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The Binding of Terbium Ions to Tubulin Induces Ring Formation[1]

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The intrinsic fluorescence excitation and emission spectra of chicken brain tubulin showed the characteristic tryptophan fluorescence. The emission spectrum of Tb^{3+} in the presence of tubulin and GTP excited at 295 nm, showed four peaks, with the maxima at 490, 545, and 586 nm and a minor peak around 620 nm. Titration of tubulin with Tb^{3+} was followed by the increment in luminescence at 545 nm and showed a sigmoidal curve where the initial lag interval and the maximal luminescence intensity depended on tubulin concentration. The presence of Mg^{2+}, Co^{2+}, and Zn^{2+} diminished both the sigmoidicity of the curve and the maximal luminescence intensity. Titration of tubulin with Tb^{3+} also produced a sigmoidal increase in turbidity, which was shifted to the left with respect to the luminescence curve. The dependence of turbidity on the wavelength of the Tb^{3+}-induced polymers revealed that the large structures formed were not microtubules. Electron microscopy of the aggregates induced by Tb^{3+} showed mainly a lattice of double rings with side-by-side contacts. These results indicate that Tb^{3+} induces principally double ring formation and that these rings (33 ± 2 nm external diameter) aggregate in large-ordered arrays. The luminescence of Tb^{3+} seems to be induced mainly by the aggregation of rings.

Tubulin is an asymmetric α–β dimer protein that self assembles *in vitro* into microtubules and other structures like rings and sheets of protofilaments, depending on both the concentration and the nature of the divalent metal used. Divalent cations play an important but poorly understood role in the assembly of microtubules. Microtubule assembly *in vitro* is promoted by Mg^{2+} (1), Mn^{2+} (2), and Al^{3+} (3) and inhibited by Ca^{2+} (4). Zn^{2+} and Co^{2+} induce tubulin to form sheets with considerably more than 13 protofilaments (5, 6). Zn^{2+} alters the alignment of protofilaments from a parallel to an antiparallel arrangement (7). Working with the purified protein and applying the Wyman linkage expression (8), Lee and Timasheff (9) found that microtubule growth was accompanied by the binding of 1 mol of Mg^{2+} per tubulin dimer added. The same holds true for the tubulin self-association to double rings (10, 11). These authors cautioned, however, that these results in themselves did not establish the direct participation of this ion in the microtubule growth process, since the ion binding may simple reflects an alteration of the electrostatic free energy of the protein upon polymerization. Gaskin (12) suggested that Zn^{2+}-induced structures are not due to a Zn^{2+}–GTP complex and that Mg^{2+} does not promote microtubule assembly only through a Mg^{2+}–GTP complex. Since its isolation from brain tissue by Weisenberg *et al.* (13), it has been known that tubulin contains two guanylyl nucleotide binding sites per 110,000 molecular weight dimer. One site has GTP tightly bound to it and it has been called nonexchangeable site, and the other site (E-site) can freely exchange GTP and GDP. Tubulin has 1 mol of Mg^{2+} tightly bound per mole of dimer (14) and several low affinity binding sites for this cation (10, 11).

Tb^{3+} luminescence has been widely used to investigate Mg^{2+} and Ca^{2+} binding sites of proteins (15). The luminescence emission intensity of Tb^{3+} undergoes a tremendous enhancement when this lanthanide binds to proteins (16). The present study was initiated to determine the effect of Tb^{3+} on tubulin assembly and its use as a probe for the binding of Mg^{2+} and other divalent cations such as Zn^{2+}, Co^{2+}, and Mn^{2+}, at sites different from that of the high affinity E-site. The luminescent properties of Tb^{3+} allowed the study of ring formation and ring aggregates by the interaction of this lanthanide with tubulin, mimicking the behavior of tubulin in the presence of Mg^{2+}.

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MATERIALS AND METHODS

Reagents. Terbium chloride was obtained from Aldrich Chemical Co., GTP (type III), glycerol, Mes[3] Sephadex G-25, DEAE-Sephadex A-50, and guanidine hydrochloride were purchased from Sigma Chemical Co. Guanidine hydrochloride was recrystallized by the procedure described by Nozaki and Tandford (17) and had a molar absorbance of 0.068 at 275 nm. Magnesium chloride and all the other divalent cations were analytical grade obtained from Merck AG Darmstadt.

