Bis-ANS as a Specific Inhibitor for Microtubule-Associated Protein Induced Assembly of Tubulin†

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ABSTRACT: 5,5′-Bis[8-(phenylamino)-1-naphthalenesulfonate] (bis-ANS), the fluorescent probe which binds to tubulin, inhibits its assembly into microtubules [Horowitz et al. (1984) *J. Biol. Chem. 259*, 14647–14650]. The results described in this paper demonstrate that bis-ANS is quite distinct from other well-known microtubule inhibitors in its specificity of action. The inhibitory potentials of bis-ANS and its three structural analogues ANS, Prodan [6-propionyl-2-(dimethylamino)naphthalene], and NSA (naphthalenesulfonic acid) have been compared. It is found that they can be arranged in the following order according to their polymerization inhibitory potentials: bis-ANS ≃ Prodan ≫ ANS > NSA. Interestingly, the naphthalene nucleus is sufficient to cause inhibition of polymerization. Detailed experiments were carried out to examine the mode of assembly inhibition by aminonaphthalenes at the molecular level, using bis-ANS as a representative. It was found that there was little or no effect of bis-ANS on the assembly of tubulin when polymerization was induced by assembly promoters like taxol, DMSO, or glutamate, or on the assembly of subtilisin-digested protein (tubulin S), for all of which half-maximal inhibition could not be achieved even at 120 μM bis-ANS. On the contrary, bis-ANS acts as an inhibitor in the case of MAP- (MAP2 and tau) and poly(L-lysine)-induced assembly of tubulin, with half-maximal inhibitory concentrations ranging from 1.5 to 7.6 μM. Our results place bis-ANS as a novel inhibitor, which seems to specifically inhibit C-termini-mediated assembly. Of all assembly inhibitors known so far, none exhibits such selection. We further confirmed that the inhibition was not due to the disruption of MAP–tubulin interaction by bis-ANS.

Agents which are well-known to inhibit the self-assembly of brain tubulin can be broadly classified into the following categories: (a) colchicine and its structural analogues such as colcemid and podophyllotoxin; (b) vinblastine and its analogues like vincristine and maytansine; and (c) the metal ions Ca^{2+}, Cu^{2+}, and Hg^{2+} (Wilson & Bryan, 1974; Dustin, 1978). From the literature, it is apparent that the different agents bind to tubulin at different sites and arrest self-assembly.

In this connection, it is interesting to explore the possibilities of another class of compounds, viz., the aminonaphthalenes, as potential inhibitors. As extrinsic fluorescent probes, 8-anilino-1-naphthalenesulfonate (ANS)[1] and bis-ANS are well-known (Bhattacharyya & Wolff, 1975; Lee et al., 1975; Prasad et al., 1986a,b). The effect of bis-ANS on tubulin-causing inhibition of MAP-induced assembly has also been reported (Horowitz et al., 1984). Binding of bis-ANS to tubulin does not affect the binding of colchicine, vinblastine, or calcium (unpublished results), indicating that it binds to yet another site and causes inhibition.

It may be noted that usually assembly inhibition takes place irrespective of which inducer is used to promote assembly. Colchicine, vinblastine, nocodazole, and their analogues each inhibit MAP-, tau-, DMSO-, taxol-, and glutamate-induced self-assembly (Olmsted & Borisy, 1975; Sloboda & Rosenbaum, 1979; Kumar, 1981; Schiff & Horowitz, 1981; Robinson & Engelborghs, 1982; Wilson et al., 1982).

In this paper, we report that aminonaphthalenes like ANS, bis-ANS, Prodan, etc. act as inhibitors of self-assembly. What is most striking, even naphthalenesulfonic acid can inhibit assembly. Further, we have carried out detailed studies using bis-ANS as a representative dye of this class. Our results indicate an interesting mode of inhibition by bis-ANS, not observed so far for any assembly inhibitor: it specifically inhibits the assembly induced by MAP2 or tau but not those promoted by DMSO, taxol, etc.

