Thermodynamics of Tubulin Polymerization into Zinc Sheets: Assembly Is Not Regulated by GTP Hydrolysis†

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ABSTRACT: The thermodynamics of tubulin assembly into Zn sheets have been studied, with special emphasis on the role of bound nucleotide and of GTP hydrolysis in polymerization. In contrast to microtubules, Zn sheets could be assembled from GDP–tubulin as well as from GTP–tubulin. Accordingly, no appreciable destabilization of the Zn sheets was observed following GTP hydrolysis and P_i release, indicating that the binding of Zn^{2+} to tubulin has abolished the regulatory switch role played by GTP hydrolysis in tubulin assembly. As a consequence, the critical concentration for assembly of Zn sheets did not increase with tubulin concentration, a feature characteristic of microtubule assembly. Zn sheets do not bind P_i analogs, indicating that the γ-phosphate binding locus of the E-site of tubulin is occluded following GTP hydrolysis in these GDP–tubulin polymers. Nonlinear van't Hoff plots were obtained for assembly of Zn sheets in the presence of either GTP or GDP, consistent with a change in heat capacity. Enthalpy, entropy, and heat capacity changes had values similar to those reported for assembly of microtubules or polymerization of tubulin–colchicine, indicating that hydrophobic tubulin–tubulin interactions are of comparable size in these different polymers.

The tubulin molecule is characterized by its ability to undergo self-assembly into a variety of polymeric structures. Microtubules represent the biologically functional tubulin polymer in vivo, and they can be reassembled in vitro under well-defined ionic conditions (Weisenberg, 1972; Lee & Timasheff, 1975). However, tubulin exhibits a broad polymorphism of assembly into other polymerized structures such as double rings (Frigon & Timasheff, 1975a,b; Howard & Timasheff, 1986), curly polymers of tubulin–colchicine (Saltarelli & Pantaloni, 1982), helical spirals and ribbons induced by the binding of vincaalkaloids (Himes et al., 1976), and flat sheets induced by the binding of Zn^{2+} or Co^{2+} ions (Larsson et al., 1976; Gaskin & Kress, 1977; Haskins et al., 1980; Gaskin, 1981). All these different polymers can be generated by the self-assembly of protofilaments in variable numbers and with different curvatures and relative polarities. While microtubules result from the parallel arrangement of 13 ± 2 adjacent protofilaments of identical polarity into a closed cylinder (Beese et al., 1987a,b), diffraction of electron micrographs of negatively stained Zn sheets and subsequent image reconstruction show that these flat bidimensional polymers are made of protofilaments arranged in an alternating antiparallel fashion (Baker & Amos, 1978; Crépeau et al., 1977; Tamm et al., 1979; Ceska & Edelstein, 1984); the three-dimensional structure of tubulin could be derived from such sheets with a resolution of $\sim$20 Å.

The dynamics of microtubules is known to be regulated by GTP hydrolysis [for a review see Carlier (1989)]. A large destabilization of tubulin–tubulin interactions in the polymer accompanies the release of P_i in the medium, following cleavage of the γ-phosphate; the loss in free energy is not sufficient for the GDP subunits in contact with four neighbors inside microtubules to dissociate from the body of the polymer but is revealed by the rapid depolymerization that occurs when GDP subunits are exposed to the solvent at microtubule ends. It is thought that the lateral rather than the longitudinal interactions between tubulin subunits are weakened following GTP hydrolysis (Melki et al., 1989). Similarly, the tubulin–colchicine polymer breaks down following total hydrolysis of GTP in the medium and cannot be assembled from GDP–tubulin–colchicine (Saltarelli & Pantaloni, 1982). The dynamics of the Zn sheets has not been studied in great detail, although treadmilling of Zn sheets has been reported (Lee et al., 1983). Little is known about the nature of lateral tubulin–tubulin contacts in this type of polymer. In this report, the thermodynamics of assembly of Zn sheets is analyzed with special emphasis on the role of bound nucleotide, of GTP hydrolysis, and of colchicine binding in the dynamics of this polymer, and a comparison with microtubules is derived.

MATERIALS AND METHODS

Chemicals. MES [2-(*N*-morpholino)ethanesulfonic acid] was purchased from Calbiochem. EGTA (ethylene glycol bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid was from Sigma. GTP (guanosine 5′-triphosphate) came from Boehringer. Taxol was a gift from Dr. D. Guénard (ICSN, Gif-sur-Yvette, France). [^{3}H]GTP, [γ-^{32}P]GTP, ^{65}Zn, ^{7}Be, and monoiodinated ^{125}I-Bolton–Hunter reagent came from Amersham. All other chemicals were analytical grade from Prolabo and Merck.

Tubulin Purification and Labeling. Pure tubulin was prepared from fresh pig brain by three assembly–disassembly cycles according to Shelanski et al. (1973), followed by phosphocellulose (Whatman P11) chromatography (Weingarten et al., 1975). Tubulin was concentrated by ultrafiltration and stored at –80 °C in MG buffer (0.05 M MES, pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 3.4 M glycerol, and 200 μM GTP) at a concentration of 7–10 mg/mL.

Tubulin was ^{125}I-labeled using Bolton–Hunter reagent as described (Carlier et al., 1980).

Preparation of GTP–Tubulin. Tubulin (90–100 μM) in MG buffer was incubated at 0 °C for 1.5 h in the presence of 0.5 mM GTP (γ-^{32}P-labeled when desired). The 1:1 GTP–tubulin complex was isolated by chromatography through

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Table I: Identification of Nucleotides Bound to Tubulin in GTP–Tubulin and GDP–Tubulin 1:1 Complexes[a]

sample	total nucleotide bound to tubulin	
	% GTP	% GDP
	46	54
GDP–tubulin complex	46	54
	49	51
	49	51
GTP–tubulin complex	94	6
	93	7

[a] GTP–tubulin or GDP–tubulin 1:1 complexes were prepared at 70–110 μM, and perchloric extracts were performed as described in Materials and Methods. UV spectrophotometric measurements showed that the concentration of guanine nucleotides in the extracts corresponded to 2.1 ● 0.1 GNP per tubulin. An amount of 2.25–3.0 nmol of nucleotide was injected in the HPLC column. GDP and GTP came off the column at times 7.5 and 14 min, respectively. The percentages of GDP and GTP present in the extracts were derived from measurements of the areas under the peaks of GDP and GTP. no GMP was found. about equal amounts of GTP (bound to N site) and GDP (bound to E site) were found in the GDP–tubulin samples, while essentially GTP (bound to E and N sites) was found in the GTP–tubulin samples.

Sephadex G-25 (PD-10 Pharmacia) equilibrated in buffer A (100 mM MES, pH 6.8, and 30% glycerol).

The concentration of tubulin was determined spectrophotometrically using an extinction coefficient of 1.2 mg·cm^2 at 277 nm (Detrich & Williams, 1978) and a molecular weight of 100 000 (Krauhs et al., 1981).

