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Magnesium-induced Structural Changes in Tubulin*

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Exogenously added Mg^{2+}, in the intracelluar concentration range, has significant effect on the conformation of tubulin as monitored by tryptophan fluorescence. This magnesium-induced conformational change is reflected in the change of the slope and intercept of the Lehrer plot. The off-rate of colchicine from colchicine-tubulin complex is also decreased severalfold upon the addition of exogenous magnesium. The conformational change is magnesium-specific, and magnesium can only be replaced by manganese. GTP, $CaCl_2$, and NaCl have no effect. Magnesium, however has no effect on tryptophan accessibilities of tubulin-S. Implications of these results are discussed in terms of C-terminal tail-body interaction.

Magnesium is known to be an essential component in many biological processes (1). Its effects on tubulin self assembly are well known, and without it no tubulin polymerization can occur (2). Magnesium requirement for polymerization is dependent on the solution conditions (3) and on the type of nucleotide used in the polymerization reaction (4–6). Magnesium could be replaced by manganese and aluminum (7). In some cases, a very high concentration of magnesium has been used to induce polymerization instead of microtubule-associated proteins, dimethyl sulfoxide, and taxol etc. (3). Magnesium effect on disassembly has also been reported (8).

The effects of magnesium on tubulin-nucleotide interaction are well established (9–12). It is known that tubulin binds 1 mol/mol magnesium at the nonexchangeable GTP site (13). In addition, one strong magnesium binding site is observed at the exchangeable GTP site (12). Although the functional effects of magnesium are fairly well documented, its effect on tubulin conformation has not been reported.

Tryptophan fluorescence has been used widely to monitor conformational changes in proteins (14). Conformational changes may lead to change of tryptophan environments, which may lead to change of quantum yield, emission maximum, or solvent accessibilities. Collisional quenchers such as acrylamide have been widely used to resolve classes of accessible and inaccessible tryptophans and could be used to monitor conformational changes effectively (15). Here we report the effect of exogenously added magnesium on the conformation of tubulin as monitored by acrylamide quenching of tryptophan fluorescence. The tubulin preparations chosen for this study contained 2–3 mol of magnesium/dimer, suggesting that both the N and E nucleotide sites are saturated. Thus, the conformational effect observed in this study is induced by magnesium at a site other than N or E nucleotide binding sites.

EXPERIMENTAL PROCEDURES

Materials—GTP, PIPES,[1] EGTA, phenylmethylsulfonyl fluoride, and subtilisin BPN were from Sigma. Colchicine and podophyllotoxin were purchased from Aldrich. Podophyllotoxin was recrystallized several times from ethanol before use. All other chemicals used in this study were of analytical grade. AC was a gift from Dr. T. J. Fitzerald (Florida A & M University).

Tubulin and Tubulin-S Preparation—Tubulin was prepared from goat-brain by two cycles of temperature-dependent assembly-disassembly in PIPES assembly buffer (50 mM PIPES, pH 6.8, containing 0.5 mM $MgCl_2$ and 1 mM EGTA) with 1 mM GTP followed by two more cycles in 1 M glutamate, pH 7.0 with 1 mM GTP (16). Tubulin prepared by this method does not contain any microtubule-associated proteins. Purified microtubule was stored at −70 °C and used after depolymerization.

Mg^{2+}-depleted tubulin was prepared by two different methods. 1) Tubulin was polymerized in the presence of 1 mM GTP and 0.5 mM $MgCl_2$ at 37 °C in 50 mM PIPES, pH 7, for 30 min. Microtubules were pelleted by spinning at 35,000 rpm for 1 h at 2 °C. The pellet was washed once with 0.1 M PIPES, pH 7.0, and dissolved in the same buffer. 2) In the other method, tubulin was gel-filtered over a (13 × 1 cm) Sephadex G-50 column in 0.1 M PIPES, pH 7.0, at 4 °C. The appropriate protein fractions were pooled and centrifuged at 18,000 rpm for 15 min at 4 °C. Magnesium and GTP-free tubulin (from the E-site) have previously been shown to polymerize upon the addition of magnesium and GTP, and irreversible changes occur only upon hours of incubation (17). Similar results were obtained by us (see below).

Tubulin-S was prepared by digesting tubulin with subtilisin at 30 °C for 30 min in 0.1 M PIPES, pH 7.0, containing 1 mM GTP and 0.5 mM $MgCl_2$ (18). Subtilisin was used in the ratio of tubulin/subtilisin, 100:1 (w/w). The reaction was terminated by the addition of 1% (v/v) of 1% phenylmethylsulfonyl fluoride stock in dimethyl sulfoxide. To prepare magnesium-depleted tubulin-S; the digested protein, after depolymerization at 4 °C and centrifugation, was loaded on a Sephadex G-50 column (13 × 1 cm) and eluted with 0.1 M PIPES, pH 7.0, at 4 °C.

Fluorescence Methods—All fluorescence experiments were done in a Hitachi F-3000 spectrofluorometer equipped with a computer for the addition and subtraction of spectra. All fluorescence experiments were performed in a water-circulated thermostated cell, which was set at 25 ± 1 °C, unless mentioned otherwise. The excitation wavelength was at 295 nm, and the emission at 340 was noted. A 5-nm band pass was used for both excitation and emission. Appropriate buffer blanks were subtracted from all the fluorescence values. For determination of the off-rate of colchicine from tubulin-colchicine complex, excitation was set at 350 nm, and emission at 430 nm was monitored.

Circular Dichroism—Circular dichroism spectra were measured in a JASCO J600 spectropolarimeter at ambient temperatures in 1-cm path length cell. The gel-filtered tubulin in 0.1 M PIPES buffer, pH 7.0, was diluted to 5 μM concentration, and the CD spectrum was recorded. Immediately after that, an aliquot of 0.5 M magnesium chloride was added to a final concentration of 0.5 mM, and the CD spectrum was recorded. Each spectrum shown is an average of 10 spectra.

