Tubulin Conformation and Dynamics: A Red Edge Excitation Shift Study

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ABSTRACT: The fluorescence emission maximum of a polar fluorophore in viscous medium often shows a dependence on excitation wavelength, a phenomenon which is named red edge excitation shift (REES). We have found that the fluorescence spectra of the tubulin tryptophans exhibit a REES of about 7 nm. Also, their steady state fluorescence polarization and mean lifetimes show a dependence on both excitation and emission wavelengths. These results indicate that the average tryptophan environment in tubulin is motionally restricted. Although the tryptophan(s) responsible for the observed REES effect could not be localized, it could be concluded from energy transfer experiments with the tubulin−colchicine complex that the tryptophan(s) participating in energy transfer with bound colchicine probably does not contribute to the REES. A REES of 7 nm was also observed in the case of colchicine complexed with tubulin. However, such a REES was not seen in similar studies with the B-ring analogs of colchicine, viz. 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (called AC because it lacks the B ring of colchicine) and deacetamidocolchicine (which lacks the acetamido substituent at the C-7 position of the B ring). There may be two possible reasons to explain these data. (1) Structural differences between colchicine and its analogs may give rise to differences in their excited state dipole moments which will directly affect the extent of REES, and (2) The B-ring substituent, hanging outside the colchicine binding site on the β-subunit of the tubulin dimer, probably makes contact with the α-subunit of tubulin and imparts a rigidity to that region of the protein, which facilitates the REES.

Fluorescence spectroscopy represents a sensitive approach for investigating both structural and molecular dynamic properties of systems of biological interest. The fluorescence emission spectrum for most polar fluorophores in nonviscous medium is generally observed to be independent of excitation wavelength. However, when such a fluorophore is in a polar, viscous environment, i.e., where its mobility is restricted, the wavelength of maximum fluorescence emission frequently shows a dependence on excitation wavelength. Excitation at the extreme red edge of the absorption spectrum causes a red-shifted emission in these cases. This phenomenon is popularly known as the red edge excitation shift (REES)[1] (Chen, 1967; Galley & Purkey, 1970; Castelli & Forster, 1973; Itoh & Azumi, 1975; Demchenko, 1982; Lakowicz & Keating-Nakamoto, 1984; Mukherjee & Chattopadhyay, 1995).

For a polar fluorophore, there exists a statistical distribution of solvation states based on their dipolar interactions with the solvent molecules in both the ground and excited states. A polar fluorophore, in the ground state, interacts with the surrounding solvent dipoles to remain in an energetically favorable orientation. Upon excitation, the dipole moment of the fluorophore changes, and consequently, the solvent dipoles have to reorient themselves around the excited state fluorophore in order to reattain the energy minima (solvent relaxation). This reorientation process consists of two components: (i) electron redistribution in the solvent molecules which occurs within 10^{-15} s and (ii) the physical reorientation of the solvent molecules. This latter process is slow and dependent on the viscosity of the medium. In a nonviscous solvent, the solvent dipoles can reorient around the excited state polar fluorophore within 10^{-12} s, i.e., within its excited state lifetime (10^{-9} s). Hence, no matter what the excitation wavelength is, emission always occurs from the solvent-relaxed state. In a viscous solvent, solvent relaxation is restricted and occurs on the time scale of 10^{-9} s or longer. This results in differential extents of solvent reorientation around the excited state fluorophore, with each excitation wavelength selectively exciting a different average population of fluorophores. Thus, excitation at the red edge of the absorption spectrum (by lower-energy quanta) selectively excites those fluorophores which interact more strongly with solvent molecules in the excited state, i.e., those around which solvent molecules are oriented in a way similar to that found in the solvent-relaxed state. Emission from this state will be red-shifted. On the other hand, excitation at a wavelength central to the absorption band selects for those fluorophores around which solvent relaxation has not yet occured and a blue-shifted emission is observed. This, in brief, is the principle underlying the REES phenomenon (Macgregor & Weber, 1982; Lakowicz & Keating-Nakamoto, 1984; Demchenko, 1986, 1988a; Demchenko & Ladokhin, 1988; Mukherjee & Chattopadhyay, 1995).

The origin of the REES thus lies in the change in fluorophore−solvent interaction in the ground and excited states, brought about by a change in dipole moment of the

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[1] Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine triphosphate; REES, red edge excitation shift; AC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; DAAC, deacetamidocolchicine.