Preparation of GDP–Tubulin. Before each experiment, phosphocellulose tubulin was cycled in MG buffer supplemented with 6 mM $MgCl_2$. Microtubules were pelleted at 300000*g*, 37 °C, for 15 min. Microtubules were suspended in buffer A and depolymerized at 0 °C for 30 min. The solution was then clarified by centrifugation at 300000*g*, 4 °C, for 6 min. The supernatant containing GDP–tubulin 1:1 complex was supplemented with 150 μM GDP.

The nature of the nucleotide bound to the E site in the 1:1 GTP–tubulin and GDP–tubulin complexes, prepared as described above, was checked by HPLC of the perchloric extracts of the samples. Briefly, tubulin (50–100 μM) was denatured by 5% $HClO_4$ at 0 °C, followed by addition of 0.4 M sodium acetate to bring the solution to pH 4. Nucleotides were separated by anion-exchange HPLC using a Synchropak AX300 column (25 × 0.4 cm, Synchrom, Inc.) and isocratic elution at 1 mL/min by 0.35 M KH_2PO_4 and 1.3 M NaCl, pH 3.5. The percentages of bound GTP and GDP were derived from measurements of the areas under the peaks of the elution pattern at 254 nm. Table I summarizes the amounts of bound GTP and GDP in several preparations of GTP–tubulin and GDP–tubulin.

Preparation of Tubulin–Colchicine Complex. Tubulin–colchicine complex was prepared according to Sherline et al. (1974). In brief, tubulin (70 μM) in MG buffer was incubated for 40 min at 37 °C with a 15-fold excess of colchicine. The tubulin–colchicine complex was isolated by chromatography through Sephadex G-25 (PD-10 Pharmacia) equilibrated in buffer A. The amount of colchicine bound per tubulin molecule was determined spectrophotometrically and was found to be 1:1 using an extinction coefficient of 16.74×10^3 M^{-1} cm^{-1} at 350 nm for bound colchicine.

Preparation of Subtilisin-Treated Tubulin (α_s β_s). The C-terminal region of tubulin was removed by subtilisin treatment as described (Sackett et al., 1985; Melki et al., 1991). Briefly, tubulin (20 μM) in 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM GTP was digested at 30 °C by 20 μg/mL subtilisin (Carlsberg) for 45 min. Microtubules were sedimented and resuspended in buffer A, and the solution was clarified by centrifugation at 300000*g* for 6 min and processed as described for uncleaved tubulin.

Polymerization Measurements. Tubulin polymerization was monitored turbidimetrically at 350 nm at 37 °C in a spectrophotometer equipped with a 120-μL thermostated cuvette (light path 0.5 cm). The amount of polymerized tubulin at steady state was determined using a sedimentation assay carried out at 170000*g* for 4 min in an Airfuge (Beckman) thermostated at 30 °C by a flow of warm air. Protein present in the supernatant was assayed according to Lowry et al. (1951) or Bradford (1976).

GTPase Activity Measurements. GTP hydrolysis was measured in the time course of tubulin assembly by extraction of the [^{32}P]phosphomolybdate complex formed in 1 N HCl, as described previously (Carlier & Pantaloni, 1981).

Electron Microscopy. Samples of tubulin (10 μM) polymerized in buffer A supplemented with 160 μM $ZnCl_2$ and 50 μM GTP or GDP were negatively stained (without fixation) on carbon-coated grids with 1% uranyl acetate and examined in a Philips EM 410 electron microscope.

Zn^{2+} Binding to Dimeric Tubulin and to Tubulin Assembled in Zn Sheets. Equilibrium binding studies were performed using $^{65}ZnCl_2$ and the gel-filtration method of Hummel and Dreyer (1962). Columns of Sephadex G-25M (PD-10, Pharmacia) (5 × 1.8 cm) were equilibrated at 4 °C in buffer A containing 5–15 μM GTP or GDP, 0 or 100 μM DTT, and various concentrations of $^{65}ZnCl_2$ in the range 2.5–700 μM. Tubulin (0.5 mL, 20–30 μM) containing $^{65}ZnCl_2$ at the same concentration as the column buffer was applied to the column following a 20-min incubation at 4 °C. Elution of the column was carried out at 4 °C with the equilibrating buffer. Fractions of 0.3 mL were collected. Protein concentration and radioactivity were quantitated in each fraction. Radioactivity measurements were made in a Packard CA2000 scintillation spectrometer. Concentrations of Zn–nucleotide and free Zn^{2+} in the elution buffer were calculated by using equilibrium dissociation constants of 11 and 100 μM for Zn–GTP and Zn–GDP, respectively (Sigel, 1977; Correia et al., 1987). Binding of Zn^{2+} ions to tubulin assembled in Zn sheets was carried out at 4 °C using a sedimentation assay. Because spontaneous polymerization, which is rate-limited by nucleation, is very slow at 4 °C, Zn sheets were first assembled at 37 °C in the presence of GTP or GDP and $^{65}ZnCl_2$ in the range 25–500 μM, then brought to 4 °C for 10 min and sedimented at 4 °C. Because this experiment might be biased due to the change in temperature, causing Zn sheets to be in a metastable state at 4 °C, the binding of $^{65}Zn^{2+}$ to Zn sheets was also studied in an isothermic experiment carried out at 20 °C. The data were analyzed according to Scatchard (1949). The concentration of Zn^{2+} bound to pelleted Zn sheets was equal to the difference between the total concentration of Zn^{2+} ions, $[Zn]_0$, and the concentration of Zn^{2+} ion measured in the supernatants, $[Zn]_s$. The concentration of free Zn^{2+} ions in $[Zn]_s$ was calculated taking into account the binding of Zn^{2+} to dimeric tubulin, present at 1–2 μM in the supernatant. The proportion of Zn^{2+} bound to dimeric tubulin in $[Zn]_s$ was always lower than 10% and could be neglected at $[Zn^{2+}]_s$ concentrations higher than 100 μM.

CrGTP Synthesis. β,γ-Bidentate CrGTP was synthesized as described (McNeal & Purich, 1978; Carlier et al., 1991). The purity of CrGTP was assessed by anion-exchange HPLC with a Synchropak AX300 column (Synchrom, Inc.) and