Chicken brain tubulin purification. Chicken brains were dissected from freshly slaughtered animals, kept on ice, and used within 2 h. Tubulin was purified by the method of Weisenberg *et al.* (13, 18), as modified by Lee *et al.* (19). The stock protein was stored at −70°C in the presence of 1 M sucrose (20). The experimental samples were prepared by batch equilibration of the stock protein with 5–10 g of dry packed Sephadex G-25 (fine), equilibrated in the experimental buffer (0.1 M Mes, 3.4 M glycerol, pH 6.4) at 4°C, followed by filtration at 10°C through a Sephadex G-25 column, and equilibrated with the same buffer (14).

Fluorescence measurements. The steady-state fluorescence excitation and emission spectra were made on a Perkin–Elmer spectrofluorometer MPF-2A, with a 5-nm slit width for both excitation and emission. The instrument was operated in the ratio mode. The excitation light source was a high-pressure xenon lamp. In general, excitation and emission were scanned from 250 to 300 nm and from 300 to 600 nm, respectively. Samples were placed in a temperature-controlled cuvette holder, and the temperature was maintained at 20°C. Tubulin solutions were titrated with Tb^{3+} by addition of 1 μl of 0.01 or 0.1 M $TbCl_3$ stock solutions. Polymerized tubulin solutions at the upper concentration range of $TbCl_3$ used in these experiments showed turbidity. To measure the intrinsic tubulin fluorescence, a front-face cuvette was used to eliminate the inner filter effects due to light scattering. These particular experiments were done in a Perkin-Elmer LS 50 luminescence spectrometer and no filters were used. When luminescence was measured at 545 nm, interposition of filters (F31 or F43) in the emission beam were used to cut off the excitation wavelength (under 290 or 400 nm, respectively), eliminating possible errors due to scattering of light by turbidity of the sample. All the fluorescence intensity measurements were done at the same time after the addition of $TbCl_3$ aliquots (5 min). The final volume increased less than 2% in the entire titration experiments.

Other methods. Tubulin concentrations were determined measuring the absorbance of aliquots of the samples diluted 10-fold in 6 M guanidine hydrochloride, using an absorptivity value of 1.03 liter g^{-1} cm^{-1} at 275 nm (21). GTP bound to tubulin was determined precipitating at 0°C the protein with 0.5 N perchloric acid and centrifuging for 10 min in a microfuge. The supernatant was treated with $NaHCO_3$ until pH 1.0 was reached, and the GTP spectrum was recorded in a Hewlett-Packard 8452A diode array spectrophotometer. GTP concentration was estimated using a molar absorption coefficient at 256 nm of 12,400 M^{-1} cm^{-1}. The absence of protein in the supernatant was checked. Electron microscopy studies were done using a Philips EM-300 microscope. Samples were negatively stained with 2% uranyl acetate (22). Tb^{3+} content of solutions was determined by the fluorimetric assay described by Barela and Sherry (23), using dipicolinic acid.

Data analysis. The curves presented in Fig. 5 were fitted to the experimental data through a nonlinear regression program (kindly provided by Dr. O. Alvarez), on a IBM AT personal computer.

RESULTS

Intrinsic tubulin fluorescence. The fluorescence studies were performed with pure chicken brain tubulin. Electrophoresis in sodium dodecyl sulfate–polyacrylamide gels at high protein concentration showed only two bands corresponding to the α- and β-tubulin subunits. The corrected emission spectrum of tubulin (2 mg/ml), excited at 295 nm, revealed the characteristic tryptophan emission of the protein, with a fluorescence maximum at 326 nm (not shown). The excitation spectrum presented a maximum at 288 nm (not shown) indicating that the fluorescence was due to tryptophan residues, because at this wavelength tyrosine and phenylalanine have no significant absorption, and tryptophan is the only fluorophor stimulated. Similar maxima (with a slight shift to the blue) for the excitation and emission spectra of tubulin in the presence of Tb^{3+} were observed. These experiments were done using front-face optics, because when a square cuvette was used there was a decrease in the fluorescence intensity, probably due to inner filter effects produced by the high optical density after the addition of $TbCl_3$. The emission maximum (326 nm) was shifted to the blue with respect to tryptophan in solution, indicating that most of the eight tryptophan residues of the tubulin dimer are in a hydrophobic environment, which is in agreement with the findings of Steiner (24) using human platelet tubulin.

