Energy Transfer Studies of the Distances between the Colchicine, Ruthenium Red, and BisANS Binding Sites on Calf Brain Tubulin†

Larry D. Ward,‡ Robert Seckler,§ and Serge N. Timasheff*

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254-9110

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ABSTRACT: Fluorescence energy transfer experiments were performed in order to measure the spatial separation between the colchine and Ruthenium Red binding sites, the high-affinity bisANS and Ruthenium Red sites, and the allocolchicine and high-affinity bisANS sites on calf brain tubulin. Energy transfer was observed between both colchicine and allocolchicine and Ruthenium Red, resulting in a distance of 40–45 Å between these sites on the tubulin molecule. No detectable energy transfer could be observed when allocolchicine was used as fluorescence donor and bisANS as acceptor or when bisANS was used as donor and Ruthenium Red as acceptor. This indicates that the distance of separation between the allocolchicine and bisANS sites is greater than 50 Å, while that between the bisANS and Ruthenium Red sites is greater than 72 Å. On the basis of these and previous distance measurements (Ward & Timasheff, 1988), two triangles of binding sites have been defined (colchicine–bisANS–E-site and colchicine–bisANS–Ruthenium Red). Since the dihedral angle between them is not known, a schematic model has been drawn with all the sites located in a single plane. Incorporation of the recently identified location of the colchicine site on the β-subunit (Shearwin & Timasheff, 1994), the assignment of the exchangeable GTP binding site to the N-terminal region of the β-subunit distant from the αβ interface (Kirchner & Mandelkow, 1985), and the proposed chemical environments of the various sites result in a model in which the Ruthenium Red binding site is on the α-subunit close to the strongly anionic C-terminal region, the colchicine site is on the β-subunit with ring A oriented toward the αβ intersubunit interface, the nucleotide E-site is in the N-terminal domain of the same subunit in the region of formation of the longitudinal bond in protofilament assembly, and the high-affinity bisANS (or ANS) site is in a hydrophobic region of the same domain.

The microtubule-forming protein tubulin has the propensity to assemble into a variety of polymeric forms (Timasheff & Grisham, 1980) that exhibit a broad structural polymorphism. The geometry of the end products is modulated via allosteric controls of the protein conformation by the binding of a variety of ligands at particular sites. Tubulin is a noncovalent dimer of two similar subunits, α and β, each having a molecular weight of approximately 54 000 (Lee et al., 1973). The dimeric protein contains two guanine nucleotide binding sites, one of which can freely exchange with exogenous nucleotide, the E-site, and the other cannot, the N-site (Weisenberg et al., 1968). The exchangeable site is located on the β-subunit (Geahlen & Haley, 1977; Hesse et al., 1987). The N-site, which is on the α-subunit, does not become exchangeable even when the two subunits are caused to dissociate from each other (Shearwin et al., 1994). Occupancy of the E-site by guanosine 5′-triphosphate (GTP)[1] properly coordinated to a magnesium ion favors assembly into microtubules (Shearwin & Timasheff, 1992). On the other hand, if the E-site is occupied by GDP, polymerization in the presence of magnesium ions leads to the formation of double rings that consist of 28 ± 2 αβ-tubulin dimers (Frigon & Timasheff, 1975a; Howard & Timasheff, 1988; Diaz et al., 1994). These observations have led to the conclusion that tubulin exists in two fundamental conformational states in equilibrium: the microtubule-forming, or "straight", conformation and the ring-forming, or "curved", conformation (Howard & Timasheff, 1988; Melki et al., 1989). This equilibrium is controlled allosterically by E-site occupancy: Mg^{2+}-liganded GTP induces the "straight" conformation, while GDP or Mg-free GTP favors the "curved" conformation (Shearwin et al., 1994). In light of this, the "curved" state has been identified as the ground state of tubulin (Shearwin et al., 1994), i.e., the storage form of the protein (Weisenberg et al., 1976), while the "straight" form is the activated, biologically functional one.

A number of other ligands can also modulate the state of self-assembly of tubulin. Calcium ions inhibit the formation of microtubules (Weisenberg, 1972), while Zn^{2+} or Co^{2+} induces the formation of flat sheets (Larsson et al., 1976; Gaskin & Kress, 1977; Eagle et al., 1983) in which the tubulin appears to exist in the ground, or "curved", conformation (Melki & Carlier, 1993). The binding of vinca alkaloids to tubulin inhibits microtubule formation substoichiometrically (Wilson et al., 1976; Owellen et al., 1976; Himes et al., 1976) and induces tubulin to undergo a linear isodesmic polymerization (Lee et al., 1975; Na & Timasheff, 1980; Prakash & Timasheff, 1991) that leads to spirals and ribbons (Himes et al., 1976). Colchicine, which binds essentially irreversibly to a single site on tubulin (Garland, 1978), inhibits microtubule formation substoichiometrically (Margolis & Wilson, 1977) by binding as the tubulin–colchicine complex to the growth

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* Correspondence should be addressed to this author at the Graduate Department of Biochemistry, Brandeis University, 415 South St., Waltham, MA 02254-9110.

‡ Present address: AMRAD Laboratories, 3 Guest St., Hawthorn, Victoria 3122, Australia.

§ Present address: Universität Regensburg, Institut für Biophysik & Physikalische Biochemie, Universitätsstrasse 31, D-8400 Regensburg, Germany.

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; MAP, microtubule-associated protein; ANS, 8-anilino-1-naphthalenesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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end of a microtubule (Skoufias & Wilson, 1992). The binding of colchicine to GTP tubulin in the presence of Mg^{2+} reorients the protein self-assembly from the formation of microtubules to that of curly arrays, or sheets of protofilaments, while maintaining the thermodynamic characteristics of the polymerization identical with those of microtubule formation (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b). This polymerization is inhibited by Ca^{2+} ions and does not occur when the E-site is occupied by GDP (Andreu et al., 1983). Another consequence of colchicine binding is the induction in tubulin of a GTPase activity (David-Pfeuty et al., 1977) that hydrolyzes the E-site GTP to GDP. This is similar to the assembly-induced GTPase in microtubules (Weisenberg et al., 1976; Zeeberg & Caplow, 1981; Carlier, 1982) and Zn^{2+}-sheets (Melki & Carlier, 1993), although in the case of colchicine this enzymatic activity is independent of polymerization (Andreu et al., 1983). The binding of colchicine to tubulin induces a conformational change that activates the GTPase activity and can be strongly enhanced by a variety of co-solvents known to promote microtubule formation (Perez-Ramirez & Timasheff, 1994). Consistent with this is the earlier observation that the metal ion that is tightly bound to the E-site GTP becomes less accessible to solvent when the tubulin–colchicine complex is placed into a 1 M sucrose medium (Ward & Timasheff, 1988).

