Divalent Cation-Nucleotide Complex at the Exchangeable Nucleotide Binding Site of Tubulin*  

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Tubulin was first treated with alkaline phosphataseagarose to vacate the exchangeable nucleotide binding site and then reacted with manganese binding sites by Mn(II) EPR. Buttlaire et al. (1980) J. Biol. Chem. 255, 2164-2168 have shown that high affinity manganese binding occurs at a single site normally occupied by magnesium. We report that the number of high affinity manganese binding sites per mol of tubulin depends on the number of occupied exchangeable nucleotide binding sites. Thus, removal of nucleotides results in a loss of high affinity manganese binding sites. The EPR spectra of manganese bound to tubulin and to GTP are found to be qualitatively similar. These data indicate that high affinity manganese binding is the result of the formation of a metal-nucleotide complex at the exchangeable nucleotide binding site. In addition it was found that zinc, cobalt, and magnesium bind with approximately equal affinity to this site whereas calcium binds only weakly.

Microtubules are intracellular organelles found in eucaryotic cells. Morphologically they are characterized as long, hollow, cylindrical structures of approximately 25-nm outside diameter. They are known to be involved in cell motility as components of cilia and flagella, in intracellular movement as components of the axonal transport system and the mitotic apparatus, and in maintenance of cell shape as a cytoskeletal element (for reviews, see Refs. 1 and 2).

The principal protein component of microtubules, tubulin, is a $M_r = 110,000$ dimer of a subunit and $M_r = 55,000$. Tubulin, isolated from mammalian brain, has been extensively studied and characterized. It has two guanine nucleotide binding sites (3). The two sites are distinguishable by the exchangeability of the nucleotides. Thus, the nonexchangeable site contains GTP that does not exchange with free nucleotide whereas the exchangeable site nucleotide, usually either GTP or GDP, can be exchanged with free nucleotides under certain conditions (4). The two sites are further distinguished by the fact that during the polymerization of tubulin into microtubules, the exchangeable site GTP is hydrolyzed to GDP with the release of phosphate while the nonexchangeable site GTP is unaltered (4-5). Therefore, the exchangeable nucleotide binding site is involved in regulating tubulin polymerization and may be an important in vivo control point for microtubule formation. In addition to the nucleotide sites, tubulin has binding sites for other low molecular weight molecules such as colchicine and vinblastine, for proteins such as the microtubule-associated proteins, and for divalent cations such as magnesium, calcium, and zinc.

The divalent cation binding sites may also be important regulators of tubulin polymerization. For example, magnesium is required for the in vitro assembly of microtubules (6, 7), while zinc induces tubulin to assemble into alternate polymer structures such as sheets (8). Finally, calcium inhibits tubulin polymerization (6, 7). Tubulin contains one tightly bound magnesium (6) that is probably exchangeable because manganese replaces magnesium at this site (9). Since manganese is paramagnetic its binding can be studied by electron paramagnetic resonance. Manganese can be used as a probe for magnesium binding sites on tubulin because it supports polymerization and it replaces the tightly bound magnesium (9).

We have determined from manganese EPR studies that the high affinity magnesium binding site consists of a metal-nucleotide complex at the exchangeable nucleotide site. Removal of the exchangeable nucleotide results in the loss of the high affinity magnesium binding site. In addition, we have characterized the interaction of zinc, cobalt, and calcium with this high affinity site.

**EXPERIMENTAL PROCEDURES**

**Tubulin Isolation**—Tubulin was isolated from bovine brain extracts by ammonium sulfate fractionation and DEAE-Sephadex ion exchange chromatography as described by Weisenberg et al. (3, 10), followed by magnesium precipitation (11). Briefly, tubulin was precipitated from brain extracts by 30% and 41% saturated ammonium sulfate (4°C), adsorbed to DEAE-Sephadex A-50 and eluted from the resin with a KCl step gradient of 0.4 to 0.8 M KCl. At this stage, protein was concentrated by ammonium sulfate precipitation and stored at -80°C. Pellets were resuspended in 10 mM sodium phosphate, pH 6.8, 5.0 mM MgCl$_2$, and 0.1 mM GTP, desalted into this solution, and precipitated with 28 mM MgCl$_2$ as described by Lee et al. (11). The purified tubulin was dialyzed against 10 mM Na-Pipes, pH 6.8, 0.5 mM MgCl$_2$, and 0.1 mM GTP, dialyzed into this solution, and precipitated with 28 mM MgCl$_2$ as described by Lee et al. (11). The purified tubulin was dialyzed against 10 mM Na-Pipes, pH 6.8, 5.0 mM MgCl$_2$, and 0.1 mM GTP, and 1.0 mM sucrose and stored at -20°C. Samples were clarified by centrifugation and desalted into 50 mM Na-Pipes, pH 6.8 as needed.