Emission spectrum of the Tb^{3+}-tubulin complex. Figure 1 shows the emission spectrum of Tb^{3+} in the presence of tubulin and GTP. The corrected emission spectrum revealed the characteristic Tb^{3+} luminescence maxima at 490, 545, 586, and around 620 nm. Tb^{3+} luminescence intensity in the experimental buffer was negligible. These results indicate that the luminescence intensity of Tb^{3+} is enhanced when this lanthanide interacts with tubulin.

Excitation spectrum of the Tb^{3+}-tubulin complex. In order to determine if the binding of Tb^{3+} to GTP enhances

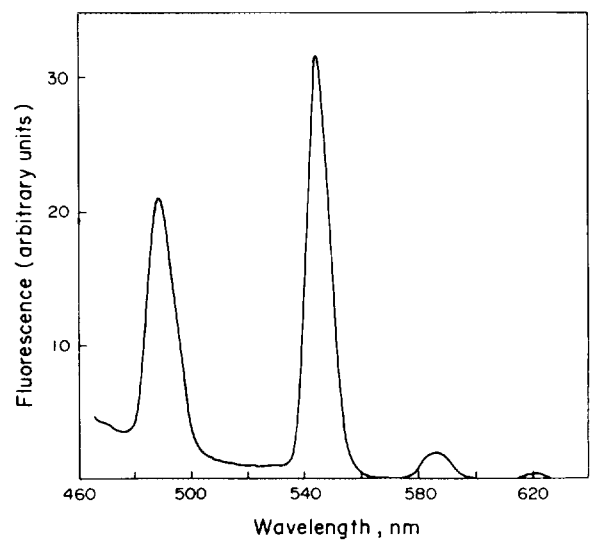

FIG. 1. Emission spectrum of Tb^{3+}–tubulin complex. Luminescence was measured with an excitation wavelength of 295 nm. The sample contained 0.2 mg/ml of tubulin, 0.2 mM $TbCl_3$, and 0.1 mM GTP. The buffer used was 0.1 M Mes, 3.4 M glycerol, pH 6.4.

[3] Abbreviation used: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

Tb^{3+} luminescence, and if in turn, this interferes with the luminescence measurements of the Tb^{3+}–tubulin complex (which has 2 mol of GTP bound per mole of tubulin, determined as described under Materials and Methods), the excitation spectra of the Tb^{3+}–tubulin complex, Tb^{3+}–tubulin complex plus free GTP, and Tb^{3+}–GTP complex were recorded. Figure 2 shows that the presence of free GTP mainly enhances the luminescence intensity of the Tb^{3+}–tubulin complex, which is not due to a significant emission produced by the Tb^{3+}–GTP complex. The luminescence enhancement of the Tb^{3+}–tubulin complex in the presence of an excess of free GTP may be explained by the exchange of a fraction of the Mg^{2+} bound at the E-site by Tb^{3+} (25). This explanation is supported by the finding that Mg^{2+} at the E-site was partially replaced by Tb^{3+}, as determined after removing the free and weakly bound Tb^{3+} through Chelex-100 (14), and determining the Tb^{3+} and Mg^{2+} tightly bound to tubulin (not shown).

Tb^{3+} titration of tubulin. Titration of tubulin with Tb^{3+} resulted in an increase of the emission intensity at 545 nm with a sigmoidal behavior (Fig. 3B). When several-fold excess of Tb^{3+} to tubulin concentration was added the sample was turbid. Addition of Tb^{3+} to the assay solution without tubulin produced no such turbidity over the range of Tb^{3+} concentration of interest. When the Tb^{3+}–tubulin complex was treated with EDTA, and an excess of Mg^{2+} was added to the solution, tubulin was able to polymerize into microtubules, indicating that the structure of tubulin was not severely altered. Figure 3A shows the plot of turbidity at 350 nm versus Tb^{3+} concentration. Turbidity follows an apparent sigmoidal saturation curve with a sharp increase in turbidity in a narrow range of Tb^{3+} concentration. Since this process was time dependent, tubulin was incubated with the different $TbCl_3$ concentrations until the increase in turbidity reached the plateau (≤5 min). The increase in turbidity at lower concentrations of Tb^{3+} with respect to the increase in luminescence at 545 nm indicates that the enhancement of luminescence is produced after tubulin polymerization. As expected, an increase in tubulin concentration led to an increase in the sigmoidicity and the maximal values of the Tb^{3+} titration curves measuring turbidity or luminescence. At all tubulin concentrations tested, the plateau of turbidity was reached at lower Tb^{3+} concentrations than the maximal values of luminescence (not shown).

