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Interaction of Prodan with tubulin

A fluorescence spectroscopic study

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The compound 6-propionyl-2-(*N*,*N*-dimethyl)-aminonaphthalene (Prodan), an efficient fluorescent probe for proteins, is shown to bind to tubulin. Detailed experiments on fluorescence enhancement, anisotropy and energy transfer were carried out to unravel the nature of Prodan-tubulin interaction and the Prodan-binding site on tubulin. It was found that Prodan binds to tubulin at a rigid site, with a stoichiometry of 1:1 and a dissociation constant of 20 μM. Competition experiments using 1,8-[^{3}H]anilinonaphthalenesulfonate ([^{3}H]ANS) indicated that the three aminonaphthalenes, ANS, bis(1,8-anilinonaphthalenesulfonate) and Prodan probably bind to a common region on the tubulin molecule.

The aminonaphthalenes, 1,8-anilinonaphthalenesulfonate (ANS) and bis(1,8-anilinonaphthalenesulfonate) (bis-ANS) (Fig. 1) have been used extensively as fluorescent probes for hydrophobic sites in proteins [1–3]. Several interesting results have been reported on the interaction of these compounds with tubulin. Hydrophobic sites (to which bis-ANS molecules bind) are generated in tubulin, related to ageing of the protein, with little change in the overall conformation [4]. Bis-ANS is an effective inhibitor of microtubule assembly [5].

Prodan (Fig. 1), another aminonaphthalene designed as a better hydrophobic marker [6], has been used in some studies with apomyoglobin and membrane proteins [7, 8]. Absence of a charge and a greater spectral dependence on solvent polarity make Prodan a more attractive probe as compared to ANS or bis-ANS. In a separate study, we have examined the role of different aminonaphthalenes on the inhibition of self-assembly of tubulin into microtubules. It was found that Prodan is as effective as bis-ANS in inhibiting self-assembly of tubulin. According to their inhibitory potentials, the aminonaphthalenes can be arranged as 6-propionyl-2-(*N*,*N*-dimethyl)aminonaphthalene) (Prodan) ≈ bis-ANS > ANS as assembly inhibitors (unpublished results). However, there is no study available in the literature on the interaction of Prodan with tubulin. In view of the structural and functional similarities between bis-ANS and Prodan, the study of Prodan-tubulin interactions assumes importance. Another strong reason for carrying out such a study is the absence of clear data about how bis-ANS inhibits self-assembly. There are several binding sites for bis-ANS on tubulin, with different stoichiometries; the association constants of binding of which also vary.

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Abbreviations. ANS, 1,8-anilinonaphthalenesulfonate; bis-ANS, bis(1,8-anilinonaphthalenesulfonate); Prodan, 6-propionyl-2-(*N*,*N*-dimethyl)aminonaphthalene.

In this paper, we report a detailed study of Prodan-tubulin interactions using fluorescence spectroscopy. The parameters of binding are determined using different methods. Prodan is found to bind to a single site on tubulin with a dissociation constant of 20 μM. Attempts were made to find whether Prodan, bis-ANS and ANS bind to a common site on tubulin. Results of our experiment, using ^{3}H-labeled ANS, point to the sharing of a common region of binding of the three probes.

MATERIALS AND METHODS

GTP, Pipes and EGTA were obtained from Sigma. Phosphocellulose (P11) was from Whatman. ANS, bis-ANS and Prodan were obtained from Molecular Probes (Junction City, USA). All other reagents were analytical grade.

[^{3}H]ANS was a kind gift from Dr Hans J. Cahnmann, National Institute of Health, Bethesda, USA. Specific activity of [^{3}H]ANS was 64 Ci/mol.

Preparation of tubulin

Tubulin was prepared by phosphocellulose column chromatography of microtubule proteins purified from goat brain by two cycles of temperature-dependent polymerisation in Pipes assembly buffer (100 mM Pipes, pH 6.9, 0.5 mM $MgCl_2$, 1 mM EGTA) with 1 mM GTP [9]. Following column chromatography, the protein was concentrated to 5–10 mg/ml using CF 50A membrane cones and stored in liquid nitrogen. Protein concentration was determined by the method of Lowry et al. [10].