To date, little is known about the spatial distribution of these various sites on tubulin and of their location relative to the tubulin–tubulin contacts in the $\alpha\beta$ dimer, as well as in the higher polymers. Such knowledge is needed when extending the various controls identified in solution studies to structural features of tubulin involved in its assembly into microtubules and other structures, as well as in the generation of the GTPase activity. The best characterized loci are the GTP E-site and, most recently, the colchicine binding site. Using a cross-linking and limited proteolysis approach, Kirchner and Mandelkow (1985) have concluded that in dimeric tubulin the intersubunit contacts are made between the N-terminal region of the α-subunit and the C-terminal region of the β-subunit, while in the association of tubulin dimers into protofilaments the longitudinal contacts are between the N-terminal region of the β-subunit and the C-terminal region of the α-subunit. The E-site has been shown to be located in the N-terminal region of the β-subunit (Geahlen & Haley, 1977; Hesse et al., 1987; Sternlicht et al., 1987; Shivanna et al., 1993), at a locus which according to Kirchner and Mandelkow (1985) is close to the longitudinal bond forming site in protofilament assembly and thus distal from the α–β-subunit interface. This assignment, however, need not be unique, since the nonaccessibility to solvent of the E-site in microtubules could be the consequence of a conformational change, as well as of shielding by the formation of the longitudinal bond. The locus of the colchicine binding site has been established recently in a solution thermodynamic study of the linkage between colchicine binding and the dissociation of tubulin into the α- and β-subunits with the E-site occupied, in turn, by GTP and GDP (Shearwin & Timasheff, 1994). It was found that colchicine must be bound necessarily to the subunit that contains the exchangeable GTP site. This is known to be the β-subunit (Geahlen & Haley, 1977; Hesse et al., 1987). Furthermore, it was shown that any contribution that the α-subunit can make to the free energy of binding must be less than 10% of the total (Shearwin & Timasheff, 1994). On the basis of literature reports of photolabeling and photoaffinity studies that suggest the involvement of both subunits (Williams et al., 1985; Floyd et al., 1989; Wolff et al., 1991), it has been proposed that the colchicine binding site is close to the $\alpha\beta$-subunit interface (Shearwin & Timasheff, 1994). This would be supported by the recent report that, in a photolabeling experiment carried out in a large excess (5–10-fold) of colchicine, the 15–25% of the tubulin that had become covalently attached to colchicine carried 25% of the label in the α-subunit and 75% in the β-subunit (Uppuluri et al., 1993). It must be cautioned, however, that this study had not established that the reacted colchicine was that complexed in an essentially irreversible manner to tubulin rather than that present freely in the medium. The location of the colchicine binding site close to the $\alpha\beta$ interface would place it in a region of the β-subunit that according to Kirchner and Mandelkow (1985) involves principally the C-terminal domain. In agreement with this, Sackett and Varma (1993) have concluded that the binding of colchicine affects the folding of the carboxy-terminal region of the β-subunit of tubulin and renders it more susceptible to proteolytic cleavage. Uppuluri et al. (1993), on the other hand, have reported that photolysis in the presence of a 5–10-fold excess of colchicine resulted in the labeling of a number of bands on their peptide gels. From among these they have isolated two peptides, both located in the N-terminal domain of the β-subunit close to the E-site. Because of low yields, however, they could not identify which amino acids were missing from these reported sequences as a consequence of their having reacted with colchicine. The apparent contradiction between the conclusions of Sackett and Varma (1993) and Uppulavi et al., (1993) could possibly be reconciled if the folding of tubulin into its three-dimensional structure brought the C- and N-terminal regions of the β-subunit close to each other and into the vicinity of the $\alpha\beta$ interface. Juxtaposition of the large C and N domains has, in fact, been observed by Little and Luduena (1985, 1987), who have cross-linked Cys-239 with Cys-354, and Cys-12 with Cys-211/201, both of which span from the N to the C domain of the β-subunit. Nevertheless, juxtaposition of the colchicine and the GTP E-sites would be at odds with the conclusion that the GTP E-site is distal from the α–β interface (Kirchner & Mandelkow, 1985), while all the evidence on colchicine binding locates its binding site close to that interface [summarized in Shearwin and Timasheff (1994)].

The positioning of the colchicine and exchangeable GTP binding sites at loci on the β-subunit distant from each other is fully consistent with the finding that the colchicine site must be removed by more than 24 Å from the high-affinity metal bound to the E-site GTP, as measured by fluorescence nonradiative energy transfer (Ward & Timasheff, 1988). Furthermore, it has been shown in an NMR study (Monasterio, 1987) that the high-affinity metal (Mg^{2+}, Mn^{2+}) is directly coordinated to the GTP molecule, most probably at the γ-phosphate. Since the colchicine chromophore is the C-ring, these measurements lead to the conclusion that the distance between the colchicine C-ring binding subsite and the E-site GTP γ-phosphate is greater than 24 Å. Therefore, GTPase activation of tubulin by the binding of colchicine or its analogues (Andreu et al., 1991) must be exercised via long-range allosteric control.

In a continuation of the attempts at triangulation of various sites on the $\alpha\beta$-tubulin dimer, we have examined the fluorescent probes ANS and bisANS and the chromophoric probe Ruthenium Red. The tubulin was in the GTP state, i.e., in the "straight" conformation. ANS has been shown to bind to tubulin (Bhattacharyya & Wolff, 1974; Lee et al., 1975; Ward & Timasheff, 1988) at what has been proposed to be

