Excimer fluorescence of pyrene-maleimide-labeled tubulin

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Excimer-forming cysteines in tubulin are detected by the presence of excimer fluorescence in N-(1-pyrenyl)maleimide-labeled tubulin. The ratio of excimer/monomer fluorescence of labeled protein remained unchanged upon its dilution. These results indicating that both partner of each pair(s) of cysteine are located in the same subunit. The excimer fluorescence is insensitive to prior treatment of tubulin with either colchicine or GTP, indicating that pairs of cysteines protected by those drugs are not involved in excimer formation. This excimer fluorescence of N-(1-pyrenyl)maleimide-labeled tubulin disappeared upon treatment with SDS, guanidinium chloride (GdmCl) and urea. Studies with GdmCl induced unfolding of N-(1-pyrenyl)maleimide-labeled tubulin showed that the loss of excimer fluorescence preceeds subunit dissociation. The loss of both colchicine-binding activity and the excimer fluorescence with increasing temperature indicates a major conformational change of the tubulin molecule at elevated temperatures.

Tubulin is a heterodimeric protein composed of two subunits, α and β, each containing about 450 amino acid residues [1, 2]. There are 12 and 8 cysteines in the α and β subunits, respectively [1, 2]. Cysteines play important roles both in colchicine binding and tubulin self assembly. Kuriyama and Saki obtained total inhibition of tubulin polymerization by the alkylation of two cysteines [3]. According to them, 70 – 90% inhibition of colchicine binding to tubulin requires modification of its 5 – 6 cysteine residues [3].

Cysteine is an important amino acid residue in proteins which is often used as an attachment point for external fluorophores used for conformational studies as well as to gain insight into protein structure. Tubulin, being a multicysteine-containing protein, is probably not ideal for the above purposes because identification of functional cysteine(s) and labeling of a single cysteine with fluorophore is difficult. Nevertheless, it is interesting to know how cysteines are distributed in the tubulin molecule, their proximity and distances from each other, etc. Little and Luduena [4] reported the presence of two pairs of cysteines in the β subunit, which could be cross-linked with N',N'-ethylenebis(iodoacetamide) (EBI). From their cross-linking study, they concluded that the distance between two cysteines in each pair is within 0.9 nm or less.

In the present study, we have tested whether cysteines in the tubulin molecule are capable of forming excimers using N-(1-pyrenyl)maleimide fluorophore.

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Abbreviations. GdmCl, guanidine chloride; EBI, N',N'-ethylenebis(iodoacetamide); PM-tubulin, N-(1-pyrenyl)maleimide-labeled tubulin.

MATERIALS AND METHODS

Materials

PIPES, EGTA, GTP, colchicine and SDS were from Sigma, (1-pyrenyl)maleimide on celite from Molecular Probe, guanidinium chloride (GdmCl) from Aldrich Chemical Company. All other chemicals used were of analytical grade.

Methods

Tubulin purification

Tubulin was prepared by phosphocellulose chromatography of microtubule protein, purified from goat brain, after two cycles of temperature-dependent polymerization in Pipes assembly buffer (100 mM Pipes, pH 6.8, 0.5 mM $MgCl_2$ and 1 mM EGTA with 1 mM GTP) [5]. Following the column chromatography, protein was concentrated to 5 – 10 mg/ml using an Amicon CF-50A membrane cone and stored at −70°C. The protein concentration was determined by the method of Lowry et al. [6].

