Local Unfolding and the Stepwise Loss of the Functional Properties of Tubulin

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ABSTRACT: Tubulin exhibits a number of characteristic functions that can be used to identify it. They include the ability to polymerize to microtubules, GTPase activity, and the binding of numerous antimitotic drugs and fluorophores. These functions can be differentially modified by low (0.1–1.0 M) urea concentrations, and such urea-induced modifications are stable over time periods of minutes to hours. These intermediate states suggest the existence of restricted regions in the protein each of which is associated with a function and its own urea sensitivity. In order of decreasing sensitivity to urea these effects are decreased *rate* of polymerization of tubulin to microtubules > decreased *extent* of polymerization ≈ decreased GTPase activity > enhanced fluorescence of a rapidly binding analogue of colchicine-MTPT [2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone] ≈ decreased proteolysis by trypsin (after αArg339) and by chymotrypsin (after βTyr281) > enhanced fluorescence of 1-anilino-8-naphthalenesulfonic acid (ANS). Additional evidence for the independent behavior of the restricted regions stems from the markedly different time dependence of the response to urea. These low urea concentrations do not induce significant changes in tryptophan fluorescence, suggesting that the observed effects are due to local unfolding. At higher urea concentrations (2–4 M), the enhanced fluorescence of the ligands is abolished; MTPT fluorescence decreases at lower urea concentrations than ANS fluorescence. Moreover, tubulin becomes highly susceptible to proteolysis at multiple sites, and tryptophan emission shows a red-shift, as expected. Multistep unfolding in response to denaturants has been reported for some other proteins. Tubulin appears to be an extreme example of such *local* responses that proceed under milder conditions than the *global* transition to the unfolded state.

Tubulin is the most abundant protein of microtubules. It is a noncovalent heterodimer of two related, but not identical, monomers termed α and β tubulin. Numerous functional properties characterize the heterodimer. These include (1) the ability to polymerize in a GTP- and temperature-dependent manner in the presence of certain cosolvents or with the aid of microtubule-associated proteins (MAPs), (2) the ability to bind and hydrolyze GTP, (3) the binding of antimitotic drugs on at least two distinct sites with profound consequences for the polymerization process, GTP hydrolysis, and other properties, (4) interactions with a number of polarity sensitive fluorescent dyes, and (5) charge-based interactions with MAPs and certain other proteins.

Attempts have been made to assign these properties to specific domains of the α and β monomers. Thus, using cross-linking the *intra*subunit contacts have been identified for the dimer, as have the *inter*subunit contacts of the microtubule (Kirchner & Mandelkow, 1985; Serrano & Avila, 1985). There are two GTP binding sites in the dimer, a nonexchangeable GTP binding site on α tubulin and an exchangeable GTP-binding site on β tubulin with a major GTP interaction at βCys12 (Shivanna *et al.*, 1993). Colchicine binding occurs primarily on β tubulin (Wolff *et al.*, 1991), and we have recently identified two regions involved in this binding—one near the N-terminus, the other on the center of the sequence of the β monomer (Uppuluri *et al.*, 1993). Binding results in local unfolding of a helical region near residue 390 of β tubulin (Sackett & Varma, 1993). The binding locus is sufficiently close to α tubulin to permit secondary interactions with the latter (Wolff *et al.*, 1991), which suggests a location near the α/β contact region of the dimer. A similar argument has been made for the binding of the polarity-sensitive dye, Nile red, whose fluorescence depends on the association of the two monomers, and which is lost upon protein dilution resulting in subunit dissociation (Sackett *et al.*, 1990). Charge-based MAP-binding domains have been localized to the highly acidic C-termini of both monomers (Littauer *et al.*, 1986; Melki *et al.*, 1991) which extend from the surface of the microtubule (Sackett & Wolff, 1986). A number of structural domains of α and β tubulin have been identified by limited proteolysis with trypsin, chymotrypsin, and subtilisin (Brown & Erickson, 1983; Mandelkow *et al.*, 1985; Sackett & Wolff, 1986). X-ray diffraction from oriented microtubule gels has provided evidence for multiple domains in α and β tubulin and indicated the potential for significant *intrasubunit* flexibility between these domains as well as significant *intersubunit* flexibility (Beese *et al.*, 1987). Association of particular structural domains or domain junctions with characteristic properties of tubulin has, however, only begun; it is not even clear how to map the proteolytically defined regions onto those found by X-ray diffraction. Nevertheless, it seems likely that particular domains and domain junctions will have differing roles in defining various properties of the native tubulin dimer.

An additional way of identifying at least some of these domains can be by demonstration of stepwise denaturation of tubulin. Ideally, discrimination of different structural states by denaturants is accomplished by the demonstration of equilibrium states that differ as a function of denaturant concentration. This approach is not appropriate for tubulin because even native tubulin is not in equilibrium because it is unstable. The spontaneous "aging" of tubulin is well known (Wilson, 1970; Barton, 1978) and is manifested by a time-dependent loss of characteristic functions even at low

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temperatures. Different properties of native tubulin decay with different time constants. Thus, colchicine binding decays with a half life of 4–7 h (Wilson, 1970), whereas polymerization activity is lost more rapidly (Barton, 1978); when colchicine binding has been lost, tubulin can still bind ANS like the native protein (Bhattacharyya & Wolff, 1975). Moreover, the properties of tubulin are quite sensitive to the method of preparation (Clark *et al.*, 1981). Indeed, this differential loss of properties with time suggested the existence of multiple nonnative states which the present study examines in detail using urea as a denaturant. To avoid long times and elevated temperatures, we have generally used urea for short times (<1 h) and in most cases at ≤25 °C. These studies demonstrate differential urea sensitivities of tubulin properties. Several properties appear to be lost following unfolding of limited regions of the protein with unusually high sensitivity to denaturation; this occurs at lower urea concentrations than required to show significant spectral changes.

MATERIALS AND METHODS

Materials. Microtubule protein was isolated from rat brains as previously described (Sackett *et al.*, 1991). Tubulin was purified from this material by sequential selective polymerizations in 0.4 M piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES) + 10% dimethyl sulfoxide (Himes *et al.*, 1977) followed by sodium glutamate polymerization (Hamel & Lin, 1984) as described in Sackett and Varma (1993). Tubulin, at 25 g/L in Mes assembly buffer [0.1 M morpholinoethanesulfonic acid, 1 mM $MgCl_2$, 1 mM ethylene glycol bis-(β-aminoethyl ether), pH 6.9] was drop frozen and stored in liquid nitrogen. Taxol was a gift from Dr. Matthew Suffness, National Cancer Institute. Fatty acid-free bovine serum albumin was from Sigma Chemical Co., and all other reagents used were reagent grade. [γ-^{32}P]GTP (30 mCi/mmol) and [^{3}H]colchicine were obtained from New England Nuclear. Highly purified ANS (Ferguson & Cahnmann, 1975) was a gift of Dr. Hans Cahnmann.

Urea. Urea was UltraPure from Schwarz-Mann (Cleveland, OH); it was made up to 8 M in Mes assembly buffer, drop frozen, and stored in liquid nitrogen until use. In freshly made urea, or preparations stored for >1 year, the cyanate content was undetectable (<3 mM in 8 M urea solution) by the modified Werner method (Marier & Rose, 1964), whereas cyanate was readily detectable in urea solutions heated for a brief period.

Polymerization. Taxol-induced polymerization was monitored by turbidity at 350 nm using a thermostated Cary Model 219 spectrophotometer. Tubulin was diluted to 7.5 μM in Mes assembly buffer + 1 mM GTP and polymerization induced by addition of taxol to 10 μM at 25 °C. Absorbance was recorded for 50 min or until a clear plateau was reached.

