Reversible Dimer Dissociation of Tubulin S and Tubulin Detected by Fluorescence Anisotropy†

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ABSTRACT: Concentration-dependent dissociation of dimers of goat brain tubulin S and tubulin was studied by fluorescence anisotropy. Upon dilution, assembly-competent fluorescein 5′-maleimide labeled dimers of tubulin S and tubulin show a progressive decrease in fluorescence anisotropy. That this lowering of anisotropy results from the dissociation of tubulin S dimers into monomers was shown by dilution experiments with unlabeled homologous and heterologous proteins. A nonlinear least-squares fit of the data gave a dissociation constant of 7.1×10^{-8} M for tubulin S compared to 7.2×10^{-7} M for tubulin at 25 °C in 0.1 M PEM buffer, pH 7.0. van't Hoff plots of dimer–monomer dissociation of tubulin S and tubulin also show considerable differences in ΔH and ΔS. Effects of ionic strength and colchicine on the equilibrium constants are also substantially different for tubulin and tubulin S. The implications of these observations on the influence of C-terminal tails on tubulin structure are discussed.

Tubulin (MW 100 000), the major protein in microtubules, is a heterodimer of two nonidentical subunits, α and β. Recent experiments suggest that each subunit is composed of a large N-terminal domain of about 300 amino acids and a small C-terminal domain of about 140 amino acid residues (Sackett & Wolff, 1986; de la Vina et al., 1988). In addition, each subunit has a negatively charged flexible C-terminal tail (Krauhs et al., 1981; Postingl et al., 1981; Ringel & Sternlicht, 1984). The tails are about 15–20 amino acid residues long. They are susceptible to proteolytic cleavage (Serrano et al., 1984b; Sackett et al., 1985; Bhattacharyya et al., 1985) and are binding sites for several agents such as MAPs,[1] Ca^{2+}, dyes, etc. (Serrano et al., 1984a,b, 1985, 1986; Kanazawa & Timasheff, 1989). Recent experiments have established that these tails play important roles in the regulation of self-assembly of tubulin by MAPs, τ, and Ca^{2+} (Serrano et al., 1984a,b, 1986). It has also been shown that these agents bind at the tails for their functions and removal of these tails by subtilisin removes the binding sites of these agents from the protein (Serrano et al., 1984b; Sackett et al., 1985; Bhattacharyya et al., 1985). A recent report on the regulation of colchicine binding to tubulin by the C-terminal tails is another example that underlines their important role in tubulin function (Mukhopadhyay et al., 1990). Removal of these tails by subtilisin produces tubulin S, which polymerizes very efficiently without any assembly inducers such as MAPs, DMSO, taxol, etc. (Bhattacharyya et al., 1985). The critical concentration of tubulin S polymerization is at least 10 times lower than that of the native tubulin polymerization by MAPs or τ (Bhattacharyya et al., 1985). As the critical concentration measures the strength of protein–protein interactions, the lowering of critical concentration upon removal of C-terminal tails suggests higher affinity among dimers of tubulin S compared to that occurring among native tubulin molecules of the microtubule. These results also suggest that the surface properties of tubulin dimers are altered significantly upon removal of the C-terminal tails. Thus, a study of dissociation of tubulin S dimer may throw light on some aspects of the role of the tails in protein–protein interactions.

Fluorescence anisotropy of protein molecules, covalently labeled with a fluorescent dye, is a convenient and rapid method to study the monomer–dimer equilibrium, provided that both dimer and monomer exhibit characteristic anisotropy values depending on their rotational correlation times (Weber, 1953). Fluorescence anisotropy measurements were used to demonstrate dissociation of tubulin dimers and to calculate thermodynamic parameters (Mejillano & Himes, 1989). Reversible dissociation of tubulin dimers was also demonstrated by equilibrium ultracentrifugation (Detrich & Williams, 1978; Detrich et al., 1982). Recently, a kinetic method using proteolysis (Sackett et al., 1989), and modified equilibrium ultracentrifugation, were used to study the dissociation of tubulin dimers (Sackett & Lippoldt, 1991). In the present study, tubulin and tubulin S were labeled with either fluorescein 5′-maleimide (FM) or 7-(diethylamino)-4-methylcoumarin 3-maleimide (CM) in polymerized conditions. The advantage of labeling with FM and CM is that they are donor–acceptor pairs suitable for energy transfer study; in addition, their high quantum yields allow accurate measurement of anisotropy at nanomolar concentrations. We report below a study of the monomer–dimer equilibrium of tubulin S by measurement of anisotropy as well as energy transfer.

MATERIALS AND METHODS

Materials. Fluorescein 5′-maleimide and 7-(diethylamino)-4-methylcoumarin 3-maleimide were purchased from Molecular Probes Inc. PIPES, EGTA, BPN′ subtilisin, PMSF, GTP, BSA, and colchicine were purchased from Sigma Chemical Co. Sephadex G-25 was from Pharmacia, Sweden.

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[1] Abbreviations: MAPs, microtubule-associated proteins; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PEM, 0.1 M PIPES, pH 7.0, 1 mM EGTA, and 1 mM $MgCl_2$; FM, fluorescein 5′-maleimide; FM-tubulin, FM-labeled tubulin; CM, 7-(diethylamino)-4-methylcoumarin 3-maleimide; CM-tubulin, CM-labeled tubulin; BSA, bovine serum albumin; bis-ANS, bis(8-anilinonaphthalene-1-sulfonate); DTAF, 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein; α_S, α subunit of tubulin S; β_S, β subunit of tubulin S; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; EM, electron microscope.