Chemical modification

Tubulin 3 mg/ml was incubated with 15 times its concentration of pyrene maleimide in the dark at 25°C for 1 h. Stock solutions of pyrene maleimide were made in acetone. The acetone concentration in the reaction mixture was kept below 2% (by vol.). The reaction was stopped by adding excess 2-mercaptoethanol. The label protein was separated from free N-(1-pyrenyl)maleimide by passing the reaction mixture through a G-25 column (15 cm × 1.2 cm), previously equilibrated with 20 mM sodium phosphate, pH 7, 0.5 mM $MgCl_2$ and 1 mM EGTA. The concentration of N-(1-pyrenyl)maleimide-labeled tubulin (PM-tubulin) was determined by the
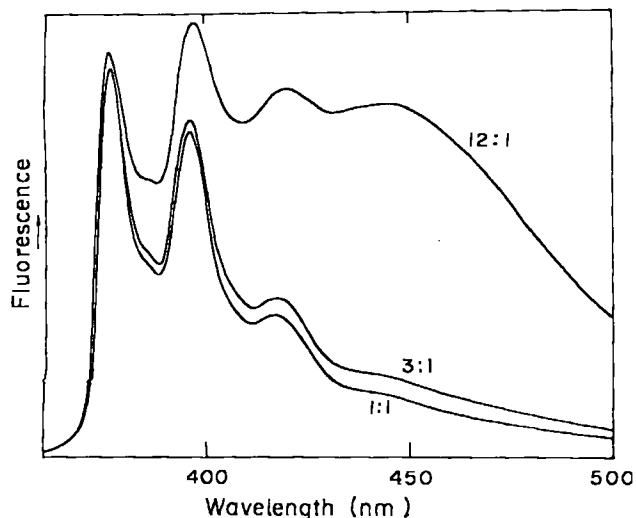
Fig. 1. Emission spectra of tubulin labeled with different amount of *N*-(1-pyrenyl)maleimide. Tubulin (1 μM) was incubated with different amount of *N*-(1-pyrenyl)maleimide (the molar ratio of pyrene maleimide/tubulin is given in the figure) at 25°C for 1 h in buffer A. All emission spectra were normalized at 377 nm. The excitation was 343 nm in all cases.

Fig. 2. The ratio of the fluorescence intensity of excimer/monomer (F_{450}/F_{377}) as a function of the *N*-(1-pyrenyl)maleimide concentration. The tubulin concentration was 1 μM in all cases (pyrene maleimide/tubulin varies over 0.5—20). All other conditions were same as in Fig. 1.

Lowry method using bovine serum albumin as a standard [6]. Incorporation of *N*-(1-pyrenyl)maleimide was determined from the absorbance at 343 nm using a molar absorption coefficient 30000 $M^{-1} \cdot cm^{-1}$. The degree of incorporation was obtained from the incorporated maleimide/protein concentration.

Fluorescence measurements

Fluorescence spectra were recorded on a Hitachi F-3000 spectrofluorometer fitted with a water-circulating cell holder connected to a constant-temperature circulating waterbath. All experiments were performed at 25°C. Polarization experiments were performed using Hitachi polarization accessories. The fluorescence-intensity components (I_{vv}, I_{vh}, I_{hv}, I_{hh}), in which the subscripts refer to the horizontal (h) or the vertical (v) positioning of the excitation and emission polarizers, respectively, were used to calculate steady-state fluorescence polarization using the equation

$$P = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}},$$

where G is the correction factor for the transmission efficiency of the emission monochromator to light-polarized parallel and light-polarized perpendicular to the grating. The band pass of both excitation and emission monochromators were 3 nm unless otherwise stated. All experiments were carried out in 20 mM sodium phosphate, pH 7.0, 0.5 mM $MgCl_2$ and 1 mM EGTA (buffer A).

