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Tubulin Dimer Dissociation Detected by Fluorescence Anisotropy†

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ABSTRACT: We have demonstrated a concentration-dependent dissociation of bovine brain tubulin dimer covalently labeled with 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF) or with fluorescein isothiocyanate (FITC) by fluorescence anisotropy and size-exclusion HPLC. The fluorescence anisotropy values decreased to a limiting value upon dilution of tubulin from 10^{-5} to 8×10^{-8} M. A dissociation constant in 0.1 M Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$ at 20 °C was estimated to be $(8.4 \times 10^{-7}) \pm (0.4 \times 10^{-7})$ M. Control experiments using monomeric and other dimeric proteins, urea-denatured tubulin, and DTAF-tubulin diluted into solutions of bovine serum albumin or unlabeled tubulin were consistent with the finding that the changes in anisotropy upon dilution are due to protein dissociation. These results were supported by size-exclusion HPLC data where an increase in the elution volume of DTAF-tubulin and FITC-tubulin was observed with decreasing protein concentrations. Reversibility of the dissociation process and the lack of denaturation at high dilution were shown by the ability of reconcentrated protein to assemble into microtubules to about the same extent as undiluted protein. Fluorescent lifetimes and limiting anisotropy values were found to be approximately identical at different tubulin concentrations, indicating that the anisotropy changes reflect changes in size or rotational correlation time of the protein. Studies on the effects of tubulin ligands and promoters or inhibitors of assembly demonstrated that their effects on tubulin dimer–monomer equilibria are small but reproducible. Increasing the temperature to 36 °C resulted in about a 2-fold higher K_d value while lowering it to 10 °C caused a 2-fold decrease. Thermodynamic constants for the association reaction were calculated to be $\Delta H° = -9.5$ kcal/mol, $\Delta S° = -4.6$ eu, and $\Delta G°(20\ °C) = -8.1$ kcal/mol.

The major protein of microtubules, tubulin, is a heterodimer of two noncovalently linked subunits, α and β. The dimer binds two guanine nucleotides, divalent cations, colchicine, vinca alkaloids, and other mitotic inhibitors. To have a better understanding of the nature of the tubulin dimer under a variety of in vivo and in vitro conditions, it is important to understand the monomer association reaction. To date, only two physical studies of the reversible dissociation of bovine brain tubulin have been reported in the literature (Detrich & Williams, 1978; Detrich et al., 1982). In these studies, ultracentrifugal techniques and small zone gel filtration were used to calculate a dissociation constant of $(7-10) \times 10^{-7}$ M at 5 °C. Although there was a good fit of the centrifugation data with theoretical curves in these studies, the actual experimental points were somewhat limited at lower protein concentrations. We felt that it was important to examine the dissociation process by another physical technique which allowed the use of lower protein concentrations.

In this study, fluorescence anisotropy measurements were employed to investigate the tubulin monomer–dimer equilibrium. This method provides a highly sensitive and convenient tool for detection and analysis of oligomeric protein dissociation (Weber, 1953). The extent of dissociation of a protein covalently labeled with a fluorescent dye can be readily followed provided that the dimer and monomer exhibit characteristic anisotropy values depending on their rotational correlation times.

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Previous studies have shown that tubulin can be labeled with the fluorescein analogue 5-[(4,6-dichlorotriazin-2-yl)amino]-fluorescein (DTAF)[1] and other fluorescent derivatives and that the labeled tubulin can assemble into microtubules in vitro and in vivo (Keith et al., 1981; Wadsworth & Sloboda, 1983; Leslie et al., 1984; Vigers et al., 1988). In this work, we have used DTAF- and fluorescein isothiocyanate (FITC)-labeled tubulin as probes to study the concentration-dependent dissociation of the dimer using fluorescence spectroscopic methods and size-exclusion HPLC.

Experimental Procedures

Materials. DTAF, fluorescein isothiocyanate, α-chymotrypsin, ribonuclease A, malate dehydrogenase, GTP, vinblastine sulfate, colchicine, and EGTA were obtained from Sigma Chemical Co. Pipes was purchased from Research Organics Inc. The gel exclusion HPLC standards were from Bio-Rad.

Preparation of Tubulin. Bovine brain microtubule protein (MTP) was prepared by a modified procedure of the assembly–disassembly method of Shelanski et al. (1973). Tubulin was purified from microtubule-associated proteins by chromatography on a Biogel P-10–phosphocellulose (Whatman P11) piggyback column (Algaier & Himes, 1988). Purified tubulin was drop-frozen into liquid nitrogen and stored at −70 °C in PEM buffer (0.1 M Pipes, 1 mM EGTA, and 1 mM $MgSO_4$), pH 6.9.

Microtubule Assembly Assay. Tubulin was polymerized into microtubules by incubating 1.2–5.0 mg/mL tubulin with 0.5 mM GTP and 10% DMSO in PEM buffer, pH 6.9. The reaction components were mixed at 4 °C, and the assembly was initiated by adding the cooled solution to a cuvette at 37 °C. The reaction was monitored by the increase in the apparent absorbance at 350 nm in a temperature-controlled spectrophotometer.

