Bis(1,8-anilinonaphthalenesulfonate)
A NOVEL AND POTENT INHIBITOR OF MICROTUBULE ASSEMBLY*

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Two related compounds, 1,8-anilinonaphthalenesulfonate (1,8-ANS) and bis(1,8-anilinonaphthalenesulfonate) (Bis-ANS), are useful fluorescent probes for hydrophobic areas on protein molecules. Using fluorescence, we examined the binding of these compounds to bovine brain tubulin and found that Bis-ANS and 1,8-ANS bound to tubulin with \( K_i \) values of 2 and 25 \( \mu M \), respectively. Bis-ANS potently inhibited the polymerization of tubulin into microtubules in vitro. In the presence of microtubule-associated protein 2, half-maximal inhibition of assembly was obtained at 3 \( \mu M \) Bis-ANS. In the presence of tau protein, half-maximal inhibition was obtained at 15 \( \mu M \) Bis-ANS. Surprisingly, 1,8-ANS, even at 200 \( \mu M \), did not inhibit assembly. Scatchard analysis indicated one binding site for Bis-ANS on tubulin. Previous reports of 1,8-ANS binding to tubulin may have been influenced by the presence of Bis-ANS which until recently was a common contaminant of commercial supplies. Because of its intense fluorescence in addition to its potent inhibitory effects, Bis-ANS appears to be a useful probe to study microtubule assembly and other interactions involving tubulin.

Microtubules are long cylindrical organelles that play critical roles in a variety of cellular processes. The formation of microtubules involves the precise assembly of tubulin subunits (1). The detailed nature of the tubulin-tubulin interactions that lead to the formation of microtubules is not completely understood, although some involvement of hydrophobic interactions might be postulated based on recent elegant studies of drug binding (2). If these interactions were indeed involved, hydrophobic probing reagents might inhibit assembly and would, in addition, provide informative probes for these critical regions and could be used to study the interactions of tubulin with other ligands.

Anilinonaphthalenesulfonates such as 1,8-anilinonaphthalenesulfonate and bis(1,8-anilinonaphthalenesulfonate) are fluorescent compounds that greatly increase their fluorescence in media of low dielectric constant and are widely used as sensitive reporters of solvent-accessible apolar regions on the surface of proteins (3). Comparisons between 1,8-ANS and Bis-ANS would be particularly interesting because of the obviously close structural relationship between these molecules.

The interaction of 1,8-ANS with tubulin has been studied previously (4), and it has been reported to bind to a single site on the tubulin protomer. However, no work has been reported that relates the binding of this or any other apolar probe to the functional events of microtubule assembly.

In the present work, we have studied the interaction of tubulin with 1,8-ANS and Bis-ANS and have found that Bis-ANS binds strongly to tubulin at a single site and can potently inhibit assembly at concentrations approaching those at which better known antimitotic drugs are effective. In contrast, 1,8-ANS, whose binding to tubulin is an order of magnitude weaker than Bis-ANS, is completely unable to block assembly even at concentrations eight times higher than its \( K_i \). It is therefore proposed that Bis-ANS because of its strong binding, potentially intense fluorescence, and apparently specific interaction with tubulin is likely to be a useful probe for the study of microtubule assembly.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,8-ANS and Bis-ANS were obtained as pure compounds from Molecular Probes, Inc. (Junction City, OR) and were pure as judged by thin-layer chromatography in chloroform:methanol:acetic acid (14:5:1). GTP was from Sigma. All other reagents were analytical grade.

**Tubulin Preparation**—Cow brains were obtained from Roegelein Co., San Antonio, TX. Microtubule protein was prepared from the cerebral cortex by the method of Fellous et al. (5). Tubulin was purified from microtubule protein by chromatography on phosphocellulose (Whatman P-11). Thermostable microtubule-associated proteins were prepared and resolved into tau and microtubule-associated protein 2 by filtration on Ultrogel ACA 34 (LKB Instruments, Rockville, MD). Protein concentrations were determined during the purification by the method of Lowry et al. (6) using bovine serum albumin as a standard. For the fluorometric binding studies, the protein concentrations were determined spectrophotometrically using an extinction coefficient at 280 nm of 1.2 ml/mg·cm (7). The molecular weight of tubulin was taken to be 100,000.

**Microtubule Assembly Assays**—The ability of tubulin to polymerize was measured by either turbidity or by centrifugation.

**Turbidity Assay**—The ability of tubulin to polymerize was assayed by placing microtubule protein solutions in cuvettes and measuring the time course of the development of turbidity at 350 nm in a Gilford 250 spectrophotometer with an automatic cuvette programmer connected to a 37 ℃ water bath. All experiments were done in buffer A (100 mM Mes, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM MgCl₂, 1 mM GTP, and 1 mM β-mercaptoethanol). The rates of microtubule formation were estimated by drawing the steepest tangents to the turbidity progress curves.

