Sulfhydryls of tubulin

A probe to detect conformational changes of tubulin

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The 20 cysteine residues of tubulin are heterogeneously distributed throughout its three-dimensional structure. In the present work, we have used the reactivity of these cysteine residues with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as a probe to detect the global conformational changes of tubulin under different experimental conditions. The 20 sulfhydryl groups can be classified into two categories: fast and slow reacting. Colchicine binding causes a dramatic decrease in the reactivity of the cysteine residues and causes complete protection of 1.4 cysteine residues. Similarly, other colchicine analogs that bind reversibly initially decrease the rate of reaction; but unlike colchicine they do not cause complete protection of any sulfhydryl groups. Interestingly, in all cases we find that all the slow reacting sulfhydryl groups are affected to the same extent, that is, have a single rate constant. Glycerol has a major inhibitory effect on all these slow reacting sulfhydryls, suggesting that the reaction of slow reacting cysteines takes place from an open state at equilibrium with the native. Ageing of tubulin at 37 °C leads to loss of self-assembly and colchicine binding activity. Using DTNB kinetics, we have shown that ageing leads to complete protection of some of the sulfhydryl groups and increased reaction rate for other slow reacting sulfhydryl groups. Ageing at 37 °C also causes aggregation of tubulin as indicated by HPLC analysis. The protection of some sulfhydryl groups may be a consequence of aggregation, whereas the increased rate of reaction of other slow reacting sulfhydryls may be a result of changes in global dynamics. CD spectra and acrylamide quenching support such a notion. Binding of 8-anilino-1-naphthalenesulfonate (ANS) and bis-ANS by tubulin cause complete protection of some cysteine residues as indicated by the DTNB reaction, but has little effect on the other slow reacting cysteines, suggesting local effects.

Keywords: ageing; colchicine; cysteine; DTNB; tubulin.

Tubulin, a heterodimeric protein ($\alpha\beta$), has 20 cysteine residues, of which 12 are in the α subunit and eight in the β subunit [1,2]. The distribution and static solvent exposure of cysteine residues in tubulin are shown in the crystal structure (Fig. 1) [3], which indicates that both the environments and the exposure of the cysteines are highly heterogeneous. Cysteine has been used extensively as an attachment point of external flourophores, which can not only be used to study conformational transitions, but also to glean insights into protein structure. However, in many such applications site-selectivity is of utmost importance. Tubulin, being a multicysteine protein, is not an ideal one for this purpose as identification of functional cysteine(s) and labelling of a single cysteine with a fluorophore is difficult. However, a few unique cysteines have been identified in tubulin: these are two pairs in the β subunit of the protein, Cys12–Cys201/211 and Cys239–Cys354, which could be cross-linked with N,N'-ethylene bis(iodoacetamide) [4,5]. Despite such drawbacks, the cysteines can still be exploited to study the conformational changes of tubulin by binding ligands of different kinds to different sites of the protein and

thereby protecting the sulfhydryl groups. This is possible because many of the cysteines are distributed globally throughout the primary sequence of both the subunits. In the present study, we have attempted to detect the conformational changes of tubulin due to the binding of antimitotic drugs, the ageing of tubulin at 37 °C, and the binding of anilinonaphthalene sulfonates to tubulin, using the reactivity of sulfhydryl residues as a probe.

MATERIALS AND METHODS

Materials

Pipes, EGTA, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), MgCl$_2$, GTP and colchicine were obtained from Sigma. Acrylamide was obtained from Spectrochem (Calcutta, India). Glycerol was obtained from Merck. 8-Anilino-1-naphthalenesulfonate (ANS) and bis-ANS were from Molecular Probes. 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC) and desacetamido-colchicine (DAAC) were a gift from S. Bane Hastie (SUNY, Binghamton). Sodium dihydrogen orthophosphate was obtained from BDH industries. All other reagents were of analytical grade. Radioactive colchicine ([^{3}H] ring C-methoxy, 70.0 Ci·mmol^{-1}) was obtained from New England Nuclear.

Tubulin preparation and estimation

Goat brain tubulin free of microtubule-associated proteins was prepared by two cycles of temperature-dependent
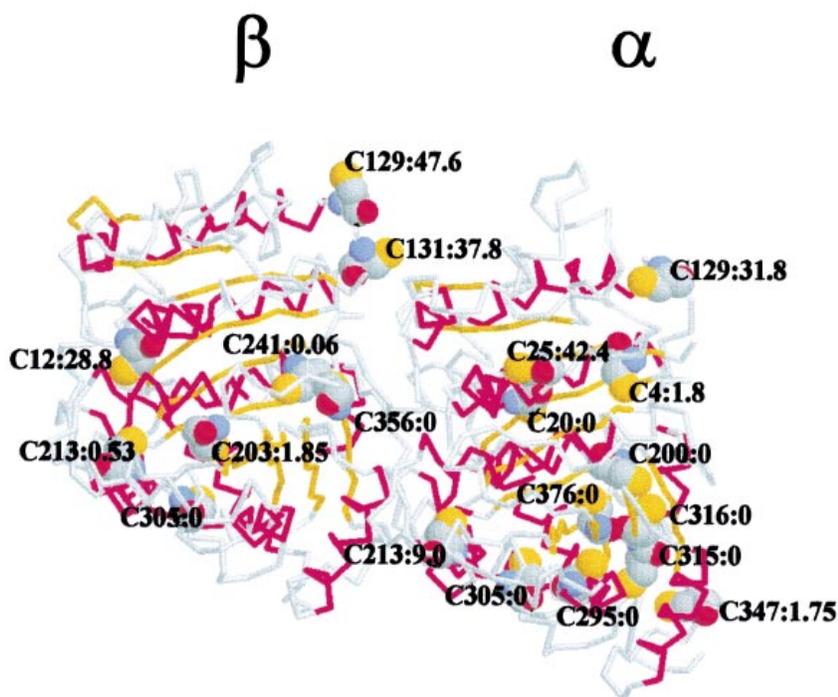
Fig. 1. The distribution and static solvent exposure of cysteine residues in tubulin. The secondary structural elements are shown in color (magenta for helices and yellow for β-sheets) and cysteine residues are shown as Corey–Pauling–Kendrew (CPK) models. The first part of the labels denotes the position of the cysteine and the second part is the solvent accessible surface area [25] of the sulfur atom in Å^2. The surface area was calculated using the GETAREA program [26]. The figure was generated using the program RASMOL (R. Sayle, Glaxo Research and Development, Greenford, Middlesex, UK) using the PDB coordinates for tubulin (1tub).