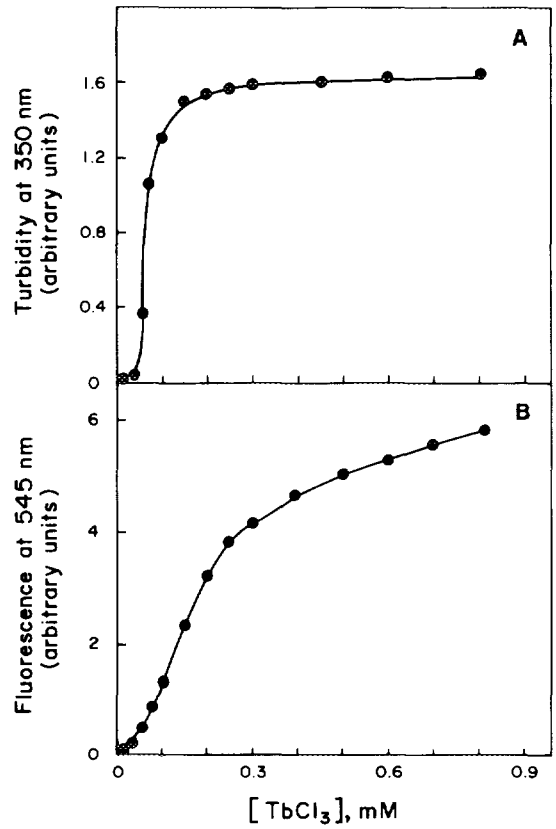

FIG. 3. Effect of Tb^{3+} on tubulin turbidity and on the luminescence of the Tb^{3+}–tubulin complex. Aliquots of 1 μl of stock solutions of 0.01 or 0.1 M of $TbCl_3$ were successively added to 1 ml of a sample containing 2 mg of tubulin in the buffer described in the legend to Fig. 1. After completion of time-dependent changes, (A) turbidity was recorded at 350 nm; (B) luminescence emission was followed at 545 nm with excitation at 295 nm.

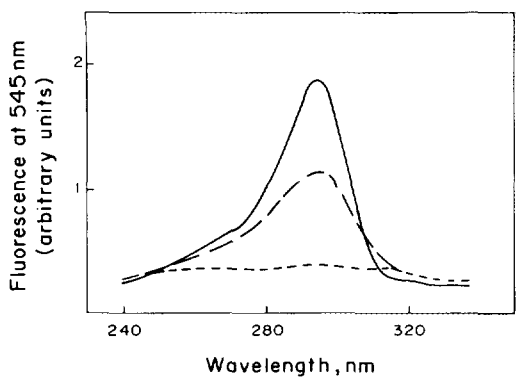

FIG. 2. Effect of free GTP on the excitation spectrum of Tb^{3+}–tubulin complex. Luminescence emission was recorded at 545 nm. The excitation spectra of 0.2 mM $TbCl_3$ plus: (- - -) 0.1 mM GTP; (——) 0.1 mM GTP and 2 mg/ml tubulin; (– –) 2 mg/ml tubulin. The buffer used was the same as that described in the legend to Fig. 1.

Dependence of turbidity on wavelength for the Tb^{3+}-induced polymers. For particles with dimensions smaller than the wavelength of light, turbidity shows an inverse fourth-power dependence on the wavelength and is a complex function of the size and shape of the scattering particles. For very long rod-like particles, like microtubules, the situation is different. As shown by Berne (26), if a limiting condition is reached in which the rods can be considered to be very long in comparison with the wavelength of light, turbidity shows an inverse third-

power dependence on the wavelength. When this limit is reached, the turbidity is a function only of the total weight concentration of scattering particles.

The inset of Fig. 4 shows the wavelength dependence (between 300 to 450 nm) of the Tb^{3+}–tubulin aggregates at different $TbCl_3$ concentrations. The dependence of ΔA at 0.04 mM $TbCl_3$ (slope 1 which presents low values of turbidity) was inverse to 3.48 power of the wavelength. This means that the structures formed are smaller than the wavelength and are probably rings, as will be shown later. In contrast, when higher $TbCl_3$ concentrations were used (0.8 mM, slope 3 with the highest values of turbidity), the dependence of ΔA was inverse to 1.9 power of the wavelength. This value is clearly different from those of microtubules, indicating that the polymerization of the Tb^{3+}–tubulin aggregates does not follow the Berne theory (26) and the large polymers formed are not microtubules. The size of these structures are probably comparable to the wavelength, and, as will be shown later, they correspond to ring aggregates.

