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The Carboxy Terminus of the α Subunit of Tubulin Regulates Its Interaction with Colchicine†

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ABSTRACT: Controlled proteolysis of goat brain tubulin by subtilisin was carried out to investigate regulatory aspects of the binding of colchicine to tubulin. Tubulin S, obtained by the cleavage of the carboxyl termini of both the α- and β-subunits of tubulin by subtilisin, exhibited the following differences compared to native tubulin: (a) Reaction with colchicine, which has an optimum pH of 6.8, becomes independent of pH (in the range 5.7-8.0). (b) The colchicine-binding site, which is labile at 37 °C ($t_{1/2}$ = 4-5 h), becomes highly stable ($t_{1/2}$ > 12 h). (c) The affinity for colchicine is lowered. (d) This lowering of affinity arises from a faster dissociation (higher off rate) of the complex. The above characteristics of tubulin S were not shown by a partially digested hybrid in which the C-terminus of the β-subunit alone was cleaved. The hybrid behaved very much like the undigested native protein. These results strongly suggest that the regulatory switch for colchicine-tubulin interaction is located in a small region (about 15 residues) of the C-terminus of the α-subunit of tubulin. Possibilities of the C-termini being involved in nonbonded contacts with the main body of tubulin are also noticed from the change in conformation between tubulin and tubulin S.

Limited proteolysis is a powerful tool in the study of the structures and functions of proteins as well as of regulatory mechanisms of proteins in vivo (Jacobson, 1964; Mihalyi, 1978). This classical technique has been applied to tubulin for the identification of domains for the binding of colchicine (Serrano et al., 1984b; Avila et al., 1987), nucleotides (Maccioni & Seeds, 1983), calcium (Serrano et al., 1986) and microtubule-associated proteins (MAPs)[1] (Serrano et al.,

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1984a,c, 1985). It has also been used to throw light on the three-dimensional structure of tubulin (Sackett & Wolff, 1986; de la Viña et al., 1988).

Tubulin is a heterodimer containing two subunits, α and β, each of which has been sequenced and is composed of about 450 amino acids (Krauhs et al., 1981; Postingl et al., 1981). Both the subunits are acidic, with the β-subunit slightly more acidic than α (apparent pI = 5.3 and 5.4, respectively) (Berkowitz et al., 1977). The C-terminal end of each is remarkably rich in acidic residues, especially in glutamic acid (Krauhs et al., 1981; Ponstingl et al., 1981). These regions (residues 410–451 in α and 400–445 in β) typically contain about 40% of all the glutamates and about 20% of all the aspartates in tubulin. At physiological pH, the sequences are highly charged and stay in an extended conformation (Sackett & Wolff, 1986). Proteolysis of tubulin with subtilisin has revealed that small regions at the C-terminal ends of both subunits are exposed to the solvent and are readily cleaved (Serrano et al., 1984c; Sackett et al., 1985). Digestion of the C-termini is little affected in taxol- or Zn-induced polymers (Sackett & Wolff, 1986), suggesting that these regions extend into the solvent away from the surface of the polymer. This view is supported by NMR data, which indicate that the C-terminal ends of tubulin are relatively mobile (Ringel & Sternlicht, 1984).

With a review to probing the functional features of the C-terminal end of tubulin, we have used selective proteolysis of subunits by subtilisin, which specifically cleaves the C-terminal ends (Serrano et al., 1984c; Sackett et al., 1985; Bhattacharyya et al., 1985). In this paper we report that several properties of colchicine–tubulin complexes (viz., pH sensitivity, off and on rates of complex formation, and the stability of the colchicine-binding site) are regulated by the C-terminal tail of the α-subunit of tubulin and that this regulation works through an alteration in the off rate of complex formation. It may be mentioned that while this work was in progress, Avila et al. (1987) have reported that binding of colchicine to porcine or lamb brain tubulin is modulated by the C-terminal region.

