Tubulin Exchanges Divalent Cations at Both Guanine Nucleotide-binding Sites*

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The tubulin heterodimer binds a molecule of GTP at the nonexchangeable nucleotide-binding site (N-site) and either GDP or GTP at the exchangeable nucleotide-binding site (E-site). Mg²⁺ is known to be tightly linked to the binding of GTP at the E-site (Correia, J. J., Baty, L. T., and Williams, R. C., Jr. (1987) J. Biol. Chem. 262, 17278–17284). Measurements of the exchange of Mn²⁺ for bound Mg²⁺ (as monitored by atomic absorption and EPR) demonstrate that tubulin which has GDP at the E-site possesses one high affinity metal-binding site and that tubulin which has GTP at the E-site possesses two such sites. The apparent association constants are 0.7–1.1 × 10⁶ M⁻¹ for Mg²⁺ and ~4.1–4.9 × 10⁶ M⁻¹ for Mn²⁺. Divalent cations do bind to GDP at the E-site, but with much lower affinity (2.0–2.3 × 10⁴ M⁻¹ for Mg²⁺ and 3.9–6.6 × 10⁴ M⁻¹ for Mn²⁺). These data suggest that divalent cations are involved in GTP binding to both the N- and E-sites of tubulin. The N-site metal exchanges slowly (Kₑₑ = 0.020 min⁻¹), suggesting a mechanism involving protein “breathing” or heterodimer dissociation. The N-site metal exchange rate is independent of the concentration of protein and metal, an observation consistent with the possibility that a dynamic breathing process is the rate-limiting step. The exchange of Mn²⁺ for Mg²⁺ has no effect on the secondary structure of tubulin and that tubulin which has GTP at the E-site exchanges Mg²⁺ strongly when it is occupied by GDP and weakly when occupied by GDP. A second high affinity site, most likely the GDP-occupied N-site, binds divalent cations strongly, but exchanges them very slowly (τₑ = 35 min). Thus, the binding of divalent cations to tubulin is best described as occurring at four possible classes of noninteracting sites. It involves high affinity coordination with GDP at the E-site and with GTP at the N-site, as well as low affinity coordination with GDP at the E-site and with other weak binding sites most likely associated with the acidic carboxyl-terminal region of both subunits. These results have important consequences for the detailed mechanism of divalent cation release from microtubules after GTP hydrolysis.

Tubulin has two guanine nucleotide-binding sites: the N-site, at which a GTP is permanently bound, and the E-site, at which either GDP or GTP is exchangeably bound. It is known that GDP binding to the E-site is strongly linked to the binding of Mg²⁺, whereas GDP binding is Mg²⁺ independent (1). These results are consistent with a recent model of nucleotide binding to tubulin that is based upon homologies with known nucleotide-binding proteins (2). This difference in Mg²⁺ linkage may be due to the presence of two different conformations of tubulin allosterically induced by the binding of GDP or GTP at the E-site (2, 3). Other divalent cations are known to exchange for bound Mg²⁺ on tubulin and to promote tubulin polymerization. Mn²⁺ (4, 5), Co²⁺ (6, 7), and Zn²⁺ (6, 8, 9) are believed to compete for the same high affinity site, although Co²⁺ and Zn²⁺ induce aberrant assembly of tubulin into sheets of protofilaments. The binding of Mn²⁺ to tubulin has been described as occurring at two classes of noninteracting sites: one high affinity site (Kₑ = 6.3 × 10⁶ to 4.0 × 10⁶ M⁻¹) and eight weak sites (Kₑ = 2.2–2.6 × 10⁴ M⁻¹) (4, 5). The fractional number of high affinity sites per heterodimer varies with the nucleotide content of tubulin, suggesting that bound nucleotide is part of the site. GTPcr also binds to the E-site and promotes microtubule assembly (6, 10). Recently, a tight metal-binding site has been shown to be within 6–8 Å of the γ-phosphate of E-site GTP (11). These results strongly suggest that guanine nucleotides bind to the E-site of tubulin as a metal complex.

