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Spectroscopic and Kinetic Features of Allocolchicine Binding to Tubulin†

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ABSTRACT: Allocolchicine is a structural isomer of colchicine in which colchicine's tropone C ring is replaced with an aromatic ester. In spite of the structural differences between the two ligands, the association parameters for both molecules binding to tubulin are quite similar. The association constant for allocolchicine binding to tubulin was determined by fluorescence titration to be 6.1×10^5 M^{-1} at 37 °C, which is about a factor of 5 less than that of the colchicine–tubulin association. In particular, analysis of the kinetics of the association of allocolchicine with tubulin yielded nearly equivalent activation parameters for the two ligands. The activation energy of the allocolchicine binding reaction was found to be 18.4 ± 1.5 kcal/mol, which is only slightly less than the activation energy for colchicine binding to tubulin. This finding argues against conformational flexibility of the C ring as the structural feature of colchicine responsible for the slow kinetics of colchicinoid–tubulin binding reactions. Tubulin binding promotes a dramatic enhancement of allocolchicine fluorescence. Unlike colchicine, the emission energy and intensity of the tubulin-bound allocolchicine fluorescence can be mimicked by solvent, and a general hydrophobic environment for the ligand binding site is indicated. The excitation spectrum of the protein-bound species, however, is shown to possess two bands which center at higher and lower energy than the energy maximum of the spectrum of the ligand in apolar solvents, indicating that properties of the colchicine binding site in addition to a low dielectric constant contribute to the fluorescence of the bound species. It is suggested that a π-stacking interaction between allocolchicine and an aromatic amino acid in the binding site may account for the unusual excitation spectrum of allocolchicine liganded to tubulin.

Many pharmacologically active substances that interfere with microtubule function exert their biological effects by binding to tubulin, a 100-kDa heterodimer which is the major component of microtubules. The most thoroughly studied agent in this class is colchicine, an alkaloid isolated from the Autumn crocus. Colchicine binds to a single high-affinity site on the tubulin heterodimer, and the resulting colchicine–tubulin complex substoichiometrically inhibits normal assembly of the tubulin subunits into the microtubule (Dustin, 1984).

The colchicine binding site appears to recognize two flanking aryl or conjugated rings which possess or may adopt a biaryl angle on the order of 55–85°. The stereochemical relationship of the rings is of critical importance in binding activity. The phenyl–tropone system in colchicine is in an *S* configuration, and ligands which bind to tubulin at this site must possess the same stereochemical arrangement in their biaryl rings as colchicine (Zavala et al., 1978; Yeh et al., 1988; Brossi et al., 1988).

An important structural feature of colchicine is the α-methoxytropone that constitutes the C ring portion of the molecule. The tropone ring of colchicine has been implicated in several aspects of the ligand binding mechanism. For example, the electronic properties of colchicine complexed to tubulin are altered in unique fashions which have resisted precise duplication in the absence of the protein. The near-UV absorption band of the molecule, which is comprised of electronic transitions of the tropone ring, is shifted to lower energy upon tubulin binding. Lowering the dielectric constant of the medium in the absence of tubulin shifts this band to higher energy. Also, the quantum yield of colchicine in aqueous solution and solvents at room temperature is so low that colchicine luminescence is difficult to detect, while the fluorescence of colchicine in the tubulin–colchicine complex is dramatically enhanced (Bhattacharyya & Wolf, 1974, 1984). Finally, colchicine displays a negative circular dichroic band centering at 340 nm in aqueous solution that has been shown to arise primarily from the interaction of the tropone C ring with the trimethoxyphenyl A ring (Yeh et al., 1988). When colchicine is bound to tubulin, the ellipticity at 340 nm of the ligand is reduced to nearly zero (Detrich et al., 1981).

The tropone ring is also thought to be responsible for the unusual kinetic features of the association of colchicinoids with tubulin. The binding of colchicine to tubulin is remarkably slow: the second-order rate constant is $\sim 10^2$ M^{-1} s^{-1} at 37 °C, and the activation energy for binding has been reported to be 20–25 kcal/mol (Cortese et al., 1977; Garland, 1978; Lambeir & Engelborghs, 1981). It has been suggested that both the spectroscopic properties of the bound ligand (Detrich et al., 1981) and the kinetic parameters for complex formation (Bane et al., 1984; Hastie et al., 1989) may be a result of conformational properties of the tropone ring. Specifically, both colchicine and tubulin may undergo simultaneous conformational readjustments during the asssociation reaction, yielding a second, more planar colchicine molecule as the bound species.

In order to more fully explore the role of the C ring in the mechanism of colchicine binding to tubulin, the spectroscopic and kinetic properties of the association of allocolchicine with tubulin have been studied (see Figure 1 for structures). Allocolchicine is a structural isomer of colchicine in which the tropone ring has been substituted with an aromatic ester. X-ray crystallographic analysis of *N*-acetylcolchicinol, a closely related structural analogue of allocolchicine, indicates that the biaryl moiety of allocolchicine possesses the proper configuration for binding to tubulin (Margulis & Lessinger, 1978). This assumption is further supported by CD[1] spectral analysis

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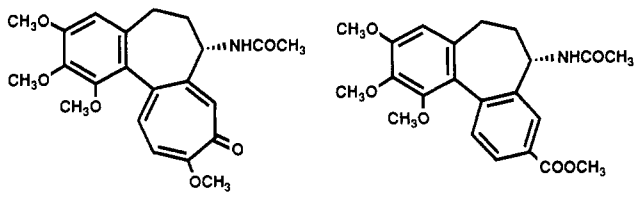

Colchicine Allocolchicine

FIGURE 1: Structures of colchicine (**1**) and allocolchicine (**2**).

of allocolchicine (Hrbek et al., 1982) and by the observations that allocolchicine inhibits tubulin assembly and is a competitive inhibitor of [^{3}H]colchicine binding (Fitzgerald, 1976; Dienum et al., 1981). In allocolchicine, however, the C ring is aromatic and is therefore unable to exhibit the conformational flexibility ascribed to the C ring of colchicine. Thus, if C ring conformational features are responsible for the high activation energy and spectroscopic alterations of colchicine binding, the association properties of allocolchicine should be quite different than those of colchicine.