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fluorophore upon excitation, and the rate at which solvent molecules reorient around the excited state fluorophore. Since the REES is observed only under conditions of restricted mobility, it has been used as a potential tool to estimate the fluorophore (both intrinsic and extrinsic) environment in organized biological assemblies such as membranes, micelles, and proteins. Various membrane phenomena have already been studied by incorporating lipids labeled with fluorescent reporter groups such as 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) into model membranes (Chattopadhyay & Mukherjee, 1993) and micelles (S. S. Rawat, S. Mukherjee, and A. Chattopadhyay, unpublished observations). The fluorescent probe 6-(*p*-toluidinyl)-2-naphthalenesulfonic acid (TNS) shows a REES when bound to proteins such as apomyoglobin, β-lactoglobulin, β-casein, and several serum albumins (Demchenko, 1982; Lakowicz & Keating-Nakamoto, 1984; Albani, 1992). A REES has also been reported in proteins like mellitin (Chattopadhyay & Rukmini, 1993), gramicidin (Mukherjee & Chattopadhyay, 1994), the intact eye lens (Rao et al., 1989), and cytochrome b_5 (Ladokhin et al., 1991). The REES of tryptophan fluorescence is usually not observed in proteins having either very short (307−323 nm) or very long (341−350 nm) wavelength emission maximum, but it is usually observed in proteins that emit at intermediate wavelengths (Demchenko, 1988b). Our protein, tubulin, falls in this latter category. There are eight tryptophan residues per tubulin dimer, and on excitation at 280 nm, their emission maximum is at 333 nm. It is thus of interest to examine whether the REES takes place in tubulin. In this paper, we report some observations upon red edge excitation of the tubulin tryptophans. We have also done REES studies with the antimitotic drug colchicine and its structural analogs when bound to tubulin. These drugs have a single, specific binding site on the protein, and our study provides useful information about not only the dynamic properties of the region of the protein containing the binding site but also the involvement of different parts of the colchicine molecule in the binding phenomenon.

MATERIALS AND METHODS

Materials. PIPES, EGTA, GTP, and colchicine were obtained from Sigma Chemical Co. Urea was from Aldrich Chemical Co., and deacetylcolchicine from Molecular Probes. Other colchicine analogs used in this study were gifts from T. J. Fitzgerald, Florida A & M University, Tallahassee, FL. All other reagents were of analytical grade.

Purification of Tubulin. Tubulin was isolated from goat brains by two cycles of GTP and temperature dependent assembly and disassembly in buffer containing 50 mM PIPES, 1 mM EGTA, and 0.5 mM $MgCl_2$ (pH 7), followed by two further cycles in 1 M glutamate buffer (Hamel & Lin, 1981). The purified tubulin, freed from microtubule-associated proteins, was stored in aliquots at −70 °C. Protein concentrations were estimated by the method of Lowry et al. (1951).

Complexes of colchicine with tubulin were prepared by incubating the protein (5 μM) and drug (10 μM) at 37 °C for 1 h. All experiments were done in buffer containing 20 mM sodium phosphate, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 7.0 and 25 °C, unless otherwise mentioned.

Fluorescence Spectroscopic Studies. Steady state fluorescence measurements were done on a Hitachi F-3000 fluorescence spectrophotometer, fitted with a circulating water bath for maintaining constant temperature in the cell holder. A 1 cm path length quartz cuvette was used for all experiments. For experiments that monitor changes in emission maxima with excitation wavelength, excitation and emission slits with a band-pass of 5 nm were used. For polarization measurements, excitation and emission slits corresponding to band-passes of 5 and 10 nm, respectively, were used. Polarization experiments were performed using Hitachi polarization accessories. The fluorescence intensity components (I_{vv}, I_{vh}, I_{hv}, and I_{hh}), in which the subscripts refer to the horizontal (h) or vertical (v) positioning of the excitation and emission polarizers, respectively, were used to calculate steady state fluorescence polarization using the following equation (Chen & Bowman, 1965)

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

where G is the grating correction factor and is equal to I_{hv}/I_{hh}. In all cases, background intensities of the buffer were subtracted from the sample spectra to eliminate errors due to scattering artifacts.

Fluorescence lifetimes were calculated from time-resolved decay of fluorescence intensity using a Photon Technology International LS-100 luminescence spectrophotometer operated in the time-correlated, single-photon-counting mode. A thyraton-gated nanosecond flash lamp filled with nitrogen (16 ± 1 in. of mercury vacuum) was used and the machine run at 22−25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox as the scatterer. All experiments were performed using slit widths of 6 nm or less. To optimize the signal to noise ratio, 5000 counts were collected in the peak channel. The sample and scatterer were alternated after 10% acquisition to minimize errors due to shape and timing drifts during data collection. The data obtained were analyzed on an IBM PC/AT computer, and the intensity decay curves were fitted as a sum of exponential terms:

$$F(t) = \alpha_i \exp(-t/\tau_i) \quad (2)$$

where α_i is the preexponential factor representing the fractional contribution to the time-resolved decay by the component having a lifetime of τ_i. The decay parameters were obtained using a nonlinear least-squares, iterative program based on the Marquardt algorithm (Bevington, 1969). The goodness of each fit was checked from the chi-square values, the weighted residuals (Lampert et al., 1983), and the autocorrelated function of the weighted residuals (Grinvald & Steinberg, 1974). A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a chi-square value generally not exceeding 1.5. Mean lifetimes (τ) for biexponential decays of fluorescence were then calculated using the equation (Lakowicz, 1983)

$$\tau = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (3)$$

RESULTS

Red Edge Excitation of the Tubulin Tryptophans. Figure 1A shows the fluorescence emission spectra of the tubulin tryptophans upon excitation at different wavelengths. As