Here we have reinvestigated the exchange of Mn²⁺ for bound Mg²⁺ on tubulin and show that the number of high affinity (K > 10⁶ M⁻¹) metal sites is correlated with the total GTP content of tubulin and not with the GDP content. We have found that the E-site exchanges the two cations rapidly, binding Mn²⁺ strongly when it is occupied by GTP and weakly when occupied by GDP. A second high affinity site, most likely the GTP-occupied N-site, binds divalent cations strongly, but exchanges them very slowly (τₑ = 35 min). Thus, the binding of divalent cations to tubulin is best described as occurring at four possible classes of noninteracting sites. It involves high affinity coordination with GTP at the E-site and with GDP at the N-site, as well as low affinity coordination with GDP at the E-site and with other weak binding sites most likely associated with the acidic carboxyl-terminal region of both subunits. These results have important consequences for the detailed mechanism of divalent cation release from microtubules after GTP hydrolysis.

MATERIALS AND METHODS

Reagents—Pipes, EGTA, diithioerythritol, GDP (Type I), and GTP (Type II-S) were from Sigma. MgSO₄ and MnCl₂ were ACS reagent-grade from Fisher. Magnesium and manganese atomic absorption standard solutions were from Sigma.

Preparation of Tubulin—Microtubule protein was purified from bovine brain by three assembly/disassembly cycles according to the method of Shelanski et al. (12), with modifications described by Williams and Lee (13). ATP (to 2.5 mM) was added to the supernatant of the first high speed centrifugation (14) to increase the yield. Tubulin was separated from microtubule-associated proteins by chromatography on Whatman P-11 phosphocellulose in 0.1 M Pipes, 2 mM EGTA, 2 mM diithioerythritol, 1 mM MgSO₄, pH 6.9, plus 0.1 mM GDP (1, 15); MgSO₄ (to 1 mM) was added to fractions of the eluate that contained tubulin (16). Each preparation was checked for purity

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‡ The abbreviations used are: N-site, tubulin's nonexchangeable nucleotide-binding site; E-site, tubulin's exchangeable nucleotide-binding site; HPLC, high performance liquid chromatography; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EGTA, [ethylenebis(oxyethylenenitrito)]tetraacetic acid.
by sodium dodecyl sulfate gel electrophoresis on heavily overloaded gels, frozen dropwise in liquid nitrogen, stored at -75 °C, and used within 3 months. The GDP content of this tubulin is typically 46-49% of the total bound nucleotide, and this protein is designated TBGD, i.e. tubulin with GDP bound at the E-site. To make tubulin with predominantly GTP at the E-site (TBGT), a solution of tubulin purified by phosphocellulose chromatography (PC-tubulin) was brought to 1 mM GTP and 1 mM MgSO4, incubated for 5 min, and column-centrifuged (17) into 0.1 M Pipes, 1 mM MgSO4, pH 6.9, and the process was repeated. The tubulin was exposed to 1 mM GTP and MgSO4, a third time and column-centrifuged into 0.1 M Pipes, pH 6.9. All steps were performed at 0-4 °C. The bound nucleotide content of this material is typically 5-10% GDP, or 0.1-0.2 molecules of GDP/E-site. Prior to an experiment, TBGD or TBGT in Pipes buffer was diluted to the desired experimental concentration, and other buffer components (dithioerythritol, Mn2+, Me, glycerol, etc.) were added.

Determination of Concentrations—The concentrations of Mn2+ and Mn2+ in stocks and buffers and tightly bound to tubulin were determined by atomic absorption on a Perkin-Elmer Model 603. The concentration of tubulin was determined from its absorbance: ε278.5 = 1.23 ml mg-1 cm-1 (18). The molecular weight of the dimer was taken to be 100,000 (19-21). The number of moles of guanine nucleotide bound to 1 mol of tubulin dimer (denoted GXP/TB) was determined as described by Croom et al. (22). Tubulin was freed of unbound nucleotide (and weakly bound metal) by column centrifugation (17); the protein was precipitated with perchloric acid at 0 °C, and the amount of guanine nucleotide was determined from the absorption of the supernatant: ε256 = 1.24 × 104 M-1 cm-1, applicable near pH 1.