In the present study, the absorption and fluorescence properties of allocolchicine as a function of solvent and tubulin binding were investigated. Like colchicine, allocolchicine was only weakly fluorescent in aqueous solution, and its fluorescence was dramatically enhanced by tubulin binding. Unlike colchicine, the emission properties of the tubulin-bound ligand could be mimicked by a low dielectric constant solvent. The excitation spectrum of allocolchicine bound to tubulin, however, could not be duplicated in the absence of tubulin, indicating that the binding site environment is more complex than a simple hydrophobic pocket in the protein. It is proposed that the spectroscopic data reveal the presence of a π-stacking association between the ligand and an aromatic amino acid in the binding site.

The enhancement of allocolchicine fluorescence upon tubulin binding was used to evaluate association parameters of the ligand with tubulin. The association constant and activation energy for the allocolchicine binding to tubulin were very similar to the values for colchicine. These findings indicate that conformational flexibility of the C ring is not responsible for the high activation energy of colchicine binding to tubulin.

Experimental Procedures

Materials. Allocolchicine was synthesized by treatment of colchicine with sodium methoxide (Fernholz, 1950). The recrystallized product was analyzed by thin-layer chromatography and its structure confirmed by infrared and proton NMR spectroscopy and mass spectrometry. No residual colchicine was detected by these techniques.

Pipes, EGTA, and GTP (type II-S) were obtained from Sigma. Quinine sulfate was obtained from Aldrich. All experiments with tubulin were performed in PMEG buffer (0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM GTP, pH 6.90 at 25 °C). Spectrograde solvents were used in absorption and fluorescence spectroscopy.

Tubulin Purification and Protein Determination. Bovine brain tubulin, free of microtubule-associated proteins, was prepared by two cycles of assembly/disassembly followed by phosphocellulose chromatography as described previously and stored in liquid nitrogen (Williams & Lee, 1982). Tubulin obtained by this procedure is routinely >98% pure when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Prior to use, the frozen pellets were gently thawed, centrifuged at 5000*g* for 10 min at 4 °C, and then desalted into PMEG buffer on 1-mL Sephadex G-50 columns according to the method of Penefsky (1977). Tubulin concentrations were determined spectrophotometrically by the use of an extinction coefficient at 278.5 nm of 1.23 $(mL/mg)^{-1}$ cm^{-1} in PMEG buffer (Detrich & Williams, 1978). Allocolchicine concentrations in aqueous solution were also determined spectrophotometrically with an extinction coefficient which was determined at 290 nm of 1.534×10^4 M^{-1} cm^{-1} in PMEG buffer and 10% DMSO in PMEG buffer.

Absorption Spectroscopy. Absorption spectra were measured on a Hewlett-Packard Model 8451A diode array spectrometer. For difference spectra, tandem cells (0.437-cm path length in each compartment) were used in a Perkin-Elmer Model 559A absorption spectrophotometer in the following manner: the front compartments of each were filled with equal volumes of 5.2 μM tubulin in 3% DMSO in PMEG, and the rear compartments were filled with equal volumes of 72 μM allocolchicine in the same buffer. The temperature of the sample cell was held constant at 37 °C; the reference cell was at ambient (≈23 °C) temperature. A base line was obtained at half full scale with the automatic base-line feature (0.2 nm/s scan rate, 1-nm spectral band width, 0.25 absorbance unit full scale). To obtain the difference spectrum, the sample cuvette was removed, mixed by inversion, incubated at 37 °C for 1 h in the sample chamber, and scanned.

Fluorescence Spectroscopy. Excitation and emission spectra was obtained on a SLM 8000 spectrofluorometer operated in the ratio mode. The temperature was held at 25 °C with a circulating water bath except where otherwise noted. Excitation spectra were corrected by a rhodamine solution in the reference channel of the spectrometer; emission spectra are uncorrected. All samples were measured in a 2 × 10 mm fluorescence cell oriented such that the excitation beam passed through the shorter path. The effective path length of the cell was determined to be <1 mm in our instrument by analyses of emission intensity as a function of absorption at the excitation wavelength. Solution concentrations in excitation and emission spectra were chosen on the basis of this determination to avoid inner-filter effects.

Data were collected at 1-nm intervals with an IBM-PC interfaced to the instrument. Background spectra of the solvent in the absence of the fluorophore were also collected and subtracted from the appropriate spectrum on the PC.

Allocolchicine–tubulin solutions for fluorescence analysis were prepared by mixing the appropriate amounts of allocolchicine in PMEG containing 10% DMSO and tubulin. The final DMSO concentration was 10% or less for each experiment. It has been shown previously that concentrations of DMSO of up to 15% do not adversely effect tubulin's ability to bind colchicine (Head et al., 1985). We obtained identical excitation and emission spectra of allocolchicine in the presence and absence of tubulin with varying DMSO concentrations of up to 10%. The solution of allocolchicine and tubulin was incubated at 37 °C for 60 min prior to measurements.

