Eur. J. Biochem. 216, 757–761 (1993)

A study of colchicine tubulin complex by donor quenching of fluorescence energy transfer

Anusree BHATTACHARYYA[1], Bhabatarak BHATTACHARYYA[2] and Siddhartha ROY[1]

[1] Department of Biophysics and
[2] Department of Biochemistry, Bose Institute, Calcutta, India

(Received February 25/May 27, 1993) – EJB 93 0299/2

The utility of collisional quenching of energy donors in fluorescence energy transfer is described. In multi-donor single acceptor systems, which contain different classes of donors (as distinguished by their accessibility towards a collisional quencher), donor quenching may be used to assess the fraction of energy transfer from each class of donor. The tubulin-colchicine complex was used as a donor-acceptor system to show that two inaccessible tryptophans are at or near the colchicine binding site.

Tubulin (a heterodimer $\alpha\beta$), the major component of microtubules, binds the plant alkaloid colchicine, specifically and quasi-irreversibly at a single site and inhibits tubulin polymerization [1]. In spite of the intensive study of colchicine–tubulin interaction, there is no consensus about the location of the colchicine binding site on tubulin. Some studies have placed the colchicine binding site on the α subunit [2], some on the β subunit [3] and some at the interface [4]. Luduena and co-workers [5] have used cross linkers to cross-link two sulfhydryl groups which are at the colchicine binding site. They have shown that these two sulfhydryl groups are protected from chemical modification by colchicine and its analogs and identified them as Cys239 and Cys354 of the β subunit [6, 7].

One approach for localizing a binding site on a protein is to measure distances from fixed points in proteins by fluorescence energy transfer. Colchicine, which is non-fluorescent in solution, fluoresces upon binding to tubulin [8]. Its excitation spectrum overlaps well with the emission spectrum of tryptophan, thus forming a good donor–acceptor pair. The presence of eight tryptophans in tubulin, however, makes it difficult to have even a qualitative assessment of the involvement of various tryptophan residues in energy transfer to bound colchicine.

Donor quenching of fluorescence energy transfer has previously been used to estimate distance distribution between a donor–acceptor pair [9]. In this experiment, a collisional quencher is used which quenches donor fluorescence causing a reduction in R_0 and hence energy transfer efficiency. A study with model peptide and protein with single indole and dansyl moieties has firmly established the feasibility of this approach [9]. In a multi-tryptophan protein, where classes of tryptophan residues differ in accessibility towards a collisional quencher, donor quenching may be used to assess the degree of energy transfer from different classes. We have explored this concept in the colchicine-tubulin complex using acrylamide as the collisional quencher.

Correspondence to S. Roy, Department of Biophysics, Bose Institute, P 1/12, C. I. T. Scheme VII M, Calcutta, India 700 054

Abbreviations. LADH, liver alcohol dehydrogenase; Me_2SO, dimethyl sulfoxide; IAEDANS, 5-[2-(Iodoacetyl)aminoethylamino]-naphthalene-1-sulfonic acid.

EXPERIMENTAL PROCEDURES

Materials

Horse liver alcohol dehydrogenase (LADH), NADH and colchicine were purchased from Sigma Chemical Co. (St Louis, Mo, USA). The enzyme was dialyzed twice against 0.1 M potassium phosphate pH 7.5 overnight at 4°C before use. Tubulin was prepared from goat brain by two cycles of temperature-dependent polymerization in 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 7.0 containing 1 mM GTP [10]. Podophyllotoxin was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and was recrystallized five times from ethanol before use. Succinimide was of analytical grade and recrystallized from alcohol/water. Four-times-recrystallized acrylamide was purchased from Spectrochem (India). 5-[2-(Iodoacetyl)-aminoethyl]aminonaphthalene-1-sulfonic acid (IAEDANS) was purchased from Molecular Probes Inc. (Eugene, OR, USA). All other reagents were of analytical grade.