Preparation of DTAF- and FITC-Labeled Tubulin. Purified tubulin (5.0 mg/mL) was assembled into microtubules at 37 °C for 15 min, and then DTAF or FITC was added at a final dye to protein molar ratio of 50:1. The assembled microtubules were labeled for 15 min at 37 °C. Two 500-μL aliquots of the reaction mixture were each layered onto 2 mL of warm 40% sucrose–PEM containing 10% DMSO. The mixture was centrifuged at 37 °C in a Beckman TLA-100.3 rotor at 200000*g* for 12 min. After removal of the supernate, warm PEM buffer was added to cover the pellet and then removed. The pellet was then resuspended in 0.2–0.3 mL of ice-cold PEM buffer with the use of a glass homogenizer, and the suspension was kept on ice for 15 min to induce microtubule disassembly. The resulting solution was cleared by centrifugation at 27000*g* and 4 °C for 10 min. The supernatant was centrifuged by using a clinical centrifuge through 1.0 mL of Sephadex G-25 in PEM buffer packed into a 1.0-mL plastic syringe. This additional step removes any remaining unbound dye. A control sample of tubulin was carried through the same procedure but the labeling step was omitted. Protein concentration and label incorporation in the samples were quantitated, and the samples were tested for their ability to assemble to microtubules.

Preparation of Other Fluorescently Labeled Proteins. For control experiments, chymotrypsin, RNase A, and malate dehydrogenase were used. Labeling of chymotrypsin and RNase was performed by incubating 5.0 mg/mL protein in PEM buffer, pH 6.9, with a 50-fold molar excess of DTAF at 37 °C for 30 min. The mixture (200 μL) was centrifuged through 1 mL of Sephadex G-25 (in a plastic syringe) in the same buffer.

Porcine mitochondrial malate dehydrogenase was labeled with FITC according to the method of Shore and Chakrabarti (1976). A suspension of the enzyme in $(NH_4)_2SO_4$ was centrifuged, and the pellet was dissolved in 0.05 M Tris–acetate, pH 8.0. The enzyme was labeled with the dye by adding FITC on Celite at a 5:1 dye to protein molar ratio. The solution (0.5 mL) was stirred at 4 °C for 30 min and centrifuged through 2 mL of Sephadex G-25.

Determination of Protein and Fluorescent Label Concentrations. The concentrations of the proteins used were determined by the Bradford assay (Bradford, 1976). For tubulin, the protein concentration was also measured from the absorbance at 275 nm using an ϵ value of 1.13 mg^{-1} mL cm^{-1}. The amount of protein-bound DTAF was determined spectrophotometrically from its absorbance at 492 nm using a molar ϵ value of 5.9×10^4 M^{-1} cm^{-1}. We determined this value for free DTAF in PEM buffer, pH 6.9. FITC bound to malate dehydrogenase was quantitated from its absorbance at 490 nm using a molar ϵ value of 5.2×10^4 M^{-1} cm^{-1}. The amount of FITC bound to tubulin was determined by using a molar ϵ of 6.9×10^4 M^{-1} cm^{-1} at 490 nm as measured for the unbound FITC in PEM, pH 6.9.

SDS–Polyacrylamide Gel Electrophoresis and Electroelution of DTAF-Tubulin. DTAF-tubulin was precipitated with 10% $HClO_4$ followed by several H_2O washes of the pellet. The labeled protein was then carboxymethylated under reducing conditions as described by Allen (1981). The solution was dialyzed at 5 °C overnight against 0.1 M NH_4HCO_3. Preparative SDS–PAGE was performed according to a modified procedure of Laemmli (1970) using a 7.5% running gel and a 3% stacking gel. A band of DTAF-tubulin sample (1 mg) was applied across a 1.5-mm slab gel. The α and β subunits were well resolved, and the Coomassie-stained bands corresponding to the each subunit were removed by slicing the gel. Electrophoretic elution of the protein in each subunit was done at room temperature on the basis of a published procedure (Hunkapiller et al., 1983). The elution buffer used was 25 mM NH_4HCO_3 with 0.025% SDS, and the dialysis buffer was a 10-fold diluted solution of the elution buffer. The amount of protein in each electroeluted band was determined by the Bradford assay. The recovery of the protein from the gel following electroelution was about 60%.

To determine the distribution of the DTAF label in the α and β subunits, the fluorescence intensity of the electroeluted protein was measured after dilution (300-fold). The amount of DTAF present in each subunit was estimated from a calibration curve of fluorescence measurements of DTAF-tubulin of known concentration. That Coomassie dye did not interfere in the fluorescence measurements was verified by the addition of Coomassie stain to a sample of DTAF-tubulin.

Fluorescence Spectroscopic Studies. Fluorescence anisotropy of labeled protein solutions was measured by using a Perkin-Elmer MPF-44B fluorescence spectrophotometer attached to a differential corrected spectra-2. Anisotropy was measured as

$$A = [I_{\parallel} - I_{\perp}(G)]/[I_{\parallel} + 2I_{\perp}(G)] \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ represent the emission intensities parallel and

[1] Abbreviations: DTAF, 5-[(4,6-dichlorotriazin-2-yl)amino]-fluorescein; FITC, fluorescein isothiocyanate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PEM buffer, 100 mM Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PB, pyrene butyrate; Bis-ANS, bis(8-anilinonaphthalene-1-sulfonate).

perpendicular to the plane of polarization, respectively, and G is a correction factor for the transmission efficiency of the emission monochromator to light polarized parallel and perpendicular to the grating.

