺ (see "Results"). Because tubulin solutions are depleted of Mg²⁺ during chromatography on phosphocellulose, substitution of GDP for GTP should ensure occupancy of the E-site by a nucleotide and thus preserve a larger fraction of active tubulin. This procedure may be preferable to Mg²⁺ saturation of the phosphocellulose column (31) since an unsaturated column more efficiently removes MAPS. As isolated, GDP-eluted tubulin contains 47-49% GDP, a figure compatible with full occupancy of the N-site by GTP and nearly complete occupancy of the E-site by GDP.

Results

Effect of Mg²⁺ on Nucleotide Binding—The alteration by Mg²⁺ of the relative affinity of tubulin for GDP and GTP was explored in a competition experiment. Binding of nucleotides was assayed after incubation of tubulin in buffers that contained differing concentrations of Mg²⁺ and constant, nearly equal, amounts of GDP and [³²P]GTP. As shown in Fig. 1, the affinity of the E-site for GTP is much greater than its affinity for GDP when the Mg²⁺ concentration is small. As the Mg²⁺ concentration increases, the amounts of the two bound nucleotides become approximately equal. The steepest change in relative affinity occurs at Mg²⁺ concentrations below 500 μM, although a plateau has not yet been reached by 2 mM Mg²⁺. Thus, the competition of GDP and GTP for tubulin depends strongly on the Mg²⁺ concentration.

This Mg²⁺-dependent competition is apparent throughout a range of relative nucleotide concentrations (Fig. 2). As the Mg²⁺ concentration increases, the amounts of the two bound nucleotides become approximately equal. The steepest change in relative affinity occurs at Mg²⁺ concentrations below 500 μM, although a plateau has not yet been reached by 2 mM Mg²⁺. Thus, the competition of GDP and GTP for tubulin depends strongly on the Mg²⁺ concentration.
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FIG. 1. Mg$^{2+}$ dependence of the relative affinity of tubulin for GDP and GTP. Samples of tubulin (30 μM, as estimated by the method of Bradford (64)) were incubated at 0 °C for 30 min in PM buffer + GDP and [3H]GTP, to which different amounts of Mg$^{2+}$ had been added. The ratio of GDP concentration to GTP concentration was 1.14, and the total concentration of GDP and GTP was 1 mM. Values of GDP*/TB (O), of the ratios of bound GTP to bound GDP, and of total bound nucleotide were determined. Each value of the number of molecules of GDP bound per tubulin dimer (Δ) was estimated as: (bound GDP/tubulin)/(bound GTP/tubulin). The dashed lines represent the trend of the data and are not fitted curves.

FIG. 2. Variation of the amount of bound [3H]GTP/tubulin when the ratio of free GDP to free GTP is varied. Samples of tubulin (30 μM, Ref. 64) were incubated for 30 min at 0 °C in mixtures of [3H]GTP and GDP at a constant total nucleotide concentration of 1 mM and then column centrifuged to remove unbound nucleotide. The amount of bound GTP was then determined by scintillation counting. ♦, experiment performed in PD buffer (i.e. –Mg$^{2+}$); ☐, experiment performed in PMD buffer (i.e. +Mg$^{2+}$). The dashed lines represent the trend of the data and are not fitted curves.

FIG. 3. Number of moles of [3H]GDP or GDP bound to tubulin as a function of the ratio of free GDP to free GTP, (GDP/GTP)$^0$. α, samples of tubulin (30 μM) were incubated for 30 min at 0 °C in mixtures of [3H]GDP and GTP at a constant total nucleotide concentration of 1 mM and then column centrifuged to remove unbound nucleotide. The amount of bound nucleotide was then determined by counting or by PCA extraction and HPLC. The solid lines (1–6) represent the best fits of the total data set in each panel as reported in Table 1. A few points (6) corresponding to (GDP/GTP)$^0$ values >3.0 were included in the fits but are not plotted. Total Mg$^{2+}$ concentrations: 2.025 mM (Δ), 0.503 mM (☐), 0.277 mM (●), 0.126 mM (◊), 0.0726 mM (☐), and 0.0259 mM (○). b, same as a except the GDP/TB values were determined as the number of moles of GDP bound per tubulin by HPLC only. This method assumes all N-site nucleotide is GTP and avoids error due to the determination of specific activity. In both analyses, the estimates of total nucleotide concentration include the measured amounts of E-site nucleotides (GDP and GTP) initially bound to the tubulin and exclude the N-site GTP. Total Mg$^{2+}$ concentrations were measured in the incubation mixture and include the Mg$^{2+}$ carried into the solution by tubulin. A small fraction (<10%) of the tubulin appeared to be inactive, not binding nucleotide. On the assumption that the inactive molecules had empty N-sites as well as empty E-sites, the concentration of active E-sites was obtained by multiplying the total tubulin concentration (30 μM) by one-half of the total number of nucleotide molecules/tubulin molecule.