Fluorescence methods

All fluorescence experiments were done in a Hitachi F-3000 spectrofluorometer at ambient temperatures, unless mentioned otherwise. Donor quenching of LADH/NADH/Me_2SO complex was done with 5 μM LADH, 5 μM NADH in 0.1 M phosphate pH 7.5 containing 0.5% dimethylsulfoxide (Me_2SO). At those concentrations, most of the NADH and LADH is associated as a ternary complex (K_d = 0.1 μM) [11]. The excitation wavelength was at 295 nm and emissions at 340 nm and 525 nm were noted. The 525-nm emission was chosen for energy-transfer measurements because there is very little emission from direct excitation of tryptophan. The blank fluorescence values from a control containing

0.5% Me_2SO and appropriate concentrations of succinimide was subtracted from each value.

Fluorescence experiments with colchicine-tubulin complex were done at 5 μM colchicine and 1 μM tubulin; 50 μM colchicine and 10 μM tubulin were incubated at 37 °C for 1 h. The complex was then diluted in 0.1 M Pipes pH 6.9 containing 0.5 mM $MgCl_2$ to appropriate concentrations. The excitation wavelength was 295 nm and emission wavelength was 525 nm. All fluorescence values from quenching experiments were corrected for dilution and inner-filter effect. The formula used for the inner-filter effect was

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

Energy transfer efficiency, E, was calculated from excitation spectra using following equation [12]

$$F_{D+A}/F_A = 1 + \varepsilon_D c_D/\varepsilon_A c_A\, E$$

where $F_{D+A}$is the fluorescence of the donor-acceptor (i.e. protein-ligand) complex, and F_A is the fluorescence of the acceptor (in this case the ligand) only at the same wavelenghts; ε_A is the absorption coefficient of the acceptor, ε_D is the absorption coefficient of the donor and c_A and c_D are the concentrations of the acceptor and the donor respectively. Since free colchicine is not fluorescent in solution, F_A, i.e. acceptor-only fluorescence, cannot be determined directly. The absorption spectrum of a fluorophore, however, is identical to its excitation spectrum and can be substituted for its excitation spectrum. The absorption spectrum of the acceptor (i.e. the ligand) bound to the protein was taken as the excitation spectrum of the acceptor without donor. The absorption spectrum of the acceptor was obtained by subtracting the absorption spectra of the protein from that of the protein-ligand complex at the same protein concentrations. F_A was calculated after normalizing and matching absorption and excitation spectra at 350 nm. c_D/c_A is assumed to be the fractional occupancy of the ligand. This was calculated from a Scatchard plot for binding of colchicine to tubulin ($n = 0.6$). Such sub-stoichiometric binding of colchicine to tubulin is well known [8, 13]. Absorption coefficients of tryptophan and colchicine at 295 nm were calculated as 2200 and 3664 $M^{-1}cm^{-1}$ using the standard absorption coefficient values from [14] and [15], respectively.

The distance between colchicine and inaccessible tryptophans of the tubulin was determined by the following equation

$$R = R_o \cdot (E^{-1} - 1)^{1/6}$$

where R is the distance between donor and the acceptor, R_o is the distance where the energy transfer efficiency is 50% and E is the energy transfer efficiency. R_o is given by:

$$R_o = (J\kappa^2 Q\eta^{-4})^{1/6} \cdot (9.79 \times 10^3)\ \text{cm}$$

where J is a measure of spectral overlap (the overlap integral), Q is the quantum yield of the donor, η is the refractive index and κ is the orientation factor. The overlap integral was calculated according to the method of Wu and Stryer [16]. Value of κ^2 was taken for random orientation i.e. 2/3. The quantum yield of tryptophan was taken as 0.2 [12].

