

The colchicine-binding and pyrene-excimer-formation activities of tubulin involve a common cysteine residue in the β subunit

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Colchicine binding and pyrene excimer fluorescence of tubulin have been used to identify cysteine residue(s) essential for the colchicine binding activity of the protein. We report here that both the colchicine binding activity and the ability to form pyrene excimers of tubulin decay at an identical rate when the protein ages at 37°C. Glycerol, which stabilizes the colchicine binding site also stabilizes the excimer formation equally. Thus, these two properties of tubulin are correlated and are likely to belong to the same structural domain. In an attempt to identify the excimer-forming Cys residues, we found that incubation of tubulin with *N,N'*ethylenebis(iodoacetamide) causes a significant inhibition of excimer fluorescence. Incubation of tubulin with colchicine prior to this treatment fully retains excimer-forming ability. It is known that Cys239 and Cys354 of β -tubulin, which are about 0.9 nm apart in the native structure, are protected from ethylenebis(iodoacetamide) cross-linking by incubation of tubulin with colchicine [Luduena, R. F. & Roach, M. C. (1981) *Pharmacol. Ther.* 49, 133–152]. These residues must therefore be responsible for the excimer formation of tubulin with pyrene maleimide. Incubation of tubulin with ethylenebis(iodoacetamide) decreases the colchicine binding activity and the excimer formation at an identical rate. Since the alkylation of Cys239 of β -tubulin (responsible for tubulin self-assembly) has no effect on colchicine binding [Bai, R., Lin, C. M., Nguyen, N. Y., Liu, T. & Hamel, E. (1989) *Biochemistry* 28, 5606–5612], our results suggest that excimer formation and the colchicine binding site of tubulin share Cys354 of the β -subunit. Determination of the number of essential Cys residue(s) for colchicine binding activity, using the statistical method of Tsou [Tsou, C. L. (1962) *Sci. Sin.* 11, 1535–1558], also shows only one essential Cys residue.

Keywords: cysteine; colchicine; aging; pyrene maleimide; excimer.

Tubulin, a 100-kDa heterodimeric protein, undergoes self polymerisation to form microtubules which participate in various cellular functions. The drug colchicine binds to tubulin and inhibits this polymerisation [1, 2]. Tubulin loses both these properties, viz. polymerisability and colchicine binding activity, as a function of time, when left in solution – a phenomenon referred to as aging. Despite their importance, the sites on the protein involved in polymerisation and in colchicine binding have not been well characterised.

The two subunits, α and β of tubulin, each comprises about 450 amino acid residues. It is known that Cys residues of tubulin are essential for both polymerisation as well as colchicine binding [3]. However, dimeric tubulin is a multicysteine protein with 12 Cys in the α and 8 in the β subunit [4, 5]. With so many, it is difficult to identify functional Cys or to selectively label some with a fluorophore. However, a few unique Cys have been identified in tubulin: here are two pairs in the β subunit of the protein (Cys12-Cys201/Cys211 and Cys239-Cys354, see Fig. 1) which could be cross-linked with *N,N'*ethylenebis(iodoacetamide), indicating that the residues in each pair are not more than 0.9 nm apart [6, 7].

Polymerisation is very sensitive to modification of Cys residues. Alkylation of the single Cys239 of β tubulin results in a

complete loss of polymerisation [8]. No such essential Cys has yet been identified for the colchicine binding activity and even the total number involved in the colchicine binding activity is not known. Titration of Cys with *p*-chloro-mercuribenzoate revealed that about 60% inhibition of colchicine binding could be achieved when 10–12 Cys of tubulin were modified. The drug binding remained virtually unchanged when 5 residues were modified [9]. It is the purpose of this paper to identify the Cys residue(s) involved in the colchicine binding activity of tubulin. Several studies have clearly established that the colchicine binding site is located in the C-terminal domain of β -tubulin (β C, from Tyr281, in Fig. 1). These include direct photoaffinity labeling [10, 11]; linkage of binding and the exchange of guanine nucleotides [12]; disruption of ethylenebis(iodoacetamide) induced Cys239-Cys354 cross-linking by the binding of colchicine [13] and colchicine-induced proteolysis at around Arg390 [14]. There are, however, only two Cys in β C: Cys303 and Cys354.