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[*ring C, methoxy*-^{3}H]Colchicine (specific activity 4.2 Ci/mmol) was obtained from Amersham, U.K. DE-81 filter discs were purchased from Whatman, U.K. All other chemicals were of analytical grade.

Isolation of Tubulin. Tubulin was isolated by phosphocellulose chromatography of microtubule-associated proteins, purified from goat brain by two cycles of temperature-dependent polymerization in a PIPES assembly buffer (0.1 M PIPES, pH 6.9, containing 1 mM EGTA and 1 mM $MgCl_2$) with 1 mM GTP (Sloboda & Rosenbaum, 1982). Following phosphocellulose chromatography, the protein was concentrated to 6–10 mg/mL by using Amicon CF-50A membrane cones, and aliquots were stored at −70 °C.

Chemical Modification. Tubulin (6 mg/mL) was polymerized in the presence of 1 M glutamate and 1 mM GTP at 37 °C for 30 min. The assembled tubulin was then fluorescein labeled with a 10-fold molar excess of FM (stock FM solution was in 0.1 M PEM buffer, pH 7.0). The labeling reaction was carried for 25 min at 37 °C and quenched with excess β-mercaptoethanol to a final concentration of 2 mM. The labeled tubulin was then centrifuged at 120000*g* for 1 h at 27 °C. The pellet was first washed with PEM buffer and then homogenized in the same buffer with the help of a glass homogenizer. The homogenized protein solution was kept in ice for 30 min and then centrifuged for 10 min at 15 000 rpm using a Sorvall SS-34 rotor at 4 °C. The free dye was separated from labeled protein by passing the protein solution through a Sephadex G-25 column (1.2 × 15 cm), previously equilibrated with 0.1 M PEM buffer, pH 7.0, at 4 °C.

The procedure for labeling tubulin with CM was the same as for FM labeling, except that a 5-fold molar excess of CM was used [stock CM solution was in DMF and the final concentration of DMF in the reaction mixture was kept below 1.5% (v/v)]. Protein concentrations were determined by the Bradford assay (Bradford, 1976) using BSA as the standard. The incorporation ratio of FM in tubulin was calculated using a molar extinction coefficient of 66 000 cm^{-1} M^{-1} at 495 nm in 0.1 M PEM buffer, pH 7.0. Similarly, the incorporation ratio of CM in tubulin was calculated using a molar extinction coefficient of 30 000 cm^{-1} M^{-1} at 383 nm in PEM buffer, pH 7.0.

Preparation of Tubulin S. Subtilisin was stored in aliquots as a stock solution of 1 mg/mL in water, at −70 °C and used only once after thawing. Digestion of FM-tubulin and CM-tubulin with subtilisin was performed at 30 °C for 30 min in a 0.1 M PEM buffer, pH 7.0, containing 1 mM GTP. Subtilisin was added to a solution of FM-tubulin at a ratio of 1:100 (w/w). The reaction was terminated by the addition of 1% (v/v) of 1% (w/v) PMSF in DMSO. Digestion at 30 °C resulted in the cleavage of the C-termini of both the subunits, giving rise to the $\alpha_s\beta_s$ dimer which is called tubulin S (Mukhopadhyay et al., 1990). The solution containing labeled tubulin S was then passed through a Sephadex G-25 column (1.2 × 15 cm) previously equilibrated with 0.1 M PEM buffer, pH 7.0.

Fluorescence Studies. Steady-state fluorescence was measured in a Hitachi F-3000 spectrofluorometer equipped with a computer. The band pass for the excitation and emission monochromators was 5 nm unless stated otherwise. Anisotropy was calculated from the formula

$$A = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

$$G = \frac{I_{hv}}{I_{hh}} \quad (2)$$

where I_{vv}, I_{vh}, I_{hh}, and I_{hv} are fluorescence intensity components; the subscripts refer to the horizontal (h) or vertical (v) positioning of the excitation and emission polarizer, respectively. G is the grating factor that corrects for wavelength-dependent distortion of the polarizing system. The fluorescence anisotropies of FM-tubulin S and FM-tubulin were determined at various protein concentrations by dilution with 0.1 M PEM buffer, pH 7.0, except where the effect of ligand or salt on dimer dissociation was determined. In such cases, the appropriate ligand or salt was included in the buffer. In each case, a buffer blank was subtracted from each experimental value. Computer averaging was performed when needed to increase the signal to noise ratio. The excitation wavelength used was 495 nm, and fluorescence was measured at 520 nm.

Data Analysis. Observed anisotropy in a dimer–monomer equilibrium system can be related to monomer anisotropy, dimer anisotropy, dissociation constant, and total protein concentration. The principle of additivity of anisotropy is used to relate the measured anisotropy to the dissociation constant. If dimer and monomer are present in solution, then

$$A = A_d f_d + A_m f_m \quad (3)$$

where A is the observed anisotropy, A_d and A_m are the limiting anisotropies of the dimer and monomer, and f_d and f_m are the mole fractions of each species present. Combining eq (3) with the expression for dissociation constant, we can write

$$A = \frac{K_d[-1 - (1 + 4P_0/K_d)^{1/2}][2A_m + A_d[-1 + (1 + 4P_0/K_d)^{1/2}]]}{4P_0}$$

where K_d is the dissociation constant and P_0 is the total protein concentration. Tubulin concentrations are always expressed in terms of dimer concentration, unless stated otherwise.

The three unknown parameters were systematically varied within a given range, and χ^2 statistics were used to judge the quality of the fit with experimental data. The best-fit curve was taken to be that for which a minimum χ^2 value was obtained.