Effect of divalent cations on the luminescence of the Tb^{3+}–tubulin complex. To determine if the sites titrated with Tb^{3+} correspond to the low affinity binding sites for divalent cations, such as Mg^{2+}, Mn^{2+}, Zn^{2+}, or Co^{2+}, which promote tubulin polymerization, metal competition experiments were performed. The concentration used for Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} were 10, 3, 6, and 28 times their dissociation constants, respectively (11, 2, 27, 28). As shown in Fig. 5A, Mg^{2+} diminished the sigmoidicity (inset Fig. 5) and lowered the maximal luminescence. The sigmoidicity of the curve almost completely disappeared with Zn^{2+}, and the maximal luminescence was substantially reduced. Co^{2+} had a behavior similar to that of Mg^{2+}, and Mn^{2+} showed no effect but a slight increase in the maximal luminescence. The Hanes–Woolf representation (Fig. 5B) shows the degree of sigmoidicity of the curves. In all cases tested, the sigmoidicity became less apparent at a low concentration of tubulin (0.4 mg/ml), while at the highest concentration tested (1.4 mg/ml) the sigmoidicity was enhanced (not shown). The effect of Ca^{2+} on Tb^{3+} luminescence was also studied, and the curves presented no changes with respect to the control without additions (not shown).

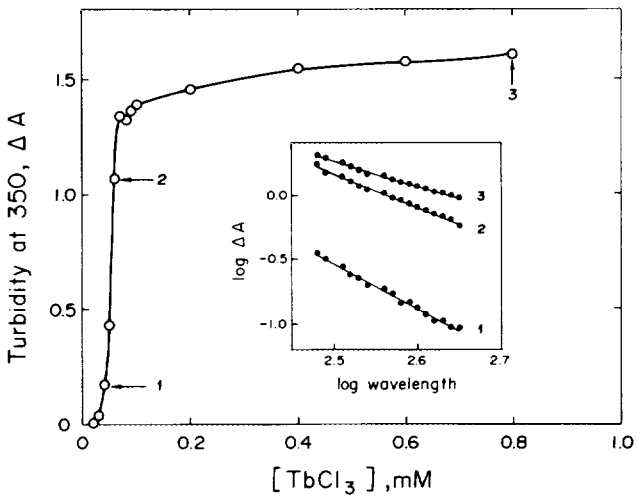

FIG. 4. Dependence of turbidity on the wavelength of the Tb^{3+}-induced polymers. The titration was done as indicated in the legend to Fig. 3. The sample contained 0.6 mg/ml of tubulin in 50 mM triethanolamine, 100 mM KCl, 3.4 M glycerol, pH 7.0, and turbidity was recorded at 350 nm. At the points indicated in the figure (1, 0.04 mM $TbCl_3$; 2, 0.06 mM $TbCl_3$; 3, 0.8 mM $TbCl_3$) the absorbance was measured at different wavelength (300 to 450 nm). The inset shows the dependence of turbidity (ΔA) on wavelength at different $TbCl_3$ concentrations. The slopes in 1, 2, and 3 were −3.48, −2.63, and −1.90, respectively.