RESULTS

Pyrene maleimide (20 μM) has very low fluorescence and it increased 90—100-fold in the presence of 1 μM tubulin. Fig. 1 shows the emission spectra of PM-tubulin at increasing fluorophore concentrations. Excitation is at 343 nm and emission is monitored over the 360—550-nm range. It is immediately apparent from the large, broad emission peak
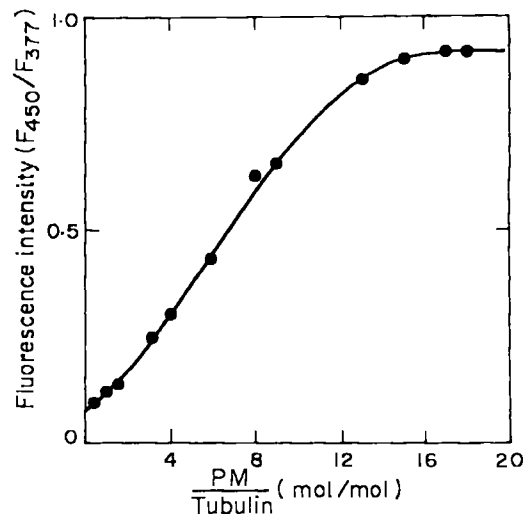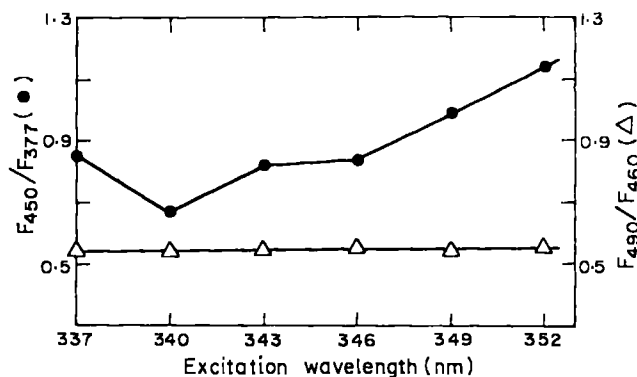

Fig. 3. Ratio of fluorescence emission intensities at different wavelengths of emission as a function of the excitation wavelength. PM-tubulin (1 μM), having incorporated 7 mol *N*-(1-pyrenyl)maleimide/mol tubulin was excited at different wavelengths and emission spectra were generated. From those spectra, the ratio of the emission intensities at 450 nm and 377 nm (○), 490 nm and 460 nm (△) were plotted as a function of the excitation wavelength.

centered near 450 nm, that a large portion of the total fluorescence arises from pyrene excimers. The amount of excimers, measured as a fluorescence ratio (fluorescence$_{450}$/fluorescence$_{377}$), increases with increasing maleimide concentration at a fixed tubulin concentration of 1 μM (Fig. 2). The fluorescence-emission spectra shown in Fig. 1 arises from two types of subpopulation of protein-bound fluorophores. If it arises from one type of population, then the ratio of the emission intensities at any two wavelengths should be independent of the excitation wavelength. This hypothesis has been tested and the results are presented in Fig. 3. It is evident from this figure that the ratio fluorescence$_{490}$/fluorescence$_{460}$ is independent of the excitation wavelength, indicating that the spectra arise from the same population of fluorophore. The ratio of fluorescence$_{450}$/fluorescence$_{377}$ is, however, dependent on the excitation wavelength (Fig. 3), indicating that the spectra originated from two different populations. Thus, in Fig. 1, the early part of the emission-spectra (370—390 nm) fluorescence
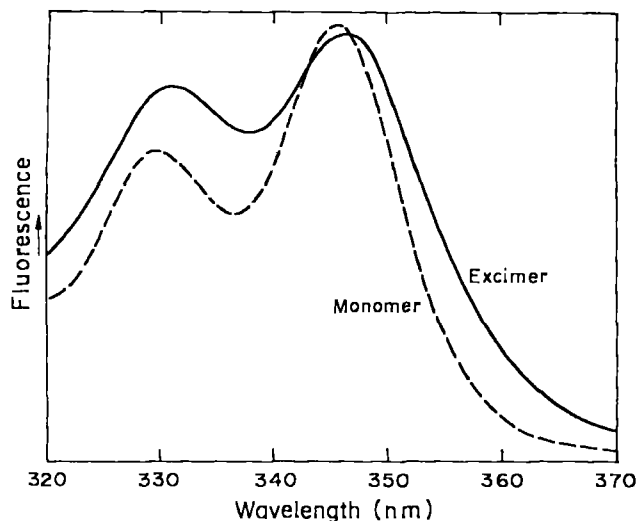
Fig. 4. Fluorescence-excitation spectra of PM-tubulin, having incorporated of 7 mol N-(1-pyrenyl)maleimide/mol tubulin. Emissions were at 377 nm and 450 nm for the monomer and the excimer, respectively.