TB + GDP $\rightleftharpoons$ TB·GDP

TB + GDP·Mg $\rightleftharpoons$ TB·GDP·Mg

TB + GDP·Mg $\rightleftharpoons$ TB·GDP·Mg

GTP + Mg$^{2+}$ $\rightleftharpoons$ GTP·Mg

GDF + Mg$^{2+}$ $\rightleftharpoons$ GDP·Mg

TB·GTP + Mg$^{2+}$ $\rightleftharpoons$ TB·GTP·Mg

TB·GDP + Mg$^{2+}$ $\rightleftharpoons$ TB·GDP·Mg

EGTA + Mg$^{2+}$ $\rightleftharpoons$ EGTA·Mg

Two of the equilibrium constants (e.g. $K_1$ and $K_5$) can be calculated from the others. Measurements carried out at 0 °C in Pipes buffer, as described under "Materials and Methods," gave $K_5 = 2.83 \times 10^5$ M$^{-1}$ and $K_6 = 6.75 \times 10^4$ M$^{-1}$. From data given by Ringbom (40), $K_3 = 3.2$ M$^{-1}$, a negligibly small

determines the ratio of nucleotides that are bound to tubulin's E-site.

Framework for Analysis—To analyze these effects quantitatively, some assumptions are necessary. Guanine nucleotide binding to tubulin involves a tight binding of Mg$^{2+}$, associated with the occupancy of the E-site (15). The nonexchangeable site is occupied by GTP. Both GDP and GTP bind to the E-site in a rapidly reversible equilibrium (16, 17). The following set of equilibria represent the simplest description of the experimental system.

\[ K_1 \]

\[ TB + GDP \rightleftharpoons TB \cdot GDP \]
number in the present context. Thus, only the first four equilibrium constants need to be determined by fitting of nucleotide-binding data. This framework does not include the large number (approximately 8) of weak Mg$^{2+}$-binding sites ($K < 2 \times 10^4$ M$^{-1}$) that are thought to exist (15, 57, 58), possibly located in the carboxyl-terminal regions of both subunits.

Quantitative Fitting of GDP-GTP Competition Data—To explore the observed Mg$^{2+}$ dependence more fully, quantitative competition experiments were performed over a wide range of Mg$^{2+}$ concentrations (25 μM to 2 mM, the minimum amount being that carried into the solutions by the tubulin). Binding was assessed both by the use of [3H]GDP as an indicator and by direct measurement of unlabeled GDP by HPLC. The points in Fig. 3 show the results. The entire set of data were fitted simultaneously to Reactions 1–6, as represented in the following fitting function.

$$\text{GDP/TB} = \frac{K_4 \text{[GDP]} + K_{24} \text{[GDP]} \text{[Mg$^{2+}$]}}{1 + K_4 \text{[GTP]} + K_3 \text{[GDP]} + K_{24} \text{[GTP]} \text{[Mg$^{2+}$]} + K_0 \text{[GDP]} \text{[Mg$^{2+}$]}} (10)$$

Since these experiments measure only nucleotide exchange, they can yield no data that would allow determination of an absolute equilibrium constant for the binding of any nucleotide to tubulin, but they do allow accurate estimates of the ratios of constants. To establish the correct range, we fixed the value of $K_0$ to that determined by Zeeberg and Caplow (16): $K_0 = 1.6 \times 10^7$ M$^{-1}$. Table 1 shows the values of $K_0$, $K_2$, and $K_4$ obtained. The solid lines in Fig. 3 show the fitted curves resulting from these values of the constants. Although small systematic deviations of the curves from the data are present, the fitted curves approximate the data quite well overall. Since the data were obtained from different preparations of protein, some of the small observed differences may have arisen from variations in biochemical properties of the protein.