polymerization and depolymerization in Pipes/EGTA/Mg^{2+} buffer in the presence of 1 mM GTP, followed by two more cycles in 1 M glutamate buffer (pH 7.0) [6] and stored at −70 °C. The concentration of tubulin was determined by the method of Lowry *et al.* [7] using bovine serum albumin as standard.

Fluorescence measurements

To measure the tryptophan fluorescence intensity of tubulin, the samples were excited at 280 nm and emission was observed at 335 nm. Slit widths of 5 nm were chosen for both excitation and emission. All the fluorescence experiments were performed in Pipes/EGTA/Mg^{2+} buffer at 25 °C. The fluorescence spectra were recorded on a Hitachi F-3000 spectrofluorometer fitted with a constant-temperature cell holder.

Circular dichroism

CD spectra in the far UV region were recorded on a JASCO J-600 spectropolarimeter using a cuvette of 0.1 cm pathlength. The wavelength range for the far UV region was 200–260 nm. A bandwidth of 1 nm and a time constant of 2 s were used for these measurements. All the CD experiments were performed in phosphate buffer (20 mM phosphate, pH 7, 0.5 mM MgCl$_2$) at 25 °C.

Gel-filtration chromatography

Gel-filtration chromatography was performed with a Waters HPLC instrument using a Bio SEC-250 silica column (600 × 7.5 mm; Bio-Rad) at 22 °C; 100 mM phosphate buffer, pH 7, was used as the mobile phase; the flow rate was 1 mL·min^{-1}. Typical elution volumes for tubulin and aggregated tubulin were 7.68 and 5.35 mL, respectively.

Radioactive assay

Tubulin (3 μM) in Pipes/Mg^{2+} buffer was incubated with 3 μM of [^{3}H]colchicine for 1 h at 37 °C. Colchicine binding activity

was evaluated by the DE 81 (Whatman) filter disc method of Weisenberg *et al.* [8].

Measurement of DTNB kinetics

The formation of a thio-nitrobenzoate anion by DTNB reaction with free sulfhydryl groups of tubulin was monitored by measuring absorbance at 412 nm ($\varepsilon_{412} = 13\ 600$ M^{-1}·cm^{-1}) over time [9]. All the absorbance measurement experiments were carried out in Pipes/Mg^{2+} buffer at 25 °C. Each tubulin sample was mixed with excess DTNB in a quartz cuvette and the absorbance was recorded continuously at 412 nm using a Shimadzu UV-2401PC spectrophotometer.

Native and aged tubulin. Aliquots of tubulin (1.54 μM) in Pipes/Mg^{2+} buffer were incubated at 37 °C separately for 0 min, 30 min, 45 min, 60 min and 90 min. Immediately after the incubation, each protein sample was left until at 25 °C, then mixed with DTNB at a final concentration of 400 μM in a quartz cuvette and the absorbance was measured at 412 nm continuously over time. A 0 min incubation means tubulin was thawed and then kept at 25 °C just until it had attained that temperature and then mixed with DTNB.

Tubulin-drug complexes. Tubulin–colchicine, tubulin–DAAC and tubulin–AC complexes were prepared separately by mixing 0.6 μM tubulin and 10 μM of the respective drugs in Pipes/Mg^{2+} buffer and then incubating at 37 °C for 60 min. Immediately after the incubation, each sample was brought to 25 °C, mixed with DTNB at a final concentration of 400 μM in a quartz cuvette and the absorbance was measured at 412 nm continuously over time.

Tubulin–ANS and tubulin–bis-ANS complexes were prepared by mixing 0.6 μM tubulin and 10 μM of the respective fluorophores in Pipes/Mg^{2+} buffer and then incubating at 25 °C for 5 min. Immediately after the incubation, each sample was mixed with DTNB at a final concentration of 400 μM in a quartz cuvette and the absorbance was measured at 412 nm continuously over time.