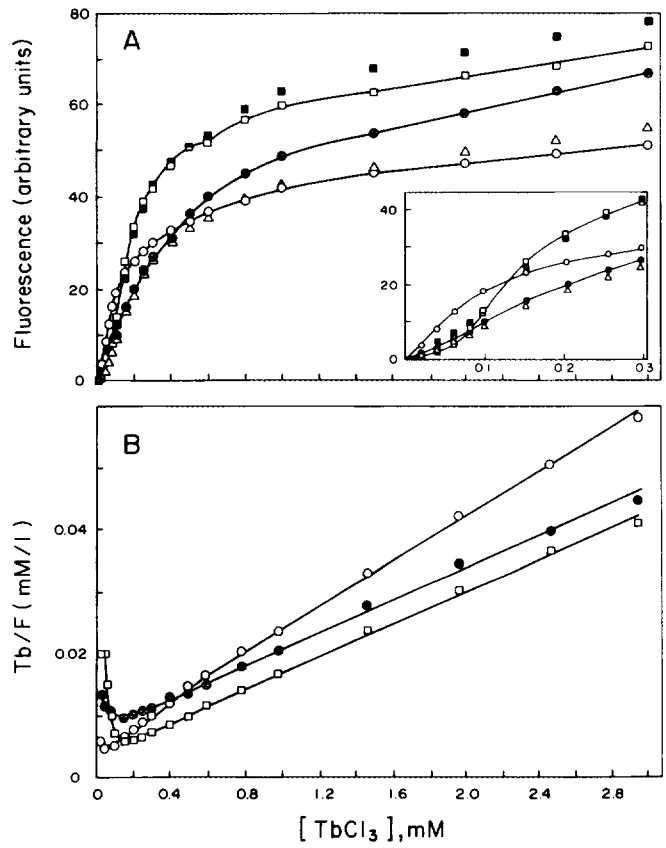

FIG. 5. Effect of divalent cations on the binding of Tb^{3+} to tubulin. The assay contained 1.2 mg/ml of tubulin in the buffer described in the legend to Fig. 1 and one of the following compounds, in a final volume of 1 ml: (●) 90 mM $MgCl_2$; (○) 0.5 mM $ZnCl_2$; (■) 1 mM $MnCl_2$, (△) 0.5 mM $CoCl_2$; (□) no addition. Aliquots of 1 μl of a stock solution of 0.01 or 0.1 M $TbCl_3$ were successively added. The samples were excited at 295 nm and the emission was followed at 545 nm. (A) For clarity of the figure, only the lines corresponding to the presence of Zn^{2+}, Mg^{2+}, and no addition were drawn. The inset shows in detail the initial part of the curves. (B) Hanes–Woolf representation of the data presented in A.

Polymerization products induced by Tb^{3+}. Figure 6 shows a negatively stained sample of the tubulin polymerization products induced by Tb^{3+}. The polymers

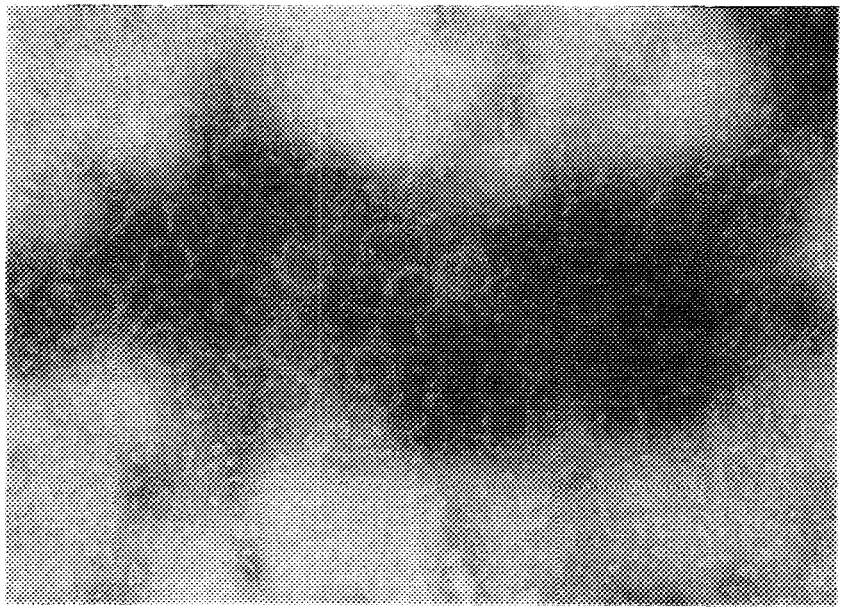

FIG. 6. Polymers of tubulin assembled in the presence of Tb^{3+}. 3 mg/ml of tubulin, 0.2 mM $TbCl_3$, 0.1 mM GTP, 0.1 M Mes, 3.4 M glycerol, pH 6.5, in a final volume of 1 ml were incubated at 10°C. The polymerization was followed measuring the absorbance at 350 nm until the plateau was reached. The polymerized material was visualized by electron microscopy. Samples were treated as indicated under Materials and Methods. ×38,300.

formed were double rings which aggregated mainly in large ordered arrays, in a honeycomb form. The size of the rings was 33 ± 2 nm.

DISCUSSION

The binding of Tb^{3+} to tubulin induces double rings which aggregate in a two-dimensional array. The overall process is accompanied by an enhancement of Tb^{3+} luminescence and by an increase in the turbidity of the solution.