Fluorescence measurements

All steady-state fluorescence measurements were made on an F-3000 model Hitachi Spectrofluorometer equipped with a

data processor. The band pass of both excitation and emission monochromators were 5 nm. Binding studies with ANS, bis-ANS and Prodan were performed in buffer A (50 mM Pipes, pH 6.9, 0.5 mM $MgCl_2$, 1 mM EGTA) at room temperature. Purified tubulin was used for titrations in the absence of any microtubule-associated proteins.

Measurement of binding parameters of Prodan-tubulin complex by fluorescence titration

The fluorescence intensity of the tubulin-bound Prodan was assessed by adding increasing amounts of tubulin (0.1 – 15 μM) in a cuvette containing a fixed amount of Prodan (0.5 μM). Extrapolating the straight line obtained from a plot of $1/F$ vs. 1/[tubulin] to infinite tubulin concentration, the value of F_{max} was obtained. This extrapolated fluorescence value was used to determine the concentration of Prodan bound to tubulin. The observed fluorescence values were always corrected for dilution. In a second titration, tubulin at fixed concentration (0.1 μM) was titrated with increasing amounts of Prodan. In both titrations, signal averaging was used to improve the signal-to-noise ratio. Binding parameters were determined by various methods, as given below.

Binding of Prodan to tubulin

The binding of Prodan to tubulin was measured in buffer A at room temperature. The samples were excited at 360 nm and emission was measured at 450 nm. The concentration of Prodan-tubulin complex at each point was determined by the method of Mas and Colman [11] using the F_{max} value obtained from the previous titration. The fluorescence of Prodan was measured in the presence (F) and absence (F_0) of tubulin, and their ratio (F/F_0), was used to calculate $[\text{Prodan}]_{bound}$ from the equation,

$$[\text{Prodan}]_{bound} = \frac{\text{Prodan}_{total}}{Q-1}(F/F_0-1),$$

where $[\text{Prodan}]_{total}$ is the total concentration of Prodan, $[\text{Prodan}]_{bound}$ the concentration of Prodan bound to tubulin and Q is the fluorescence-enhancement factor (for bound Prodan). The concentration of free Prodan was obtained from the difference of $[\text{Prodan}]_{total}$ and $[\text{Prodan}]_{bound}$. The data were analysed according to the Lineweaver-Burk equation [12],

$$1/r = 1/n + K_d/n[\text{Prodan}]_{free},$$

where r represents the molar ratio of Prodan bound/tubulin, n the number of binding sites for Prodan/molecule tubulin, and K_d the dissociation constant for the tubulin-Prodan complex. K_d was determined from the slope of the straight line obtained by plotting $1/r$ against $1/[\text{Prodan}]_{free}$, using the value of n determined from the intercept on the $1/r$ axis.

Dissociation constant for Prodan-tubulin

The dissociation constant for the Prodan-tubulin complex was also determined from fluorescence anisotropy measurements, by plotting $1/A$ against 1/[tubulin], according to [13].

Job plot

The stoichiometry of binding was determined from the Job plot constructed by using the fluorescence induced when Prodan binds to tubulin, keeping the total concentration of Prodan plus tubulin at 5 μM [14]. The samples were excited at 360 nm and emission was observed at 450 nm.

Polarisation measurements

Fluorescence-polarisation measurements were made on F-3000 model Hitachi spectrofluorometer using Hitachi (650-10/40) polarisation accessory. The fluorescence intensity components [I_{vv}, I_{vh}, I_{hv}, I_{hh} in which the subscripts refer to the horizontal (h) or vertical (v) positioning of the excitation and emission polarisers, respectively] were used to calculate the steady-state fluorescence anisotropy (A),

$$A = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh}),$$

where G (I_{hv}/I_{hh}), the grating factor which corrects for the wavelength-dependent distortions of the polarising system, was separately determined under our conditions.