In a solution containing several molecular species, the observed anisotropy may be determined from the expression:

$$A = \sum_i A_i f_i / \sum_i f_i \quad (2)$$

where A_i is the emission anisotropy of the ith species and f_i is the concentration of the ith species (Weber, 1952). Thus, if dimer and monomer are present in solution, the anisotropy measured is

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

where A_d and A_m are the contributions to the observed anisotropy of the dimer and monomer, respectively, and f_d and f_m are the molar fractions of each species.

DTAF-labeled tubulin was diluted to different concentrations from 10^{-5} to 8×10^{-8} M and incubated on ice for 1 h prior to anisotropy measurements. The excitation wavelength used was 492 nm with a spectral bandwidth of 4 nm, and the emission wavelength was 517 nm with a 4-nm bandwidth. Studies were done at 20 °C in PEM buffer, pH 6.9. The same procedure was used for anisotropy measurements of labeled chymotrypsin and RNase A. For polarization and anisotropy studies of FITC-labeled malate dehydrogenase or tubulin, the samples were diluted to various concentrations, and the fluorescence was measured at 520 nm using an excitation wavelength of 490 nm.

The lifetimes of DTAF-tubulin at different concentrations were measured at 20 °C using an SLM 4800 cross-correlation phase fluorometer as described by Lackowicz (1983). The sample was excited at 492 nm, and the intensity was modulated at 18 or 30 MHz. Lifetimes were determined from the relative modulation of the fluorescence sample relative to a glycogen reference solution. Emission was monitored through Schott cut-on RG630 filters and KV490 filters for the reference and sample chambers, respectively. Lifetime heterogeneity analysis was also performed to determine the purity of the fluorescent species (Weber, 1981).

To determine the limiting anisotropy, A_0, a Perrin plot of $1/A$ vs T/η as defined by eq 4 (Weber, 1952) was extrapolated

$$1/A = (1/A_0)[1 + (\tau_f/\tau_c)] = (1/A_0)[1 + (\tau_f kT/V_h\eta)] \quad (4)$$

to infinite viscosity. In this equation, τ_f is the fluorescent 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 (20 °C) by the addition of sucrose in PEM buffer, pH 6.9. Literature values for the viscosity of sucrose (Weast, 1977) were used assuming negligible effects of the buffer components.

Gel Exclusion HPLC of DTAF-Labeled Tubulin. DTAF-Labeled tubulin diluted to different concentrations with PEM buffer, pH 6.9, was incubated on ice for 1 h. The diluted samples were injected into a 200-μL loop and chromatographed at 20 °C on a TSK 3000 SW size-exclusion HPLC column equilibrated with the same buffer. The elution was monitored by the absorbance of the protein at 280 nm, and 0.5-mL fractions were collected. The fluorescence intensity of each fraction was also measured using 517 nm as emission and 492 nm as excitation wavelengths. The same procedure was used for chromatography of the control sample, DTAF-chymotrypsin. The elution volumes of DTAF-tubulin and DTAF-chymotrypsin were compared to the following standards: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). FITC-tubulin and unlabeled tubulin at 5.0, 0.8, and 0.1 μM were also chromatographed on a gel exclusion HPLC column. The fluorescence of each fraction was measured at 520 nm (490-nm excitation) for FITC-tubulin and at 330 nm (280-nm excitation) for unlabeled tubulin.

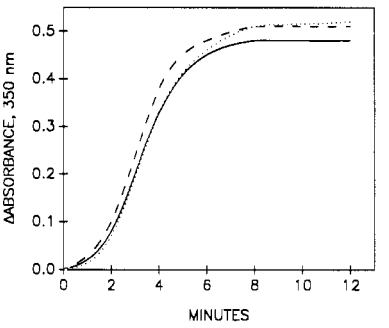

FIGURE 1: Assembly of fluorescently labeled tubulin. DTAF-tubulin (—), FITC-tubulin (···), and unlabeled tubulin (- - -). Labeled and unlabeled tubulin at 1.2 mg/mL was incubated with 0.5 mM GTP and 10% DMSO in PEM buffer, pH 6.9 at 4 °C. Assembly to microtubules was initiated by placing the precooled solution in a cuvette at 37 °C.