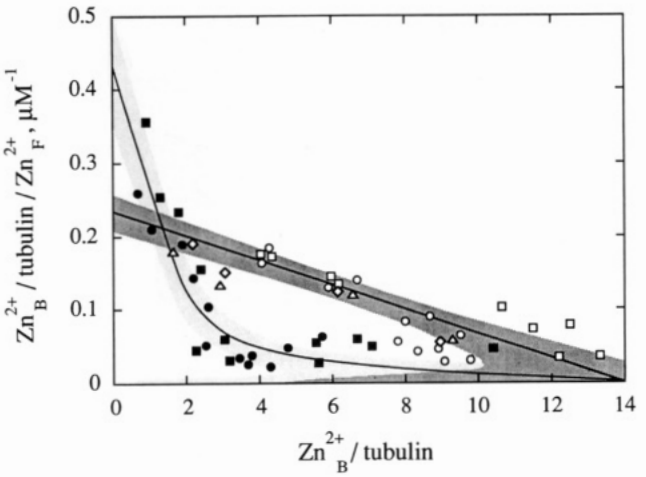


FIGURE 1: Scatchard representation of the binding of $^{65}Zn^{2+}$ to dimeric and assembled tubulin. Closed symbols: Binding of Zn^{2+} to dimeric GTP–tubulin (■) or GDP–tubulin (●) at 4 °C. Open symbols: Binding of Zn^{2+} to tubulin assembled into Zn sheets from either GTP–tubulin (□, △) or GDP–tubulin (○, ◇). Measurements were done as described in Materials and Methods at 4 °C (□, ○) or 20 °C (△, ◇). The curves are theoretical and represent binding of Zn^{2+} to two classes of n sites and m sites with equilibrium dissociation constants K_1 and K_2, as described by the equation $\bar{\nu} = \{n/[1 + (K_1/[Zn]_{free})]\} + \{m/[1 + (K_2/[Zn]_{free})]\}$ where $\bar{\nu}$ represents the number of Zn^{2+} bound per dimeric tubulin. The curves are drawn with n = 2, K_1 = 5 μM, m = 12, and K_2 = 400 μM for dimeric tubulin and n = 2, K_1 = 60 μM, m = 12, and K_2 = 60 μM for Zn sheets. The gray zones account for the theoretical standard deviation calculated by assuming a 3% error in measurements of $[Zn]_{total}$ and $[Zn]_{free}$.

isocratic elution by 0.25 M KH_2PO_4, pH 3.5, and 1.3 M NaCl. The CrGTP solution (7 mM) was stored at –20 °C at pH 3.

Interaction of Beryllium Fluoride and Aluminum Fluoride with Zn Sheets. Binding of beryllium fluoride to polymers was measured by sedimentation using ^7Be as described (Carlier et al., 1988). Tubulin (30 μM) in buffer A containing 100 μM GTP, 5 mM NaF, and different amounts of ^7Be-labeled beryllium was assembled by addition of 100 μM $ZnCl_2$ or 6 mM $MgCl_2$ in order to obtain Zn sheets or microtubules, respectively. The samples were brought to 37 °C for 20 min and spun at 170000g for 4 min in the airfuge. The amount of beryllium bound to polymers was derived from measurements of total ^7Be radioactivity present in solution before centrifugation and remaining in the supernatant. Control experiments were carried out in the absence of NaF or tubulin. The binding of AlF_4^- and $BeF_3^-\cdot H_2O$ to polymers was determined using a fluoride-specific electrode (PF-4L, Tacussel, Solea) as described previously (Combeau & Carlier, 1989).

RESULTS

Binding of Zn^{2+} to Tubulin and Linkage to Polymerization of Tubulin into Zn Sheets. Equilibrium binding of Zn^{2+} ions to GTP–tubulin or GDP–tubulin was measured at 4 °C in buffer A using $^{65}ZnCl_2$ and the Hummel and Dreyer technique. The results, which are shown in Figure 1, indicate that Zn^{2+} ions bind to two high-affinity and 12 low-affinity sites on dimeric tubulin with K_Ds of 5 μM and 400 μM, respectively. Binding of Zn^{2+} took place to practically the same extent on either GTP–tubulin or GDP–tubulin. Both the binding of Zn^{2+} to tubulin and the assembly into Zn sheets were inhibited by DTT, suggesting that Zn^{2+} ions may bind to either cysteine or histidine residues on tubulin.

Analysis of Zn^{2+} binding to tubulin assembled in Zn sheets was performed at 4 and 20 °C as described in Materials and Methods. The data, also shown in Figure 1, are consistent with the binding of Zn^{2+} to a single category of 14 sites with K_D = 60 μM. Although no Zn sheets can be formed in a range of low concentrations of Zn^{2+} ions in which Zn^{2+} is bound to tubulin at the high-affinity sites only, it was not possible to fit the data by assuming the existence of the high-affinity sites on the polymer. The data displayed in the Scatchard representation, however, clearly show that in the range of 2–14 Zn^{2+} ions bound per tubulin, the binding of Zn^{2+} is about 7–fold tighter to the polymer than to dimeric tubulin, and globally an increased binding of Zn^{2+} to the polymer accompanies the formation of Zn sheets, in agreement with the linked function theory (Wyman & Gill, 1991).

Polymerization of GTP–Tubulin and GDP–Tubulin into Zn Sheets. Tubulin was polymerized at different concentrations in the presence of 150 μM $ZnCl_2$ and 150 μM GTP in buffer A. Polymerization was monitored by turbidity and by a sedimentation assay (see Materials and Methods). The results, displayed in Figure 2, show evidence for a nucleated polymerization and a critical concentration of 1.5 μM. When the same experiment was done with GDP–tubulin in the presence of 150 μM $ZnCl_2$ and 150 μM GDP, very similar results were obtained, except that the lag time for Zn sheet assembly was longer than in the presence of GTP at a given tubulin concentration. Accordingly, the critical concentration for Zn sheet assembly from GDP–tubulin was slightly higher than from GTP–tubulin: a value of 2.0 μM was found. As was previously observed in turbidimetric measurements of microtubule assembly (Carlier & Pantaloni, 1978), the change in turbidity versus total tubulin concentration displayed a slight downward deviation from linearity (Figure 2C,D), consistent with the nonideality of the polymer solution. These data show that, in contrast to microtubule assembly, the presence of GTP is not strictly necessary for the assembly of Zn sheets. It was found, using the sedimentation assay described in Materials and Methods, that the tubulin concentration in the supernatant, i.e., the critical concentration, at a given Zn^{2+} concentration (150 μM) was practically constant at tubulin concentrations in the range 2.5–40 μM at both 30 and 37 °C. This behavior is strikingly different from that of microtubules assembled in the presence of GTP. The difference between microtubules and Zn sheets is illustrated in Figure 3. Microtubules exhibit a linear increase in apparent critical concentration upon increasing total concentration of tubulin. It has been shown (Carlier, 1989) that this linear increase is not due to denatured tubulin but corresponds to the steady-state accumulation of unpolymerizable dimeric GDP–tubulin, resulting from the rapid depolymerization of stretches of GDP microtubules and the relatively slow exchange of GTP for GDP on dimeric tubulin. The general equation accounting for this reaction is

$$[\text{T–GDP}] = [\text{P}]\frac{k_{-\text{D}}}{k_e + k_{+\text{D}}[\text{P}]} \quad (1)$$

where [P] represents the number concentration of polymer, k_{-D} is the monomolecular rate constant for dissociation of GDP–tubulin from the polymer, k_{+D} is the bimolecular rate constant for association of GDP–tubulin to the polymer, and k_e is the rate constant for nucleotide exchange on dimeric tubulin. In the case of microtubules, $k_{+D} = 0$, and eq 1 reduces to

$$[\text{T–GDP}] = [\text{P}]\frac{k_{-\text{D}}}{k_e} \quad (2)$$

Equation 2 describes the linear relationship between the steady-state concentration of T–GDP and the number of