The rotational correlation time (t_c) of Prodan bound to tubulin was determined by measuring the dependence of anisotropy on the solvent viscosity at a constant temperature of 25 °C. Measurements in solvents of various viscosities were achieved by repeating the experiments in solutions containing increasing concentrations of sucrose, upto 61.5 g/100 ml. A plot of $1/A$ against T/η, where T is the absolute temperature and η the viscosity of the solvent, gives the value A_0 of the limiting anisotropy, upon extrapolating to $T/\eta = 0$. 12 μM protein was complexed with 1 μM Prodan, the concentrations chosen to allow 80% binding of the probe at 25 °C. The binding of Prodan remained unaffected by the addition of sucrose. The rotational correlation time T_c was determined from the Perrin equation:

$$1/A = 1/A_0 \cdot (1 + t_f/t_c) = 1/A_0 \cdot (1 + t_f kt/\eta V_h). \quad [15]$$

Fluorescence lifetimes (t_f) were measured in the picosecond single-photon-counting apparatus at the Physical Chemistry Department of the Indian Association for the Cultivation of Science, Calcutta, as described in [16].

[^{3}H]ANS binding to tubulin

To study [^{3}H]ANS binding to tubulin, Sephadex G-25 column of 1.2 cm × 0.9 cm (spin-x, Coaster, USA) was used. A complex of 2 μM tubulin and 2 μM [^{3}H]ANS was formed in buffer A. 50 μl complex was passed through the Sephadex column and the material eluted from the column was taken in a microfuge and centrifuged rapidly for 10 – 12 s. The sample was collected from the outer tube for measurement of radioactivity. Under this condition [^{3}H]ANS was found in elute only when tubulin was present. Addition of increasing concentrations of unlabeled ANS to the [^{3}H]ANS-tubulin complex was found to result in progressive decrease of the bound [^{3}H]ANS in the eluate. Addition of increasing concentrations of bis-ANS or Prodan in the [^{3}H]ANS-tubulin complex was also found to cause progressive lowering of radioactivity in the eluate.

RESULTS

Fluorescence properties of Prodan

A convenient monitor of the interaction of the aminonaphthalene group of drugs with tubulin is the enhanced fluorescence of the bound probe. ANS and bis-ANS has negligible fluorescence emission in buffer when excited at 350 nm and 340 nm, respectively. Unlike these probes, Prodan shows sig-

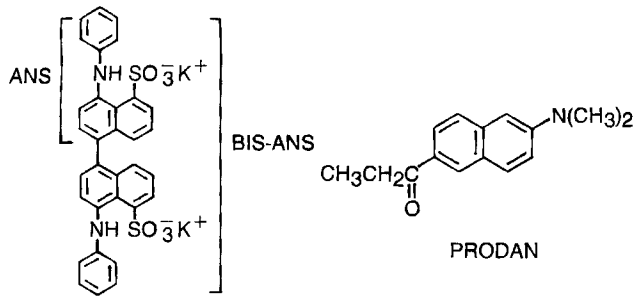

Fig. 1. Structure of anilinophthalenes.