**GTP Concentration**—The total concentration of guanine nucleotides was measured by ultraviolet absorption spectroscopy of 5% perchloric acid-soluble material. Protein samples were adjusted to 5% perchloric acid and then maintained at 4°C for 15 min. The resulting precipitate was removed by centrifugation at 2000 $g$ for 10 min and if necessary the supernatant was clarified with a second centrifugation. The concentration of guanine nucleotides was calculated from the absorbance at 256-nm wavelength using a molar extinction coeff.

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Most of the magnesium found appears to be tightly bound. When tubulin, initially in buffer with 10 mM EDTA, is desalted into 50 mM Na-Pipes, pH = 6.8, and analyzed for magnesium, we find approximately 0.9 mol of magnesium/mol of tubulin, as shown in Table I. However, when tubulin was treated with 1.0 mM MnCl₂ in place of EDTA and then desalted and analyzed for magnesium only, 0.1 mol/mol of tubulin is detected. These results indicate that although the magnesium is tightly bound it is probably exchangeable. The 0.1 mol of magnesium/mol to tubulin reported above is probably not significant. We find that when tubulin is treated with 6 mM guanidine and 10 mM EDTA a small signal is observed during atomic absorption spectroscopy, as shown in Table I.

Removal of GTP from the Exchangeable Nucleotide Site of Tubulin—We wanted to explore the possibility that the exchangeable nucleotide binding site and the high affinity magnesium-manganese binding site are related. With this in mind, we prepared samples of tubulin with the exchangeable nucleotide binding site partially vacated. This can be accomplished by alkaline phosphatase treatment (15). Since the samples were to be used for manganese binding studies it was essential to avoid contamination of the protein with soluble alkaline phosphatase. Therefore, an agarose-coupled preparation of the enzyme was used. While GTP is removed by this treatment, the amount depends on the incubation time and on the amount and activity of the coupled enzyme. The incubation times required are longer than those reported for the soluble enzyme. Therefore, we included glycerol in the reaction mixture to prevent protein denaturation during the incubation period. The data presented in Table II indicate that glycerol does protect against denaturation. Samples of tubulin were incubated with alkaline phosphatase agarose either with or without 3.4 mM glycerol, and subsequently desalted into 50 mM Na-Pipes, pH = 6.8. Portions of each

| Table I |
| Tubulin-bound magnesium |
| Tubulin in 50 mM Na-Pipes, pH = 6.8, was either desalted directly into 50 mM Na-Pipes as a control or treated with either 10 mM EDTA or 1.0 mM MnCl₂ or 10 mM EDTA and 6 mM guanidine HCl and subsequently desalted into 50 mM Na-Pipes, pH = 6.8. The amount of tubulin-bound magnesium was measured by atomic absorption spectroscopy and is reported as the number of moles of magnesium/mol of tubulin along with a range that reflects the uncertainty in the magnesium concentration. |
| Treatment | Amount |
|---|---|
| Control | 1.13 ± 0.08 |
| 10 mM EDTA | 0.88 ± 0.03 |
| 1.0 mM MnCl₂ | 0.06 ± 0.02 |
| 10 mM EDTA, 6 M guanidine HCl | 0.11 ± 0.05 |

| Table II |
| GTP binding to alkaline phosphatase agarose-treated tubulin: effect of glycerol |
| Tubulin was treated with alkaline phosphatase agarose in 50 mM Na-Pipes, pH = 6.8, 0.03 mM ZnCl₂, 0.2 mM NaCl, 1.0 mM GDP-CP, and either with 3.4 mM glycerol or without glycerol (control). The samples were desalted into 50 mM Na-Pipes, pH = 6.8, and portions of each were analyzed for GTP. The results are shown in the second column. To the remainder of the samples, GTP was added to 1.0 mM. The samples were desalted and analyzed for GTP with the results shown in the third column. As a reference, tubulin not treated with alkaline phosphatase but processed otherwise as described above contains between 1.8 and 1.95 mol of GTP/mol of tubulin. |
| Before GTP addition | After GTP addition |
| mol GTP/mol tubulin |
| Control | 1.34 | 1.60 |
| Glycerol-treated | 1.31 | 1.93 |
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Fig. 1. Scatchard plot of manganese binding to alkaline phosphatase agarose-treated tubulin. Tubulin was treated with alkaline phosphatase agarose in order to remove GTP from the exchangeable nucleotide binding site. Two samples were prepared with 1.15 (crosses) and 1.83 (closed circles) mol of GTP/mol of tubulin and tested for manganese binding sites as described under "Experimental Procedures." The data are presented as a Scatchard plot. The curves through the data are calculated from the equation

