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Fluorescence energy transfer measurement of distances between ligand binding sites of tubulin and its implication for protein–protein interaction

ANUSREE BHATTACHARYA,[1] BHABATARAK BHATTACHARYYA,[2] AND SIDDHARTHA ROY[1]

[1]Department of Biophysics and Bose Institute, P 1/12 C.I.T Scheme VII M Calcutta 700054, India
[2]Department of Biochemistry, Bose Institute, P 1/12 C.I.T Scheme VII M Calcutta 700054, India

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Abstract

9-(Dicyanovinyl) julolidine (DCVJ) is a fluorescent probe, which binds to a unique site on the tubulin dimer and exhibits different properties that are dependent upon its oligomeric state (Kung & Reed, 1989). DCVJ binds to tubulin, the tubulin–colchicine complex, and the tubulin–ruthenium red complex equally well, but binds tighter to the ANS–tubulin complex than to tubulin alone. The energy transfer studies indicate a small amount of energy transfer with colchicine, but a significant energy transfer with ANS. It was shown previously that ruthenium red binds near the C-terminal tail region of the α-subunit. Ruthenium red causes major quenching of fluorescence of the tubulin–DCVJ complex, suggesting proximity of binding sites.

The derived distances are consistent with DCVJ binding near the $\alpha\beta$ interface, but on the opposite face of the colchicine binding site. Location of the binding site correlates with the observed effect of a different polymerized state of tubulin on the DCVJ spectroscopic properties. The effect of dimer–dimer association on DCVJ binding, at high protein concentrations (Kung & Reed, 1989), suggests that such an association may occur through lateral contacts of the elongated tubulin dimer, at least in a significant fraction of the cases. Transmission of ANS-induced conformational change to the DCVJ binding site, which is near important dimer–dimer contact sites, makes it possible that such conformational changes may be responsible for polymerization inhibition by anilino-naphthalene sulfonates.

Keywords: ANS; colchicine; DCVJ; fluorescence energy transfer; protein–protein interaction; ruthenium red; tubulin

Accessible hydrophobic surfaces are often the sites of functional importance in proteins, for example, domain–domain interfaces, subunit interfaces, and ligand binding sites. The environment sensitive fluorescence probes, ANS, bis-ANS, and PRODAN, which preferentially bind to hydrophobic surfaces, have emerged as very important probes for analysis of protein structure–function relationships. Tubulin is involved in many protein–protein contacts and, therefore, has been usefully investigated by many such hydrophobic probes (Stryer, 1965; Brand & Golkhe, 1972; Bhattacharyya & Wolff, 1975). Many members of this class of probes are polymerization inhibitors (Mazumdar et al., 1992a), suggesting that they affect protein–protein contact. In an important study, Kung and Reed (1989) have shown the utility of DCVJ, also a hydrophobic molecule, in the study of local mobility changes in tubulin that occurs upon protein associations of different types. In addition, the ability of DCVJ to detect conformational changes, which has been reported by Kung and Reed (1989) and in this article, highlights the need to localize its binding site.

Localization of fluorescent ligand binding sites are of importance in the study of protein structure–function relationships. The spatial relationships with other fluorescent ligands may be elucidated using fluorescence energy transfer. This not only helps to map the ligand binding sites by generating self-consistent distances, but also may act as future reference points for an energy transfer to other fluorescent ligands. Localization of binding sites of these conformation sensitive probes also helps to clarify the nature of the conformational changes these probes often detect during functional cycles. The spectral properties of DCVJ have been reported to be differentially sensitive to various protein–protein contacts of the tubulin dimer (Kung & Reed, 1989). Thus, localization of the DCVJ binding site on the tubulin dimer may lead to a better understanding of the protein–protein interaction in

Reprint requests to: Siddhartha Roy, Department of Biophysics, Bose Institute, P 1/12, C.I.T Scheme VII M, Calcutta 700054, INDIA; e-mail: siddarth@boseinst.ernet.in.

Abbreviations: DCVJ, 9-(dicyanovinyl) julolidine; PIPES, Piperazine-N,N-bis (2-ethane sulfonic acid); ANS, 1,8-anilino-naphthalene sulfonate; Bis-ANS, (1,1′-bi(4-anilino)naphthalene-5,5′disulfonic acid; Prodan, 6-propionyl-2-dimethyl amino naphthalene

tubulin. In the present study, we have localized the DCVJ binding site on the tubulin dimer, which in turn, sheds light on the nature of protein–protein interaction in tubulin and microtubules.

Results

Ward et al. (1994) have measured distances between several fluorescent ligands of tubulin. Based on their studies, we have chosen three ligands, ruthenium red, ANS, and colchicine, which are located in the α, β, and the α/β interface, respectively. The overlap integrals of these probes with DCVJ are also excellent, giving rise to large R_0 values. Figure 1 shows the absorption/excitation and emission spectra of all the ligands used in this study.

Distance measurement from the anilino-naphthalene sulfonate site to the DCVJ site

Figure 2 shows the emission spectra of 0.5 μM DCVJ in the presence of 0–5 μM tubulin at 12 °C. With increasing protein concentrations the fluorescence intensity increases, which is accompanied by a moderate blue shift of the emission maxima from approximately 504 nm to approximately 493 nm. This indicates binding of DCVJ to tubulin. The inset shows the double reciprocal plot of the binding data, and a dissociation constant of 12 μM was obtained. Scatchard analysis, using the fluorescence enhancement value obtained from the double reciprocal plot, gave a similar K_d value and a stoichiometry of 0.75 (data not shown). The K_d and stoichiometry values of DCVJ for tubulin at 12 °C are not unlike that obtained by Kung and Reed (1989) at 4 °C. The inset also shows the double reciprocal plot of DCVJ binding to tubulin in the presence of saturating concentration of ANS (50 μM). ANS has no absorption in the excitation and emission wavelengths of DCVJ used in these experiments. The plot yields a dissociation constant of 4.3 μM, suggesting synergistic ligand binding.