It has been reported from our laboratory that tubulin has the unique property of excimer formation when treated with pyrene maleimide [15]. This excimer-forming ability is retained even in the dissociated, monomeric state of tubulin, indicating that the excimer-forming Cys residues belong to the same subunit. Interestingly, full colchicine binding activity of tubulin is retained in the monomeric state [12, 16]. It has also been reported that both colchicine binding activity and the excimer fluorescence of tubulin are lost at identical rates with increasing temperature [15].

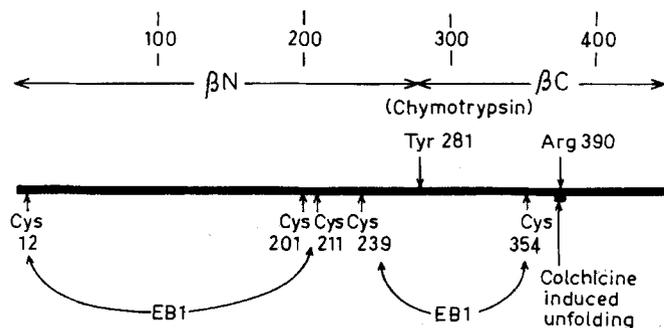


Fig. 1. Schematic diagram of β -tubulin. Residue numbers are indicated at the top. The chymotrypsin cleavage site at Tyr281 is shown by an arrow (I) and the resulting N-terminal domain (β N) and C-terminal domain (β C) of β -tubulin are indicated. Two Cys pairs which can be cross-linked with ethylenebis(iodoacetamide) (EB1) and the site of the colchicine-induced unfolding (Arg390) are shown below. Cys239 has been identified as essential for polymerisation [8]. Further details and references are given in the introduction.

Thus it is possible that Cys residues involved in excimer formation and in colchicine binding could be common. As mentioned above, the colchicine binding site is located in region of the β subunit, but the location of excimer-forming Cys is not known. On the basis of distances, however, the two pairs of Cys which undergo cross-linking with ethylenebis(iodoacetamide) (see Fig. 1) are likely candidates for excimer formation.

We have tested this possibility in the present study; using the statistical method of Tsou [17], we have also determined that only one Cys is essential for the colchicine binding activity of tubulin. In order to identify this residue, a series of experiments have been carried out which strongly indicate that a common Cys is shared by the colchicine binding site and the excimer formation by pyrene-maleimide-labeled tubulin.

MATERIALS AND METHODS

Materials. Pipes, EGTA, GTP and dithiothreitol were obtained from Sigma. *N*-(1-Pyrene)maleimide on celite from Molecular Probes and *N*-ethylmaleimide from Fluka. All other chemicals used were of analytical grade. 2-Methoxy-5-(2',3',4'-trimethoxy phenyl)troponone was kindly provided by Dr T. J. Fitzgerald (Florida, A & M University) and *N,N'*-ethylenebis(iodoacetamide) by Dr Richard F. Luduena (The University of Texas, Health Science Center, San Antonio, Texas).

Methods. Microtubular proteins were isolated from goat brain by two cycles of temperature-dependent polymerisation and depolymerisation in the buffer containing 50 mM Pipes pH 6.9, 1 mM EGTA and 0.5 mM $MgCl_2$ in the presence of 1 mM GTP and 4M glycerol. Pure tubulin, free of microtubule-associated proteins, was isolated from the microtubular proteins by two more cycles of temperature-dependent polymerisation and depolymerisation using 1 M glutamate buffer for the assembly [18]. The protein was stored at $-70^\circ C$. The concentration of tubulin was determined by the method of Lowry et al. [19].

Modification of Cys residues of tubulin. (a) Tubulin (3 μM) and *N*-ethylmaleimide (60 μM) were mixed together in 20 mM sodium phosphate pH 7.0 at $25^\circ C$ in a spectrophotometric cuvette. The extent of modification was calculated from the changes in absorbance at 300 nm with time, using $\epsilon = 620 M^{-1} cm^{-1}$ at 300 nm in water [20]. The reaction was quenched at different time interval with excess dithiothreitol. (b) 1 μM tubulin was labeled with 20 μM pyrene maleimide in 20 mM sodium phosphate pH 7.0 at $25^\circ C$ for 45 min. The reaction was

quenched using excess 2-mercaptoethanol and the incorporation of pyrene maleimide calculated using $\epsilon = 30000 M^{-1} cm^{-1}$ at 343 nm [15]. (c) Ethylenebis(iodoacetamide) was prepared in dimethylsulfoxide according to Luduena and Roach [21] and 150 μM was mixed with 1 μM tubulin in 20 mM sodium phosphate pH 7.0 and incubated for 30 min at $25^\circ C$. The reaction was quenched with excess 2-mercaptoethanol.