Fig. 5. Effect of SDS on PM-tubulin (containing 7 mol N-(1-pyrenyl)maleimide/mol tubulin). Curve 1, emission spectra of PM-tubulin; curve 2, emission spectra of PM-tubulin in the presence of 0.1% SDS. The PM-tubulin concentration was 1.5 μM in both cases.

arises from monomers and, similarly, the emission intensity in the latter part of the spectra (450 — 550 nm) is purely from the excimers. The area inbetween the emission spectra is contributed by both monomers and excimers. Fluorescence excitation spectra of the monomer and the excimer are obtained using emissions of 377 nm and 450 nm, respectively (Fig. 4). The excimer-excitation peak is broader and red-shifted compared to that of the monomer. Broadening and red-shifting of the excitation peak are characteristic of excimer formation and were reported by others in the case of N-(1-pyrenyl)maleimide-labeled tropomyosin [7, 8].

Perturbation studies

Excimers form only when excimer-forming groups are within 0.3 nm or less of each other [9]. GdmCl, urea and SDS are commonly used denaturing agents which perturb the protein tertiary structure. As a result, the groups which are in close proximity to each other are no longer at their proximal distance. In the case of PM-tubulin, the observed excimer fluorescence at 450 nm disappear almost completely in the presence of 0.1% SDS (Fig. 5, curve 2). The effect of GdmCl on the excimer of PM-tubulin is shown in Fig. 6. Here we measure the loss of excimer fluorescence of PM-tubulin with increasing denaturing agent. We also observed that this loss of excimer fluorescence in the presence of added GdmCl is not due to the subunit dissociation of tubulin which occurs at a much higher concentration of GdmCl. Polarization measurement of a N-(1-pyrenyl)maleimide-labeled protein molecule at various dilutions provides a sensitive and convenient method for the detection and analysis of its subunit association [10]. Here we have used PM-tubulin to determine its GdmCl-induced subunit dissociation. The transition midpoint for subunit dissociation of 5 μM tubulin occurs at 2.9 M GdmCl (Fig. 6B). At 0.7 μM tubulin, the transition midpoint is at 2.3 M GdmCl (Fig. 6B). Lowering of the transition-midpoint concentrations in response to lowering the protein concentrations are characteristics of subunit dissociation. Under identical conditions, when excimer fluorescence is measured with increasing GdmCl, both protein concentrations (5 μM and 0.7 μM) show identical transition midpoints at 1.9 M GdmCl (Fig. 6A). Our
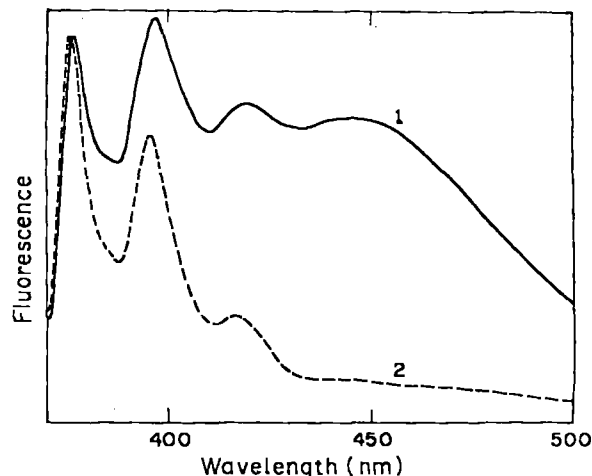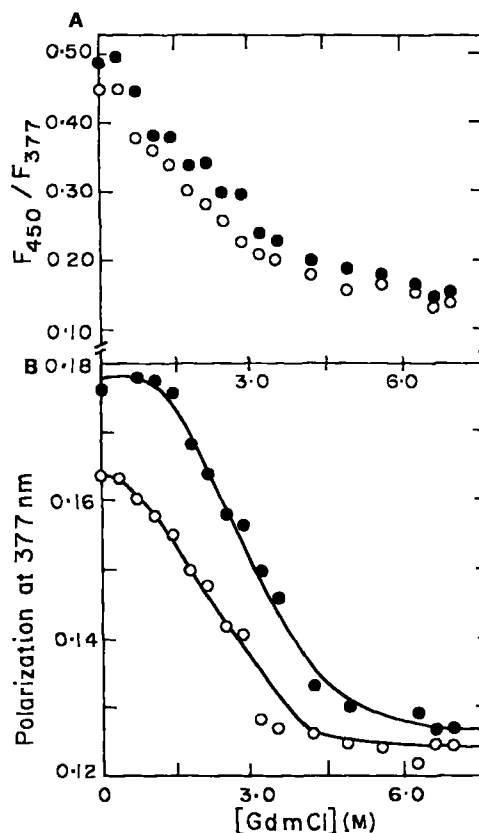