The fitting process yielded optimum values of $K_4$ that were very small in comparison to the other association constants. Analysis of the difference in the goodness of fit produced by variation of $K_4$ showed that values of $K_4$ between 0 and 1.4 $\times$ 10$^3$ M$^{-1}$ were almost indistinguishable. Thus, $0 < K_4 < 1.4 \times 10^3$ M$^{-1}$.

**Linked Equilibria**—The relative values of the constants in Table 1 reveal several interesting aspects of the linkage between Mg$^{2+}$ concentration and nucleotide binding. 1) In the complete absence of Mg$^{2+}$, the affinity of tubulin’s E-site for GDP is more than 1100-fold greater than its affinity for GTP. 2) In saturating concentrations of Mg$^{2+}$ (several millimolar), however, the E-site’s affinity for GTP is 2.5–3.3 times greater than its affinity for GDP. 3) The presence of saturating Mg$^{2+}$ raises the effective association constant of GTP more than 4600-fold above its value in the absence of Mg$^{2+}$. 4) Saturating Mg$^{2+}$ has only a small (less than 2-fold) effect on the effective association constant of GDP (see "Discussion").

Here, the effective association constant for GTP and tubulin is defined as

$$K_{\text{eff}}^{\text{GTP}} = \frac{[[\text{GTP}]]}{[[\text{GTP}-\text{Mg$^{2+}$}]^{\text{GTP}}]} (11)$$

The value of $K_0$, the equilibrium constant for the binding of Mg$^{2+}$ to the TB-GDP complex, is small enough that nearly complete loss of Mg$^{2+}$ would be expected to occur upon gel filtration of GDP-tubulin into Mg$^{2+}$-free buffer (see "Results"). The consistent observation that approximately 0.83 mol of Mg$^{2+}$ accompanies 1 mol of GDP-tubulin dimer is, therefore, puzzling. We suggest that some of it may be bound at the N-site in a slowly reversible fashion (J. J. Correia, A. Beth, and R. C. Williams, Jr., manuscript in preparation).

### Table 1

<table>
<thead>
<tr>
<th>Analysis by scintillation counting (Fig. 4a)</th>
<th>Analysis by HPLC (Fig. 4b)</th>
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<tbody>
<tr>
<td>$K_0$ (M$^{-1}$)</td>
<td>$K_0$ (M$^{-1}$)</td>
</tr>
<tr>
<td>$1.6$</td>
<td>$143^a$</td>
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</tr>
<tr>
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<td>$4.6 \times 10^6$</td>
</tr>
<tr>
<td>$1.7$</td>
<td>$1.6$</td>
</tr>
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$^a$These values represent the best fitting approximations to $K_0$. The experimental uncertainty in their determination, as discussed in the text, allows us to specify only that $0 < K_0 < 1.4 \times 10^4$ M$^{-1}$.

$^b$The value of $K_0$ was fixed, $K_0 = 2.83 \times 10^4$ M$^{-1}$, and $K_0 = 7.65 \times 10^6$ M$^{-1}$ as described in the text.

$^c$The values of these ratios are based on $0 < K_0 < 1.4 \times 10^4$ M$^{-1}$.

and the effective association constant for GDP is defined analogously.

**Accuracy of the Derived Constants**—With these definitions, the origins of the experimental uncertainty in $K_0$ become clear. In essence, the measurement process yields values of these effective association constants, because it does not distinguish directly between those nucleotide-tubulin complexes that have Mg$^{2+}$ associated with them and those that do not; the distinction is inferred from the Mg$^{2+}$ dependence of $K_{\text{eff}}^{\text{GTP}}$. Assuming a value of $K_0 = 6.4 \times 10^7$ and $[\text{Mg$^{2+}$}] = 8$ μM (appropriate to 25 μM total Mg$^{2+}$), in the limit of $K_0 = 0$, $K_{\text{eff}}^{\text{GTP}} = 1.417 \times 10^7$. At $K_0 = 1.417 \times 10^3$, $K_{\text{eff}}^{\text{GTP}} = 1.431 \times 10^8$, a 1% change in $K_{\text{eff}}^{\text{GTP}}$. Likewise, at 2 mM Mg$^{2+}$, $K_{\text{eff}}^{\text{GTP}} = 5.439 \times 10^7$ at the smaller value of $K_0$ and $5.4392 \times 10^7$ at the larger value.