Fluorescence. All fluorescence assays were performed using a Perkin-Elmer MPF-66 spectrofluorimeter in ratio mode, equipped with a red-sensitive Hamamatsu R928 photomultiplier. Microcells with a working volume of 250 μL and a path length of 5 mm were used for most assays. The emission slit was set to 5 nm, and the excitation slit was set to 2 nm, to minimize irradiation of MTPT. Data were collected with a dedicated Perkin-Elmer Series 7300 computer, and spectral analysis and manipulation were performed using software (PECLs-III) from Perkin-Elmer. Colchicine-photosensitized covalent tubulin dimer formation was carried out with 0.2 mM colchicine and 0.05–0.1 mM tubulin. After incubation for 30 min at 36 °C in the dark, the mixture was irradiated for 8 or 10 min at 4 °C under 2.0 cm of 20% $CuSO_4 \cdot 5H_2O$ solution with an ultraviolet source at 89 W lamp power (Wolff *et al.*, 1992). Irradiated samples were separated by SDS gel electrophoresis. Stained 8% SDS gels were quantified on a Beckman DU-8 scanner.

Binding of MTPT and ANS. Fluorescence of 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (MTPT) bound to tubulin was monitored following excitation at 350 nm. Emission was recorded between 390 and 490 nm, and appropriate blanks were subtracted. Integrated emission intensity was taken as a measure of bound MTPT. When single emission wavelength measurements were made, excitation and emission wavelengths were 350 and 430 nm, respectively. Fluorescence of anilinonaphthalenesulfonic acid (ANS) was measured with excitation at 350 nm. Emission spectra were recorded between 400 and 550 nm, and single wavelength emission was measured at 480 nm. MTPT binding kinetics were measured with the MPF-66 in time mode and excitation and emission set to 350 and 430 nm, respectively. The kinetics of binding was evaluated by measuring the increase in fluorescence of 5 μM tubulin following the addition of 10 μM MTPT. The data collection rate was 5 s^{-1}, and typically the fluorescence was monitored for 30 s before addition of MTPT and 60 s following addition. The kinetics of debinding were evaluated by monitoring the fluorescence of a solution containing 5 μM tubulin and 5 μM MTPT for 2 min, adding 50 μM podophyllotoxin, and measuring the rate of loss of fluorescence over the following 10 min with a data collection rate of 2 s^{-1}. Equilibrium binding was measured by a rapid gel filtration assay. Small columns (0.7 mL) of Bio-Gel P-10, equilibrated in Mes assembly buffer with or without 0.75 M urea, were prepared in Spin-X microcentrifuge filter unit tubes (0.45 μM cellulose acetate, Costar, Cambridge, MA) and centrifuged 30 s at 1000*g* to remove the excluded volume of the column. The filtered liquid was removed from the bottom of the tube, samples of 75 μL were applied to the column, and the tube was recentrifuged for 30 s at 1000*g*. The filtered sample was removed, mixed with an equal volume of 5 M guanidine HCl, and the absorbance spectrum was recorded between 265 and 420 nm using 1-cm pathlength, 0.15-mL microcuvettes (Hellma Cells, Jamaica, NY). Matched blanks were run over each spin column with no tubulin, followed by five washes with 0.3 mL of buffer with or without urea, prior to loading the tubulin containing sample. The efficiency of the columns at removing free MTPT was 99% from Mes buffer. This decreased to about 97% at 6 M urea. The small blank absorbances were subtracted from the matched tubulin samples, correcting for differences in efficiencies of removal. MTPT concentration was estimated from the absorbance at 345 nm using a molar extinction coefficient of 1.7×10^4. Tubulin was estimated from absorbance at 278 nm with a molar extinction coefficient of 1.05×10^5.

GTP Hydrolysis. Hydrolysis of [^{32}P]GTP was monitored by extraction of the liberated inorganic [^{32}P]phosphate into isobutanol/toluene by a micromethod based on that of Shacter (1984). Assay mixtures contained 100 μM GTP, 10 μM tubulin, and [^{32}P]GTP at about 2000 dpm/μL in Mes assembly buffer with urea added to various concentrations. Time 0 was defined by the addition of tubulin to tubes containing all other components. Aliquots of 10 μL were removed at various times between 0 and 30 min and placed in extraction tubes containing 0.4 mL of 5 mM silicotungstic acid in 1 mM H_2SO_4. A 0.6-mL aliquot of isobutanol/toluene (1:1, v/v) and a 0.08-mL aliquot of ammonium molybdate (3.5% in 2 M H_2SO_4) were added. The tube was vortexed hard for 10 s and centrifuged for 2 min at ~2500*g*. A sample (0.35 mL) of the

upper organic layer was carefully removed and counted after addition of 5 mL of Ultima Gold counting solution (Packard Instruments, Downers Grove, IL). In the absence of urea, the hydrolysis rate was about 0.025 mol of GTP per mol of tubulin per min at room temperature (23 °C).

Proteolysis. Two types of proteolysis experiments were performed: continuous and pulse proteolysis. In continuous proteolysis, tubulin was diluted to 10 μM in Mes assembly buffer ± urea, and trypsin or chymotrypsin were added at 1:50 (w/w) to tubulin; proteolysis was allowed to proceed at room temperature of about 22 °C. Aliquots were removed at various times, and proteolysis was stopped by addition of 0.2 mM phenylmethanesulfonylfluoride for chymotrypsin or 0.01 mM leupeptin for trypsin. Samples were then prepared for electrophoresis. Following electrophoresis, gels were scanned and the rate constant for digestion was determined by plotting the fraction uncleaved versus time as described in (Sackett *et al.*, 1989). For pulse proteolysis experiments, tubulin was diluted to 10 μM in Mes assembly buffer plus different concentrations of urea. At various times, aliquots were removed and chymotrypsin or trypsin were added to a concentration of 1:25 (w/w) to tubulin. Proteolysis was allowed to proceed for 5 min at room temperature. The reactions were stopped and samples were processed for electrophoresis. Coomassie blue-stained gels were scanned, and the fraction undigested was plotted as a function of the time of exposure of tubulin to the buffer/urea solution before addition of enzyme. The effect of urea on the intrinsic activities of trypsin and chymotrypsin was assayed with chromogenic esters and with casein.

Esterolytic Activity. Esterolytic activity was determined from the increase in the absorbance, at 400 nm, from 0.1 mM succinylalanylalanylprolylphenylalanyl-*p*-nitroanilide for chymotrypsin and 0.5 mM benzoylarginyl-*p*-nitroanilide for trypsin.

Proteolytic Activity. Proteolytic activity was determined with casein and denatured tubulin as substrates. Casein hydrolysis was assayed by the increase in trichloroacetic acid (5%) soluble absorbance at 414 nm following cleavage of 1 mg/mL azocasein in Mes buffer alone or with added urea. Denatured tubulin hydrolysis was assayed by fluorescence. Tubulin (50 μM in Mes buffer) was reacted for 20 min at room temperature with 500 μM eosin maleimide. Remaining tubulin sulfhydryls were blocked by 15 min incubation with 3 mM iodoacetic acid at 37 °C. Labeled protein was precipitated at −20 °C with four volumes of acetone, pelleted, washed with 80% cold acetone, and dried.

The dried pellet was resuspended in Mes buffer + 0.05% SDS and sonicated to clarity, diluted 50-fold to 50 μg/mL in Mes buffer with or without urea for assay, and proteolyzed as above. An equal volume of 5 M guanidine-HCl was added, and the sample was centrifuged through an Amicon Microcon 10 microconcentrator. The small fluorescent peptide products in the filtrate were detected by emission at 545–550 nm following excitation at 520 nm.