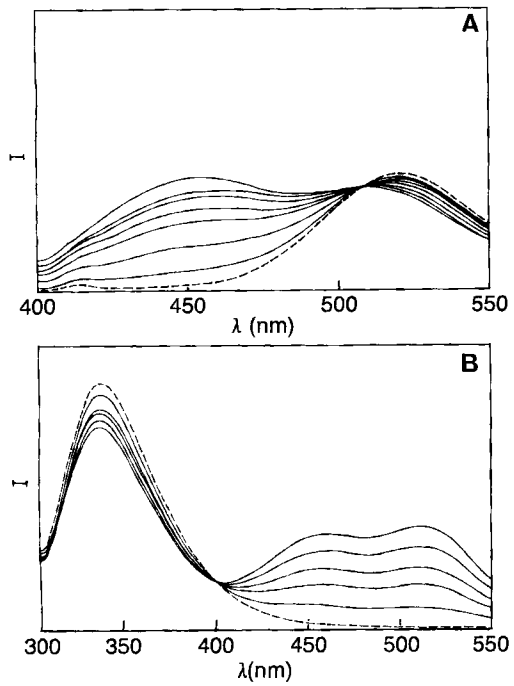

Fig. 2. Fluorescence properties of Prodan. (A) Fluorescence emission spectra of tubulin bound Prodan. The broken line shows the fluorescence spectrum of 0.5 μM Prodan in buffer A. Phosphocellulose-purified tubulin at concentrations 0.25, 0.5, 2, 4.5, 6, 7.5 and 9 μM was mixed with 0.5 μM Prodan and excited at 360 nm. An isoemission point appeared at 507 nm. (B) Energy transfer between tryptophan residues of tubulin and bound Prodan. Tubulin (0.5 mg/ml) in buffer A was titrated with increasing concentrations of Prodan (0.5, 1.0, 1.5, 2.0, and 2.5 μM; top to bottom) and fluorescence-emission spectra recorded. Excitation was at 295 nm. The broken line represents the tryptophan fluorescence-emission spectrum of 0.5 mg/ml tubulin.

nificant intrinsic fluorescence in the range 450 – 580 nm with λ_{max} at 520 nm, upon excitation at 360 nm. Fig. 2A shows the emission spectra of free Prodan in buffer and that of Prodan bound to tubulin. Upon excitation of a solution of Prodan at 360 nm, an emission maximum appears at 520 nm in the buffer. However, there is a significant increase in the fluorescence intensity at 450 nm upon formation of Prodan-tubulin complex, when increased amounts of tubulin are added to a fixed concentration of Prodan (Fig. 2A). It is noteworthy that a single isoemission point appears at 507 nm as the fluorescence intensity at 450 nm increases significantly and that of 520 nm decreases marginally (Fig. 2A). When excited at 295 nm, tubulin shows a characteristic emission maximum at 338 nm for tryptophan. When Prodan is added to tubulin and excited at 295 nm, there is a decrease in emission at 338 nm and a concomitant increase in fluorescence at 450 nm characteristic of bound Prodan (Fig. 2B). This suggests an energy transfer between tryptophans of tubulin and bound Prodan (Fig. 2B). Moreover, the occurrence of an isoemission point (at 407 nm) indicates a single class of binding site of Prodan on tubulin.

An interesting observation on bis-ANS – tubulin interaction was the appearance of hydrophobic sites on the protein as a function of time, leading to binding of several molecules of bis-ANS to a single molecule of tubulin [4]. This results in a time-dependent increase of fluorescence emission of bis-ANS when complexed with tubulin. This type of secondary site is not generated for binding of ANS [4]. Prodan behaves like ANS in this respect. The secondary sites for bis-ANS have lower affinity for binding to tubulin compared to its single high-affinity site [4]. Probably, small structural changes take place on the tubulin molecule as the protein ages, leading to generation of low-affinity binding sites for bis-ANS. These changes are not detected by circular dichroism, implying a maintenance of the secondary structures and the overall conformation. However, the nature of these hydrophobic sites excludes the binding of the analogous compounds: ANS or Prodan.

