Aging of Tubulin Monomers Using 5,5′-Bis(8-anilino-1-naphthalenesulfonate) as a Probe†

Nabanita Sarkar, K. Mukhopadhyay, Pradip K. Parrack, and B. Bhattacharyya*

Department of Biochemistry, Bose Institute, Centenary Building, Calcutta 700 054, India

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ABSTRACT: The fluorescent probe bis(8-anilino-1-naphthalenesulfonate) (bis-ANS) has been used to monitor the time- and temperature-dependent aging of tubulin, whereby new hydrophobic binding sites of lower affinity are generated on the protein [Prasad, A. R. S., et al. (1986a) *Biochemistry 25,* 739−742]. We carried out a detailed analysis of this phenomenon and found that, in addition to antimitotic drugs like colchicine or vinblastine, other parameters, viz., low temperature and protein stabilizers (e.g., glycerol and sucrose), inhibit the extent of enhanced binding of bis-ANS. Moreover, the generation of additional bis-ANS binding sites are also suppressed at high concentrations of tubulin. Cleavage of the carboxy-termini of tubulin (bound to bis-ANS) by subtilisin causes a significant reduction in the enhanced fluorescence, but has no effect on the high-affinity binding site of bis-ANS. All of these observations can be explained by the correlation of the presence of additional binding with the dissociation of heterodimeric tubulin into monomers. Enhanced binding of bis-ANS is due to tubulin dimers that have undergone dissociation, resulting in a loosening of its tertiary structure with the generation of a plethora of hydrophobic sites.

A unique property of dimeric tubulin ($\alpha\beta$) is its self-assembly into microtubules. Subsequent to the discovery of in vitro conditions of tubulin self-assembly, at least two groups of drugs have been well established as in vitro inhibitors of tubulin self-assembly. These include colchicine, podophyllotoxin, combretastatin, steganacin, nocodazole, and their structural analogues (Wilson et al., 1975; Bhattacharyya & Wolff, 1974; Cortese et al., 1977; Lin et al., 1988, 1989; Schiff & Horowitz, 1981; Schiff et al., 1978; De Brabander et al., 1976). The second group includes vinblastine, vincristine, maytansine, etc. (Wilson, 1970; Bhattacharyya & Wolff, 1976, 1977; Gerzon 1980; Wilson et al., 1982; Kupchan et al., 1972, 1974). Recently, a third group of drugs, the aminonaphthalenes (e.g., ANS, bis-ANS, and PRODAN),[1] has been reported to bind to dimeric tubulin and inhibit self-assembly at substoichiometric concentrations (Horowitz et al., 1984; Mazumdar et al., 1992). Members of the same group bind to the same site on tubulin, and the binding site is different for each group. Although all inhibit protein−protein interaction, their binding sites are unrelated.

Of the preceding antimitotic drugs, the interaction of colchicine with tubulin has been studied rather extensively, and its mechanism of binding is fairly well understood (Detrich et al., 1981; Garland, 1978; Bane et al. 1984; Pyles & Hastie, 1993). Among the rest, the binding of bis-ANS to tubulin is of special importance due to the unique specificity of this drug in inhibiting tubulin self-assembly mediated by the carboxy-termini of the protein (Horowitz et al., 1984; Mazumdar et al., 1992). Bis-ANS has two classes of binding sites on tubulin: a high-affinity site in the N-terminal domain of β-tubulin, which is responsible for the inhibition of tubulin self-assembly, and a number of low-affinity sites in the flexible region of the protein, whose exact number varies in reports by different groups (Prasad et al., 1986b; Ward & Timasheff, 1994). Thus, the earlier study reported six secondary bis-ANS binding sites using spectroscopic techniques (Prasad et al., 1986b), whereas recently 40−50 secondary bis-ANS binding sites were observed using equilibrium methods (Ward & Timasheff, 1994). Our own determinations of the number of low-affinity sites yielded variable results, from 25 to 50 (unpublished observations). The evaluation of the number of sites is extremely difficult using spectroscopic techniques, as all sites may not have the same quantum yield (Ward & Timasheff, 1994).

The spontaneous "aging" of tubulin is well-known and is manifested by a time-dependent loss of characteristic functions (Wilson, 1970). Different properties of native tubulin decay with different time constants. Thus, colchicine binding activity decays at 37 °C with a half-life of 4−6 h, whereas the polymerization activity is lost more rapidly (Wiche et al., 1977). Tubulin devoid of colchicine binding activity has been found to lose its higher affinity vinblastine binding site, whereas the lower affinity binding site remains unaffected (Bhattacharyya & Wolff, 1976). Prasad et al. (1986a) have shown that the decay of tubulin is accompanied by an increase in bis-ANS binding with six low-affinity binding sites appearing on the protein with time, in addition to a high-affinity site that is always present. Bis-ANS has been used to detect structural correlates to understand tubulin decay.