Electrophoresis. Samples were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis (Sackett *et al.*, 1989; Sackett, 1989). Coomassie blue R-stained gels were scanned using a Beckman DU-8 gel scanner. Integration of band intensity was performed by the programs provided with the DU-8. Alternatively, some gels were scanned with a Micotek 600G flatbed scanner controlled by Photostyler software (Aldus Corp., Seattle, WA) and operated at 200 dpi resolution. Integration of band intensity was performed using Quantiscan software (Biosoft, Ferguson, MO).

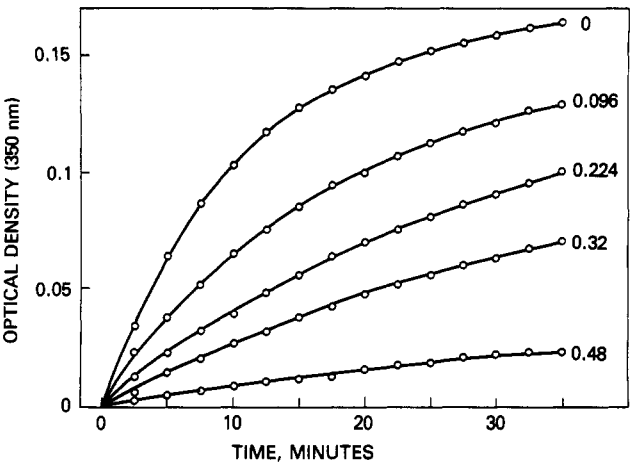

FIGURE 1: Urea inhibits taxol-induced polymerization of tubulin. Tubulin was diluted to 7.5 μM in Mes assembly buffer + 1 mM GTP, supplemented with urea as indicated, and baseline absorbance recorded. Taxol was added to 10 μM, and polymerization at 25 °C was monitored by optical density at 350 nm. The addition of taxol was taken as time 0. The inhibitory effect of added urea is demonstrated. Polymerization in the presence of 0, 0.096, 0.224, 0.32, and 0.48 M urea (top to bottom) is shown.

RESULTS

Polymerization. Tubulin polymerization to microtubules is sensitive to numerous variables such as solvents, the critical protein concentration, associated proteins, pH, Mg^{2+} and GTP concentrations, Ca^{2+} and other salts, temperature, and drugs. Many of these become less perturbing when polymerization is promoted by taxol, a diterpenoid that binds to and stabilizes the polymerized state, and we have chosen these conditions to focus on the effects of urea. As seen in Figure 1, very low urea concentrations hinder polymerization, and a drop in assembly is already seen at <0.10 M urea. With 1.0 M urea, assembly is completely blocked (data not shown). It should be noted that the urea effect on the *initial rate* of polymerization is more sensitive than the urea effect on the final *amount* of polymer obtained at 45 min. The effect of urea on the polymerization rate and final extent are shown in Figure 2. Similar results were observed when polymerization was carried out at 37 °C, although slightly greater urea concentrations were required to achieve the same degree of inhibition as seen at 25 °C (data not shown). Incubation of tubulin with 0.5 M urea for 15 min at 25 °C, followed by 10-fold dilution before measurement of taxol-induced polymerization (also at 25 °C), results in polymerization comparable to that obtained in the presence of 0.05 M urea, which is only slightly less than the polymerization in urea-free buffer (results not shown). Since 0.5 M urea results in near-total inhibition of polymerization (see Figure 2), this result shows that low concentration urea-induced inhibition of polymerization is reversible. We conclude that urea produces some effect on the conformation of the dimer/dimer interface that interferes with the addition of new dimers. This effect is not restricted to urea since the same inhibition can be obtained with low concentrations of guanidine hydrochloride (data not shown).

GTPase Activity. Tubulin has a low intrinsic GTPase activity. This is stimulated by polymerization and by binding of colchicine (Hamel, 1992; Correia, 1991). Intrinsic GTPase activity is sensitive to urea at low concentrations and is completely inhibited by 1.0 M urea (see Figure 2). At urea concentrations up to 0.5 M, the inhibition of GTPase activity is of similar magnitude as the decrease in the extent of polymerization (Figure 1). The inhibition of GTPase activity

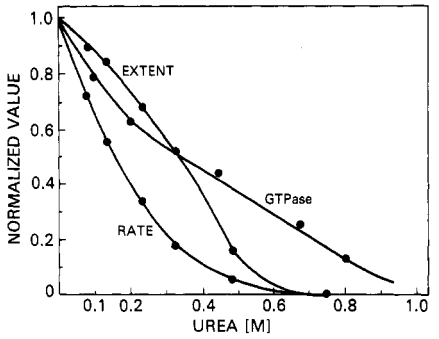

FIGURE 2: Low urea concentrations inhibit polymerization and GTPase activity. Assembly initial rates and assembly extent at 45 min are from the experiments shown in Figure 1. Hydrolysis of GTP was measured during 30 min of incubation at room temperature by release of $^{32}PO_4$ as described under Materials and Methods. Values were averaged, and results obtained at different concentrations of urea were normalized to the values obtained in buffer alone.

by low urea concentrations is not due to displacement of bound GTP, since even 1.0 M urea causes little loss in measured bound nucleotide (results not shown). Thus, urea must affect structures in tubulin required for hydrolysis of GTP rather than structures required for binding *per se*.

MTPT Binding. Perhaps the second most characteristic property of tubulin is the ability to bind colchicine and related drugs. Such binding is accompanied by enhanced fluorescence deriving from the tropolone chromophore (Bhattacharyya & Wolff, 1974). Because colchicine binding is very slow, it is convenient to use a rapidly binding analogue [called MTPT or 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone] in order to study binding without the time constraints imposed by colchicine kinetics (Bane *et al.*, 1984). To our surprise, 1.0 M urea markedly *enhanced* the fluorescence of MTPT bound to tubulin (Figure 3A). On the basis of the areas under the curves, this amounts to a 1.6-fold increase. This enhancement is not the result of a shift in the polarity of the binding environment as shown by the fact that when the peak emission intensities of the two curves were set equal, emission spectra are superimposable (Figure 3B). At higher urea concentrations there is a reduction and finally, abolition, of the fluorescence of MTPT. Moreover, there is a clear time dependence for the fluorescence behavior upon exposure of tubulin to urea, as depicted in Figure 4. The urea concentrations required to produce maximal enhancement of MTPT fluorescence decreased over an exposure time from 15 to 270 min; however, the maximal intensity attained occurs at the shortest time interval. Closer inspection of Figure 4, and additional short-term observations (not shown), reveals that the "enhancing" effect of low urea concentrations occur relatively quickly, whereas the inhibitory effects are slow and cause a progressive decrease in maximal enhancement concomitantly with a decrease in the urea concentration required for maximal stimulation of fluorescence. Incubation of tubulin with 0.75 M urea for 15 min, followed by a 10-fold dilution with buffer alone, resulted in fluorescence intensity similar to that seen in the presence of 0.075 M urea, which, in turn, was only slightly enhanced over the fluorescence observed in the absence of urea (data not shown).