Measurement of Colchicine Off-rate—Tubulin-colchicine complex was prepared by incubating 18 μM tubulin with saturating concentration, *i.e.* 180 μM, of colchicine in 0.1 M PIPES, pH 7.0, at 37 °C for 90 min. Free colchicine was separated from protein-bound colchicine by passing over

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[1] The abbreviations used are: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); AC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; HPLC, high performance liquid chromatography.

a Sephadex G- 50 column (12 × 1 cm) equilibrated with 0.1 M PIPES, pH 7.0, at 4 °C. The protein-bound colchicine was split into two equal parts. To one part, 50 μM colchicine was added, and to other part 50 μM podophyllotoxin was added. Incubation at 37 °C was continued and fluorescence at 430 nm was monitored as a function of time (19).

Tubulin Polymerization—The polymerization of gel-filtered tubulin and tubulin-S was monitored by turbidity measurement at 350 nm in a Shimadzu UV-160 spectrophotometer. The polymerization was carried out in 0.1 M PIPES, pH 7.0, containing 1 mM GTP in a water-circulated thermostated cell at 37 °C. The polymerization was initiated by the addition of 10% dimethyl sulfoxide. Both the polymers were depolymerized at 4°C. But there was a small residual absorbance at 350 nm. The absorbance of the cold stable polymer was subtracted from the polymerization profile of both tubulin and tubulin-S.

Size Exclusion HPLC of Tubulin and Tubulin-S—Molecular weights of tubulin and tubulin-S in the absence and in the presence of 0.5 mM $MgCl_2$ were measured by size exclusion HPLC. The column used was a Protein Pak 300 SW having a fractionation range of 10,000–400,000 Da. The experiments were done at 25 °C. The following proteins were used to calibrate the column: 1) apoferritin (443,000 Da), 2) β amylase (200,000 Da), 3) yeast alcohol dehydrogenase (150,000 Da), 4) bovine serum albumin (66,000 Da), and 5) carbonic anhydrase (29,000 Da). Blue dextran and tryptophan were used to determine the void and the column volumes, respectively. In each case, 50 μl of 2 mg/ml sample was injected, and the elution was carried out in 0.1 M PIPES, pH 7.0, containing 0.5 mM $MgCl_2$ or no magnesium. $K_{average}$ was defined as $(v_e - v_o)/(v_t - v_o)$. v_e was the elution volume of the macromolecules, v_o was the void volume of the column, and v_t was the elution volume of the small molecule. $K_{average}$ values of the standard proteins were plotted against the corresponding logarithms of molecular weights. Magnesium-depleted tubulin and tubulin-S were prepared by passing through a Sephadex G-50 column (19 × 1 cm) equilibrated with 0.1 M PIPES, pH 7.0, at 4 °C. Eluted tubulin and tubulin-S were centrifuged at 18,000 rpm for 15 min at 4 °C. For experiments in magnesium-containing buffer, $MgCl_2$, to a final concentration 0.5 mM was added, and 50 μl of this sample was injected. The molecular weight of the tubulin and tubulin-S in the presence and the absence were determined from the calibration curve. The molecular weights of tubulin in the presence of acrylamide were measured as follows. The magnesium-free tubulin was incubated in 0.1 M PIPES buffer, pH 7.0, containing 0.1 M acrylamide in the presence and absence of magnesium for 15 min and then injected on to the size-exclusion HPLC column equilibrated with 0.1 M PIPES, pH 7.0, containing 0.1 M acrylamide, either in the presence or absence of 0.5 mM magnesium. The recoveries are consistently high, around 80% or more.

Atomic Absorption Measurement—Atomic absorption experiment was done in AA-575, ABQ (Varian A.G.) atomic absorption spectrophotometer. A magnesium hollow cathode lamp was used. The lamp current was 3.5 mA, and the flame used to atomize was air-acetylene (fuel). The excitation was done at 285.2 nm, which is specific for magnesium. 0.5-nm band pass was maintained.

Nonlinear Least Square Fit—In order to determine the binding constant of exogenously added magnesium to tubulin, the fluorescence quenching data were fitted to a single binding site equation by a nonlinear least squares fit procedures. In this method, the initial fluorescence, the fluorescence at the infinite ligand concentration, and the dissociation constant were systematically varied within a given range (grid search). The set of values that gave the lowest χ^2 was chosen as the best-fit curve.

Time-resolved Fluorescence Experiment—Fluorescence life time of tryptophans in tubulin in the presence and absence of magnesium were determined in an Applied Photophysics single photon counting apparatus using laser (Coherent antares, 76 yag) as an excitation source. Excitation was done at 296 nm, and emission was measured at 340 nm. The repetition rate was 0.95 MHz, and the response time was 60 ps. The data was fitted using a Applied Photophysics nonlinear least square fit program.

Electron Microscopy Study—For the electron microscopy, the samples were prepared using the procedure of Horowitz *et al.* (20).

RESULTS

Magnesium is known to be essential for tubulin polymerization (1). Tubulin depleted of magnesium by extensive dialysis is unable to polymerize. Addition of excess exogenous magnesium restored full polymerizability (2). These experiments were, however, done with MAP containing microtubules.