The concentrations of the ligands employed in this study were determined spectrophotometrically in their aqueous solutions at their respective absorption maximums. The extinction coefficients used were as follows: colchicine at 352 nm, $\varepsilon = 16900$ $M^{-1} \cdot cm^{-1}$; AC at 341 nm, $\varepsilon = 18840$ $M^{-1} \cdot cm^{-1}$; DAAC at 352 nm [10], $\varepsilon = 16200$ $M^{-1} \cdot cm^{-1}$; ANS at 370 nm, $\varepsilon = 6800$ $M^{-1} \cdot cm^{-1}$; bis-ANS at 385 nm, e = 16790 $M^{-1} \cdot cm^{-1}$ [11].

Nonlinear least squares fit

Nonlinear least squares fits were done using SIGMA PLOT and fitted to the equation:

$$A = A_o + n \cdot c \cdot \varepsilon (1 - e^{(-kt)})$$

where, A is absorbance, A_o is the absorbance at zero time, n is the number of slow reacting sulfhydryl groups, c is the concentration of tubulin, ε is the extinction coefficient of thionitrobenzoate (TNB^-), k is the rate constant, and t is time.

RESULTS AND DISCUSSIONS

Reactivity of the cysteines of tubulin and the effect of drug binding

The kinetics of DTNB reaction with sulfhydryl groups of tubulin are shown in Fig. 2. When the data were fitted to a pseudo first-order rate equation with one rate constant and an initial offset (for fast reacting sulfhydryl groups that react within a mixing time of about 15 s), a perfect correlation coefficient was obtained ($R^2 = 1.0$). The residuals also show no bias, suggesting that apart from the fast reacting sulfhydryl groups, the rest of the cysteines react with rate constants that are identical or nearly so. This uniformity of reaction rates is surprising and suggestive of a common underlying mechanism (see below).

It is known that colchicine and its B-ring analogs AC and DAAC induce conformational changes in tubulin. The bound drug stimulates the intrinsic GTPase activity of tubulin and generates an additional chymotrypsin site in its β subunit [12,13]. Are these conformational changes also reflected in the reactivity of the cysteines of tubulin towards DTNB? We have examined the DTNB reaction with tubulin alone and in complexes with colchicine and its analogs. Tubulin (0.6 μM) was incubated with 10 μM of the various drugs at 37 °C in Pipes/Mg^{2+} buffer and then the drug–protein complex was reacted with 400 μM of DTNB (Fig. 2).

Multiple effects are seen upon drug binding that are dependent on the nature of the drug. The extent of reaction is different for the different cases. In the cases of the AC and DAAC complexes, all the 20 sulfhydryl groups were reactive, while in the case of colchicine bound tubulin, around 18.6 cysteine groups reacted with DTNB at infinite time, suggesting that about 1.4 sulfhydryl groups were protected due to the conformational change induced upon colchicine binding. It is known that colchicine protects one sulfhydryl group from
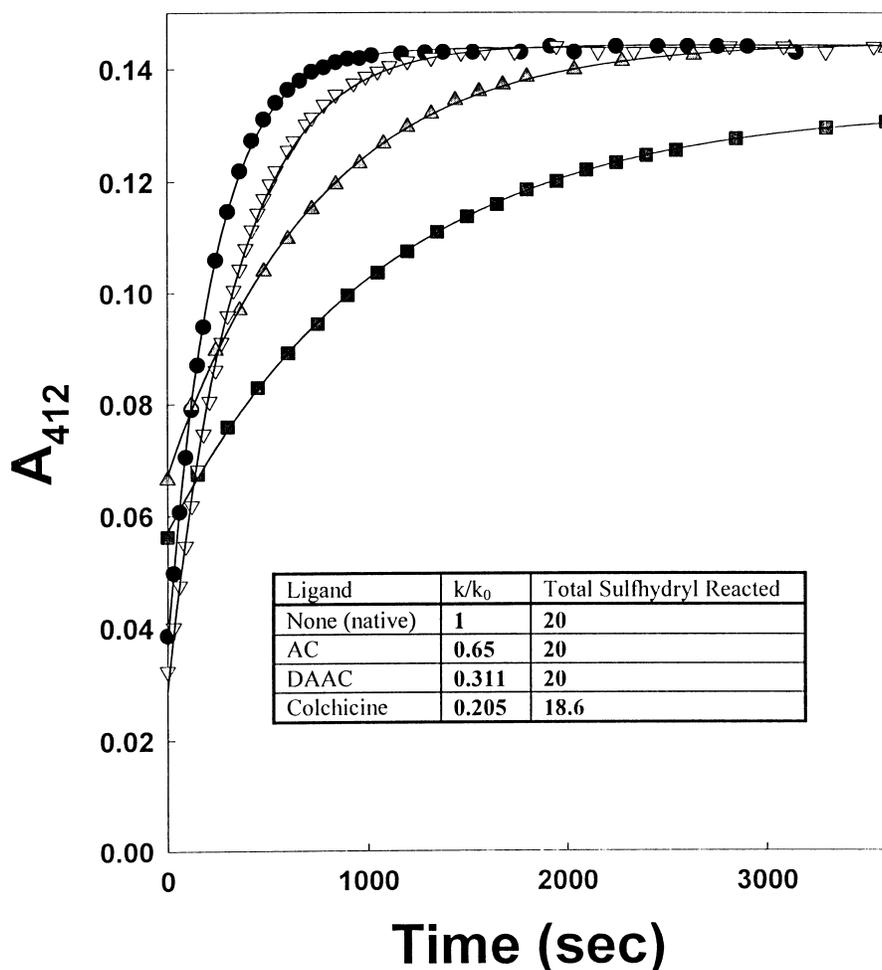