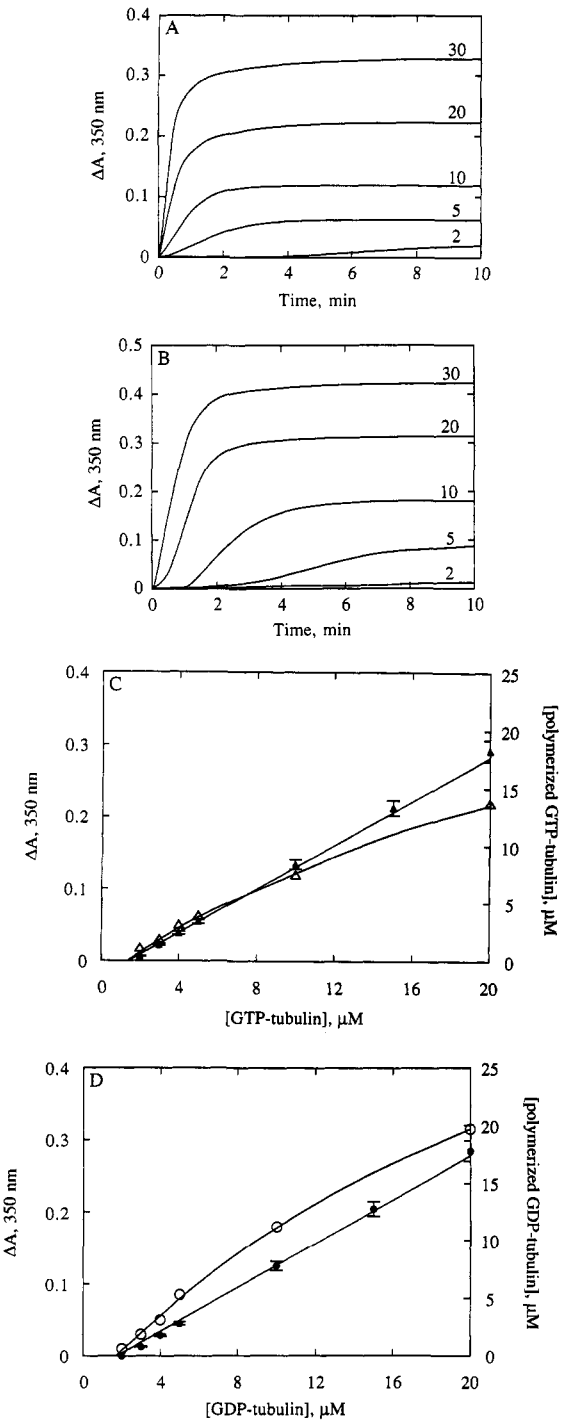


FIGURE 2: Polymerization of GTP–tubulin and GDP–tubulin into Zn sheets. GTP–Tubulin (panel A) or GDP–tubulin (panel B) was polymerized at 37 °C at the indicated concentrations in buffer A containing 150 μM $ZnCl_2$. Turbidity was recorded at 350 nm (light path 0.5 cm). The critical concentration for Zn sheet assembly from either GTP–tubulin (panel C) or GDP–tubulin (panel D) was derived from the maximum extent of turbidity change (open symbols) as well as from sedimentation assays (closed symbols). Note the slight curvature of the turbidity plots (see text) and also the larger slope of the turbidity plot in the presence of GDP, consistent with the larger size of Zn sheets (see Figure 4C–F).

microtubules in solution, which increases with total tubulin concentration. The above interpretation was confirmed by showing that the high-affinity analog of inorganic phosphate, $BeF_3^-{\cdot}H_2O$, reconstitutes a very stable GDP–P_i microtubule and abolishes the large increase in apparent critical concentration with total tubulin concentration [see Figure 6 in Carlier et al. (1988)]. In the case of Zn sheets, GDP–tubulin released

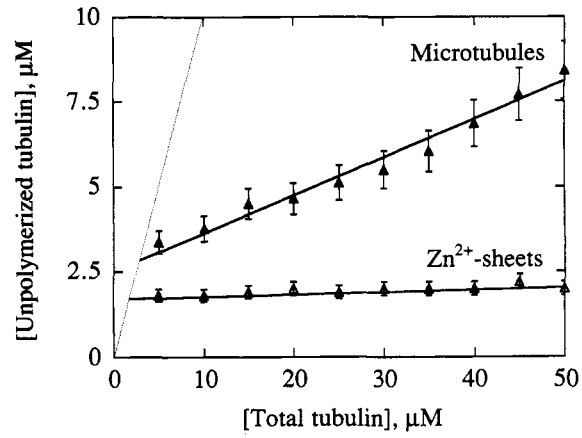
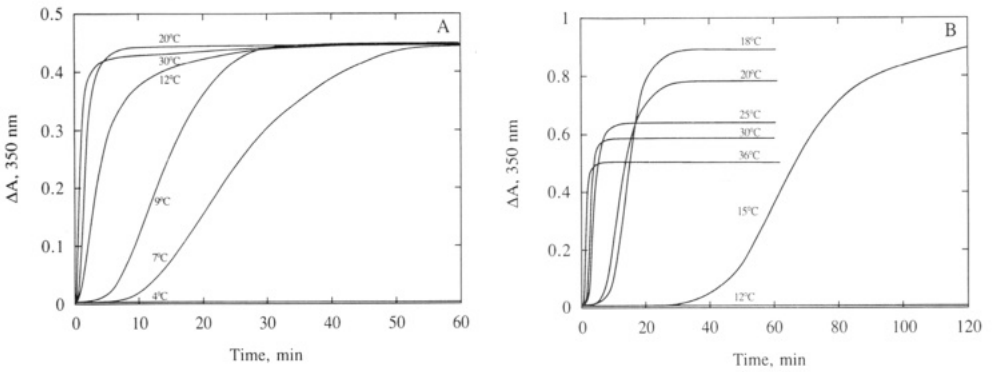
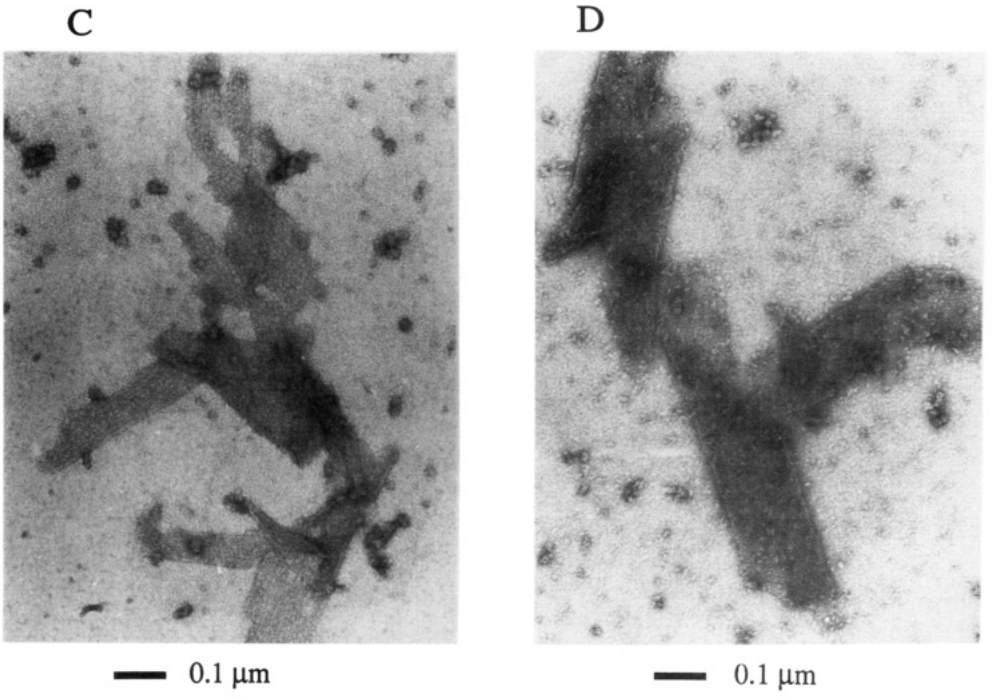