In an effort to understand the basis of this urea-induced fluorescence enhancement, the kinetics of binding and debinding were investigated. The decrease in fluorescence of 5 μM MTPT in the presence of 5 μM tubulin was compared in the absence and presence of 1.0 M urea following addition of excess podophyllotoxin to block rebinding of MTPT. The value for k_{off} obtained, 0.014 s^{-1} in buffer alone, is somewhat

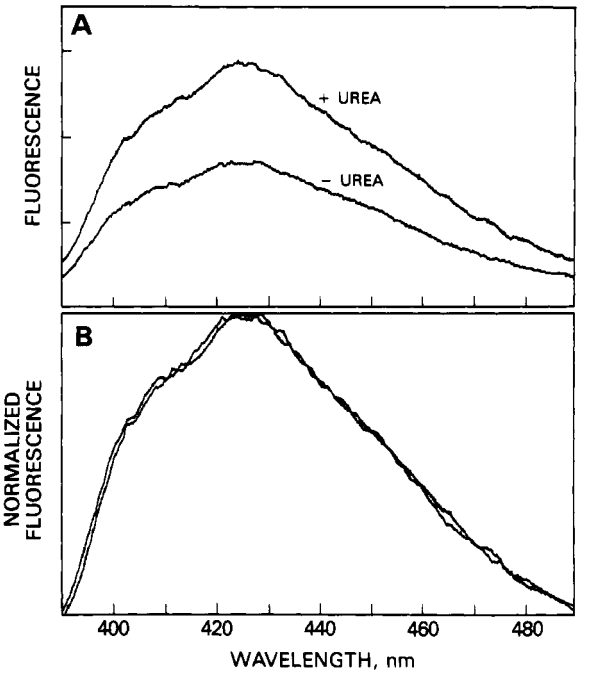

FIGURE 3: Urea enhances the fluorescence of tubulin-bound MTPT. MTPT (10 μM) was added to 5 μM tubulin in Mes assembly buffer with or without 1 M urea. The fluorescence emission spectra were recorded 5 min after mixing and are presented in panel A. Based on integrated areas, emission intensity is enhanced about 1.6-fold in the presence of urea. These spectra are shown peak-normalized (peak emission intensities set equal) in panel B, demonstrating that enhancement does not alter wavelength dependence of emission intensity.

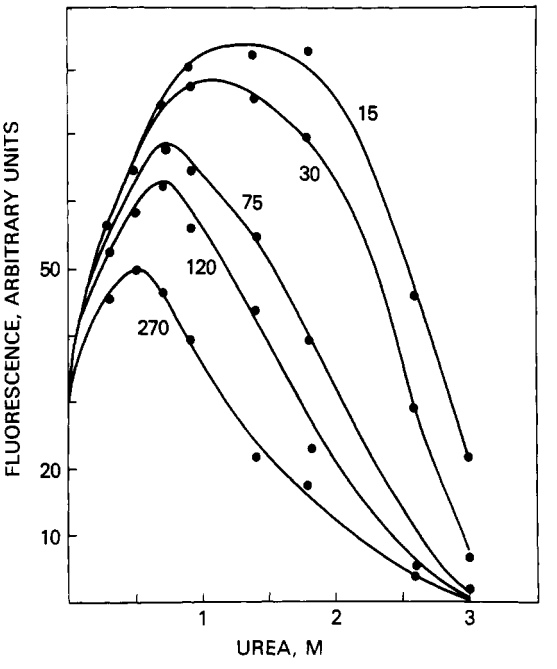

FIGURE 4: Time- and urea concentration dependence of MTPT fluorescence enhancement. Solutions of 4 μM tubulin with 10 μM MTPT were prepared in Mes assembly buffer plus various urea concentrations. The fluorescence emission intensity at 430 nm upon excitation at 350 nm was monitored at various times following mixing. Plots of fluorescence intensity versus urea concentration are presented. These were taken at 15, 30, 75, 120, and 270 min after mixing (top to bottom).

less than the value reported for k_{off} by dilution alone, 0.065 s^{-1} (Bane *et al.*, 1989). The initial rate of dissociation of MTPT was slowed by a factor of 1.4 in the presence of 1.0 M urea (Figure 5A). Although *on* rates for MTPT binding

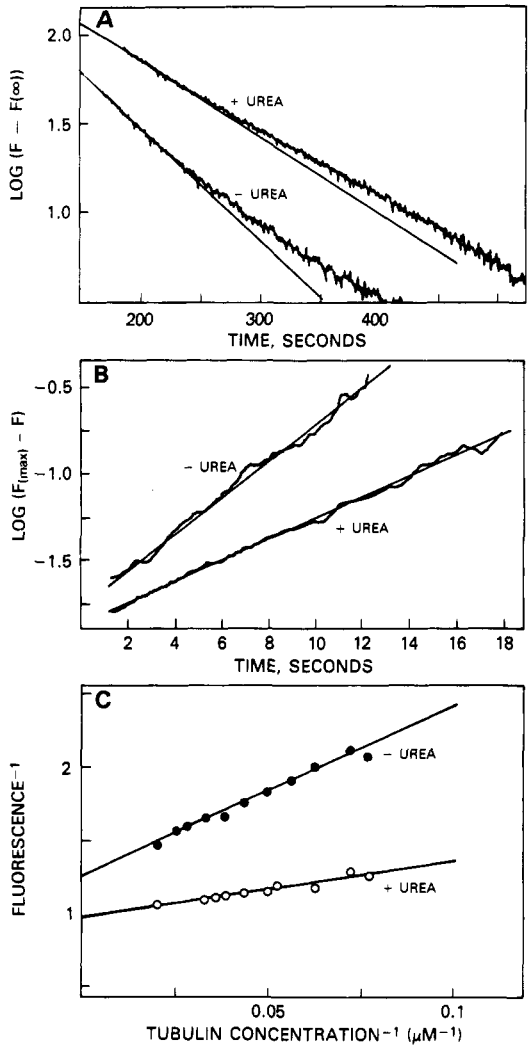

FIGURE 5: Urea effects on colchicinoid binding and fluorescence. (Panel A) Urea lowers the dissociation rate of the tubulin–MTPT complex. The fluorescence of a solution of 5 μM tubulin and 5 μM MTPT prepared in the presence or absence of 1 M urea was monitored beginning 5 min after mixing. Both solutions exhibited steady fluorescence emission. Excess podophyllotoxin (50 μM) was added to block all binding sites as they became available, thereby allowing measurement of dissociation of MTPT from tubulin. The dissociation rate in the absence of urea is about 1.4 times that in the presence of 1 M urea (0.014 s^{-1} at 0 M versus 0.010 s^{-1} at 1 M). (Panel B) Urea lowers the association rate of MTPT with tubulin. Tubulin (5 μM) was mixed with 10 μM MTPT, and fluorescence was monitored in the presence or absence of 1 M urea. The apparent association rate constant under these conditions was 0.22 s^{-1} at 0 M versus 0.15 s^{-1} at 1 M urea. The rate of increase in fluorescence in the absence of urea is about 1.5 times that in the presence of 1 M urea. (Panel C) The quantum yield of the tubulin-MTPT complex is compared in the presence and absence of urea. A solution of 2 μM MTPT in Mes assembly buffer with or without 1 M urea was titrated by addition of 2–28 μM tubulin. The resulting fluorescence in the presence and absence of urea is presented in double-reciprocal form. In all three panels, "+urea" indicates a urea concentration of 1 M.

are fast, it was possible in a darkened room with an open instrument to obtain measurements after 2–3 s and obtain acceptable estimates of the initial rate. The apparent rate constant obtained by treating this as pseudo-first-order binding was 0.22 s^{-1}, slightly less than the 0.52 s^{-1} reported by Bane *et al.* (1989). As shown in Figure 5B, 1.0 M urea decreased the on rate such that the ratio of the slopes without urea/with urea was 1.47. The equivalent slowing of both the on and off rates suggest that 1.0 M urea did not significantly alter the equilibrium binding constant. However, the change in quantum yield (see below) and the time dependence of the

Table 1: Equilibrium Binding of MTPT to Tubulin in the Presence and Absence of Urea

	av bound (mol/mol)[a]	SD	*n*	*P*[b]	$K_{d,app}$
10 μM MTPT					
control	0.69	0.08	8		3.0
+0.75 M urea	0.81	0.04	5	<0.01	1.5
100 μM MTPT					
control	1.17	0.24	9		
+0.75 M urea	1.23	0.31	7	>0.33	

[a] Bound MTPT was measured from absorbance spectra following separation of bound from unbound MTPT by rapid centrifugal gel filtration as described under Materials and Methods. Conditions were 5 μM tubulin and 10 or 100 μM MTPT as indicated. [b] Probability that the difference between urea and control samples would occur by chance.

enhancement (Figure 4) make calculations of the affinity constant from these rate constants unreliable.