Ligand	k/k_0	Total Sulfhydryl Reacted
None (native)	1	20
AC	0.65	20
DAAC	0.311	20
Colchicine	0.205	18.6

Fig. 2. The effects of the binding of colchicine and its analogs to tubulin on the kinetics of DTNB. Native tubulin (●); tubulin–AC complex (▽); tubulin–DAAC complex (▲); tubulin–colchicine complex (■). Protein concentration was 0.6 μM and the concentration of the drugs was 10 μM in all cases. The concentration of DTNB was 400 μM. The experiments were carried out in Pipes/Mg^{2+} buffer and spectra were recorded at 25 °C. The details of the sample preparations and experiments are given in Materials and methods.
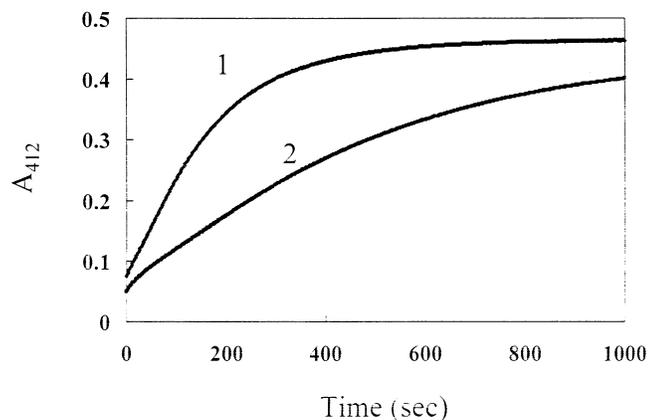
Fig. 3. The effects of glycerol on the DTNB reactivity of the sulfhydryl groups of tubulin. Trace 1, tubulin in Pipes/Mg^{2+} buffer; trace 2, tubulin in the same buffer containing 20% glycerol. Tubulin (2 μM) was mixed with DTNB (400 μM) and the absorbance was recorded at 412 nm continuously at 25 °C.

reaction with other sulfhydryl reagents [14]. However, it is possible that several sulfhydryl groups are partially reacted and this number reflects the sum of partial protection of several groups.

The interesting observation is that unlike colchicine, neither DAAC nor AC shows differential protection of sulfhydryl groups. The protection of sulfhydryl group(s) from reaction with DTNB by colchicine could be due either to the presence of sulfhydryl groups in the binding site and consequent steric blockage or to a change of accessibility resulting from a substrate-induced conformational change. Since the difference between DAAC and colchicine is only the presence of an acetamido group in the latter compound, it is likely that the additional protection offered by colchicine is due to conformational change induced by it.

It has been reported that despite relatively small differences in the molecular structures of DAAC and colchicine, their thermodynamic and kinetic parameters of binding to tubulin are dramatically different. DAAC and AC bind reversibly with negative ΔH, whereas colchicine binds irreversibly with positive ΔH. The activation energy of this reaction is also significantly different (21 kcal·mol^{-1} for colchicine versus 12 kcal·mol^{-1} for DAAC and AC), suggesting a major difference in the mode of interaction [10,15,16]. Differences are also seen in the number of fast reacting sulfhydryl groups. This number is much higher for the DAAC–tubulin and colchicine–tubulin complexes compared to native tubulin and the AC–tubulin complex. How these are related to the properties of each of the drugs is difficult to explain at this stage. The differences may be the result of the interaction of their B-rings with tubulin.

The question may be asked whether the reaction of DTNB with the fast reactive sulfhydryl groups could alter the accessibility of the other sulfhydryl groups to DTNB. It is difficult to design an experiment to obtain an unambiguous
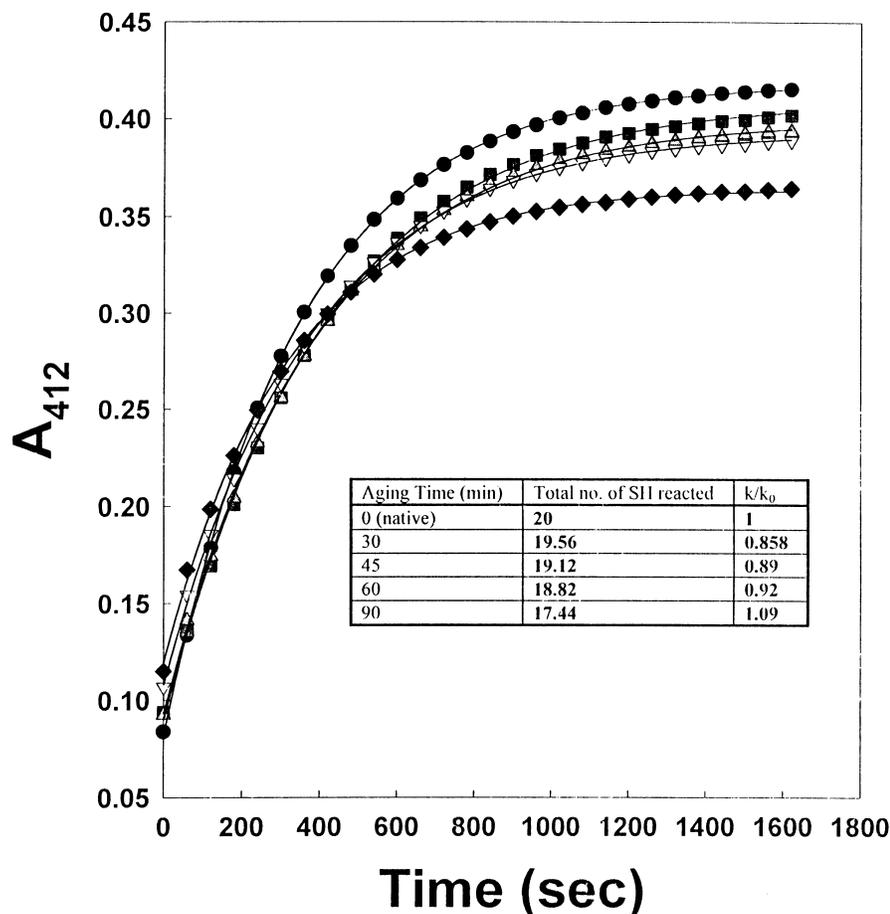