Fig. 6. Effect of GdmCl on the excimer and polarization of PM-tubulin (containing 7 mol N-(1-pyrenyl)maleimide/mol tubulin). The GdmCl effect on the excimer of PM-tubulin was measured by the ratio of fluorescence intensities at 450 nm and 377 nm as a function of the GdmCl concentration (A). The GdmCl effect on polarization of PM-tubulin is shown in B. The PM-tubulin concentrations were 5 μM (●) and 0.7 μM (○) in both experiments.

results thus show that the loss of excimer fluorescence preceeds subunit dissociation, because the transition midpoint of the former is at 1.9 M GdmCl, compared to 2.9 M for that of subunit dissociation.
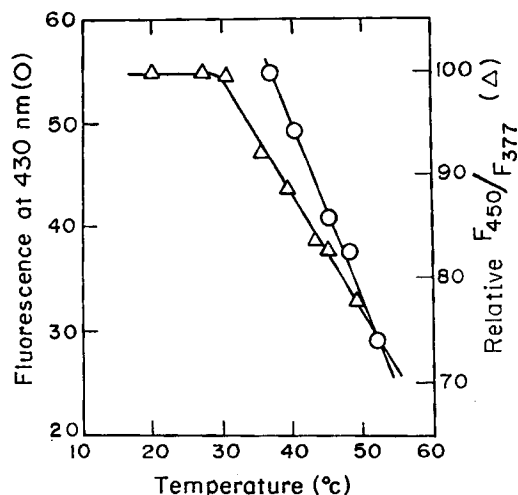
Fig. 7. Effect of temperature on the excimer of PM-tubulin and colchicine binding activity of unlabeled tubulin. Temperature effect on excimers of PM-tubulin was monitored by the ratio of fluorescence intensities at 450 nm and 377 nm as a function of temperature ($\triangle$). The temperature was varied from 20−55°C. The temperature effect on the colchicine-binding activity of tubulin was measured by incubating tubulin (4 µM) and colchicine (8 µM) together in buffer A for 1 h at various temperature ranging over 37−55°C ($\bigcirc$). After the incubation, all samples were brought to 25°C. The extent of colchicine-binding activity was measured from the emission intensities at 430 nm according to Mukhopadhyay et al. [11]. Excitation was at 340 nm in all cases.

Effect of temperature

The tertiary structure of tubulin can also be perturbed by heating tubulin at high temperatures. We measured the colchicine-binding activity of unlabeled tubulin and the excimer fluorescence of PM-tubulin with increasing temperature, and the results are shown in Fig. 7. The fluorescence of the colchicine-tubulin complex at 430 nm increases up to 37°C (data not shown), then decreases sharply with further increases in the temperature, as shown in Fig. 7 [11]. These results indicate conformational changes in the colchicine-binding domain of tubulin, leading to a decline in drug binding. Surprisingly, the ratio of $\text{fluorescence}_{450}/\text{fluorescence}_{377}$ of PM-tubulin with increasing temperature (37−55°C) followed the same trend as that of the colchicine-binding activity shown in Fig. 7.