In the present study, we have made an attempt to understand the aging of tubulin quantitatively by using bis-

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; bis-ANS, 5,5′-bis(8-anilino-1-naphthalene-sulfonate); PRODAN, 6-propionyl-2-(dimethylamino)naphthalene; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; C-termini, carboxy-termini.

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ANS as a probe. Our results indicate that the decay of tubulin in terms of colchicine binding activity or in terms of tubulin self-assembly, observed at higher protein concentrations and reported in the literature, is distinct from the decay of tubulin observed at concentrations of 1−2 μM at 37 °C using bis-ANS as a probe.

EXPERIMENTAL PROCEDURES

Materials. PIPES, PMSF, GTP, and EGTA were purchased from Sigma. Bis-ANS was obtained from Molecular Probes. Subtilisin BPN′, colchicine, and vinblastine were obtained from Sigma. All other reagents were of analytical grade.

Tubulin Preparation and Estimation. Goat brain tubulin free of microtubule-associated proteins was prepared by two cycles of temperature-dependent assembly and disassembly in PEM buffer [50 mM PIPES (pH 6.9), 1 mM EGTA, and 0.5 mM $MgCl_2$] in the presence of 1 mM GTP, followed by two more cycles in 1 M glutamate buffer (Hamel & Lin, 1981), and stored at −70 °C. The concentration of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Fluorescence Measurements. To measure the fluorescence intensity of bound bis-ANS, the samples were excited at 385 nm and emission was observed at 490 nm. Slit widths of 5 nm were chosen for both excitation and emission. The fluorescence values were recorded on a Hitachi F-3000 spectrofluorometer fitted with a constant-temperature cell holder. All of the experiments with bis-ANS were performed in PEM buffer [100 mM PIPES (pH 6.9), 1 mM EGTA, and 0.5 mM $MgCl_2$].

Enzymatic digestion was done with subtilisin BPN′ at a concentration of 1% (w/w) of tubulin. Enzyme stock solution 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.

The enzymatic reaction was terminated by the addition of 1% by volume of 1% (w/v) PMSF in DMSO.

Gel Electrophoresis. Electrophoresis of samples was performed in vertical sodium dodecyl sulfate−polyacrylamide slab gels using a modification of the method of Laemmli (1970). Gels contained 9% and 0.6% acrylamide (FMC) and were cast using Gelbond PAG (FMC). The lower gel buffer pH was 9.2, and the SDS in the electrode buffer (0.1%) was Sigma "lauryl sulfate" or the equivalent (i.e., containing significant tetradecyl sulfate), in order to increase the separation of α- and β-tubulins (Best et al., 1981). Samples were prepared by mixing with SDS loading solution [1% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, and 0.01 M Tris-HCl (pH 6.8), with bromophenol blue as the tracking dye] and boiling immediately for 1 min. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250, destained, soaked in 5% glycerol, and air-dried.

RESULTS

Figure 1 illustrates the effect of incubation of tubulin with bis-ANS. Tubulin (1 μM) was mixed with bis-ANS (10 μM) and incubated at 37 °C. The fluorescence intensity at 490 nm was measured as a function of time. Excitation was at 385 nm. Within the time of mixing of protein and bis-ANS,

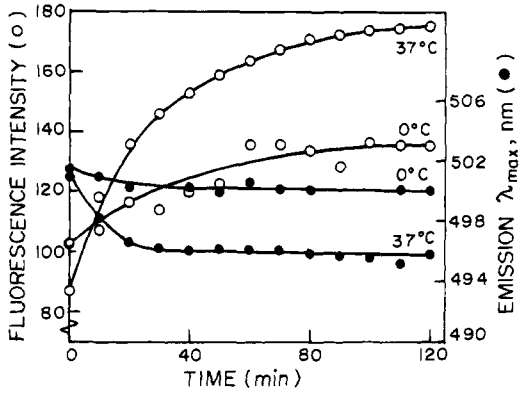

FIGURE 1: Binding of bis-ANS to tubulin: effect of time and temperature. 1 μM tubulin was mixed with 10 μM bis-ANS and incubated at 0 or 37 °C. Fluorescence intensity (○) and λ_{max} values (●) were measured at specified intervals of time for 120 min. Excitation and emission wavelengths were 385 and 490 nm, respectively.