$$
\bar{v} = n_1 K_1 F/(1 + K_1 F) + n_2 K_2 F/(1 + K_2 F)
$$

where the $n$ refers to the number of sites in a particular class and $K$ is the association constant. The high affinity sites are assigned subscript 1, and $F$ is defined by "Experimental Procedures." Terms with subscript 2 represent binding to the lower affinity class of sites. Since the data presented in Fig. 1 are restricted to low values of $\bar{v}$, they do not contain much information about the lower affinity class. Therefore, in a separate experiment using a higher protein concentration we obtained data (not shown) at higher values of $\bar{v}$ ($\bar{v} = 1.0$ to 5.0). Using the curve fitting programs of Reed et al. (17) we obtained best fit parameters for the low affinity class of $n_1 = 8.0$, $K_1 = 2.22 \times 10^5$ M$^{-1}$, and for the sample with 1.83 mol of GTP/mol of tubulin (closed circles) and $n_1 = 0.20$, $K_1 = 4.0 \times 10^6$ M$^{-1}$ for the data points indicated by $n_2 = 0.65$, $K_2 = 8.0 \times 10^5$ M$^{-1}$, $n_2 = 8.0$, $K_2 = 8.0 \times 10^5$ M$^{-1}$ for the points indicated by the closed circles. A third alkaline phosphatase-treated sample initially containing 1.11 mol of protein-bound GTP/mol of tubulin and desalted to remove unbound nucleotides. This sample, now containing 1.56 mol of GTP/mol of protein was tested for manganese binding sites. The data are shown as the open circles.

The extent to which GTP rebinds to alkaline phosphatase-agarose treated tubulin varies but is enhanced when glyceral is employed.

Manganese Binding—We prepared two samples of GTP-deficient tubulin and tested them for manganese binding sites. The data are presented in Fig. 1 as a Scatchard plot. The closed circles are for tubulin with $1.83 \pm 0.20$ mol of protein-bound GTP/mol of tubulin and the crosses are for tubulin with $1.15 \pm 0.20$ mol of protein-bound GTP/mol of tubulin. Both data sets indicate manganese binding to at least two classes of sites. The lines drawn through the data sets were calculated assuming that the system contains two classes of noninteracting sites. The equation representing this situation is:

$$
\bar{v} = n_1 K_1 F/(1 + K_1 F) + n_2 K_2 F/(1 + K_2 F)
$$

where $n$ refers to the number of sites in a particular class and $K$ is the association constant. The high affinity sites are assigned subscript 1, and $F$ are defined under "Experimental Procedures." Terms with subscript 2 represent binding to the lower affinity class of sites. Since the data presented in Fig. 1 are restricted to low values of $\bar{v}$, they do not contain much information about the lower affinity class. Therefore, in a separate experiment using a higher protein concentration we obtained data (not shown) at higher values of $\bar{v}$ ($\bar{v} = 1.0$ to 5.0). Using the curve fitting programs of Reed et al. (17) we obtained best fit parameters for the low affinity class of $n_1 = 8.0$, $K_1 = 2.22 \times 10^5$ M$^{-1}$. These values were subsequently used to obtain best fit parameters for the high affinity sites. We find $n_1 = 0.65$, $K_1 = 8.0 \times 10^5$ M$^{-1}$, $n_2 = 8.0$, $K_2 = 2.22 \times 10^5$ M$^{-1}$ for the sample with 1.83 mol of GTP/mol of tubulin (closed circles) and $n_1 = 0.20$, $K_1 = 4.0 \times 10^6$ M$^{-1}$, $n_2 = 8.0$, $K_2 = 8.0$ for the data points indicated by $n_2 = 0.65$, $K_2 = 8.0 \times 10^5$ M$^{-1}$, $n_2 = 8.0$, $K_2 = 8.0 \times 10^5$ M$^{-1}$ for the points indicated by the closed circles. A third alkaline phosphatase-treated sample initially containing 1.11 mol of protein-bound GTP/mol of tubulin and desalted to remove unbound nucleotides. This sample, now containing 1.56 mol of GTP/mol of protein was tested for manganese binding sites. The data are shown as the open circles.