Materials and Methods

Tubulin was prepared by phosphocellulose chromatography of microtubule protein purified from goat brain by two cycles of temperature-dependent polymerization in PIPES assembly buffer (100 mM PIPES, 0.5 mM $MgCl_2$, and 1 mM EGTA, pH 6.8) with 1 mM GTP (Sloboda & Rosenbaum, 1982). Following column chromatography, the protein was concentrated to 5–10 mg/mL by using Amicon CF 50A membrane cones and stored in liquid nitrogen. Protein concentration was determined by the method of Lowry et al. (1951).

GTP, PIPES, EGTA, PMSF, and subtilisin BPN were from Sigma. Colchicine, podophyllotoxin, and dimethyl sulfoxide (DMSO) were obtained from Aldrich. All other chemicals used in this study were reagent grade.

Subtilisin was stored as stock solutions of 1 mg/mL in water, frozen in aliquots at −70 °C, and thawed once only. Digestion of tubulin with subtilisin was performed at 30 °C in PIPES assembly buffer with 1 mM GTP except as noted. Subtilisin was taken in the ratio enzyme:protein = 1:100 (w/w). The reaction was terminated by the addition of 1% by volume of 1% (w/v) PMSF in DMSO. When the digestion was carried out at 4 °C, only the C-terminus of the β-subunit was cleaved (Bhattacharyya et al., 1985) and the product $\alpha\beta_s$ is called hybrid tubulin. Digestion at 30 °C resulted in the cleavage of the C-termini of both the subunits, giving rise to the cleaved product $\alpha_s\beta_s$, which is called tubulin S. In the digested product $\alpha_s\beta_s$, no trace of any low molecular weight fragment was noticed on SDS gel electrophoresis (Figure 1). De la Viña et al. (1988) and Kanazawa and Timasheff (1989) have reported an internal cleavage on the α-subunit of rat brain tubulin when digestion is done with subtilisin, in phosphate buffer. However, under our experimental conditions, cleavage occurred predominantly on the C-termini, as is evident from Figure 1.

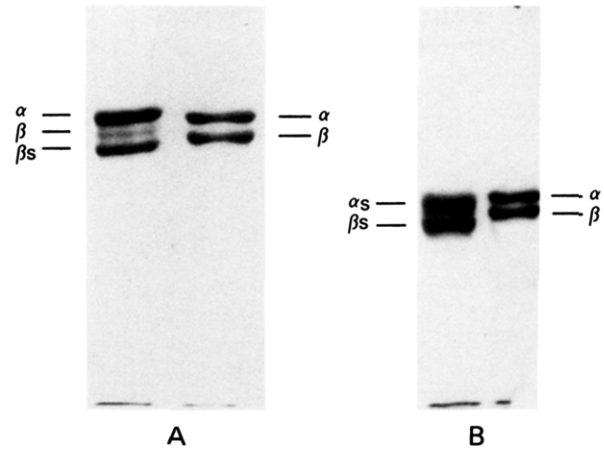

FIGURE 1: SDS gel electrophoresis of $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ tubulins. Samples were prepared as described under Materials and Methods. Gels were run according to a modification of the Laemmli (1970) method as used by Sackett et al. (1985). (A) $\alpha\beta_s$ (left) and $\alpha\beta$ (right), (B) $\alpha_s\beta_s$ (left) and $\alpha\beta$ (right).

For fluorescence assay of colchicine binding, excitation was done at 350 nm and emission was measured at 430 nm in all cases, with slit widths of 5 nm each. Fluorescence spectra were recorded on a Hitachi F-3000 spectrofluorometer fitted with a constant-temperature cell holder connected to a constant-temperature circulating water bath. All experiments were carried out at 25 °C in PIPES or PM (10 mM phosphate and 10 mM $MgCl_2$, pH 6.8) buffers. All the kinetics experiments were carried out in PM buffers of appropriate pH.