In order to evaluate urea-induced changes in equilibrium binding, a rapid gel filtration assay was employed to measure tubulin-bound MPTP. This assay was performed with low MTPT concentrations (10 μM, the same as in Figure 3) to evaluate urea-induced changes in affinity under conditions similar to those of Figures 3 and 4. The results are given in Table 1. In the absence of urea, MTPT binding of 0.69 mol per mol of tubulin was found. This corresponds to an apparent K_d of 3 μM, which agrees well with reported values of 2.9 μM (Bane *et al.*, 1984), 3.4 μM (Engelborghs & Fitzgerald, 1986), and 2.5 μM (Choudhury *et al.*, 1983). In the presence of 0.75 M urea, binding is increased by slightly less than 1.2-fold, to 0.81 mol per mol of tubulin. Part of the increase in binding may be due to the slower dissociation rate in the presence of urea, resulting in an artifactually higher fraction of drug–tubulin complex remaining together after the column. However, the binding measured in the control agrees quite well with published equilibrium binding studies, suggesting that losses during centrifugal filtration were not large enough to greatly bias the results. These measurements were repeated with a high concentration of MTPT (100 mM) to examine the possibility that increased binding might represent exposure of a second binding site. At this concentration of MTPT, the variance in the measurements is increased due to the higher blank value. Despite this difficulty, the data clearly indicate that there is one binding site for MTPT per dimer of tubulin in the absence and in the presence of 0.75 M urea. Thus, urea does not affect the binding capacity of tubulin for MTPT.

Measurements of the fluorescence quantum yields of 2.0 μM MTPT were carried out in the presence of a substantial molar excess of rat brain tubulin such that all of the ligand would be bound. The effect of 1.0 M urea was examined. Extrapolation of the double-reciprocal plots to infinite tubulin concentration revealed that urea caused a significant increase in the quantum yield, Φ, such that $\Phi_{urea}/\Phi_{control} = 1.23$, (Figure 5C). This increase is not, however, sufficient to explain all of the fluorescence enhancement by itself. It is clear, therefore, that the fluorescence enhancement produced by urea cannot be easily explained by a single mechanism.

The urea effect on MTPT (and colchicine) binding has been observed under two other conditions. Irradiation of the MTPT–tubulin complex leads to covalent attachment of the ligand to tubulin (Wolff *et al.*, 1991). The yield of adduct is increased by urea with a maximum at 1.0–1.3 M urea and then declines. A second effect of such irradiation is the photosensitization by colchicine or MTPT of the covalent cross-linking of α and β tubulin to form dimers, trimers, and tetramers in decreasing order of concentration and time of

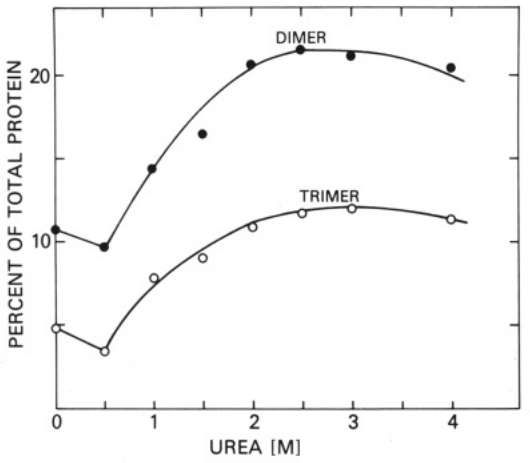

FIGURE 6: Irradiation of the tubulin–colchicine complex in the presence of urea. Rat brain tubulin (50 μM) and 300 μM colchicine were incubated in assembly buffer with or without urea (as indicated) at 37 °C for 30 min protected from room light. Ice-water cooled samples were irradiated under 2.5 cm of 20% $CuSO_4 \cdot 5H_2O$ (as far ultraviolet and infrared filter) for 5 min at 95-W lamp power. Stained 8% gels were quantified on a Beckman DU-8 gel scanner as described (Wolff *et al.*, 1992).

appearance, suggesting stepwise monomer addition (Wolff *et al.*, 1992). As shown in Figure 6, dimer and trimer formation is enhanced in the presence of urea with half-maximal urea concentrations ranging from 1.0 to 1.5 M. Tetramers are not depicted because they are difficult to quantitate.

Proteolysis. The colchicine binding studies suggested that low urea concentrations might release some structural constraint at the site that prevent optimal binding. The slower kinetics k_{on} and k_{off} (Table 1) suggested that urea might induce a "tightening" of the binding domain and, perhaps, also in other regions of the dimer. If such changes do occur, they might well have an effect on the proteolytic susceptibility of tubulin to trypsin or chymotrypsin. We have shown previously that α tubulin and β tubulin possess an internal cleavage site for trypsin and chymotrypsin, respectively (Sackett & Wolff, 1986). The kinetics of cleavage exhibit straight line plots of log undigested protein versus time, and the pseudo-first-order rate constants so obtained reflect various perturbations. As shown in Figure 7, low urea concentrations reduce the rates of both trypsin and chymotrypsin hydrolysis of tubulin. As expected, early trypsin hydrolysis is on α tubulin (Figure 7A,B), while early chymotrypsin hydrolysis is on β tubulin (Figure 7A,C). It should be pointed out that this urea effect is not on the catalytic efficiency of the proteases since 4.0 M urea led to extensive tubulin hydrolysis by both proteases (Figure 7A, lanes 3 and 6). The conclusion that reduced proteolysis is due to a urea effect on tubulin rather than the protease activity is confirmed by studies using alternative protein substrates. While 1–2 M urea did decrease esterolytic activity (particularly for trypsin), the hydrolysis of casein, a protein in random coil conformation (Krescheck, 1965), was increased for both enzymes at these urea concentrations, particularly for chymotrypsin (data not shown). Moreover, tubulin previously denatured by means independent of urea (see Materials and Methods) showed no change in proteolysis rate under the influence of these concentrations of urea. Therefore, the decrease in tubulin proteolysis observed above is likely to be due to conformational effects of urea on native tubulin which, in turn, change the accessibility of the cleavage sites of tubulin. In contrast to the time-dependent change with MTPT, there is only a small effect of increasing time of exposure to urea on the reduction in the rate of proteolysis of the two tubulin monomers as shown in Figure 8 for trypsin-