The correlation time and limiting anisotropy were determined from the slope and the extrapolated intercept of the isothermal Perrin plot of $1/A$ versus T/η (Weber, 1952). The Perrin equation is

$$1/A = 1/A_0[1 + \tau_f/\tau_c] = 1/A_0[1 + (\tau_f kT/V_h\eta)]$$

In this equation τ_f is the fluorescence lifetime, τ_c is the rotational correlation time, k is the Boltzmann constant, T is the temperature, V_h is the molecular volume, and η is the viscosity of the solution. Viscosity was varied at constant temperature by the inclusion of sucrose in 0.1 M PEM buffer, pH 7.0. Literature values for the viscosity of sucrose solution were used (Weast, 1977). Fluorescence lifetimes were determined in an Applied Photophysics single photon counting apparatus, using a nanosecond lamp (nitrogen) based system. The lifetimes of FM-tubulin S were determined at 3.3 μM and 0.009 μM FM-tubulin S concentrations; at these concentrations the tubulin S is mostly in dimeric and monomeric form, respectively, at 25 °C in 0.1 M PEM buffer, pH 7.0.

Fluorescence Energy Transfer Study. The energy transfer efficiency (E) was defined as

$$E = \frac{F^{d+a}_{530}/F^{d+a}_{465}}{(F^{d}_{530} + F^{a}_{530})/(F^{d}_{465} + F^{a}_{465})}$$

$$E = \frac{F^{d+a}_{530}(F^{d}_{465} + F^{a}_{465})}{F^{d+a}_{465}(F^{d}_{530} + F^{a}_{530})}$$

where F^{d+a}_{530} and F^{d+a}_{465} are the fluorescence of the acceptor in the presence of the donor at 530 and 465 nm, respectively, F^{a}_{530} and F^{a}_{465} are the fluorescence of the acceptor in the absence of the donor at 530 and 465 nm, respectively, and F^{d}_{530} and F^{d}_{465} are the donor fluorescence in the absence of the acceptor at 530 and 465 nm, respectively. The experiment was performed in three sets as follows: First, 1.1 μM CM-tubulin S was progressively diluted to different concentrations by 0.1 M PEM buffer, pH 7.0, and for each protein concentration, an emission spectrum was taken. From the emission spectra at different CM-tubulin S concentrations, F^{d}_{465} and F^{d}_{530} were noted. Second, 1.1 μM FM-tubulin S was similarly diluted to various concentrations. From the emission spectra at different FM-tubulin S concentrations, F^{a}_{465} and F^{a}_{530} were noted. Third, 1.1 μM FM-tubulin S was mixed with 1.1 μM CM-tubulin S. The mixture, which will contain 50% hybrid tubulin S, was then diluted to different concentrations using 0.1 M PEM buffer, pH 7.0. From the emission spectra of hybrid tubulin S at different concentrations, F^{d+a}_{465} and F^{d+a}_{530} were recorded. In all cases, the excitation wavelength was 420 nm, and excitation and emission band pass was 3 nm. In each case, the buffer blank was subtracted from the original values, and computer averaging was done to increase the signal to noise ratio, when required.

Size-Exclusion HPLC of Tubulin S. Tubulin S was chromatographed on a Waters HPLC at 25 °C on Protein Pak 300 SW column having a fractionation range 10 000–400 000 Da. The following proteins were used to calibrate the column: (i) apoferritin (MW 443 000), (ii) β-amylase (MW 200 000), (iii) yeast alcohol dehydrogenase (MW 150 000), (iv) bovine serum albumin (MW 66 000), and (v) egg albumin (MW 45 000). Blue dextran and tryptophan were used to determine the void and column volumes, respectively. In each case 50 μL of an 0.8 mg/mL sample was injected, and the elution was carried out in 0.1 M PEM buffer, pH 7.0. $K_{average}$ is defined as the ratio $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of a macromolecule, V_0 is the void volume of the column, and V_t is the elution volume of a small molecule. $K_{average}$ values of the proteins were plotted against the corresponding logarithms of molecular weights. From the calibration curve and the elution volumes of tubulin and tubulin S, the molecular weights were approximately determined.

Electron Microscopy Study. For the electron microscopy study, samples were prepared using the procedure of Horowitz et al. (1984).

Colchicine Binding Assay. Protein (2 μM) was incubated at 37 °C, for 1 h, with varying concentrations of colchicine, ranging from 0.2 to 40 μM, containing [^{3}H]colchicine. Then the colchicine binding assay was done by the filter disc method of Borisy (1972). The dissociation constants and stoichiometries of binding were calculated from Scatchard plots according to the procedure of Roychowdhuri et al. (1983).

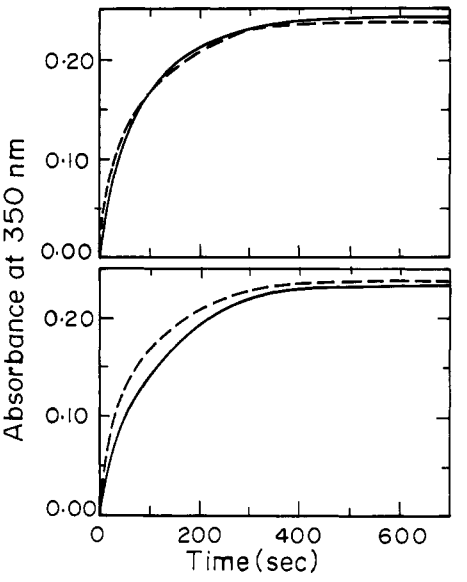

FIGURE 1: Assembly of native and maleimide-labeled tubulin. Assembly curves for FM-tubulin (—) and native tubulin (- - -) are shown in the upper panel. Assembly curves for CM-tubulin (—) and native tubulin (- - -) are shown in the lower panel. Assembly reaction was in 0.1 M PEM buffer, pH 7.0, containing 1 mM GTP and 10% DMSO at 37 °C. Tubulin concentration was 1.4 mg/mL in all cases. Assembly was monitored by measuring absorbance at 350 nm as a function of time.