FIGURE 3: Dependence of the apparent critical concentration on total tubulin concentration, for microtubule and Zn sheet assembly, on a comparative basis. Tubulin, at the indicated concentrations, was polymerized either in microtubules at 37 °C for 20 min in buffer A supplemented with 0.25 mM GTP and 6 mM $MgCl_2$ (▲) or in Zn sheets under the same conditions except buffer A was supplemented with 0.15 mM GTP and 150 μM $ZnCl_2$ (△). Samples were sedimented at 37 °C, 400000*g* for 5 min. The concentration of tubulin in the supernatant was determined by the Lowry assay.

in the medium in the polymer turnover process is able to polymerize (Figure 2) with a critical concentration k_{-D}/k_{+D} very close to that of GTP–tubulin; therefore, according to eq 1 and as demonstrated by the data in Figure 3, the change in critical concentration upon increasing total tubulin concentration is extremely small. These results hence provide additional support to the previously proposed interpretation for the increase in C_c.

Another difference between Zn sheets and microtubules is the temperature dependence of the polymerization reaction. Zn sheets assembled at 37 °C from 20 μM tubulin depolymerized slowly and to a small extent (10%) upon cooling of the solution to 4 °C. In contrast, Zn sheets rapidly depolymerized upon addition of 1 mM EDTA. Depolymerization promoted by the addition of EDTA was faster at 37 °C than at 4 °C. The temperature dependence of the polymerization into Zn sheets was analyzed by turbidimetry and sedimentation assays, in the presence of either GTP or GDP. Results are displayed in Figure 4. The rate of polymerization increased with temperature in the presence of either GTP or GDP. In the presence of GTP, the extent of turbidity change remained practically constant in the temperature range studied (7–37 °C). In contrast, in the presence of GDP, the extent of turbidity change increased upon decreasing the temperature at which assembly was monitored, in the range 15–37 °C (Figure 4B). Electron microscopy (Figure 4C–F) showed that in the presence of GTP the general morphology of Zn sheets was unchanged in the range 12–37 °C, but sheets were broader at low temperature, consistent with the larger specifc turbidity change and slow nucleation process. In the presence of GDP, Zn sheets of the same morphology as in GTP were formed at 37 °C. Superimposable optical diffraction patterns were obtained from electron micrographs of sheets assembled at 37 °C from either GTP–tubulin (Figure 4C) or GDP–tubulin (Figure 4E), indicating that protofilament spacing is identical in Zn sheets assembled from either GDP–tubulin or GTP–tubulin. At a given temperature, Zn sheets assembled from GDP–tubulin were broader than those assembled from GTP–tubulin, consistent with the larger specific turbidity apparent in Figure 2C. However, as temperature decreased, another very large flat structure, in which the characteristic striations of protofilaments were not apparent, became





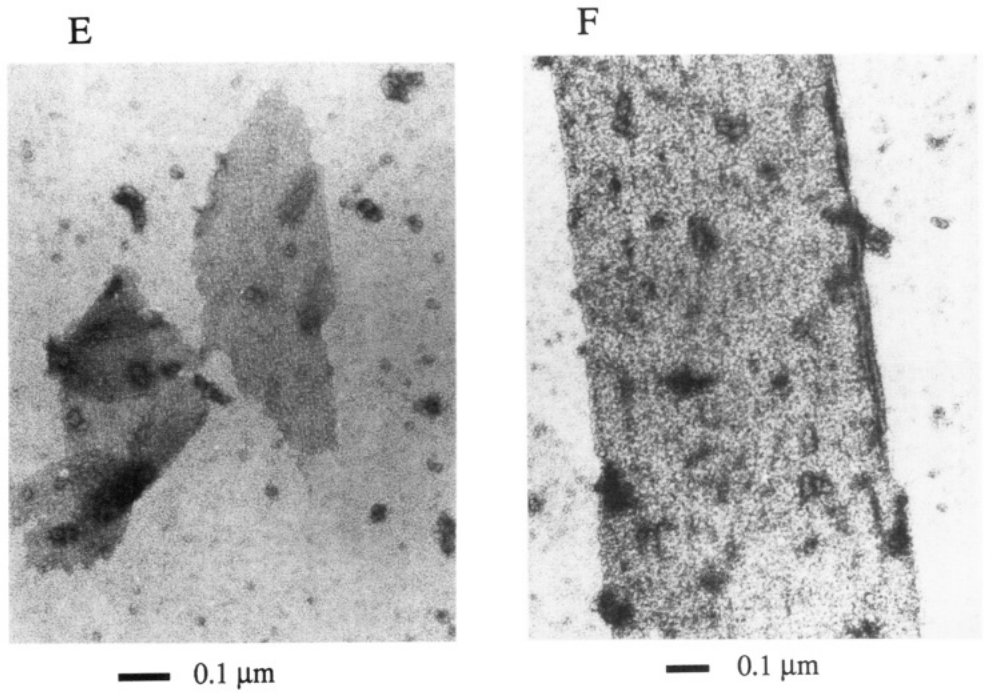


FIGURE 4: Effect of temperature on the assembly of tubulin induced by the binding of Zn^{2+}. GTP–tubulin (panel A) or GDP–tubulin (panel B) (25 μM) was assembled in buffer A containing 150 μM $ZnCl_2$ and 150 μM GTP or GDP at the indicated temperature. Polymerization was monitored by turbidity at 350 nm (light path 0.5 cm). Panels C–F shows electron micrographs of Zn sheets assembled from GTP–tubulin (panels C and D), and GDP–tubulin (panels E and F) at 37 °C (panels C and E) or 15 °C (panels D and F); bar = 0.1 μm.