ANS binds to tubulin at a single site and inhibits polymerization (Mazumdar et al., 1992a). The inset of Figure 3 shows the effect of increasing concentrations of ANS on the intensity of the excitation spectra at 476 nm (emission was set at 500 nm) of the DCVJ–tubulin complex. The fluorescence intensity increases substantially upon increasing ANS concentrations but saturates soon. Because ANS has no fluorescence emission with excitation at 450 nm and beyond, this fluorescence increase must be due to an indirect effect of binding of ANS on the bound DCVJ fluorescence, mediated by the protein. This enhancement could be due to an increased association constant, an increase in stoichiometry, or an increase in quantum yield. Whatever may be the cause, the enhancement indicates that ANS binds to a site on the protein that is distinct from DCVJ, and induces a conformational change that affects DCVJ binding. The dissociation constant estimate of ANS from the DCVJ–tubulin–ANS complex (from the half-maximum

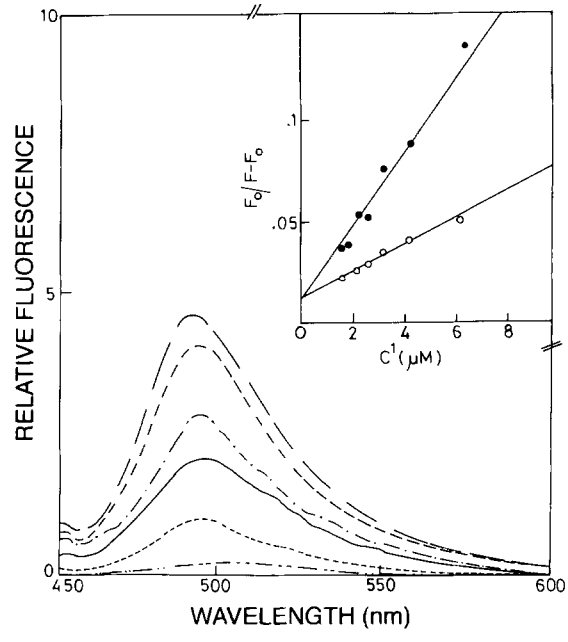

Fig. 2. Emission spectra of DCVJ in the presence of several concentrations of tubulin. Concentrations are 0 (- - — - -), 1 (- - - -), 2 (—), 3 (- — -), 4.8 (— — —) and 5.1 (— — —) μM tubulin from bottom to top. The inset shows the double reciprocal plot of reverse titration of DCVJ with tubulin (filled circles) and DCVJ with tubulin in the presence of 50 μM ANS (open circles). The solution conditions were 12 °C in 0.1 M PIPES, pH 7 containing 1 mM EGTA and 0.5 mM $MgCl_2$. The excitation and emission wavelengths were 430 and 495 nm, respectively. The excitation and emission bandpasses were 5 nm.

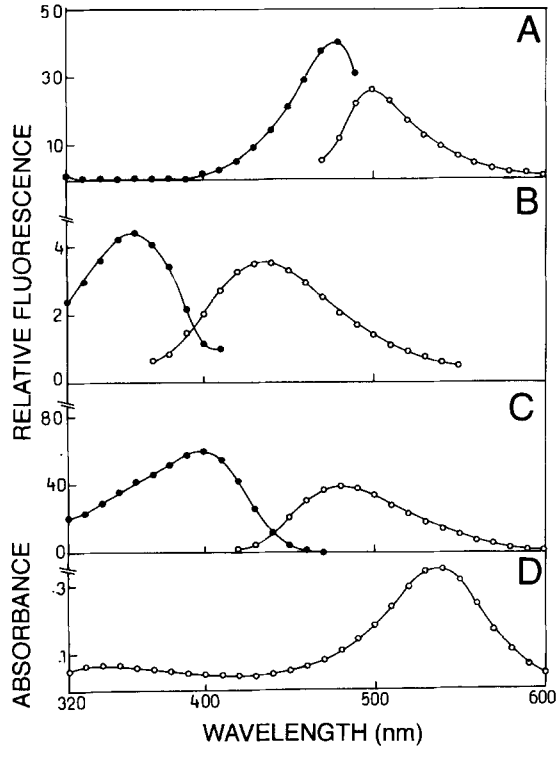

Fig. 1. **(A)** (filled circles) Excitation spectra of a 1:1 complex of tubulin and DCVJ. The protein and DCVJ concentration each were 5 μM. Emission wavelength was 500 nm. (open circles) Emission spectra of the above-mentioned complex. Excitation wavelength was 500 nm. **(B)** (filled circles) Excitation spectra of the 5 μM tubulin and colchicine complex. Emission wavelength was set at 430 nm. (open circles) Emission spectra of the above-mentioned complex. **(C)** (filled circles) Excitation spectra of a 1:1 complex of tubulin and ANS. Tubulin and ANS concentrations were 5 μM each. Emission wavelength was 480 nm. (open circles) Emission spectra of the above-mentioned complex. Excitation wavelength was 372 nm. **(D)** Absorption spectra of the tubulin–ruthenium red complex. Band passes were 5 nm for both emission and excitation for all the complexes and the temperature was 12 °C.