Results

pH Sensitivity of Colchicine Binding. Colchicine–tubulin interaction is strongly influenced by pH. Wilson (1970) reported that in the case of the 100000*g* supernatant of chick embryo brain tubulin the maximum colchicine-binding activity is obtained at pH 6.7–6.8. In this study, we also observed a similar optimum pH (6.8) for purified goat brain tubulin (Figure 2A, curve 1), with the colchicine binding decreasing on both the lower (pH < 6.8) and higher (pH > 6.8) sides. The pH profile, however, gives no indication as to why the binding declines above and below this optimum pH. The decline may result from (a) the formation of an improper ionic form of tubulin, (b) inactivation of tubulin, or (c) a combination of both (a) and (b). From Figure 2A (curve 2) it is also observed that preincubation of tubulin (for 45 min, at 37 °C) at different pH values in the range 5.7–8.0 has no effect on the colchicine-binding activity provided that the protein is brought back to pH 6.8 during the binding assay. Thus, the change in the ionization state and associated structural changes (if any) must be responsible for the loss of colchicine-binding activity at pH ≠ 6.8. These changes, however, are fully reversible, since complete binding activity is regained when

[1] Abbreviations: MAPs, microtubule-associated proteins; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

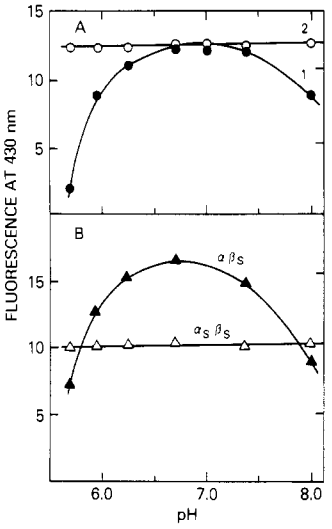

FIGURE 2: (A) Curve 1 (●): colchicine binding of native ($\alpha\beta$) tubulin as a function of pH, as monitored by fluorescence. Tubulin (5 μM) was incubated in 100 mM PIPES containing 0.5 mM $MgCl_2$ and 1 mM EGTA, at the pH indicated on the figure, for 30 min at 37 °C in the presence of 10 μM colchicine. Curve 2 (○): effect of preincubation of the protein at different pH values on colchicine binding. The protein was incubated at the indicated pH for 45 min (as for curve 1) without colchicine. Thereafter, the solution was brought to pH 6.8 by dialyzing against PIPES buffer (as above), pH 6.8, before adding colchicine and incubating at 37 °C for 30 min. (B) Fluorescence assay of colchicine binding for $\alpha\beta_s$ (▲) and $\alpha_s\beta_s$ (△) tubulins as a function of pH. Conditions were the same as for curve 1 in (A).

the activity is measured at pH = 6.8.

However, when the colchicine-binding activity of tubulin S was measured at different pH values, a significant result was obtained. As shown in Figure 2B, the binding activity was little influenced by pH in the range pH = 5.7–8.0. In order to identify the possible role of the C-terminus of one of the subunits, a similar experiment was carried out with hybrid tubulin ($\alpha\beta_s$). Surprisingly, this showed a pH sensitivity akin to that shown by native ($\alpha\beta$) tubulin (Figure 1B). These results suggest that the C-terminus of the α-subunit of tubulin is responsible for the pH sensitivity of colchicine–tubulin interaction, in spite of the fact that the subtilisin-cleaved C-terminus of the α-subunit is outside the colchicine-binding site (Avila et al., 1987). This result is in conformity with a recent report (Morgan et al., 1985) on the relationship between the C-terminus of the α-subunit and the cholchicine-binding site, which showed that the colchicine–tubulin complex is a less efficient competitor for the peptide antibody (raised against seven C-terminal amino acids of the α-subunit) than tubulin alone, suggesting that the binding of colchicine to tubulin has induced a conformational change in the C-terminal end of the α-subunit of tubulin.