a large fluorescence enhancement was observed that increased further with the time of incubation, reaching a plateau in 100−120 min. The initial rate of increase was very high, and almost 50% of the total enhancement occurred within the first 20 min of incubation. This was accompanied by a 3−4 nm shift of the emission peak toward blue, indicating the involvement of hydrophobic pockets of tubulin in the binding process. When the same experiment was carried out at 0 °C, the extent of fluorescence increase with time was much less, and the λ_{max} shift was 1−2 nm. Prasad et al. (1986a) had suggested that this increased binding of bis-ANS to tubulin upon incubation at 37 °C was due to the appearance of new hydrophobic pockets on the protein. They also reported a decrease in the rate and extent of bis-ANS binding when tubulin was preincubated with other antimitotic drugs, such as colchicine, podophyllotoxin, or vinblastine. We confirmed their observations with colchicine and vinblastine (Figure 2A). It is well-known that the binding of either colchicine or vinblastine stabilizes tubulin against the decay of colchicine binding activity (Wilson, 1970). Therefore, the phenomenon of enhanced bis-ANS binding to tubulin upon incubation at 37 °C could be related to protein stability. We observed that even the inclusion of general protein stabilizers such as sucrose or glycerol in the incubation mixture significantly decreased both the rate and the extent of bis-ANS binding (Figure 2B).

The stability of a protein is related to its concentration, and it is well-known that a protein rapidly loses its functional activity when present in low concentrations. Figure 3A shows the effect of tubulin concentration on enhanced bis-ANS binding upon incubation at 37 °C. As is evident from the figure, the extent of fluorescence enhancement upon incubation at 37 °C decreases drastically upon increasing protein concentrations (compare curves a, b, and c where tubulin concentrations were 1, 5, and 10 μM, respectively). Since tubulin has two types of bis-ANS binding sites (a primary site responsible for the inhibition of microtubule assembly with a K_d of 4.5 × 10^5 M^{-1} and 40−50 lower affinity binding sites) (Ward & Timasheff, 1994), this lowering of percent fluorescence upon increasing protein concentrations could be due to the formation of a lesser number of bis-ANS binding sites in tubulin. It is also possible that, due to the high protein concentration, all bis-ANS molecules are bound to the high-affinity site and there

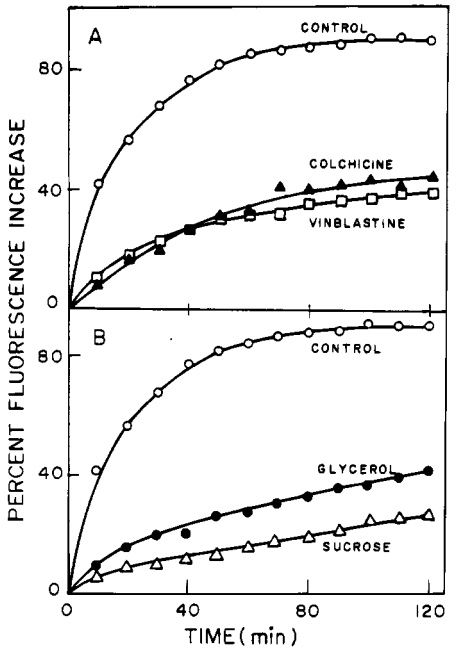

FIGURE 2: Effect of bound drugs and solvents on the binding of bis-ANS. (A) Tubulin (1 μM) was preincubated at 37 °C with 10 μM colchicine (▲) for 30 min or with 10 μM vinblastine (□) for 10 min prior to the addition of bis-ANS (10 μM) and further incubation. Fluorescence intensity at 490 nm was monitored at different times for 120 min, counted from the point of addition of bis-ANS. (B) Tubulin (1 μM) was mixed with bis-ANS (10 μM) in the presence of 4 M glycerol (●) or 1 M sucrose (△). Fluorescence intensity was measured at different times for 120 min at 37 °C.

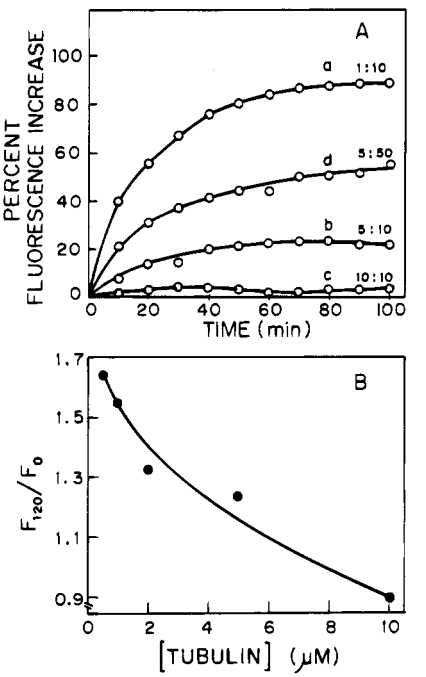

FIGURE 3: Effect of different concentrations of tubulin and bis-ANS upon time- and temperature-dependent bis-ANS binding to tubulin. (A) Tubulin and bis-ANS at the indicated concentrations (e.g., 5:10 means 5 μM tubulin + 10 μM bis-ANS) were incubated at 37 °C. Fluorescence intensity was monitored at different time intervals for 100 min. (B) Tubulin at different concentrations (0.5–10 μM) was incubated at 37 °C for 0 and 120 min. Thereafter, each of the protein samples was diluted to 0.5 μM before the addition of bis-ANS (10 μM) and measurement of fluorescence. Excitation and emission wavelengths were 385 and 490 nm, respectively.