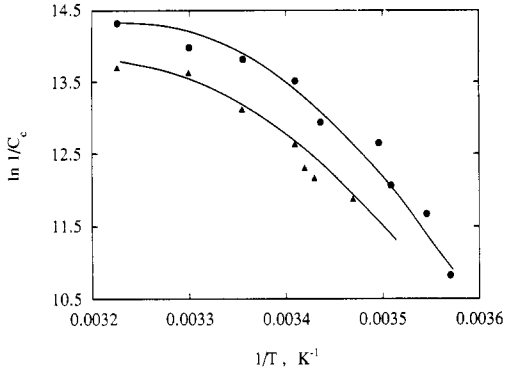


FIGURE 5: Temperature dependence of the critical concentration for Zn sheet formation in the presence of GTP or GDP. Tubulin (25 μM) was assembled at different temperatures in buffer A containing 150 μM $ZnCl_2$ and GTP or GDP. When the plateau was reached, Zn sheets were sedimented and the critical concentration C_c was determined as the concentration of dimeric tubulin in the supernatant. The data obtained in the presence of GTP (●) or GDP (▲) were plotted according to the van't Hoff equation displayed in the text.

gradually predominant over Zn sheets. Both structures coexisted at 20 °C, and no Zn sheets were formed from GDP–tubulin below 12 °C. The formation of the large undefined structure accounts for the very large turbidity change observed in the presence of GDP at low temperature. This structure also rapidly and totally disassembled upon addition of EDTA. Because of the dependence of the turbidity of Zn sheets on the size of the polymers, the thermodynamic parameters for Zn sheet assembly were more conveniently studied using asedimentation assay. The temperature dependence of the critical concentration for Zn sheet formation in the presence of either GTP or GDP is shown in Figure 5. The data were plotted according to the van't Hoff equation:

$$\ln K_p = \ln (C_c^{-1}) = -\frac{\Delta G}{RT} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (3)$$

where C_c is the critical concentration derived from the sedimentation assay, T is the absolute temperature, and ΔG, ΔH, and ΔS represent the changes in free energy, enthalpy, and entropy linked to the assembly of Zn sheets. The van't Hoff plots were not linear in both GTP and GDP, showing that the enthalpy change of the reaction varies with temperature, consistent with an apparent change in heat capacity $\Delta C_p = d\Delta H/dT$. The values of ΔH, ΔS, and ΔC_p were derived by fitting the data shown in Figure 5 to a limited development of the integrated van't Hoff equation (Glasstone, 1947; Lee & Timasheff, 1977):

$$\ln 1/C_c = a + b/T + c \ln T \qquad (4)$$

where

$$\Delta C_p = Rc \qquad \Delta H = R(cT - b) \qquad (5)$$

Similar nonlinear van't Hoff plots have previously been reported for microtubule assembly from pure tubulin in the presence of 16 mM $MgCl_2$ (Lee & Timasheff, 1977) and for the polymerization of tubulin–colchicine, also in the presence of Mg^{2+} ions (Saltarelli & Pantaloni, 1982; Andreu et al., 1983). On the other hand, Robinson and Engelborghs (1982) found linear van't Hoff plots for microtubule assembly in the presence of dimethyl sulfoxide (DMSO). The thermodynamic parameters for Zn sheet assembly are summarized in Table II. Comparison with the corresponding values reported for microtubules (Lee & Timasheff, 1977; Hinz & Timasheff, 1986) and tubulin–colchicine polymers readily shows that the enthalpy and entropy changes associated with the assembly of Zn sheets are very similar to those associated to the polymerization of tubulin or tubulin–colchicine in the presence of magnesium ions; in particular, the same change in heat capacity, ΔC_p, of −1200 ± 200 cal/(mol K), was found. In addition, changes in ΔH values appear to counterbalance those of $T\Delta S$. This is in agreement with the observations made by Andreu et al. (1983) in a comparison of microtubule assembly and tubulin–colchicine polymerization. Finally, the values of ΔH and ΔC_p are practically the same for the assembly of Zn sheets from GTP–tubulin or from GDP–tubulin. The fact that the exact same value of ΔC_p is found for polymerization of tubulin into microtubules, Zn sheets, or tubulin–colchicine polymer, although under different ionic conditions, indicates that the binding of Mg^{2+} ions is not involved in the change in heat capacity, since Mg^{2+} ions are not necessary for Zn sheet formation, and suggests that the hydrophobic areas involved in tubulin–tubulin contacts are of comparable size in all three polymers.

Finally, in agreement with Gaskin (1981), Zn sheets were also obtained when GTP was replaced by CrGTP, a stable analog of the Mg–GTP complex (Mac Neal & Purich, 1978; Carlier et al., 1991). Zn sheets could also be successfully assembled from subtilisin-treated tubulin ($\alpha_S\beta_S$) in which the acidic C-terminal region of tubulin has been removed (Sackett et al., 1985; Melki et al., 1991). Collectively, all these data indicate that the formation of Zn sheets is not very sensitive to the nature of E-site-bound nucleotide GTP/GDP, complexed or not with a divalent metal ion, and that Zn ions bind at sites different from the nucleotide E-site and from the acidic C-terminus, as proposed (Gaskin, 1981; Eagle et al., 1983).

Colchicine binding to tubulin is known to inhibit its incorporation into microtubules; however, tubulin–colchicine retains the ability to self-assemble into nonmicrotubular large

Table II: Thermodynamics of Zn Sheet Growth in the Presence of GDP–Tubulin or GTP–Tubulin[a]

T (°C)	ΔG(GDP) (kcal/mol)	ΔH(GDP) (kcal/mol)	ΔS(GDP) (eu)	ΔG(GTP) (kcal/mol)	ΔH(GTP) (kcal/mol)	ΔS(GTP) (eu)
7				−6.07	40	164.5
9				−6.6	37.2	155.3
12				−6.9	33	140
13				−7.2	31.6	135.7
15	−6.8	28.4	122.2			
18	−7.1	24.8	109.6	−7.5	24.6	110.3
19	−7.2	23.6	105.5			
20	−7.4	22.4	101.7	−7.9	21.8	101.3
25	−7.8	16.4	81.2	−8.2	14.8	77.2
30	−8.3	10.4	61.7	−8.5	7.8	53.8
37	−8.5	2.0	33.9	−8.9	−2	22.2
	ΔC_p(GDP) = −1200 ± 100 cal/(mol K)			ΔC_p(GTP) = −1400 ± 100 cal/(mol K)		

[a] The values of ΔG were derived from the measurements of the critical concentration at different temperatures. The values of ΔH and ΔC_p were derived by fitting the data from Figure 4 to eqs 2 and 3. The values of ΔS were calculated as $(\Delta H - \Delta G)/T$ at each temperature.