Excimer fluorescence. Fluorescence spectra were recorded in a Hitachi F-3000 spectrofluorometer fitted with a water-circulating cell holder connected to a constant-temperature circulating water bath. The excimer fluorescence emission spectra were measured over 370–600 nm at an excitation wavelength of 343 nm. The excitation and emission bandpass were 3 nm in each case. The peaks corresponding to 377 nm and 450 nm are for monomers and excimers respectively.

Native and aged tubulin. Tubulin (1 μM) in 20 mM sodium phosphate pH 7.0 without or with 4 M glycerol was incubated at $37^\circ C$ for several hours. Aliquots of tubulin solution were withdrawn at different time intervals, mixed with 20 μM pyrene maleimide and incubated at $25^\circ C$ for 45 min. Excimer fluorescence spectra were recorded as described above.

Labeled tubulin. Native tubulin, tubulin-colchicine complex or tubulin-GTP complex were labeled by incubating each with 150 μM ethylenebis(iodoacetamide) in 20 mM sodium phosphate pH 7.0 at $25^\circ C$ for 30 min. Tubulin-colchicine complex was prepared by mixing 1 μM tubulin in 20 mM sodium phosphate pH 7.0 and 5 μM colchicine, and incubating at $37^\circ C$ for 1 h. Tubulin-GTP complex was prepared by mixing 1 μM tubulin with 1 mM GTP in 20 mM sodium phosphate pH 7.0 followed by a 1-h incubation at $37^\circ C$. Labeled samples were then mixed with 20 μM pyrene maleimide and incubated at $25^\circ C$ for 45 min. Excimer fluorescence spectra were recorded as given above.

[3H]colchicine binding activity. Native and aged tubulin. Tubulin (1 μM) in 20 mM sodium phosphate pH 7.0, with and without 4 M glycerol, was incubated at $37^\circ C$ for several hours. Aliquots were withdrawn at different times, mixed with 5 μM [3H]colchicine and incubated further at $37^\circ C$ for 45 min. The [3H]colchicine binding was assayed using a DEAE-cellulose filter disc (DE-81) as described by Weisenberg et al. [22].

Labeled tubulin. Tubulin (1 μM) was mixed with either 20 μM pyrene maleimide or 150 μM ethylenebis(iodoacetamide) in 20 mM sodium phosphate pH 7.0 and incubated at $25^\circ C$. The labeling reaction was quenched with 1 mM 2-mercaptoethanol at different times. Labeled tubulin was mixed with 5 μM [3H]colchicine and then incubated further at $37^\circ C$ for 45 min and the binding was assayed as described above.

Binding activity of labeled tubulin. To measure the colchicine binding activity of *N*-ethylmaleimide-modified tubulin, the colchicine analog 2-methoxy-5-(2',3',4'-trimethoxyphenyl)troponone was used as it binds instantaneously. The wavelength used to excite the complex was 360 nm and excitation and emission bandpass were 5 nm in each case.

RESULTS

Determination of number of essential Cys residues for colchicine binding. Two analytical approaches available for determining the number of the essential groups involved in the active site of proteins were proposed by Ray and Koshland [23] and Tsou [17]. The former is a kinetic method while the latter, a simple statistical method, relies on the measurement of the progressive loss of activity as a function of the modification of the residue, e.g. Cys [24], Arg [25], carboxyl group [26] etc. Let a biopolymer containing a total of n groups of type Q, all equally