The efficiency of energy transfer (E) was calculated from the relative fluorescence intensity at 330 nm of tubulin in the presence (F_{da}) and absence (F_d) of allocolchicine by (Stryer, 1978)

$$E = 1 - F_{da}/F_d \quad (1)$$

The emission intensity of the complex was corrected for con-

[1] Abbreviations: AC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide; PMEG buffer, 0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, and 0.1 mM GTP, pH 6.90 at 23 °C.

tribution of allocolchicine emission in the following manner. Emission spectra of the tubulin–allocolchicine complex were measured at exciting wavelengths of 280 and 315 nm. The latter spectrum, which shows essentially no tubulin emission, was normalized such that the amplitudes of the two emission spectra at 400 nm were equal. The intensity of the normalized emission spectrum at 330 nm was taken to approximate the contribution of ligand fluorescence at this wavelength and was subtracted from the fluorescence intensity of the complex prior to the transfer efficiency calculation.

The excitation spectrum of tubulin-bound allocolchicine was found by correcting the excitation spectrum of the complex for intrinsic tubulin emission and energy transfer. The spectrum of tubulin in PMEG buffer (which is small relative to that of the complex) was digitally subtracted from the spectrum of the complex to remove contribution due to tubulin emission. To account for energy transfer, the excitation spectrum of unliganded tubulin was normalized to 38% of the intensity of the complex at 280 nm and also digitally subtracted.

The quantum yields of the allocolchicine–tubulin complex were calculated by comparison of the quantum yield of the quinine sulfate in 0.1 M H_2SO_4 as described by Demas and Crosby (1972). Low-temperature measurements were performed on an Oxford Instruments Model DN1704K variable-temperature liquid nitrogen cryostat fitted with synthetic sapphire inner windows and quartz outer windows. Solution samples were contained in a fused quartz 1-cm cell. The ether–isopentane–ethanol (EPA) solution at 5:5:2 ratio was made up from rigorously dried solvents which had been repeatedly distilled to remove emitting impurities. The temperature of the sample was monitored to ±0.2 K by means of an Oxford Instruments Model 3120 controller. The excitation and emission wavelengths were 290 and 450 nm, respectively.

Fluorescence lifetimes of allocolchicine were determined by multiple-frequency phase-modulation fluorometry (Gratton et al., 1984). An ISS GREG 200 phase fluorometer was used. The output of a 300-W xenon-arc lamp was amplitude modulated by means of a Pockel's cell driven by a Hewlett-Packard frequency synthesizer. A second frequency synthesizer, locked in phase with the first, provided a radio-frequency signal to modulate the photomultiplier tube (Hamamatsu R928) response. The two synthesizers differed in frequency by 40 Hz, which is the cross-correlation frequency. Samples were excited at 290 nm, and the emitted light was passed through a Schott 435-nm cutoff filter. A solution of *p*-terphenyl in ethanol (lifetime = 0.98 ns) was used as the lifetime reference. Modulation frequencies were varied from 10 to 100 MHz in 10-MHz increments. The data were analyzed by means of both least-squares and continuous distribution analyses (Lakowicz et al., 1984; Alcala et al., 1987).

Kinetic Measurements. The kinetics of the association of allocolchicine with tubulin were measured under pseudo-first-order conditions on the SLM 8000. The temperature was controlled with a circulating water bath. The excitation and emission wavelengths were 330 and 400 nm, respectively. The excitation wavelength was chosen to avoid interference of protein emission in the kinetic data. The experiments were performed as follows: a solution of allocolchicine in PMEG containing 10% DMSO was equilibrated in the instrument in the 2 × 10 mm fluorescence cell. Tubulin was added (at a volume <5% of the final sample volume) while the shutters were closed. The sample was rapidly mixed without removing the cell from the compartment, and the shutters were opened to monitor the fluorescence. The data were collected with the IBM-PC and corrected for the time in which the shutters were closed.

The data of increasing ligand fluorescence as a function of time were fit to

$$F_t = Ae^{-\alpha t} + Be^{-\beta t} + C \quad (2)$$

where F_t is the fluorescence at time t, α and β are the observed pseduo-first-order rate constants of the fast and slow phases, respectively, A and B are the amplitudes of these two phases, and C is an integration constant (Lambeir & Engelborghs, 1981). The parameters A, B, α, and β were obtained by nonlinear curve fitting with the program KINFIT from On-Line Instrument Systems, Inc. (Jefferson, GA).

Equilibrium Measurements. The association constant at 37 °C was determined by fluorescence titration. Individual samples of tubulin (2 μM) and varying amounts of allocolchicine were incubated at 37 °C for 60 min. The emission intensity at 400 nm (excitation at 300 nm) was measured for each sample at 37 °C with the SLM 8000 spectrofluorometer. The concentration of allocolchicine in all samples was low enough to avoid any inner-filter effect.

The data were analyzed according to the following equation which can readily be derived from the law of mass action:

$$\alpha/[\text{allo}]_{\text{free}} = -K\alpha + nK \quad (3)$$

where α = fraction of tubulin bound, or [complex]/T_0, where T_0 is the total tubulin concentration, $[\text{allo}]_{\text{free}}$ = the concentration of the unbound ligand, or $[\text{allo}]_{\text{free}} = [\text{allo}]_0 - \alpha T_0$, where $[\text{allo}]_0$ is the total allocolchicine concentration, n = number of binding sites, and K = association constant.

The fraction of tubulin bound was determined from

$$(F_{\max} - F)/F_{\max} = \alpha \quad (4)$$

where $F_{\max}$ = fluorescence at binding saturation and F = fluorescence measured at any allocolchicine concentration. It is noted that this method of analysis requires that the number of binding sites on the protein is equal to 1. Since allocolchicine bound to tubulin was found to have a single fluorescence lifetime (vida infra), the assumption of a single binding site on the protein as detected by fluorescence was deemed appropriate.