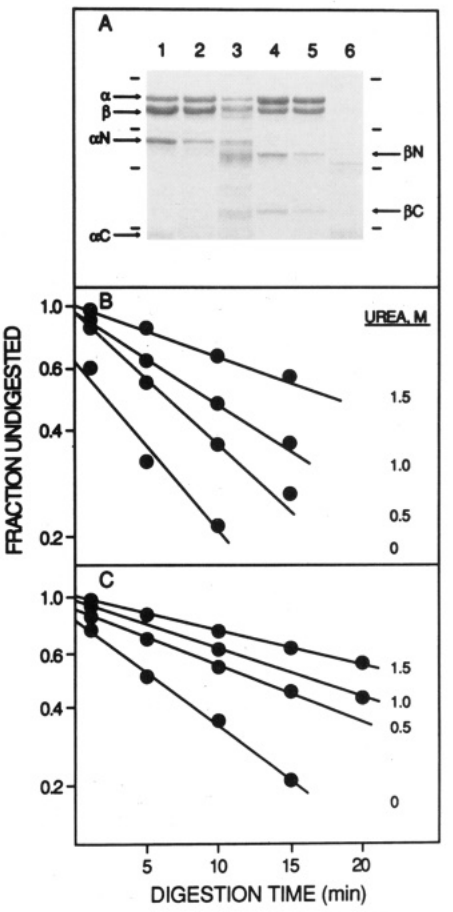

FIGURE 7: Urea reduces proteolytic cleavage of α and β tubulin. Tubulin, 10 μM in Mes assembly buffer, was digested with trypsin or chymotrypsin at room temperature for increasing periods of time in the presence of various concentrations of urea. Digestions were terminated, and reaction products were analyzed by SDS gel electrophoresis as described under Materials and Methods. In panel A, all digestions were for 10 min, and enzyme was added to 2% w/w to tubulin. Arrows show the position of α and β tubulin and of the amino (N) and carboxyl (C) domains produced by cleavage. The tic marks show the positions of molecular standards (66, 45, 31, 20 kDa, top to bottom; see Materials and Methods). Lanes 1–3 contain reactions with trypsin, which digests α tubulin after Arg339. Lanes 4–6 contain reactions with chymotrypsin, which digests β tubulin after Tyr281. Lanes 1 and 4 are control digests which contain no urea. Lanes 2 and 5 are digests with 1 M urea. The reduced cleavage compared to controls is evident by comparing the intensity of the band labeled α-N in lanes 1 and 2 and the intensity of the band labeled β-N in lanes 4 and 5. Reduced cleavage is not due to inhibition of enzyme activity, since cleavage in 4 M urea is very extensive and is shown in lanes 3 and 6. The urea dependence of trypsin digestion of α tubulin and chymotrypsin digestion of β tubulin are shown in panels B and C, respectively. Reactions were performed at room temperature with 10 μM tubulin, 3% (w/w) enzyme, and urea at 0–1.5 M, as indicated. Samples were prepared, separated, and quantitated as described under Materials and Methods. The rate constant for digestion of α tubulin by trypsin is lowered from 0.11 min^{-1} at 0 M urea to 0.04 min^{-1} at 1.5 M urea. The rate constant for digestion of β tubulin by chymotrypsin is lowered from 0.07 min^{-1} at 0 M urea to 0.02 min^{-1} at 1.5 M urea.

and chymotrypsin-catalyzed cleavage. Thus, it is not likely that the conformational changes produced by 1.0 M urea for these two effects are identical. Nonetheless, these observations are consistent with the proposal that low urea concentrations "tighten" the structure of tubulin, leading to enhancement of fluorescence on the one hand, and reducing proteolytic accessibility on the other.

ANS Fluorescence. Tubulin has a high-affinity binding site ($K_D \sim 32$ μM, $\Phi = 0.48$) for 1-anilino-8-naphthalene sulfonate (ANS) that is independent of the colchicine binding

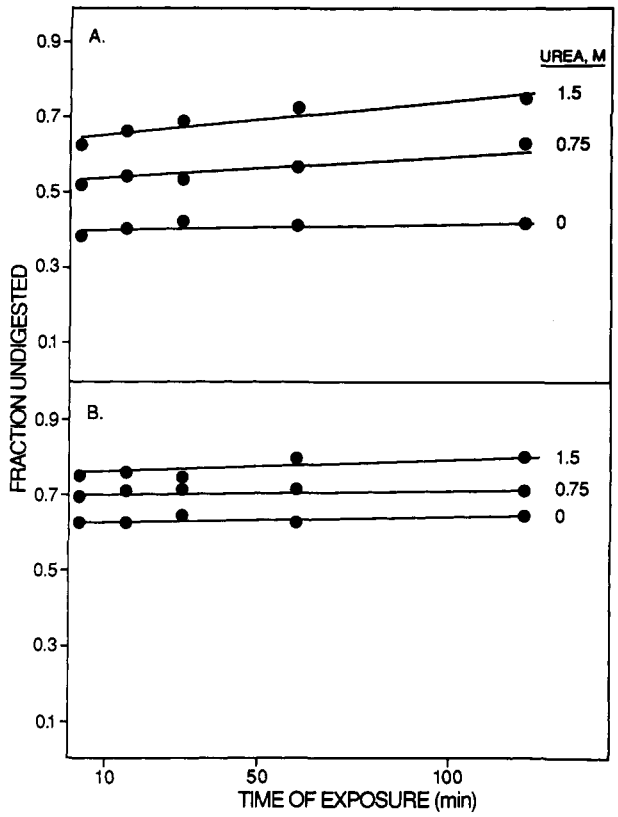

FIGURE 8: Time course of urea effects on tubulin assayed by pulse proteolysis. Tubulin (10 μM) was incubated at room temperature in Mes assembly buffer with 0, 0.75, or 1.5 M urea, as indicated. At the indicated times, an aliquot was removed, the indicated protease was added to 4% (w/w to tubulin), and digestion was allowed to proceed for 5 min. Digestion was stopped, all samples were separated by SDS gel electrophoresis, and gels were stained, destained, and scanned as described under Materials and Methods. The fraction undigested was determined as $\alpha/(\alpha + \alpha N)$ in panel A and $\beta/(\beta + \beta N)$ in panel B, where α and αN refer to the integrated area of the bands corresponding to α tubulin and the 38-kDa amino-terminal domain cleaved by trypsin, respectively. Similarly, β and βN refer to the integrated area of β tubulin and the 34-kDa amino-terminal domain cleaved by chymotrypsin. The absolute value of $\alpha + \alpha N$ and $\beta + \beta N$ decreased with extended incubation and increased urea, indicating the addition of further digestion reactions. (Panel A) Trypsin digestion of α tubulin. (Panel B) Chymotrypsin digestion of β tubulin.

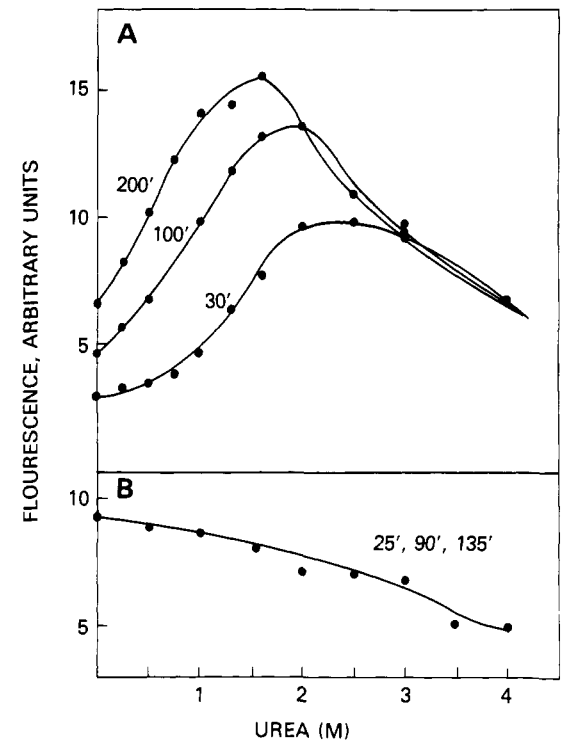

FIGURE 9: Urea effects on fluorescence of ANS bound to tubulin or albumin. (A) Tubulin (5 μM) was incubated with 5 μM ANS in the presence of various concentrations of urea as indicated. The fluorescence emission at 480 nm (excitation at 350 nm) was measured at various times after mixing. (B) Albumin (bovine serum albumin, fatty acid-free) at 5 μM was incubated with 5 μM ANS in the presence of various concentrations of urea and fluorescence measured with time exactly as in panel A.

site (Bhattacharyya & Wolff, 1975). The fluorescence is enhanced by calcium and vinblastine but not by colchicine, and the ANS has no effect on colchicine binding. Low concentrations of urea promote a time-dependent increase in ANS fluorescence (Figure 9A) that is more intense and occurs at lower concentrations when the urea was present for 200 rather than 30 min (with concentrations of half-maximal enhancement at 0.6 and 1.3–1.4 M, respectively). There is no change in the λ_{max} at these urea concentrations, and normalized emission curves are entirely superimposable (data not shown). At higher urea concentrations the enhancement is abolished (Figure 9A). By contrast, the fluorescence of an ANS–bovine serum albumin complex (Figure 9B) shows no enhancement in the presence of urea and falls progressively with increasing urea concentrations.