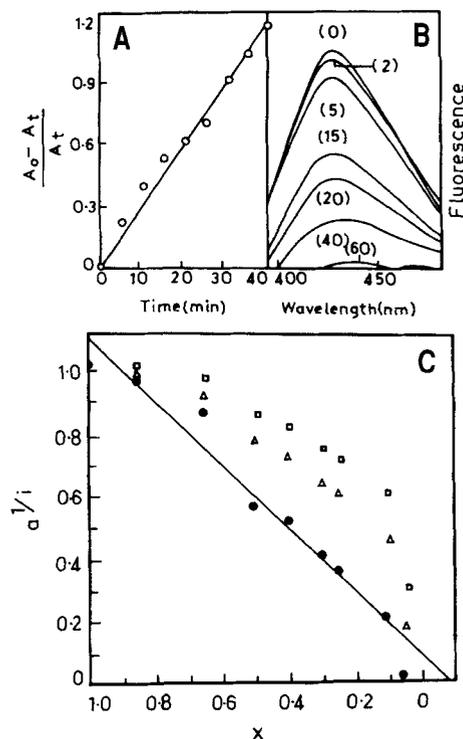


Fig. 2. Determination of number of essential Cys residues for colchicine binding activity of tubulin. 3 μM tubulin was mixed with 60 μM *N*-ethylmaleimide in 20 mM sodium phosphate pH 7.0 and the decrease in absorbance at 300 nm was measured with time. To measure the colchicine binding activity of modified tubulin at different times, aliquots were withdrawn, the reaction was quenched with dithiothreitol and the colchicine binding activity was measured using the tropon as described in Materials and Methods. (A) Monophasic kinetics of tubulin/*N*-ethylmaleimide second-order reaction, ensuring the equal rate of modification, a prerequisite for the statistical analysis. (B) Fluorescence emission spectra of modified tubulin/tropon complexes. Numbers in the parentheses indicate the time (min) of modification with *N*-ethylmaleimide. The fluorescence at 430 nm was used to determine the colchicine binding activity (a). (C) Relation between the fraction of activity remaining (a) and the fraction of Cys remaining unmodified (X) for the reaction of *N*-ethylmaleimide with tubulin. Data are plotted for (\bullet) $i = 1$, (Δ) $i = 2$ and (\square) $i = 3$. The straight line drawn through the points for $i = 1$ is that given by linear least-square fit (linear correlation coefficient 0.92).

reactive towards the modifying agent, but only i of which are essential. The fraction of each essential group remaining unmodified is therefore equal to the overall fraction X , of the type-Q groups remaining unmodified. Assuming that the modification of any one of the essential groups in the protein leads to complete loss of activity, the fraction of the activity remaining after partial modification, a , is given by

$$a = X^i$$

or

$$a^{1/i} = X.$$

The value of i (which will normally be a small positive integer) is found from the plot of $a^{1/i}$ versus X , which gives the best straight line, using several trial values of $i = 1, 2, 3$ etc.

To measure the number of essential Cys residues for the colchicine binding activity, 3 μM tubulin was mixed with 60 μM *N*-ethylmaleimide in 20 mM sodium phosphate pH 7.0 (at 25°C). A small aliquot of the reaction mixture was taken at different times and the number of Cys groups modified (Fig. 2A), as well as the colchicine binding activity of the modified protein, were determined (Fig. 2B). Since, 2-methoxy-5-

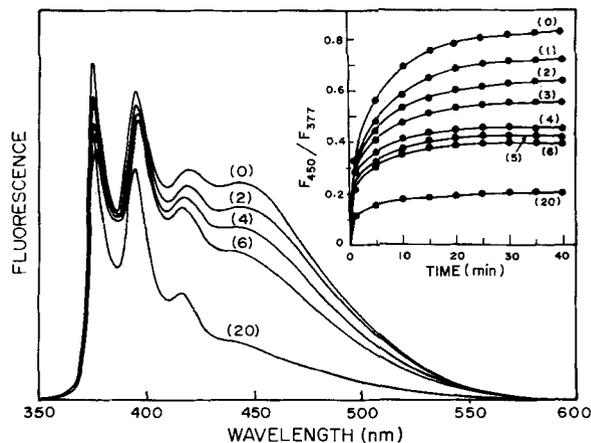


Fig. 3. Effect of aging at 37°C on excimer fluorescence. Tubulin (1 μM) was allowed to age by incubation in 20 mM sodium phosphate pH 7.0 at 37°C. Aliquots were withdrawn at different times, mixed with 20 μM pyrene maleimide and incubated at 25°C. The emission spectra shown are for a reaction time of 45 min. The inset shows the change of excimer fluorescence measured as the ratio of excimers and monomers (F_{450}/F_{377}) with reaction time. The numbers in the parentheses correspond to the time (h) of aging.