Assessment of Purity of GTP and GDP—Guanine nucleotides were separated on a SynChromax AX-300 anion-exchange column (SynChrom, Inc.) with 1 M NaCl, 0.35 M NaH2PO4, solvent system, pH 5.0. This solvent resolves GMP, GDP, and GTP clearly. Samples of GTP were found to contain 95-96% GTP and 4-5% GDP. The sample of GDP contained less than 1% GTP and 3-4% GMP.

To assay the relative amount of GDP or GTP bound to tubulin after column centrifugation, an aliquot of the perchloric acid extract used to determine the nucleotide concentration was mixed with 4 M KCH3COO (12%, v/v) to precipitate the perchloric acid and centrifuged. The resulting solution of GTP and GDP was then analyzed by HPLC to determine the fractional amount of each nucleotide.

Circular Dichroism—Far-UV CD spectra were measured on a Jasco Model 500 at 2 °C in 0.1 M Pipes, 0.1 mM dithioerythritol, pH 6.9, at a protein concentration of 5 μM in a 0.1-cm jacketed cell. Molar residue ellipticities were calculated assuming a mean residue weight of 110.

Electron Paramagnetic Resonance—EPR spectra were collected on a Varian E-112 X-band spectrometer interfaced to a FDP 11/73 microcomputer, which drove the spectrometer magnetic field and collected data digitally. A Varian E-257 temperature control unit regulated the temperature of the Varian E-388 TM-39 mod. high volume aural cavity. The spectra for Fig. 3 were recorded at 2 °C in 50-1 borosilicate capillaries at 9.42 GHz, 60-milliwatt microwave power, and 5-G modulation amplitude (peak to peak) with a filter time constant of 0.005 s. The field was scanned over 1000 G in 1024 increments, and each point was averaged over 22 recordings. Since the X-band EPR spectrum of Mn2+ bound to tubulin is severely broadened by virtue of its characteristic six-line spectrum, each scan was blank-corrected prior to determining the free Mn2+ concentration by comparison with the spectrum of a known concentration of free Mn2+ (23). Kinetic measurements (Fig. 2b) were performed in a TE-flat cell at 9,42 GHz and 30-milliwatt microwave power. The appropriate Mn2+ concentration (~100 μM) was mixed with the protein (~40 μM), and the cell was filled and allowed a few minutes to cool in the cavity. A single peak-to-peak data region was collected (usually the third or the fifth pair) every 5 min until the signal no longer changed.

Measurement of Binding of Mn2+ by GTP and GDP—The association constants for the binding of Mn2+ to GDP and GTP under the conditions of the experiments were measured by EPR (2 °C) and by the resin-binding method (2 °C) of Walaas (24), as modified by Jenkins (25). Bio-Rad AG MP-1 resin was used in place of the Dowex 1-2X2 recommended by Jenkins. The apparent equilibrium constants were calculated for contaminating GMP in the GTP samples and the binding of Mn2+ by contaminating GDP in the GTP samples. The following results were obtained: KDGD = 6.5 × 108 M-1 (EPR) and KDGP = 5.6 × 104 M-1 (EPR) - 5.2 × 104 M-1 (resin).