Materials and Methods

GTP, PIPES, EGTA, and poly(L-lysine) (average MW 40 000) were obtained from Sigma. Phosphocellulose (P-11) was from Whatman. ANS, bis-ANS, and Prodan were obtained from Molecular Probes (Junction City, OR). All other reagents were analytical grade.

MAP2 and tau fractions, prepared according to Hamel and Lin (1984), were a kind gift from Dr. Ernst Hamel, NCI, NIH, Bethesda, MD.

Preparation of Tubulin. Tubulin was prepared by phosphocellulose column chromatography of microtubule proteins purified from goat brain by two cycles of temperature-dependent polymerization in PIPES assembly buffer (100 mM PIPES, pH 6.9, 0.5 mM $MgCl_2$, and 1 mM EGTA) with 1 mM GTP (Sloboda & Rosenbaum, 1982). 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. (1951).

Preparation of Tubulin S. Phosphocellulose-purified tubulin was digested with subtilisin BPN at a concentration of 1% (w/w) of tubulin (Bhattacharyya et al., 1985; Sackett et al., 1985; Mukhopadhyay et al., 1990). Enzyme stock solution

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; bis-ANS, 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate]; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; NSA, naphthalenesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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was prepared by dissolving subtilisin at 1 mg/mL in water. Aliquots of this solution were frozen and stored at −70 °C and thawed once only, as and when required. Tubulin was digested in assembly buffer containing 1 mM GTP at 37 °C. The digestion was terminated by the addition, at 0 °C, of 1% by volume of 1% (w/w) PMSF in DMSO when the polymer formation reaches a plateau.

Turbidity Assay for Microtubule Assembly. Polymerization of tubulin was assayed by incubating microtubule protein (1.5 mg/mL) solutions in cuvettes at 37 °C in a thermostated SHIMADZU UV 160 spectrophotometer and measuring the absorption at 350 nm with time. All experiments were done in buffer A (50 mM PIPES, pH 6.9, 1 mM EGTA, and 0.5 mM $MgCl_2$). Polymerization was always initiated with the addition of 2 mM GTP. In order to examine the effect of the drugs on self-assembly, the microtubule protein was preincubated at 37 °C for 5 min with the respective drug (ANS, bis-ANS, Prodan, or NSA) before the addition of GTP. For inhibition studies of assembly induced by MAP2 (0.25 mg/mL), tau (0.3 mg/mL), and poly(L-lysine) (10 μg/mL), concentrations of tubulin (mg/mL) used were 0.75, 1.0, and 1.5, respectively.

For electron microscopy, microtubule proteins were prepared in the absence and presence of different concentrations of ANS and bis-ANS prior to negative staining and examination as described in Horowitz et al. (1984).

Centrifugation Assay. Six hundred microliter samples of different concentrations (0.5–1.2 mg/mL) of purified tubulin plus either taxol (10 μM), DMSO (10%), or glutamate (1 M, pH 7.0) were prepared in buffer A at 0 °C. To observe the effect of bis-ANS on polymer formation induced under these conditions, different concentrations of bis-ANS were added to aliquots of tubulin in buffer A before the addition of either taxol/DMSO/glutamate. The samples were incubated at 37 °C for 30 min after addition of 1 mM GTP to induce polymerization (except for tubulin S, which was prepared as described, with an initial protein concentration of 2 mg/mL and incubated without further addition of GTP). Control samples were examined in each case under identical conditions without adding bis-ANS. Assembled tubulin was isolated after centrifugation at 133000*g* for 15 min at 30 °C. The protein present in the sedimented pellet was quantified by the method of Lowry et al. (1951).