(2',3',4'-trimethoxy phenyl)tropon, a structural analog of colchicine, binds to the colchicine site of tubulin instantaneously, we used it instead of colchicine for measuring the remaining activity. The fraction of the activity remaining (a) and the fraction of Cys remaining unmodified (X) was then plotted as $a^{1/i}$ versus X (Fig. 2C). Three cases with $i = 1, 2$ and 3 ($a^1, a^{1/2}$ and $a^{1/3}$), corresponding to one, two and three essential group(s), produce the curves shown in Fig. 2C. The best linear fit was obtained for $i = 1$ (linear correlation coefficient = 0.92). A straight line was obtained only when $i = 1$ indicating that there is only one essential Cys for the colchicine binding activity of tubulin.

Effect of aging on excimer formation and colchicine binding.

Tubulin exhibits the unique property of excimer formation with pyrene maleimide. Higher temperatures ($> 37^\circ\text{C}$) drastically reduce the excimer-forming ability of tubulin [15]. The loss of colchicine binding activity of tubulin upon aging at 37°C is well known [27]: 4–5 h of incubation at 37°C results in a 50–60% loss. The structural changes occurring in tubulin upon aging are not known. Circular dichroism and tryptophan fluorescence of tubulin remain virtually unchanged upon aging, indicating no global structural changes [28, 29]. In the present study, tubulin in 20 mM sodium phosphate pH 7.0 was aged at 37°C for several hours. At different time intervals, aliquots were withdrawn and their excimer-forming ability determined. Results of such an experiment are shown in Fig. 3. The inset shows the extent of excimer formation, measured as F_{450}/F_{377} , when aged tubulin was allowed to react with pyrene maleimide for various times. It may be noted that while pyrene excimer emission peak is generally observed in the range 470–490 nm [30, 31], the emission maximum of pyrene-maleimide-labeled tubulin is found to be centered near 450 nm. This emission peak is independent of reaction time and the pH of the reaction mixture (pH 6.0–7.5; unpublished results). Thus, in the present study the intensity was measured at 450 nm for the excimer fluorescence, in conformity with our earlier measurements [15]. The results shown in Fig. 3 indicate that excimer formation requires the native structure of tubulin. The aging process probably alters the close proximity of Cys residues responsible for excimer formation. Interestingly, the extent of excimers measured as the fluorescence ratio $F_{450}/$

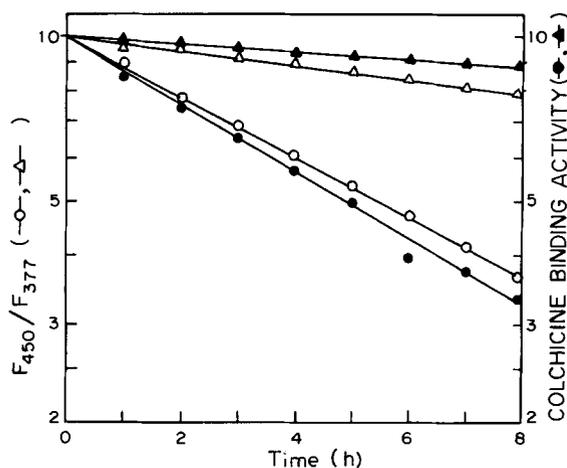


Fig. 4. Effect of aging at 37°C on colchicine binding activity and the excimer fluorescence of tubulin. Tubulin (1 μ M) was incubated in 20 mM sodium phosphate pH 7.0 at 37°C in the presence and absence of 4M glycerol. Aliquots were withdrawn from each reaction mixture at different times and divided into two: one part was assayed for [3 H]colchicine binding activity as described in Materials and Methods. The other part was mixed with 20 μ M pyrene maleimide, incubated for 45 min at 25°C and excimer fluorescence was measured. (●, ▲) Colchicine binding activity of tubulin in (●) absence and (▲) presence of 4M glycerol; (○, △) excimer fluorescence measured as the ratio (F_{450}/F_{377}) in (○) absence or (△) presence of glycerol shown as a function of time of aging.