are not enough bis-ANS molecules left free to bind to the lower affinity sites that are generated. To test the latter possibility, we carried out the same experiment with bis-ANS concentrations 10 times higher than those of tubulin. Even with 5 μM tubulin and 50 μM bis-ANS, the extent of enhanced fluorescence was less than that observed with the sample containing 1 μM tubulin and 10 μM bis-ANS, although the ratio of tubulin:bis-ANS was same (Figure 3A, compare curves a and d), indicating that the observed phenomenon is due to the temperature-induced changes taking place on the protein itself and is pronounced when the protein concentration is kept low.

Figure 3B shows the results of another experiment where protein concentrations were kept high during the period of incubation at 37 °C. In this experiment, aliquots of tubulin of different concentrations in the range 0.5–10 μM were incubated for 120 min at 37 °C. All protein samples were then diluted to the same concentration of 0.5 μM and mixed with 10 μM bis-ANS, and the fluorescence intensity at 490 nm (F_{120}) was measured immediately after mixing. These fluorescence values were compared with that of a 0.5 μM sample of tubulin that was complexed with bis-ANS without any incubation (F_0), and the ratio F_{120}:F_0 was plotted against the concentration of tubulin incubated alone. As is evident from the figure, this ratio decreases with an increase in protein concentration, indicating that the sample containing maximum tubulin concentration during the incubation period has minimum bis-ANS binding capacity. In other words, small changes take place on tubulin during incubation at 37 °C. These changes are an intrinsic property of the protein and are suppressed at higher protein concentrations or lower temperatures. Bis-ANS binding merely detects these changes, which are not detected in the CD spectra of tubulin (Prasad et al., 1986a).

Figure 4A shows the titration of 1 μM tubulin with increasing concentrations of bis-ANS at 25 °C. The spectra clearly reveal the presence of different classes of bis-ANS binding sites on tubulin. There is a gradual red shift of the emission λ_{max} of bis-ANS fluorescence as a function of the concentration of bis-ANS, indicating the increasing occupancy of more exposed sites of lower affinity. The next experiment was designed to find the relation between fluorescence increase (with gradual blue shift) upon incubation and the lower affinity bis-ANS binding sites. Tubulin–bis-ANS complexes were prepared (as in Figure 4A) with 1 μM tubulin and increasing bis-ANS concentrations, i.e., 0.25, 0.5, 1.0, 5.0, and 10 μM. λ_{max} values of the complexes were noted immediately after the bis-ANS and tubulin were mixed. We observed a gradual red shift of λ_{max} with increasing bis-ANS concentrations, as shown in the inset of Figure 4B (λ_{max} values were 482 and 499 nm when bis-ANS concentrations were 0.25 and 10 μM, respectively). Each of these complexes was incubated at 37 °C for the indicated time period, and the λ_{max} was noted again. In each case, the incubation caused a blue shift of λ_{max}, and this blue shift is maximum when the bis-ANS concentration is highest. Thus, the blue shifts were 0.6 and 4 nm when bis-ANS concentrations were 0.25 and 10 μM, respectively.

Since the small protein to bis-ANS ratio causes major occupancy of bis-ANS at lower affinity sites, the preceding results indicate that temperature has a larger effect on bis-ANS binding to the lower affinity sites than on the binding