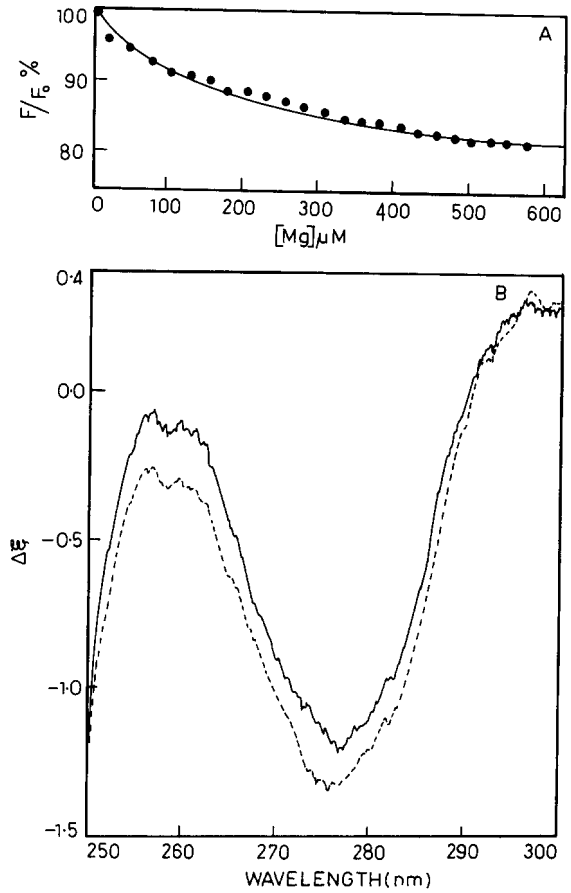

FIG. 1. *A*, determination of dissociation constant of $MgCl_2$ binding to tubulin. Magnesium titration was performed on 1 μM tubulin in 0.1 M PIPES, pH 7.0, at ambient temperature. Excitation wavelength was 295 nm, and the emission wavelength was 340 nm. Both excitation and emission bandpass were 5 nm. *B*, near UV CD spectra of tubulin in the absence (——) and in the presence (– – –) of 0.5 mM magnesium. Tubulin was gel filtered in 0.1 M PIPES buffer, pH 7.0, and CD spectra were measured as described under "Experimental Procedures." Each spectrum is an average of 10 measurements.

Magnesium-depleted tubulin was prepared by gel filtration in a buffer devoid of magnesium. All of the data reported in this article are with gel-filtered tubulin without microtubule-associated proteins. The magnesium content of gel-filtered tubulin was measured by atomic absorption spectrometry. A value of approximately 2.1 mol of magnesium/mol of tubulin dimer was obtained. For comparison, a milder method was also used to prepare magnesium-depleted tubulin. In the latter method, the tubulin is polymerized, pelleted, and washed in buffer devoid of magnesium followed by dissolution in the same buffer. All of the results described in this paper were with tubulin depleted of magnesium by gel filtration, but for comparison, every experiment was repeated with pelleted tubulin. The results in both cases are very similar, suggesting no major untoward effect of gel filtration on tubulin structure.

Binding of ligands to proteins often lead to changes in the fluorescence emission spectra of tryptophans and can be used to measure binding constants (21). Fig. 1*A* shows the fluorescence intensity of tryptophans of tubulin as a function of added magnesium ions. The fluorescence intensity decreases as increasing amounts of magnesium are added but quickly levels off at about 500 μM. Beyond 500 μM, no significant change in fluorescence can be seen. A nonlinear least square fit to a binding equation with single class of binding site yields a dissociation constant of 180 μM (21). Since the binding of magnesium leads to change of environment of one or more tryptophan resi-

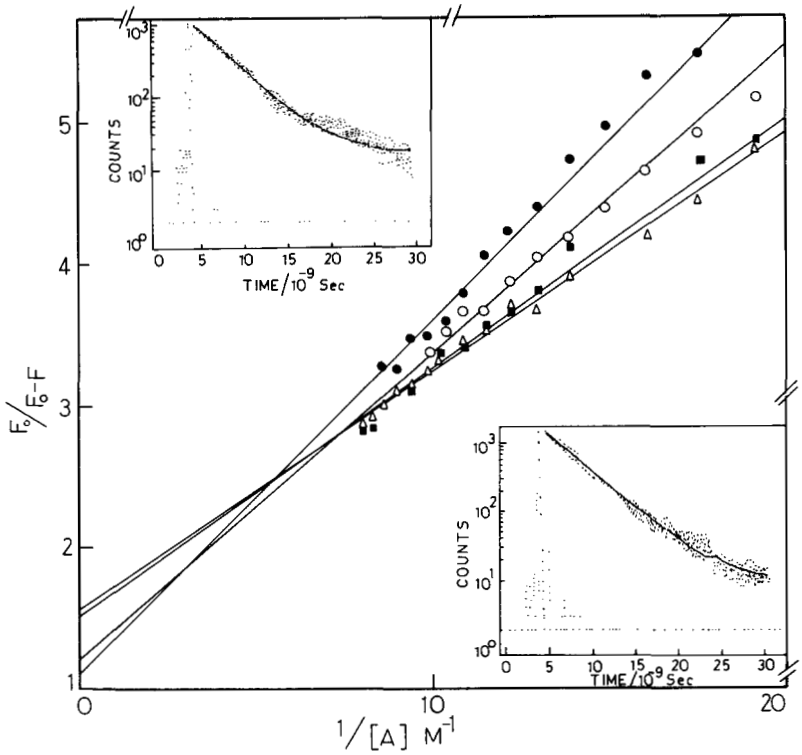

FIG. 2. **Lehrer plot of acrylamide quenching of 1 μM magnesium depleted tubulin prepared by gel filtration at 25 °C in 0.1 M PIPES, pH 7.0, 1) in the absence of $MgCl_2$ (△), 2) in the presence of 100 μM $MgCl_2$ (○), 3) in the presence of 500 μM $MgCl_2$ (♦), and 4) in the presence of 500 μM magnesium chloride followed by the addition of 1 mM EDTA (■).** Excitation and emission wavelengths were 295 and 340 nm, respectively. The *inset* shows the real time fluorescence decay of tubulin in the absence (*upper left*) and in the presence (*lower right*) of 0.5 mM magnesium chloride. The solution conditions and magnesium removal method were same as Fig. 1.

dues, it may also lead to an altered side chain CD spectra. Fig. 1*B* shows the near UV side chain CD spectra of tubulin in the absence and in the presence of 0.5 mM magnesium. There are significant differences in the two spectra providing independent support for change of tryptophan environment upon magnesium binding.