RESULTS

The time course of the exchange of Mn2+ for Mg2+ bound to tubulin was investigated to test for complete and rapid binding. PC-tubulin (in the absence of excess nucleotide) with predominantly GTP at the E-site (TBGT) and tubulin with

![Fig. 1. Exchange of Mn2+ for endogenous Mg2+ bound to tubulin versus time.](image-url)

The solid line through the GXP/TB data is a least-squares fit of the data (X, O); the other lines represent trends of the data. A composite kinetic analysis of nine experiments monitoring the change in GXP/TB versus time at 0 °C in the presence of Mg2+, Mn2+, or no added metal gives a first-order rate constant of 8.55 × 10-10 min-1, b, the total bound metal (Mg2+ + Mn2+) per total bound GTP/(N- and E-sites) is plotted versus time. The line is a least-squares fit of the data. By assuming first-order kinetics, these data give an apparent rate of loss of metal of 6.92 × 10-7 min-1.
Divalent Cations by Tubulin

Exchange of Divalent Cations by Tubulin

FIG. 2. Kinetic analysis of apparent Mg2+ dissociation rate and apparent Mn2+ association rate. a, the log [Mg/TB] versus time (minutes) from the data in Fig. 1. The slopes are the Mg2+ dissociation rate for TBGDP (A; 0.0323 min-1) and TBGTP (A; 0.0203 min-1). A mean value of 0.0294 ± 0.0022 min-1 from four experiments was obtained. b, the log [Mn2+] versus time (minutes) from a series of EPR kinetic experiments. A, C, 45.10 µM TBGDP; ○, 43.01 µM TBGDP with 100 µM total Mn2+. The apparent Mn2+ association rate is independent of [protein] (25.20–58.46 µM) and [Mn2+] (50 µM to 1.0 mM). When analyzed as a first-order process, the apparent Mn2+ dissociation rate is 0.0494 ± 0.0063 min-1 for these three experiments and 0.0388 ± 0.0093 min-1 when 10 experiments (including the data from Fig. 1) with both TBGTP and TBGDP are averaged. When corrected for protein denaturation (8.55 × 104 min-1), the apparent Mn2+ association rate (0.0212 min-1) approaches the apparent Mn2+ dissociation rate. Thus, the apparent on rate for Mn2+ is kinetically limited by the apparent off rate for Mg2+.

will be presented elsewhere.3) The values of the Mn2+ affinity for the E-site that is inferred from those experiments are 4.1–4.9 × 1012 M-1 for Mn2+ binding to TBGTP and 3.9–6.6 × 1012 M-1 for Mn2+ binding to TBGDP. As anticipated, these affinities are stronger than the corresponding Mg2+ affinities.4 They are also consistent with the data in Fig. 3.

That the slow exchange and binding of Mn2+ to tubulin do not involve a significant conformational change in the protein

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4 The Mg2+-tubulin association constants were originally determined by fitting competitive binding data (1) to a scheme that did not include the N-site or seven weak sites. Refitting those data to the complete scheme at the same total Mg2+ concentrations gives the following values: K_{TTP} = 2.2–5.0 × 1012 M-1 for Mn2+ binding to TBGTP and 3.9–6.6 × 1012 M-1 for Mn2+ binding to TBGDP. As anticipated, these affinities are stronger than the corresponding Mg2+ affinities.

The qualitative conclusions of this study are not affected by these considerations.
Each spectrum was scanned four times to obtain an average spectrum. The experiment was repeated on two successive days, but in reverse order to correct for time variations. The two experimental spectra for each sample were then averaged to give the final spectra shown. The maximum deviation at 350 nm represents the initial protein concentration (C_0) for Mg^{2+} (O), and Mn^{2+} (X)-containing solutions (see inset). Within the accuracy of this method, these results are considered to be identical. The extrapolated intercept is at 0.509 mg/ml.