Aging Time (min)	Total no. of SH reacted	k/k$_0$
0 (native)	20	1
30	19.56	0.858
45	19.12	0.89
60	18.82	0.92
90	17.44	1.09

Fig. 4. Effects of ageing on DTNB kinetics. Tubulin (1.54 μM) was aged at 37 °C for different times: 0 (●); 30 min (■); 45 min (▲); 60 min (▽); 90 min (◆) and then reacted with DTNB (400 μM). The experiments were carried out in Pipes/Mg^{2+} buffer and spectra were recorded at 25 °C. The details of the experiments are given in Materials and methods.

answer, as it is not possible to selectively modify the fast reactive sulfhydryl groups without also modifying the remaining sulfhydryl groups. We carried out an experiment where tubulin was first allowed to react with an amount of DTNB calculated to be stoichiometrically equivalent to the fast reactive sulfhydryl groups and kept for 15 min at 25 °C before excess DTNB was added to monitor the rate of the reaction. We found no difference in the rate of the DTNB reaction with the slower reacting sulfhydryls, suggesting that modifications of the fast reactive sulfhydryls do not cause a time-dependent denaturation that affects the slower reacting ones (data not shown).

In all cases, upon drug binding, the rate of the DTNB reaction with the slow reacting sulfhydryls decreased to an extent that is dependent on the nature of the drug. Non-linear regressions show that in all cases the slow phase of the reaction can be fitted to a single exponential with a correlation coefficient of 1.0. The extracted values of rate constants decrease in the following order: tubulin > AC–tubulin > DAAC–tubulin > colchicine–tubulin. With colchicine-bound tubulin, the rate decreased to one-fifth of the value obtained for the native protein.

The most interesting observation in this study is that 15 slow reacting sulfhydryl groups react with DTNB at identical or near-identical reactivity. It is even more surprising that the binding of ligands such as DAAC or AC changes the reactivity of all the slow reacting sulfhydryls to the same extent. This is even true for colchicine, which shows protection of 1.4 sulfhydryl groups with the rest having identical reactivity. A correlation coefficient of 1 and no bias in the residuals (data not shown) suggest that this fit is not due to any artifact of curve fitting procedure.

Identical reactivity for all the 15 or so cysteines would be too much of a coincidence unless some underlying mechanism is in operation. One possible explanation is that native tubulin is in equilibrium with one or more open states and the slow reacting sulfhydryls react with DTNB when tubulin is in the open state such that:

$$T_c \rightleftharpoons T_o + DTNB \rightarrow T_o{\cdot}TNB + TNB^-$$
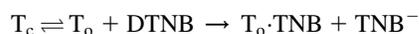
where, T_c is the closed state of the protein, that is the native form, and T_o is the open state of the protein. Similar mechanisms are known for other reactions, a well-known case being amide proton exchange [17,18]. Depending on the magnitude of the rate constants of the two steps, such reactions can be limited either by the rate of protein opening or by the rate of reaction.

As the inherent rate of reaction of cysteine with DTNB is very fast, it is likely that the pseudo-first order rate constants of around 0.003 s^{-1} reported here for the slow reacting sulf-hydryls are a reflection of the reaction being limited by the rate of protein opening. Thus, if the binding of a ligand affects the opening rate, it would affect the reaction rates of all the sulfhydryl groups at once. In general, the reaction from such open states involves large amplitude motions and significant exposure of backbone and sidechain atoms that are buried in the native state. Under such circumstances, it is likely that glycerol would have a very significant inhibitory effect on protein opening and consequently on sulfhydryl reaction rate, either due to the increased viscosity or because the transfer of free energy from buried atoms would be more unfavorable in glycerol compared to water [14,19]. The effect of glycerol on the reactivity of sulfhydryl groups of tubulin is shown in Fig. 3.