Such magnesium-induced change in tryptophan environments may be probed in greater details by collisional quenching with neutral quenchers such as acrylamide. Fig. 2 shows the Lehrer plot (a modified Stern-Volmer plot) (22) of acrylamide quenching of tryptophan fluorescence of magnesium-depleted tubulin and with 100 and 500 μM added magnesium. The Lehrer plot for magnesium-depleted tubulin cuts the *y* axis at 1.53 ± 0.07 (Table I), indicating that approximately 34% of the initial fluorescence is nonquenchable by low concentrations of acrylamide (15). The Stern-Volmer constant of the quenchable tryptophans are 8.8 ± 0.5 M^{-1}. As increased amounts of exogenous magnesium are added, the intercept values decrease and approach a limiting value of approximately 1.1 ± 0.07 at 0.5 mM Mg^{2+} and beyond. There is a parallel reduction in Stern-Volmer constants from 8.8 ± 0.5 to 4.75 ± 0.13 M^{-1} for the quenchable tryptophans. In the limit, approximately 10–15% of the initial fluorescence remains nonquenchable at 0.5 mM Mg^{2+} and beyond. The addition of excess EDTA completely reverses the magnesium-induced changes in the Lehrer plot indicating the effect of magnesium to be fully reversible (Fig. 2). This indicates that some nonaccessible tryptophans in magnesium-depleted tubulin becomes accessible to acrylamide upon the addition of magnesium, and at the same time the accessibilities of previously accessible tryptophans diminish. Change in accessibilities of many tryptophan residues suggests that the conformational change may affect both the subunits (tubulin has 8 tryptophans, 4 in each subunit). Such major changes of environments of many tryptophans are likely to be a result of a global conformational change. This conformational change, however, does not lead to any significant change in the far UV circular dichroism spectrum, indicating no significant change in the secondary structure (data not shown).

TABLE I
Effect of various ligands on K_{sv} and intercept of Lehrer plot

Ligands added	K_{sv}	Intercept
	M^{-1}	
None	8.8 ± 0.5	1.53 ± 0.07
None (monomeric)[a]	30	1
None (5 μM tubulin)	9.7	1.85
$MgCl_2$ (100 μM)	5.5	1.25
$MgCl_2$ (500 μM)	4.75 ± 0.13	1.1 ± 0.07
$MgCl_2$ (500 μM + 1 mM EDTA)	8.57	1.5
$MgCl_2$ (500 μM, 5 μM tubulin)	4.7	1.15
$MgCl_2$ (1 mM)	3.85	1.15
$MnCl_2$ (500 μM)	5.3	1.1
NaCl (0.2 M)	8.8	1.65
$CaCl_2$ (100 μM)	8	1.55
GTP (200 μM)	10	1.7

The quenchings were all carried out in 0.1 M PIPES buffer, pH 7.0, at ambient temperature. The protein concentrations were 1 μM except in the cases mentioned explicitly.

[a] The protein concentration was 0.1 μM.

Magnesium-induced ring and higher order structure formation has been noted previously by Timasheff and co-workers (23, 24), and in tubulin-S by Peyrot *et al.* (25). Although such structures are formed at much higher magnesium and protein concentrations, we have measured the molecular weights of tubulin and tubulin-S in the presence and in the absence of 0.5 mM magnesium by size-exclusion HPLC. The results indicate the molecular weights of tubulin in the absence of magnesium, in the presence of 0.5 mM $MgCl_2$ tubulin-S in the absence of magnesium and in the presence of 0.5 mM $MgCl_2$ are 120,000, 100,000, 85,000, and 90,000 Da, respectively. The recoveries under these conditions are high, suggesting only minor presence of other forms, if any. This indicates that under the conditions where magnesium-induced changes are seen in the Lehrer plot, no association to higher order structure of occurs.

In order to determine if magnesium-induced changes in tryptophan accessibilities also result in changes of fluorescence life times, we have measured fluorescence life time of gel-filtered

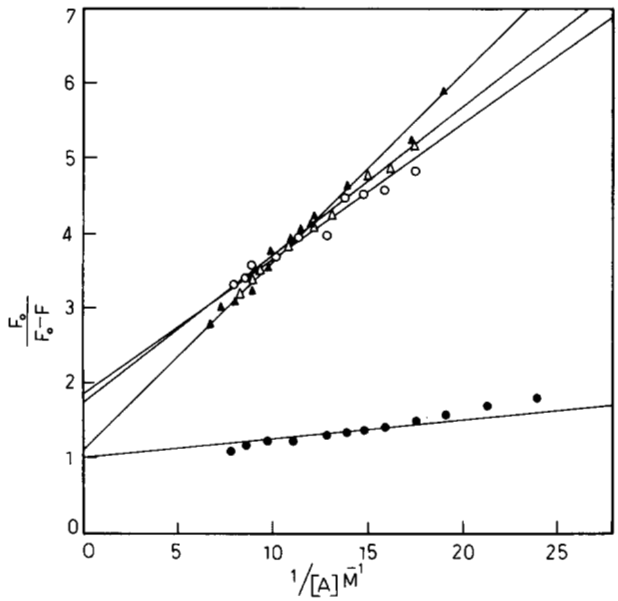

FIG. 3. **Lehrer plot of acrylamide quenching of 1) 1 μM tubulin in 0.1 M PIPES buffer, pH 7.0, containing 0.2 M NaCl (△), 2) 0.1 μM tubulin (●), 3) 5 μM tubulin (○) in 0.1 M PIPES, pH 7.0, and 4) 5 μM tubulin in 0.1 M PIPES buffer pH 7.0 containing 0.5 mM magnesium (▲) at 25 °C.** Excitation wavelength was 295 nm, and the emission wavelength was 340 nm.