The **error bars** in Fig. 3a were estimated by uncorrelated propagation of the measured standard deviations of the four factors that were used to determine values of GDP*/TB: 1) the number of disintegrations/min in the incubation mixture; 2) the concentration of GDP in the incubation mixture; 3) the purity of the [3H]GDP; and 4) the number of disintegrations/min in the final sample. Uncertainties in the active tubulin concentration, both random and systematic, will increase the magnitude of the limits of error. The data in Fig. 3b are subject to much less experimental uncertainty (error bars would be smaller than the symbols on the graph), because the number of sources of experimental error is smaller. Here, GDP content of tubulin was analyzed directly by HPLC. The analysis is associated with a standard deviation of ±0.25% and with systematic uncertainty of <0.5% due to hydrolysis of GTP subsequent to the precipitation of tubulin with PCA. Any systematic error due to the overestimation of active GDP to tubulin.

The values of $K_0$, $K_2$, and $K_4$ yielding the best least squares fit of the data to the model were found by successive approximations employing the algorithm of Marquardt (61). The values of the concentrations of the free species at each data point (and, consequently, the value of the fitting function) were determined by an iterative scheme similar to that of Storer and Cornish-Bowden (62) or to algorithm II described by Goldstein (63).
were equilibrated with a buffer identical to PMD but without EGTA. The mixtures were column centrifuged to remove bound nucleotide. GTP*/TB is plotted as a function of the concentration of ATP in the presence of 1 mM $\text{Mg}^{2+}$.

The absolute values of the constants were set by fixing the value of $K_a$ to the value found in Zeeberg and Caplow (16): $K_a = 1.6 \times 10^7$ M$^{-1}$. The buffer employed in that study was 0.1 M Mes, 0.5 mM MgCl$_2$, 1 mM EGTA, pH 6.8. Mes binds Mg$^{2+}$, $K_a = 6.3 \times 10^7$ M$^{-1}$ (42), and the estimated lower free Mg$^{2+}$ concentration (~245 $\mu$M) produced by that binding, coupled with the weak affinity of GDP for Mg$^{2+}$, allows us to calculate that Zeeberg and Caplow (16) observed TB-GDP-Mg. Thus, their value is a reasonable approximation to $K_a$. Fishback and Yarbrough (17) reported nearly the same apparent value, $1.2 \times 10^7$ M$^{-1}$, for GDP binding to tubulin in 0.1 M Pipes, 1 mM MgCl$_2$, and 25% glycerol. Hamel et al. (43) have also reported pH-dependent values of nucleotide-binding constants in the same range. Hence, the values of $K_a$, $K_b$, and $K_c$ obtained in this study are likely to have absolute, as well as relative, meaning.

**DISCUSSION**

**Linkage of Mg$^{2+}$ and Nucleotide Binding**—Binding of GDP and GTP to the E-site of tubulin is linked to the concentration of Mg$^{2+}$. At low Mg$^{2+}$ concentration, tubulin overwhelmingly prefers GDP. Its affinity for GTP rises as the Mg$^{2+}$ concentration increases until, at concentrations above about 0.5 mM, GTP is somewhat preferred over GDP. The relative values of the equilibrium constants obtained can be compared with those to be found in the literature. Zeeberg and Caplow (16) found the ratio of the effective association constants for GTP and GDP to be 2.8 at 22 °C in a buffer of pH 6.8 and in the presence of 0.5 mM Mg$^{2+}$. Fishback and Yarbrough (17) in competitive displacement studies with thio-GTP and thio-GDP in a buffer of pH 6.8 and in the presence of 1 mM Mg$^{2+}$ and 25% glycerol found a value of 4.9 for the same ratio. From the constants in Table I, one calculates that the ratio of effective association constants $K_{\text{GTP}}/K_{\text{GDP}}$ (see Equations 11 and 12) in 0.5 mM Mg$^{2+}$ is 1.5 to 2.1, in reasonably good agreement with the values cited above.