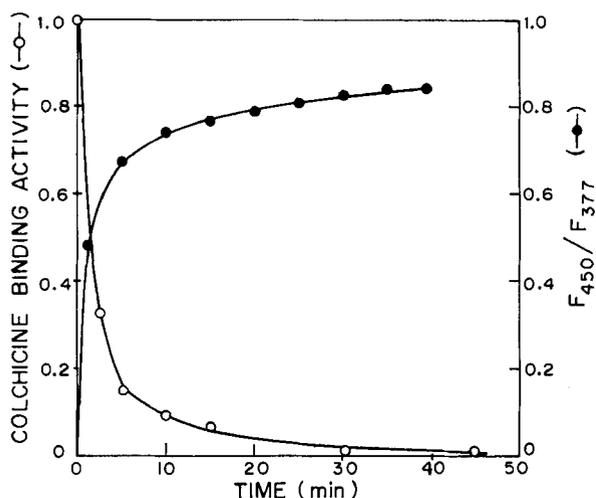


Fig. 5. Effect of pyrene maleimide labeling of tubulin on its colchicine binding activity and excimer formation. 1 μ M tubulin was treated with 20 μ M pyrene maleimide in 20 mM sodium phosphate pH 7.0 at 25°C. Aliquots were withdrawn at different times and the reaction was quenched with 1 mM 2-mercaptoethanol. One part of this aliquot was assayed for [3 H]colchicine binding activity (○) as described in Materials and Methods. Excimer fluorescence of the other part (●) was measured as ratio of excimers and monomers (F_{450}/F_{377}).

F_{377} , and the colchicine binding activity of tubulin, are found to decline at an identical rate upon aging at 37°C (Fig. 4). Further, it was observed that solution conditions which stabilize the colchicine binding site of tubulin also stabilize its excimer-forming ability equally well. Thus, a high concentration of glycerol, which is known to stabilize the colchicine binding site of tubulin in solution [32], also stabilizes the excimer-forming ability of the protein equally well (Fig. 4). The loss of both the colchicine binding activity and the excimer-forming capacity of tubulin in

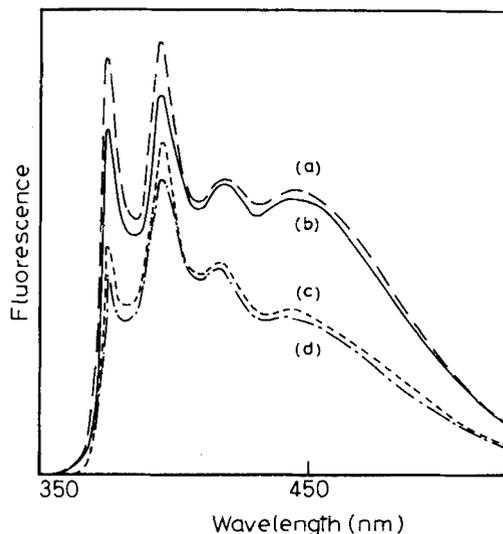


Fig. 6. Effect of incubation of tubulin with ethylenebis(iodoacetamide) on its excimer fluorescence. Curves (a, c) Samples containing tubulin (1 μ M) without (curve a) and with 150 μ M ethylenebis(iodoacetamide) (curve c) were incubated for 30 min at 25°C. Excimer fluorescence of both samples was then measured as described in Materials and Methods. Curves (b and d) samples containing tubulin (1 μ M) were incubated with either 5 μ M colchicine (curve b) or with 1 mM GTP (curve d) at 37°C for 1 h. 150 μ M ethylenebis(iodoacetamide) was then added to both samples and incubated further for 30 min at 25°C. Excimer fluorescence of both samples was then measured as above.

4M glycerol is very small, to the extent of only 10–20% in 8 h. These results indicate that aging induces conformational changes in the colchicine binding domain of tubulin, resulting in the decline in colchicine binding activity and the alteration of proximity of the Cys residue(s) responsible for the excimer fluorescence. The close relationship between the colchicine binding activity and the excimer-forming Cys of tubulin is further evident from Fig. 5. Labeling of Cys which induces excimer fluorescence caused a concomitant decrease in the colchicine binding activity. These findings strongly indicate that the colchicine binding site and the Cys residues responsible for excimers are part of the same domain.

Involvement of a common Cys residue for the colchicine binding and the excimer formation of tubulin.