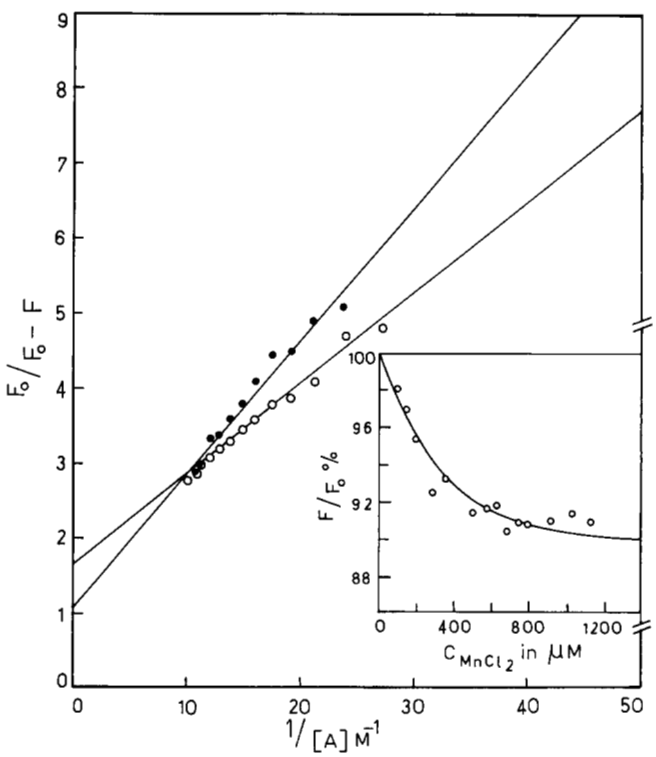

FIG. 4. **Lehrer plot of acrylamide quenching of 1 μM tubulin in 0.1 M PIPES buffer, pH 7.0, 1) in the absence (○) and 2) in the presence (●) of 500 μM $MnCl_2$.** The *inset* shows the manganese titration of 1 μM magnesium-depleted tubulin. The excitation and emission wavelengths were 295 and 340 nm, respectively.

tubulin in the presence and absence of added magnesium. The decays are probably complex and multiexponential. We have fitted the decay to a single exponential to determine the average life time. The life times are 3.9 ± 0.03 and 3.76 ± 0.039 ns for without and with added magnesium, respectively (Fig. 2, *insets*), suggesting small but significant change in tryptophan fluorescence life times.

Since acrylamide quenching is the principal tool used in this article to probe magnesium-induced conformational change, it is important to explore the effect of acrylamide on tubulin structure. After magnesium depletion by gel filtration, tubulin was incubated with 0.1 M acrylamide for 15 min at 25 °C (typical time needed to complete a Lehrer plot), and then magnesium was added to a final concentration of 0.5 mM. In a parallel experiment, magnesium and acrylamide were added immediately after gel filtration to a final concentration of 0.5 mM and 0.1 M, respectively, and incubated for 15 min. At this point, polymerization ability, near UV side chain CD, and molecular weight by size-exclusion HPLC were measured. The polymerization profiles of both the acrylamide-treated samples are similar to the native tubulin (see Fig. 8). The near UV side chain CD spectra also do not differ significantly (data not shown). Electron microscopy of polymerized tubulin in the presence of acrylamide showed formation of microtubules (see Fig. 9*B*). Size exclusion HPLC in the presence of 0.1 M acrylamide shows no significant change in the elution profile from tubulin chromatographed without acrylamide (data not shown). These results certainly suggest that 0.1 M acrylamide, in the presence and in the absence of magnesium under conditions similar to the quenching experiments, has no significant effect on tubulin structure and function.

In order to test whether the effect of Mg^{2+} is due to increased ionic strength, we have looked into the effect of NaCl on Lehrer plot of tubulin. Effect of NaCl is shown in Fig. 3. The Lehrer plot passes through 1.65 with a K_{sv} of 8.8 M^{-1} sec^{-1} indicating that the presence of 0.2 M NaCl has no significant effect on tryptophan accessibilities.

In order to see if the change in dimer-monomer dissociation is in any way responsible for the observed change in Lehrer plot, we have done the Lehrer plot at protein concentrations that are significantly below and above the dissociation constant. At 0.1 μM concentration, the Lehrer plot when extrapolated; it cuts the y axis at 1 with a Stern-Volmer constant of 30 M^{-1}. This indicates very high degree of exposure for all of the tryptophans in the monomeric state. The enhancement of accessibilities of all tryptophans in the monomeric state could be due to location of the tryptophans at the subunit interface or conformational change of the subunits upon dissociation or both. Fig. 3 also shows the Lehrer plot of tubulin at concentration of 5 μM in the absence and presence of 0.5 mM magnesium. At 5 μM concentration, in the presence of magnesium, the K_{sv} and intercept values are similar to those at protein concentration of 1 μM in the presence of magnesium. The Lehrer plot in the absence of magnesium at 5 μM protein concentration is similar to that at 1 μM protein concentration, in the absence of magnesium (Fig. 3 and Table I). This suggests that even if magnesium induces a change in subunit dissociation constant; it is not responsible for the change in slope and intercept of the Lehrer plot. In many biological systems, Mn^{2+} can replace Mg^{2+} effectively (26). Fig. 4 shows the Lehrer plot for acrylamide quenching of tubulin, with or without Mn^{2+}. Like Mg^{2+}, the addition of 500 μM Mn^{2+} causes a decrease of y axis intercept to 1.1 and the K_{sv} of 5.3. The quenching of tryptophan fluorescence upon Mn^{2+} addition shows the saturation with a binding constant of 200 μM (*inset*). Thus, Mn^{2+} behaves very similarly to Mg^{2+} in its ability to induce the conformational change.