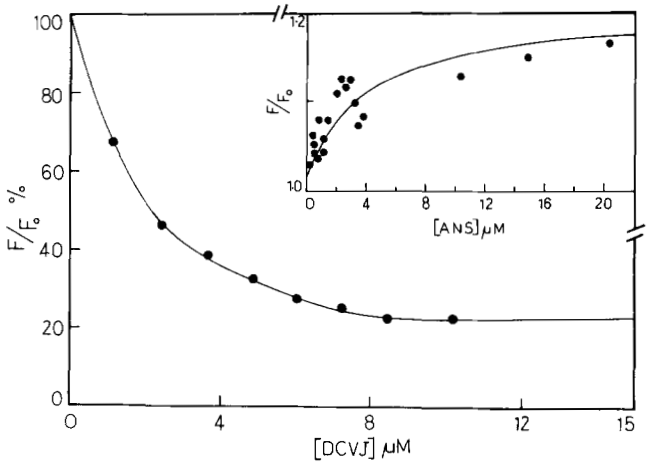

Fig. 3. Effect of DCVJ binding on the fluorescence emission of 1 μM tubulin–50 μM ANS complex at 550 nm. The excitation wavelength was at 400 nm. The excitation and the emission wavelength at 400 nm and 550 nm were chosen in order to minimize the inner-filter effect due to both DCVJ and ANS and to minimize direct excitation of DCVJ. The inset shows the direct titration of the 5 μM DCVJ–tubulin complex with increasing concentrations of ANS. The emission was at 500 nm and the excitation at 476 nm was recorded. The buffer used was 0.1 M PIPES, pH 7 containing 1 mM EGTA, and 0.5 mM $MgCl_2$. The temperature was maintained at 12 °C.

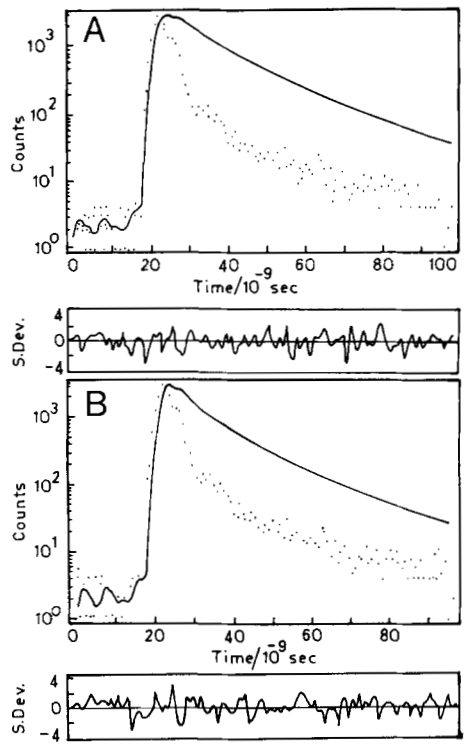

Fig. 4. Decay of tubulin-bound ANS (5:5 μM) fluorescence in the absence (A) and in the presence (B) of 5 μM DCVJ. Each channel was 0.44 ns. The excitation was at 372 nm and emission wavelength was set at 450 nm. The buffer used was 0.1 M PIPES, pH 7 containing 1 mM EGTA, and 0.5 mM $MgCl_2$. The temperature was maintained at 12 °C.

point) appears to be significantly lower than 20–25 μM, reported for the ANS–tubulin complex (Horowitz et al., 1984). The concentration of ANS at the half-maximal point is around 5 μM. When the 1 μM tubulin–50 μM ANS complex is titrated with increasing concentrations of DCVJ (Fig. 3), a large quenching of ANS fluorescence is observed. The excitation was at 400 nm to avoid excitation of DCVJ fluorescence, and emission was at 500 nm to minimize the inner filter effect due to an increased DCVJ concentration. The concentration of DCVJ at the half-maximal point is about 2–3 μM. As shown previously, the DCVJ binding to the ANS–tubulin complex is consistent with a dissociation constant, which may be lower than that of the tubulin–DCVJ complex, suggesting a synergism. If the ligands enhance each other's binding, then the large quenching of ANS fluorescence observed upon the binding of DCVJ is likely to be a consequence of decreased quantum yield, either due to altered environment at the ANS binding site, or energy transfer, or both.

Such interacting sites makes the determination of the energy transfer efficiency, by steady-state fluorescence alone, difficult. Because free ANS has very little fluorescence in water, the measurement of donor lifetime (ANS), in the ANS–tubulin complex, in the presence and in the absence of acceptor (DCVJ), may give an estimate of the energy transfer efficiency. Such measurements are free of interference from effects such as synergistic ligand binding and an inner filter effect. Figure 4 shows the decay of fluorescence of the ANS–tubulin complex in the absence (A) and in the presence of the acceptor, DCVJ (B). Both the decays can be fitted to bi-exponential decays and have different lifetimes. The obtained lifetimes are 14 ns (relative amplitude = 0.138) and 5.3 ns (relative amplitude = 0.18) for the tubulin–ANS complex and 13 ns (relative amplitude = 0.11) and 1.98 ns (relative amplitude = 0.5) for the tubulin–ANS–DCVJ complex. The calculated energy transfer efficiency from the first-order average is 0.54. Although energy transfer estimates obtained this way are free from many other factors that plague the steady-state measurements, it may still be an overestimate or underestimate, because the lifetime of the donor may change due to ligand-induced conformational change.