Chemical modification

Tubulin (1.5 mg/ml) was incubated with a 10-fold molar excess of 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone, at room temperature for 5 min. A 30-fold molar excess of *N*-ethylmaleimide was then added and the mixture incubated at 4 °C for 20 min in the dark. The reaction was then quenched with 2-mercaptoethanol (100-fold molar excess over *N*-ethylmaleimide). The reaction mixture was then dialyzed against 20 mM phosphate pH 7.0 for 2 h with four changes. The dialyzed solution was then divided into two equal parts. To one part 10-fold molar excess of IAEDANS and to another part, 10-fold molar excess of iodoacetamide was added. The reactions were then incubated for 20 min at 4 °C in the dark before being loaded onto a Sephadex G-15 column (1 × 18 cm). The modified tubulin was eluted with 20 mM phosphate pH 7.0. Protein concentration was measured by the method of Lowry [17]. Incorporation ratio of IAEDANS-labeled tubulin was calculated using a molar absorption coefficient of $6.3 \times 10^3\ M^{-1}cm^{-1}$ for IAEDANS at 337 nm [18]. $MgCl_2$ was added before acrylamide quenching experiments to a final concentration of 0.5 mM.

Theory

We will refer to the collisional quencher, which quenches the fluorescence of the energy donor but not that of the acceptor, as a donor quencher. The energy transfer efficiency of a donor-acceptor pair in the absence of a donor quencher (E_o) and in its presence (E) may be defined as [12],

$$E_o = \frac{k_T}{k_T + k_F^D + k_{ic}^D + k_{is}^D} \tag{1}$$

and

$$E = \frac{k_T}{k_T + k_F^D + k_{ic}^D + k_{is}^D + k_q[Q]} \tag{2}$$

where k_T is the rate constant for energy transfer, k_F^D is the rate constant for fluorescence of the donor, k_{ic}^D is the rate constant for the internal conversion of the donor, k_{is}^D is the rate constant for the intersystem crossing of the donor, k_q is the bimolecular rate constant for the collisional quenching of the donor and [Q] is the collisional quencher concentration.

Combining Eqns (1) and (2), we obtain

$$E_o/E = 1 + \tau_{D,A} k_q[Q] \tag{3}$$

where $\tau_{D,A}$ is the lifetime of the donor in the presence of the acceptor, but in the absence of the donor quencher.

If the energy transfer efficiency is measured at a fixed emission wavelength, where no significant fluorescence of the donor is seen, and at a fixed excitation wavelength, where the donor has substantial absorption, then in the absence of donor quencher [12]

$$\frac{F_{D+A}^o}{F_A^o} = 1 + \frac{\varepsilon_D C_D}{\varepsilon_A c_A} \cdot E_o \tag{4}$$

where F_{D+A}^o is the fluorescence of the donor-acceptor complex and F_A^o is the fluorescence of the acceptor only, in the absence of donor quencher. ε_A and c_A are the absorption coefficient and concentration of the acceptor, respectively, and ε_D and c_D are the absorption coefficient and concentration of the donor, respectively.

Since acceptor-only fluorescence is not affected in the presence of the donor quencher, then in the presence of the quencher

$$\frac{F_{D+A}^o}{F_A^o} = 1 + \frac{\varepsilon_D C_D}{\varepsilon_A c_A} \cdot E \tag{5}$$

where F_{D+A} is the fluorescence of the donor-acceptor complex in the presence of the quencher.

Combining Eqns (4) and (5) and simplifying,

$$\frac{F^{\circ}}{F} = \frac{F^{\circ}_{D+A} - F^{\circ}_{A}}{F_{D+A} - F^{\circ}_{A}} = \frac{E^{\circ}}{E} = 1 + \tau_{D.A} \cdot k_q \cdot [Q] \qquad (6)$$

provided the acceptor is not quenched by the collisional quencher.

Eqn (6) formally resembles the Stern-Volmer equation. Thus in a multi-donor, single-acceptor system, the equation may be recast in the form of a Lehrer equation [19].

$$F_o/(F_o - F) = 1/f_a + 1/(f_a \cdot K_{av} \cdot [Q])$$

where f_a is the contribution of total fluorescence of the emission which is quenched.