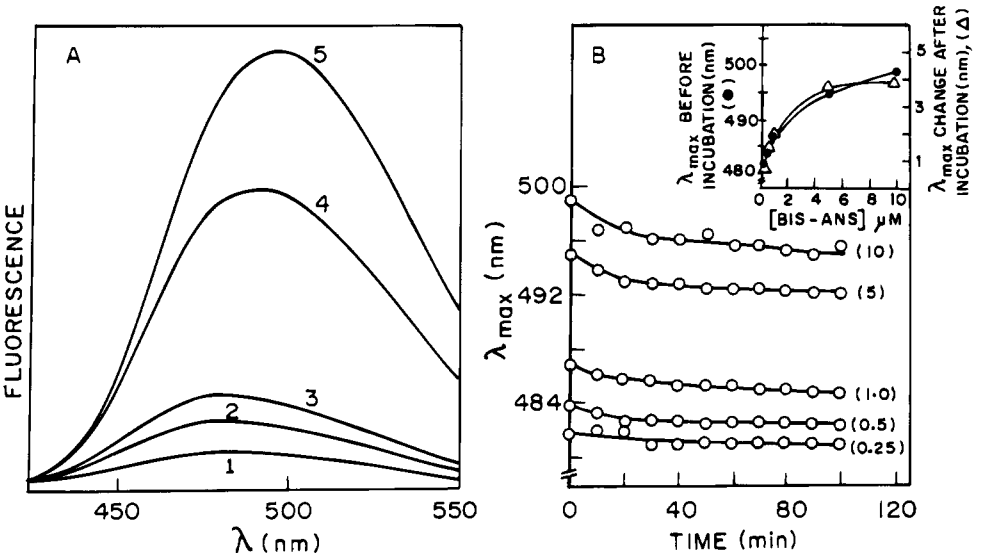

FIGURE 4: Effect of increasing bis-ANS concentration on the shift of emission maxima of the bis-ANS−tubulin complex. (A) Tubulin (1 μM) was titrated with increasing concentrations of bis-ANS (0.25, 0.5, 1, 5, and 10 μM), as indicated by curves 1−5. Fluorescence emission spectra between 425 and 550 nm are shown. Note the red shift of the emission maximum (482 nm at 0.25 μM, 499 nm at 10 μM). (B) Tubulin (1 μM) was incubated at 37 °C with increasing concentrations of bis-ANS (0.25−10 μM), and the λ_{max} value for each complex was monitored as a function of time. The inset shows the red shift in λ_{max} (●) prior to incubation at 37 °C with increasing bis-ANS concentration. λ_{max} shifts (in nm) toward blue (△) of the same complex after incubation at 37 °C are also shown as a function of bis-ANS concentration.

to the higher affinity site. It is interesting to note that both higher and lower affinity complexes are formed when sufficient bis-ANS is added to tubulin, even before incubation at 37 °C (Figure 4A, curve 5). The incubation of this complex causes a change in the conformation of the sites of tubulin where bis-ANS is bound with lower affinity, thereby causing a blue shift of λ_{max}. This change in conformation probably alters the quantum yield, affinity, and number of binding sites of the drug−protein complex. Interestingly, the incubation of the complex at 37 °C does not affect the conformation of the site of tubulin where bis-ANS is bound with higher affinity (Figure 3A, curve c).

We have previously reported that the cleavage of carboxy-termini of tubulin by subtilisin stabilizes the colchicine binding site on tubulin (Mukhopadhyay et al., 1990). Even the neutralization of negative charges of the carboxy-termini with polycations like poly(lysine) brings about such stabilization (Roychowdhuri et al., 1983). It would be interesting to examine whether $\alpha_s\beta_s$-tubulin and poly(lysine)-bound $\alpha\beta$-tubulin show less enhanced bis-ANS binding compared to $\alpha\beta$ upon incubation at 37 °C. It was, however, found that both $\alpha_s\beta_s$ and poly(lysine)-bound $\alpha\beta$-tubulin underwent aggregation when incubated with high concentrations of bis-ANS, making it difficult to draw a meaningful conclusion from these experiments. Interestingly, we observed that the addition of subtilisin, which cleaves the C-termini of tubulin, to a bis-ANS−tubulin complex (preincubated at 37 °C for 120 min) drastically decreased the fluorescence at 490 nm. This decrease in fluorescence intensity was accompanied by a large red shift in λ_{max} (Figure 5). We observed very different results when subtilisin was added to a freshly prepared tubulin−bis-ANS complex without incubation. In this case, the fluorescence intensity remained unchanged during the process of cleavage of carboxy-termini. Emission maxima underwent a small blue shift instead of a red shift (inset, Figure 5). This result indicates that the cleavage of carboxy-termini of tubulin largely affects the changes occurring on the complex upon incubation at 37 °C.

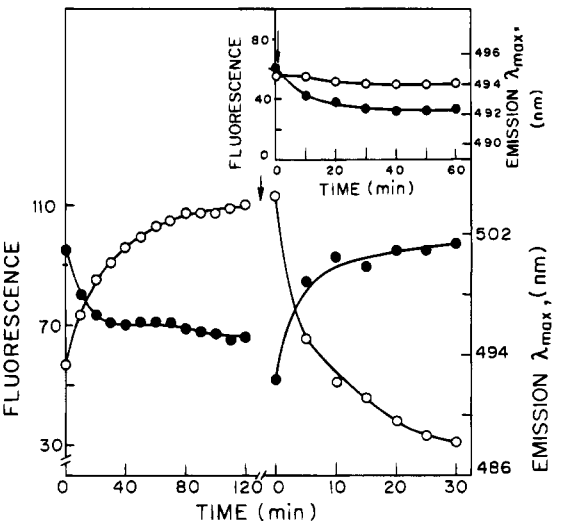

FIGURE 5: Effect of cleavage of C-termini in the bis-ANS−tubulin complex with and without incubation at 37 °C. 1 μM tubulin was incubated with 10 μM bis-ANS for 2 h at 37 °C. The fluorescence (○) and the λ_{max} values (●) were noted at specified intervals of time. At the end of 2 h, subtilisin BPN′ (protein:enzyme = 100:1) was added, and the fluorescence and λ_{max} values were noted for another 30 min at intervals of 5 min; the values are shown at the right side of the figure. The inset shows the results of a separate experiment, in which 1 μM tubulin was mixed with 10 μM bis-ANS at 25 °C, and the fluorescence values (○) and λ_{max} values (●) were noted. Subtilisin BPN′ was added immediately, and the fluorescence and λ_{max} values were noted for 60 min at intervals of 10 min. Arrows indicate the point of addition of subtilisin. Excitation and emission wavelengths were 385 and 490 nm, respectively.