We have attempted to estimate the energy transfer component by steady-state measurements. After correction of the inner filter effect, the comparison of excitation spectra of the acceptor alone (F_A) with that of the donor–acceptor complex (F_{D+A}) should yield the energy transfer efficiency (Cantor & Schimmel, 1980). The major difficulty in estimating energy transfer efficiency by this route is the proper subtraction of fluorescence intensity due to direct donor excitation. If the transfer efficiency is large or the binding constant of the donor is different in the binary and ternary complexes, then it is not possible to subtract the fluorescence of an equal concentration of the donor–protein complex as the contribution of direct donor excitation. Both energy transfer and a change in complex concentration would lead to a change in the net fluorescence intensity contributed by the donor through direct excitation. The most straightforward way is to find an emission wavelength that has very little direct donor emission. This was not possible in the ANS–DCVJ pair, as in all emission wavelengths tried, DCVJ and ANS emissions overlap.

One way to find the contribution due to direct excitation of the donor in the energy transfer is to use an internal standard. Figure 5A shows the excitation spectra of the DCVJ–tubulin complex and the DCVJ–ANS–tubulin complex. Figure 5B shows the emission spectrum of the ANS–tubulin complex. At 420 nm, the ANS–tubulin has significant emission intensity, but DCVJ has none. Because it is expected that both energy transfer and a change in

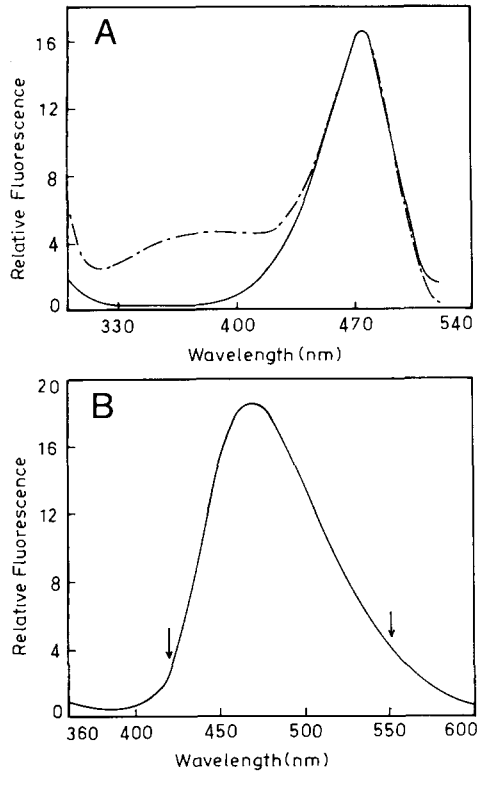

Fig. 5. **(A)** Excitation spectra of the tubulin–DCVJ complex (5:5 μM) (—) and the tubulin–DCVJ–ANS complex (5:5:5 μM) (— - —). The emission wa set at 550 nm. **(B)** Fluorescence emission spectrum of the 5 μM tubulin–5 μM ANS complex. The excitation was set at 330 nm. In both the cases, the buffer used was 0.1 M PIPES, pH 7.0 containing 1 mM EGTA, and 0.5 mM $MgCl_2$. The temperature was maintained at 12 °C.

complex concentration would lead to a proportionate increase or decrease of spectral intensity at all wavelengths, the emission intensity at 420 nm can be used as an internal standard of direct ANS excitation. When such an internal correction is used (see Materials and methods for experimental details), the calculated energy transfer efficiency is 0.81. The corresponding distances derived from time-resolved and steady-state data are 47 and 38 Å, with R_0 calculated with an assumption of $\kappa^2 = 2/3$. This suggests that the quenching of ANS fluorescence upon DCVJ binding is largely due to a fluorescence energy transfer. Because both ANS and DCVJ have high emission anisotropy values, the distance estimate using an assumption of $\kappa^2 = 2/3$, may yield values that are significantly different than the real distance.

Bis-ANS is a dimer of ANS, which has been shown to bind to the ANS binding site under high-affinity conditions (Mazumdar et al., 1992b). It has been noted by Prasad et al. (1986) that the emission anisotropy of bis-ANS bound to the high-affinity site of tubulin is low. This may be due to electronic effects or flexibilty of the probe itself. Under such circumstances the uncertainity in distance estimates is reduced considerably. We have shown that bis-ANS behaves very similarly to ANS with respect to ligand–ligand interaction with DCVJ (data not shown). The range of distance estimate derived from time-resolved data and limiting anisotropy values (Eisinger et al., 1981) is 50–70 Å. This value is somewhat longer than the ANS–DCVJ pair, but due to inherent uncertainity in the ANS–DCVJ distance estimate, they are not mutually exclusive. Based on both ANS and bis-ANS data, an estimate of 50–60 Å between the anilino-naphthalene binding site and DCVJ binding site is reasonable.