The predominant effect of increased glycerol concentration is a very significant slow down of the reaction rate (about four times) of the slow reacting sulfhydryls with all the rate constants decreasing at the same time; under similar conditions the reactivity of free cysteine is only slowed down by 50%. Based on the above data, we suggest that the fast reacting sulfhydryl groups are the ones that have high static exposure in the crystal structure and the slow reacting ones are the buried ones reacting through one or more open states limited by the opening rate. We note that five sulfhydryl groups in the native structure have high solvent exposure and 15 are buried, consistent with the numbers of fast and slow reacting sulfhydryls. One can thus form a working hypothesis that the change in the number of fast reacting sulfhydryls reflects a change in static exposure, the change in rate constants reflects a change in the opening rate and protection of sulfhydryls reflects a change in the mode of opening.
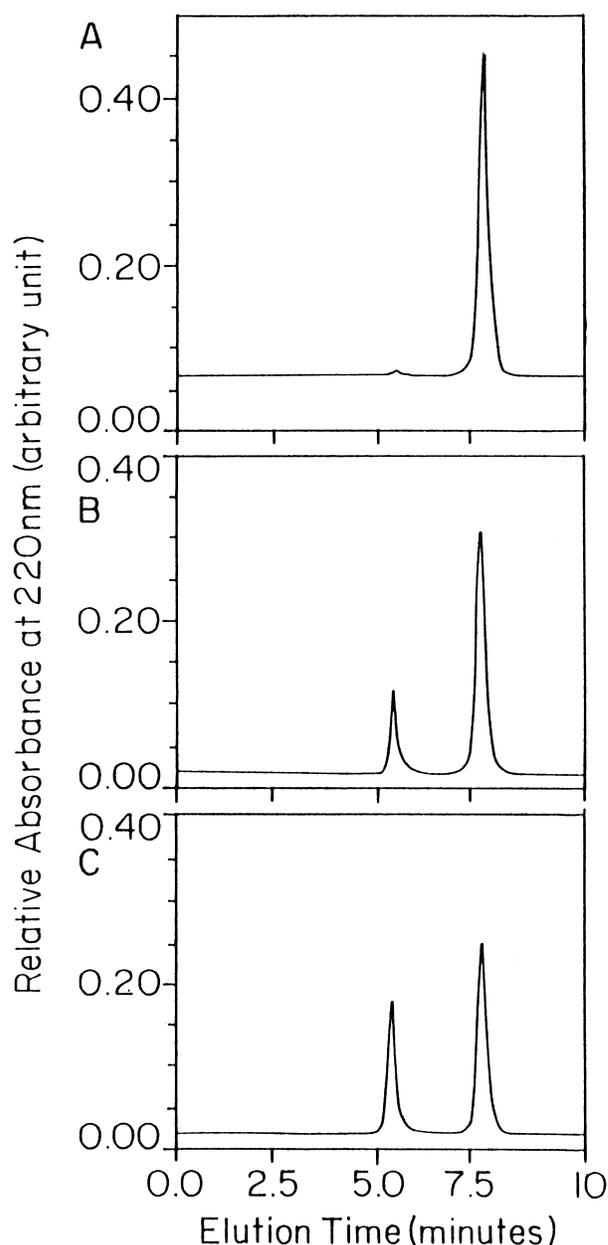

Fig. 5. Aggregation of tubulin due to ageing at 37 °C. (A) Native tubulin, (B) tubulin aged for 1 h; (C) tubulin aged for 2 h. Gel filtration and a colchicine binding assay were carried out as described in Materials and methods. Tubulin concentration was 3 μM in all cases.
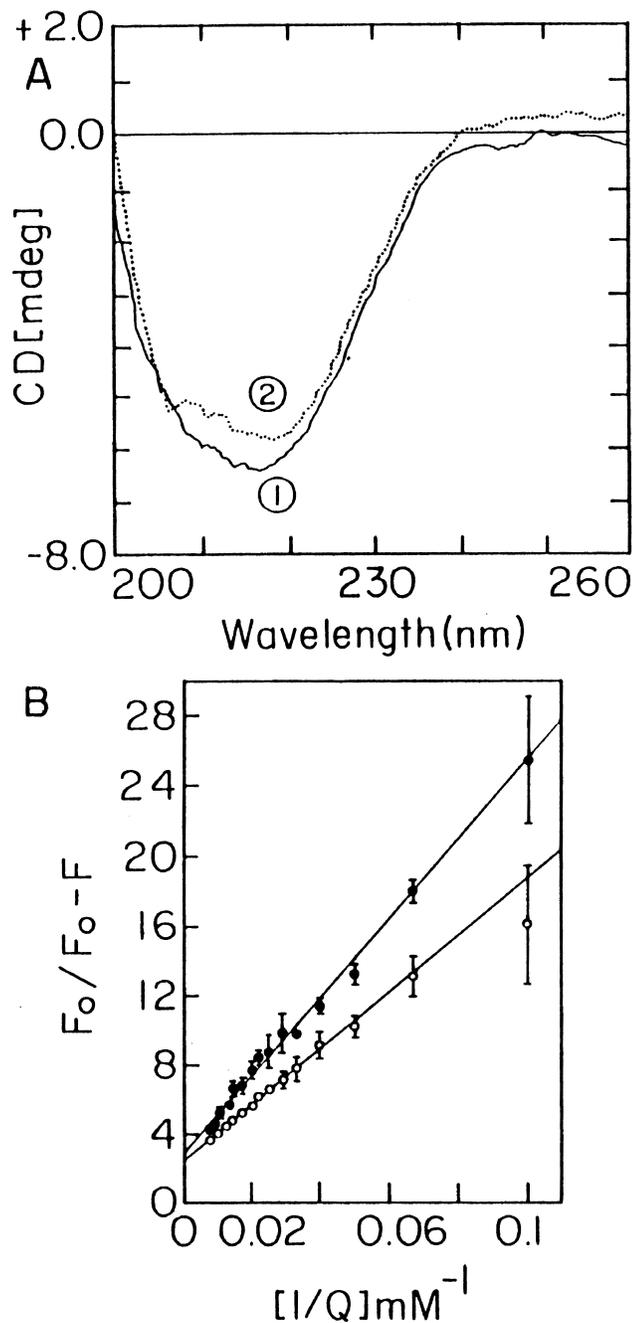
Fig. 6. Far UV CD spectra of native and aged tubulin (A) and Lehrer plots for the acrylamide quenching of native and aged tubulin (B). (A) The spectra show 1 μM native tubulin (trace 1) and the same concentration of tubulin aged for 2 h at 37 °C (trace 2) in Pipes/Mg^{2+} buffer. Each spectrum was recorded as an average of two scans. A spectral width of 1 nm and a time constant of 2 s were used for these measurements. Spectra were recorded at 25 °C. (B) Lehrer plots for the acrylamide quenching of native and aged tubulin showing 1 μM native tubulin (○) and the same concentration of tubulin aged for 2 h at 37 °C (●) in Pipes/Mg^{2+} buffer. After each addition of acrylamide (in the same buffer), samples were excited at 280 nm and emission was observed at 335 nm. Slit widths of 5 nm were chosen for both excitation and emission. Spectra were recorded at 25 °C.