Binding parameters for Prodan-tubulin interaction

The physical parameters characterising a binding interaction are the dissociation constant (K_d) and the stoichiometry of binding, by means of which a true comparison of the interaction of different probes with a single macromolecule/protein can be made. These were determined for the Prodan-tubulin interaction by the double-reciprocal plot of $1/r$ vs $1/[\text{Prodan}]_{\text{free}}$, as mentioned under Materials and Methods. Poor solubility of Prodan in water limits achieving binding ratios beyond $r = 0.2$, making the Scatchard plot method of analysis unsuitable in this case. Fig. 3A shows this plot: a straight line, which cuts the $1/r$ axis at 0.8 (giving a stoichiometry of 1:1) and a slope which corresponds to K_d = 20 μM (in buffer A at 25 °C). Similar K_d values have been reported for the lower-affinity binding sites of bis-ANS to tubulin [4]. Reported K_d values for ANS, which has only 1 binding site/tubulin dimer, range over 3 – 100 μM [4, 17, 18]. We confirmed the K_d for Prodan-tubulin interaction by reverse titration, where 0.1 μM Prodan was titrated with excess tubulin and the fluorescence anisotropy was measured. The data was analysed by plotting $1/A$ vs 1/[tubulin] (Fig. 3B), as described under Materials and Methods. This gave a K_d value of 23 μM, which is in good agreement with the earlier determination. As a further test of the binding stoichiometry, fluorescence data were collected by the method of continuous variation for both tubulin and Prodan (Job plot; Fig. 3C). The plot is consistent with there being a single binding site for Prodan on tubulin, as indicated from Fig. 3A.

Polarisation measurements

An earlier report [19] indicated that while ANS binds to a rigid portion of tubulin; bis-ANS, attached to a flexible part of the protein. Hence it was of interest to examine the nature of the Prodan-binding site in this regard. Fig. 4 shows the Weber-Perrin plot which gives information about the average rotational mobility of the bound probe for Prodan-tubulin interaction. Extrapolation of the plot to $T/\eta = 0$ gave a value of 0.30 for the limiting polarisation, indicating a relatively

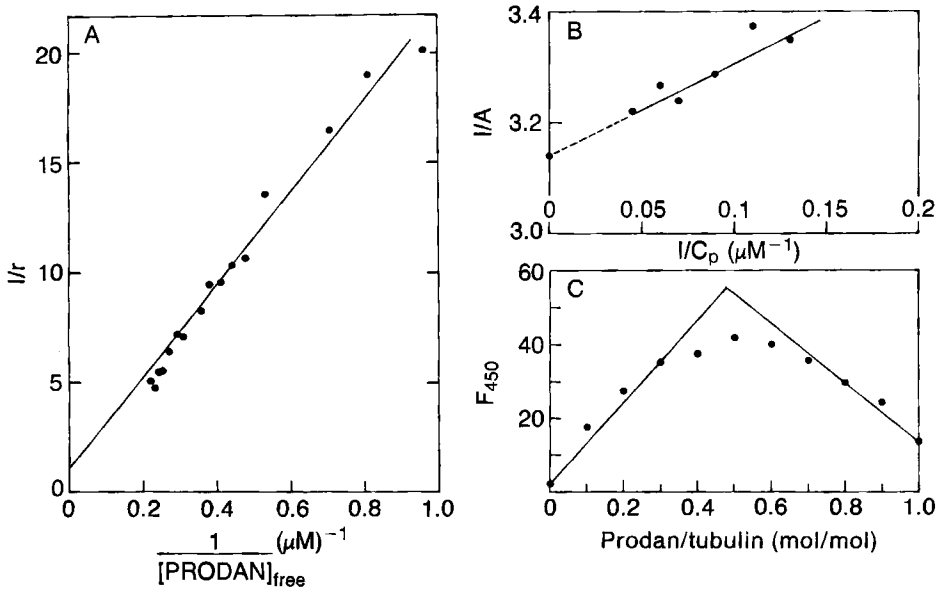

Fig. 3. Determination of binding parameters for Prodan-tubulin interaction. (A) Double-reciprocal plot derived from the Lineweaver-Burk equation. Tubulin (1 μM) in buffer A was titrated with increasing concentrations of Prodan (0.1 – 5 μM) and concentrations of free and tubulin-bound Prodan were calculated as described under Materials and Methods. Dissociation constant and stoichiometry of binding were calculated from this plot. (B) Double-reciprocal plot of fluorescence anisotropy (A) vs tubulin concentration (C_p). Aliquots of tubulin (0.5 – 15 μM) were added to 0.1 μM Prodan in buffer A, and the fluorescence anisotropy measured after each addition. (C) Job plot for the determination of stoichiometry of binding. The observed fluorescence at 450 nm is plotted against the molar ratio of Prodan/tubulin; concentrations of Prodan and tubulin were varied, keeping the total concentration [Prodan] plus [tubulin] at 5 μM.