Gel Filtration Chromatography. Gel filtration chromatography was carried out to observe the effect of bis-ANS on the MAP2–tubulin interaction. ^{3}H-Tyrosylated tubulin [prepared according to Raybin and Flavin (1977)] (1 mg/mL) was incubated with MAP2 (1 mg/mL) at 37 °C for 10 min. The incubation was done in the absence of GTP to prevent assembly of tubulin into microtubules; 0.75 mL of this complex was chromatographed on a G-200 column (1 × 40 cm) equilibrated with 50 mM PIPES (pH 6.9), 0.5 mM $MgCl_2$, and 1 mM EGTA at 25 °C. When the effect of bis-ANS on the MAP2–tubulin interaction was to be observed, the above complex was prepared in the presence of 10 μM bis-ANS, and the same column was equilibrated with the same buffer containing 10 μM bis-ANS. To determine the elution profile of tubulin and MAP2, the same amount (1 mg/mL) of each protein was chromatographed. The elution for MAP2 was monitored by its fluorescence at 340 nm (excited at 295 nm). In the case of ^{3}H-labeled tubulin or for the tubulin–MAP2 complex, the cpm was taken.

Results and Discussion

Inhibition of Microtubule Polymerization by Aminonaphthalenes. The biological activity of tubulin is best manifested in its ability to polymerize into microtubules, and any agent which inhibits this activity is important for use as an antimitotic agent. Figure 1 shows the effect of Prodan, bis-ANS, ANS, and NSA on the assembly of tubulin into microtubules using turbidity assay. When preincubated at 37 °C with different concentrations of dye before initiation of assembly with the addition of GTP, it is observed that all four fluorescent probes inhibit polymerization. The observation that even NSA can inhibit microtubule polymerization points out for the first time that the naphthalene nucleus is enough for inhibitory activity. It has been reported earlier that while bis-ANS is a potent inhibitor, ANS does not inhibit assembly at all (Horowitz et al., 1984). However, it is noteworthy that at very low concentrations (1–15 μM), ANS decreases the polymer concentration from 0.3 to 0.2 mg/mL, an inhibition of 33% (in presence of MAP2). This is evident from the inset in Figure 3 of Horowitz et al. (1984). Our experiments with ANS gave similar results. While it was effective at lower concentrations, above 15 μM ANS had no effect on the self-assembly of tubulin. From electron microscopy, it was observed that the polymers formed in the presence of ANS concentrations >15 μM are morphologically quite different (linear aggregates, with no well-defined structures) compared to normal microtubules formed in the absence and presence of low (<15 μM) concentrations of ANS (data not shown). Similar aggregates, which are amorphous in nature, were also reported for the polymers of the colchicine–tubulin complex (Andrue & Timasheff, 1982; Andrue et al., 1983). The reason for such aberrant behavior of ANS is not clear at present.

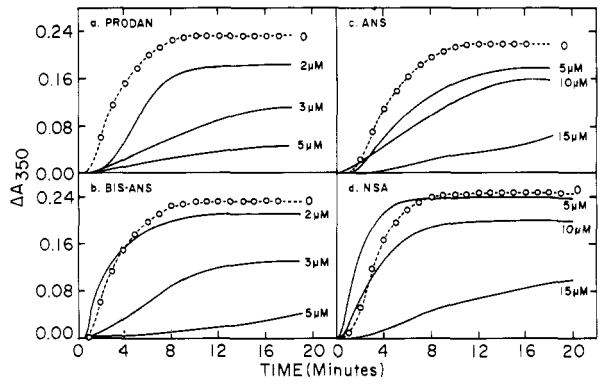

FIGURE 1: Effect of aminonaphthalenes on microtubule assembly as measured by turbidimetry. Aliquots of microtubule protein (1.5 mg/mL) were incubated in the presence of (a) Prodan, (b) bis-ANS, (c) ANS, and (d) NSA for 5 min in buffer A, at 37 °C, before initiation of polymerization with GTP. The concentrations of the drugs are as indicated on the curves. The dashed line (O---O) represents the control (tubulin alone).

In order to determine the half-maximal inhibitory concentrations for assembly, the extent of polymerization was measured at different concentrations of the drugs, and the results are presented in Figure 2. Due to its poor solubility in water, the maximum concentration tested for Prodan was 5 μM. The half-maximal concentrations in presence of each of these agents follow the order NSA > ANS ≫ Prodan ≃ bis-ANS, indicating a reverse order for their inhibitory potentials: bis-ANS ≃ Prodan ≫ ANS > NSA.