Effects of ageing at 37 °C on the reactivity of the cysteines of tubulin

Based on such a working model, we have explored the effects of protein ageing and other ligand binding on the reactivity of tubulin's sulfhydryls. Tubulin loses the ability to self-assemble and colchicine-binding activity after ageing at 37 °C. These activities are sensitive to solution conditions as the inclusion of GTP/Mg^{2+} and glycerol stabilizes both [19]. The polymerization activity is more fragile compared to colchicine-binding activity, with $t_{1/2}$ values of 53–58 min and 4–5 h, respectively [20]. These results indicate that these two activities of tubulin involve two different parts of tubulin. Unfortunately, not much is known about the structural changes occurring in tubulin due to ageing.

We were interested in finding whether ageing causes any significant global change leading to the burial or exposure of the cysteine residues of tubulin. We used DTNB kinetics to detect the changes. When tubulin (1.54 μM) in Pipes/Mg^{2+} buffer was aged for various time periods and then treated with DTNB (400 μM), we observed that the rate constant of the DTNB reaction decreased initially and then increased steadily. This increase in rate constant, is accompanied by a steady decrease in the total number of sulfhydryl groups reacted and a steady increase in the number of fast reacting sulfhydryl groups (Fig. 4). The loss of reactivity of some sulfhydryl residues during ageing is not due to disulfide bond formation or oxidation of cysteine residues as full reactivity is seen in 8 M urea. Interestingly, however, at all times during ageing the reaction profile of the slow reacting sulfhydryl groups can be fitted to a single rate constant with a correlation coefficient of 1.0. Clearly, the change in the number of fast reacting and unreactive sulfhydryls suggests that more than just a localized change occurs upon ageing.

Based on the previous model, we suggest that ageing leads to a change in the static structure, resulting in an increase in the number of fast reacting sulfhydryl groups as well as changes in the dynamic modes of protection and the rate constants of protein opening. The putative change in static structure is supported by the fact that ageing leads to the exposure of a hydrophobic site in tubulin as indicated by the enhanced binding of bis-ANS [21–23].

In many cases, the exposure of hydrophobic surfaces leads to aggregation. We have thus explored whether ageing leads to the aggregation of tubulin. Gel-filtration column chromatography shows one peak at 7.68 min for native tubulin. However, aged tubulin shows a distinct, well-resolved peak at 5.35 min, suggestive of an aggregated form. The peak height at 5.35 min increased with a concomitant decrease of the peak height at 7.68 with increased ageing (Fig. 5). Ageing of tubulin caused exposure of hydrophobic areas leading to the aggregation of tubulin. Thus, aggregation may lead to burial of some of the reactive cysteine residues in the dimer–dimer or higher order interface. It is known that tubulin loses its colchicine binding

Table 1. Aggregation and the loss of colchicine binding activity (CBA) of tubulin upon ageing at 37 °C.