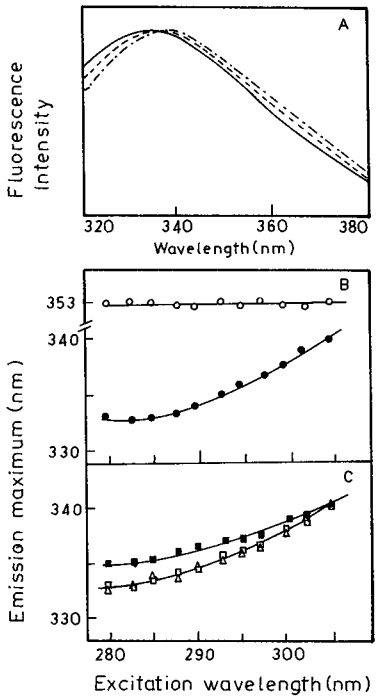

FIGURE 1: (A) Fluorescence emission spectra of tubulin, at 25 °C, in buffer containing 20 mM sodium phosphate, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 7.0 at different excitation wavelengths. The excitation wavelengths were 280 nm (—), 290 nm (- - -), and 300 nm (— • —). All spectra were normalized at the emission maximum, and the tubulin concentration was 2 μM. (B) Fluorescence emission maximum of 2 μM tubulin plotted as a function of excitation wavelength in the absence (●) and presence (○) of 8 M urea, at 25 °C. (C) Changes in the wavelength of maximum emission of 2 μM tubulin upon red edge excitation at different temperatures: 15 °C (△), 25 °C (□), and 37 °C (■).

seen from the figure, the emission spectra of the tryptophans in tubulin show considerable dependence on excitation wavelengths. The shift in emission maximum as a function of excitation wavelength is shown in Figure 1B. On excitation at 280 nm, the fluorescence emission maximum was at 333 nm. As the excitation wavelength was gradually shifted toward longer wavelengths, the emission maxima also showed a concomittant red shift. On excitation at 305 nm, the emission maximum was obtained at 340 nm. This corresponds to a REES of 7 nm. Such a red edge excitation shift was however not visible in the case of the protein that was denatured with 8 M urea. The observed REES thus implies that the environment experienced by the tryptophans in tubulin is motionally restricted, i.e., offers considerable restriction to the reorientation of the solvent dipoles in the excited state and is induced by the native conformation of tubulin. At higher temperatures, the rate of solvent reorientation will be enhanced and emission will be dominated from the solvent-relaxed states (Lakowicz & Keating-Nakamoto, 1984). Thus, the magnitude of the REES is expected to decrease as the temperature is raised. Results of such an experiment are shown in Figure 1C. As seen from the figure, at 37 °C, the extent of the REES is decreased to 5 nm, as compared to 7 nm at lower temperatures, indicating that solvent relaxation around the motionally restricted tryptophans does occur at an enhanced rate at this temperature.

Fluorescence polarization is also known to show a dependence on excitation wavelength in viscous solutions and condensed phases. There are many reports that show

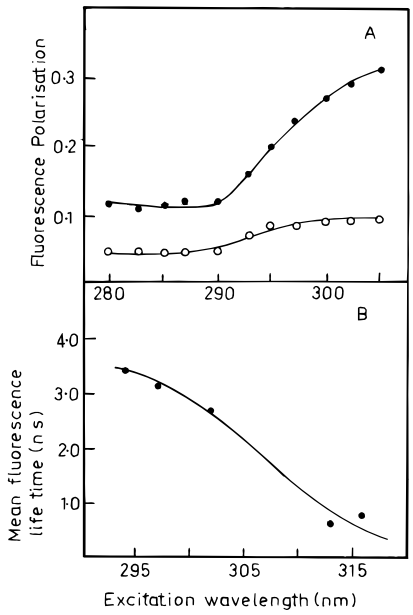

FIGURE 2: Effect of changing the excitation wavelength on (A) steady state polarization of tubulin tryptophans in the absence (●) and presence (○) of 8 M urea, keeping the emission wavelength at 333 nm, and (B) mean fluorescence lifetimes of tubulin, the emission wavelength being 340 nm. The tubulin concentration was 2 μM, and the temperature was 25 °C.

that fluorescence polarization increases with increasing excitation wavelength, when the emission wavelength is kept constant (Weber, 1960a,b; Lynn & Fasman, 1968; Weber & Shinitzky, 1970; Valeur & Weber, 1977a,b, 1978; Lakowicz, 1983, 1984; Demchenko, 1986; Chattopadhyay & Mukherjee, 1993). As discussed above, red edge excitation selects for the solvent-relaxed fluorophores, i.e., those that interact strongly with the surrounding solvent dipoles. Due to such strong interactions with the polar solvent molecules in the excited state, these fluorophores will rotate more slowly compared to the unrelaxed ones (selectively excited at lower wavelengths), giving rise to higher polarization at longer excitation wavelengths (Lakowicz, 1984). Figure 2A shows the change in fluorescence polarization of the tubulin tryptophans with increasing excitation wavelength, keeping the emission wavelength constant at 333 nm. The polarization increases sharply upon red edge excitation with a characteristic dip at 290 nm (Mukherjee & Chattopadhyay, 1994). Such an increase in polarization occurs to a much lower extent in the case of the protein treated with 8 M urea. This is further indicative of the motional restriction experienced by the tryptophans in tubulin.