Lability of the Binding Site. Lability of the colchicine-binding site of tubulin is well-known and has been reported by several authors (Wilson, 1970; Bhattacharyya & Wolff, 1975a,b, 1977). Although the precise reason for this lability is unclear, several agents are known to stabilize the colchicine-binding site. These include vinblastine (Wilson, 1970; Bhattacharyya & Wolff, 1975a,b, 1977), polylysine (Roychowdhuri et al., 1983), MAPs (Wiche & Furtner, 1980), and NaCl (Wilson, 1970). It is interesting to note that all of these are either cations or behave as cations under solution conditions, suggesting the involvement of ionic interactions in the lability of the colchicine-binding site on tubulin. As seen in Figure 3, the colchicine-binding site on tubulin S is remarkably stable: no significant decline in colchicine-binding activity is

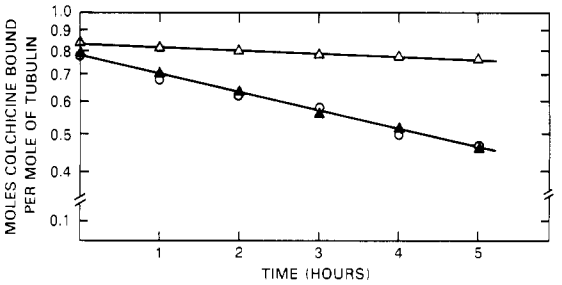

FIGURE 3: Stability of colchicine binding, measured by fluorescence as a function of time. Tubulin (5 μM) was incubated in PM buffer (10 mM phosphate and 10 mM $MgCl_2$, pH 6.8) at 37 °C for the indicated times before colchicine addition. Thereafter, preincubated tubulin was incubated with 40 μM colchicine for 90 min at 37 °C, and fluorescence was measured at 430 nm. Excitation wavelength was 380 nm, and emission intensity was corrected for inner filter effect.

detected even after an incubation for 5 h at 37 °C. Under identical conditions, native and hybrid tubulins showed about a 35–40% decrease in the colchicine-binding activity. Thus the C-terminus of the α-subunit is probably responsible for the lability of the colchicine-binding site. It is worth mentioning here that, among the stabilizing agents, MAPs and polylysine are known to bind to tubulin at the C-termini and enhance polymerization (Serrano et al., 1984a,c, 1985). The polymerization of tubulin alone or in the presence of MAPs is sensitive to rather low concentrations of NaCl and is strongly inhibited by 100 mM NaCl (Bhattacharyya et al., 1985). In contrast, polymerization of tubulin S remains unaffected even at 240 mM NaCl (Bhattacharyya et al., 1985). These results indicate that the salt sensitivity of tubulin involves its C-termini.

On and Off Rates. As shown in Figure 2B, the colchicine-binding activity of $\alpha_s\beta_s$ is always less than that of $\alpha\beta$ or $\alpha\beta_s$, and unlike the latter ones, the activity of $\alpha_s\beta_s$ is independent of pH between pH 5.7 and 8.0. It is, therefore, important to investigate what are the precise reasons for this lower colchicine-binding activity of $\alpha_s\beta_s$. As tubulin has only one colchicine-binding site (Bhattacharyya & Wolff, 1974), the lowering of stoichiometry does not explain the above. Decay of the binding activity is also ruled out, since the colchicine-binding site of $\alpha_s\beta_s$ tubulin is stabler compared to that of $\alpha\beta$ and $\alpha\beta_s$ (Figure 3). Thus, among several possibilities, the lower affinity constant for $\alpha_s\beta_s$–colchicine interaction as compared to that for $\alpha\beta$–colchicine appears to be the most likely explanation. We measured the binding parameters such as association rate constant (k_1) and dissociation rate constant (k_{-1}) for the interaction of colchicine with native and hybrid tubulins as well as with tubulin S under various pH conditions and determined the affinity constant (K_a) from their ratio. Since tubulin undergoes rapid decay during prolonged incubation (Wilson, 1970), the apparent dissociation rate constant k_{off} of colchicine–tubulin interaction is composed of two components: the true dissociation rate (k_{-1}) of the complex and the rate of decay (k_{decay}) of the colchicine-binding site due to denaturation. Thus $k_{off} = k_{-1} + k_{decay}$ was determined from the equation $0.693/t_{1/2} = k_{off}$. k_{-1} and k_{decay} were independently determined according to Garland and Teller (1975). Assuming that the binding of tubulin to colchicine is a bimolecular reaction, the association rate constant (k_1) can be written

$$k_1 = \frac{d[CT]}{dt}[C]^{-1}[T]^{-1}$$

where $d[CT]/dt$ is the rate of formation of the colchicine–tubulin complex and [C] and [T] denote concentrations of free