is apparent in Fig. 4, where the CD spectra of tubulin with Mn^{2+} or Mg^{2+} and with GDP or GTP are compared. All four spectra are nearly identical. Similar experiments with 16 mm Mg^{2+}, 100 mm GDP or GTP, and 5 mm tubulin also showed no significant change in the CD spectrum (data not shown). This result is in contrast to the differences observed by Howard and Timasheff (26). Buffers or temperature may account for the discrepancy. The protein is assembly-competent in the presence of Mn^{2+}. In a 2 mm glycerol assembly system, Mn^{2+} tubulin and Mg^{2+}-tubulin react identically to warm and cold stimulus, giving rise to identical rates of assembly and disassembly as well as overall changes in turbidity per milligram polymerized (Fig. 5). The relative affinity of GTP for the tubulin E-site versus GTPMg (or GTPMn) (1) clearly implies that the concentration of GTPMg (or GTPMn) is the critical species in inducing microtubule assembly. The only polymers visible by electron microscopy at the plateau were microtubules (data not shown). Buttlaire et al. (4) had reported different rates of assembly (10% dimethyl sulfoxide and 1 mm EGTA) in the absence of any divalent cation, but similar extents of assembly, with Mg^{2+} and Mn^{2+}. The changes in turbidity they observed (>0.43 ΔT_A200/ mg polymerized) are larger than those shown here (0.23 ΔT_A200/ mg polymerized) and may represent the presence of some nonmicrotubular forms (27). When tubulin is cycled in the presence of Mg^{2+}, Mn^{2+}, or Mg^{2+} and Mn^{2+} (Table 1), the final assembly-competent protein contains amounts of bound metal, (Mg^{2+} + Mn^{2+})/bound GTP, that are slightly larger than those shown in Fig. 1, but still consistent with high affinity metal-binding sites being at or near GTP-binding sites on both subunits. Metal-free GTP at the N-site may exist transiently prior to denaturation, and it may be removed by cycling. Similar levels of Mn^{2+} binding to tubulin after cycling were also observed by Buttlaire et al. (4).

The slow exchange of Mn^{2+} for Mg^{2+} at the N-site is consistent with a mechanism involving protein "breathing", where Mn^{2+} binding (exchange) apparently involves the diffusion of Mn^{2+} through the protein or along the αβ-interface, the dissociation rate of Mg^{2+} binding from the protein surface, the association rate of Mn^{2+} binding to the protein surface, and the diffusion of Mn^{2+} to the N-site. The Mg^{2+} dissociation rate is extremely slow (0.020 min^{-1}) and approximately equal to the Mn^{2+} association rate, suggesting that the exchange process is kinetically limited by a slow isomerization involving the protein, possibly a local unfolding of the structure (28).
Exchange of Divalent Cations by Tubulin

**TABLE I**

| Tubulin-bound nucleotides and metals |
|--------------------------------------|
| Tubulin (22.69 μM) was equilibrated into 0.1 M Pipes, pH 6.9, with 2 mM glycerol, 0.5 mM EGTA, 1 mM GTP, and 1 mM MgSO₄, 1 mM MnCl₂, or 500 μM each MgSO₄ and MnCl₂. The samples were polymerized for 20 min at 35 °C, pelleted at 27,000 × g for 20 min, resuspended in the same buffer without glycerol, spun at 2,000 × g for 10 min to clarify, and then column-centrifuged into 0.1 M Pipes, pH 6.9. Bound Mg²⁺ and Mn²⁺ were determined by atomic absorption of the protein solution. Bound nucleotide was determined as described under "Materials and Methods."|

| Treatment | GXP/TB | GDP/TB | GTP/TB | Mg²⁺/TB | Mn²⁺/TB | (Mg²⁺ + Mn²⁺)/GTP |
|-----------|--------|--------|--------|---------|---------|-----------------|
| Initial   | 1.909  | 0.811  | 1.698  | 1.170   | 1.066   |
| Mg²⁺     | 1.795  | 0.428  | 1.367  | 1.381   | 1.010   |
| Mn²⁺     | 1.910  | 0.162  | 1.748  | 0.303   | 1.622   | 1.101           |
| Mg²⁺ + Mn²⁺ | 1.964  | 0.190  | 1.774  | 0.520   | 1.255   | 1.001           |