Effect of subunit dissociation on excimer fluorescence

The purpose of this experiment is to see whether intersubunit or intrasubunit SH-groups are involved in excimer formation. The results of such an experiment are shown in Table 1. To our surprise, it was observed that the ratio $\text{fluorescence}_{450}/\text{fluorescence}_{377}$ remain almost unchanged upon dilution of tubulin from 5−0.008 µM, where tubulin is predominantly in its dimeric and monomeric form, respectively [12]. These results indicate that both partner of each pair(s) of SH groups involved in excimer formation are located in the same subunit of tubulin.

DISCUSSION

One of the most interesting fluorescence properties of pyrene and its derivatives is excimer formation. In solution, this

Table 1. Effect of dilution on excimer fluorescence of PM-tubulin. Dilution of PM-tubulin to the indicated concentrations was carried out in buffer A at 25°C. Emission intensities were measured at 377 nm and 450 nm, upon excitation at 343 nm. F, fluorescence.

Protein concentration	F_{450}/F_{377}
µM	
5	0.63
2.5	0.63
1.25	0.623
0.36	0.597
0.102	0.57
0.05	0.59
0.025	0.59
0.013	0.62
0.008	0.61

excimer formation occurs only when the fluorophore concentration is greater than 0.1 mM [13]. In the presence of a macromolecule which has two or more binding sites for pyrene, this excimer fluorescence is observed at a much-lower pyrene concentration. In the present study, PM-tubulin is exclusively present in the dimeric form and no high molecular mass aggregates were observed by gel-filtration analysis (Protein Pak column 300 SW waters) using HPLC. In this study, where the PM-tubulin concentration is 3 µM or less, the presence of excimer fluorescence indicates the proximity of two or more pyrene molecules in the same tubulin molecule. The gel filtration by HPLC ruled out the possibility that the excimer is caused by interaction between pyrene groups in different tubulin molecules. The fact that subunit dissociation does not alter the excimer fluorescence, indicates that the excimer-forming partner of each pair(s) of SH groups are probably located in the same subunit. However, we do not know the number of SH groups involved in excimer formation, or their locations. The fact that loss of excimer fluorescence preceeds subunit dissociation, indicates that a local conformational change takes place and represent one of the partially unfolded intermediates of PM-labeled tubulin in the 0−2 M GdmCl range. The loss of both colchicine-binding activity and the excimer fluorescence with increasing temperature indicates a major conformational change of the tubulin molecule at elevated temperature. The excimer fluorescence is conveniently exploited in studies of protein conformation where SH proximity is suspected. The two cysteine residues of tropomyosin are in closed proximity, as shown by their ability to form a disulphide bond [14−16]. Betcher-Lange and Lehrer [7] verified the proximity of those cysteines of tropomyosin with the observation of excimer fluorescence from N-(1-pyrenyl)maleimide covalently attached to them. Similar specific structural information cannot be expected for a protein molecule where a large number of reactive cysteines are present. Tubulin is an example of such a protein. However, it is important to know how these cysteines are distributed in the protein. In tubulin, it was reported that there are two pairs of cysteines in the β subunit which could be cross-linked with EBI [4, 17]. As the length of the EBI molecule is 0.9 nm, it was concluded that these cysteines are within 0.9 nm or less of each other. Our study reported here indicates that there are cysteines which are closer to each other and should be within 0.3 nm or less. We also observed that the presence of colchicine, which protects one of the pairs from EBI modification [17], has no effect on excimer formation (data not shown).

Similarly GTP, which protects another pair from EBI cross-linking [4] also has no effect on excimer intensity (data not shown). Finally, it would be interesting to know whether the cysteines which participate in the excimer formation can form disulphide bonds among themselves, as was observed in case of tropomyosin [7]. To answer the question raised above, we tried to oxidize tubulin with several agents such as a mixture of oxidized and reduced glutathione [18] and Cu^{2+} in the presence of 1,10-phenanthroline [19]. We observed no disulphide-bond formation, nor did these agents affect the excimer formation. The unique strategy utilized to localize the pair of SH groups protected by colchicine was also attempted here [4]. We are unable to find any such agent which would specifically bind those SH groups or protect them, when the SH groups were treated with sulfhydryl modifying agents.

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