It is apparent that the two curves in Fig. 1 are shifted along the $\bar{v}$ axis and this behavior is explained by a difference in the average number of high affinity sites. Furthermore, the average number of high affinity sites depends on the moles of protein-bound GTP/mol of protein. More specifically, the number of high affinity manganese sites correlates with the occupancy of the exchangeable nucleotide binding site. Thus, for the sample containing $1.15 \pm 0.20$ mol of GTP/mol of tubulin, the GTP is distributed into 1.0 mol at the nonexchangeable and 0.15 mol at the exchangeable nucleotide binding sites and the number of high affinity manganese sites is 0.20.

It is possible that GTP-deficient tubulin loses high affinity manganese binding sites because of irreversible denaturation of tubulin in the absence of GTP. However, we believe that this is not the case because we have shown that GTP-deficient tubulin rebinds GTP. To further eliminate the possibility of denaturation we performed the following experiment. An excess of GTP was added to a sample of alkaline phosphatase agarose-treated tubulin originally containing 1.11 $\pm 0.20$ mol of GTP/mol of tubulin. The sample was then desalted to remove unbound nucleotide, analyzed for protein-bound GTP, and tested for an increase in high affinity manganese binding sites.

GTP analysis showed that the tubulin now contained 1.56 $\pm 0.20$ mol of GTP/mol of tubulin. This is evidence for GTP binding to approximately 50% of the initially vacated exchangeable nucleotide binding sites. The manganese binding data are shown in Fig. 1 as open circles. Fig. 1 shows that manganese binding to this tubulin sample with 1.56 mol of GTP/mol of protein is characterized by an intermediate number of high affinity manganese sites.

EPR Spectra of Bound Manganese—The results of the manganese-binding studies indicate that the average number of high affinity manganese binding sites in the tubulin sample depends on the occupancy of the exchangeable nucleotide binding site. It is possible that manganese bound to the high affinity site is coordinated to the phosphate groups of GTP or GDP located at the exchangeable nucleotide binding site. If this is the case we then expect that the EPR spectra of manganese bound to GTP or GDP should be similar to the spectrum of manganese bound to tubulin. Therefore, we examined the EPR spectra of manganese bound to GTP, GDP, and tubulin.

The EPR spectra of solutions containing 50 mM Na-Pipes, pH = 6.8, 1.0 mM MnCl$_2$, and 10 mM of either GTP or GDP were recorded. The spectrum of Mn(II)-GTP is shown at the top of Fig. 2. Mn(II)-GDP gives a qualitatively similar result and is not shown. The bottom trace is the spectrum of 1.0 mM MnCl$_2$ in 100 mM Na-Pipes, pH = 6.8.

The EPR spectrum of manganese bound to tubulin is shown directly below Mn(II)-GTP in Fig. 2. This tubulin-bound manganese sample was prepared by first resuspending a magnesium-precipitated tubulin pellet in 10 mM Na-Pipes, pH = 6.8, 1.0 mM sucrose, 0.1 mM GTP, and 0.1 mM MnCl$_2$, and then dialyzing it against a 500-fold volume excess of this solution at 4 °C for 8 h. The sample was then dialyzed for 16 h against buffer with sucrose and 0.01 mM GTP and 0.01 mM MnCl$_2$ and for 16 h against buffer with sucrose and GTP (but no manganese). These steps were performed first to exchange manganese for magnesium and second to lower the free manganese concentration. The third trace from the top in Fig. 2 is the EPR spectrum of the final dialysis buffer where no significant free manganese is apparent.