The effects of time on ANS fluorescence should be compared with those on MTPT (Figure 4): peak enhancement for ANS occurs at higher urea concentration than is seen for the urea-enhanced fluorescence of MTPT; moreover, maximum emission enhancement for ANS occurs at the *latest* time interval measured, whereas that for MTPT occurs at the *earliest* time measured. Thus, the longer exposure to urea favors ANS fluorescence but decreases MTPT fluorescence, and the mechanism of the enhancement is, thus, likely to be different for the two fluorophores.

Tryptophan Fluorescence. Changes in the emission from tryptophanyl residues have frequently been used as sensitive indicators of structural transitions or local environments of the chromophore. Because tubulin contains four tryptophans per monomer, it is not possible to localize domains from emission data alone. However, it seemed useful to attempt to correlate the above functional changes with changes in the overall fluorescence of tryptophan produced by the same concentrations of urea. In a number of dimeric proteins urea-induced changes can be described by a single transition between the folded and unfolded states (e.g., Bowie & Sauer, 1989; Gittleman & Matthews, 1990). However, the spectral transition of tubulin proved to be more complex. This will be detailed in a later paper. For the purposes of the present study, however, we want merely to demonstrate that only very small changes can be detected in the intrinsic fluorescence at urea concentrations of $\leq$1.0 M (Figure 10) where the functional changes resulting from urea are well underway (or are complete in the case of tubulin polymerization and GTPase activity). These curves were obtained 15 min after exposure to urea but remained the same for 2 h. These small fluorescence changes at low urea concentrations should be compared with the decreased intensity and red-shift occurring at 4–8 M urea. Even at 1.5 M urea only a 1-nm red-shift is observed compared to a >20-nm red-shift at 8 M urea. Because tubulin contains eight tryptophan residues, a significant change in the emission of one of these would contribute only a small change in the total emission. Clearly, significant changes in the bulk of the tryptophan emission require large urea concentrations.

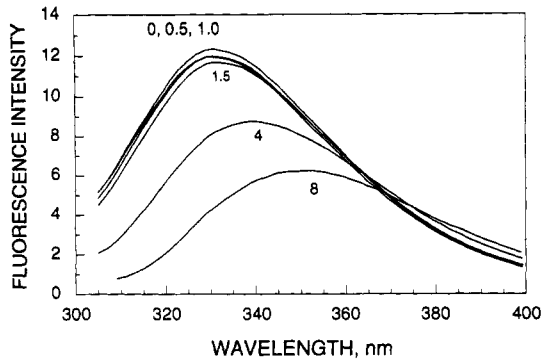

FIGURE 10: Effect of urea on tubulin tryptophan fluorescence. Tubulin was diluted to 2 μM in Mes assembly buffer plus various concentrations of urea. Tryptophan emission spectra were recorded after 15 min at room temperature. Excitation was at 295 nm. The numbers on the spectra are molar urea.

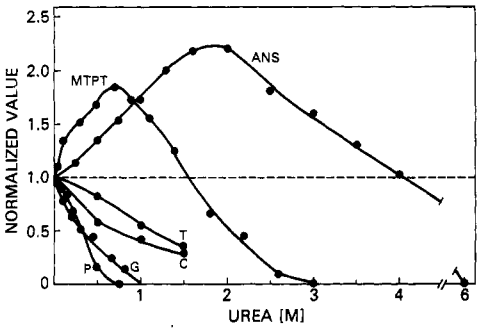

FIGURE 11: Summary of urea effects on several properties of tubulin. The results of various assays are presented as a function of urea concentration by setting the value obtained in each assay in the absence of urea equal to 1 and scaling the other values accordingly. The curves are labeled as follows: P, polymerization; G, GTPase activity; C, chymotryptic cleavage; T, tryptic cleavage; MTPT, MTPT fluorescence at 430 nm; ANS, ANS fluorescence at 480 nm.

It is of interest that enhancement of intrinsic tryptophan fluorescence has been observed in a number of proteins upon exposure to mild denaturing conditions (Hill *et al.*, 1988; Herold & Kirschner, 1990; Leon *et al.*, 1991; Pace & Laurents, 1989); this has been ascribed to possible relief of quenching by nearby amino acid side chains. It does not occur in tubulin, and this is perhaps surprising, since energy transfer between tryptophan and MTPT has been demonstrated (Bane *et al.*, 1984).

DISCUSSION

Despite overlap in the effective urea concentrations needed, it is possible to arrange the functional effects of urea on tubulin in the following order of decreasing urea sensitivity: decreased *rate* of polymerization > decreased *extent* of polymerization ≈ decreased GTPase activity > enhanced MTPT fluorescence ≈ decreased proteolytic susceptibility > enhanced ANS fluorescence ≈ decreased MTPT fluorescence > decreased ANS fluorescence. These changes are summarized as a function of urea concentration for each test in Figure 11, wherein all values are normalized to those obtained in the absence of urea.

The monotonic inhibition of tubulin polymerization is half-maximal at <0.5 M and is complete at <1.0 M urea. The *rate* is more sensitive than the *extent* of polymerization, as shown in Figure 2. This urea effect presumably implies a subtle, but critical, change in one of the interdimer contact surfaces; these contacts have been thought to be the most sensitive to manipulation in other systems (Mayo *et al.*, 1992). One can envision a conformational change in a restricted region of the dimer that is remote from reporter groups and hence not detected spectroscopically; when present in the growing end of the tubule in an "incorrect" orientation, it prevents further dimer addition and thus interrupts elongation. A similar proposal has been made for drug-liganded tubulin some years ago (Saltarelli & Pantaloni, 1982; Andreu *et al.*, 1983) and has recently been shown for colchicine-induced changes in β tubulin (Sackett & Varma, 1993). GTPase activity was similar in urea sensitivity to the *extent* of polymerization (Figure 2). It should be noted that the ability of dilute urea solutions to interfere with microtubule polymerization has been observed *in vivo*. Exposure to 0.8 M urea prevented repolymerization of cold-depolymerized microtubules of the amoeba *Chaos* (Roth, 1967), and 0.15 M urea caused retraction of axonemal microtubules of the axopodium of *Echinosphaerum*; removal of urea led to rapid repolymerization (Shigenaka *et al.*, 1971).

Another characteristic property of tubulin is the highly specific binding of various drugs to the colchicine binding site, localized mainly on the β-monomer (Wolff *et al.*, 1991). MTPT, used here as a rapidly binding colchicine analogue, is almost as sensitive to urea as microtubule assembly. The response to urea is complex in that it is the sum of two opposing effects that have different time constants. The initial enhancement of fluorescence is rapid; this becomes quenched by a slower mechanism upon longer exposure to, or at higher concentrations of, urea (Figure 4). The loss of fluorescence, and hence probably binding, at higher urea concentrations or longer exposure times, likely reflects loss of local structure in the binding site.