RESULTS

Characterization of FM-Tubulin and CM-Tubulin. Tubulin has 20 sulfhydryl groups and these are distributed among α and β subunits in the ratio of 12:8 (Krauhs et al., 1981; Postingl et al., 1981). Sulfhydryl groups play a critical role in tubulin self-assembly, and modification of only two sulfhydryl groups completely inhibits assembly (Kuriyama & Saki, 1974). Sulfhydryl labeling, by FM and CM, was performed under polymerized conditions, to avoid modification of the two essential sulfhydryl groups. Under the conditions described in Materials and Methods, the incorporation of fluorescein was 0.6 ± 0.1 mol/mol of tubulin and that of coumarin was 0.65 ± 0.15 mol/mol of tubulin. Both FM-tubulin and CM-tubulin gave two closely spaced bands on SDS–PAGE, having mobilities identical to those of the α and β subunits of unlabeled tubulin. Upon exposure of the gel to UV light from a transilluminator, two fluorescent bands corresponding to α and β subunits were observed for FM-tubulin and CM-tubulin (data not shown). Thus, the incorporation of FM and CM occurred in both α and β subunits of tubulin. The assembly profiles of FM-tubulin (upper panel) and CM-tubulin (lower panel) with unlabeled tubulin are shown in Figure 1. As we can see from that figure, both FM-tubulin and CM-tubulin exhibit assembly profiles very similar to that of native tubulin. We further observed that when the cuvette containing assembled protein was placed on ice, the absorbance dropped almost to zero in all samples, indicating disassembly of the microtubules and absence of any nonspecific aggregates. EM studies (Figure 2) indicate that FM-tubulin and CM-tubulin assemble into microtubules having a nativelike structure. We have also measured the binding isotherms of colchicine to tubulin, tubulin S, FM-tubulin, and FM-tubulin S. The dissociation constants and stoichiometries of binding are presented in Table I. These results show that tubulin and FM-tubulin are indistinguishable with respect to their colchicine binding activity. We may thus conclude that FM and CM modification of tubulin, under

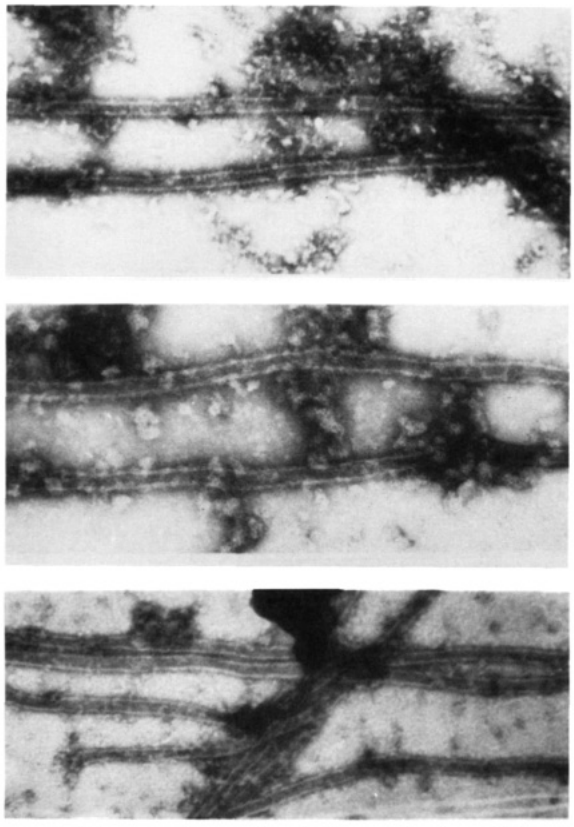

FIGURE 2: Electron micrographs of microtubules formed from (top) unlabeled tubulin (55200×), (middle) FM-tubulin (86400×), and (bottom) CM-tubulin (55200×). Protein (1.4 mg/mL) was polymerized in 0.1 M PEM buffer, pH 7.0, containing 1 mM GTP and 10% DMSO at 37 °C. Samples were prepared by using the procedure of Horowitz et al. (1984).

Table I: Comparison of Colchicine-Binding Properties of Tubulin, FM-Tubulin, Tubulin S, and FM-Tubulin S

	dissociation constant (μM)	stoichiometry
tubulin	0.50	0.76
FM-tubulin	0.52	0.75
tubulin S	1.10	0.73
FM-tubulin S	1.10	0.77

the restrictive conditions mentioned above, does not lead to any significant perturbation of its native structure.

The fluorescence anisotropy of a fluorophore attached to a protein molecule is a complex function of the molecular weight of the protein, and no direct relationship can be established. Since we have used fluorescence anisotropies to follow the dissociation of tubulin S, an independent method for the determination of molecular weight, and hence the state of aggregation for tubulin S, was desirable. Size-exclusion HPLC was used to determine the state of aggregation of tubulin and tubulin S. The molecular weights of tubulin and tubulin S are estimated to be, approximately, 110 000 and 100 000, respectively. This indicates that under these HPLC conditions tubulin S, like tubulin, is a dimer.