Results

Characterization of DTAF- and FITC-Labeled Tubulin. The molar ratio of DTAF and FITC bound to tubulin after centrifugation through sucrose and Sephadex G-25 in several experiments ranged from 0.3 to 0.4. A wide range of values for the amount of DTAF bound to tubulin has been reported by others: 0.01–0.1 (Wadsworth & Sloboda, 1983), 0.1 (Vigers et al., 1988), 0.5 (Keith et al., 1981), and between 1.0 and 1.8 mol of DTAF per mole of tubulin (Leslie et al., 1984). In part, this is probably due to the different extinction coefficients used for DTAF by different groups: 2.7×10^4 (Leslie et al., 1984), 5.7×10^4 (Vigers et al., 1988), and 5.7×10^5 M^{-1} cm^{-1}, (Keith et al., 1981). In this work, we determined the ϵ for free DTAF to be 5.9×10^4 M^{-1} cm^{-1} which is in good agreement with that measured by Vigers et al. (1988).

The behavior of labeled and unlabeled tubulin on SDS–PAGE was identical, and good separation of the α and β subunits was achieved following carboxymethylation under reducing conditions. The amount of DTAF on each subunit determined after electroelution was found to be essentially equal. Previously, Leslie et al. (1984) also found labeling of both subunits but concluded from qualitative comparison of the fluorescence image of both bands from two-dimensional gels that the β subunit was more heavily labeled than the α subunit.

The assembly profiles of unlabeled tubulin and tubulin labeled with DTAF or FITC are shown in Figure 1. It is evident that the labeling procedure did not affect the assembly properties of tubulin. When the assembled labeled tubulin samples were placed on ice for 10 min, the absorbance at 350 nm dropped to near zero, indicating disassembly of the microtubules and the absence of nonspecific aggregated material in both labeled and unlabeled samples. Assembled DTAF-tubulin, after collection by centrifugation, was shown to contain the same amount of DTAF as the original DTAF-tubulin.

Fluorescence Anisotropy Studies. The anisotropy of fluorescence of tubulin labeled with DTAF or FITC was determined at various protein concentrations. A decrease in the anisotropy values was observed upon dilution of the protein from 10^{-5} to 8×10^{-8} M. A plot of anisotropy vs log of tubulin concentration (Figure 2A,B) produced a sigmoidal curve, in-

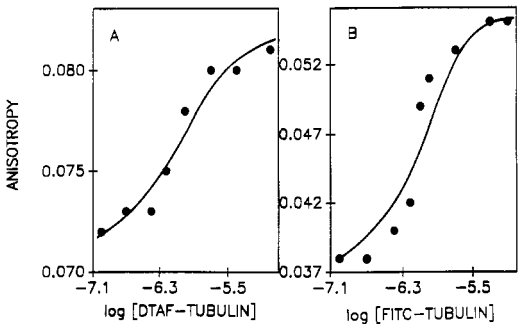

FIGURE 2: Concentration-dependent change in anisotropy of fluorescently labeled tubulin. (A) Fluorescence anisotropy of DTAF-tubulin. (B) Fluorescence anisotropy of FITC-tubulin. Measurements were made as described under Experimental Procedures. The curved lines are drawn to show the trend of the data.

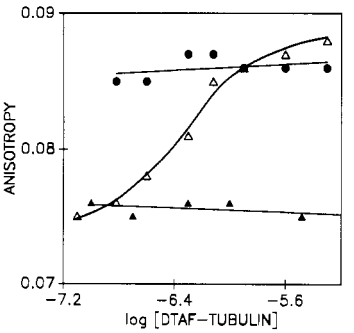

FIGURE 3: Effect of dilution on the fluorescence anisotropy of DTAF-tubulin. DTAF-tubulin was diluted in the presence of unlabeled tubulin (●), in the presence of bovine serum albumin (△), and in the presence of 7.2 M urea (▲). Details are described under Experimental Procedures. The curved lines are drawn to show the trend of the data.

dicating dissociation of the tubulin dimer upon dilution. Both plots give a dissociation constant of approximately 8×10^{-7} M. From several experiments, a value of $(8.4 \times 10^{-7}) \pm (0.4 \times 10^{-7})$ M was calculated. Fluorescence polarization of labeled tubulin at different concentrations was also measured. The values corresponded to the anisotropy readings as verified by the relationship of anisotropy to polarization:

$$A = 2P/(3 - P) \quad (5)$$

The K_d obtained from the polarization data was about 1×10^{-6} M.

To determine if tubulin is denatured at the low concentrations used in the anisotropy experiments, the fluorescence emission spectrum of tubulin was measured. Tubulin at 1.0, 0.1, and 0.05 μM, diluted in PEM buffer, pH 6.9, was incubated on ice for 1 h prior to recording the spectra. A λ_{max} at 330 nm was observed at all these concentrations. For tubulin denatured by 7.2 M urea, there was a shift in the λ_{max} to 350 nm. These results indicate that tubulin at concentrations as low as 0.05 μM is still in the native conformation.

In order to ensure that the observed changes were due to dissociation of the dimer upon dilution and not to some artifact of measurements at low protein concentrations, a number of positive and negative controls were done. As a positive control, previous fluorescence polarization studies of subunit dissociation of dimeric mitochondrial malate dehydrogenase labeled with FITC (Shore & Chakrabarti, 1976) were repeated. As the labeled enzyme was diluted 200-fold, both polarization and anisotropy decreased in a sigmoidal fashion in a plot of anisotropy or polarization vs log of enzyme concentration. A dissociation constant of approximately 1.0×10^{-6} M was calculated from the anisotropy data and 8×10^{-7} M from the polarization studies compared to the reported value of 2×10^{-7} M (Shore & Chakrabarti, 1976). Negative control experiments were also performed which utilized nondissociating proteins such as α-chymotrypsin and RNase A. These proteins contained covalently bound DTAF and were diluted to the same concentration range as DTAF-tubulin. There was no change in anisotropy even up to a 100-fold dilution for both DTAF-chymotrypsin and DTAF-RNase A, indicating the lack of protein dissociation.