The protein concentration of the sample is 0.144 mM (15.8
peting against magnesium for this site. Once manganese is such concentration of added divalent cation required to displace half by magnesium. Thus manganese binds by successfully com-

bound it can in turn be displaced by other divalent cations scanned time, 8 min, time constant, 1.0 s; gain, 4 x 10^7 at a modulation frequency of 100 kHz. The second trace from the top is the spectrum of manganese bound to tubulin. Magnesium-precipitated tubulin was resuspended in a solution containing 10 mM GTP, and 0.1 mM MnCl_2 and dialyzed against a 500-fold excess of this solution for 8 h at 4 °C. Dialysis was continued for 16 h against a similar solution containing 0.01 mM GTP (and no MnCl_2). The sample was clarified by centrifugation. EPR spectra of the tubulin sample and the final dialysate are shown as free Mn^2+. The EPR spectrum of manganese bound to GTP is as top truce. This spectrum was recorded from a solution containing 10 mM Na-Pipes, pH = 6.8. The bottom trace represents the spectrum of 0.1 mM MnCl_2 in 100 mM Na-Pipes, pH = 6.8. These two spectra were recorded at room temperature in a Varian E-231 cavity under the following conditions: field setting, 3000 G, range, 2000 G, modulation amplitude, 10 G, scan time, 8 min; time constant, 1.0 s; gain, 4 x 10^7 at a modulation frequency of 100 kHz.

The exchangeable nucleotide binding site is involved in tubulin polymerization because hydrolysis of GTP normally occurs at this site during microtubule formation (4, 5). The substrate for hydrolysis is probably magnesium ATP. This is reasonable because many enzymes that hydrolyze ATP use magnesium ATP as the substrate (18). In addition, magnesium is required for tubulin polymerization (6, 7) and magnesium binds tightly to tubulin at a single site (6, 9).

Buttlaire et al. (9) first showed that manganese can be used to study magnesium binding sites on tubulin. This study established that manganese both supports polymerization and binds to the high affinity magnesium site. In the study presented here we establish that the high affinity magnesium site and the exchangeable nucleotide binding site are related.

Tubulin, pretreated with alkaline phosphatase agarose to alter the occupancy of nucleotide at the exchangeable nucleotide binding side (15), was tested for manganese binding sites. The manganese binding curves that are presented as Scatchard plots in Fig. 1 indicate the presence of at least two classes of manganese binding sites. We are interested in the high affinity sites which are responsible for the region of each curve with a steep slope at low values of v. It is apparent that the curves are shifted relative to one another and this behavior can be explained by a variation in the number of high affinity binding sites/mol of tubulin.

FIG. 2. X band EPR spectra of Mn(II)-GTP, Mn(II)-tubulin, and free Mn(II). The EPR spectrum of manganese bound to GTP is shown as the top trace. This spectrum was recorded from a solution containing 10 mM GTP and 1.0 mM MnCl_2 in 50 mM Na-Pipes, pH = 6.8. The bottom trace represents the spectrum of 0.1 mM MnCl_2 in 100 mM Na-Pipes, pH = 6.8. These two spectra were recorded at room temperature in a Varian E-231 cavity under the following conditions: field setting, 3000 G, range, 2000 G, modulation amplitude, 10 G; scan time, 8 min; time constant, 1.0 s; gain, 4 x 10^7 at a modulation frequency of 100 kHz. The second trace from the top is the spectrum of manganese bound to tubulin. Magnesium-precipitated tubulin was resuspended in a solution containing 10 mM Na-Pipes, pH = 6.8, 1.0 mM sucrose, 0.1 mM GTP, and 0.1 mM MnCl_2 and dialyzed against a 500-fold excess of this solution for 8 h at 4 °C. Dialysis was continued for 16 h against a similar solution containing 0.01 mM GTP and 0.01 mM MnCl_2. Finally the sample was dialyzed for 16 h against a solution containing 0.01 mM GTP (and no MnCl_2). The sample was clarified by centrifugation. EPR spectra of the tubulin sample and the final dialysate are shown as the second and third traces, respectively. The protein concentration is 15.8 mg/ml. The protein sample contains 9.9 x 10^{-4} M Mn bound to tubulin. The spectra were recorded at room temperature in a Varian E-238 cavity under the following conditions: field setting, 3000 G; range, 2000 G; modulation amplitude, 12.5 G; scan time, 8 min; time constant, 2 s; gain, 2.5 x 10^7 at a modulation frequency of 100 kHz.

mg/ml; 110,000 daltons). Additionally we measured the total manganese concentration by EPR spectroscopy on a 5% perchloric acid extract of the sample and found 9.9 x 10^{-3} \pm 0.15 x 10^{-3} M manganese which corresponds to 0.7 mol of manganese/mol of tubulin.

Divalent Cation Specificity—The high affinity binding site observed in the manganese binding studies is initially occupied by magnesium. Thus manganese binds by successfully competing against magnesium for this site. Once manganese is bound it can in turn be displaced by other divalent cations such as magnesium, zinc, cobalt, and calcium. The total concentration of added divalent cation required to displace half of the bound manganese is a measure of the relative affinity of the cation for this site.