Fluorescence Anisotropy Studies. Fluorescence anisotropy of FM-tubulin and FM-tubulin S were determined at various protein concentrations. A decrease in anisotropy values was observed upon dilution of FM-tubulin S in the range of 1.6 μM–2 nM and of FM-tubulin in the range 10 μM–11 nM. Plots of anisotropy versus the logarithm of protein concentration produced sigmoidal dissociation curves for both tubulin S (Figure 3A) and tubulin (Figure 3B), indicating a dissociation of the dimeric protein upon dilution. Dissociation

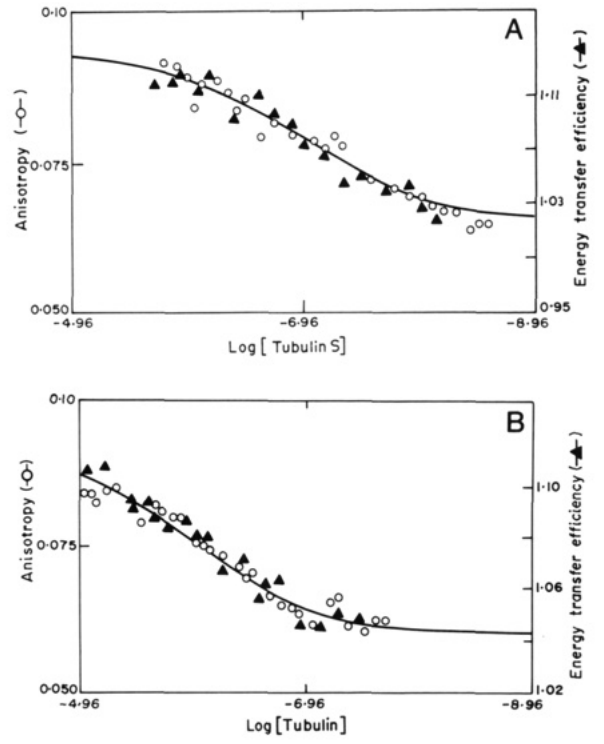

FIGURE 3: Concentration-dependent changes in anisotropy and energy transfer efficiency of fluorescently labeled tubulin S and tubulin. (A) O indicates fluorescence anisotropy of FM-tubulin S and ▲ indicates energy transfer efficiency of hybrid tubulin S (prepared by mixing equimolar concentrations of FM-tubulin S and CM-tubulin S). (B) O represents fluorescence anisotropy of FM-tubulin and ▲ represents energy transfer efficiency of hybrid tubulin (prepared by mixing equal concentrations of FM-tubulin and CM-tubulin). Measurements and data analysis were made as described in Materials and Methods.

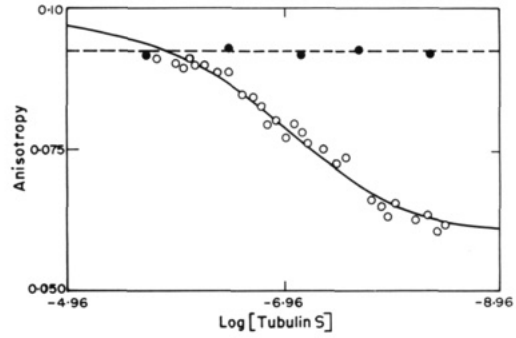

FIGURE 4: Effect of dilution on the fluorescence anisotropy of FM-tubulin S. FM-tubulin S was diluted with unlabeled tubulin S (●), keeping the total tubulin S concentration at 1.6 μM. FM-tubulin S was diluted with BSA (O), keeping the total protein concentration at 1.6 μM. Measurements and data analysis were made as described in Materials and Methods.

constants determined from nonlinear least-squares fitting of the data were 7.1×10^{-8} M and 7.2×10^{-7} M for FM-tubulin S and FM-tubulin at 25 °C, respectively.

Control experiments were performed to demonstrate that the observed decreases in the values of anisotropy upon dilution are not an artifact of the measurement but are actual changes due to dissociation of the tubulin S dimer. The results of these control experiments, using FM-tubulin S, are presented in Figure 4. When FM-tubulin S was diluted with unlabeled tubulin S to keep the total tubulin S concentration constant at 1.6 μM, there was no change in observed anisotropy. However, when bovine serum albumin was added to maintain total protein concentration at 1.6 μM, the anisotropy decreased with dilution of the tubulin S dimer. The reversibility of the

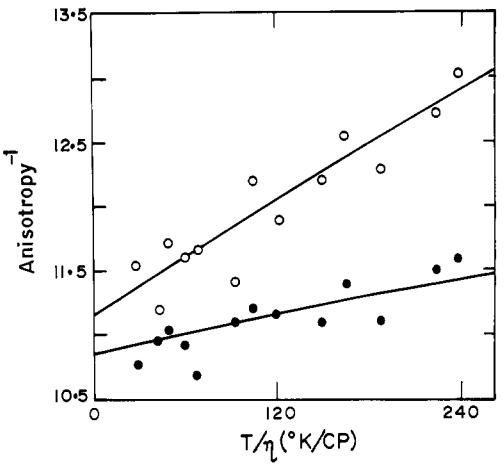

FIGURE 5: Isothermal Perrin plot of FM-tubulin S at two different concentrations. FM-tubulin S concentrations were 0.009 μM (○) and 1.6 μM (●). Viscosity was varied isothermally at 25 °C by inclusion of sucrose in 0.1 M PEM buffer, pH 7.0. Measurements and data analysis were made as described in Materials and Methods.

dissociation was shown by diluting the FM-tubulin S from 1.6 to 0.005 μM and then adding unlabeled tubulin S to a final concentration of 1.6 μM. The anisotropy value, which was 0.091 at 1.6 μM, changed to 0.063 at 0.005 μM. Upon addition of unlabeled tubulin S, the anisotropy value returned to 0.086, indicating the reversibility of the dissociation process. In a separate experiment, the anisotropy of FM-tubulin S, which was 0.063 at 0.003 μM, increased to 0.083 when this diluted protein was concentrated to 1.1 μM using Amicon ultrafiltration membrane cones at 4 °C, indicating complete reversibility of the process.