The effect of other ligands on the Lehrer plot is shown in Table I. As can be seen from the table, added GTP and $CaCl_2$ have no effect on the slope or intercept of the Lehrer plot, indicating no significant change in the accessibilities of the tryptophans of tubulin. In contrast, both Mg^{2+} and Mn^{2+} have significant effect as described previously.

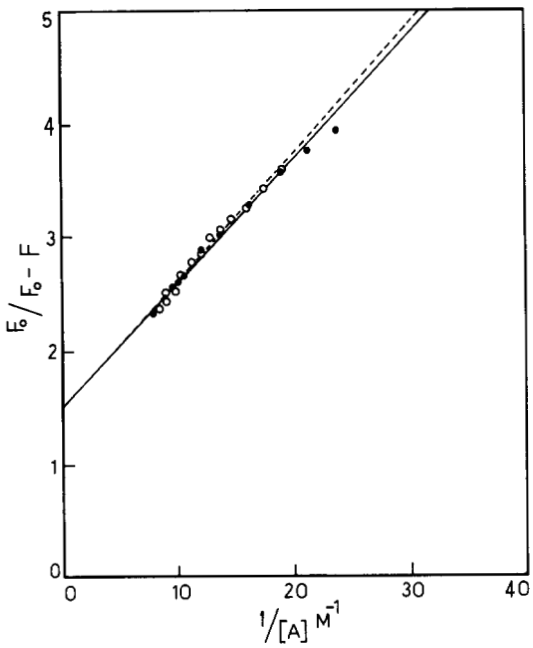

FIG. 5. **Lehrer plot of acrylamide quenching of 1 μM tubulin-S in the presence (○) and in the absence (●) of 500 μM $MgCl_2$ in 0.1 M PIPES buffer, pH 7.0, at 25 °C.** The excitation and the emission wavelengths were 295 and 340 nm, respectively. Method of preparation of tubulin-S is as described under "Experimental Procedures."

The C-terminal tail of tubulin is known to be flexible and highly negatively charged. Recent work from our laboratory (19) indicated that the C-terminal tail exerts a profound effect on the rest of the tubulin structure and properties. The C-terminal tail is postulated to be a site of divalent ion binding (27). We have investigated the possibility that Mg^{2+}-induced changes may involve, in some way, the C-terminal tail of tubulin. Fig. 5 shows the Lehrer plot for tubulin-S in the presence and absence of Mg^{2+}. Both of the plots are similar and cut the y axis at approximately 1.5 with a K_{sv} of 10 and 11 M^{-1}. K_{sv} and y axis intercepts of the plots appear to be similar to magnesium-depleted tubulin. This lack of magnesium effect in tubulin-S suggests a role for the C-terminal tails in the Mg^{2+}-induced conformational change.

A crucial property of tubulin that is affected by removal of the C-terminal tail of tubulin is the dissociation rate of colchicine from colchicine-tubulin complex (19). In the absence of the tail, *i.e.* in tubulin-S, the dissociation rate of colchicine is significantly enhanced. Fig. 6 shows a typical dissociation kinetics of colchicine-tubulin complex in the absence of magnesium. In the absence of magnesium, the off-rate from colchicine-tubulin complex is $1.43 \pm 0.33 \times 10^{-3}$ min^{-1} (average of four determinations). In the presence of magnesium, the off-rate from colchicine-tubulin is lowered 3–4-fold (0.5×10^{-3} min^{-1}). This rate is very similar to that reported by Mukhopadhyay *et al.* (19), 0.35×10^{-3} min^{-1}. In contrast, under identical conditions, off-rate from colchicine-tubulin-S complex was reported to be 1.2×10^{-3} min^{-1} (19). It is noteworthy that the off-rate from tubulin-colchicine complex in the absence of magnesium is similar to that of the colchicine-tubulin-S complex.

Another property of tubulin on which the C-terminal tails have an effect, is the rate of loss of colchicine binding activity upon incubation at 37 °C. We have used a rapidly equilibrating colchicine analog AC to study the rate of loss of colchicine binding activity of tubulin and tubulin-S in the presence and absence of Mg^{2+}. Fig. 7 shows the loss of AC binding as a function of incubation time. Tubulin in the absence of magnesium, loses AC binding capacity fairly rapidly with a $t_{½}$ of

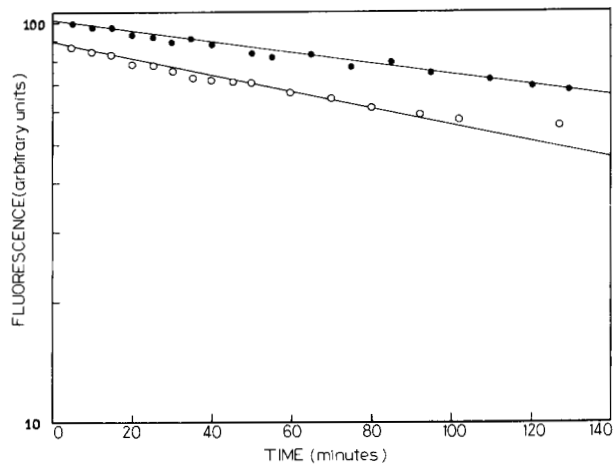

FIG. 6. **Dissociation kinetics of Colchicine-tubulin complex in the absence of magnesium.** 18 μM tubulin and 180 μM colchicine were incubated at 37 °C for 90 mins in 0.1 M PIPES, pH 7.0, containing 0.5 mM magnesium. The complex was then gel filtered in 0.1 M PIPES buffer, pH 7.0. The peak protein fraction was diluted into two solutions containing 1 μM tubulin-colchicine complex. To one, 50 μM colchicine (○) was added, and to another, 50 μM podophyllotoxin (●) was added; fluorescence values were recorded for about 3 h. Excitation wavelength was 350 nm, and the emission wavelength was 430 nm.