It is known that tryptophan has two overlapping $S_0 \rightarrow S_1$ electronic transitions (^1L_a and ^1L_b) which are almost perpendicular to each other (Weber, 1960a; Song & Kurtin, 1969; Yamamoto & Tanaka, 1972; Eftink, 1991). Both $S_0 \rightarrow {}^1L_a$ and $S_0 \rightarrow {}^1L_b$ transitions occur in the 260−300 nm range. In nonpolar solvents, ^1L_a has a higher energy than ^1L_b. However, in polar solvents, the energy level of ^1L_a is lowered, making it the lowest-energy state. This inversion is believed to occur because the ^1L_a transition has a higher dipole moment (as it is directed through the ring NH group) and can have dipole−dipole interactions with polar solvent molecules. Whether ^1L_a or ^1L_b is the lowest S_1 state, equilibration between these two states is believed to be very fast (on the order of 10^{-12} s) so that only emission from the lower S_1 state is observed (Ruggiero et al., 1990). In a

Table 1: Lifetimes of Tryptophans in Tubulin as a Function of Excitation Wavelength[a]

excitation wavelength (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2
294	0.68	1.02	0.32	4.50	0.96
297	0.78	0.73	0.22	4.51	1.04
302	0.85	0.68	0.15	4.38	0.93
313	0.99	0.50	0.01	2.48	1.64
316	0.98	0.53	0.02	3.06	1.21

[a] Emission wavelength was 340 nm.

motionally restricted polar environment, absorption at the red edge photoselects the lowest-energy S_1 (^1L_a in this case), and thus, the polarization is high since only depolarization due to small angular differences between the absorption and emission transition moments and solvent reorientation, if any, occurs. Excitation at the shorter wavelengths, however, populates both ^1L_a and ^1L_b states. Equilibration between these two states produces a depolarization due to the approximately 90° angular difference between ^1L_a and ^1L_b moments. Thus, near 290 nm, there is a dip in polarization due to maximal absorption by the ^1L_b state. Figure 2A shows such a characteristic dip around 290 nm in the excitation polarization spectrum of tubulin. Thus, the sharp increase in polarization toward the red edge of the absorption band is probably because the extent of depolarization in tubulin is reduced at the red edge not only due to the decreased rotational rate of the fluorophore in a solvent-relaxed state but also due to photoselection of predominantly ^1L_a transition, which in turn reduces the contribution to depolarization because of $^1L_b \rightarrow {}^1L_a$ equilibration.

In addition to steady state polarization, fluorescence lifetimes are also known to change with excitation wavelengths in motionally restricted media (Castelli & Forster, 1973; Conti & Forster, 1974; Valeur & Weber, 1978; Demchenko, 1985; Mukherjee & Chattopadhyay, 1994). When a fluorophore in a restricted environment is excited at its mean excitation wavelength, a majority of fluorophores will emit at the steady state fluorescence emission maximum for that particular excitation wavelength. Also, a majority of fluorophores will have a mean lifetime associated with them, if the decay of fluorescence intensity is monitored at these mean excitation and emission wavelengths. However, for lifetime measurements upon red edge excitation, keeping the emission wavelength (although it is now no longer the emission maximum) unchanged, preselection of only that small population of fluorophores will occur which have emitted early, i.e., around which solvent relaxation has not yet taken place and shifted their emission toward the mean, red-shifted emission maximum for that excitation wavelength. Thus, fluorescence lifetimes decrease across the excitation spectrum of a fluorophore under conditions where it shows a REES. Table 1 shows the lifetimes of the tryptophan residues in tubulin as a function of excitation wavelength, keeping the emission wavelength constant at 340 nm. The emission wavelength was fixed at 340 nm in order to eliminate interference due to scattering at longer excitation wavelengths. The fluorescence decay of the tryptophans of tubulin could be fitted to a biexponential function at all excitation wavelengths, with a short lifetime component, the preexponential factor associated with which increased at the red edge of excitation; and a relatively longer lifetime component, the preexponential factor for which decreased