Distance measurement from colchicine to the DCVJ site

It is generally accepted that colchicine binds to a single site on tubulin and inhibits polymerization (Olmstead & Borisy, 1973). We have attempted to measure the distance of the DCVJ binding site from the colchicine binding site. The DCVJ binding is largely unaffected by binding of colchicine. Scatchard analysis of binding of DCVJ to the colchicine–tubulin complex shows a similar dissociation constant and stoichiometry (data not shown). Figure 6 shows the excitation spectra of the DCVJ–colchicine–tubulin complex (B) and for comparison, direct excitation spectra of the colchicine–tubulin complex (C) and the DCVJ–tubulin complex (A) at the same concentrations and wavelengths. The excitation spectra are indicative of a small amount of energy transfer. Because the K_d value of DCVJ in tubulin and the tubulin–colchicine complex is similar and energy transfer efficiency is small, the same concentration of the colchicine–tubulin complex may be subtracted to remove the direct excitation component of the donor. After subtraction of the direct excitation spectrum of the colchicine–tubulin complex from the DCVJ–colchicine–tubulin complex, the difference spectrum (B-C) may be compared to the excitation spectrum of the acceptor (A) for energy transfer efficiency calculation (Cantor & Schimmel, 1980). The intensity at 350 nm (peak of the colchicine absorption band) of the (B-C) spectrum (see the inset) is only slightly larger than that of the DCVJ–tubulin complex (A), and the calculated energy transfer efficiency is only about 0.03.

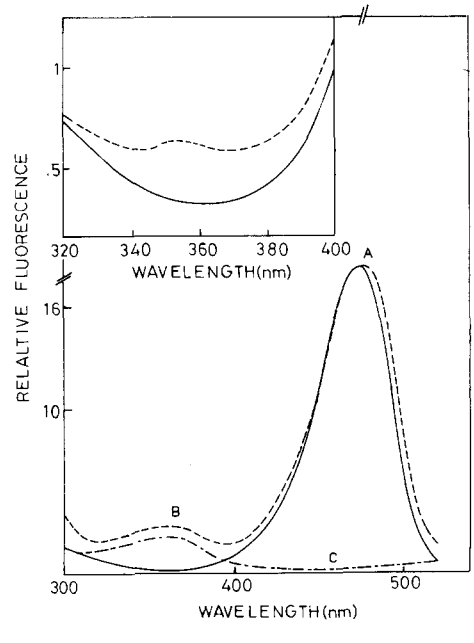

Fig. 6. Excitation spectra of the 5 μM DCVJ–5 μM tubulin complex (—), the 5 μM colchicine–DCVJ–tubulin complex (- - - -), and the 5 μM colchicine–tubulin complex (- — -). The emission was set at 550 nm. The buffer used was 0.1 M PIPES, pH 7 containing 1 mM EGTA, and 0.5 mM $MgCl_2$. The temperature was maintained at 12 °C. The inset shows the excitation spectra of the tubulin–colchicine complex (—), the tubulin–DCVJ–colchicine complex from which direct excitation of colchicine has been subtracted, as described in Materials and methods.

With a calculated R of 30Å, this translates to a distance of 54 Å between the colchicine and DCVJ site, assuming a κ^2 value of 2/3 for random orientation. We have measured the limiting anisotropy values of the DCVJ–tubulin complex and the colchicine–tubulin complex by the Perrin plot (data not shown). The limiting anisotropy values for the colchicine–tubulin complex and the DCVJ–tubulin complex are 0.14 and 0.4, respectively. The derived limits of κ^2 are 0.225 and 2.3, corresponding to distance limits of 70 and 48 Å (Eisinger, 1981).

Distance measurement from ruthenium red to DCVJ

Recently, it has been shown that ruthenium red binds to a single site on the C-terminal domain of the α subunit and its binding site may involve part of the acidic C-terminal tail region (Ward et al., 1994). We have thus attempted to determine the distance of the DCVJ binding site from the ruthenium red binding site. The emission spectrum of DCVJ overlaps well with the absorption spectra of ruthenium red, making it a good pair for determination of distance by the quenching of emission by a Forster mechanism. Figure 7 shows the emission spectra of the DCVJ–tubulin complex (corrected for an inner filter effect at each wavelength) as a function of added ruthenium red. The spectral intensity decreases rapidly, but the decrease levels off at around 6–7 μM ruthenium red concentration. The free DCVJ spectrum at the same concentration is shown for comparison. The inset shows the F/F_0 plot as a function of ruthenium red concentration. The leveling off is fairly sharp, indicating very tight binding of ruthenium red. The leveling off point is close to 5 μM, suggesting a 1:1 stoichiometric complex as observed before (Ward et al., 1994). Because the residual fluorescence is much higher than that of the free DCVJ fluorescence at the same concentration, it is not likely that the quenching is due to competitive displacement of the bound DCVJ. The magnitude of quenching suggests an approximate 50% reduction in quantum yield.

To confirm that binding of DCVJ to the tubulin–ruthenium red complex is similar to the binding of DCVJ to native tubulin, we have titrated tubulin and the tubulin–ruthenium red complex with DCVJ. The inset of Figure 8 shows the direct titration of tubulin and the tubulin–ruthenium red complex with DCVJ under identical conditions. The binding shows saturation behavior in both cases, and the half-maximal concentrations are similar in both cases, which is around 5–6 μM. The saturation levels, however, are significantly different, with the tubulin–ruthenium red complex reaching only about half the value of the tubulin. This strongly suggests that binding of DCVJ to the tubulin–ruthenium red complex is similar to tubulin but with reduced quantum yield.