A Lehrer plot of $F^{\circ}/(F^{\circ} - F)$ versus 1/[Q], where emission and excitation wavelengths are chosen such that direct fluorescence of the donor is negligible compared to the energy transfer, would reveal the proportion of energy transfer from quenchable and non-quenchable donors.

RESULTS AND DISCUSSION

The utility of donor quenching of fluorescence energy transfer in estimating distance distribution and dynamics had been demonstrated by Gryczynski et al. [9]. They utilized systems which contain single donor/acceptor pairs. The situations are more complex in multi-donor/single-acceptor systems, which are commonplace in natural macromolecules. We have studied horse liver alcohol dehydrogenase (LADH) and its ternary complex with NADH and Me_2SO as a model system for donor quenching of fluorescence energy transfer in a multi-donor/single-accpetor system. LADH is a dimer of identical subunits. Each subunit has two tryptophan residues, Trp15 and Trp 314, whose fluorescence properties have been thoroughly investigated [11, 20]. Trp15 is accessible to collisional quenchers and Trp314 is completely buried and inaccessible. In the ternary complex with NADH and Me_2SO, the fluorescence energy transfer to bound coenzyme NADH occurs primarily, if not exclusively, from Trp314 [20]. It is thus anticipated that there will be very little donor quenching of tryptophan−NADH energy transfer. We have chosen succinimide (which does not quench NADH fluorescence) as the collisional quencher, since the acceptor NADH fluorescence is quenched by the two commonly used collisional quenchers, acrylamide and iodide. Fig. 1 shows the plot of F°/F versus [succinimide] for quenching of fluorescence energy transfer. The plot is absolutely flat, having zero slope, indicating no donor quenching of fluorescence energy transfer. The fluorescence energy transfer to bound coenzyme NADH causes quenching of tryptophan fluorescence. If Trp314 is mainly involved in energy transfer and consequent quenching, the residual emission of the LADH/NADH/Me_2SO complex, primarily coming from Trp15, should be quenchable to a greater extent than in the free enzyme. The inset shows the Lehrer plot of quenching of tryptophan fluorescence of free LADH and LADH/NADH/Me_2SO complex by succinimide. The intercept on the y-axis is 2.1 for the free enzyme and 1.5 or the complex. The relative contribution of the Trp314 emission to the total emission spectra of LADH at 340 nm decreases from 53% to 33%. This is fully consistent with a large energy transfer from and differential quenching of Trp314.

Tubulin has eight Trp residues, some of which are inaccessible to collisional quenchers [21]. Bane et al. [22] and Garland [23] have previously noted that binding of colchicine leads to significant quenching of tryptophan fluorescence. Fig. 2 shows the emission spectra of tubulin (I), tubulin-colchicine complex (II) and the difference sprectra (III), when excited at 295 nm. The emission maximum of the free protein (I) is at 336.8 nm. The tryptophan emission of the colchicine-tubulin complex (II) is significantly quenched compared to the free protein. The tryptophan emission maximum of colchicine-tubulin complex shows a slight red-shift