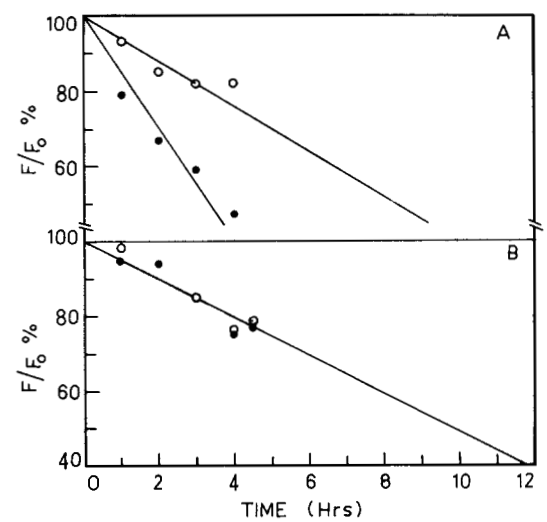

FIG. 7. **Loss of AC binding to tubulin as a function of time.** Magnesium free tubulin (*A*) or tubulin-S (*B*) was eluted from Sephadex G-50 column and incubated in either (●) 0.1 M PIPES buffer, pH 7, or (○) in 0.1 M PIPES buffer, pH 7, containing 0.5 mM magnesium at 37 °C. At desired times, an aliquot of tubulin was withdrawn and mixed with 20 μM AC, and fluorescence was measured at 450 nm at 25 °C. Excitation wavelength was 350 nm.

approximately 4 h. Addition of magnesium stabilizes the protein significantly against this loss of binding activity with $t_{½}$ becoming approximately 10 h. Tubulin-S on the other hand, is more stable toward the loss of AC binding ability, and the addition of magnesium has no effect on $t_{½}$ ($t_{½}$ = 12 h). Such increased stability of tubulin-S has been noted before (19). These results also reinforces the role of the C-terminal tail in Mg^{2+} tubulin interaction.

The role of Mg^{2+} in polymerization of tubulin has been studied before. We have compared the effect of Mg^{2+} on polymerization of tubulin and tubulin-S in the concentration range where magnesium has significant effect on tubulin structure and also in the presence of acrylamide. Fig. 8 shows the effect of magnesium on polymerization of tubulin. In the absence of added magnesium, the rate and extent of polymerization of magnesium-depleted tubulin is insignificant. Addition of mag-

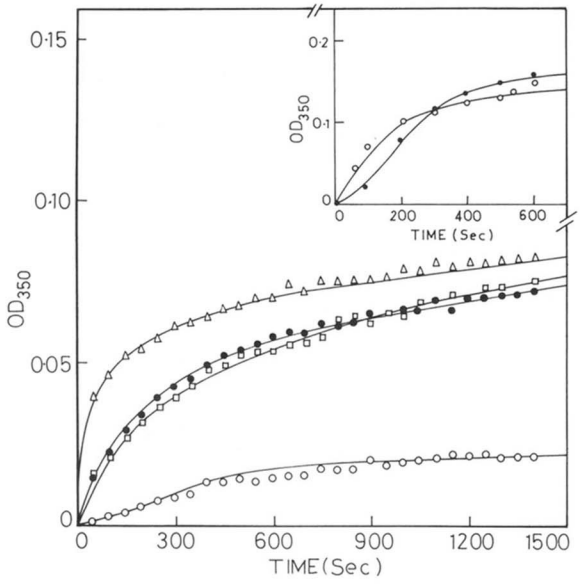

FIG. 8. **The effect of magnesium on the polymerization of 7 μM magnesium depleted tubulin (prepared by gel filtration), in 0.1 M PIPES, pH 7.0, containing 1 mM GTP.** Polymerization was induced by 10% dimethyl sulfoxide, and the experiment was carried out at 37 °C and monitored at 350 nm. ○, in the absence of magnesium; □, in the presence of 100 μM; △, in the presence of 500 μM magnesium after incubation in magnesium free condition for 30 minutes; and ●, in presence of 0.1 M acrylamide and 0.5 mM magnesium after incubation in magnesium-free buffer in the presence of 0.1 M acrylamide for 15 min. The *inset* shows the 10 μM taxol-induced polymerization profile at 350 nm for 6.4 μM tubulin-S, freed from magnesium by gel filtration, in the absence (●) and in the presence (○) of 0.5 mM magnesium, at 37 °C.