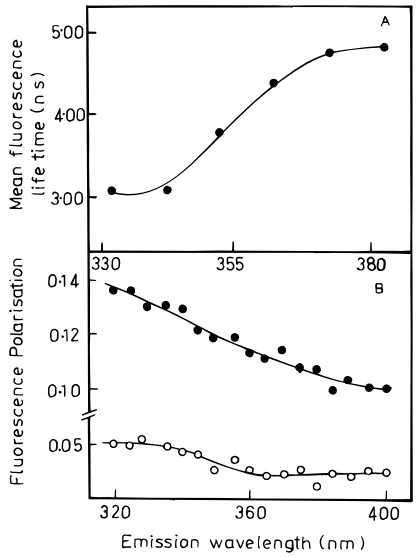

FIGURE 3: Effect of changing the emission wavelength on (A) mean fluorescence lifetimes of tubulin tryptophans upon excitation at 297 nm and (B) fluorescence polarization in the absence (●) and presence (○) of 8 M urea, keeping the excitation wavelength constant at 280 nm. The tubulin concentration was 2 μM, and the temperature was 25 °C.

Table 2: Lifetimes of Tryptophans in Tubulin as a Function of Emission Wavelength[a]

emission wavelength (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2
330	0.22	4.82	0.78	1.11	1.30
340	0.22	4.51	0.78	0.73	1.04
350	0.39	4.81	0.61	1.25	0.99
360	0.35	5.81	0.65	2.99	1.02
370	0.34	6.34	0.66	3.24	0.99
380	0.58	2.62	0.42	6.10	0.94

[a] Excitation wavelength was 297 nm.

concomitantly. Mean lifetimes were calculated according to eq 3 and plotted as a function of excitation wavelength in Figure 2B. As seen from the figure, mean lifetimes of tubulin decreased steadily (77%) as the excitation wavelength was increased from 294 to 316 nm.

On the other hand, fluorescence lifetimes for a polar fluorophore in viscous medium are often found to increase with increasing emission wavelength when the excitation wavelength is kept constant (Easter et al., 1976, 1978; Lakowicz & Cherek, 1980; Matayoshi & Kleinfeld, 1981; Lakowicz et al., 1983a,b; Demchenko & Shcherbatska, 1985; Mukherjee & Chattopadhyay, 1994). Figure 3A shows the change in the mean lifetime of tubulin with the change in emission wavelength, keeping the excitation wavelength constant at 297 nm. At the emission wavelength was gradually increased from 330 to 380 nm, the mean lifetime of tubulin increased by 52%. The decay of fluorescence intensity at all emission wavelengths was fitted to a biexponential function, and the preexponential factors associated with the two lifetime components are shown in Table 2. Such increasing lifetimes across the emission spectrum of a fluorophore may be interpreted as follows. Shorter wavelengths of emission select predominantly for unrelaxed fluorophores which are decaying rapidly both at the rate of fluorescence emission at that excitation wavelength and also at the rate of solvent reorientation to longer emission wavelengths. Hence, observed lifetimes at these emission

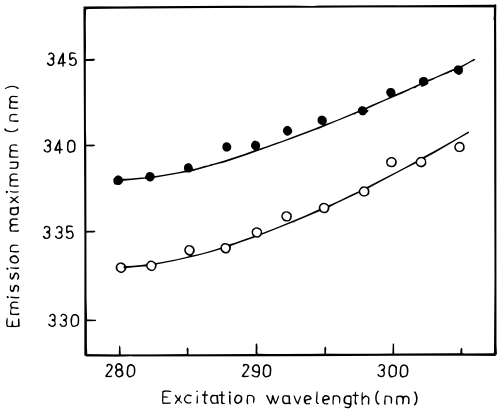

FIGURE 4: Fluorescence emission maxima of tryptophans as a function of excitation wavelength in free tubulin (○) and in the tubulin−colchicine complex (●). The tubulin concentration was 5 μM, and it was incubated with 10 μM colchicine at 37 °C for 1 h to obtain the tubulin−colchicine complex. Fluorescence emission maxima were monitored at 25 °C.

wavelengths will be shorter compared to that obtained at the red edge of emission. Here, solvent-relaxed fluorophores are selected preferentially which have spent enough time in the excited state to allow increasingly larger extents of dipole reorientation around them; i.e., lifetimes are longer.

It follows from the above argument that this subclass of solvent-relaxed fluorophores, having a longer excited state lifetime compared to that of the unrelaxed ones, should thus have more time to rotate in the excited state; i.e., polarization should decrease across the emission spectrum. It has been seen that fluorescence polarization decreases across the emission spectrum of a fluorophore when it is excited at its mean excitation wavelength (Matayoshi & Kleinfeld, 1981; Lakowicz et al., 1983b; Demchenko & Shcherbatska, 1985; Sommer et al., 1990; Chattopadhyay & Mukherjee, 1993). Figure 3B shows the variation in polarization of tryptophans as a function of emission wavelength on excitation of tubulin at 280 nm. While the polarization of the tubulin tryptophans decreases steadily across the emission spectrum, that of the tryptophans in unfolded tubulin remains practically invariant.