a hydrophobic locus related to E-site GTP binding (Hanssens et al., 1990). Our earlier fluorescence nonradiative energy transfer measurements have shown its binding site to be located at a distance greater than 28 Å from the GTP E-site (Ward & Timasheff, 1988). We have now carried out similar measurements of the colchicine–bisANS, colchicine–Ruthenium Red, and bisANS–Ruthenium Red distances. These ligands bind to single spatially independent sites on tubulin that have been identified with certain regions on the three-dimensional structure of the protein and may, therefore, be used as markers. BisANS, which inhibits microtubules substoichiometrically (Horowitz et al., 1984), has been reported to bind at 1 strong and 6 weaker sites on tubulin (Prasad et al., 1986a), although the number of weak sites is, in fact, 40–50 (Ward & Timasheff, 1994). Ruthenium Red binds strongly to tubulin and inhibits microtubule formation stoichiometrically (Deinum et al., 1981). Since Ruthenium Red is a highly positively charged metal ion complex, it has been proposed (Deinum et al., 1985) that it binds to one of the highly negatively charged carboxy-terminal domains of tubulin (Ponstingl et al., 1981; Krauhs et al., 1981). This is supported by the observation (Kanazawa & Timasheff, 1989) that removal of the C-terminal domains from $\alpha\beta$-tubulin by limited proteolysis greatly reduced the binding affinity of Ruthenium Red to the protein. The new distance measurements have permitted us to extend the map of site locations on GTP–$\alpha\beta$-tubulin, and the findings are reported in this paper.

MATERIALS AND METHODS

Chemicals. Colchicine and GTP[1] were Sigma products. Allocolchicine was kindly prepared for us by Dr. M. J. Gorbunoff. Bis(8-anilino-1-naphthalenesulfonic acid) (bisANS)[1] was obtained from Molecular Probes and used without further purification. Ultrapure guanidine hydrochloride was obtained from Heico. Ruthenium Red was a Fluka product.

Preparation of Tubulin and Stable Tubulin–Colchicine and Tubulin–Allocolchicine Complexes. Calf brain tubulin was purified by the modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973) with the small changes described by Prakash and Timasheff (1983). The stable tubulin–colchicine complex was prepared by the method described by Andreu and Timasheff (1982a), except that 20 mM PIPES/0.1 mM GTP, pH 7.0, was used as the buffer system. The tubulin–allocolchicine complex was prepared in a similar manner with modifications developed by Ward and Timasheff (1988). Because of the poor solubility of allocolchicine, tubulin could not be incubated in the presence of 1 mM allocolchicine. Consequently, allocolchicine was added at room temperature to tubulin to a final concentration of 4×10^{-4} M in 2 μL aliquots of a 12 mM solution over a period of 30 min. This permitted the binding of each aliquot prior to further addition of the drug and prevented precipitation. To remove any unbound drug, the complex was passed down a short dry G-25 Sephadex column that had been equilibrated with buffer and then a 12 cm wet column, and the peak fractions were pooled. The concentrations of tubulin and tubulin–colchicine were determined using, respectively, molar absorption coefficients of 1.03 (Na & Timasheff, 1982) and 1.16 $mL \cdot mg^{-1} \cdot cm^{-1}$ (Andreu & Timasheff, 1982a) in 6 M guanidine hydrochloride. The concentration of tubulin–allocolchicine was measured by a modified Lowry procedure (Peterson, 1977) using tubulin as the protein standard.

Preparation of Ruthenium Red. Ruthenium Red was purified by recrystallization in 0.5 N ammonia (Fletcher et al., 1961). After being dried, it was stored at –20 °C. Ruthenium Red solutions were prepared for use just prior to experimentation by dissolving in the experimental buffer and filtering through a 0.22 μm Millipore filter (Deinum et al., 1981). It was found to be free of any impurities due to Ruthenium Brown or Ruthenium Violet, as judged by its absorbance spectra in water and 0.1 M ammonium acetate (Fletcher et al., 1961; Luft, 1971). The concentration of Ruthenium Red in the stock solution was calculated on the basis of a molar absorption coefficient of 6.80×10^4 M^{-1} at 533 nm in 0.1 M ammonium acetate (Luft, 1971).

Preparation of Stable Complexes of Ruthenium Red with Tubulin and Tubulin–Colchicine. Tubulin–Ruthenium Red and tubulin–colchicine–Ruthenium Red complexes were routinely prepared by mixing Ruthenium Red with tubulin or the corresponding tubulin–drug complex. The solution was then passed at 4 °C through a 7 × 1 cm dry Sephadex G25 column equilibrated in 20 mM PIPES/0.1 mM GTP, pH 7.0, under the influence of a centrifugal field (Na & Timasheff, 1982) and then a 26 × 1 cm Sephadex G25 column to remove loosely bound Ruthenium Red. One colored peak fraction was collected, and the absorbances of binary and ternary complexes in 6 M guanidine hydrochloride were measured. The concentration of Ruthenium Red associated with tubulin was calculated on the basis of a molar absorption coefficient of 7.03×10^4 M^{-1} at 544 nm. Ruthenium Red had a small absorbance contribution at 275 nm described by a molar absorption coefficient of 1.91×10^3 M^{-1}. Consequently, the concentration of protein in this complex was calculated from its absorbance at 275 nm after subtracting the absorbance due to Ruthenium Red at this wavelength.

Preparation of the Tubulin–Allocolchicine–Ruthenium Red Complex and Measurement of Energy Transfer. The tubulin–allocolchicine–Ruthenium Red ternary complex was prepared by addition of 60 μL of a 0.3 mg/mL Ruthenium Red stock solution to 0.6 mL of the binary tubulin–allocolchicine complex in the 20 mM NaPIPES/0.1 mM GTP, pH 7.0, buffer. After a brief incubation, the mixture was passed down a dry column and then a long (25 cm) wet column of Sephadex G25 at 4 °C. One colored peak fraction was collected. The concentrations of the binary and ternary complexes used in the energy transfer experiments were essentially identical. The two samples were transferred simultaneously to a 20 °C water bath, and fluorescence readings were taken at regular intervals over a period of 1 h. The excitation wavelength was 320 nm; the slits were set at 5 nm. The readings, taken in the RATIO mode, were corrected for buffer background. The emission was recorded by scanning spectral segments about the maximum (400 nm). There was a slight blue shift in the emission spectra of the ternary complex (ca. 7 nm) relative to the tubulin–allocolchine complex. Between readings, the samples were kept in the dark at 20 °C.

UV/Visible Spectroscopy. All spectral measurements were performed on a Perkin-Elmer Lambda 3B UV/visible spectrophotometer after base-line correction. The UV/visible tubulin–bisANS and the tubulin–Ruthenium Red difference spectra were recorded using (0.4 + 0.4) cm tandem cells.