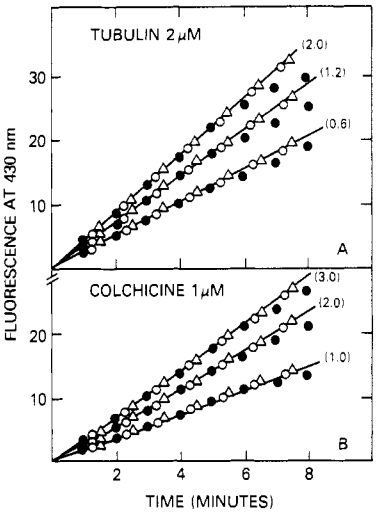

FIGURE 4: Rate of colchicine binding as a function of concentrations of colchicine and tubulin. Buffer conditions were the same as in Figure 3. (A) Tubulin was held constant at 2 μM. Numbers in parentheses refer to colchicine concentrations (μM). (B) Colchicine was held constant at 1 μM. Numbers in parentheses represent tubulin concentrations (μM). Results of experiments with the three varieties of tubulin, viz., $\alpha\beta$ (Δ), $\alpha\beta_s$ (O), and $\alpha_s\beta_s$ ($\bullet$), are presented.

colchicine and unbound tubulin, respectively. Conditions were adjusted in such a manner that less than 10% of the reactants were consumed during the linear portion of the binding curves (Figure 4). In one set of experiments, the protein concentrations were held constant (2 μM) and the rate curves were linear for about 6 min over a concentration of 0.6–2 μM colchicine (Figure 4A). In the other set, when the concentration of colchicine was 1 μM, the curve showed linearity for about 6 min, over a protein concentration of 1–3 μM (Figure 4B). The association rate constants (k_1) determined from these experiments for colchicine binding with $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ tubulins were found to be very similar. Furthermore, the k_1 values did not change with pH in the range 6.0–8.0 and were restricted to 2.2–2.7 mM^{-1} min^{-1} (see Figure 6A).

The apparent dissociation rate constant k_{off} and the decay constant k_{decay} were also determined similarly, and the true dissociation rate constant k_{-1} was evaluated therefrom as shown in Figure 5. k_{-1} and k_{decay} at various pH values are plotted in Figure 6, panels B and C, for each of the species $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$. Interestingly, for both native and hybrid tubulins, k_{-1} showed a minimum value of (0.3–0.4) $\times$ 10^{-3} min^{-1} at pH = 6.8, while for tubulin S k_{-1} = 1.2 $\times$ 10^{-3} min^{-1}, independent of pH. Thus it is evident that the lower colchicine-binding activity of $\alpha_s\beta_s$ arises from its higher rate of dissociation. Results presented in Figure 6C demonstrate the pH stability of $\alpha_s\beta_s$: the decay constant remains unchanged at the low value of $\sim$0.05 $\times$ 10^{-3} min^{-1} as compared to native and hybrid tubulins, for which k_{decay} increases from 0.05 $\times$ 10^{-3} min^{-1} to about 0.5 $\times$ 10^{-3} min^{-1} as the pH is raised from 6.0 to 8.0.

Affinity constants K_a ($=k_1/k_{-1}$) presented in Figure 6D again show that tubulin S is little affected by pH. In contrast, there is a sharp maximum at pH = 6.8 for native and hybrid tubulins. The affinity constant K_a = 7 $\times$ 10^6 M^{-1} for $\alpha\beta$ and $\alpha\beta_s$, as compared to about 2 $\times$ 10^6 M^{-1} for tubulin S. Thus the selective cleavage of the C-terminal tail of the α-subunit causes, through an enhanced dissociation rate, a 3.5-fold decrease in the affinity constant for colchicine binding at pH = 6.8.