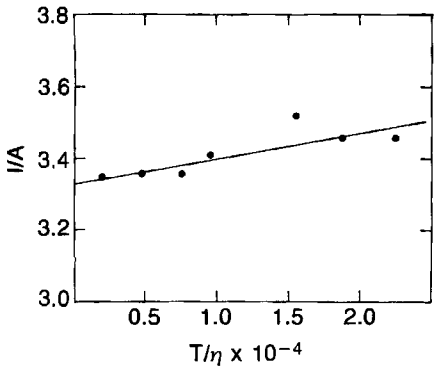

Fig. 4. Perrin-Weber plot for Prodan bound to tubulin. Fluorescence anisotropy was measured as a function of viscosity at 25°C. Tubulin (12 μM) was incubated at 25°C for 30 min with 1 μM Prodan. Excitation was at 360 nm and emission was at 450 nm.

rigid environment for the probe on tubulin. The correlation time for Prodan, calculated from this plot, was found to be 54 ns, a value that agrees well with the expected value of t_c for a spherical protein molecule of molecular mass 110 kDa. This reaffirms that the binding site for Prodan on tubulin is indeed a rigid one. Under identical conditions, t_c for ANS was found to be 46 ns. These values have been calculated after measurement of fluorescence lifetimes of the probes in the presence of tubulin, in the appropriate buffer (see Table 1).

Solute-quenching experiments

Emission maxima for Prodan, ANS and bis-ANS in the bound state (Table 1) indicate that the binding sites of all the three probes are shielded from the solvent. To further examine the extent of their accessibilities, solute-quenching experiments with KI were carried out. It may be pointed out here that unlike acrylamide, KI is an effective quencher for aminonaphthalenes. Surprisingly, in all four cases the bound probe seemed to be totally inaccessible to KI (data not shown), which was ineffective even up to 0.2 M. Higher concentrations of KI were not used since KI binds to tubulin at high concentrations [20].

Competition experiments using [^{3}H]ANS

In view of the above results, it is interesting to ask whether the three probes, ANS, bis-ANS and Prodan, share a common binding site on tubulin. However, experiments relating to competitive interaction among the three probes at a common site on the protein cannot be carried out using fluorescence because of the spectral overlap of emission of the three fluorophores. Therefore, competition experiments with ^{3}H-labeled ANS were carried out to investigate the above question, as described below.

A complex of 2 μM tubulin and 2 μM [^{3}H]ANS was formed in buffer A. To a solution of this complex, increasing concentrations of unlabeled ANS, bis-ANS and Prodan were added in three separate experiments. Each of the compounds gradually displaced the bound [^{3}H]ANS from the complex (Fig. 5). While ANS and bis-ANS displaced [^{3}H]ANS almost completely (90%) at 100 μM, a similar total removal of [^{3}H]ANS by Prodan could not be accomplished due to the poor solubility of Prodan beyond 5 μM. The trend, however, clearly indicates that both bis-ANS and Prodan compete with ANS for binding to tubulin implying that they bind to the protein at a common region.

DISCUSSION

Our study of Prodan-tubulin interactions provides a basis for comparison of the properties of the different amino-

Table 1. Summary of the tubulin-binding properties of aminonaphthalenes. ID_{50}, concentration of compound at which inhibition of microtubule polymerisation was 50% of control. λ_{max}, fluorescence emission peak of probe bound to tubulin. P_0, limiting polarisation value of probe bound to tubulin, as determined from Perrin's equation [14]. Values for bis-ANS correspond to high-affinity binding. Polarisation measurements were not performed in this case for reasons explained in the text.