Results

Absorption and Fluorescence Spectroscopy. Allocolchicine displays three absorption maxima in aqueous solution: 206, 235 (shoulder), and 290 nm ($\epsilon = 1.534 \times 10^4$ M^{-1} cm^{-1}). The maximum of the long-wavelength band is essentially invariant with solvent (Table I), indicating that the transition is benzenoid in character (Rao, 1969).

The absorption and luminescence properties of allocolchicine as a function of solvent are shown in Table I. Like colchicine, allocolchicine is only weakly luminescent in aqueous solution, and its emission is difficult to detect. Unlike colchicine, the luminescence of allocolchicine is dramatically enhanced by decreasing solvent polarity. The emission spectrum is highly dependent on solvent, and the wavelength maximum ($\lambda_{\max}$) varies from 399 nm in dioxane to almost 500 nm in water. Due to the large Stokes shift (~9000 to ~14 000 cm^{-1}), it was unclear whether the luminescence was fluorescence or phosphorescence. Purging samples with nitrogen did not alter emission intensity (data not shown). Furthermore, the excited-state lifetime of allocolchicine in dioxane was determined by the phase modulation method as described under Experimental Procedures to be 5.1 ± 1.0 ns, indicating that the luminescence is fluorescence.

Table I: Absorption and Fluorescence Properties of Allocolchicine

solvent	$E_T(30)^a$	A_{max} (nm)[b]	ex_{max} (nm)[c]	em_{max} (nm)[d]	quantum yield
dioxane	36	292	288	399	0.32
ethyl acetate	38.1	290	283	401	0.26
dimethyl-form-amide	43.8	292	289	438	0.23
dimethyl sulfoxide	45	292	292	448	0.18
acetonitrile	46	290	283	448	0.13
2-propanol	48.6	290	284	448	0.065
ethanol	51.9	290	286	464	0.015
methanol	55	290	284	476	0.012
formamide	56.6	290	283	490	0.0011
water	63.1	290	292[e]	496[e]	0.0006[e]
tubulin		280[f]	280, 305	400	0.28

[a] Values from Dimroth et al. (1963). [b] Absorption maximum. [c] Excitation maximum. [d] Emission maximum. [e] Difficult to detect. [f] Separate absorption maximum for allocolchicine not observable.

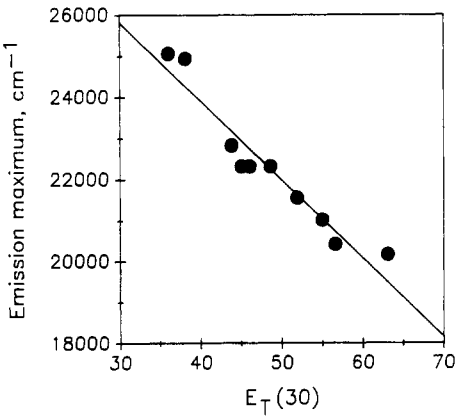

FIGURE 2: Frequency of fluorescence maximum of allocolchicine as a function of solvent $E_T(30)$ values. Data for this plot were taken from Table I. The solid line was calculated by linear regression analysis.

The effect of solvent composition on the fluorescence emission maximum of allocolchicine is shown in Figure 2. The essentially linear dependence of the emission energy on solvent polarity suggests that the observed spectral shifts are due to general solvent effects and not to specific solvent–fluorophore interactions such as hydrogen bonding (Lakowicz, 1983).

The effect of tubulin binding on allocolchicine fluorescence is shown in Figure 3. When tubulin-bound allocolchicine is excited at 290 nm (the absorption maximum of the ligand), two bands centering at 335 and 400 nm are observed in the emission spectrum. The two bands were assigned to tubulin and tubulin-bound allocolchicine, respectively, by examining the emission spectra as a function of excitation wavelength. As the excitation wavelength was moved to lower energy, the 335-nm band disappeared, leaving only allocolchicine emission.

Allocolchicine binding also quenches intrinsic tubulin fluorescence (curve b of Figure 3). The absorption spectrum of allocolchicine possesses sufficient overlap with the emission spectrum of tubulin for energy transfer between the protein and the ligand to occur (data not shown). From the relative emission intensities of tubulin in the presence and absence of allocolchicine, the efficiency of energy transfer was calculated to be 38 ± 4%.

Fluorescence lifetime measurements of allocolchicine liganded to tubulin yielded a single-exponential decay of the excited state with a lifetime of 5.2 ± 1.0 ns. Thus, the quantum yield, emission energy, and excited-state lifetime of the tubulin-bound species are very similar to those of allocolchicine in dioxane, indicating that allocolchicine is bound to a hydrophobic region of the tubulin dimer. It is noted that the quantum yield found for tubulin-bound allocolchicine is in good agreement with the value previously determined by Ward and Timasheff (1988).

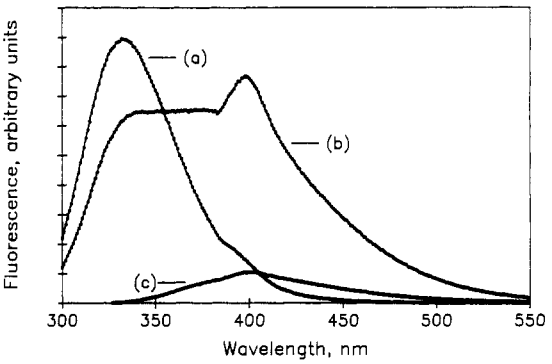

FIGURE 3: Emission spectra of tubulin and allocolchicine bound to tubulin. Curves a and b are the emission spectra of 2.2 μM tubulin in the absence and presence, respectively, of 9.6 μM allocolchicine. The excitation wavelength was 290 nm. Curve c is the emission spectrum of 2.2 μM tubulin plus 9.6 μM allocolchicine when excited at 315 nm. Samples were incubated at 37 °C for 1 h prior to analysis.