**Centrifugation Assay**—Duplicate 250-μl samples of tubulin plus either tau or MAP 2 were prepared at 0 ℃ in buffer A containing glutathione at a final concentration of 4 mM. Other additives were indicated below under "Results." The samples were incubated at 37 ℃ for 30 min and then centrifuged at 39,000 × g for 40 min at 30 ℃. The supernatants were removed, and the clear, glassy pellets
were resuspended in 100 μl of deionized water. Duplicate samples of the supernatant and pellet suspensions were analyzed for protein content by the method of Lowry (6).

Fluorescence Measurements—All fluorescence measurements were made on an SLM Model 4800 spectrophotofluorometer (SLM Instruments, Urbana, IL). For Bis-ANS, the fluorescence excitation was at 385 nm and the emission was observed at 500 nm. Binding studies with Bis-ANS were performed using buffer A at 37 °C. Microtubule formation was eliminated by performing the titrations at low tubulin concentrations in the absence of tau or MAP 2 proteins. In fact, no turbidity was detected in the course of these titrations. The fluorescence intensity of Bis-ANS bound to tubulin was assessed by adding increasing amounts of tubulin (0.4 mg/ml) to a cuvette containing a fixed concentration of Bis-ANS (1 μM) and extrapolating a double-reciprocal plot of this data to infinite tubulin concentration. This extrapolated fluorescence value was used to determine the concentration of Bis-ANS bound to tubulin in the course of a titration after correcting the observed fluorescence values for dilution and the inner filter effect. The absorbance at the exciting wavelength was always below 0.1, and the inner filter correction never exceeded 10%. Thus, in a typical titration, 2 ml of a tubulin solution at 5 μM (0.5 mg/ml) were titrated with 0–40 μM Bis-ANS by adding microliter amounts of a concentrated stock solution of Bis-ANS (200 μM). The corrected fluorescence intensities were used to measure the concentrations of Bis-ANS bound to tubulin which in turn were used to determine the concentrations of free dye. These values together with the spectrophotometrically determined concentration of tubulin were then used to assess the binding parameters for the interaction of Bis-ANS with tubulin. The binding constant for the interaction of tubulin with 1,8-ANS was determined using an excitation wavelength of 380 nm and an emission wavelength of 465 nm. The binding constant in this case was assessed from a linear least-squares fit to a double-reciprocal plot of the fluorescence data. In all of the binding studies reported here, the titrations were begun within 30 min of the elution of the tubulin from the phosphocellulose column to minimize any possible decay of binding as has been widely observed for the interaction of tubulin with colchicine and to control for any time dependence of the ligand binding (8).

The quantum yield for Bis-ANS bound to tubulin was measured by comparing the area under the fluorescence emission spectra for 1 μM Bis-ANS in 1-propanol with the areas under emission spectra obtained with an equally absorbing sample of Bis-ANS bound to tubulin. To ensure that only bound Bis-ANS contributed to the reported quantum yields, the areas under the Bis-ANS-tubulin emission spectra were measured with tubulin concentrations of 0.3, 0.75, 1.50, and 3.00 mg/ml. The reciprocals of the resulting areas were plotted versus the reciprocals of the corresponding tubulin concentrations and the data were extrapolated to infinite tubulin concentration. This limiting value was used to calculate the quantum yield of bound Bis-ANS using a value for the quantum yield of Bis-ANS in 1-propanol of 0.67 (9).

RESULTS

Fig. 1 shows a Scatchard plot (10) for the binding of Bis-ANS to tubulin at 0.39 mg/ml in buffer A with the concentrations of bound and free Bis-ANS determined as described under “Experimental Procedures.” The line drawn through the data points corresponds to a $K_d = 2.03$ μM and a nominal number of binding sites of 0.75 Bis-ANS sites/molecule of tubulin. These results are shown in Table I along with the corresponding values for the binding of 1,8-ANS to tubulin which appears to have considerably weaker binding as evidenced by a $K_d = 25$ μM. The quantum yield for Bis-ANS bound to tubulin was measured as described under “Experimental Procedures” and gave a value of 0.47.

Fig. 2a shows the effect of Bis-ANS on the assembly of tubulin into microtubules using the turbidity assay. Bis-ANS clearly inhibits both the rate and extent of microtubule assembly, and 50 μM Bis-ANS (curve 4) has almost complete inhibition of assembly. Electron microscopy was used to test whether microtubules could form in the presence of Bis-ANS. Unfractionated microtubule protein containing tubulin, tau, MAP 1, and MAP 2 was allowed to polymerize after partial inhibition by 20 μM Bis-ANS. Negatively stained samples of this preparation were examined by electron microscopy as previously described (11) and clearly showed the presence of 0...