Two pairs of Cys in the β subunit of tubulin can be cross-linked with ethylenebis(iodoacetamide). The first pair involving Cys12 and Cys201 or Cys211 [7], is protected from cross-linking if the protein is first incubated with GTP or with vinblastine [33]. Similarly cross-linking of the second pair (between Cys239 and Cys354) is impaired after incubation of tubulin with colchicine or podophyllotoxin [21, 33–35]. Formation of cross-links implies that the Cys residues in each pair are within 0.9 nm [7], a distance ideal for excimer formation with pyrene maleimide. To test if indeed any of these pairs form excimers upon treatment with pyrene maleimide, 1 μ M tubulin was incubated with 150 μ M ethylenebis(iodoacetamide) for 30 min at 25°C and then labeled with 20 μ M pyrene maleimide. We observed about 44% inhibition of excimer formation (Fig. 6, curve c) compared to an untreated sample (Fig. 6, curve a). Incubation of tubulin with colchicine before the cross-linking caused no inhibition in excimer formation with pyrene maleimide (Fig. 6, curve b). On the other hand, incubation of tubulin with GTP before the cross-linking does not provide such protection and 40–50% inhibition of excimer formation compared to control sample is observed

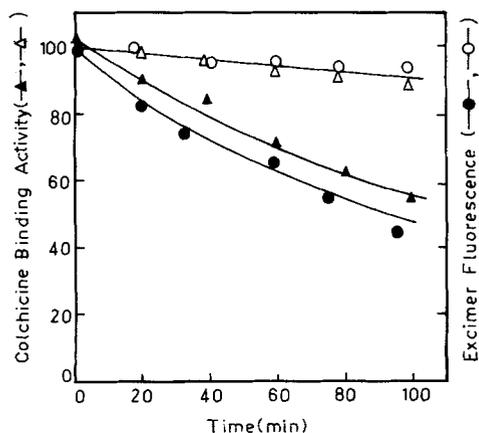


Fig. 7. Effect of ethylenebis(iodoacetamide) labeling on the colchicine binding activity and the excimer fluorescence of tubulin. Tubulin (1 μ M) was incubated with 150 μ M ethylenebis(iodoacetamide) in 20 mM sodium phosphate pH 7.0 at 25°C. At different times, aliquots were withdrawn and the [3 H]colchicine binding activity (\blacktriangle) and the excimer fluorescence (\bullet) were measured as described in Materials and Methods. The straight line at the top corresponds to the control experiments for excimer fluorescence (\circ) and colchicine binding activity (\triangle) without ethylenebis(iodoacetamide).

(Fig. 6, curve d). Thus, it is clear that the pair of Cys239 and Cys354, which is protected by colchicine from cross-linking, is also involved in excimer formation with pyrene maleimide. Furthermore, the other pair (Cys12 and either Cys201 or Cys211) is definitely not involved in excimer formation. The extent of inhibition of excimer formation after incubation with ethylenebis(iodoacetamide) can be increased by incubating tubulin for longer periods. We have, however, restricted our incubation time to within 30 min as excimer formation of tubulin is also sensitive to aging (Figs 3 and 4, a 30-min incubation retains more than 95% of excimer-forming ability).

It may be questioned that, if colchicine protects the Cys239–Cys354 pair from cross-linking, how does it allow modification of the same residues by pyrene maleimide resulting in excimer formation? Incubation of tubulin with colchicine has not been found to have any effect on excimer formation [15] (Fig. 6, curve b). To resolve this anomaly, the ability of pyrene maleimide to displace colchicine from its binding site on the protein was tested. A complex was formed between tubulin and [3 H]colchicine. Addition of pyrene maleimide to this complex displaced the bound [3 H]colchicine from tubulin (data not shown), indicating that pyrene maleimide is a more reactive Cys-modifying agent than ethylenebis(iodoacetamide).

Results presented in Figs 3–5 clearly indicate that colchicine binding and excimer fluorescence (in the presence of pyrene maleimide) of tubulin are inter-related and probably part of the same domain of tubulin. Of the two residues (Cys239 and Cys354) involved in excimer formation, Cys239 has been reported to be responsible for the self assembly of tubulin, while it has no effect on the colchicine binding activity of the protein [8]. Consequently, Cys354 is the only common residue involved in both the colchicine binding activity and the excimer formation with pyrene maleimide. If so, treatment of tubulin with ethylenebis(iodoacetamide) should affect both colchicine binding and excimer formation at the same rate. When simultaneous measurements of both these properties were made as a function of time (Fig. 7), treatment of tubulin with ethylenebis(iodoacetamide) did indeed inhibit both the colchicine binding activity and the excimer-forming ability of tubulin at an identical rate.