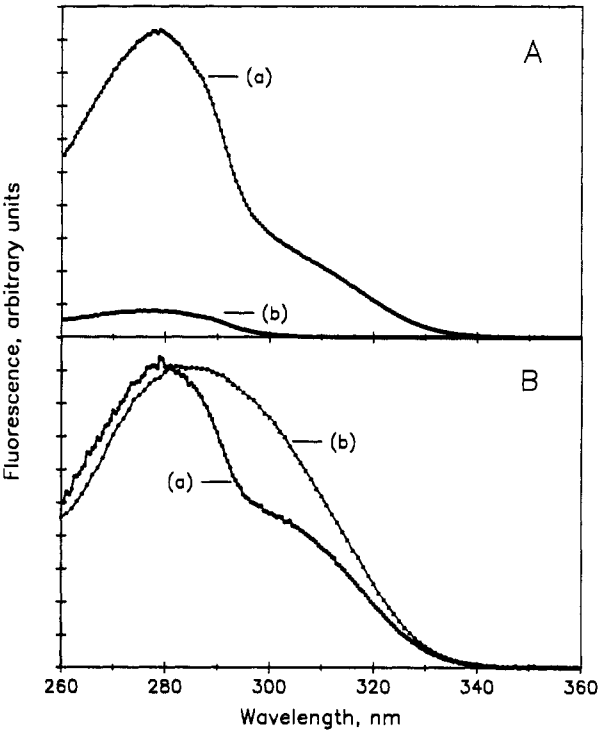

FIGURE 4: (A) Excitation spectra of tubulin and allocolchicine bound to tubulin. Curve a is the excitation spectrum of 9.6 μM allocolchicine in the presence of 2.2 μM tubulin; curve b is the excitation spectrum of 2.2 μM tubulin. The emission wavelength was 400 nm for both spectra. (B) Excitation spectra of tubulin-bound allocolchicine (a) and allocolchicine in dioxane (b). The excitation spectrum of the protein-bound ligand is corrected for intrinsic protein fluorescence and energy transfer as described under Experimental Procedures. The emission wavelength is 400 nm for both spectra, and the amplitudes of the two spectra have been normalized.

Figure 4A shows the excitation spectrum of tubulin in the presence and absence of allocolchicine. Several features of the spectrum should be noted. First, the complex shows two bands centering at 280 and 305 nm. The intensity of the 280-nm band in the complex is much greater than that of free tubulin, demonstrating that the higher energy band is not solely due to protein emission. Also, a significant component of the intensity of the 280-nm band of the complex is attributable

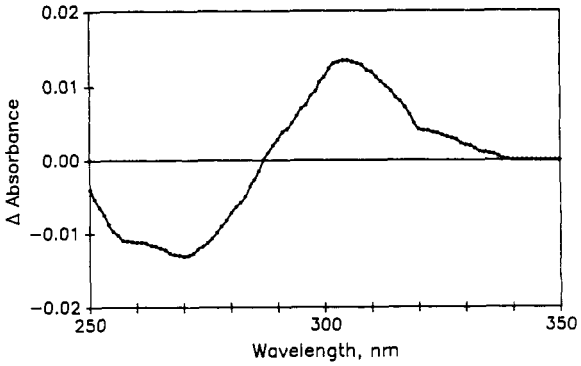

FIGURE 5: UV difference spectrum of allocolchicine and tubulin. The concentrations of allocolchicine and tubulin before mixing were 5.2 and 72 μM, respectively. The spectrum was recorded at 37 °C after the sample was incubated for 1 h at this temperature.

to energy transfer as found in the emission spectra (Figure 3). That both excitation bands lead to emission at 400 nm was established by analyzing tubulin and tubulin–allocolchicine solutions as a function of emission wavelength. The intensity of the 280-nm band of unliganded tubulin was found to decrease with increasing emission wavelength. In the allocolchicine–tubulin complex, however, the intensities of both the 280- and 305-nm bands increased with emission wavelength to a maximum intensity at ~400 nm.

When the excitation intensity of the allocolchicine–tubulin complex was adjusted for contributions from intrinsic tubulin fluorescence and energy transfer as described under Experimental Procedures, an excitation spectrum corresponding to the tubulin-bound ligand resulted (Figure 4B). The excitation spectrum of the bound species clearly displays bands at both 280 and 305 nm.

One explanation for the appearance of two excitation bands in the tubulin-bound species is that these are vibrational bands of an electronic transition which are obscured in more polar environments (Lakowicz, 1983). In order to investigate this possibility, the excitation spectrum of allocolchicine as a function of solvent was examined. It is seen in Table I that a larger variation in excitation maxima than in absorption maxima was observed in the corresponding solvents, but in no case was structure in the absorption band observed. As an illustration, the excitation spectrum of allocolchicine in dioxane is compared to the tubulin-bound spectrum in Figure 4B.

Vibrational structure in fluorescence spectra may also be observed by increasing the rigidity of the chromophore environment (Lakowicz, 1983). The quantum yield of colchicine is greatly enhanced by raising the viscosity of the medium, but is relatively unaffected by other solvent properties, leading to the hypothesis that tubulin binding serves to "immobilize" colchicine in a manner similar to increased viscosity (Bhattacharyya & Wolff, 1984). The effect of medium rigidity on allocolchicine fluorescence was therefore assessed by collecting excitation and emission spectra of allocolchicine at 78 K (−195 °C) as described under Experimental Procedures. The low-temperature emission spectrum ($\lambda_{max} \simeq 470$ nm) displayed very weak shoulders at ~440 and ~500 nm, but no vibrational structure was apparent in the excitation spectrum (data not shown).