C-Termini-Mediated Assembly: Specificity of Bis-ANS. Do the aminonaphthalenes inhibit polymerization the same way as done by other inhibitors like colchicine, vinblastine, etc.? To answer this question, it was necessary to carry out a detailed study of the mode of inhibition by this group of drugs. Since polymerization of tubulin is strongly dependent on solution conditions and different polymorphic forms are readily obtained for microtubules formed under such conditions

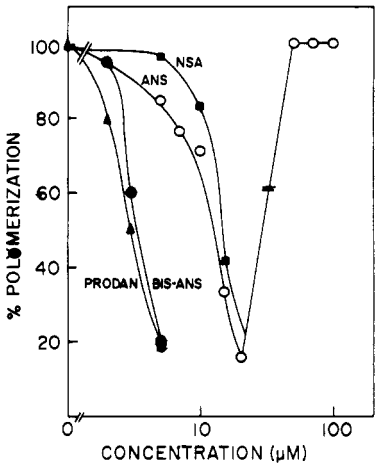

FIGURE 2: Determination of half-maximal inhibitory concentrations for Prodan (▲), bis-ANS (●), ANS (○), and NSA (■). Percent polymerization of microtubule proteins (1.5 mg/mL) at indicated concentrations of the compounds was calculated from polymerization inhibition curves (Figure 1) and is plotted in a semilogarithmic curve to obtain the 50% polymerization inhibitory concentration for each compound.

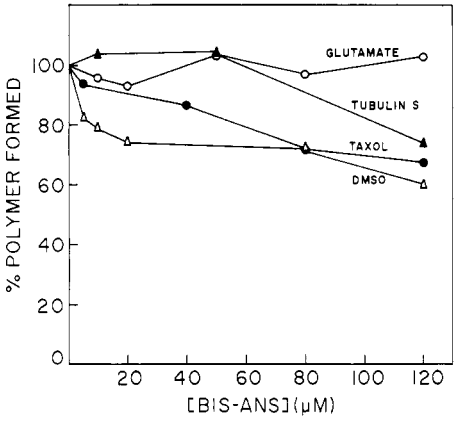

FIGURE 3: Effect of bis-ANS concentration on the assembly of tubulin induced by (a) taxol, (b) DMSO, and (c) glutamate and (d) using the subtilisin-digested protein tubulin S. Aliquots (600 μL) of protein (0.5–2 mg/mL) were incubated with taxol, DMSO, or glutamate in the presence of different concentrations of bis-ANS (0–120 μM) at 37 °C for 30 min, after adding GTP, as described under Centrifugation Assay (see Materials and Methods). Microtubule assembly was measured as described, and expressed as a fraction of the control (incubated under identical conditions, without adding bis-ANS).

(Matsumura & Hayashi, 1976; Hamel et al., 1982; Robinson & Engelborghs, 1982; Serrano et al., 1984), it appeared logical for us to test the inhibitory activity of aminonaphthalenes with different assembly promoters or buffer components. In the present study, only bis-ANS was used for its high inhibitory activity and water solubility.

The assembly of low concentrations of tubulin has been achieved with agents with taxol, DMSO, or water-structuring buffer components like sodium glutamate. Apart from these inducers, tubulin S (in which the C-termini are enzymatically removed) also shows increased ability to polymerize at low protein concentrations in the absence of MAPs (Bhattacharyya et al., 1985; Sackett et al., 1985). We tested bis-ANS for its inhibitory potential with respect to the above-mentioned assembly conditions.