DISCUSSION

The present study was initiated to gather information about the Cys residue(s) involved in the colchicine binding activity of tubulin. The study includes the determination of the number of Cys involved in the colchicine binding activity and identification of these Cys with respect to the known domain structure of tubulin using spectroscopic techniques. For a protein like tubulin, which has 20 Cys residues distributed in two subunits, this task is by no means simple. The statistical method of Tsou [17] has been applied successfully in different systems for the determination of the number of essential residue(s): e.g. the number of catalytically essential carboxyl groups in pepsin [26], the number of essential Arg residues for the activity of the purine nucleoside phosphorylase [25], or the number of Cys responsible for the activity of UDP-glucose 4-epimerase [24]. One important requirement of this analysis is to select a modifying agent which is equally reactive to all Cys present in the protein. In tubulin, even though all of the 20 Cys are neither in an identical environment nor equally exposed to solvents, *N*-ethylmaleimide reacts with all of them at the same rate (Fig. 2A), making it possible to perform such an analysis. It is evident from this analysis that there is just one essential Cys responsible for the colchicine binding activity of tubulin (Fig. 2C). However, previous studies reported about 60% inhibition of initial colchicine binding activity when 10–12 Cys of platelet tubulin were modified with *p*-chloromercuribenzoate [9]. It should be noted that the above conclusion was drawn on the basis of titration of different concentrations of platelet tubulin with varying concentrations of *p*-chloromercuribenzoate at a single time and neither kinetic nor statistical analysis were performed. Furthermore, platelet tubulin used in this study was purified by two cycles of polymerisation/depolymerisation only and contained 24 Cys/dimer.

Another objective of the present study was to identify the Cys involved in the colchicine binding. One of the important findings of the present study is the relation between colchicine binding activity and the excimer fluorescence of tubulin (Figs 4 and 5). It was observed that aging tubulin at 37°C caused the decay of its colchicine binding activity and excimer-forming ability to an equal extent (Fig. 4). Furthermore, glycerol, which is known to prevent the decay of colchicine binding activity, also prevents the decay of excimer-forming ability of tubulin equally well (Fig. 4). These results strongly suggest that the colchicine binding site and the excimer-forming Cys of tubulin are part of the same domain of tubulin. Recent studies have indicated that the colchicine binding site is located in the C-terminal domain of β -tubulin [10–14]. Interestingly, this part of tubulin contains only two Cys (Cys303 and Cys354) [5]. This, of course, does not exclude the possibility of the participation of Cys from other domains of tubulin. The possibility that long-range interactions or other indirect phenomena are responsible for the above results cannot be ruled out. The decay of colchicine binding activity of tubulin has been known for the last two decades [27]. However, it was not known (a) what structural changes occur upon decay, (b) in which part of tubulin do these occur and (c) how to monitor these structural changes. It had been reported that the far-ultraviolet circular dichroism and the tryptophan fluorescence of tubulin remain virtually unchanged upon aging [28, 29] implying that no global structural changes occurred on tubulin. Prasad et al. [29] have shown that the decay of tubulin at 37°C is accompanied by an increase in 5,5'-bis(8-anilino-1-naphthalenesulfonate) binding with six low-affinity sites appearing in the protein with time, in addition to a high-affinity site that is always present. One of the structural changes we have observed is the alteration of the proximity of Cys responsible for excimer formation. This change in the structure of tubulin

upon aging can be easily monitored by fluorescence spectroscopy (by measuring the excimer-forming ability of tubulin). Regarding the identification of these Cys, it is evident from the results of the experiments (Figs 6 and 7) presented here that Cys239 and Cys354, a pair protected by colchicine from cross-linking by ethylenebis(iodoacetamide), is responsible for the excimer formation as well as for the colchicine binding activity. On the basis of cross-linking experiments, it could be predicted that Cys354 is involved in the colchicine binding activity of tubulin. Furthermore, while Cys239 is involved in the tubulin self assembly, there is no inhibition of the colchicine binding activity if Cys239 is alkylated [8]. Thus, on the basis of experiments presented here and the cross-linking experiments of Luduena and Roach [13], we conclude that Cys354 is the only essential cysteine residue involved in the colchicine binding activity of tubulin. Recent experiments of Bai et al. [36], using a reactive thiocolchicine analog binding with tubulin, indicated that the covalently labeled colchicinoid is within 0.3 nm of the sulphur atom of the Cys354 residue [36]. Thus, two different studies with the same objective but entirely different approaches lead to the same conclusion.

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