Variations in anisotropy upon dilution of proteins can also result from the changes in lifetimes. We therefore measured the fluorescence lifetime of FM-tubulin S at 3.3 and 0.009 μM, representing the dimeric and the monomeric form of the protein, respectively. The lifetimes of tubulin S under the dimeric and monomeric conditions are 4.2 and 4.5 ns, respectively. Thus the lifetimes are very similar, indicating that changes in anisotropy are not caused by changes in lifetimes. Perrin plots of FM-tubulin S, at dimeric (1.6 μM) and monomeric (0.009 μM) conditions, are shown in Figure 5. In both cases, the limiting anisotropy is approximately 0.09. The observed limiting anisotropy is substantially less than the theoretical limiting anisotropy of a totally rigid system (0.4). In practice, this limit is rarely reached and a value of 0.2 is not uncommon (Yguerabide, 1972). The A_0 for pyrene butyrate bound bovine serum albumin ranges from 0.03 to 0.09, depending on the ratio of fluorophore to protein (Knopp & Weber, 1969). The limiting anisotropies are 0.167 for bis-ANS-tubulin (Prasad et al., 1986) and 0.086 for DTAF-tubulin (Mejillano & Himes, 1989). The small A_0 value indicates flexibility of the probe at the site of modification or flexibility of that part of the macromolecule to which the probe is attached (Cantor & Schimmel, 1981; Mejillano & Himes, 1989). The rotational correlation time was obtained from the value of the slope of Perrin plot (Figure 5) and the fluorescence lifetime by using Perrin's equation. The rotational correlation times are 55 and 20.6 ns for dimeric and monomeric tubulin S, respectively.

Fluorescence Energy Transfer. The fluorescence energy transfer between properly matched donor and acceptor pairs is widely used to determine distances in the range 10–80 Å. If a dimeric protein is labeled with a donor in one subunit and an acceptor in the other, fluorescence energy transfer may occur. Upon dissociation, however, the intersubunit distance would be too large for energy transfer to take place. Thus, in an appropriately labeled protein, the loss of energy transfer may be taken as an indicator of subunit dissociation (Shore & Chakraborty, 1976). We used this technique to verify our data on the dissociation constant of monomer–dimer equilibrium of tubulin S determined by fluorescence anisotropy. When a solution of FM-tubulin S is mixed with a solution of CM-tubulin S, in equimolar proportions, hybrid tubulin S dimers are formed in which the α_s and β_s subunits are labeled with different fluorophores. If energy transfer occurs in this hybrid molecule, it is expected to decline gradually and then disappear upon dissociation. We have measured the energy transfer efficiency of hybrid tubulin S dimers in the concentration range where fluorescence anisotropy measurements indicated that subunit dissociation was taking place. As expected, we observed a lowering of energy transfer efficiency with dilution. A plot of energy transfer efficiency (E) vs the logarithm of tubulin S concentration produced a sigmoidal curve, which superimposes well with that obtained from the change in anisotropy.

Thermodynamics of Dimer Dissociation. The effect of temperature on the dimer–monomer equilibrium of tubulin S was studied at four different temperatures ranging from 15 to 37 °C (Figure 6A). The van't Hoff plot (Figure 6A, inset) is linear, and the value of −5.9 kcal/mol was obtained for ΔH for the association reaction. ΔS and ΔG values at 25 °C are +13.1 eu and −9.8 kcal/mol, respectively. Thus, monomer association is favored by both enthalpy and entropy. The effect of temperature on the dimer–monomer equilibrium of tubulin was also determined at four different temperatures (Figure 6B). The van't Hoff plot (Figure 6B, inset) was linear and the value of −8.6 kcal/mol was obtained for ΔH for the association reaction. The ΔS and ΔG values are −0.6 eu and −8.4 kcal/mol, respectively, at 25 °C.

Effect of Ionic Strength on Dimer Dissociation. The dependence of the association constant on ionic strength was examined in order to elucidate the importance of hydrophobic and ionic interactions in the association reaction of α_s and β_s. The dissociation constant was determined in the presence of 0.3 M NaCl. This condition clearly favors the dimeric form, shifting the K_d from 7.1×10^{-8} M to 2.9×10^{-9} M. As the monomer association is favored at higher ionic strengths, we could conclude that hydrophobic interactions play an important role in the formation of tubulin S dimer (Ross & Subramanian, 1981; Sackett & Lippoldt, 1991). The association constant of α and β, determined in the presence of 0.3 M NaCl, was 1.5 times the value without the added salt.

Effect of Colchicine on Dimer Dissociation. Colchicine is a mitosis inhibitor which inhibits tubulin self-assembly. Binding of colchicine to tubulin causes conformational changes of the tubulin molecule (Garland, 1978; Detrich et al., 1982). The binding of colchicine to tubulin causes a stabilization of the colchicine binding site of the protein (Wilson, 1970; Bhattacharyya & Wolff, 1975a,b, 1977; Wiche & Furtner, 1980). In order to see the effect of colchicine on dissociation of the tubulin S dimer, FM-tubulin S dimer was mixed with a 5-fold molar excess of colchicine and incubated for 1 h. The complex was diluted to lower concentrations in a 0.1 M PEM buffer containing the same concentration of colchicine, and K_d was determined by curve-fitting as described in Materials and Methods (Figure 7A). The dissociation constant decreased 1.3 times, as compared to tubulin S without colchicine, at 25 °C. Similar results were obtained with a 10-fold molar excess of colchicine. The effect of colchicine on tubulin