This observed lowering of the fluorescence of the tubulin−bis-ANS complex upon subtilisin digestion is not an artifact in our experiment, as the addition of PMSF at any point of subtilisin digestion arrested further lowering of fluorescence instantaneously (Figure 6A). It was reported that the high-affinity bis-ANS binding site is in the N-terminal domain of β-tubulin (Ward et al., 1994), whereas the lower affinity

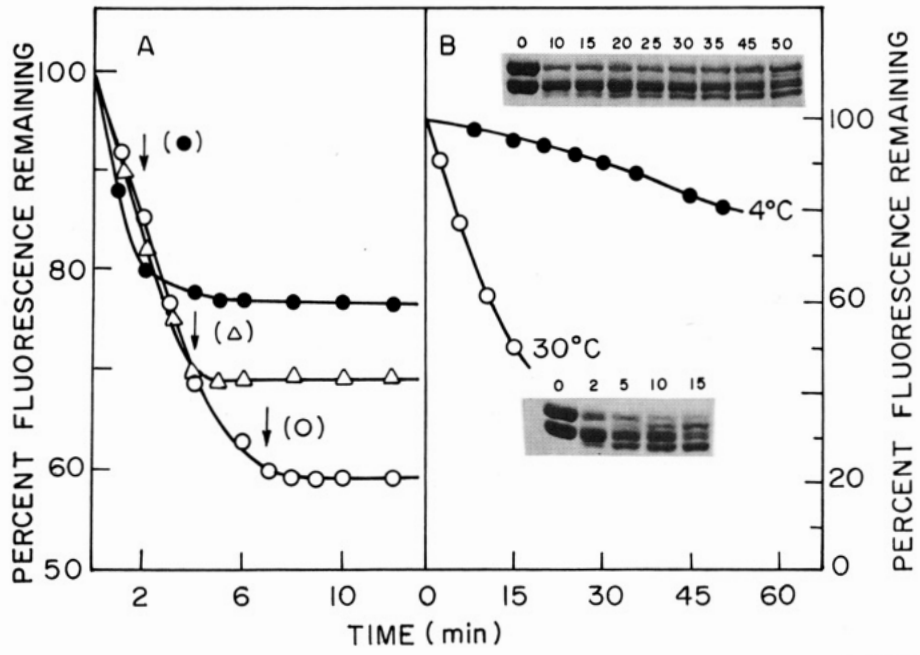

FIGURE 6: Effect of protease inhibitor and temperature on the subtilisin-induced fall in fluorescence of the bis-ANS−tubulin complex. (A) 1 μM tubulin was incubated with 10 μM bis-ANS at 37 °C for 2 h. The complex was digested with 1% (w/w) subtilisin at 30 °C, and the fall in fluorescence intensity at 490 nm was monitored. The protease activity was quenched with 1% (v/v) PMSF in DMSO at the end of 2, 4, and 8 min, as indicated by arrowheads. (B) A complex of bis-ANS (10 μM) with tubulin (2 μM) was prepared by incubation for 2 h at 37 °C. This complex was digested at the indicated temperatures with 1% (w/w) subtilisin. Fluorescence at 490 nm was measured at different time intervals following subtilisin addition. The cleavage patterns of α- and β-subunits (SDS−PAGE) at these time intervals are also shown for each temperature. The percent fluorescence prior to subtilisin addition was assumed as 100%.

multiple sites are in the flexible part of the protein (Ward et al., 1994; Prasad et al., 1986b). In order to examine the contribution of digestion of individual subunits upon the lowering of the fluorescence of the tubulin−bis-ANS complex, the digestion was performed at 30 and 4 °C when both α- and β-subunits and only β-subunit, respectively, were digested. Digestion, as well as the lowering of fluorescence, is slow at 4 °C, and 20−25% reduction in fluorescence intensity occurred when about 50% of the β-subunit was digested. On the other hand, at 30 °C digestion, as well as the lowering of fluorescence, is fast: about 50% lowering of fluorescence is observed when both α- and β-subunits are digested. These results suggest that both subunits contribute toward the lowering of fluorescence of the bis-ANS−tubulin complex.