The above results also bring home an important aspect of colchicine–tubulin interaction: the reversibility of the interaction is dependent upon the pH of the reaction. Reversibility is determined by the activation energy for dissociation of the complex, ΔA_d, which is the sum of the activation energy of associated reaction, ΔA_a, and the difference in equilibrium free energy, ΔF, between the reactants and the product:

$$\Delta A_d = \Delta A_a + \Delta F$$

Colchicine–tubulin interaction has a rather large activation energy ($\Delta A_a \simeq$ 20 kcal/mol) and the exothermicity of the process (ΔF) is known to be −9 to −10 kcal/mol (Bane et al.,

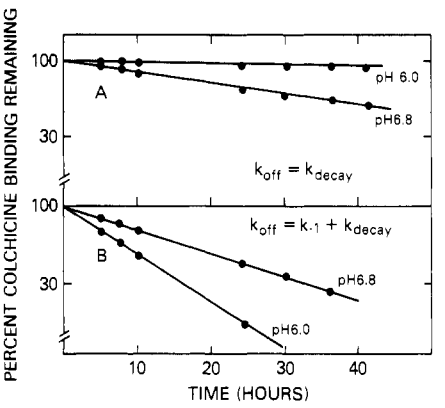

FIGURE 5: Measurement of off rates for colchicine–tubulin interaction in the presence of (A) excess colchicine or (B) excess podophyllotoxin. Results for tubulin ($\alpha\beta$) at two different pH's are shown. Similar measurements for $\alpha\beta_s$ and $\alpha_s\beta_s$ tubulins (not shown) were carried out at several pH values. The protein (10 μM) was incubated with a saturating concentration of colchicine (100 μM) in 10 mM phosphate, 10 mM $MgCl_2$, and 0.1 mM GDP, pH 6.8, for 90 min at 37 °C. Free colchicine was separated from protein-bound colchicine by passing the incubation mixture over a Sephadex G-75 column (20 $\times$ 1 cm). The protein-bound colchicine, which eluted at the void volume, was then collected and split into two equal parts. To one part was added 50 μM colchicine. To the other was added the same concentration of podophyllotoxin. Incubation was continued and both the samples were assayed for bound colchicine by fluorescence measurement at different time intervals, as shown. The loss of bound colchicine in the presence of excess colchicine represents the irreversible denaturation of the protein and is characterized by the rate k_{decay} (A), while in (B), the loss of bound colchicine in the presence of an excess podophyllotoxin gives the rate of loss as k_{off}.

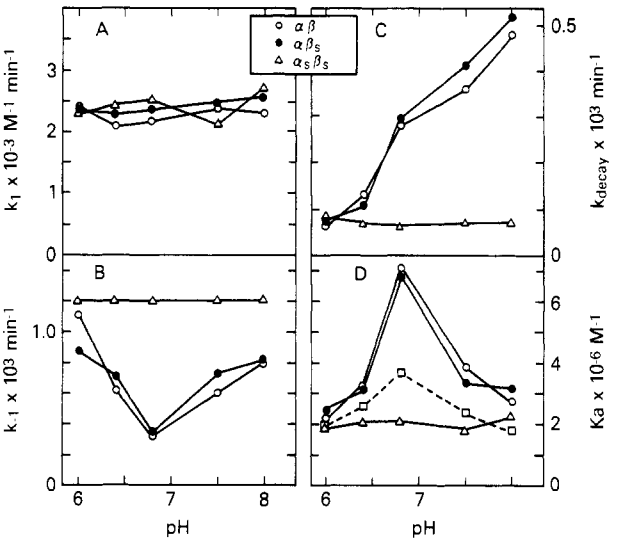

FIGURE 6: On rates (k_1) (A), off rates (k_{-1}) (B), decay constants (k_{decay}) (C), and association constants (K_a) (D) for $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ tubulins at various pH values. Determinations were done as described in the text. K_a determined from $K_a = k_1/k_{decay}$ for tubulin ($\alpha\beta$) is shown in panel D with broken lines as an example to demonstrate the importance of separation of k_{decay} from k_{off} to arrive at the true dissociation rate, k_{-1}.