The REES studies (both steady state and time-resolved) reported above thus prove that the tryptophan residues in tubulin are present in a viscous environment where mobility is restricted and solvent relaxation dynamics occurs on the nanosecond time scale or longer. Tubulin being a multi-tryptophan protein, our data are indicative of the average environment to which the eight tryptophans of tubulin are exposed and it can only be said that there is at least one tryptophan which is sufficiently restricted for the REES of fluorescence to occur. However, it is difficult to exactly localize the tryptophan(s) responsible for the REES by spectroscopic studies alone.

In an attempt to identify which of the eight tryptophans of tubulin are the major contributors to the REES effect reported above, we carried out REES studies of the tryptophans in the colchicine−tubulin complex. Figure 4 shows a plot of the emission maxima of the tryptophans in the tubulin−colchicine complex and also in free tubulin with changing excitation wavelength. As reported above, in tubulin, the emission maximum shifts by about 7 nm (from 333 to 340 nm) upon changing excitation wavelength from 280 to 305 nm. We found that the fluorescence emission maximum of the tubulin−colchicine complex is at 338 nm

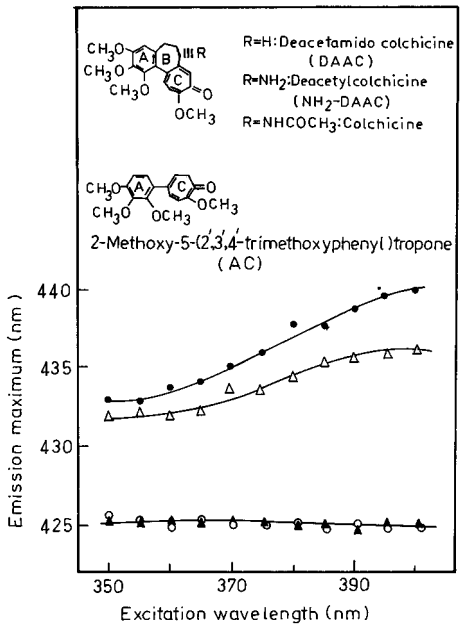

FIGURE 5: Red edge excitation shifts of drug fluorescence in complexes of tubulin with colchicine (●), deacetylcolchicine (△), deacetamidocolchicine (▲), and AC (○). Tubulin and drug concentrations were 5 and 10 μM, respectively, and the temperature was 25 °C. Structures of AC compound, colchicine, and its B-ring analogs used in this study are also shown in the figure.

when it is excited at 280 nm. Changing the excitation wavelength to 305 nm causes a shift in the emission maximum to 344 nm, corresponding to a REES of 6 nm. Thus, although the REES in the tubulin−colchicine complex occurs to almost the same extent as in tubulin, the emission maximum values at each excitation wavelength are red-shifted in the former compared to those in the latter. Now, it is known that the colchicine binding site on tubulin is in close proximity to one or more tryptophan(s) and that energy transfer, from the tryptophan(s) to bound colchicine, occurs (Garland, 1978; Andreu & Timasheff, 1982). Such energy transfer to colchicine from one or more tryptophan(s) near its binding site will make these tryptophans optically silent as far as emission is concerned. These tryptophans are probably present in a very hydrophobic environment, i.e., emit at a lower wavelength compared to more exposed ones, as shutting off of fluorescence emission from them causes an overall red shift in the emission maximum of tubulin. Since a polar environment is a primary criterion for the REES effect to take place, it is expected that the tryptophan(s) present near the colchicine binding site on tubulin and involved in energy transfer with bound colchicine will not contribute significantly to the observed REES.

Red Edge Excitation of Colchicine Complexed with Tubulin. The REES of colchicine fluorescence also was observed in colchicine−tubulin complexes. A plot of emission maxima of colchicine against different excitation wavelengths is given in Figure 5. When the colchicine−tubulin complex was excited at 350 nm, which is the wavelength central to the absorption band of colchicine, the emission maximum was at 433 nm. The emission maximum got progressively red-shifted upon red edge excitation until it was at 440 nm on excitation at 400 nm, corresponding to a REES of 7 nm. We have stated above that the tryptophan(s) of tubulin, which are very near to the colchicine binding site and participate in energy transfer with bound colchicine,

are exposed to a nonpolar environment. However, the REES of colchicine fluorescence in the tubulin−colchicine complex indicates that, unlike the environment of these tryptophan(s), the environment of bound colchicine is significantly polar and viscous.

An interesting observation was noted when REES studies were carried out in complexes of tubulin with some B-ring analogs of colchicine, viz. 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC), deacetamidocolchicine (DAAC), and deacetylcolchicine (structures shown in Figure 5). The REES of fluorescence emission maxima was totally absent when both AC (a biphenyl analog of colchicine that lacks the B ring) and DAAC (which lacks the side chain at the C-7 position of the B ring) bound to tubulin were excited at the red edge of their absorption bands (Figure 5). On the other hand, the fluorescence of deacetylcolchicine (having an amino substituent at the C-7 position of the B ring) bound to tubulin showed a REES; the emission maximum shifted from 432 to 436 nm as the excitation wavelength was increased from 350 to 400 nm.