It has been reported that the circular dichroism spectrum of tubulin was essentially unchanged after incubation for various times up to 1 h at 37 °C, suggesting that no major changes took place in the secondary structure of tubulin (Prasad et al., 1986a). However, Prasad et al. (1986a) observed a correlation between the appearance of low-affinity bis-ANS binding sites and the decay of tubulin's ability to form microtubules. In order to check whether this enhanced binding of bis-ANS to tubulin at 37 °C also correlated with the decay of colchicine binding activity, we have examined the time and temperature dependence of the ability of tubulin to bind colchicine. It has been observed that the $t_{1/2}$ for the decay of colchicine binding activity varied between 4 and 6 h for 1, 3, or 5 μM tubulin (data not shown). These data are very similar to those reported in the literature (Wilson, 1970). Therefore, it appears that the enhanced bis-ANS binding at 37 °C, which has a $t_{1/2}$ of 18−20 min (Figure 1), is unrelated to the decay of colchicine binding activity.

The enhanced bis-ANS binding of tubulin upon incubation at 37 °C takes place at tubulin concentrations that are fairly low, when a substantial amount of the protein would be in the (dissociated) monomeric form. To examine whether the enhanced bis-ANS binding was related to the presence of tubulin monomers, bis-ANS fluorescence enhancement was measured at various tubulin concentrations, as shown in Figure 7. For the purpose of comparison, the percent of monomer and dimer at each tubulin concentration calculated from the dissociation constant for the $\alpha\beta \rightleftharpoons \alpha + \beta$ equilibrium (Panda et al., 1992) are also plotted. It is apparent that both the enhanced bis-ANS fluorescence upon incubation at 37 °C and the fraction of tubulin in the monomeric form present in the sample have similar patterns of reduction with increasing tubulin concentrations. Thus, the fluorescence changes being related to the appearance of tubulin monomers cannot be ruled out.

DISCUSSION

The present study is intended to better understand the phenomenon of enhanced bis-ANS binding observed at low tubulin concentrations with time of incubation at 37 °C (Prasad et al., 1986a). We find that this enhanced bis-ANS binding is protein concentration-dependent and is closely related to protein stability. The observed phenomenon is inhibited at higher tubulin concentrations, and there is no detectable enhancement of bis-ANS binding upon incubation at tubulin concentrations above 10 μM. Likewise, lower temperatures, drugs such as colchicine and vinblastine, or stabilizers such as glycerol and sucrose inhibit this process.

Enhanced binding of bis-ANS to tubulin has recently been reported. It may be noted that this enhanced binding was observed at 20 °C with increasing drug concentrations in the range $(0-4) \times 10^{-4}$ M (Ward & Timasheff, 1994), using 10 μM tubulin (when about 80% of the protein is in the dimeric form). We observed enhanced binding with 10 μM

bis-ANS, at 37 °C, and 1 μM tubulin (where 56% of the protein would be present as monomers) as a function of time. Further, when protein and drug concentrations were 10 μM each, no time-dependent enhancement was seen (Figure 3A). Under the latter conditions, bis-ANS would bind to the higher affinity site with a 1:1 stoichiometry (Ward & Timasheff, 1994). Thus, the observed time-dependent binding of 1 μM tubulin with 10 μM bis-ANS is very likely to be a consequence of binding of the drug molecules to an increased population of tubulin monomers present at 1 μM.

It is important to ask how a large fluorescence enhancement could be obtained upon the binding of bis-ANS to dissociated subunits of tubulin. Ward and Timasheff (1994) have recently pointed out the very unusual nature of the binding of bis-ANS to tubulin. It is unusual both in terms of the high number of binding sites as well as in terms of the positive cooperative nature of the binding process. The mechanism proposed to explain this phenomenon involves conformational changes induced by ligand binding, which leads to enhanced binding of subsequent ligand molecules. A similar mechanism could explain our results, viz., the enhancement of fluorescence of bis-ANS−tubulin complexes at low protein concentrations. Ward and Timasheff's proposal requires that the binding domain of the protein must be flexible and must possess multiple ligand binding sites in the vicinity of each other, allowing interactions among neighboring bound ligands. According to our results, these flexible binding domains could very well be the monomers of tubulin, which could harbor lower affinity binding sites of bis-ANS. These monomers have lesser tertiary structure compared to native tubulin ($\alpha\beta$) and are capable of undergoing time-dependent conformational changes with incubation at 37 °C. An alternative mechanism that could explain this kind of phenomenon involves the interactions between free ligand molecules themselves. However, the concentration of bis-ANS used here is 10 μM, which is probably too low for self-association among free ligands to take place (Ward & Timasheff, 1994).