Compound	ID_{50}	No of binding sites on tubulin	K_d	λ_{max}	P_0	t_f	t_c
	μM		μM	nm		ns	
ANS	12	1	3–100[a]	465	0.30	9	46
bis-ANS	3	1	2	470	–	–	–
Prodan	–	1	20	450	0.30	4	54

[a] Values obtained from literature [3, 17, 18].

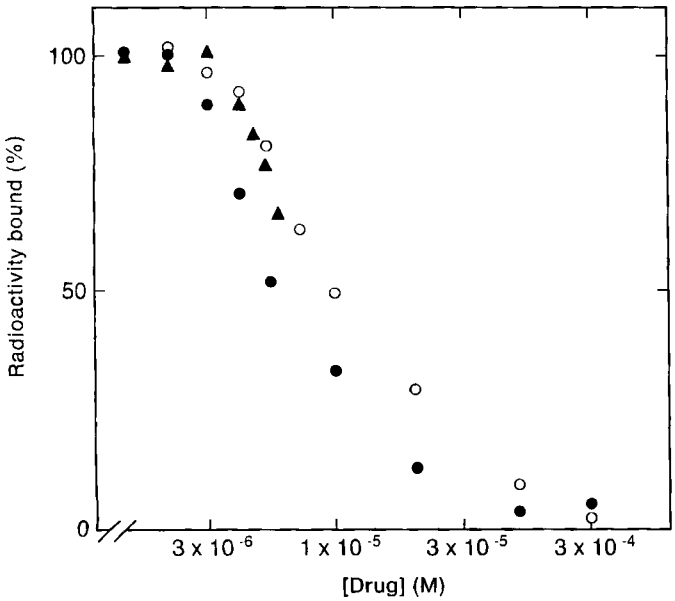

Fig. 5. Displacement of [^{3}H]ANS bound to tubulin by unlabeled ANS (●), bis-ANS (○) and Prodan (▲). Tubulin–[^{3}H]ANS complex was formed as described in the text; the radioactivity given by this complex alone was taken as 100%.

naphthalenes with respect to their interaction with tubulin. The different data for the three aminonaphthalenes, ANS, bis-ANS and Prodan, are collected in Table 1. Prodan binds to the protein with a lower affinity than the high-affinity binding of bis-ANS. However, a true comparison of the strength of binding is not possible in this case, as bis-ANS has multiple binding sites on tubulin, and the number of binding sites changes with time [4] (unpublished results). Prodan, like ANS, binds to a single site on tubulin, with a dissociation constant of 20 μM.

As evidenced from the limiting polarisation, as well as the value of correlation time, t_c (Table 1), both ANS and Prodan bind to a site on tubulin that is more or less rigid. Thus t_c for ANS or Prodan binding yield values close to the expected correlation time for tubulin, 46 ns (assuming tubulin to be a spherical molecule of molecular mass 110 kDa). The theoretical value of the limiting polarisation for a totally rigid and immobilised site would be 0.4. The measured limiting polarisation, P_0, for Prodan and ANS is 0.3, which is close to the above value. Our results for ANS are in agreement with an earlier report on the measurement of the rotational correlation time of ANS bound to tubulin [19]. The same study by Prasad et al. [19] indicated that, unlike ANS, bis-ANS binds to a flexible part of the tubulin molecule. This result for bis-ANS contradicts our conclusion of a common binding site based upon the competition experiment (Fig. 5). However, any straightforward conclusion regarding the nature of binding sites for bis-ANS is difficult to draw, since at the conditions of the experiment of Prasad et al. [19], both the high-affinity and low-affinity sites would be occupied, and t_c would not reflect the nature of any particular binding site. The different types of binding of bis-ANS and their role in the inhibition of polymerisation is a problem which merits an independent, in-depth study which is beyond the scope of the present paper. The outcome of our experiments put forward a possibility: the high-affinity binding site of bis-ANS shares a common region with the binding site of ANS and of Prodan, binding to which can cause inhibition of tubulin self-assembly.

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