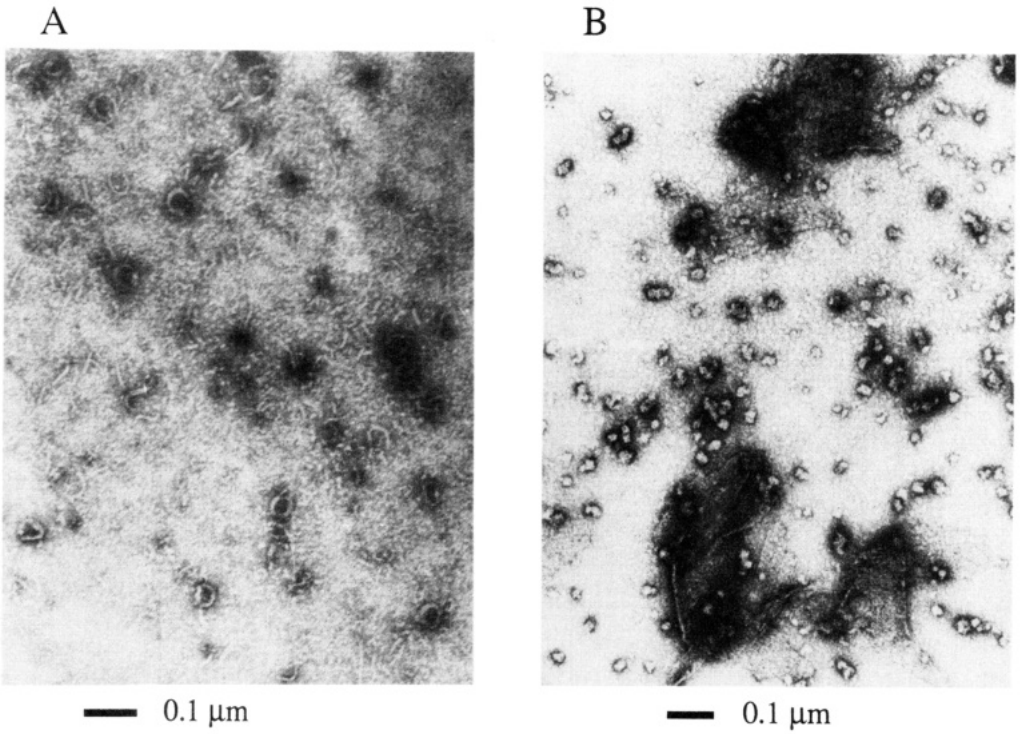


FIGURE 6: Electron micrographs of tubulin–colchicine polymers in the presence of Mg^{2+} or Zn^{2+}. Tubulin–colchicine 1:1 complex (70 μM) in 0.1 M MES, 30% glycerol, and 150 μM GTP was incubated at 37 °C in the presence of 10 mM $MgCl_2$ (panel A) or 200 μM $ZnCl_2$ (panel B). The bar indicates 0.1 μm.

aggregates which were characterized as irregular associations of curly filaments (Saltarelli & Pantaloni, 1982, 1983). Alternatively, formation of large sheets has been reported (Andreu & Timasheff, 1982, 1983). It was interesting to examine whether lateral contacts between the antiparallel protofilaments forming bidimensional Zn sheets are inhibited by colchicine binding. In the presence of GTP and Mg^{2+} ions, the tubulin–colchicine complex assembled into curved protofilaments, in agreement with Saltarelli and Pantaloni (1982). No flat sheets were observed in the presence of Mg^{2+} ions. When Mg^{2+} was replaced by Zn^{2+} in the polymerizing solution, characteristic in flat Zn sheets were observed. These sheets were narrower than sheets made of unmodified GTP–tubulin or GDP–tubulin. Electron micrographs of polymers of tubulin–colchicine obtained in the presence of either 10 mM Mg^{2+} or 200 μM Zn^{2+} are displayed in Figure 6. The critical concentration for assembly of tubulin–colchicine in Zn sheets was only 10-fold larger than for assembly of unliganded tubulin (data not shown). This result suggests that the lateral tubulin–tubulin interactions taking place between antiparallel protofilaments in Zn sheets are not abolished by colchicine binding and therefore are of a different nature from lateral contacts between protofilaments in the microtubular lattice, which are thought to be inhibited by colchicine binding.

Hydrolysis of GTP upon Zn Sheet Assembly. The hydrolysis of [γ-^{32}P]GTP was assayed during polymerization of tubulin (30 μM) into Zn sheets. Figure 7a shows that cleavage of the γ-phosphoester bond (as acid-labile $^{32}P_i$) occurs during polymerization of tubulin in Zn sheets. When the polymerization plateau was reached, a steady-state rate of GTP hydrolysis of 0.46 μM P_i/min was measured, consistent with a monomer–polymer exchange process taking place between dimeric tubulin and the Zn sheet polymer. When the 1:1 [γ-^{32}P]GTP–tubulin 1:1 complex (30 μM) was assembled into Zn sheets, Figure 7B shows that no appreciable spontaneous depolymerization of the sheets accompanied GTP consumption, contrasting with the observation made on microtubules (Carlier & Pantaloni, 1978; Carlier et al., 1987).

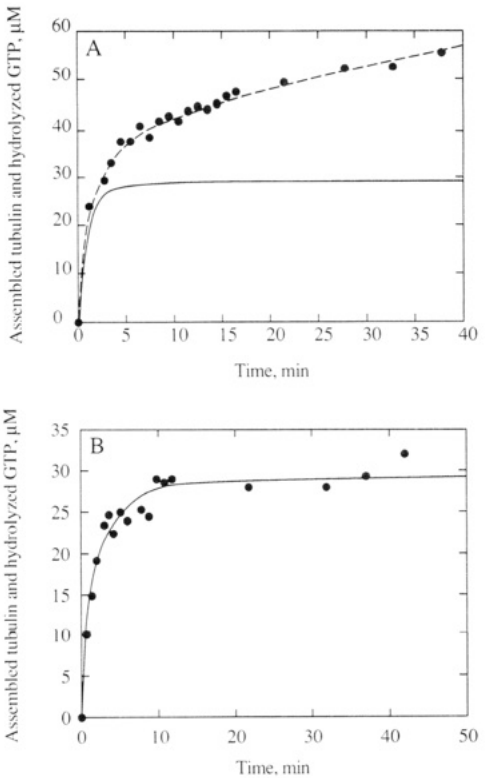


FIGURE 7: Cleavage of the γ-phosphate of GTP accompanying tubulin polymerization into Zn sheets. A solution of [γ-^{32}P]GTP–tubulin (1:1 complex, 30 μM) was assembled at 37 °C in the preheated 1-mL cuvette of the spectrophotometer in buffer A containing 150 μM $ZnCl_2$ in the presence (panel A) or absence (panel B) of 200 μM [γ-^{32}P]GTP. Turbidity was recorded (light path 1 cm) while aliquots of the solution were withdrawn from the cuvette at time intervals for determination of acid-labile $^{32}P_i$ (●). The solid line represents the amount of polymerized tubulin derived from turbidity recording.