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In a study by the binding of Al$^{3+}$ to tubulin, Macdonald et al. (46) measured values of several of the association constants reported here, using an indirect analysis based on kinetic measurements. Although their value for $K_{s1} - K_{s2}$ (denoted by them $K_{s1} - K_{s2}$) is in excellent agreement with that reported here, their value of $K_1$ (denoted by them $K_1$) is more than 3 orders of magnitude larger than the value found in the present work. We can offer no certain explanation for this discrepancy. A value of $K_1$ this large would imply, contrary to observed fact (26, 44), that GTP would not be lost upon gel filtration in an Mg$^{2+}$-free buffer and that inhibition of assembly by GDP would not become more pronounced at low Mg$^{2+}$ concentrations (45).
Fig. 5 shows the concentrations of free and associated tubulin and nucleotide species present in solution, at 1 mM total nucleotide and a GDP/GTP ratio of 1, over a wide range of Mg2+ concentrations. (Similar plots, of course, can be constructed for other nucleotide ratios.) At low Mg2+ concentrations the principal tubulin-containing molecular species are TB-GDP and TB-GDP-Mg2+. Only at high Mg2+ concentration does TB-GTP-Mg2+ predominate. The concentration of TB-GTP, over the whole range of Mg2+ concentration, is far too small to appear on the scale of Fig. 5. The results graphically demonstrate both the strong Mg2+ dependence of affinities and tubulin's virtual inability to bind uncomplexed GTP.

Effects on Polymerization of Tubulin—The stimulating effect of Mg2+ on microtubule assembly (e.g. Refs. 9, 10, and 13) must be mediated at least in part through the linkage described here. Increasing the Mg2+ concentration produces more GTP-tubulin dimer, which participates more readily than GDP-tubulin dimer in microtubule assembly (47-54), possibly because of a nucleotide-dependent conformational change (55, 56). This interpretation is reinforced by the findings of Huang et al. (45) that the ability of GDP to inhibit microtubule formation increases as the Mg2+ concentration decreases. Our measurements provide a potential quantitative basis for understanding this observation.

Other effects of Mg2+ on tubulin and microtubules are unlikely to be mediated through this mechanism. A conformational change has been suggested as being important in Mg2+-dependent formation of oligomers by GDP-tubulin (44) and by GTP-tubulin (57). This change occurs at Mg2+ concentrations above 4 mM and may involve weak nucleotide-independent metal-binding sites such as those described by Buttlaire et al. (58) and Himes et al. (59). The origin and importance of these effects as well as possible participation of Mg2+ in tubulin-MAP interactions (perhaps through binding in the carboxylate-rich C-terminal region of tubulin) remain to be investigated.

Location of the Mg2+-binding Site—Buttlaire et al. (58) and Jemio and Grisham (15) have identified a metal-binding site in close proximity to one of the tubulin-bound nucleotides, suggesting that Mg2+ may bind as part of a GTP-Mg complex. The values of the association constant were somewhat less than 10^8 M⁻¹, lying between the values we have obtained for the binding of Mg2+ to TB-GDP (K = 1.1 x 10^9 M⁻¹) and for the binding of Mg2+ to TB-GTP (K > 1.8 x 10^10 M⁻¹), as one would expect if they had observed equilibria involving a mixture of TB-GDP and TB-GTP. Therefore, although our results do not specify the location of the bound Mg2+, they are compatible with the idea that it is close to the nucleotide. The ratio of K₅ to K₆ (4.1). This striking difference suggests that Mg2+ is much more strongly coordinated to the protein in GTP-tubulin than in GDP-tubulin. This observation is compatible with a model (60) that rationalizes both the Mg2+ dependence of GTP binding and the Mg2+ independence of GDP binding in terms of sequence homologies between tubulin and known nucleotide-binding proteins.

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