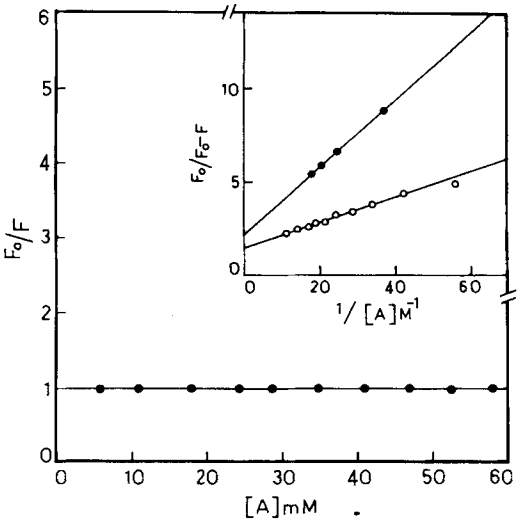

Fig. 1. Donor quenching of tryptophan to NADH energy transfer of HLAD/NADH/Me_2SO complex by succinimide. The quenching was done in 0.1 M potassium phosphate pH 7.0 containing 5 μM LADH, 5 μM NADH, 0.5% Me_2SO at 25°C. The excitation wavelengh was 295 nm and the emission wavelength was 525 nm. The inset shows the Lehrer plot of quenching of free LADH (●) and LADH/NADH/Me_2SO complex (○) by succinimide. The conditions were the same as above, except the emisseion wavelength was 340 nm. A = succinimide (quencher).

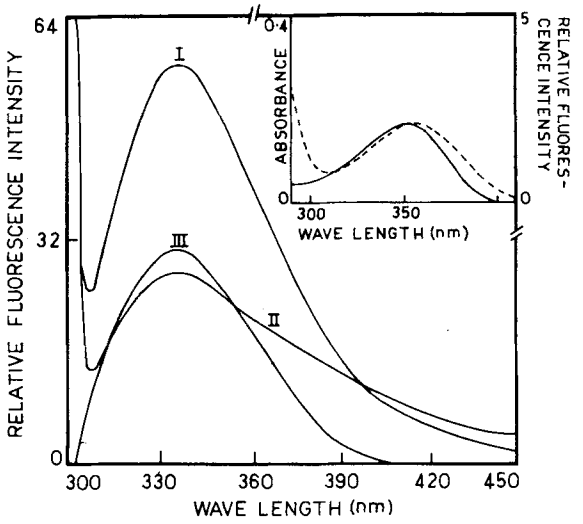

Fig. 2. Fluorescence emission spectra of 1 μM tubulin (I), colchicine-tubulin complex (1 μM/5 μM) (II) and the difference spectra (III = I–II). The excitation wavelength was 295 nm. The solution conditions were 0.1 M Pipes pH 6.9 containing 0.5 mM $MgCl_2$ at 25°C. The inset compares the absorption spectrum of colchicine (——) with the excitation spectrum of colchicine-tubulin complex (- - -). The emission wavelength was 525 nm and rest of the conditions were as above.

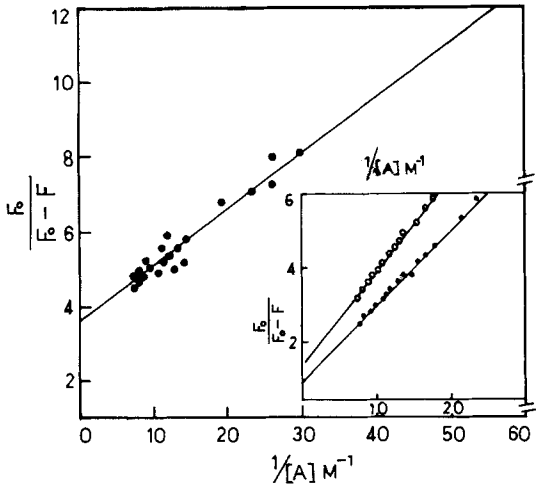

Fig. 3. Donor quenching of fluorescence energy transfer of 1 μM colchicine-tubulin complex by acrylamide. The emission wavelength was 525 nm and the fluorescence was monitored at the excitation wavelength of 295 nm. The solution contitions were same as in Fig. 2. The inset shows the Lehrer plot of free 1 μM tubulin (○) and 1 μM tubulin-colchicine complex (●). A = acrylamide (quencher).