One of the ways to estimate distance between a donor and an acceptor is to compare the quantum yield of the donor in the presence and in the absence of acceptor. Figure 8 shows the spectra of DCVJ bound to tubulin and the tubulin–ruthenium red complex under identical conditions. The ratio of integrated fluorescence intensities (equivalent to the quantum yield ratio) is 0.42. The R_0 value of DCVJ and ruthenium red is 30 Å (calculated as described in the Materials and methods). The calculated distance between the two is 33 Å. For two reasons, distance estimate between the DCVJ site and the ruthenium red site is likely to have small errors due to uncertainity in the κ^2 value. Ruthenium red binds to the C-terminal tail region of the α subunit, which is known to be flexible (Ward et al., 1994). Secondly, pseudo-spherical symmetry of the metal

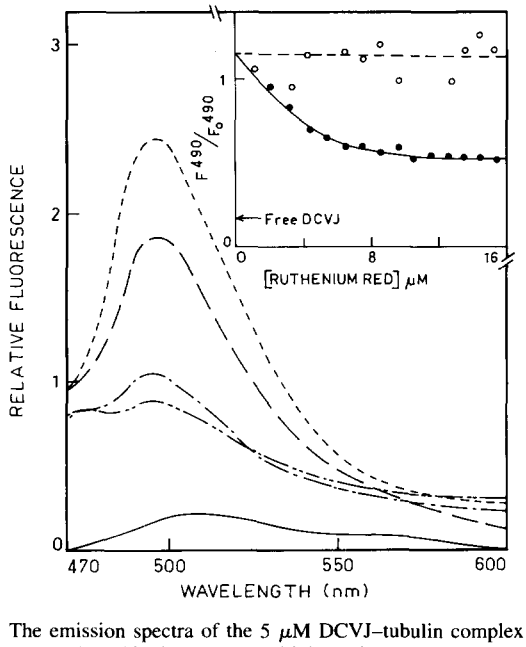

Fig. 7. The emission spectra of the 5 μM DCVJ–tubulin complex in the absence (- - - -), and in the presence of 3.87 μM (– –), 7.68 μM (— - —), and 15.15 μM (— - - —) ruthenium red. The free DCVJ spectrum (—) at the same concentration is shown for comparison. The inset shows the F/F_0 plot of free DCVJ (open circles) and the DCVJ–tubulin complex (filled circles) as a function of ruthenium red concentration. Excitation wavelength was 400 nm and emission values were monitored at 490 nm. The buffer used was 0.1 M PIPES, pH 7 containing 1 mM EGTA, and 0.5 mM $MgCl_2$. The temperature was maintained at 12 °C.

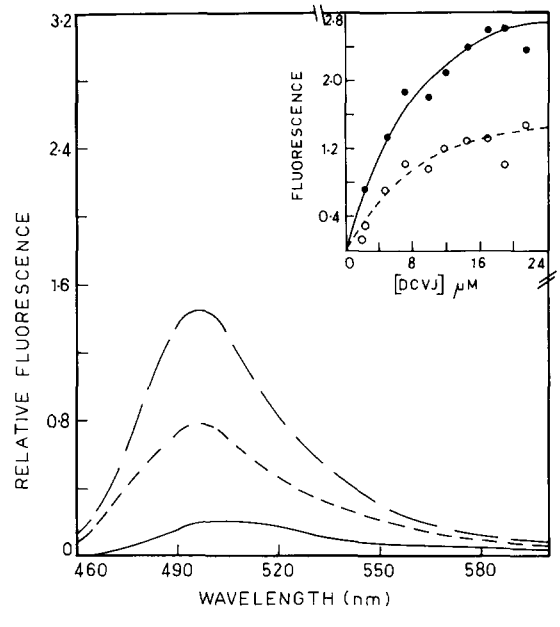

Fig. 8. The fluorescence emission spectra of the 5 μM tubulin–DCVJ complex (— —) alone, in the presence of 5 μM ruthenium red (– –), and 5 μM DCVJ (—) alone. The inset of Figure 7 shows the direct titration of tubulin (filled circles) and the tubulin–ruthenium red complex (open circles) with DCVJ under identical conditions. The excitation wavelength was kept at 400 nm and emission was at 490 nm. The buffer used was 0.1 M PIPES, pH 7 containing 1 mM EGTA, and 0.5 mM $MgCl_2$. The temperature was maintained at 12 °C.

complexes may make κ^2 close to 2/3 (Rhee et al., 1981). Thus, a value of 33 Å, between the DCVJ and ruthenium red sites may be accepted with a high degree of confidence.

Discussion

Tubulin binds many classes of ligands that have different effects on functional properties of the tubulin molecule. Many of these ligands, natural and synthetic, are hydrophobic in nature and are directed towards accessible hydrophobic areas of the proteins such as subunit and domain interfaces. DCVJ is an apolar molecule that does not cause significant inhibition of polymerization but whose fluorescence properties are affected differentially by different protein–protein interactions (Kung & Reed, 1989). Anilinonaphthalene sulfonates are another class of hydrophobic probes, which also bind to tubulin, and in contrast, cause polymerization inhibition (Mazumdar et al., 1992a). Colchicine is a specific natural ligand that also causes inhibition of polymerization (Olmstead & Borisy, 1973). They all affect the protein–protein interaction in different ways and are useful in analysis of particular aspects of the tubulin structure and function. The localization of binding sites of colchicine and ANS has been investigated by several authors. Although conclusive proof is still lacking, the general consensus appears to favor the α/β interface for the colchicine binding site and N-terminal domain of the β-subunit for the ANS binding site (Fig. 9) (Uppuluri et al., 1993; Shearwin & Timasheff, 1994; Ward et al., 1994). The localization of the DCVJ binding site on the tubulin dimer had not been reported. Because spectral properties of DCVJ are sensitive to different protein–protein interactions, knowledge about its localization on the $\alpha\beta$; tubulin dimer may reveal information about protein–protein interactions in tubulin.