Several other control experiments using DTAF-tubulin are presented in Figure 3. When DTAF-tubulin was diluted into unlabeled tubulin to keep the concentration of total tubulin constant at 5 μM, there was no change in the observed anisotropy. However, when bovine serum albumin was present to maintain the total protein concentration at 5 μM, anisotropy decreased with dilution of the tubulin dimer, and a dissociation curve similar to that of DTAF-tubulin alone was obtained. In the presence of 7.2 M urea, the anisotropy values of labeled tubulin remained constant at 0.076 for different concentrations, indicating complete dissociation at all concentrations.

Table I: Fluorescent Lifetimes (τ_f) and Rotational Correlation Times (τ_c) of DTAF-Tubulin

[DTAF-tubulin] (μM)	τ_f (ns)[a]	τ_c (ns)[b]
8.0	3.35 ± 0.05	40.0 ± 6.7
0.8	3.57 ± 0.08	29.5 ± 7.7
0.08	3.26 ± 0.05	20.5 ± 1.2

[a] τ_f values were measured with a cross-correlation phase fluorometer as described under Experimental Procedures. The values are given with standard deviations. [b] τ_c values were calculated by using the Perrin equation (eq 4). The values are given with standard errors.

In attributing the changes in anisotropy upon dilution to protein dissociation, we are assuming that the changes are a result of the different rotational correlation times of the monomer and dimer (see eq 4). However, variations in anisotropy can also result from changes in fluorescent lifetimes as seen from eq 4. Furthermore, changes that affect the rotational freedom of the probe but not the macromolecule could also cause similar changes in anisotropy. Fluorescent lifetimes of DTAF-tubulin were measured at 8, 0.8, and 0.08 μM which would represent dimer, dimer + monomer, and monomer states, respectively. The lifetimes were very similar and do not differ by more than 9% (Table I), indicating that the anisotropy changes are not caused by differences in the lifetimes of the labeled tubulin. Heterogeneity analysis of the emitting species was done by cross-correlation phase and modulation measurements at several frequencies (Weber, 1981). This allows the detection of individual lifetimes of more than one fluorophore in a mixture and their fractional contributions to the total intensity. The data obtained revealed that the samples were 88–97% homogeneous.

Changes in anisotropy could also reflect conformational changes influencing the rotational environment of the labeled moiety and not changes in protein size. To verify that this was not the case, the limiting anisotropy (A_0) in the absence of macromolecular rotation was determined. This parameter was derived from the y intercept of the Perrin plot of $1/A$ vs T/η (eq 4) where η, the viscosity of the medium, was varied with sucrose concentration. A downward curvature at low values of T/η was observed in the Perrin plot shown in Figure 4. This departure from linearity has been explained by Weber (1952) to be due to two different relaxation times of rotation of an elongated ellipsoid about the long and short axes. A nonlinear Perrin plot can also be an indication of some degree of flexibility in the binding of the probe to the macromolecule. At high viscosities, there is a greater dependence of anisotropy on T/η since the protein is fairly stationary while the fluorophore is still free to move (Cantor & Schimmel, 1981). A_0

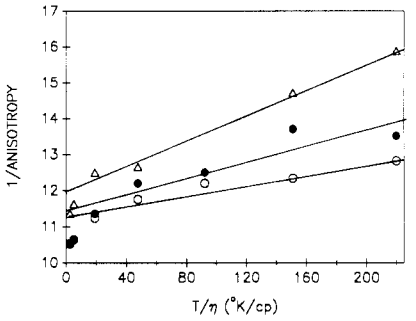

FIGURE 4: Perrin plot of anisotropy of DTAF-tubulin at different concentrations. Tubulin contained 0.4 mol of bound DTAF per mole of dimer at 8.0 μM (○), 0.8 μM (●), and 0.08 μM (△). Viscosity was varied isothermally (20 °C) by the inclusion of 10–65% sucrose in PEM buffer, pH 6.9.

was measured by extrapolation of the linear portion of the plot in Figure 4 to $T/\eta = 0$. The values were about the same within experimental error for different DTAF-tubulin concentrations, indicating the absence of large conformational changes. In addition, the slopes of the lines are related to the molecular volume by the Perrin equation (eq 4). Since the lifetimes were determined to be constant for the three concentrations of labeled tubulin, the relative molecular volumes are 1.8:1.6:1.0 for 8, 0.8, and 0.08 μM protein. By assuming that tubulin is a hydrated spherical molecule, the rotational correlation time, τ_c, can be calculated from eq 4. The τ_c value obtained for the highest concentration of DTAF-tubulin, conditions where it is assumed that the dimer exists, was 40 ns, twice that for the lowest concentration, at which tubulin is assumed to be in the monomer form (Table I).