Tubulin binding also modifies the absorption spectrum of the ligand, which is best observed in a difference spectrum (Figure 5). The complex displays a small increase in absorption centering around 305 nm; this increase in absorption is at approximately the same energy as the lower energy band in the excitation spectrum. A decrease in absorption intensity is apparent near 280 nm. Again, a major perturbation in the difference spectrum is found at wavelengths corresponding to a peak in the excitation spectrum of the bound ligand.

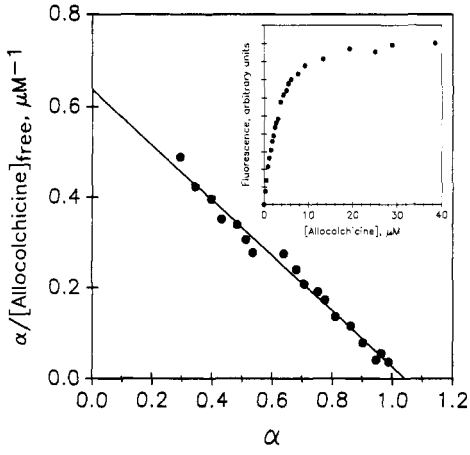

FIGURE 6: Equilibrium binding of allocolchicine to tubulin at 37 °C. A Scatchard plot of the fluorescence titration data (inset, performed as described under Experimental Procedures) is shown. The symbol α denotes the fraction of tubulin with bound ligand; $[allo]_{free}$ is the concentration of unbound ligand. The solid line was calculated by linear regression analysis.

Equilibrium and Kinetic Properties. The dramatic difference in the fluorescence intensity of the bound species over that of free allocolchicine allowed allocolchicine emission at 400 nm to be used to monitor the association of the drug with tubulin. A fluorescence titration of the binding at 37 °C was analyzed by a Scatchard plot (Figure 6), yielding an association constant of 6.1×10^5 M^{-1}.

The kinetics of the association reaction were studied under pseudo-first-order conditions by monitoring the increase in ligand emission intensity as a function of time. A typical kinetic profile is shown in Figure 7A. In Figure 7B, the data were analyzed as a single pseudo-first-order reaction. As for the colchicine and the bicyclic colchicine analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC), the association was not adequately described by a single pseudo-first-order process (Lambeir & Engelborghs, 1978; Bane et al., 1984; Engelborghs & Fitzgerald, 1987) and could be analyzed as consisting of two parallel pseudo-first-order reactions by eq 2. As with earlier studies with colchicine and AC, the amplitude of the slow phase was small relative to that of the fast phase. Only the fast phase is presented for quantitative analysis.

The association of allocolchicine with tubulin is relatively slow. At 37 °C the second-order rate constant of the fast phase was found to be 1.1×10^3 M^{-1} s^{-1}, which is approximately a factor of 10 greater than the rate constant of colchicine binding to tubulin at this temperature (Lambeir & Engelborghs, 1981) but about 50-fold less than the value found for AC binding to the protein (Bane et al., 1984; Engelborghs & Fitzgerald, 1987). The second-order rate constants for the fast phase were determined at a single allocolchicine concentration as a function of temperature, and an Arrhenius plot was constructed (Figure 8). The similarity between allocolchicine and tubulin binding kinetics is further accentuated by comparing the activation energy of 18.4 ± 1.5 kcal/mol found for allocolchicine in this study with an activation energy of 20.3 kcal/mol reported by Cortese et al. (1978).

Discussion

In spite of the structural differences in allocolchicine and colchicine, the association parameters of the the ligands with the tubulin are surprisingly similar. Allocolchicine has been previously shown to competitively inhibit colchicine binding

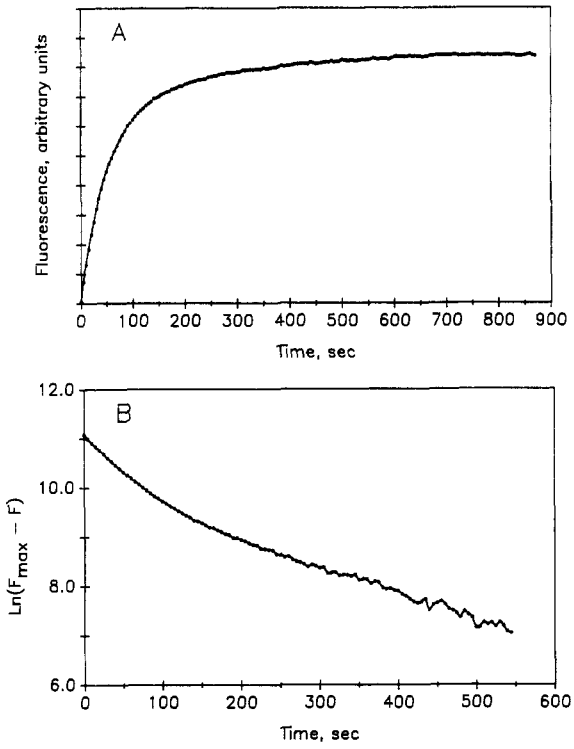

FIGURE 7: Enhancement of allocolchicine fluorescence upon tubulin binding as a function of time. (A) Tubulin was added to an allocolchicine solution to yield a final concentration of 72 μM ligand and 2 μM protein at 25 °C. The excitation and emission wavelengths were 330 and 400 nm, respectively. (B) The data from (A) plotted as a single pseudo-first-order reaction. F_{max} and F denote the maximum fluorescence and the fluorescence at time t, respectively. Note the nonlinearity of the curve in (B).