Fluorescence Spectroscopy. Fluorescence measurements were performed on a Perkin Elmer 650-40 spectrofluorometer using the RATIO mode. Background was routinely subtracted, and all spectral measurements and any instrumental variation as a function of wavelength were corrected for by generating correction factors with the use of Rhodamine B and a light diffuser.

Measurement of the Quantum Yield of Tubulin–BisANS. The quantum yield of tubulin–bisANS was obtained by comparing the integrated fluorescence emission spectrum with that of a standard with the application of the relation described by Parker and Rees (1960). The standard used was quinine sulfate in 1.0 N sulfuric acid at 25 °C; the excitation wavelength was 365 nm (Demas & Crosby, 1971; Melhuish, 1961). In the calculation of the quantum yield of the tubulin–bisANS complex, it was not possible to saturate the bisANS binding site without having such an absorbance at the excitation wavelength that inner filter effects became significant. The quantum yield of bound bisANS was calculated, therefore, by utilization of the binding constant for the tubulin–bisANS interaction under the conditions employed (Ward & Timasheff, 1994), which permitted calculation of the concentration of bound bisANS and, consequently, that of the quantum yield of tubulin–bisANS. This was found to be 0.233.

Determination of Fluorescence Energy Transfer. Distances between tubulin-bound colchicine and allocolchicine and Ruthenium Red, as well as those between allocolchicine and bisANS, and bisANS and Ruthenium Red, were measured by application of the Förster theory (1948) for fluorescence radiationless energy transfer that relates the efficiency of energy transfer to the distance between the fluorescent donor and the acceptor. The energy transfer efficiency, E, is related to the distance between donor and acceptor sites, R, by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (1)$$

$$E = (Q_D - Q_{D\text{-}A})/Q_D$$

where R_0 is the critical transfer distance at which the energy transfer efficiency is 50% and Q_D and $Q_{D\text{-}A}$ are the quantum yields of the donor in the absence and presence of the acceptor, respectively. The value of R_0 is a function of the properties of the donor and acceptor pairs and can be evaluated with the equation:

$$R_0 = (JK^2Q_Dn^{-4})^{1/6} \times (9.7 \times 10^3\ \text{Å}) \quad (2)$$

where K^2 is the orientation factor for dipole–dipole interaction between the donor and acceptor, J is the spectral overlap integral of donor fluorescence and acceptor absorption, and n is the refractive index of the medium between the donor and acceptor, taken as that of water, 1.4 (Stryer, 1978). The value of the orientation factor, K^2, was taken as $^2/_3$, which is the value when the donor and acceptor dipoles can assume all possible relative dipole orientations during the lifetime of the donor in the excited state. The value of J was calculated by numerical integration over 4 nm intervals according to the method of Jacobson and Colman (1983).

RESULTS

Distances between the bound pairs colchicine–Ruthenium Red, allocolchicine–Ruthenium Red, allocolchicine–bisANS, and bisANS–Ruthenium Red were measured by radiationless energy transfer. The spectral characteristics and results are summarized in Table 1.

Tubulin–Ruthenium Red Complex. The ability of tubulin–colchicine to form a stable complex with Ruthenium Red was tested by incubating tubulin–colchicine (9 μM) with Ruthenium Red at concentrations between 5 and 60 μM and separating free or weakly bound Ruthenium Red by gel filtration. As shown in Figure 1, at the conditions employed, a 1:1 complex of Ruthenium Red with tubulin–colchicine was obtained at total Ruthenium Red additions above a molar ratio of 2:1. Similar results were obtained whether tubulin alone or tubulin–allocolchicine was used, which indicates that the colchicine and Ruthenium Red sites are independent of each other on the tubulin molecule. Passage of the tubulin–Ruthenium Red complex through a Chelex 100 chelating resin removed the tubulin-bound Ruthenium Red. The metal ion associated with the exchangeable GTP site is known to remain associated with tubulin when treated in the same way (Monasterio, 1987; Ward & Timasheff, 1988). The binding of Ruthenium Red to tubulin results in some disturbance both of the intrinsic chromophores of tubulin and of the Ruthenium Red chromophore as indicated by the UV/visible difference spectrum shown in Figure 2. It is characterized by a peak at 273 nm, that most probably reflects perturbation of a tubulin tryptophan, and a peak and trough at 550 and 472 nm due to Ruthenium Red perturbation. As shown in Figure 3, the fluorescence emission spectrum of tubulin–Ruthenium Red, when excited at 300 nm in order to avoid complications due to inner filter effects, had the same shape as the free tubulin fluorescence emission spectrum, although the intensity had decreased by approximately 5% (Figure 3). As the Ruthenium Red absorption spectrum overlaps with the fluorescence emission spectrum of tubulin, this would be consistent with quenching via energy transfer, rather than direct perturbation of the environment of the fluorophore.

Energy Transfer between Colchicine and Ruthenium Red. The fluorescence emission spectrum of the tubulin–colchicine complex and the absorption spectrum of Ruthenium Red when bound to tubulin are shown in Figure 4. The good overlap of the two spectra makes this donor–acceptor pair suitable for energy transfer experiments. The overlap integral, J, for this

Table 1: Energy Transfer Characteristics of the Chromophoric Pairs

system[a]	Q_D	J (M^{-1} cm^3)	R_0 (Å)	E	R (Å)
COL–RR	0.023	1.206×10^{-13}	27.4	0.03–0.07	43–46
ALLO–RR	0.20	5.253×10^{-14}	34.0	0.15–0.20	40–45
ALLO–bisANS	0.20	2.70×10^{-14}	30.5	0	>50
bisANS–RR	0.233	2.19×10^{-13}	44.0	0	>72

[a] COL, colchicine; ALLO, allocolchicine; RR, Ruthenium Red.

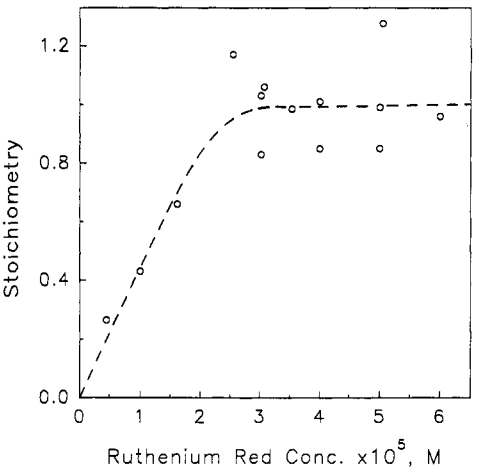

FIGURE 1: Formation of the tubulin–colchicine–Ruthenium Red complex. Tubulin–colchicine (1 mg/mL) was incubated with Ruthenium Red in a total volume of 0.5 mL in 20 mM PIPES/0.1 mM GTP, pH 7.0, buffer before separation of excess Ruthenium Red, as described under Materials and Methods. The stoichiometry was calculated from the concentrations of tubulin and Ruthenium Red measured for each point. The line represents the trend of the data.