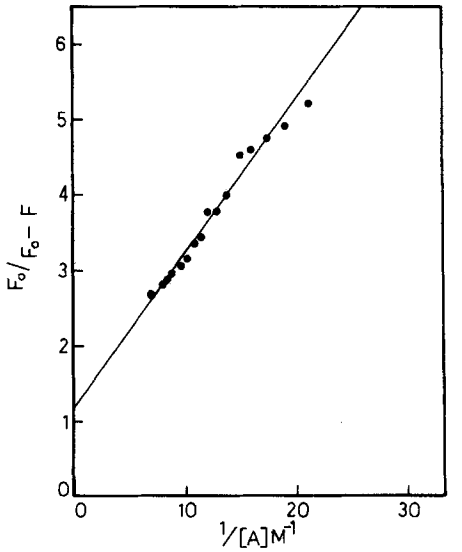

Fig. 4. Lehrer plot of acrylamide quenching of tubulin-podophyllotoxin (2 μM/4 μM) complex. The solution conditions were 0.1 M Pipes pH 6.9 containing 0.5 mM $MgCl_2$ at 25°C. The emission wavelength was 340 nm and the fluorescence values were recorded at the excitation wavelength of 295 nm. A = acrylamide (quencher).

(337.4 nm). This indicates preferential quenching of tryptophan residues that have shorter wavelength emission maxima. The degree of quenching was estimated to be approximately 35% at 340 nm, after making correction for the inner-filter effect. This value is similar to that obtained by Bane et al. [22].

The emission spectrum of tubulin-colchicine complex shows a higher intensity at wavelenghts longer than 400 nm, when compared to the tubulin-only spectrum. The long-wavelength component is likely to be due to either direct excitation of tubulin-bound colchicine or energy transfer from tryptophan to colchicine or both. The energy transfer efficiency was estimated by comparing the absorption spectrum of colchicine and the excitation spectrum of the tubulin-colchicine complex (see Experimental procedures). The superimposition of the absorption spectrum and excitation spectrum is shown in the inset. The calculated energy transfer efficiency is 2.8. For a single donor-acceptor pair, the maximum energy transfer efficiency (as given in Eqn 4) is 1. In the case of proteins, where an unknown number of tryptophan residues can participate as energy donors, the transfer efficiency can exceed 1 (since we use the molar absorption coefficient of tryptophan and not that of the whole protein). Indeed, in cases where E exceeds 1, it may be taken as an evidence of energy transfer from more than one tryptophan residue.

We have used acrylamide as a quencher to study the donor quenching of the colchicine-tubulin complex. Acrylamide is a suitable collisional quencher in this case since it does not quench colchicine fluorescence in the colchicine-tubulin complex (data not shown). The inset of Fig. 3 shows the Lehrer plot of quenching of tryptophan fluorescence by acrylamide in tubulin and tubulin-colchicine complex. The tubulin-only plot is linear and cuts the y axis at 1.3. Thus, from the Lehrer plot, we estimate that approximately 25% of the tryptophan fluorescence in tubulin is not quenchable by acrylamide. The most likely interpretation is that two tryptophan residues out of eight are inaccessible. Donor quenching of fluorescence energy transfer from tryptophan to colchicine is shown in a Lehrer-type plot in Fig. 3. The extrapolated intercept value on the y axis is 3.3, indicating only 30% of the fluorescence energy transfer to colchicine occurs from tryptophans that are quenchable by low concentrations of acrylamide. It is thus anticipated that the tryptophans which are not quenchable by acrylamide should be quenched by energy transfer to colchicine to a much greater extent than the others. Thus, a Lehrer plot of quenching of tryptophan emission in the colchicine-tubulin complex should have an intercept close to 1. The inset shows the Lehrer plot of quenching of tryptophan emission of the colchicine-tubulin complex. In contrast to free tubulin (intercept 1.3), the colchicine-tubulin complex yields an intercept of 1.