Table I: Comparison of Colchicine-Binding Properties of Tubulin $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$

	pH dependence of binding act.	rel colchicine-binding act.	$t_{1/2}$ for decay of binding site (h)	assocn const, K_a (M^{-1})	off rate const, k_{-1} (min^{-1})
$\alpha\beta$	max at 6.8	1.0	4–5	7.1×10^6	0.31×10^{-3}
$\alpha\beta_s$	max at 6.8	1.0	4–5	6.8×10^6	0.35×10^{-3}
$\alpha_s\beta_s$	independent of pH	0.6	>12	1.9×10^6	1.20×10^{-3}

1984). Therefore, the irreversibility of the colchicine–tubulin complex is generally attributed to the large activation energy of association, coupled with the thermodynamic stability of the complex, which imposes an extremely high activation energy barrier for the dissociation of the complex. Interestingly, the partial reversibility that we observe in the case of tubulin S is due to the reduced thermodynamic stability of the complex. It is worthwhile to note that B-ring analogues of colchicine such as 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone, deacetamidocolchicine, and colcemid exhibit reversibility of binding with tubulin through a different mechanism: in those cases, the activation energy of complex formation is lowered as compared to that for colchicine (Bhattacharyya & Wolff, 1974; Bane et al., 1984; Andreu et al., 1984; Banerjee & Bhattacharyya, 1979; Ray et al., 1981; Bhattacharyya et al., 1986).

Conformations. The results presented above reveal marked differences between tubulin and tubulin S with respect to various features of their colchicine-binding property: stabilization of the binding site, reduction in the pH sensitivity of binding, and increase in the off rate of complex formation. These are summarized in Table I. It is also evident that cleavage of the C-termini of both subunits is essential for this difference, as the hybrid behaves similarly to native tubulin.

Since the C-termini of the α- and β-subunits do not include the colchicine-binding site (Avila et al., 1987), it is difficult to reconcile our results with the existing belief that cleavage of the C-terminal tail of tubulin by subtilisin leaves the rest of the molecule unaffected. Before trying to find a reason for a difference in the structural organization between tubulin and tubulin S, it was therefore necessary to investigate possible conformational changes caused by subtilisin cleavage. For this purpose, we carried out the following experiments, both of which confirm our suspicion that the gross conformations of $\alpha\beta$ and $\alpha_s\beta_s$ tubulins are rather different.

First, we examined the quenching of the tryptophan residues in each of the species by acrylamide. A previous report by Maccioni et al. (1986) had shown that the removal of C-termini of tubulin is accompanied by a blue shift in tryptophan fluorescence, indicating that some tryptopans were in a more hydrophobic environment after subtilisin digestion. Quenching by acrylamide was thus expected to reveal the differences in the environments of tryptophan residues between tubulin and tubulin S. Changes in fluorescence intensities were accompanied by a blue shift in each case, as a fixed concentration of the protein was titrated with increasing concentrations of acrylamide. The results are shown in Figure 7 in the form of Lehrer plots. The intercepts on the ordinate, which are a measure of exposure of tryptophans, clearly show that in $\alpha_s\beta_s$ tryptophan residues are less available for quenching by acrylamide as compared to those in $\alpha\beta$. The accompanying blue shifts, shown in the inset, again support this result. As the acrylamide concentration is increased to 0.5 M, the fluorescence emission spectrum for $\alpha_s\beta_s$ undergoes a larger blue shift compared to $\alpha\beta$, indicating that tryptophans in $\alpha_s\beta_s$ are

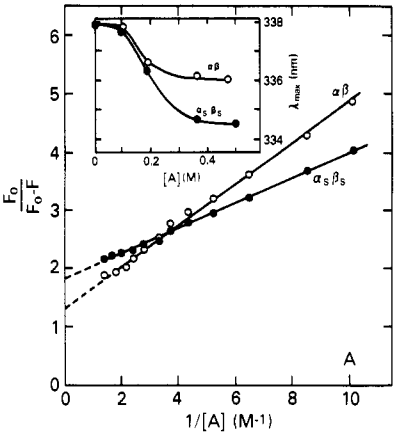

FIGURE 7: Lehrer plots for tubulin (○) and tubulin S (●), as constructed from acrylamide quenching of the tryptophan fluorescence at 340 nm. Excitation was at 295 nm. F_0, fluorescence in the absence of quencher; F, fluorescence in the presence of a concentration [A] of acrylamide. The inset shows the variation of the position of the fluorescence emission peak as a function of acrylamide concentration.