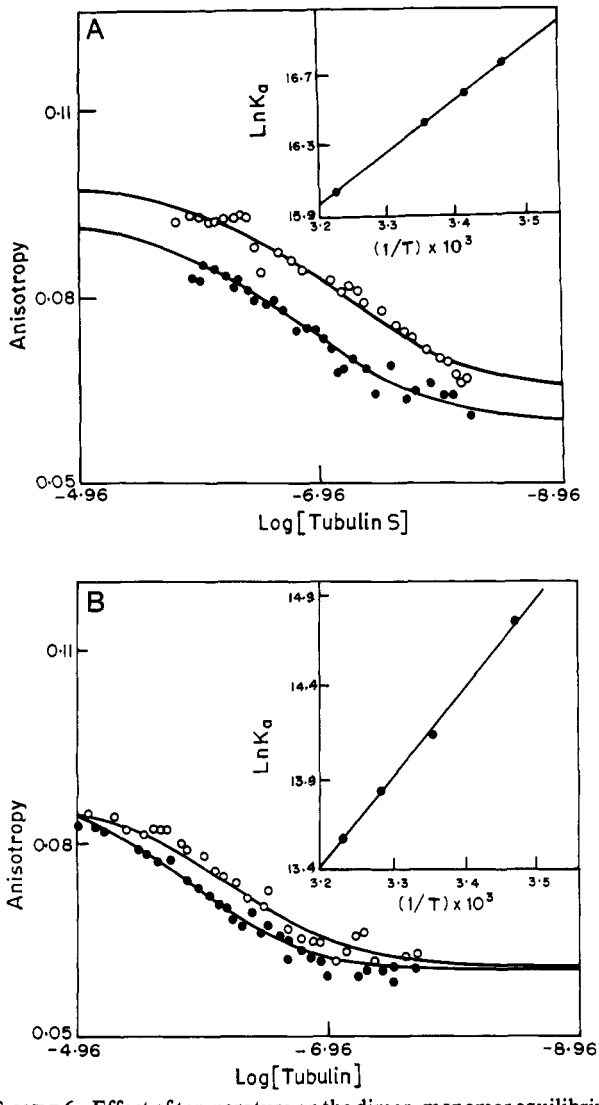

FIGURE 6: Effect of temperature on the dimer–monomer equilibrium of FM-tubulin S and FM-tubulin. (A) Concentration-dependent change in anisotropy at 15 °C (○) and 37 °C (●). The inset is a van't Hoff plot of association of FM-tubulin S for which experiments were carried out at four different temperatures. (B) Concentration-dependent change in anisotropy of FM-tubulin at 15 °C (○) and 37 °C (●). The inset is a van't Hoff plot of association of FM-tubulin for which experiments were carried out at four different temperatures. Measurements and data analysis were made as described in Materials and Methods.

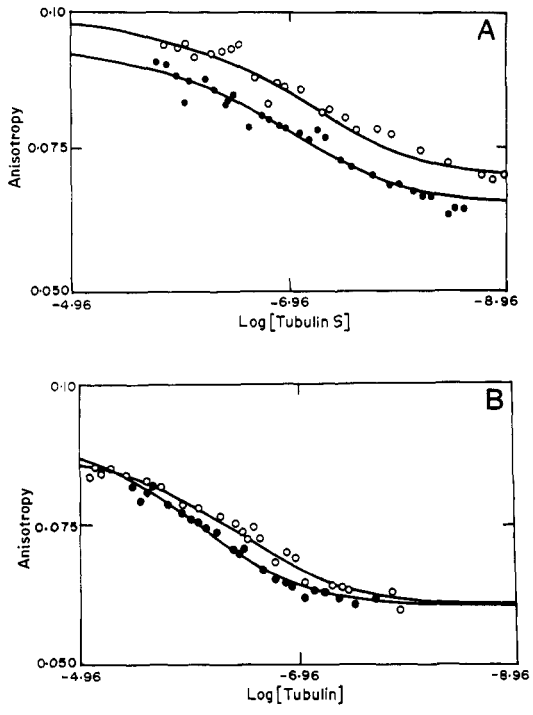

FIGURE 7: Concentration-dependent change in anisotropy of colchicine-bound FM-tubulin S and FM-tubulin. (A) FM-tubulin S was incubated with a 5-fold molar excess of colchicine for 1 h at 37 °C. Dilution of colchicine-bound FM-tubulin S was carried out in 0.1 M PEM buffer, pH 7.0, containing the same concentration of colchicine, whereas FM-tubulin S was diluted with 0.1 M PEM buffer, pH 7.0, as described in Figure 3. ○ represents the fluorescence anisotropy of colchicine-bound FM-tubulin S and ● represents the fluorescence anisotropy of FM-tubulin S. (B) FM-tubulin was incubated with a 5-fold molar excess of colchicine for 1 h at 37 °C. Dilution of FM-tubulin was carried out in 0.1 M PEM buffer, pH 7.0, containing the same concentration of colchicine, whereas FM-tubulin was diluted with 0.1 M PEM buffer, pH 7.0, as described in Figure 3. ○ represents the fluorescence anisotropy of colchicine-bound FM-tubulin and ● represents the fluorescence anisotropy of FM-tubulin S. Measurements and data analysis were carried out as described in Materials and Methods.

dissociation was also investigated. FM-tubulin was treated with a 5-fold molar excess of colchicine and incubated for 1 h at 37 °C. The experiment was carried out as usual at 25 °C. The K_d was determined to be 2.7×10^{-7} M (Figure 7B), compared to 7.2×10^{-7} M in the absence of colchicine. Thus, colchicine stabilized the tubulin dimer by a factor of 2.6. Although colchicine has a somewhat lower affinity for tubulin S than for tubulin (Table I), the calculated fractional saturations, under the above-mentioned conditions, were greater than 90% for both tubulin and tubulin S. Previously, Detrich et al. (1982) observed a 3-fold decrease and Mejillano and Himes (1989) observed a 2.5-fold decrease of dimer–monomer dissociation constant values in the presence of colchicine.