If the two inaccessible tryptophans are contributing 70% of the energy transfer, then energy transfer efficiencies must be very high for both tryptophans ($2.8 \times 0.7 = 1.96$). R_o for energy transfer from tryptophan to colchicine is 2.8 nm. Although it is hard to obtain a precise distance estimate for such very high energy transfer efficiencies, it is likely that the distances between bound colchicine and the two tryptophans are in the order of 1.0−1.5 nm or less. Although the precise size of the tubulin molecule is not known, one can calculate a diameter of approximately 6.5 nm, assuming that the tubulin molecule is a perfect sphere (assuming a partial specific volume of 0.73 $cm^3\ g^{-1}$ and a molecular mass of 110 kDa. Thus it is likely that both the tryptophan residues are situated very close to the colchicine binding site.

To show that binding of colchicine is not changing the accessibilities of the tryptophans significantly, we have used the podophyllotoxin-colchicine complex as a control for the colchicine-tubulin complex. Podophyllotoxin is a colchicine analog that binds specifically to the colchicine binding site, reversibly [24]. Its binding constant is comparable to that of colchicine, i.e. 0.5 μM [25]. It lacks, however, the 340-nm absorption band of colchicine and is not fluorescent when bound to tubulin. Thus, its binding to the colchicine site is not expected to result in fluorescence energy transfer from tryptophan and consequent quenching of tryptophan emission. Fig. 4 shows the Lehrer plot of quenching of tryptophan fluorescence of tubulin by acrylamide, in the presence of

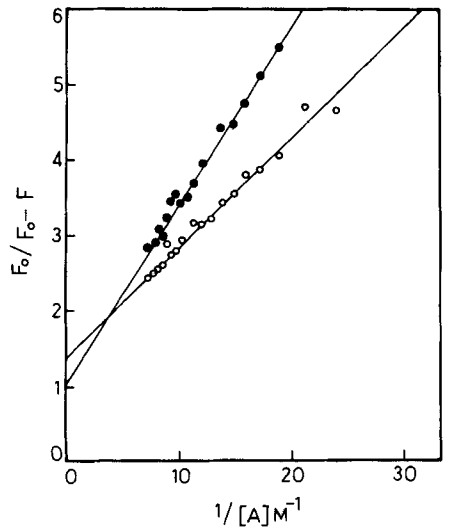

Fig. 5. Lehrer plot of acrylamide quenching of 1 μM acetamido-tubulin (○) and 1 μM AEDNS-tubulin (●). Conditions were identical to that of Fig. 4.

4 μM podophyllotoxin. The plot is linear and when extrapolated cuts the y axis at 1.25. The Stern-Volmer constant (K_{sv}) derived from the slope of the plot is 3.5 as compared to 3.3 for the free tubulin. This indicates that fluorescence and accessibility properties of the tryptophans remain unchanged in the podophyllotoxin-tubulin complex. To show that acrylamide is not affecting podophyllotoxin binding to tubulin, we have also measured podophyllotoxin binding to tubulin at 150 mM acrylamide by small (5%) but reproducible quenching of the tryptophan fluorescence of tubulin. The result indicated that podophyllotoxin binding to tubulin is not affected significantly up to 150 mM acrylamide (data not shown).

The conclusions reached above would be considerably strengthened if a verification by an independent method is demonstrated. Recently, Luduena and co-workers have demonstrated that colchicine and its analogs protect two sulfhydryl groups from modification by sulfhydryl reagents [5–7]. They have concluded that these two sulfhydryl groups are at the colchicine binding site. If those two sulfhydryl groups could be labeled with a fluorescent probe whose absorption spectra overlap well with the emission spectra of tryptophan, then the two putative tryptophans at the colchicine binding site may be preferentially quenched. We have used a reversible analog of colchicine [26] [2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone], to protect the two sulfhydryl groups while modifying the rest with a non-fluorescent maleimide (*N*-ethylmaleimide). The colchicine analog was then removed by exhaustive dialysis and the remaining two sulfhydryl groups were labeled with IAEDANS. The excess reagent was then removed by gel filtration. A control experiment was done similarly, only iodoacetamide was used to label the protected sulfhydryl groups instead of IAEDANS. Fig. 5 shows the Lehrer plot of the IAEDANS- and iodoacetamide-labeled tubulin. The intercepts on the y axis are 1.1 for IAEDANS-labeled tubulin and 1.4 for iodoacetamide-labeled tubulin. This indicates a preferential quenching of the buried tryptophans in the IAEDANS-labeled tubulin. Thus we may conclude that the sulfhydryls at the tubulin binding site are close to the two hidden tryptophans, reinforcing the conclusion that two buried tryptophans are at or near the binding site of colchicine.