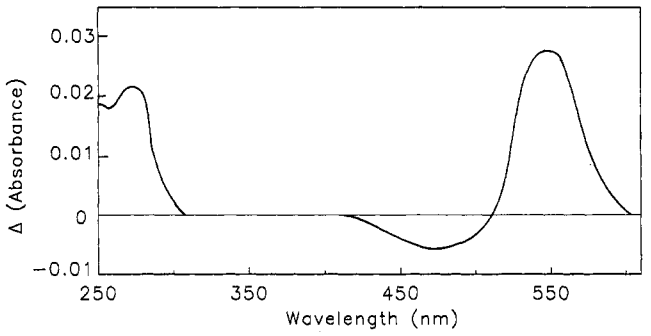

FIGURE 2: UV/visible absorption difference spectrum generated on the binding of Ruthenium Red to tubulin. The spectrum was obtained on mixing 1×10^{-5} M Ruthenium Red and 1×10^{-5} M tubulin in 20 mM PIPES/0.1 mM GTP, pH 7.0, at 20 °C. The readings were recorded relative to identical concentrations of the two reactants present in different compartments of (0.4 + 0.4) cm tandem cells.

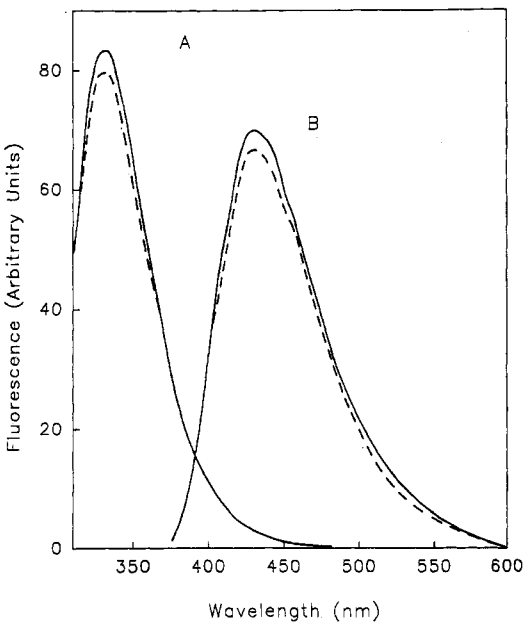

FIGURE 3: (A) Fluorescence emission spectra of tubulin (—) and tubulin–Ruthenium Red (- - -) in 20 mM PIPES/0.1 mM GTP, pH 7.0, at 20 °C. The concentrations of tubulin and tubulin–Ruthenium Red were 3×10^{-6} M. The excitation wavelength was 300 nm, and the background was subtracted in both cases. (B) Energy transfer between colchicine and Ruthenium Red bound to tubulin. The fluorescence emission spectra of tubulin–colchicine (—) and tubulin–colchicine–Ruthenium Red (- - -) were recorded in 20 mM PIPES/0.1 mM GTP, pH 7.0, buffer at 20 °C. The concentrations of the complexes were 1×10^{-6} M in both cases. The excitation wavelength was 350 nm; the excitation and emission slits were 5 and 10 nm, respectively.

interaction was calculated to be 1.206×10^{-13} M^{-1} cm^3. A value of 0.023 was used for the quantum yield of tubulin–colchicine as calculated previously (Ward & Timasheff, 1988). Assuming that the donor and acceptor rotate in a short time relative to the lifetime of the excited state, the value of K^2 was set equal to $^2/_3$, which results in $R_0 = 27.4$ Å. The fluorescence emission spectra for tubulin–colchicine and tubulin–colchicine–Ruthenium Red complexes are shown in Figure 3B, with excitation at 350 nm, i.e., that of the colchicine chromophore. A 3–7% decrease in fluorescence was observed consistently for the ternary tubulin–colchicine–Ruthenium Red complex relative to tubulin–colchicine. That this is due to energy transfer and not to perturbation of the colchicine fluorophore is indicated by the absence of any change in the shape of the fluorescence spectrum upon addition of Ruthenium Red.

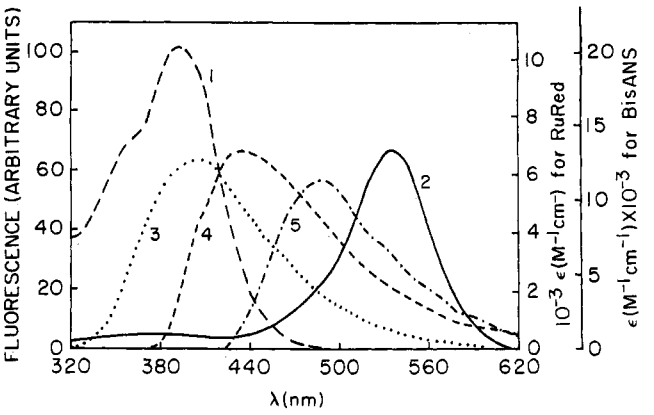

FIGURE 4: Overlaps of the absorption and fluorescence emission spectra of the various tubulin complexes. (1 and 2) Absorption spectra of the tubulin–bisANS and tubulin–Ruthenium Red complexes; (3–5) fluorescence emission spectra of the tubulin–allocolchicine, tubulin–colchicine, and tubulin–bisANS complexes. All spectra were recorded at 20 °C. The tubulin–allocolchicine and tubulin–colchicine pairs with Ruthenium Red, as well as the tubulin–bisANS pair with Ruthenium Red, were obtained in 20 mM NaPIPES/0.1 mM GTP, pH 7.0, buffer. The tubulin–allocolchicine pair with tubulin–bisANS was obtained in 10 mM sodium phosphate/0.1 mM GTP, pH 7.0, buffer. The excitation wavelengths were 350 nm for the tubulin–allocolchicine and tubulin–colchicine pairs with Ruthenium Red, 320 nm for the tubulin–allocolchicine pair with bisANS, and 400 nm for the tubulin–bisANS pair with Ruthenium Red. The excitation and emission slits were 5 nm in all cases.