DISCUSSION

We have used fluorescence anisotropy and fluorescence energy transfer to explore the dissociation of tubulin S dimer and its thermodynamic parameters, as well as the size and shape of the protein. In all these studies we have compared our results on tubulin S with those of tubulin studied by the same technique.

Tubulin S, which is a dimer at micromolar concentrations, dissociates into monomers with a dissociation constant of 7.1×10^{-8} M at 25 °C. This value is an order of magnitude lower than that of tubulin under identical conditions (7.2×10^{-7} M). There have been several reports of dissociation constant determination of tubulin by various techniques; these are presented in Table II (Detrich & Williams, 1978; Detrich et al., 1982; Sackett et al., 1989, Sackett & Lippoldt, 1991; Mejillano & Himes, 1989). At 5 °C, K_d values reported from different laboratories, by different techniques (ranges from 1.5×10^{-7} to 3.8×10^{-7} M), are comparable, although an earlier equilibrium ultracentrifugation study reported a somewhat higher value of $(7–10) \times 10^{-7}$ M. Such modest variations could be due to differences in preparation, solution, and storage conditions. An increased ionic strength favors association of tubulin monomers modestly. Association of tubulin S, however, is even more favored at higher ionic strengths. A significant increase of the association constant in tubulin S, reported in the present study, may have resulted from the removal of charge–charge repulsion of the α and β subunits in the intact tubulin.

The thermodynamic parameters of association of tubulin and tubulin S also clearly bear out the different nature of the

Table II: Dissociation Constant and Thermodynamic Parameters of Dimer–Monomer Equilibrium of Tubulin

parameter	Detrich et al. (1978)	Sackett et al. (1989)	Sackett & Lippoldt (1991)	Mejillano & Himes (1989)	present study
K_d[a] (M)	$(7–10) \times 10^{-7}$	1.5×10^{-7}	2.0×10^{-7}	3.8×10^{-7}[b]	2.6×10^{-7}[b]
ΔH (kcal/mol)			2.1 ± 0.7	–9.5	–8.6
ΔS (eu)			38.1 ± 2.4	–4.6	–0.6

[a] At 5 °C. [b] Calculated from thermodynamical data.

interactions present. Association of tubulin is enthalpy-driven (ΔH = –8.6 kcal/mol) with very little change in entropy (ΔS = –0.6 eu). Association of tubulin S, on the other hand, is favored by both enthalpy (ΔH = –5.9 kcal/mol) and entropy (ΔS = +13.1 eu). The change of entropic contribution is, almost certainly, due to an increased hydrophobic interaction present in the tubulin S dimer. The lowering of ΔH in tubulin S, as compared to that of tubulin, may be due to a loss of enthalpy-contributing interactions, such as hydrogen bonding or van der Waals interactions, in the tubulin. Thus, the α_s/β_s interface may be substantially different from the α/β interface. The values of enthalpy and entropy of association of the monomer of goat brain tubulin reported here do not agree with those of bovine brain tubulin, obtained by using equilibrium centrifugation (Sackett & Lippoldt, 1991). Our results, however, closely correspond to those of Mejillano and Himes (1989), who used fluorescence anisotropy measurements for determining the thermodynamic parameters. Whether covalent modification of the protein or the particular experimental technique used is responsible for these differences, is not clear to us. The calculated K_d value at 5 °C, however, agrees with that determined from ultracentrifugation measurements of tubulin (Sackett & Lippoldt, 1991). Furthermore, the colchicine binding activity, polymerizability, and microtubule structure of the labeled tubulin are identical to those of unlabeled tubulin. This argues against the possibility that FM modification is the source of these differences.

We also looked into the shape of the tubulin S monomer and dimer by an isothermal Perrin plot. The rotational correlation time of the tubulin S monomer is 20 ns, which is very close to the expected value for a spherical protein of that size. This value is also very close to that obtained for tubulin monomers by Mejillano and Himes (1989). This indicates that there are no gross differences in shape or size between the tubulin S monomer and the tubulin monomer. The tubulin S dimer, on the other hand, has a rotational correlation time of 55 ns. A spherical protein of the size of tubulin S is expected to have a rotational correlation time of 44 ns. The increased correlation time from the expected value is generally taken as an indication of nonspherical shape. The ratio of correlation time 55/44 = 1.25 relates to the axial ratio of the ellipsoid (Beneckey et al., 1990). This ratio indicates that tubulin S may best be represented by a prolate ellipsoid of axial ratio approximately 2.9:1 or by an oblate ellipsoid of axial ratio approximately 1.9:1. The rotational correlation time of tubulin had been derived by many groups and falls in the range of 35–46 ns, indicating an approximately spherical shape of the protein (Mejillano & Himes 1989; Mazumdar et al., 1992). Thus the α_s,β_s association not only is different in terms of the nature of interaction but also may differ in the association geometry.

The effect of the antimitotic drug colchicine on tubulin dissociation is, again, substantially different from its effect on tubulin S. Colchicine binding stabilizes the tubulin dimer substantially, whereas colchicine binding to tubulin S causes virtually no change in the dissociation constant. The different effects of colchicine binding again point toward substantial differences in the structure of tubulin and tubulin S.

We thus conclude that the removal of the C-terminal tail of tubulin affects the structure and ligand binding properties of the rest of the protein. Thus the C-terminal tail may significantly influence the structure and properties of the rest of the protein.

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