We measured the relative affinities of tubulin for the above mentioned divalent cations in competition experiments. Tubulin-bound manganese samples were prepared by desalting tubulin out of buffer with 2.0 mM MnCl_2 and 0.1 mM GTP and into 50 mM Na-Pipes, pH = 6.8. When the samples were then analyzed for manganese by EPR spectroscopy we found that, of the total perchloric acid-soluble manganese, approximately 5–10% was initially free. Upon addition of known amounts of either magnesium, zinc, cobalt, or calcium, the free manganese concentration increased indicating that the added cations displaced the protein-bound manganese. The free-manganese concentration was recorded and plotted as a percentage of the total manganese against the logarithm of the added divalent cation concentration. These results are shown in Fig. 3. Magnesium, zinc, and cobalt are of equal relative affinity with a concentration of approximately 0.05 mM (−log (5 x 10^{-5} M) = 4.3) required to displace 50% of the bound manganese. However calcium shows a 100-fold lower relative affinity. The calcium data points are shifted to a higher concentration with 50% competition occurring at approximately 5.0 mM (−log (5 x 10^{-5} M) = 2.3).

DISCUSSION

The exchangeable nucleotide binding site is involved in tubulin polymerization because hydrolysis of GTP normally occurs at this site during microtubule formation (4, 5). The substrate for hydrolysis is probably magnesium ATP. This is reasonable because many enzymes that hydrolyze ATP use magnesium ATP as the substrate (18). In addition, magnesium is required for tubulin polymerization (6, 7) and magnesium binds tightly to tubulin at a single site (6, 9).
The protein samples used in these experiments contain different amounts of GTP at the exchangeable nucleotide binding site. The variation in the number of high affinity manganese binding sites does in fact correlate with the average protein-bound GTP content of the samples. As the exchangeable nucleotide binding site is vacated, a reduction in the number of high affinity manganese binding sites is observed. This is shown in Fig. 1. Thus we conclude that the existence of a high affinity manganese binding site depends on the presence of GTP at the exchangeable nucleotide binding site.

EPR spectroscopy of manganese bound to tubulin supports this conclusion. It is likely that the manganese is coordinated to the protein at least in part through the phosphate groups of GTP. If this is the case, then the EPR spectra of manganese bound to tubulin and of manganese bound to GTP should be similar. The spectra shown in Fig. 2 indicate that manganese bound to tubulin is in a similar environment to that of manganese-GTP. Although minor differences do exist, the similarities are striking. Therefore, we conclude that the high affinity manganese binding site is made up in part of GTP bound at the exchangeable nucleotide binding site. This observation raises the question of how the nonexchangeable GTP is bound to tubulin. It appears that the nonexchangeable GTP does not bind divalent cations with high affinity. This is true for magnesium, as mentioned above. We analyzed tubulin for zinc and cobalt and found that under conditions in which tubulin contains 2 mol of GTP/mol of tubulin no zinc is found.

The only naturally abundant cation not tested is calcium, but it appears unlikely that under physiological conditions calcium binds tightly to tubulin (19).

Divalent cations other than magnesium do interact with tubulin. As examples, calcium inhibits polymerization while zinc and cobalt induce sheet formation. We tested the specificity of the high affinity magnesium binding site for these cations in order to determine whether it is likely that their mode of action in tubulin polymerization is through this site. Tubulin was prepared with manganese bound to the high affinity magnesium binding site. We then introduced known concentrations of either magnesium, zinc, cobalt, or calcium. When the added cations successfully compete with manganese for the binding site, a shift in the distribution of manganese from bound to free occurs. This can be followed by EPR spectroscopy. The concentration of added cation at which 50% of the total manganese occurs in the free form indicates the relative affinity of the cation for the site. As shown in Fig. 3, magnesium, zinc, and cobalt all bind with an apparent affinity of approximately 0.05 mM. Calcium on the other hand binds with an apparent affinity of approximately 5.0 mM.

These results may shed light on the mechanism of action of these cations. Zinc and cobalt may be inducing sheet formation by altering a tubulin-tubulin binding domain at the exchangeable nucleotide binding site. Calcium inhibition of tubulin polymerization is unlikely to occur by calcium binding to the exchangeable nucleotide binding site. The apparent affinity of calcium for this site is low and is expected to be even lower in the presence of physiological levels of magnesium.

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