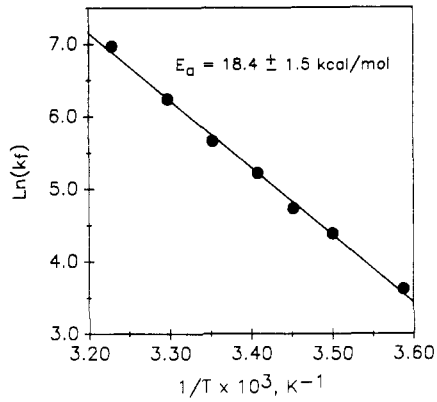

FIGURE 8: Temperature dependence of the apparent second-order rate constant, k_f, of the fast phase. Values of k_f were calculated from the pseudo-first-order rate constant of the fast phase of the association reaction. The solid line was calculated by linear regression analysis.

to tubulin with an inhibition constant of 2 μM (Dienum et al., 1981). In this work the association constant was measured directly and found to be 6.1×10^5 M^{-1} at 37 °C, about a factor of 5 less than that of colchicine at this temperature (Bhattacharyya & Wolff, 1974).

Deinum and Lincoln (1986) have studied the association of tubulin and an allocolchicine derivative in which a spin probe is linked to the allocolchicine molecule through the amide of the B ring. The allocolchicine spin probe binds to tubulin at the colchicine site with about 5-fold less affinity than was found for allocolchicine in this work and associates with the protein at one or more additional lower affinity sites. If allocolchicine itself binds to tubulin in a manner similar to that of the allocolchicine spin probe, we would expect to see curvature in the Scatchard plot (Figure 6), indicating at least two different association processes. Furthermore, it is unlikely that binding to different sites would result in identical fluorescence lifetimes of the ligand. As a single photochemical lifetime was found for allocolchicine bound to tubulin, it is concluded that if allocolchicine binds to sites other than the colchicine site on tubulin, these sites do not appear to be probed by enhancement of ligand fluorescence.

The kinetics of allocolchicine–tubulin association closely resemble the parameters found for colchicine binding to tubulin. The association is biphasic, and the rates of both phases are temperature dependent (data not shown for second phase). Each of the two phases of the colchicine binding reaction has been shown to consist of two separate steps: a rapid preequilibrium of ligand and protein to form a low-affinity species, followed by a slow, unimolecular step to produce the stable tubulin–colchicine complex (Lambeir & Engelborghs, 1981). Due to the poor solubility of allocolchicine in aqueous solution, we did not examine the concentration dependence of the association rate, which is nonlinear for both colchicine and AC and reveals the presence of multistep a process (Garland, 1978; Lambeir & Engelborghs, 1981; Fitzgerald & Engelborghs, 1987). The kinetic parameters determined for allocolchicine therefore quantitate the global process and are compared with the appropriate parameters for colchicine. Whether allocolchicine binds to tubulin in more than one step is not determined in this analysis.

It has been argued that the slow binding rate and high activation energy found for colchicine and inferred for other colchicinoids binding to tubulin are due to steric bulk of the B ring and B ring substituents (Ray et al., 1981; Bhattacharyya et al., 1986), conformational properties of the tropone C ring (Bane et al., 1984; Hastie et al., 1989), or a combination of both factors. In the latter mechanism, the second, slow step of the association reaction is ascribed to simultaneous conformational changes in both ligand and protein which generate a second, more planar conformation of colchicine. The conformational flexibility of colchicine is attributed to the tropone C ring of the molecule, which is thought to undergo a boat–boat interconversion to form the protein-bound species. This hypothesis has adequately described the kinetic behavior of colchicinoids investigated to date.

Allocolchicine, by virtue of its aromatic C ring, may be considered a conformationally rigid C ring analogue of colchicine. Allocolchicine may undergo atropisomerism about the biaryl bond, but this process is also available to the colchicine ring system and does not yield a more planar conformation for either molecule (Detrich et al., 1981). Thus, if flexibility of the C ring π system is the structural feature of the ligand responsible for the high activation energy of colchicine binding, allocolchicine would be predicted to bind to tubulin in a manner characterized by the initial rapid and reversible step of the association. The finding that the activation energy for allocolchicine binding to tubulin is only slightly less than that of colchicine argues against conformational flexibility of the C ring portion of the ligand as the structural feature of the ligand responsible for the slow kinetics of the association.

Whether a more subtle conformational readjustment of the ligand occurs in either colchicine or allocolchicine cannot be ascertained from these data. It is clear that the B ring of each molecule is intimately involved in the kinetics of complexation as bicyclic analogues of both ligands bind considerably faster than the parent molecules (Ray et al., 1981; T. J. Fitzgerald, personal communication). However, the role of the C-7 substitutent in this process remains unclear. Quantitative

examination of the energetics of B ring analogues binding to tubulin is required to sort out the role of this substituent in the mechanism.

Changing the structure of the C ring also alters the spectroscopic properties of the ligand. The absorption maximum of the near-UV band of allocolchicine is at higher energy than colchicine and is probably comprised largely of the conjugation band normally observed in bridged homobiphenyls (Rao, 1975). We have recently found that the near-UV band of colchicine consists of two π–π^* transitions which are mostly localized on the tropone ring (Hastie & Rava, 1989). Thus, the electronic properties and permutations of the spectra upon tubulin binding will not necessarily be similar for the two molecules.

The differences in electronic properties of allocolchicine and colchicine are most dramatically observed in the fluorescence spectra. Unlike colchicine, allocolchicine fluorescence is readily observed in the absence of tubulin, and the luminescent properties of the molecule in the absence of the protein are describable by solvent hydrophobic effects. The close correlation between the excited-state properites of the molecule in dioxane and bound to tubulin indicate that the colchicine binding site is an apolar region of the tubulin dimer.