In order to confirm the observations reported above, we carried out lifetime measurements of colchicine when bound to tubulin, at different excitation and emission wavelengths. The fluorescence lifetime of colchicine in its complex with tubulin was previously determined by phase fluorimetry and found to be 1.14 ns (±0.02), at an ionic strength of 0.1 M, excitation and emission wavelengths being 353 and 430 nm, respectively (Ide & Engelborghs, 1981). The decay of fluorescence intensity of colchicine was fitted to a biexponential function at all excitation and emission wavelengths studied. A decay profile with biexponential fitting and the various statistical parameters used to check the goodness of fit are shown in Figure 6A. Figure 6B shows the variation in mean lifetime of colchicine as a function of excitation wavelength. As the excitation wavelength was increased from 354 nm, the mean lifetime decreased gradually until it was almost 44% decreased when the excitation wavelength was 390 nm. The emission wavelength was kept constant at 430 nm for these measurements. The mean lifetime of colchicine was also measured at different emission wavelengths, keeping the excitation wavelength fixed at 358 nm. As seen from Figure 6C, the lifetime of the excited state fluorophore increased by about 72% with increasing emission wavelength from 430 to 500 nm. Thus, colchicine lifetimes decrease across its excitation spectrum and increase across its emission spectrum as expected in the case of a polar fluorophore in motionally restricted media. Such dependence of fluorescence lifetimes on excitation and emission wavelengths occurred to a significantly lesser extent in the case of DAAC (Figure 6B,C).

Our lifetime studies with colchicine thus support the steady state data and confirm that the colchicine binding site on tubulin is indeed polar and viscous where solvent relaxation around the excited state molecule is considerably restricted. Also, the REES of colchicine analogs appears to be significantly influenced by heterogeneity in ligand structure as proven by the fact that some structural analogs of colchicine, binding at the same site on tubulin, did not show a REES like colchicine.