FIG. 1. Binding of Bis-ANS to tubulin in the absence of MAPs. Tubulin at 0.39 mg/ml in buffer A was titrated at 37 °C with 0–12 μM Bis-ANS within 20 min of elution of the protein from a phosphocellulose column to remove MAPs. Bound and free Bis-ANS was determined as described under “Experimental Procedures” which also presents the details of all other experimental methods; the results are plotted here as a Scatchard plot (9). The line drawn through the data points gives the $K_d = 2$ μM and $n = 0.75$. B denotes bound Bis-ANS in moles/mole of tubulin, and F denotes the concentration of free Bis-ANS in moles/liter.

**Table I**

| Bis-ANS | 2 | 0.75 |
|---|---|---|
| 1,8-ANS | 25 | 1* |

*Reported in Ref. 4.

FIG. 2. Effect of Bis-ANS and 1,8-ANS on microtubule assembly as measured by turbidity. Aliquots (500 μl) of microtubule protein (1.0 mg/ml) were incubated in the presence of either Bis-ANS (a) or 1,8-ANS (b). The concentration of Bis-ANS used in a were as follows: curve 1, 0 μM; curve 2, 10 μM; curve 3, 20 μM; curve 4, 50 μM. The concentrations of 1,8-ANS used in b were as follows: curve 1, 0 μM; curve 2, 50 μM; curve 3, 100 μM; curve 4, 200 μM. Other experimental details are described under “Experimental Procedures.”
short well-formed microtubules. In contrast, as shown in Fig. 2b, 1,8-ANS had little or no effect on assembly even when present at 200 \(\mu\text{M}\) (Fig. 2b, curve 4).

The rates of polymerization were estimated in the presence of 1,8-ANS and Bis-ANS by the procedure outlined under “Experimental Procedures” from experiments similar to those shown in Fig. 2. These results show that Bis-ANS is clearly an inhibitor of the rate of assembly as well as its extent, even though the kinetics may be complicated by the possibility that the ratio of Bis-ANS to binding sites changes as polymerization proceeds. The concentration of Bis-ANS giving one-half of the maximum inhibition is approximately 26 \(\mu\text{M}\). Just as for the extent of polymerization, 1,8-ANS does not inhibit the rate of polymerization, and although the data are not precise enough for a detailed analysis, they may suggest, in fact, a slight enhancement of the rate of polymerization in the presence of 1,8-ANS.

The experiments reported above were performed with microtubule protein that contained MAP 1, MAP 2, and tau as well as tubulin. In order to diminish the possible effects of Bis-ANS and 1,8-ANS inhibition of assembly due to direct interactions with the MAPs, we undertook to investigate the effects of Bis-ANS and 1,8-ANS in a system that would permit the study of tubulin assembly under conditions where the effects of MAP 2 or tau could be separately examined. Fig. 3 shows the effect of Bis-ANS (main plot) and 1,8-ANS (inset) on microtubule assembly stimulated by tau and MAP 2 as measured by the centrifugation assay. Bis-ANS inhibited microtubule assembly in the presence of MAP 2 half-maximally at a concentration of approximately 3 \(\mu\text{M}\), which is comparable to the \(K_d\) for Bis-ANS observed with pure tubulin. It should be noted, however, that the concentration of ligand giving the half-maximal effect is not necessarily equal to the \(K_d\) for the ligand-tubulin interaction, but the comparison is used here for convenience. In the presence of tau, the half-maximal inhibition occurred at approximately 22 \(\mu\text{M}\) Bis-ANS.

Several titrations were performed to test directly for the effect of tau and MAP 2 on the affinity of Bis-ANS for tubulin. Titrations of Bis-ANS with tubulin alone (0.50 mg/ml) and in combination with either tau (0.15 mg/ml) or MAP 2 (0.3 mg/ml) gave \(K_d\) values ranging from 2.9 to 4.0 \(\mu\text{M}\). By contrast, titration with Bis-ANS of tau or MAP 2 in the absence of tubulin gave \(K_d\) values in excess of 100 \(\mu\text{M}\) in each case. As a further test, the effect of Bis-ANS on the assembly of tubulin prepared by phosphocellulose chromatography was directly tested to eliminate the possibility that the inhibition of assembly requires an interaction between Bis-ANS and MAPs. Phosphocellulose tubulin at 4 mg/ml was induced to assemble by making the solution 10\% in dimethyl sulfoxide. Bis-ANS clearly inhibited the formation of microtubules by 50\% at a concentration of 110 \(\mu\text{M}\). In contrast to the results with Bis-ANS, 1,8-ANS (Fig. 3, inset) appeared to have no effect on microtubule formation with either MAP. In some experiments, 1,8-ANS enhanced microtubule assembly. However, 1,8-ANS never inhibited microtubule assembly in any experiments. Although there was some variability in the precise effect of 1,8-ANS which either enhanced assembly or had no effect, it never caused any significant inhibition of assembly. The interaction between 1,8-ANS and Bis-ANS for binding to tubulin was examined by titrating tubulin with Bis-ANS in the presence of several fixed concentrations of 1,8-ANS (10, 20, and 30 \(\mu\text{M}\)). Under all conditions, 1,8-ANS clearly inhibited the binding of Bis-ANS. However, due to the relatively weak binding of 1,8-ANS and the spectral overlap between the compounds, the data were not precise enough to determine whether the two fluorophores are strictly competitive with respect to each other.