Thus, from the present study it appears that the observed enhanced fluorescence of the complex (with 1 μM tubulin and 10 μM bis-ANS) is due to the time-dependent conformational changes of the binding sites on monomers of tubulin and possibly not due to dimers (Figure 3, curves a and c), as dimers would require much higher concentrations of bis-ANS ($\sim 4 \times 10^{-4}$ M) to exhibit similar phenomena (Ward & Timasheff, 1994). These conformational changes of binding sites are favored at lower tubulin concentrations, and a correlation exists between the enhanced bis-ANS fluorescence and the fraction of tubulin in the monomeric form (Figure 7). Thus, it is expected that these bis-ANS binding sites are located in the dissociated subunits that have less tertiary structure, are more flexible with exposed sites, and may be more prone to undergoing conformational changes upon incubation at 37 °C than the dimer. Dissociation of dimeric tubulin into monomers causes a significant reduction in their tertiary structure as evidenced by near-UV CD spectra (data not shown). However, this reduction in the content of tertiary structure does not affect the colchicine binding activity of tubulin, and both dimers and monomers bind colchicine equally well (unpublished results).

Since increasing the protein concentrations, lowering the temperature, and binding of colchicine to tubulin all drive the monomer ⇌ dimer equilibrium toward the dimer, the

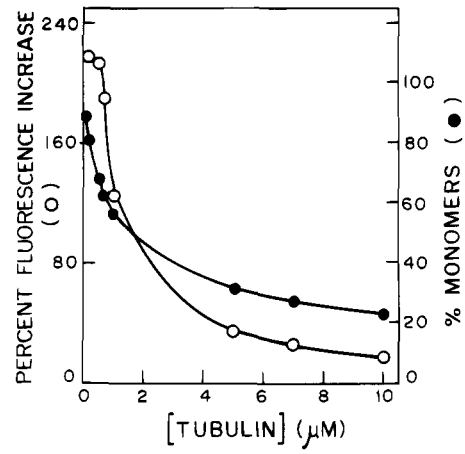

FIGURE 7: Bis-ANS−tubulin fluorescence and percent monomer in solution. Tubulin at different concentrations (0.1−10 μM) were incubated at 37 °C for 0 and 120 min. Thereafter, each of these protein samples was made 10 μM in bis-ANS, and the fluorescence at 490 nm was measured (○). The percent monomer in solution (obtained from monomer ⇌ dimer equilibrium using $K_d = 7.2 \times 10^{-7}$ M) (Panda et al., 1992) (●) for these tubulin concentrations are shown. Excitation wavelength was 385 nm.

inhibition of formation of hydrophobic sites upon treatment of tubulin with colchicine or upon lowering of incubation temperature to 0 °C is consistent with the preceding explanation. While the enhanced bis-ANS binding appears to be correlated to the enrichment of monomers in the solution of tubulin, it cannot be unequivocally ascertained whether the lower affinity sites that undergo conformational changes manifesting enhanced fluorescence upon incubation at 37 °C exist in the monomer only. Whichever form of tubulin may harbor the low-affinity bis-ANS binding sites, it is clear that that form of tubulin is significantly altered upon the digestion of C-termini (Figures 5 and 6). According to recent reports, the high-affinity bis-ANS site is in the N-terminal part of β-tubulin (Ward et al., 1994), whereas the lower affinity sites observed at high bis-ANS concentrations are predicted to be in the flexible part of $\alpha\beta$-tubulin (Prasad et al., 1986; Ward et al., 1994). In the present study, where the solution contains 1 μM tubulin and 10 μM bis-ANS, both higher and lower affinity complexes of drug−tubulin would be formed (Figure 4A,B). Subtilisin digestion of a fresh complex of bis-ANS and tubulin did not change the fluorescence, except for a few nanometers blue shift. This result indicates that the high-affinity site and even the lower affinity sites of the fresh complex are not undergoing significant conformational changes (see Figure 5, inset) upon C-termini digestion. On the other hand, incubation of the complex at 37 °C causes a significant increase in the fluorescence intensity with a concomitant blue shift (Figures 1 and 4B).

Interestingly, the subtilisin digestion of this preincubated complex causes a reverse phenomenon with a decrease in fluorescence intensity and a red shift (Figure 5). A possible explanation of this observation is that the incubation of bis-ANS−tubulin complex at 37 °C causes a dramatic change in the conformation of the lower affinity sites, which may lead to (a) changes in the affinity of the binding sites, (b) generation of new binding sites, and (c) changes in the quantum yield of the complexes. One or more of the preceding changes can arise due to the interactions among bound bis-ANS molecules caused by the incubation of the

complex at 37 °C. We believe that the C-termini of both subunits are involved in this process (Figure 6B). Thus, the digestion of C-termini causes further conformational changes in this part of the protein, affecting the interactions among bound drugs.

In conclusion, it needs to be emphasized that, although bis-ANS has been used as a hydrophobic marker in several protein studies, possible effects of bis-ANS on the conformation and stability of the protein in question have to be studied carefully.

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