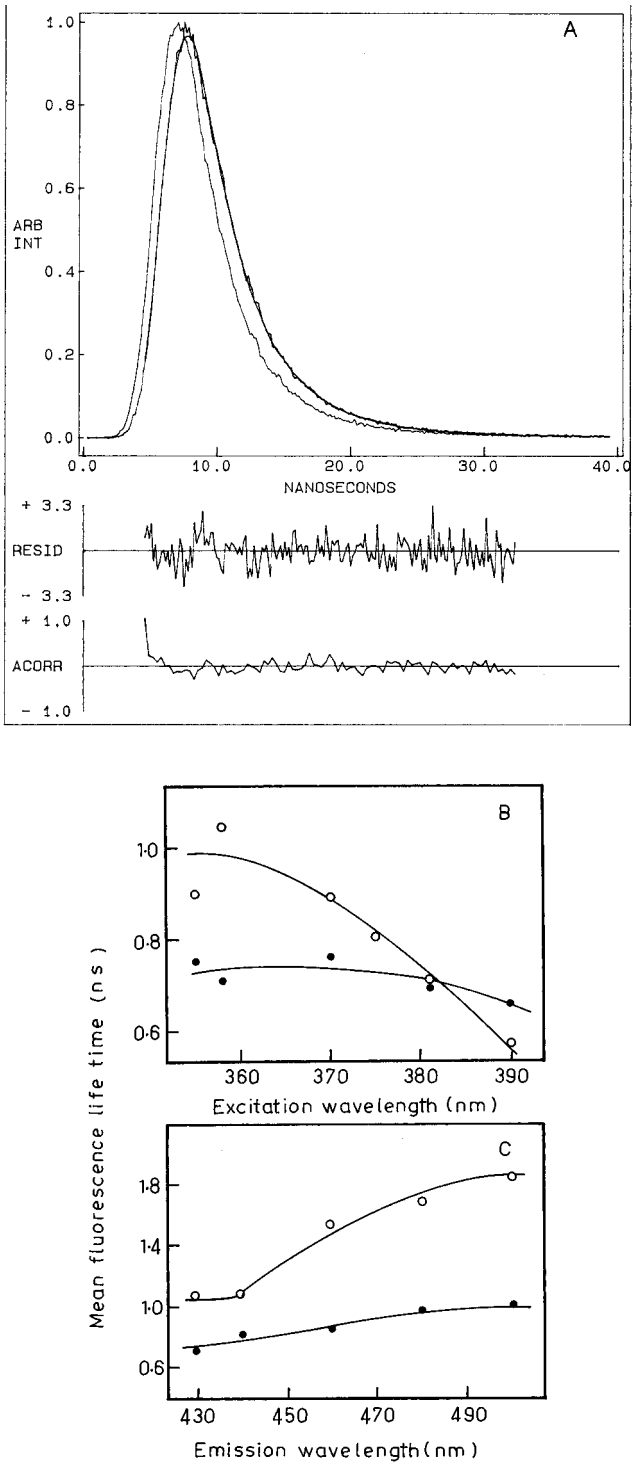

FIGURE 6: (A) Time-resolved decay of fluorescence intensity of colchicine bound to tubulin. Excitation and emission wavelengths were 355 and 430 nm, respectively. The peak on the left is the lamp profile, while that on the right represents the decay profile fitted to a biexponential function. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. (B) Changes in mean fluorescence lifetimes of colchicine (○) and DAAC (●), bound to tubulin, with changing excitation wavelength, the emission wavelength being 420 nm. (C) Mean fluorescence lifetimes of colchicine (○) and DAAC (●), bound to tubulin, as a function of emission wavelength, keeping the excitation wavelength constant at 358 nm. Tubulin and drug concentrations were 5 and 10 μM, respectively, in all cases. All data were taken at 25 °C.

DISCUSSION

Red edge excitation shifts of fluorescence emission maxima have often been used to study the organization and dynamics of biological systems such as membranes and proteins. The necessary conditions for giving rise to a REES can be summarized as follows. (i) The fluorophore should

be polar. (ii) The surrounding solvent should be polar. (iii) Upon excitation, the dipole moment of the fluorophore should change (Mukherjee et al., 1994). (iv) The solvent relaxation process around the excited state dipole should be comparable to or slower than the fluorescence lifetime; i.e., the environment should be motionally restricted.

Our results cited above prove that, among the eight tryptophans of tubulin, there is at least one present in a viscous environment that restricts the solvent relaxation process around them in the excited state. Although we were unable to localize the tryptophan(s) responsible for the REES effect, our results indicate that the tryptophan(s) present near the colchicine binding site on tubulin and involved in energy transfer with bound colchicine probably does not contribute to the REES. Also, the environment to which these tryptophans are exposed appears to be extremely hydrophobic.

Although, colchicine bound to tubulin participates in energy transfer with these tryptophan(s) present in a highly hydrophobic region of tubulin, the environment around colchicine was found to be sufficiently polar and viscous for a REES to occur. It has been reported previously that immobilization of colchicine occurs at its binding site on tubulin (Bhattacharyya & Wolff, 1984). Surprisingly, we found that fluorescence of the colchicine analogs, AC and DAAC, did not show any REES, while that of deacetylcolchicine shows a REES to a lesser extent compared to colchicine, although these drugs have the same binding site on tubulin. One possible reason for such a marked difference in behavior can be the structural differences (B-ring side chain) between the analogs, which may lead to differences in the dipole moment of these molecules in the excited state, which in turn will affect the REES. It is known that the B ring, or more specifically, the substituent at its C-7 position plays an important role in regulating the binding reaction between colchicine and tubulin (Bhattacharyya et al., 1986). The kinetic and thermodynamic parameters of the binding reaction have been found to be strictly controlled at different points of the analog structure. AC binding to tubulin is extremely rapid, highly reversible (Ray et al., 1981), and enthalpy-driven (Bane et al., 1984). Introduction of the B ring (DAAC) and inclusion of an increasingly bulky substituent at the C-7 position of the B ring [deacetylcolchicine (NH_2-DAAC), demecolcine (NHMe-DAAC), *N*-methyldemecolcine (NMe_2-DAAC), and colchicine] results in progressive lowering of on rate and off rate constants of binding and increasing of activation energy and also converts an enthalpy-driven reaction to an entropy-driven one (Chakrabarti et al., 1996).

These observations can also be explained on the basis of a simple and widely accepted model for the colchicine−tubulin complex. The colchicine site on tubulin is composed of two chemically different and independent subsites on the β-subunit of the protein: one for the A ring of colchicine and another that binds the ring C (Andreu et al., 1991; Shearwin & Timasheff, 1994). Ring B makes no contribution to the binding other than regulating the binding parameters of the reaction. It is generally believed now that the B-ring substituent of colchicine and its analogs reside outside the colchicine binding site, facing α-tubulin and also possibly making contact with it (Pyles & Bane Hastie, 1993; Wolff & Knipling, 1995). It is probable that such a contact of the C-7 substituent with the protein would restrict the flexibility of the bound drug and the environment around it at the binding site, a condition which facilitates the REES. Hence, in colchicine analogs, where there is no such substituent to make contact with the protein, no REES is observed. Our studies thus serve as further evidence for the proposed model of the colchicine−tubulin complex.

Lastly, we say that the REES phenomenon reported here can give rise to complications in spectroscopic experiments involving tubulin. In such experiments, excitation of a fluorophore is often done at the red edge of its absorption band instead of the absorption maximum (e.g., to selectively excite tryptophans or minimize inner filter effects). However, under conditions which facilitate a REES, such red edge excitation will induce a red shift in the emission maximum and a decrease in the mean lifetime, leading to the incorrect assumption of the fluorophore being more exposed to the solvent than it really is. In such cases, fluorescence characteristics should be interpreted with caution.

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