The titration curves shown in Fig. 3 indicate that the turbidity increase was due to polymer assembly induced by the binding of Tb^{3+} to tubulin. Once saturation with Tb^{3+} is obtained, having reached the maximal degree of polymerization, it is not possible to displace Tb^{3+} with high concentrations of Mg^{2+} or Ca^{2+} (not shown), which suggests that the region of Tb^{3+} binding is not exposed to the solvent in the polymer. This region should be exposed in the tubulin dimer, because Mg^{2+} prevents Tb^{3+} binding. These results support the idea that the lanthanide is involved in the mechanism of tubulin self-association, but they do not establish the direct participation in a interdimer bond formation.

The different behavior of the increment in turbidity with respect to the enhancement of luminescence intensity when tubulin was titrated with Tb^{3+} (Figs. 3A and 3B) may be explained as follows: First, at low concentrations of Tb^{3+}, double ring assembly is mainly responsible for the turbidity of the solution. Second, as Tb^{3+} concentrations are increased, rings aggregate into large two-dimensional arrays, enhancing the Tb^{3+}–tubulin luminescence intensity. This simple explanation is based on the following reaction scheme for tubulin assembly in the presence of Tb^{3+}:

$$\text{tubulin} \rightarrow \text{rings} \rightarrow \text{aggregates}.$$

Thus, if the aggregation is responsible for the increase in Tb^{3+} luminescence, then the titration curve of the luminescence intensity with respect to Tb^{3+} concentration should resemble a sigmoidal saturation curve. The initial part of the curve, at which point no substantial luminescence is produced, should be dependent on tubulin concentration since it is necessary to saturate the sites needed for ring formation before the increase of luminescence is produced. When tubulin concentration is augmented, the initial interval of titration without significant increase in luminescence is enhanced (not shown). This interpretation is compatible with the results of Frigon and Timasheff (11) for double ring assembly in the presence of Mg^{2+}.

The addition of divalent cations before the titration with Tb^{3+} (Fig. 5) modifies Tb^{3+}–tubulin equilibrium in a different fashion for each metal. Thus, both the sigmoidicity and the luminescence at Tb^{3+} saturating concentrations decrease by the addition of different divalent cations in the order $Zn^{2+} > Co^{2+} = Mg^{2+}$. We do not have a satisfactory explanation for the lack of effect of Mn^{2+}. Tb^{3+} seems to mimic Mg^{2+}, the physiological cation for tubulin assembly, and this notion is supported by the fact

that Tb^{3+}, as Mg^{2+} (22, 29), induces polymerization of rings which aggregate in a honeycomb form. Structures of similar size and distribution have been found by Peyrot *et al.* (29) using subtilisin-cleaved tubulin and by Zabrecky and Cole (30) in the presence of Mg^{2+}–ATP. Further support for the assumption that Tb^{3+} mimics Mg^{2+} is the fact that Tb^{3+} as Mg^{2+} does not stimulate the GTPase activity of tubulin as does Ca^{2+} (C. Soto and O. Monasterio, unpublished results). On the other hand, the binding of Tb^{3+} to tubulin could occur also at the Mg^{2+} sites which are different from the exchangeable nucleotide site (31) and are known to have a role in the assembly of double rings (1). Although Tb^{3+} promotes double ring formation, experimental conditions for microtubule assembly in the presence of Tb^{3+} have not been found.

Frigon and Timasheff (11) demonstrated that Mg^{2+} induced tubulin self-association favoring double ring formation. Their analysis of Mg^{2+} binding, according to the Scatchard equation, resulted in a straight line. If the behavior of Tb^{3+} is assumed to be similar to that of Mg^{2+}, the sigmoidicity in the titration curves followed by luminescence could be due to the formation of ring aggregates in the presence of Tb^{3+}. When rings and aggregate formation were determined after tubulin titration with Tb^{3+}, a good correlation was found with luminescence enhancement (O. Monasterio, unpublished results). Also, the dependence of turbidity intensity on wavelength for the Tb^{3+}-induced polymers showed that the values of the slopes were different between rings and ring aggregates. In the initial interval of titration where turbidity is still increasing, there is only a small increase in luminescence. However, at higher $TbCl_3$ concentrations when the network of rings are formed, luminescence reached the maximal values.

The apparent sigmoidal behavior of the luminescence enhancement induced by binding of Tb^{3+} to tubulin could also be interpreted as a ligand binding by a self-association system (32). However, to be rigorous, Tb^{3+} binding data are necessary, and these studies are underway.

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