Effects of Microtubule Assembly Promoters and Inhibitors on Tubulin Dissociation. Factors which influence microtubule assembly such as ionic strength, glycerol, GTP, DMSO, Mg^{2+}, Ca^{2+}, colchicine, and vinblastine may also affect the tubulin dimer–monomer equilibrium. When the effects of microtubule assembly enhancers, GTP and DMSO, on the dissociation process were examined, there was no effect on the K_d value. However, the anisotropy values were 11–18% lower in the presence of these substances than in their absence at the lowest protein concentration, but at the highest protein concentration, there was no difference. This result suggests that these compounds may cause conformational changes in the monomer that result in a less restricted rotation of the probe. In the presence of 3.7 M glycerol, the K_d was increased 2-fold.

The effect of temperature on the dimer–monomer equilibrium was also studied. The K_d determined at 10 °C was 50% of the value at 20 °C, while the K_d at 36 °C was 2-fold higher than that at 20 °C. The van't Hoff plot was linear, and a value of −9.5 kcal/mol was obtained for the $\Delta H°$ of the association reaction. $\Delta G°$ and $\Delta S°$ values at 20 °C of −8.1 kcal/mol and −4.6 eu, respectively, were calculated.

To study the effects of ionic strength on subunit dissociation, the concentration of Pipes in the PEM buffer was lowered from 0.1 to 0.01 M. This change in solution conditions resulted in about a 2-fold higher K_d, indicating that tubulin dimer dissociation is favored at a lower ionic strength. The effect of Mg^{2+} concentration was also investigated by removing the metal ion from the buffer and, in one case, by adding Mg^{2+} at a ratio of 1:1 to DTAF-tubulin. There was no change in the dissociation curves. Another factor which may influence the association–dissociation process is the presence of Ca^{2+}. The concentration of free Ca^{2+} was calculated by using an apparent K_a of EGTA with Ca^{2+} of 4.7×10^5 M^{-1} at pH 6.8 (Berkowitz & Wolff, 1981). In one case, EGTA was removed from the PEM buffer, and Ca^{2+} and EGTA were added at a constant ratio of 9:3 per mole of DTAF-tubulin. In another case, Ca^{2+} was added to labeled tubulin in PEM buffer to give a final concentration of 2 mM free Ca^{2+}. In both cases, the K_d was 2-fold higher than in the control with no Ca^{2+} added, indicating that the presence of Ca^{2+} may favor dimer dissociation.

The effects of mitotic inhibitors, vinblastine and colchicine, on tubulin dissociation equilibria were also studied. DTAF-tubulin was diluted into a 3-fold excess of colchicine in PEM buffer and incubated for at least 1 h at 4 °C. We observed a 2.5-fold decrease in the K_d at 20 °C with colchicine present. This effect of colchicine on the dissociation process of bovine brain tubulin had been previously examined using equilibrium ultracentrifugation, and a 3-fold decrease in the K_d was reported (Detrich et al., 1982). When DTAF-tubulin was preincubated with a 5-fold excess of vinblastine before dilution, the anisotropy remained constant. Previous studies (Singer et al., 1988) had shown that under such conditions, tubulin forms large and stable aggregates. We confirmed this in our present studies, and we also showed by size-exclusion HPLC that these aggregates are stable at a tubulin concentration of 0.1 μM.

Reconcentration Experiments. To demonstrate the reversible nature of tubulin dissociation to the monomer, a 0.1 μM sample of DTAF-tubulin (100 mL) was reconcentrated to 24 μM at 4 °C using Amicon ultrafiltration membrane cones. The anisotropy reading increased from 0.070 to 0.085 following reconcentration. Since the total time required for this reconcentration process was about 2 h, the stability of the diluted DTAF-tubulin samples during this time period was determined by time-dependent anisotropy measurements. The anisotropy of 5.0 and 0.1 μM DTAF-tubulin samples remained constant for 3 h. When reconcentrated DTAF-tubulin was tested for assembly competence, the sample at 1.6 mg/mL in the presence of 10% DMSO and GTP polymerized into microtubules to 82% the extent as DTAF-tubulin that had not undergone dilution and reconcentration.

Size-Exclusion HPLC. Another method used to detect subunit dissociation of the tubulin dimer was size-exclusion HPLC. Three elution profiles are presented in Figure 5A for DTAF-tubulin. The elution of the labeled protein was monitored by the fluorescence of bound DTAF. As the concentration of DTAF-tubulin injected on the column was decreased, the elution volume of the protein increased, indicating dissociation at low protein concentrations. The elution volumes were 12.3 mL for 7 μM DTAF-tubulin and 13.8 mL for 0.07 μM and correspond to molecular weights of 100K and 56K, respectively (see inset). Similarly, an increase in the elution volume of FITC-tubulin and unlabeled tubulin was also observed with dilution of the protein. On the other hand, there was no significant change in the elution volume of DTAF-chymotrypsin even at a 70-fold lower concentration (Figure 5B).