In Figure 3, the effects of bis-ANS on self-assembly of tubulin at various conditions have been shown. Tubulin was made to undergo self-assembly in the presence of different concentrations of bis-ANS (as has been used previously with microtubule proteins) when polymerization was induced by

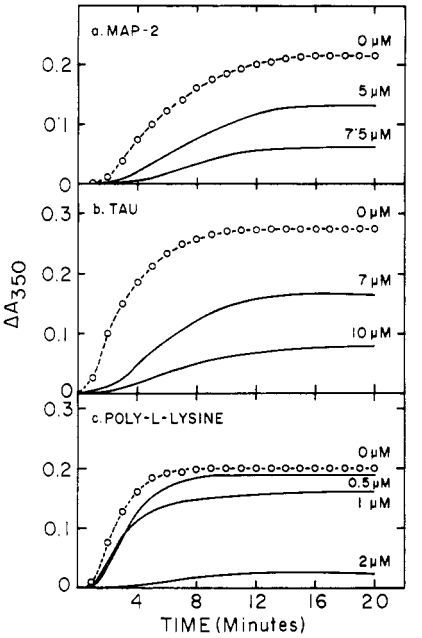

FIGURE 4: Effect of bis-ANS on the polymerization of tubulin in the presence of the assembly inducers (a) MAP2, (b) tau, and (c) poly(L-lysine), measured by turbidimetry. A mixture of tubulin and the inducing agent (concentrations as given under Materials and Methods) was incubated at each of the indicated concentrations of bis-ANS at 37 °C for 5 min before initiation of polymerization by the addition of GTP.

DMSO, taxol, or glutamate (Figure 3). Surprisingly, under these conditions, the inhibition of assembly by bis-ANS was insignificant compared to the inhibition observed in the case of MAP-induced assembly. When polymerization of tubulin S was tested, bis-ANS was again found to be ineffective (Figure 3). Thus, bis-ANS exhibited a unique specificity of inhibitory activity. While assembly of tubulin containing MAPs and tau was inhibited (as in Figure 1) by bis-ANS (80% inhibition at 5 μM bis-ANS), assembly promoted by DMSO, taxol, glutamate, or tubulin S (Figure 3) was affected marginally, even at high concentrations of bis-ANS. Microtubules induced by DMSO, glutamate, or taxol treatment, or by C-terminal cleavage of purified tubulin, were also examined by electron microscopy. Even at high concentrations, bis-ANS caused no change in the polymer structure. Clearly, the mechanism of inhibitory action of bis-ANS should be different from those of colchicine or vinblastine which inhibit self-assembly irrespective of inducers and assembly conditions.

The observation that bis-ANS is ineffective toward tubulin S prompted us to ask whether the C-termini of tubulin have any role in the assembly inhibition by bis-ANS. The fact that binding of bis-ANS to tubulin is strongly influenced by its C-termini (unpublished data of the authors) makes this question more relevant. To test this point, it was necessary to use such assembly promoters as would induce polymerization upon binding to the C-termini. We chose three such inducers, viz., the proteins MAP2 and tau (which occur in vivo in close association with tubulin) and the synthetic polypeptide poly(L-lysine) (Lee et al., 1978). MAP-2 and tau fractions and poly(L-lysine) were each separately incubated with tubulin at 37 °C, and polymerization was measured in the presence of different concentrations of bis-ANS. As can be seen in Figure 4, bis-ANS inhibited microtubule assembly in each case. The results of these experiments are summarized in Table I. The results of earlier workers (Horowitz et al., 1984) using a similar system are also shown as a comparison. These results point to a very significant feature of inhibition by

Table I: Inhibition of Self-Assembly of Tubulin by Bis-ANS

agent(s) used to induce assembly	IC_{50}[a] (μM)
poly(L-lysine)	1.5
MAP2	5.1 (3)[b]
tau	7.6 (15)[b]
unfractionated MAPs	3.5 (26)[b]
glutamate, taxol	>120
10% DMSO	>120 (110)[b]
cleavage of C-termini[c]	>120

[a] Concentration of bis-ANS at which assembly into microtubules was inhibited by 50%. [b] Results of an earlier determination by Horowitz et al. (1984) are shown in parentheses. [c] Achieved by subtilisin digestion of native tubulin to obtain tubulin S.

bis-ANS: it appears to specifically inhibit only those assembly processes that are mediated through the C-termini of tubulin. The half-maximal inhibitory concentrations for assembly inducers like MAP2, tau, or poly(L-lysine) which presumably act upon binding to the C-termini are typically at least 15–80-fold lower than for those which do not act through C-terminal binding. This conclusion is also borne out by the data of Horowitz et al. (1984) (see Table I). Polymers induced by MAP2, tau, and poly(L-lysine) were examined by electron microscopy. All three inducers under these assembly conditions induced microtubules except in the case of poly(L-lysine) where a few sheets were observed. In every case, however, the polymers formed were cold-sensitive.