Table II: Quenching of Tryptophan Fluorescence of Tubulin $\alpha\beta$ and $\alpha_s\beta_s$ by Acrylamide

	blue shift, $\Delta\lambda$ (nm)	effective Stern-Volmer constant, $K_{SV}(eff)^a$ (M^{-1})	fraction of accessible fluorescence, $f(eff)^a$
$\alpha\beta$	1.7	2.75	0.78
$\alpha_s\beta_s$	3.3	4.50	0.56

[a] Determined from Figure 6 according to Efftink and Ghiron (1981).

in a more hydrophobic environment. Thus, $\alpha\beta$ and $\alpha_s\beta_s$ tubulins are conformationally distinct as far as the tryptophan residues are concerned. Other experiments using bis(ANS) as a fluorescent probe emphasize the difference between tubulin and tubulin S (unpublished observations).

The conformational difference betwen $\alpha\beta$ and $\alpha_s\beta_s$ was also evident from the CD spectra of the proteins (not shown). It was observd that subtilisin digestion caused a reduction in the negative CD band of tubulin. Further studies are in progress to investigate this effect in more detail. We summarize our results in Table II, which establishes that a change in conformation takes place in tubulin when its C-termini are digested by subtilisin.

Discussion

The irreversibility of colchicine–tubulin interaction as well as the instability of the colchicine-binding site is poorly understood at present. The present work is the first report of its kind that examines in detail the origin of these phenomena at the protein level. The irreversible nature of the complex arises from a reduced rate of dissociation and is a function of pH. What is more important, this feature is controlled predominantly by the C-terminal tail of the α-subunit of tubulin.

Tubulin isotypes have been seen to differ mainly in their C-terminal sequences (Ponstingl et al., 1981). Binding of MAPs and Ca^{2+} takes place at the C-terminus, which regulates the binding (Serrano et al., 1984a,c, 1986). Regulation of the binding of colchicine to tubulin by the C-terminus is yet another example that underlines the importance of the C-terminal region in tubulin.

The conformations of tubulin and tubulin S appear to be very different, a fact that does not tally well with the general picture of the C-termini as loosely hanging tails from the main body of the subunit structure. Other interactions of the C-termini with the main body are thus likely to be present.

Evidence of native gel electrophoresis showing separation of the C-terminus after digestion with subtilisin (Sackett & Wolff, 1986) is usually regarded as a strong indication in favor of a loosely hanging C-terminus, since other domains need denaturation and can be separated only on SDS gels. However, when an electric field is applied, other interactions may be superseded and this could explain the greater electrophoretic mobility of the tail. It is likely that the C-terminus is involved in weak interactions between its charged residues and the rest of the protein. This conclusion is supported by the strong pH dependence of the colchicine-binding experiments. Recently, a salt bridge between a positive cluster consisting of Lys-394, His-393, and Arg-390 and the C-terminus of the α-subunit was hypothesized (Blank et al., 1986). Interestingly, these three basic amino acids constituting the positive cluster are part of the 16-kDa tryptic peptide isolated by Serrano et al. (1984b) that has colchicine-binding activity. Our results thus form another basis in support of such a model.

We have in this paper maintained that the C-terminus of the α-subunit is the responsible factor in the regulation of colchicine binding. Though the influence of the carboxy tail of the α-subunit is undoubtedly predominant, involvement of the C-terminus of the β-subunit cannot be ruled out from our experiments. In practice, it is very difficult to prepare the other analogue, $\alpha_s\beta$. However, the fact that the C-terminus of the β-subunit is first cleaved (by subtilisin) again emphasizes the difference between the C-termini of the α- and β-subunits (Bhattacharyya et al., 1985). All these findings are important in the overall structural organization of tubulin.

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