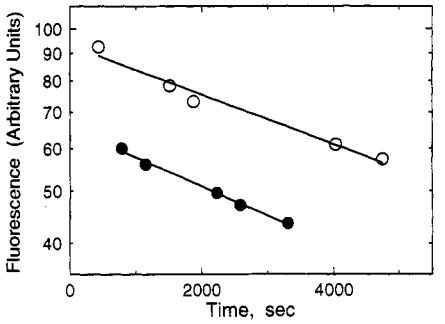

FIGURE 5: Decay of the tubulin–allocolchicine (O) and tubulin–allocolchicine–Ruthenium Red (●) fluorescence with time. Samples (1×10^{-6} M) were incubated at 20 °C in 20 mM PIPES/0.1 mM GTP, pH 7.0. Fluorescence was recorded at the indicated time intervals using an excitation wavelength of 320 nm and an emission wavelength of 400 nm with 5 nm excitation and emission slits. The samples were kept in the dark between readings.

Application of eq 1 leads to a distance of 43–46 Å between sites.

Because of the low degree of energy transfer observed for the colchicine–Ruthenium Red donor–acceptor pair, studies were also performed with the more highly fluorescent analogue of colchicine, allocolchicine. The higher quantum yield of this analogue ($Q_D = 0.2$) (Ward & Timasheff, 1988) and the good overlap of the absorption spectrum of Ruthenium Red with the fluorescence emission spectrum of tubulin–allocolchicine, $J = 5.253 \times 10^{-14}$ M^{-1} cm^{-1} (Figure 4), result in a value of $R_0 = 34.02$ Å. Consequently, a greater efficiency of energy transfer can be predicted. Since the tubulin–allocolchicine complex is known to undergo slow dissociation (Medrano et al., 1989), these experiments had to be carried out as a function of time. The time evolution of the fluorescence of the tubulin–allocolchicine and tubulin–allocolchicine–Ruthenium Red complexes is shown in Figure 5. Both display a slow decay at 20 °C with close to identical rates. The half-lives, $T_{1/2} = 95$ min (ternary complex) and $T_{1/2} = 105$ min (binary complex), are in reasonable agreement

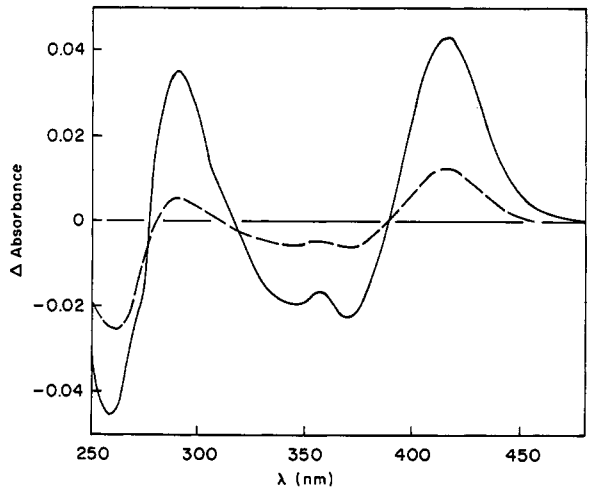

FIGURE 6: Tubulin–bisANS UV/visible difference spectra obtained on the binding of bisANS to tubulin in 10 mM sodium phosphate/0.1 mM GTP, pH 7.0, buffer at 20 °C. The spectra correspond to bisANS concentrations of 5×10^{-6} M (– –) and 1.82×10^{-5} M (—). The tubulin concentration was 1×10^{-5} M in both cases. The spectra were recorded in (0.4 + 0.4) cm tandem cells.

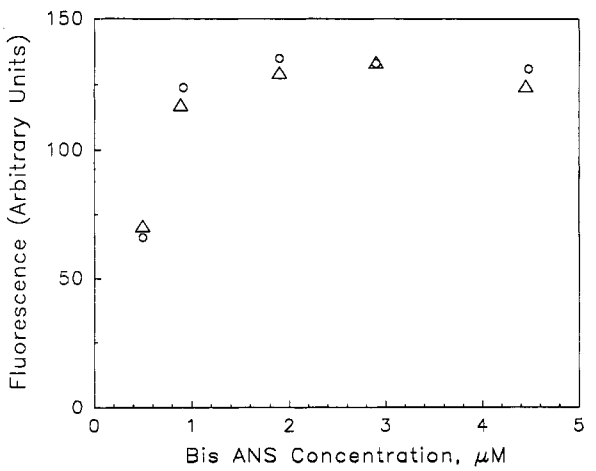

FIGURE 7: Measurement of the fluorescence energy transfer between tubulin-bound bisANS and Ruthenium Red. Tubulin–colchicine (1×10^{-6} M)(△) and tubulin–colchicine–Ruthenium red (1×10^{-6} M)-(○) were titrated with bisANS in 20 mM NaPIPES/0.1 mM GTP, pH 7.0, buffer at 20 °C. The fluorescence was measured at 485 nm using an excitation wavelength of 420 nm and 5 nm excitation and 10 nm emission slits.

with values measured at different conditions for the tubulin–allocolchicine complex (Medrano et al., 1989). As shown in Figure 5, the fluorescence intensity of the ternary complex was consistently 15–20% lower than that of tubulin–allocolchicine. Application of eq 1 leads to a distance of 40–45 Å between the allocolchicine and Ruthenium Red sites on calf brain tubulin. This is in good agreement with the distance calculated to separate colchicine and Ruthenium Red. Since, during the fluorescence measurements, the sample was exposed to light for only short periods, the observed time decay cannot be ascribed to photoinactivation of the complex, but it corresponds to the slow dissociation of allocolchicine. The difference between the emission intensities of the ternary and binary complexes cannot be explained by the washing off of allocolchicine by passage through the two additional column filtration steps involved in the preparation of the allocolchicine–Ruthenium Red complex. This was established by an additional control experiment in which tubulin–allocolchicine was subjected to an identical procedure in the absence of Ruthenium Red. When the binary complex treated in this way was used, the decrease in fluorescence emission of the tubulin–allocolchicine–Ruthenium Red complex relative to tubulin–allocolchicine remained at the level of 15–20%.