The fluorescence of the MTPT–tubulin complex is enhanced several-fold upon exposure to urea at concentrations below 2.0 M. Below 1.0 M urea the enhancement is still observed for several hours. Moreover, this fluorescence increase is reflected in the colchicine-photosensitized covalent dimerization of the α and β monomers (Figure 8). Attempts to explain the basis for this enhancement were not entirely successful. While a slight increase in affinity constant was observed (with both on and off rates reduced to about the same extent), the stoichiometry of binding is not affected (Table 1 and Figure 4A,B). The quantum yield is increased by urea (Figure 4C), but this increase can account for only $\sim^1/_2$ of the enhancement. The urea-induced increase in fluorescence thus appears to be the sum of several subtle changes that together lead to the enhanced fluorescence. A number of reports of increased enzyme activity produced by low denaturant concentrations propose that these increases result from effects on multiple constraints in the active site (Ma & Tsou, 1991; Fernandez-Velasco *et al.*, 1992; Garza-Ramos *et al.*, 1992; Rao & Nagaraj, 1991).

The effect of urea on trypsin- or chymotrypsin-mediated proteolysis shows a response opposite to the MTPT fluorescence enhancement. That is, accessibility to proteases is *reduced* by low concentrations of urea. The time dependence of the urea effect on proteolysis is also different from that observed for MTPT fluorescence. Whereas MTPT emission changes with time even at fairly low urea concentrations, the reduction in specific tryptic and chymotryptic cleavages is unchanged for at least 2 h. Since similar reduction is seen in α tubulin cleavage by trypsin (after Arg339) and in β tubulin cleavage by chymotrypsin A (after Tyr281), urea must affect α and β similarly. At higher concentrations of urea the rate of hydrolysis increases with extensive unfolding, and new bands are seen. The initial decrease in proteolysis rate occurs at nearly the same concentrations of urea as the fluorescence

enhancement of MTPT, but because of the difference in time dependence, it seems probable that this represents a separate state change of tubulin.

The slower binding kinetics and enhanced fluorescence on the one hand, and the decreased proteolysis rates on the other, strongly suggest a urea-induced conformational change that we have termed "tightening" of the tubulin structure. Such "tightening" can be viewed as restricting the accessibility both to MTPT (thus protecting against solvent-induced relaxation) and to proteases (thus reducing the rate of cleavage). It is probable that more than one exposed region of tubulin becomes "buried" or "tightened" by low urea concentrations in order to best explain the marked difference in time dependence of the binding and proteolytic responses.

The enhancement of ANS fluorescence shows the opposite time dependence from that of MTPT in that its intensity *increases* with time, suggesting either that a greater degree of tubulin unfolding is required for ANS fluorescence than for MTPT or that the ANS binding region is more resistant to urea. Enhancement of ANS fluorescence has been observed previously as a transient in the unfolding of several proteins upon addition of large concentrations of denaturant (Goldberg *et al.*, 1990; Ptitsyn *et al.*, 1990; Semisotnov *et al.*, 1991). This has been interpreted as evidence for the "molten globule" intermediate state in which certain nonpolar residues become more accessible to solvent. In the present study the enhanced fluorescence obtained at lower urea concentrations is not a transient since it lasts for prolonged periods. Whether or not this represents a molten globule tubulin intermediate that is stable at lower urea concentrations remains to be determined by independent methods. An intermediate in the thermal denaturation of tubulin has been reported (Mozo-Villarias *et al.*, 1991).

Classically, denaturation of proteins has been viewed as a cooperative transition between two states, native and unfolded, involving the bulk of the protein. The midpoint of the transition depends on the nature of the protein and on the external (solvent) conditions. Even some dimeric proteins have been thought to fit the two-state single transition in the presence of urea or guanidine HCl (e.g., Bowie & Sauer, 1989; Gittleman & Matthews, 1990). Other proteins, however, exhibit a more complex unfolding pattern with two or more transitions to the fully unfolded state. This may be seen as a loss of activity before changes in spectroscopic parameters can be demonstrated, as multiphasic spectroscopic changes, fluorescence intensity changes before red-shifts, or as dissociation between different physical parameters (Tsou, 1986; Pace & Laurents, 1989; Herold & Kirschner, 1989; Leòn *et al.*, 1991; Hill *et al.*, 1988). The effect of urea on tubulin is an example of this more complex unfolding pattern and, indeed, demonstrates a larger series of apparently independent, stepwise, functional changes in the presence of low concentrations of urea than has heretofore been seen with other proteins. Whether this is a function of our ability to measure more functional parameters or represents a unique property of tubulin remains to be explored. Many of these changes occur, or are first seen, at urea concentrations that produce no detectable changes in tryptophan fluorescence.

At higher urea concentrations, reversal of several of the above-mentioned phenomena occurs. Binding of both MTPT and ANS is decreased, and proteolysis is accelerated and changed in specificity, and changes in tryptophan fluorescence are readily observed. We are inclined to view these as *global* changes of the protein in response to urea, whereas the low-urea-induced effects, detailed above, can be viewed as *local* changes. The former will show readily measurable spectroscopic changes, whereas the *local* changes will show such spectral changes *only* when a reporter group is in the vicinity of the restricted region undergoing change.

There are a number of other examples of such local changes. Low concentrations of denaturants have changed the fluorescence of added reporter groups such as NADH [lactic dehydrogenase (Liang *et al.*, 1990) and glyceraldehyde-3-phosphate dehydrogenase (Ma & Tsou, 1991)], or *o*-phthalaldehyde bound to creatine kinase (Yao *et al.*, 1984). Intrinsic tryptophan fluorescence is modulated by low concentrations of guanidine HCl in glyceraldehyde-3-phosphate dehydrogenase (Liang *et al.*, 1990) and cytochrome oxidase (Hill *et al.*, 1988; ascribed to reduction of quenching by heme). Tryptophan phosphorescence may be more sensitive to lower denaturant concentrations than is tryptophan fluorescence; phosphorescence of alcohol dehydrogenase is decreased by concentrations of guanidine hydrochloride that have little effect on tryptophan fluorescence even though there is a loss of activity (Strambini & Gonnelli, 1986). Finally, the NMR downfield shifts of both histidine residues of the active site of ribonuclease A (but not other histidines) are influenced by low concentrations of urea or guanidine HCl (Benz & Roberts, 1975; Tsou, 1986).

Such *local* spectroscopic changes are accompanied by changes in the catalytic properties of the enzymes concerned. In most cases these changes are inhibitory (Yao *et al.*, 1984; Tsou, 1986; Hill *et al.*, 1988; Liang *et al.*, 1990; Nozais *et al.*, 1992). However, like MTPT binding to tubulin, several instances of *enhancement* of activity resulting from low concentration of urea or guanidine HCl have been reported. These include alkaline phosphatase (Rao & Nagaraj, 1991), lactic dehydrogenase (Ma & Tsou, 1991; Fernandez-Velasco *et al.*, 1992), malic dehydrogenase (Hecht & Jaenicke, 1989), and glycerol phosphate dehydrogenase (Garza-Ramos *et al.*, 1992). By definition, active sites of most enzymes are accessible to the solvent and, hence, most likely also to urea. Such sites must also be flexible to perform their catalytic functions (Huber, 1988). Thus, it is likely that the effects of low concentrations of urea are exerted on these flexible regions before the rest of the protein is altered at higher urea concentrations. In the case of tubulin it is tempting to speculate that the flexible regions correspond to the loops between structural domains, as has already been proposed for GTP hydrolysis (Amos, 1982).

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