Using double-labeled [γ-^{32}P,^{3}H]GTP, it was checked, using glass filter and sedimentation assays as described (Melki et al., 1990), that no $^{32}P_i$ was bound to the Zn sheets following cleavage of GTP. Therefore the stability of Zn sheets following cleavage of GTP is not due to P_i remaining bound to the polymer. This result is consistent with the ability of GDP–tubulin to assemble into Zn sheets and demonstrates that, in contrast to the microtubule case, no destabilization of tubulin–tubulin interaction in Zn sheets occurs upon hydrolysis of GTP and release of P_i.

The existence of a steady-state rate of GTP hydrolysis by Zn sheets was suggestive of monomer–polymer reactions occurring at steady state. The kinetics of monomer–polymer exchange was studied by performing [^{3}H]GTP and ^{125}I-tubulin pulse experiments, displayed in Figure 8. The time courses of radioactivity incorporation in Zn sheets, with the two labels, indicated a rapid turnover of Zn sheets. The kinetics of [^{3}H]-GTP incorporation was not linear with time and 60% of the process was mostly consistent with a random walk, diffusionlike process for monomer–polymer exchange (Zeeberg & Caplow, 1981), which varied linearly with $t^{1/2}$.

Analogs of Inorganic Phosphate Do Not Bind to Tubulin Assembled in Zn Sheets. It has been shown (Carlier et al., 1988, 1989) that the structural analogs of inorganic phosphate, AlF_4^- and $BeF_3^-\cdot H_2O$, bind to the site of the γ-phosphate of GTP on GDP microtubules and reconstitute a GDP–P_i-like polymer in which tubulin–tubulin interactions are strong. In contrast, AlF_4^- and $BeF_3^-\cdot H_2O$ do not bind to either GDP–tubulin dimer or linear ring oligomers of GDP–tubulin (Carlier et al., 1989). These observations indicated that in microtubules specifically, the site of the γ-phosphate of GTP remains "open". In other words, as proposed previously (Melki et al., 1989; Shearwin & Timasheff, 1992), tubulin subunits are constrained in a "straight" conformation in the microtubule polymer and adopt a "curved" conformation in the GDP dimeric or oligomeric state. It was observed that neither AlF_4^- nor $BeF_3^-\cdot H_2O$ affected the polymerization of tubulin in Zn sheets. In the presence of 5 mM NaF, no binding of ^{7}Be to Zn sheets, in the range 0–100 μM, could be detected. Accordingly, no appreciable fluoride binding to Zn sheets could be measured using a fluoride electrode as described (Combeau & Carlier, 1989).

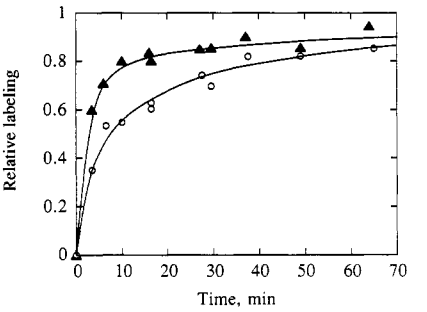


FIGURE 8: Time course of ^{125}I-tubulin and [^{3}H]GTP incorporation into Zn sheets at steady state. Tubulin (25 μM) was assembled at 37 °C in buffer A containing 150 μM $ZnCl_2$ and 200 μM GTP. ^{125}I-Tubulin (0.5 μM) or [^{3}H]GTP were added to the polymerized solution at steady state. Aliquots of the Zn sheet solution were centrifuged at different time intervals following the addition of labeled tubulin or nucleotide at 250000g, 37 °C for 4 min. The labeling of the polymer was derived from ^{125}I (▲) and ^{1}H (cop) radioactivity measurements made on the pellets of sedimented samples. the amount r of ^{125}I and ^{3}H incorporated in the polymer, at each time, was compared to the amount of r_∞ virtually incorporated at infinite time; the value of r_∞ was derived from measurements made on a control sample in which both ^{125}I-tubulin and [^{3}H]GTP were added before polymerization was started. The relative labeling is the ratio r/r_∞. Note that the two curves cannot be superimposable because, while [^{3}H]GTP specific radioactivity remains constant during the experiment, the ^{125}I-tubulin specific radioactivity gets lower and lower due to isotopic dilution in the time course of the monomer–polymer exchange process.

DISCUSSION

The present work was aimed at a better understanding of the role of GTP hydrolysis in tubulin–tubulin interactions in the Zn sheet polymer, on a comparative basis with microtubules. The main conclusion that we reach is that, in contrast to microtubules and also to the tubulin–colchicine curly polymer, the dynamics of Zn sheets is very little affected by GTP hydrolysis. Zn sheets can be assembled from GDP–tubulin almost as well as from GTP–tubulin; although GTP hydrolysis is triggered by tubulin–tubulin interactions taking place upon assembly of Zn sheets, no appreciable destabilization of the sheets is observed following phosphate release. In other words, binding of Zn^{2+} to tubulin has abolished the regulatory switch played by GTP hydrolysis in tubulin assembly. In contrast to microtubules, the Zn sheet polymer does not exert any structural constraint on the tubulin subunit, maintaining it in a "straight", GTP-binding conformation, in which the site of the γ-phosphate remains open following P_i release (Melki et al., 1989; Shearwin & Timasheff, 1992). The fact that the site on the γ-phosphate is inaccessible to phosphate analogs in Zn sheets indicates that the conformation of tubulin is, to some extent, and at least in the environment of the E-site-bound GDP, different from the conformation of tubulin in microtubules. Possible interpretations for this difference include steric hindrance of the phosphate binding site by Zn ions or the loss of the connection between GTP hydrolysis at the E site and lateral tubulin–tubulin contacts between protofilaments. It is also interesting to note that, while the binding of Mg-GTP to the nucleotide E-site is a requisite for polymerization of tubulin in microtubules, formation of Zn sheets is not affected by the presence or absence of a GTP-bound metal ion in the tubulin E-site. It is known that MgGTP and unchelated GDP are the preferred binding nucleotide species (Correia et al., 1987, 1988) and that the release of the metal ion accompanies GTP hydrolysis in microtubule assembly (Carlier et al., 1991). As has been shown to be the case for the *ras* p21 G-protein (Schlichting et al., 1990) it is possible that a change in the environment of the γ-phosphate–metal moiety of GTP following hydrolysis on microtubules is coupled to the conformational switch of the tubulin molecule responsible for the destabilization of the microtubule lattice. The role of the GTP-bound metal ion would be essential. In the assembly of Zn sheets, the loss of the regulatory switch may be correlated to the fact that the GTP-bound metal ion does not appear to be necessary for the assembly of this polymer; consequently, no evident connection appears between GTP hydrolysis and the strength of lateral interaction between tubulin subunits in Zn sheets. Nevertheless, the similarity of the thermodynamic parameters for assembly of these two polymers indicates that the hydrophobic lateral contacts are of comparable size in microtubules and Zn sheets. Given the structure of Zn sheets in alternating antiparallel protofilaments, this result is not surprising if one assumes that the resulting lateral interactions are of the same handedness as in the microtubule, i.e., the right side of one protofilament interacting with the left side of the adjacent protofilament; however, the contacts responsible for the conformational switch due to GTP hydrolysis may not be established.

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