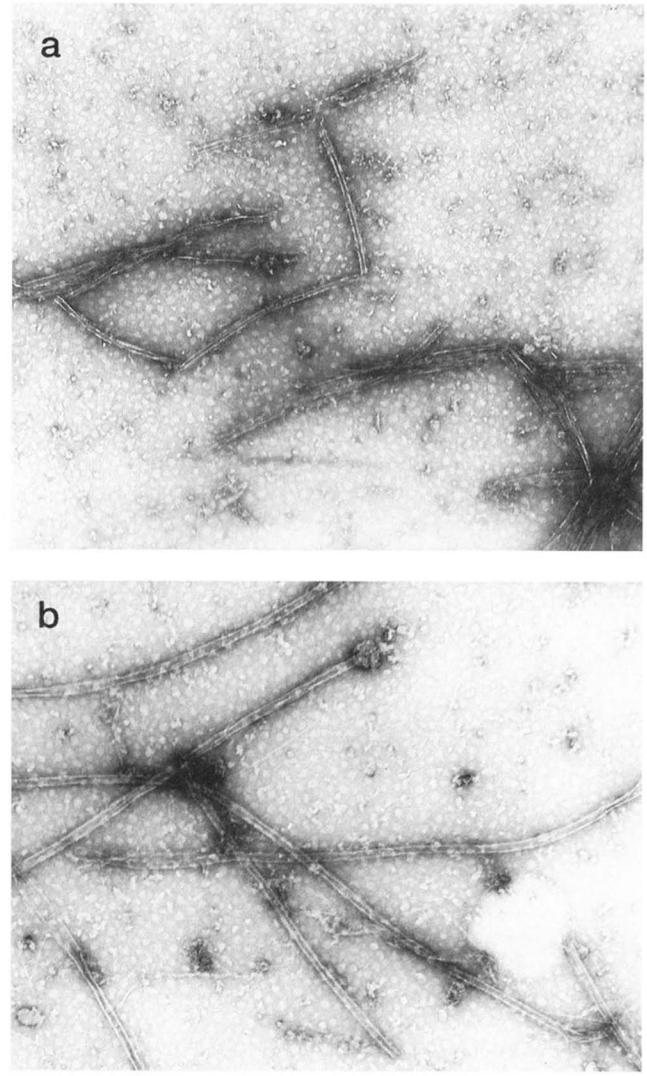

FIG. 9. **Electron micrographs of microtubules formed from gel-filtered tubulin (*a*) after immediate addition of magnesium and (× 30,000) (*b*) after a 15-min incubation in magnesium-free buffer containing 0.1 M acrylamide followed by addition of magnesium (×34,000).** Protein (approximately 1 mg/ml) was polymerized in 0.1 M PIPES buffer, pH 7.0 containing 1 mM GTP, 0.5 mM magnesium, and 10% dimethyl sulfoxide. Samples were prepared according to Horowitz *et al.* (1984).

nesium increases the rate and extent of polymerization, and 500 μM magnesium restores the full polymerizability. The figure shows that the effect of 500 μM magnesium after the gel-filtered protein was incubated in magnesium-free conditions for 30 min. The polymerization profile is very similar when the same concentration of magnesium is added immediately after the gel filtration (data not shown). Thus, the polymerizability of magnesium-depleted tubulin can be restored upon the addition of magnesium even after incubation in magnesium-free buffer up to 30 min (time tested). Even in the presence of acrylamide, this restorability of polymerization is retained at least for 15 min (roughly time taken to do a Lehrer plot) (Fig. 3), but is gradually lost beyond half an hour. All of the polymers described above are sensitive to cold, and there is only a small residual absorbance at 350 nm after depolymerization. In contrast, magnesium-depleted tubulin S without any added magnesium polymerizes well, and the addition of exogenous magnesium has only a modest effect on rate and extent of polymerization. This polymer is also cold-sensitive with a small residual absorbance at 350 nm. All of the polymers prepared above were examined under electron microscope for microtubule formation. Fig. 9, *A* and *B*, shows the electron micrographs of polymers prepared with gel-filtered tubulin with immediate addition of magnesium chloride and with gel-filtered tubulin after incubation of 15 min in magnesium-free buffer containing 0.1 M acrylamide followed by immediate addition of magnesium, respectively. Both of the polymers show clear microtubule formation.

DISCUSSION

Magnesium has long been known to be important for tubulin structure and function. It is known that one molar equivalent of magnesium is tightly bound to tubulin dimer (1, 2). However, this tightly bound magnesium is not enough to induce polymerization, and there is an absolute requirement for the additional, exogenous magnesium to initiate polymerization (1, 2, 28–30). *In vitro*, the optimum rate and extent of assembly occurs with magnesium in the range of 0.1–1 mM (2). In contrast, with tubulin in buffer containing 3.4 M glycerol (3), the extent of assembly increases by magnesium from 5 to 16 mM. However, in this buffer the morphology of the assembly products varies and is dependent on magnesium concentrations. In the present study, we have attempted to investigate any structural effects of magnesium in 0–1 mM concentration range, and we observed the structural changes on tubulin at these magnesium concentrations (<1 mM).

As shown in this article, the addition of magnesium at less than 1 mM concentration causes change of accessibilities of many of the tryptophan residues, which results in a significantly changed Lehrer plot. The effect of magnesium is specific and can only be replaced by manganese among the cation studied. This binding of magnesium also leads to change of functional properties such as off-rate of the colchicine-tubulin complex and kinetics of loss of AC binding. It is well known that the C-terminal tail of tubulin affects many properties of the rest of the protein, probably through tail-body interaction (19). Cleav-

age of the C-terminal tail enhances the dissociation rate of colchicine-tubulin complex (19), makes a significantly higher percentage of tryptophans inaccessible toward acrylamide (19), and lowers the dimer-monomer dissociation constant (altering its thermodynamic parameters as well) (31). Thus, the tubulin molecule in the absence of added magnesium behaves in some ways like tubulin-S.

The effect of added exogenous magnesium on tubulin-S is very small, if any (as judged by the Lehrer plot, polymerization rate, and decay of colchicine binding). These also suggest that much of the magnesium effect on tubulin structure and function in this magnesium concentration range may be mediated through the C-terminal tail. It has been noted that magnesium requirement for optimum polymerization of C-terminal modified tubulin is less compared with that of unmodified tubulin (12). We hypothesize that Mg^{2+} promotes tail-body interaction. Thus, tubulin in the absence of magnesium, in some ways, behaves like tubulin-S. We note that recently, Ortiz. *et al.* (32) has reported that the C-terminal tails interact with tubulin-S. Demonstration of such tail-body interaction supports our conclusions.

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