Possible Mechanisms of Inhibition by Bis-ANS. The obvious question surfacing from these studies is how bis-ANS exhibits such specific inhibition of polymerization. The following possibilities may be considered. The inhibition could be arising either through (a) binding of bis-ANS to MAP2 or tau, resulting in a change in the structure of the latter so that they can no more act as inducers, or through (b) impairment of MAP–tubulin or tau–tubulin interactions. Horowitz et al. (1984) have already shown that both tau and MAP2 have very little affinity for bis-ANS when titrated in absence of tubulin. It has also been predicted that both MAP2 (Lewis et al., 1988) and tau (Lee et al., 1988) have open and extended structure and the molecules are hydrophilic in nature. This would make bis-ANS, a hydrophobic probe, an unlikely candidate for binding to these proteins at this low concentration and causing inhibition. Additionally, our results with poly(L-lysine) (Figure 4c), another hydrophilic structure which is not expected to bind bis-ANS, indicate that the inhibitory activity is probably not mediated through binding with assembly promoters.

To test the other possibility (b) mentioned above, interactions of tyrosylated tubulin (^{3}H-labeled) and MAP2 in the presence and absence of bis-ANS were studied by gel filtration chromatography at 25 °C. When chromatographed alone on a G-200 column, MAP2 eluted at the void volume (Figure 5, middle panel), as monitored by the fluorescence at 340 nm (see Materials and Methods). At the same column, 6S tubulin appeared later (Figure 5, top panel) (monitored by radioactivity). When the labeled tubulin (1 mg/mL) was incubated with MAP2 (1 mg/mL) at 37 °C for 10 min and this complex was chromatographed, a significant fraction of radioactivity was found to coelute with MAP2 at the void volume (Figure 5, bottom panel), as may be expected from MAP2–tubulin interaction. However, when the same complex was prepared in the presence of 10 μM bis-ANS and chromatographed at identical conditions (but with the column equilibrated with buffer containing 10 μM bis-ANS), it was observed that the ratios of the areas under the two peaks where MAP2–tubulin and tubulin eluted were the same in the curves with or without bis-ANS, in Figure 5 (bottom panel). We further checked

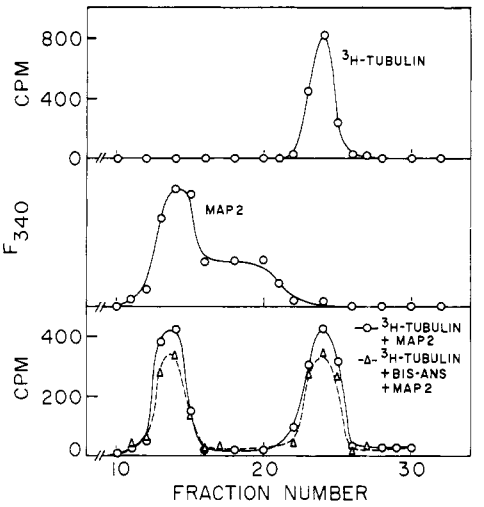

FIGURE 5: Effect of bis-ANS on tubulin–MAP2 interaction. A complex of ^{3}H-tyrosylated tubulin (1 mg/mL) and MAP2 (1 mg/mL) was formed in the presence or absence of bis-ANS (10 μM) and was chromatographed on a G-200 column in the presence and absence of 10 μM bis-ANS accordingly. (Top) Chromatogram for ^{3}H-tyrosylated tubulin, assayed by radioactivity. (Middle) Chromatogram for MAP2 alone (assayed by measuring the intrinsic fluorescence at 340 nm, after excitation at 295 nm). (Bottom) Complex of the [^{3}H]tubulin–MAP2 complex alone (○) or in the presence of 10 μM bis-ANS (Δ), assayed by measuring radioactivity.