**DISCUSSION**

The data in the present paper indicate that Bis-ANS binds tightly to tubulin with a dissociation constant that is comparable to that observed with well known antimitotic compounds such as colchicine (8, 12). This binding to tubulin appears to involve no more than one site on the protein and causes a dramatic inhibition of assembly as well as a large increase in fluorescence which is characteristic of Bis-ANS when it binds to spolar surfaces. 1,8-ANS, which also binds to tubulin, although more weakly than Bis-ANS, does not significantly inhibit assembly even at concentrations where most of the tubulin is bound to 1,8-ANS. For example, in the experiments reported here (10 \(\mu\text{M}\) tubulin, 200 \(\mu\text{M}\) 1,8-ANS and \(K_d = 25 \mu\text{M}\)), 88\% of the tubulin is complexed with 1,8-ANS. Surprisingly, 1,8-ANS is found here to bind more weakly than has been reported previously (4). Several factors might contribute to this discrepancy. First, the previous work was done with rat tubulin rather than bovine as in the present study; second, buffer conditions were different in previous studies especially in that Ca\(^{2+}\) was used, and 1,8-ANS preparations that were previously available are now known to have been contaminated with Bis-ANS (13, 14).

The fact that the measured \(K_d = 2 \mu\text{M}\) was reported here for the binding of Bis-ANS, together with the fact that Bis-ANS inhibits microtubule assembly stimulated by MAP 2 half-maximally at a concentration of 3 \(\mu\text{M}\) suggests that Bis-ANS is inhibiting microtubule assembly by binding directly to tubulin and not by binding to one of the associated proteins. However, it is quite possible that some MAPs can interfere.
with the effect of Bis-ANS on assembly and that may be the reason why weaker effects were observed with tau-stimulated microtubule assembly and that may be the reason why weaker effects were observed with tau-stimulated microtubule assembly and in a crude microtubule preparation containing tubulin together with tau, MAP 1, and MAP 2.

Whether MAPs exert their effect by competing with tubulin for Bis-ANS or whether they inhibit the Bis-ANS interaction by some other means cannot be determined from our data. The results with phosphocellulose tubulin polymerization directly show that the inhibition of microtubule formation by Bis-ANS does not require the presence of MAPs and therefore is mediated by the binding of Bis-ANS directly to tubulin.

The very different protein concentration and solvent composition needed for this latter study make it difficult to present a detailed comparison with the inhibitory effectiveness of Bis-ANS in the presence of MAPs.

The present data are consistent with the presence of a single Bis-ANS site on the tubulin protomer, which is the same conclusion drawn previously for the binding of 1,8-ANS to tubulin (4). It is conceivable that the binding sites for Bis-ANS and 1,8-ANS are different, but that is unlikely because it is difficult to imagine that a binding site that could accommodate Bis-ANS could not accommodate the chemically very similar but sterically smaller 1,8-ANS. If they bound to the same site, then the portion of the Bis-ANS site to which 1,8-ANS does not bind might include an assembly-critical region. Another possibility is that Bis-ANS, but not 1,8-ANS, causes a conformational change in the tubulin molecule which blocks microtubule assembly. It is interesting that the coplanar arrangement that is most often depicted for the Bis-ANS molecule (Fig. 4) is the most energetically unfavorable conformation because it requires that the protons on position 5 of the naphthalene rings come into contact. Binding of Bis-ANS would therefore introduce steric strains that would not apply to the binding of 1,8-ANS and could cause protein conformational effects that would be specific for Bis-ANS.

Overall, limitations in the present method make it difficult to precisely determine the type of inhibition exerted by 1,8-ANS on Bis-ANS binding, and the question of whether the sites for these ligands are identical must remain open.

In summary, Bis-ANS appears to block microtubule assembly by binding tightly to tubulin and reports this interaction by a large increase in its fluorescence quantum yield. These properties may make Bis-ANS at least as useful as the more classical microtubule inhibitors for the study of the diverse interactions that are related to the biological functions of tubulin.

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