In this article we have measured distances of the DCVJ binding site from three known ligands of tubulin: colchicine, ANS/bis-ANS, and ruthenium red. Although the crystal structure of tubulin is not known, several similar models of $\alpha\beta$ dimer structures have been proposed based on the proteolysis and the other studies (Sackett, 1995). The size of the tubulin molecule is approximately 80 × 50 × 40 Å (Sackett, 1995). In many of these structures α/β contact has been proposed to occur via the C-terminal domain of the β-subunit and the N-terminal domain of the α-subunit. Recent studies indicate that colchicine binding occurs at the C-terminal

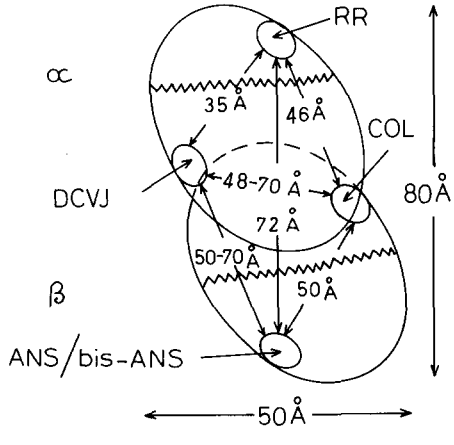

Fig. 9. Two-dimensional schematic diagram of the relative positions of various drug binding sites on the $\alpha\beta$ dimer, constructed on the basis of distance measurement using fluorescence energy transfer as a tool.

domain of the β-subunit, close to the α/β contact site (Uppuluri et al., 1993). Previously, Timasheff and co-workers (Ward et al., 1994) have measured the distance between ruthenium red and the ANS–bis-ANS binding site. The distance was estimated to be greater than 72 Å. The sum of DCVJ–ruthenium red and DCVJ–ANS distances are close (33 + 50–60 = 83–93 Å). This suggests that the DCVJ binding site is located in between the ANS and ruthenium red binding sites, more likely on the α subunit. In the model, the binding site thus may be placed near the $\alpha\beta$ interface, perhaps on the α-subunit. Because the DCVJ binding site is also 50–70 Å or so away from the colchicine binding site, it must be on the opposite face of the prolate ellipsoid.

Because the stoichiometry of DCVJ is significantly reduced by dimer–dimer association (Kung & Reed, 1989), it is likely that dimer–dimer association takes place by side-by-side contact of two prolate ellipsoids, thus masking the DCVJ binding site. Interprotofilament contacts are also located in this region, and different effects on spectroscopic properties of DCVJ upon the formation of different inter-protofilament contacts are consistent with the localization of the binding site.

Binding parameters and spectral properties of DCVJ to the colchicine–tubulin complex is very similar to that of tubulin alone. Distance measurements indicate that the DCVJ binding site, and consequently, the dimer–dimer interface, is far away from the colchicine binding site. The similarity of the dissociation constant and quantum yield enhancements in tubulin and the tubulin–colchicine complex suggests the lack of transmission of the conformational change to the DCVJ binding site, which is known to occur upon colchicine binding (Bhattacharyya & Wolff, 1984). This result is consistent with the data obtained by Detrich et al. (1982), in which they have shown that binding of colchicine has no influence on dimer–dimer association.

In contrast, the binding of ANS significantly enhances the fluorescence of the tubulin–DCVJ complex and lowers its dissociation constant, indicating a global ANS-induced conformational change. Anilino-naphthalene sulfonates are good polymerization inhibitors, but their mechanism of action is not known. Anilinonaphthalene sulfonates inhibition is conditional on polymerization agents used, suggesting indirect effects rather than direct blocking of protein–protein contact. Because the DCVJ binding site is localized near the important dimer–dimer contact site, It is possible that the detected conformational change that occurs upon ANS binding may be responsible for polymerization inhibition.

Materials and methods

Materials

9-(Dicyanovinyl) julolidine and ANS were purchased from Molecular Probes Inc. (Eugene, OR). Colchicine and ruthenium red were purchased from Sigma Chemical Co. (St. Louis, MO). Tubulin was prepared from goat brains by two cycles of temperature-dependent polymerization in a PIPES assembly buffer (50 mM PIPES, pH 6.9 containing 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM GTP) followed by two cycles of temperature-dependent polymerization in 1 M glutamate, pH 7 containing 1 mM GTP (Sloboda & Rosenbaum, 1982).

Spectroscopic methods

Absorption spectra were recorded in a Shimadzu UV-160 spectrophotometer. All fluorescence spectra were recorded in a Hitachi

F-3010 spectrofluorometer equipped with a computer for spectra addition and subtraction. All fluorescence experiments with tubulin were performed in a water-circulated thermostated cell that was set at 12 °C unless mentioned otherwise. Excitation and emission band passes were 5 nm unless mentioned otherwise. Spectrum of the appropriate buffer was always subtracted from fluorescence spectrum. Inner filter effects were corrected using the formula

$$F_{corr} = F_{obs} \times \text{antilog}[(A_{ex} + A_{em})/2]$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the observed fluorescence intensity, A_{ex} is the absorbance at the excitation wavelength, and A_{em} is the absorbance at the emission wavelength. The emission spectra of the tubulin–DCVJ and the tubulin–DCVJ–ruthenium red shown were corrected for an inner filter effect at each wavelength, after determining absorbance at each wavelength separately.