Energy Transfer between Tubulin-Bound Allocolchicine and BisANS. Studies of the binding of bisANS to tubulin in the presence and absence of colchicine have shown that the high-affinity bisANS site on tubulin is independent of the colchicine site (Prasad et al. 1986a; Ward & Timasheff, 1994). As shown in Figure 4, the spectral overlap of the fluorescence emission spectrum of the colchicine analogue, allocolchicine, and the absorption spectrum of bisANS is ideal for fluorescence energy transfer measurements. The spectrum of bisANS, shown in Figure 4, represents the spectrum of the tubulin-bound molecule. This was calculated from measured difference spectra generated on the binding of different amounts of bisANS to tubulin. As shown in Figure 6, this difference spectrum is characterized by peaks at 290 and 415 nm and troughs at 260, 348, and 370 nm. It is clear that the intensities are not proportional to the amount of bisANS added. Binding data, presented in the preceding paper (Ward et al., 1994), permitted calculation of the amount of bisANS bound under these conditions. Comparison with the spectrum of free bisANS permits deduction of the spectrum of tubulin-bound bisANS. The overlap integral for this pair, calculated by numerical integration over 4 nm intervals, was 2.70×10^{-14} M^{-1} cm^3. Use of the quantum yield for allocolchicine, Q_D = 0.2 (Ward & Timasheff, 1988), resulted in a value of R_0 = 30.5 Å. Energy transfer efficiency was measured over a bisANS concentration range of $(0–1.3) \times 10^{-5}$ M in the presence of 1×10^{-5} M tubulin, a concentration range where bisANS is bound predominantly to the high-affinity site. At 1.3×10^{-5} M, 72% of the high-affinity site is occupied. Under these conditions, no energy transfer was observed. Assuming a 5% error in such measurements, the distance of separation between the bisANS and allocolchicine sites can be estimated to be greater than 50 Å.

Energy Transfer Studies between BisANS and Ruthenium Red. The overlap of the fluorescence emission spectrum of bisANS and the tubulin-bound Ruthenium Red absorption spectrum is shown in Figure 4, from which an overlap integral of 2.19×10^{-13} M^{-1} cm^3 can be calculated by numerical integration over 4 nm intervals. This, in combination with the determined quantum yield of bisANS when it is bound to the tubulin–colchicine complex, Q_D = 0.233, resulted in a value of 44 Å for R_0. Fluorescence energy transfer was measured by titrating the tubulin–colchicine–Ruthenium Red complex with $(1–5) \times 10^{-6}$ M bisANS and comparing the observed fluorescence with that of the tubulin–colchicine complex when titrated with bisANS over the same concentration range. The results, shown in Figure 7, indicate that essentially no energy transfer takes place. If a 5% error is assigned to the measurements, these results lead to the conclusion that the bisANS and Ruthenium Red sites must be separated by a distance greater than 72 Å.

DISCUSSION

The present fluroescence energy transfer distance measurements have established the separations between the colchicine binding site, or more specifically the binding subsite of colchicine ring C, and the chromophoric markers Ruthenium Red and strongly bound bisANS, as well as the distance between the two markers. What are these sites on the tubulin molecule? All three binding sites are independent of each other, since there is no mutual positive or negative cooperativity in their bindings. Let us first take Ruthenium Red.

Ruthenium Red forms a 1:1 stable complex with tubulin, that can be separated from the free Ruthenium Red by gel filtration (Figure 1). The binding to tubulin shifts its absorption maximum from 535 to 538 nm (Deinum et al., 1981) and generates a difference spectrum (Figure 2). This shift is similar to that observed when Ruthenium Red binds to mitochondrial membranes (Reed & Bygrave, 1974), the binding locus having been assigned to negatively charged phospholipids. Being a polycation (Fletcher et al., 1961), Ruthenium Red most probably binds to tubulin via an electrostatic mechanism. That the site of interaction is not the high-affinity metal ion binding site associated with the exchangeable GTP is indicated by the fact that the high-affinity Ruthenium Red can be removed from tubulin by passage through a Chelex cation exchange column, while the high-affinity metal ion is not affected by this treatment (Monasterio, 1987; Ward & Timasheff, 1988). The ease of removal of Ruthenium Red also indicates that its binding site is fully exposed to solvent, which is not the case with the GTP-associated metal ion binding site (Ward & Timasheff, 1988). Deinum et al. (1981) have proposed that Ruthenium Red binds to one of the highly negatively charged carboxy-terminal domains of tubulin (Ponstingl et al., 1981). This proposal is supported by the observation (Kanazawa & Timasheff, 1989) that removal of these regions by limited proteolysis greatly reduced the affinity of Ruthenium Red for tubulin. The observation that Ruthenium Red acts as an inhibitor of microtubule assembly (Deinum et al., 1981) is also consistent with the binding occurring at these negatively charged regions of tubulin. Limited proteolysis with removal of the C-terminal fragments has been reported to increase the ability of tubulin to polymerize, although the structures may be different from microtubules (Serrano et al., 1984b; Bhattacharyya et al., 1985; Kanazawa & Timasheff, 1989). Similar studies have led to the conclusion that these C-terminal peptides contain the binding sites for the microtubule-associated proteins (MAPs)[1] (Serrano et al., 1984a; Maccioni et al., 1988) which promote microtubule assembly, as do a number of other polycationic molecules (Erickson & Voter, 1976; Lee et al., 1978). The fact that Ruthenium Red acts as an inhibitor indicates that, in addition to electrostatic interactions, other factors also play a role in these actions. Since only one molecule of Ruthenium Red binds to a tubulin $\alpha\beta$ dimer, which subunit contains the binding site? If it is accepted that the binding does occur at a carboxy-terminal domain, the measured spatial separation between the colchicine and bisANS sites and the Ruthenium Red site may help to answer this question. The energy transfer experiments reported in this study have established the distance between the colchicine and Ruthenium Red Sites as 40–45 Å on the tubulin dimer. Colchicine has been shown (Shearwin & Timasheff, 1994) to bind to the β-subunit close to the $\alpha\beta$ interface. Since the intersubunit contacts appear to be predominantly between the N-terminal domain of the α-subunit and the C-terminal domain of the β-subunit (Kirchner & Mandelkow, 1985), and in view of the report that colchicine binding affects the highly negatively charged C-terminal region of the β-subunit (Sackett & Varma, 1993) while it has no effect on the binding of Ruthenium Red, the measured distance makes it unlikely that Ruthenium Red could be bound to the C-terminal carboxyl-rich peptide of the β-subunit. This would locate its binding site on the similar domain of the α-subunit, which would place it at a locus distant from the $\alpha\beta$ interface.