Discussion

We have used fluorescence anisotropy measurements and size-exclusion HPLC of tubulin covalently labeled with DTAF and FITC to demonstrate that bovine brain tubulin undergoes a concentration-dependent dissociation. By using these techniques, we were able to measure the physical state of tubulin from 8×10^{-8} to 1000×10^{-8} M. From the measurements, a dissociation constant in PEM buffer, pH 6.9 and at 20 °C, of approximately $(8.4 \times 10^{-7}) \pm (0.4 \times 10^{-7})$ M was calculated. At 10 °C, the value was 5.0×10^{-7} M. A K_d at 5 °C of 3.6×10^{-7} M can be calculated by using the $\Delta H°$ value.

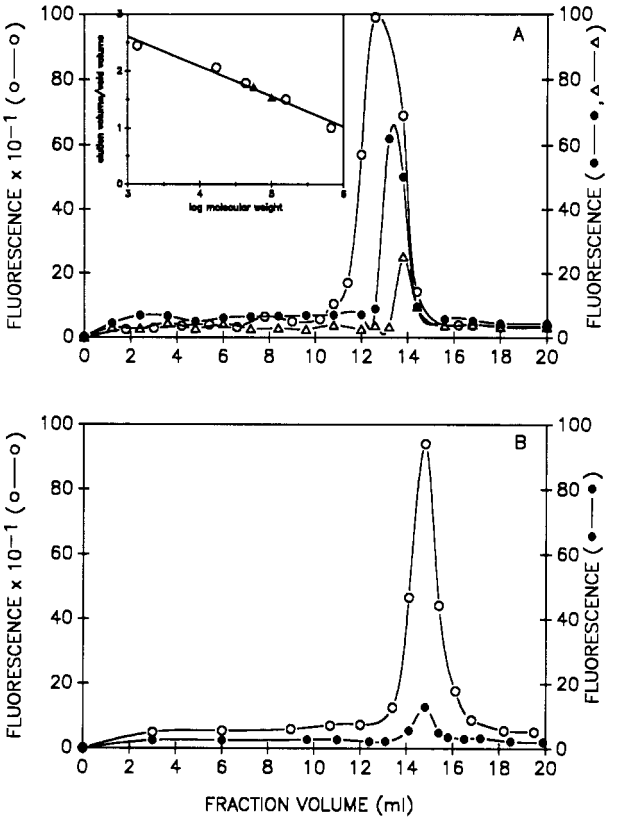

FIGURE 5: (A) Effect of dilution of DTAF-tubulin on elution behavior in size-exclusion HPLC. DTAF-tubulin (0.4 mol of DTAF per mole of dimer) was chromatographed on a TSK 3000 SW size-exclusion HPLC column. The column was equilibrated, and the fractions were developed in PEM buffer, pH 6.9. The elution of the protein was monitored by the fluorescence of the bound DTAF. The elution profiles of three different DTAF-tubulin concentrations are shown: 7.0 μM (○), 0.7 μM (●), and 0.07 μM (△). The inset is a standard curve of elution volumes as a function of molecular weight (○). The molecular weights that correspond to the elution volumes of 7.0 and 0.07 μM DTAF-tubulin (▲) were obtained from this curve. (B) Elution profile of DTAF-labeled α-chymotrypsin using 7.0 μM (○) and 0.10 μM (●) protein. The same conditions were used as in the HPLC of DTAF-tubulin.

This value is in good agreement with the value of (7–10) × 10^{-7} M determined by ultracentrifugation methods at the same temperature (Detrich & Williams, 1978; Detrich et al., 1982). In a recent article (Sackett et al., 1989), a K_d of 1.5 × 10^{-7} M was determined for rat brain tubulin. In the latter work, the authors used a kinetic approach, measuring the rate of proteolysis as a function of dilution of tubulin. With dilution appeared a fraction of β-tubulin which reacted very rapidly and was presumed to be the monomer form of the β-subunit. The advantage of using fluorescently labeled tubulin is that we were able to make measurements at a tubulin concentration 5-fold less than that used in the ultracentrifuge studies. To ensure that tubulin was not denatured upon such a high dilution, we showed that after reconcentration, the protein assembled with almost the same extent as undiluted protein. We also showed that the protein fluorescence spectrum of tubulin at 5 × 10^{-8} M was the same as at 1 × 10^{-6} M, with an emission maximum at 330 nm, while denatured tubulin had a maximum at 350 nm. One limitation of the technique used in our studies is the uncertainty of whether the chemical modification by DTAF or FITC affects the dissociation constant. However, the fact that the K_d value we obtained agrees with that determined by ultracentrifugation measurements of unmodified tubulin (Detrich & Williams, 1978; Detrich et al., 1982) argues against this possibility.

To examine the possibility that the anisotropy changes observed may be due to other factors aside from tubulin dissociation, several experiments were performed. First, the anisotropy of two monomeric proteins, chymotrypsin and RNase A, did not decrease after dilution, but dilution of dimeric malate dehydrogenase did result in a concentration-dependent decrease as had been shown earlier (Shore & Chakrabarti, 1976). Second, dilution of DTAF-tubulin into a concentration of unlabeled tubulin at which only dimer should exist prevented the decrease in anisotropy. However, in an identical experiment using bovine serum albumin to keep the total protein concentration constant, a decrease in anisotropy was observed. Third, the anisotropy values of urea-denatured tubulin did not change upon dilution. In addition, the size-exclusion HPLC results support the hypothesis that dissociation occurs upon dilution.