Energy transfer between allocolchicine and tubulin is also evident, and about 38% of the emission intensity of tubulin-bound allocolchicine can be attributed to excited-state energy transfer. These results may imply that at least one of the eight tryptophans in tubulin is in close proximity to the binding site.

Careful examination of allocolchicine excitation spectra indicates that a mechanism other that simple hydrophobic effects may contribute to the excited-state properties of the tubulin-bound species. The excitation maximum of allocolchicine shows no obvious correlation with solvent properties, but in the absence of tubulin it is consistently found between 283 and 292 nm. The excitation spectrum of the tubulin-bound ligand, however, displays two maxima at 280 and 305 nm. Although a portion of the intensity at 280 nm results from unliganded tubulin emission and energy transfer, these factors did not account for all of the intensity in this region. Furthermore, the two bands were not apparent in any solvent examined or at low temperature and are probably not vibrational bands of a single electronic transition. It is also unlikely that a specific association between tubulin and allocolchicine such as a hydrogen bond is responsible for the observed spectrum, as no evidence of a specific solvent effect was noted in the emission spectra (Figure 2).

A mechanism which could account for the unusual excitation spectrum and the absorption difference spectrum of the bound species is that the ligand is interacting with a π system of an aromatic amino acid residue at the binding site. The two bands in the excitation spectrum may be due to exciton splitting of the allocolchicine spectrum as the result of a π-stacking association between the ligand and tubulin. The difference spectrum may then be interpreted to show the hyperchroism of the lower energy band which results from the splitting and the corresponding hypochroism of the higher energy band (McRae & Kasha, 1958).

The existence of a π-stacking association between the C ring of colchicinoids and an aromatic amino acid residue in tubulin has also been postulated to explain the resonance Raman spectrum of tubulin-bound thiocolchicine (Rava et al., 1987) and the absorption spectrum of colchicine liganded to tubulin (Hastie & Rava, 1989). Such an association could also explain the differences in the efficiency of energy transfer between the colchicine–tubulin and allocolchicine–tubulin complexes. The absorption spectra of both ligands possess sufficient overlap with the tubulin emission spectrum for efficient energy transfer to occur, and both ligands quench intrinsic protein fluorescence to a similar extent, yet only a small fraction (<15%) of tubulin-bound colchicine (or AC) fluorescence may be attributed to energy transfer from tubulin (Bane et al., 1984; S. B. Hastie, unpublished results). This observation is particularly remarkable if the two ligands are oriented in essentially the same manner in the binding site and are both interacting with an aromatic amino acid, which may be a tryptophan residue. The apparent dichotomy may be rationalized if the transition dipole moments of the two ligands are oriented differently with respect to the aromatic tubulin moiety. Energy transfer efficiency is dependent on both the distance between the donor and acceptor chromophores and the relative orientation of the dipole moments (Lakowicz, 1983). Since the transitions of the two ligands arise from different states, it is possible that the geometry of the two molecules in the binding site could be very similar but the orientation of the dipole moments quite different. It may be that, in colchicine, the dipole–dipole orientation factor approaches zero and, therefore, little energy transfer is seen in the colchicine–tubulin complex.

In summary, the spectroscopic and association properties of allocolchicine binding to tubulin have been investigated. The similarities of the association constants and kinetic parameters of colchicine and allocolchicine binding to tubulin argue against the conformational properties of the C ring as the ligand structural feature responsible for the high activation energy of the colchicine association reaction. The fluorescence emission properties of allocolchicine liganded to tubulin indicate that the colchicine binding site is an apolar region of the protein, and fluorescence excitation and the absorption difference spectrum of the bound species may support the postulated existence of a π-stacking interaction between the ligand and an aromatic amino acid residue in the binding site. Whether such a mechanism can explain other unique optical properties of the colchicine–tubulin complex is currently being explored.

Added in Proof

After the manuscript was accepted, a related paper was published by Medrano et al. (1989) that includes a detailed thermodynamic analysis of the binding of allocolchicine to tubulin.

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Conformation and Dynamics of an RNA Internal Loop†

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ABSTRACT: The conformation and the dynamics of an RNA oligonucleotide (26 nucleotides) which is a model for loop E in eukaryotic 5S RNA have been investigated by one- and two-dimensional NMR. The central portion of the oligonucleotide contains two G A oppositions, a common feature of ribosomal RNAs. The exchangeable proton spectrum indicates that an internal loop separates two stems of four and five base pairs. This observation is not consistent with structures for loop E containing mismatched G·A base pairs proposed from chemical and enzymatic studies on *Xenopus laevis* 5S RNA. The nonexchangeable proton spectrum has been assigned by two-dimensional NMR. Scalar couplings from correlated experiments and interproton distances from NOESY experiments at short mixing times have been used to determine glycosidic angles, sugar puckers, and other conformational features. The conformation of the stems is very close to standard A-form RNA, and extensive base stacking continues into the internal loop. This result provides a structural basis for the large favorable enthalpy of duplex formation determined in thermodynamic studies. Unusual structural and dynamic features are localized in the nucleotides connecting the loop to the stems.

The three-dimensional structure of RNA consists of a few fundamental motifs and their interactions. Secondary structure motifs are double-helical stems, hairpin and internal loops, bulges, and mismatched base pairs. Tertiary motifs include multistem junctions, base triples, and pseudoknots. Understanding the structure, the dynamics, and the thermodynamic stability of the individual motifs will help in understanding the principles of RNA folding, the specificity of RNA–protein interactions, and the catalytic abilities of RNA.

Most information on RNA structure at the atomic detail was provided by crystallographic studies on tRNA (Saenger, 1983). However, some of the motifs of RNA structure, for example, internal loops, are not present in tRNA. Thus,

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