Further results of the present study are that the colchicine and high-affinity bisANS sites are separated by more than 50 Å, while the distance between Ruthenium Red and bisANS is greater than 72 Å. If we consider the dimensions of the tubulin molecule as deduced from small-angle X-ray scattering studies (Bordas et al., 1983; Andreu et al., 1992), the tubulin subunits appear to be elongated with axial dimensions of 7–8 nm and 5–5.5 nm. In assembled structures, the center to center distance between subunits is ca. 4.0 nm along a protofilament (Andreu et al., 1992). These dimensions can easily accommodate the intersite distances by placing the Ruthenium Red and bisANS sites on different subunits, while the colchicine site is located between them. The separation of colchicine and bisANS suggests that the bisANS site is at a locus on the β-subunit distal from the $\alpha\beta$ interface. If it is assumed that the high-affinity bisANS binding site is identical with the ANS binding site, this would place it at a distance >28 Å from the high-affinity divalent cation in the E-site of

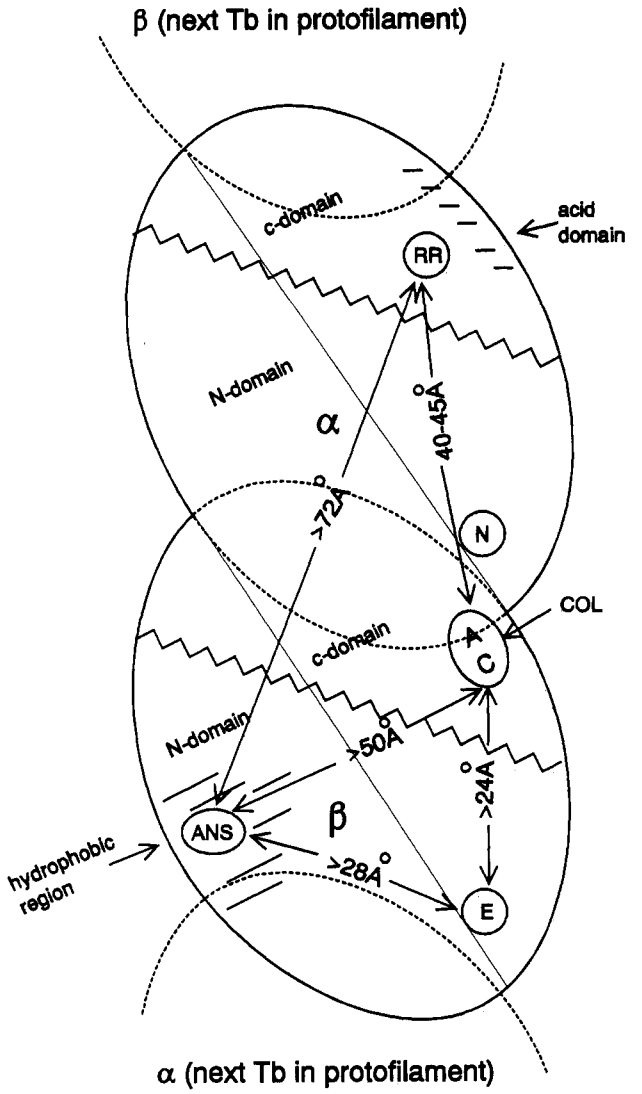

FIGURE 8: Two-dimensional schematic diagram of the relative locations of various binding sites on the $\alpha\beta$-tubulin dimer constructed on the basis of (i) distance measurements, (ii) cross-linking and peptide mapping, and (iii) the deduced chemical environment of the various binding sites. The tubulin subunits were drawn from their deduced structure within microtubules as determined by small-angle X-ray scattering (Andreu et al., 1992). The smoothed contours represent the projection viewed from outside a microtubule. The thin straight lines are the long axes of the tubulin subunits arranged diagonally relative to the axis of an assembled protofilament. The two characterized triangles are drawn coplanar in this two-dimensional diagram. Their relative orientations in the three-dimensional structure are, obviously, not known.

tubulin. The results of the distance measurements combined with the various conclusions and proposals on the nature of the sites found in the literature (see the introduction) are summarized on the schematic model of the tubulin dimer drawn in Figure 8. This is a very simple planar representation of the protein molecule whose only purpose is to help visualize some of the currently known as well as proposed or postulated features of the binding sites in question. It is possible to define two triangles of binding sites (colchicine–bisAns–E-site and colchicine–bisANS–Ruthenium Red). The colchicine–bisANS side acts as the hinge between them. The dihedral angle between their planes is not known. For simplicity, in the diagram, they are represented as coplanar. It is true that tubulin has one more axis perpendicular to the plane of the drawing. Its dimension (7–8 nm) (Andreu et al., 1992), together with the proper dihedral angle, would permit us to accommodate all of the sites within the β-subunit. This would require in turn a reexamination of some of the proposals found in the literature (see the introduction). The paucity of current knowledge, however, does not warrant a more detailed structural analysis.

In the calculation of the distances, it has been assumed in all cases that the orientation factor $K^2 = {}^2/_3$, i.e., that the donor and acceptor can assume all orientations in the lifetime of the excited state. Theoretically, K^2 can assume any value between 0 and 4 (Fairclough & Cantor, 1978) depending on the mutual orientation of the donor and acceptor. This could lead to large errors in the calculated distances. Matsumoto and Hammes (1975) have shown, however, that the error in assuming $K^2 = {}^2/_3$ becomes less than 10% if it is assumed that one member of the pair has some degree of rotational mobility. In the present study, both Ruthenium Red and bisANS would seem to have sufficient mobility. The carboxy-terminal domains of tubulin, and in particular that of the α-subunit, which is the proposed locus of Ruthenium Red binding, would be predicted to possess a fair degree of mobility based on the high proportion of acidic amino acids in these regions (Ponstingl et al., 1981), and their high susceptibility to proteolytic attack. This is supported by NMR data which indicate that the carboxy-terminal domains are relatively mobile (Ringel & Sternlicht, 1984). The bisANS site also appears to be in a flexible region of the molecule as indicated by fluorescence polarization studies (Prasad et al., 1986b). That an error in assignment of the orientation factor is not generating a large error in the measured distance between the colchicine and Ruthenium Red sites is supported by the fact that two distinct fluorophores (colchicine and allocolchicine) gave the same measured distance.

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