that 0.7 M NaCl in the column completely inhibited the MAP2–tubulin interaction and no tubulin coeluted at the void volume, ruling out any possible artifact. This experiment demonstrates that there is no impairment of the MAP2–tubulin interaction by bis-ANS. Electron microscopic examination of MAP2–tubulin association products eluted in the first peak (Figure 5, bottom panel) showed the presence of double rings and no microtubules (data not shown). To understand further the reason for this specificity of bis-ANS for a particular type of assembly, additional experiments are called for, those which can explicitly identify regions or domains on tubulin where regulation of polymerization takes place.

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Registry No. ANS, 82-76-8; Bis-ANS, 63741-13-9; Prodan, 70504-01-7; NSA, 85-47-2; poly(Lys) homopolymer, 25104-18-1; poly(Lys) SRU, 38000-06-5.

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Homo- and Heteronuclear NMR Studies of the Human Retinoic Acid Receptor β DNA-Binding Domain: Sequential Assignments and Identification of Secondary Structure Elements†

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ABSTRACT: An 80 amino acid polypeptide corresponding to the DNA-binding domain (DBD) of the human retinoic acid receptor β (hRAR-β) has been studied by ^{1}H homonuclear and ^{15}N-^{1}H heteronuclear two- and three-dimensional (2D and 3D) NMR spectroscopy. The polypeptide has two putative zinc fingers homologous to those of the receptors for steroid and thyroid hormones and vitamin D_3. The backbone ^{1}H resonances as well as over 90% of the side-chain ^{1}H resonances have been assigned by ^{1}H homonuclear 2D techniques except for the three N-terminal residues. The assignments have been confirmed further by means of ^{15}N-^{1}H heteronuclear 3D techniques, which also yielded the assignments of the ^{15}N resonances. Additionally, stereospecfic assignments of methyl groups of five valine residues were made. Sequential and medium-range NOE connectivities indicate several elements of secondary structure including two α-helices consisting of residues E26-Q37 and Q61-E70, a short antiparallel β-sheet consisting of residues P7-F9 and S23-C25, four turns consisting of residues P7-V10, I36-N39, D47-C50, and F69-G72, and several regions of extended peptide conformation. Similarly, two helices are found in the glucocorticoid receptor (GR) DBD in solution [Härd et al. (1990) *Science 249*, 157-160] and in crystal [Luisi et al. (1991) *Nature 352*, 497-505], and in the estrogen receptor (ER) DBD in solution [Schwabe et al. (1990) *Nature 348*, 458-461], although the exact positions and sizes of the helices differ somewhat. Furthermore, long-range NOEs suggest the existence of a hydrophobic core formed by the two helices.

Retinoic acid, a vitamin A derivative, has profound effects on vertebrate cellular differentiation, pattern formation, and embryonic development (Brockes, 1989; Thaller & Eichele, 1987; Maden et al., 1988; Durston et al., 1989). Retinoic acid acts through binding to the retinoic acid receptor (RAR),[1] which functions as a ligand-inducible transcription factor. The RAR was cloned and identified as a member of a nuclear receptor superfamily which comprises the receptors for steroid

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[1] Abbreviations: NMR, nuclear magnetic resonance; DBD, DNA-binding domain; RAR, retinoic acid receptor; hRAR-β; human RAR-β; GR, glucocorticoid receptor; TR, thyroid hormone receptor; ER, estrogen receptor; RE, response element; RRE, retinoic acid response element; TRE, thyroid response element; GRE, glucocorticoid response element; ERE, estrogen response element; DTT, dithiothreitol; 2D, two-dimensional; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; HOHAHA, homonuclear Hartmann-Hahn; COSY, 2D *J*-correlated spectroscopy; DQF-COSY, double-quantum-filtered COSY; HMQC, heteronuclear multiple-quantum coherence; TFIIIA, transcription factor IIIA.

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