In conclusion, we have shown that donor quenching of fluorescence energy transfer may be used in multi-tryptophan proteins to detect the degree of energy transfer from different classes (classified according to accessibility towards a collisional quencher) of tryptophans. The method has been applied to a protein containing eight tryptophan residues, tubulin, to show that two inaccessible tryptophans are close to the colchicine binding pocket.

We thank the Council of Scientific and Industrial Research (India) for a research fellowship to Anusree Bhattacharya.

REFERENCES

1. Olmsted, J. B. & Borisy, G. G. (1973) *Biochemistry 12*, 4282–4289.
2. Serrano, L., Avila, J. & Maccioni, R. B. (1984) *J. Biol. Chem. 259*, 6607–6611.
3. Wolff, J., Knipling, L., Cahnman, H. J. & Palumbo, G. (1991) *Proc. Natl Acad. Sci. USA 88*, 2820–2824.
4. Schmitt, H. & Atlas, D. (1976) *J. Mol. Biol. 102*, 743–758.
5. Little, M. & Luduena, R. F. (1985) *EMBO J. 4*, 51–56.
6. Luduena, R. F. & Roach, M. C. (1981) *Biochemistry 20*, 4437–4444.
7. Luduena, R. F. & Roach, M. C. (1981) *Biochemistry 20*, 4444–4450.
8. Bhattacharyya, B. & Wolff, J. (1974) *Proc. Natl Acad. Sci. USA 71*, 2627–2631.
9. Grychynski, I., Wiczk, W., Johhnson, M. L., Cheung, H. C., Wang, C. & Lakowicz, J. R. (1988) *Biophys. J. 54*, 577–586.
10. Sloboda, R. D. & Rosenbaum, J. L. (1982) *Methods Enzymol. 85*, 409–415.
11. Abdallah, M. A., Biellman, J. F., Wiget, P., Kuhn, R. J. & Luisi, P. L. (1978) *Eur. J. Biochem. 89*, 397–405.
12. Cantor, C. C. & Schimmel, P. R. (1980) *Biophysical chemistry*, part II, W. H. Freeman, San Francisco.
13. Bryan, J. (1972) *Biochemistry 11*, 2611–2616.
14. Demchenko, A. P. (1981) *Ultraviolet spectroscopy of proteins*, Springer-Verlag, Berlin.
15. Windholz, M. (ed.) (1976) *The Merck index*, 9th edn, p. 318, Merck & Co., Rahway NJ.
16. Wu, C. W. & Stryer, L. (1972) *Proc. Natl Acad. Sci. USA 69*, 1104–1108.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
18. Haugland, R. (1991) *Handbook of fluorescent probes and research chemicals*, p. 22, Molecular Probes Inc., Eugene OR.
19. Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
20. Strambini, G. B. & Connelli, M. (1990) *Biochemistry 29*, 196–203.
21. Mukhopadhyay, K., Parrack, P. & Bhattacharyya, B. (1990) *Biochemistry 29*, 6845–6850.
22. Bane, S., Prett, D., MacDonald, T. L. & Williams, R. C. (1983) *J. Biol. Chem. 259*, 7391–7398.
23. Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.
24. Wilson, L. (1975) *Ann. N. Y. Acad. Sci. 253*, 213–231.
25. Cortese, F., Bhattacharyya, B. & Wolff, J. (1977) *J. Biol. Chem. 2523*, 1134–1140.
26. Fitzgerald, T. J. (1976) *Biochem. Pharmacol. 25*, 1383–1387.