or heterodimer dissociation (18). To test the hypothesis that the exchange is facilitated by dissociation, experiments were conducted at tubulin concentrations near the Kᵦ for subunit dissociation (~5 μM). Rather than attempting to monitor metal exchange, we followed GDP binding by HPLC. Tubulin (initially 46% GDP) was incubated with 500 μM GDP at 0 °C. At various times, aliquots were column-centrifuged and analyzed (see "Materials and Methods") for bound GDP and bound GTP. If dimer dissociation allows metal exchange, then it should also allow GDP to exchange for GTP at the E-site. The fraction of bound GDP did not change during more than 6 h (~2.46%). When 10 mM EDTA was included, the same result was observed, but the total binding of nucleotide (GXP/TB) dropped at a rate four times faster than in the absence of EDTA. This observation suggests that removing metal from the N-site rapidly leads to denaturation of both subunits. If dimer dissociation were occurring, then either GDP cannot bind to the α-subunit, i.e. the N-site, or the subunit denatures faster than GDP binding can occur. Sternlicht et al. (2) concluded that α-tubulin lacks the guanine-specific binding loop (loop III) found in β-tubulin and suggests that "the GTP-binding domain in α-tubulin is conformationally different from that in β-tubulin." This difference may in part explain the inability of GDP to replace GTP on the α-subunit, even though both the α- and β-tubulin GTP-binding domains do appear to bind GTP in an Mg²⁺-dependent manner. Additional support for an exchange mechanism involving a slow isomerization or unfolding of the protein comes from a lack of concentration dependence in the measured apparent Mg²⁺ dissociation rate and Mn²⁺ association rates. Over a protein concentration of 25-200-68.46 μM and an Mn²⁺ concentration of 50-1000 μM, the apparent rates, when corrected for denaturation, were nearly identical. This observation supports the one-to-one exchange of Mn²⁺ for Mg²⁺ by a process that is kinetically limited by a protein isomerization step.

**DISCUSSION**

The results described above show that Mn²⁺ binds strongly to the GTP-occupied E-site and weakly to the GDP-occupied E-site of tubulin. In addition, Mn²⁺ can bind to the GTP-occupied N-site of tubulin by slowly exchanging for the Mg²⁺ usually bound there. The binding of Mg²⁺ has previously been measured by competitive binding studies (1), and its affinity for the E-site of TBGDP was found to be much weaker than its affinity for the E-site of TBGTP. On the basis of the affinities of Mn²⁺ for GDP and GTP and in the absence of steric constraints, it would be expected that Mn²⁺ will bind with the same relative affinities to the GDP- and GTP-occupied E-site. The binding data presented in Fig. 3 are consistent with this assertion. Competitive binding data for Mn²⁺, GTP, and GDP as well as nonlinear least-squares fitting and a detailed error analysis of the confidence intervals for the appropriate association constants will be presented elsewhere. Here we can summarize that the association constants for binding to GTP at the E- and N-sites are 0.7-1.1 × 10⁶ M⁻¹ for Mg²⁺ and 4.1-4.9 × 10⁵ M⁻¹ for Mn²⁺. For the binding to GDP at the E-site, the affinities are 2.0-2.3 × 10⁵ M⁻¹ for Mg²⁺ and 3.9-6.6 × 10⁵ M⁻¹ for Mn²⁺.

The evidence for binding and exchange of divalent cations at the N-site rests on two findings. First, as shown in Fig. 1b and Table I, there appears to be a one-to-one correspondence between bound GTP and bound cation. Jemiolo and Grisham (5) established that the apparent total number of high affinity sites varies with the amount of nucleotide bound to tubulin, but included no measurement of amounts of GTP or GDP. Rather, they assumed that tight binding occurred to either GTP or GDP and only when the nucleotide was bound to the E-site. Our results shows that these assumptions were too simple. Second, as shown in Fig. 3, when the E-site is occupied by GDP, tubulin still has a single high affinity metal-binding site. Because of the homology of the α- and β-subunits of tubulin, one would expect similar GTP-binding sites, i.e., sites that require Mg²⁺ coordination of comparable affinities. Both subunits contain an aspartic acid in position 205 that is in a sequence that has been identified as an Mg²⁺ coordination site by homology with other nucleotide-binding proteins (2). EDTA does not completely remove bound Mg²⁺ unless the protein is first denatured (4). Our results explain this observation as being due to the slow release of Mg²⁺ from the N-site. The finding that there is both a rapidly exchanging and a slowly exchanging site strongly supports the hypothesis that the N-site metal, unlike the N-site nucleotide, can be exchanged. This phenomenon has not been widely noted before, probably because the process of removing free metal in preparing the sample causes the dissociation of E-site GTP and the subsequent binding of GDP there (1). Himes et al. (7) have observed one tightly bound Mg²⁺ and one tightly bound Co²⁺ per tubulin. Definitive location of the divalent cation awaits application of more sensitive techniques, e.g. 35-GHz EPR (29) or NMR (11).