The anisotropy changes reflect actual changes in protein size because the fluorescent lifetimes and limiting anisotropy (A_0) values were found to be the same (within experimental error) at different tubulin concentrations. The lack of change in A_0 ruled out conformational changes which may result in greater rotational freedom for the fluorescent moiety alone, independent of the rotation of the tubulin to which it is attached. Thus, differences in anisotropy upon dilution are directly attributed to changes in the rotational correlation time which is directly proportional to the protein molecular volume.

The average A_0 value obtained from the Perrin plot (Figure 4) was 0.086, which is within the range of values found for other fluorescently labeled proteins. The reported A_0 for these proteins ranges from 0.030 to 0.089 for pyrene butyrate (PB)–bovine serum albumin (Knopp & Weber, 1969) depending on the ratio of the chromophore to protein, 0.166 for PB–phosphofructokinase (Reinhart & Lardy, 1980) and 0.167 for Bis-ANS-tubulin (Prasad et al., 1986). Although the theoretical A_0 for a totally rigid system is 0.4, in practice, this limit is never reached, and values of 0.2 are often encountered (Yguerabide, 1972). The smaller observed value for A_0 in our studies may be due to flexibility of the binding site of the fluorophore or flexibility of the macromolecule as a whole (Cantor & Schimmel, 1981), or it may be due to deviations of the protein from a spherical shape (Weber, 1952).

An investigation of the effects of various tubulin ligands and promoters of assembly showed that their effects were fairly small but reproducible. Decreased ionic strength, increased temperature, and the presence of Ca^{2+} or glycerol seemed to promote dissociation to the tubulin subunits. Mg^{2+}, however, does not have any effect on the dissociation process. At low tubulin concentrations, both GTP and DMSO seemed to cause a similar conformational change which results in more flexibility in the probe binding site and a decreased rotational relaxation time of the labeled tubulin. The antimitotic drug colchicine favored association of monomers. The effect of colchicine is in agreement with a previous proposal that colchicine binding induces a conformational change in tubulin that does not allow dissociation to its subunits to occur as readily (Detrich et al., 1982).

Most in vitro assembly studies are done at tubulin concentrations of ≥5 μM, a concentration at which essentially all of the protein is in the dimer state. However, the steady-state concentration at 37 °C often is around 2 μM, a concentration at which almost 50% of the protein would be in the monomer form. Antimitotic drug binding studies are often done at concentrations as low as 3 × 10^{-7} M (Owellen et al., 1972; Bhattacharya & Wolff, 1976). At this concentration, a mixture of dimer and monomer would also exist. It is not clear

at this time whether these ligands and others bind to both forms of tubulin or only to the dimer.

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ADP-ribosylation of Dinitrogenase Reductase in *Rhodobacter capsulatus*†

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ABSTRACT: In the photosynthetic bacterium *Rhodobacter capsulatus*, nitrogenase is regulated by a reversible covalent modification of Fe protein or dinitrogenase reductase (Rc2). The linkage of the modifying group to inactive Rc2 was found to be sensitive to alkali and to neutral hydroxylamine. Complete release of the modifying group was achieved by incubation of inactive Rc2 in 0.4 or 1 M hydroxylamine. After hydroxylamine treatment of the Rc2 preparation, the modifying group could be isolated and purified by affinity chromatography and ion-exchange HPLC. The modifying group comigrated with ADP-ribose on both ion-exchange HPLC and thin-layer chromatography. Analyses by ^{31}P NMR spectroscopy and mass spectrometry provided further evidence that the modifying group was ADP-ribose. The NMR spectrum of inactive Rc2 exhibited signals characteristic of ADP-ribose; integration of these signals allowed calculation of a molar ratio ADP-ribose/Rc2 of 0.63. A hexapeptide carrying the ADP-ribose moiety was purified from a subtilisin digest of inactive Rc2. The structure of this peptide, determined by amino acid analysis and sequencing, is Gly-Arg(ADP-ribose)-Gly-Val-Ile-Thr. This structure allows identification of the binding site for ADP-ribose as Arg 101 of the polypeptide chain of Rc2. It is concluded that nitrogenase activity in *R. capsulatus* is regulated by reversible ADP-ribosylation of a specific arginyl residue of dinitrogenase reductase.

For a number of phototrophic bacteria, mostly Rhodospirillaceae, rapid inhibition of nitrogenase activity has been reported to occur upon exposure of whole cells to ammonium ions and other sources of combined nitrogen [see Vignais et al. (1985) and Hallenbeck (1987) for reviews]. The mechanism of this regulation has been investigated at the molecular level in *Rhodospirillum rubrum* (Gotto & Yoch, 1982; Preston & Ludden, 1982; Pope et al., 1985a) and *Rhodobacter cap-*

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