Energy transfer efficiency, E, was calculated by one of the three following methods:

1. In the case of the colchicine–DCVJ pair, E was calculated from excitation spectra using the following equation (Cantor & Schimmel, 1980).

$$F_{D+A}/F_A = 1 + (\epsilon_D C_D/\epsilon_A C_A)E$$

where F_{D+A} was the fluorescence of donor–acceptor (protein–ligand ternary complex) and F_A was the fluorescence of acceptor-only (DCVJ–tubulin) at the same wavelengths; ϵ_D and ϵ_A were the extinction coefficients of the donor and the acceptor, respectively, at the same excitation wavelengths, and C_D and C_A were the concentrations of the donor and the acceptor, respectively.

2. In the case of the ANS–DCVJ pair, E, was calculated by comparing the fluorescence lifetimes of the donor in the presence of the acceptor ($\tau_{D,A}$) and in the absence of the acceptor (τ_A) (Cantor & Schimmel, 1980)

$$\tau_{D,A}/\tau_A = 1 - E$$

3. In the case of the DCVJ–ruthenium red pair, E was calculated by comparing the quantum yields, using the following equation (Cantor & Schimmel, 1980)

$$\Phi_{D+A}/\Phi_D = 1 - E$$

where Φ_D is the quantum yield of the donor and Φ_{D+A} is the quantum yield of the donor in presence of the acceptor.

The distance between donor and acceptor was determined by the following equation,

$$R = R_0 \cdot (E^{-1} - 1)$$

where R_0 is the distance between donor and acceptor when the energy transfer efficiency is 50%. The value of R can be obtained from the following equation

$$R_0 = 9.79 \times 10^3 (J\kappa^2 \Phi \eta^{-4})^{1/6} \text{ Å}$$

where J is the overlap integral, κ^2 is the orientation factor (it is taken as 2/3 for random orientation), Φ is the quantum yield of the donor, and η is the refractive index of the medium. R_0 was calculated according to Saha et al. (1992).

The quanturm yield values of the donors were taken from the following articles: ANS (Bhattacharyya & Wolff, 1975); DCVJ (Kung & Reed, 1989); colchicine (Bhattacharyya & Wolff, 1974); Bis-ANS (Horowitz et al., 1984).

Energy transfer from ANS to DCVJ by the steady-state method

Energy transfer from ANS to DCVJ was measured by the comparing excitation intensity at 370 nm (emission set at 550 nm) of the DCVJ–tubulin complex (F_A) with an equivalent concentration of the DCVJ–ANS–tubulin complex (F_{D+A}) after matching the excitation intensity of the DCVJ peak at 467 nm. Because at all wavelengths DCVJ and ANS emissions overlap, appropriate subtraction has to be made for the direct excitation of ANS. The DCVJ emission band is centered around 500 nm, whereas the ANS emission band is centered around 470 nm. At 420 nm there is significant ANS fluorescence emission but no DCVJ fluorescence emission. Thus, in a DCVJ–ANS–tubulin ternary complex, the excitation intensity at 370 nm, with an emission wavelength set at 420 nm, may be used for correction of direct excitation of the donor (ANS) after appropriate normalization. Normalization coefficient was obtained by comparing the emission intensity of the ANS–tubulin complex at 420 nm and 550 nm, with excitation at 370 nm. The relationships may be written as follows:

$$C = F_{\text{ANS}}{}^{550}/F_{\text{ANS}}{}^{420}$$

$$F_{\text{ANS-DCVJ}}{}^{420} \times C = F^*_{\text{ANS-DCVJ}}{}^{550}$$

$$F^{obs}_{\text{ANS-DCVJ}}{}^{550} - F^*_{\text{ANS-DCVJ}}{}^{550} = F^{corr}_{\text{ANS-DCVJ}}{}^{550}$$

where the subscripts refer to the nature of the complex, the superscript numbers refer to the emission wavelengths, and *obs* and *corr* stands for the observed and corrected fluorescence intensity. Excitation wavelength was always set at 370 nm and the spectra taken in the excitation mode.

Preparation of the colchicine–tubulin complex

Colchicine (30 μM) and 30 μM tubulin were incubated at 37 °C for one hour and the reaction was quenched by lowering the temperature. The complex was then diluted in 0.1 M PIPES buffer pH 7.0 containing 0.5 mM $MgCl_2$, to appropriate concentrations.

Lifetime measurements

Fluorescence lifetime measurements were made by using APL SP 70D (Applied Photophysics, UK) nanosecond fluorescence spectrometer that uses a time-correlated single-photon counting technique. A nitrogen flash lamp was used as an excitation source. Deconvolution of the decay curve was performed by using a double exponential fitting program provided by Applied Photophysics. ANS (5 μM) was mixed with 5 μM tubulin, and the lifetime of bound ANS was measured. The lifetime of bound ANS was also measured in the presence of 5 μM DCVJ. Excitation was at 372 nm and emission was at 450 nm.

Formation of the ruthenium red complex with tubulin

The tubulin–ruthenium red complex was prepared by incubating 100-fold molar excess of ruthenium red with tubulin. The complex

was then separated from excess ruthenium red by passing the solution over a Sephadex G-25 column (12 × 1 cm) equilibrated with 0.1 M PIPES, pH 7.0 containing 1 mM EGTA at 4 °C. The concentrations of bound ruthenium red and protein were calculated on the basis of molar extinction coefficients given by Ward et al. (1994).

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