Time of ageing (h)	% Aggregation	% Loss of CBA
0	0.4	0
1	18.2	9.5
2	35.4	28

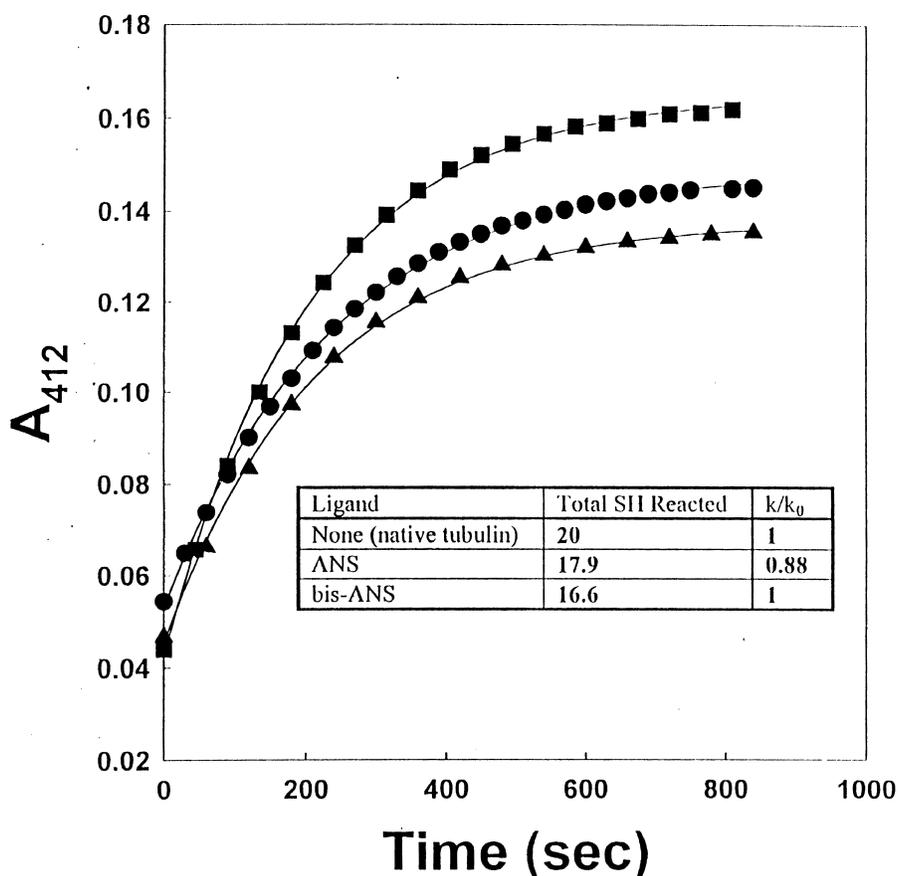| Ligand	Total SH Reacted	k/k_0
None (native tubulin)	20	1
ANS	17.9	0.88
bis-ANS	16.6	1

Fig. 7. Effect of ANS and bis-ANS binding on DTNB kinetics. Native tubulin (■); the tubulin–ANS complex (●); the tubulin–bis-ANS complex (▲). Protein concentration was 0.6 μM and the concentration of the dyes was 10 μM in each case. DTNB concentration was 400 μM in all cases. The experiments were carried out in Pipes/Mg^{2+} buffer and spectra were recorded at 25 °C.

activity upon aging at 37 °C [24]. and the results of such a study are shown in Table 1.

If changes in the static structure and opening modes occur upon ageing, it should be reflected in other spectroscopic properties. Far UV CD spectroscopy can be used to measure the secondary structure of the protein; hence any change may be a reflection of static structure. The far UV CD spectra of 1 μM native tubulin (trace 1) and 2 h aged tubulin (trace 2) at 37 °C are shown in Fig. 6A. There are some small changes in the CD spectra suggestive of changes in the static structure but no major unfolding of the protein. Fluorescence quenching of tryptophan residues in proteins by external collisional quenchers reflect the dynamics of a protein. Tubulin has eight tryptophan residues distributed throughout the primary structure. The Lehrer plot for the quenching of tryptophan fluorescence by acrylamide for 1 μM of both native tubulin and tubulin aged for 2 h is shown in Fig. 6B; the plot is linear in both cases. For native tubulin the plot cuts the *y*-axis at 2.4, which corresponds to 41% of the total fluorescence of the native tubulin being accessible to acrylamide. For aged tubulin, the Lehrer plot cuts the *y*-axis at 2.8, which indicates that 38% of the total fluorescence is accessible to the quencher. However, the K_{sv} values of the 2 h aged tubulin is 1.65 times higher than the native protein, suggestive of changes in dynamics, in agreement with the DTNB reactivity results.

Interaction of tubulin with anilinonaphthalene sulfonates

We have also tried to find out the mode of binding to tubulin of two other dyes. When ANS is bound to tubulin, the extent of the DTNB reaction decreases and the absorbance at plateau accounts for about 18 cysteine residues (Fig. 7). When bis-ANS

is bound to tubulin, the extent of the reaction decreases and the absorbance at plateau accounts for 16.6 cysteine residues (Fig. 7). These results are quite similar to the results from our ageing experiments; the burial of cysteine residues may be due to aggregation. It is generally believed that at such low concentrations anilinonaphthalene dyes bind to high affinity sites without causing major changes in protein conformation [23]. The rate of the reaction of the slow reacting sulfhydryls remains unchanged on drug binding compared to native tubulin. It has been suggested that the anilinonaphthalene sulfonate binding site is located in the β subunit, proximal to the exchangeable GTP binding site [22]. As can be seen from the modification data (Fig. 7), the DTNB reaction rate with slow reacting sulfhydryls is affected only a little upon binding of ANS/bis-ANS. We suggest that ANS/bis-ANS binding to the β subunit causes some of the sulfhydryl groups in it to be protected but does not affect the global unfolding rate of the protein, which is responsible for the slow DTNB reaction. Three putative slow reacting cysteine residues (C203, C213 and C305) in the β subunit are close in the three-dimensional structure and we speculate that these cysteine residues are completely protected upon binding of ANS/bis-ANS (see Fig. 1).

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