The CD (Fig. 4) and polymerization (Fig. 5) data clearly demonstrate no large conformational or functional changes in the protein when Mn²⁺ is exchanged for Mg²⁺ and binds to the guanine nucleotide sites. The absence of a major change in secondary structure between TBGDP and TBGTP is not necessarily inconsistent with the model hypothesized by Sternlicht et al. (2). The number of tightly bound metals per bound GDP observed after a polymerization cycle (~1.0; see Table I) also suggests that this one-to-one correspondence is typical of native, functional tubulin. These results explain the Mg²⁺ composition of TBGDP after gel filtration (1) or phosphocellulose chromatography (16) and are, in fact, consistent with the weak binding of Mg²⁺ to E-site GDP. It is not clear whether other metals will bind to the E-site...
in the same nucleotide-dependent manner. Zn$^{2+}$, Co$^{2+}$, Cr$^{3+}$, and Al$^{3+}$ (30) are all believed to bind to the tubulin E-site and to stimulate assembly. It has also been suggested that weak binding sites are required to promote the formation of both microtubules and sheets, with sheets being favored at higher metal concentrations. The data are also consistent with the binding of these metals to the N-site since slow exchange (4, 5, 7) or nonexchangeable characteristics have been described. Our data are consistent with the idea that N-site metal is directly important in protein stability, and thus only indirectly in microtubule formation. Within the microtubule, metal at both sites may be slowly exchangeable. Carlier and Pantaloni (31) have reported the slow release of P$_i$ from actin filaments subsequent to ATP hydrolysis. The mechanism may be similar to that reported here. Al$^{3+}$ apparently promotes microtubule assembly but not GTP hydrolysis (30, 32). The extra charge on Al$^{3+}$ might possibly prevent the hydrolysis step by stabilizing the TBGTP conformation over an intermediate enzymatic conformation.

In summary, high affinity divalent cation (Mg$^{2+}$ and Mn$^{2+}$) binding to tubulin occurs at GTP-occupied guanine nucleotide-binding domains. The strength of binding to GDP at the exchange of metal at the N-site may provide a new means of interpretation of more quantitative structural studies of the E-site is comparable to the strength of binding to GDP in solution and suggests little or no coordination of the metal with the protein. Metal binding to the TBGTP E-site is consistent with a coordination with the protein and possibly involves a conformational change in the protein. (Kinetically, it may actually involve GTPMg binding to tubulin.) The slow exchange of metal at the N-site may provide a new means of differentially labeling tubulin and represents a new kind of evidence of homology between the a- and b-subunits: the Mg$^{2+}$-dependent binding of GTP. This heterogeneity in high affinity metal-binding sites on tubulin may complicate the interpretation of more quantitative structural studies of the metal-nucleotide coordination environment by, for example, 35-GHz EPR. Alternatively, if the sites are identical, it may increase the sensitivity of such experiments.

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Note Added in Proof—Carlier et al. (Carlier, M.-F., Didry, D., Melki, R., Chabre, M., and Pantaloni, D. (1988) Biochemistry 27, 3555-3559) recently reported the slow release of P$_i$ from microtubules after GTP hydrolysis, and the stabilization of microtubules by millimolar concentrations of inorganic phosphate. Although no mention is made of the kinetics and the role of Mg$^{2+}$ release by these authors, their results are consistent with our suggestion that the metal ions at both nucleotide binding sites are slowly exchangeable within the microtubule.