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Deuterium oxide stabilizes conformation of tubulin: a biophysical and biochemical study

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The present study was aimed to elucidate the mechanism of stabilization of tubulin by deuterium oxide (D_2O). Rate of decrease of tryptophan fluorescence during aging of tubulin at 4°C and 37°C was significantly lower in D_2O than in H_2O. Circular dichroism spectra of tubulin after incubation at 4°C, suggested that complete stabilization of the secondary structure in D_2O during the first 24 hours of incubation. The number of available cysteine measured by DTNB reaction was decreased to a lesser extent in D_2O than in H_2O. During the increase in temperature of tubulin, the rate of decrease of fluorescence at 335 nm and change of CD value at 222 nm was lesser in D_2O. Differential Scanning calorimetric experiments showed that the T_m values for tubulin unfolding in D_2O were 58.6°C and 62.17°C, and in H_2O those values were 55.4°C and 59.35°C. [BMB reports 2008; 41(1): 62-67]

INTRODUCTION

Tubulin-microtubule is an important cytoskeletal system in eukaryotes, which is involved in number of cellular processes, including chromosome positioning and separation during mitosis and meiosis, and vesicle transport. Tubulin, the subunit of microtubules, loses its stability upon incubation in solution with consequent loss of capacity for its microtubule. Deuterium oxide (D_2O) has been known to offer stabilization effect on a number of proteins like oligomeric forms of lactic dehydrogenase, glutamate dehydrogenase (1), halophilic malate dehydrogenase (2), FtsZ (3), β-lactoglobulin (4) and various peptides (5-8). We had shown that deuterium oxide stabilizes functional properties of tubulin (9). The present study was aimed to elucidate the mechanism underlying the stabilization effect of tubulin in D_2O with particular reference to structural and conformational changes. In this work, biochemical experiments consisting of estimation of free cysteine and biophysical experiments, including fluorescence quenching, circular dichroism and differential scanning microcalorimetry were employed to study tubulin stabilization. The key factors involved in increasing the stability of tubulin were examined and the results are presented in this report.

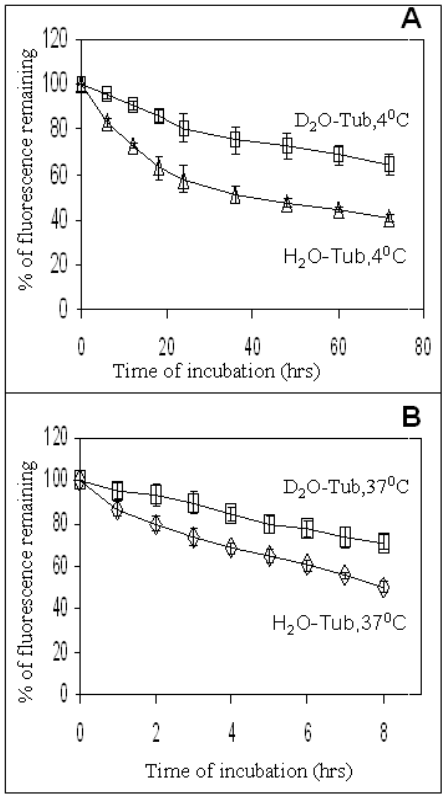

Fig. 1. Prevention of fluorescence quenching of tubulin in D_2O. Tubulin (1 μM) was incubated in 20 mM sodium phosphate buffer. (A) Fluorescence spectrum was recorded by incubating tubulin (1 μM) in 20 mM H_2O-NaPi (-▵-) and 20 mM D_2O-NaPi (-▫-) at 4°C for 72 h and data was collected 6 to 12 h intervals. (B) Tubulin (1 μM) was incubated in 20 mM H_2O-NaPi (-◇-) and 20 mM D_2O-NaPi (-▫-) at 37°C for 8 h and data was collected 1 h intervals.

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RESULTS AND DISCUSSION

Fluorescence study

The fluorescence of tryptophan residues of protein depends upon polarity of the environment (10). Any change in conformation or polarity of the environment would be reflected in the fluorescence intensity of tryptophan. Tubulin has twelve tryptophan residues. It is well known that tubulin is a labile protein that loses its polymerization ability and the colchicine binding activity upon aging at 37°C (9). We, therefore incubated 1 μM tubulin at 4°C and 37°C in both H_2O-buffer and D_2O-buffer separately. We measured the fluorescence intensity at 333 nm at indicated time intervals and the results are shown in Fig. 1(A) and 1(B). Fluorescence decreases exponentially in both H_2O-buffer and D_2O-buffer with time although the rate was higher at 37°C. Thus $t_{1/2}$ (time for 50% decrease in fluorescence intensity where that of zero time incubated protein is taken as 100%) for decay of tryptophan fluorescence was only about 8 h at 37°C whereas it was more than 45 h at 4°C. In presence of D_2O, the $t_{1/2}$ for decay increased significantly at both the temperatures (data not shown). Thus, overall the tubulin structure is stabilized globally in presence of D_2O. This decay of intrinsic tryptophan fluorescence of a protein with time at different temperature is due to the exposure of tryptophan in aqueous environment. The quenching of tryptophan fluorescence was less in presence of D_2O. These results reconfirm our previous findings and D_2O induced stabilization of protein structure (9).

Circular dichroism study

Structural changes in a protein are estimated by measuring its secondary structure using CD spectra. We therefore measured the secondary structure of tubulin using CD spectra upon ageing at 4°C both in H_2O-buffer and D_2O-buffer (Fig. 2A and 2B). The ellipticity values at 220 nm decreased to 38% in H_2O-buffer (compared to that of zero time incubated sample) whereas it was even less than 10% in D_2O-buffer under the same conditions. Further incubation at 4°C for 72 hours leads to 62% and 50% decrease in CD values in H_2O-buffer and D_2O-buffer, respectively. These results corroborate well with that observed by measuring the decay of tryptophan fluorescence (Fig. 1). The loss of fluorescence at 333 nm at 4°C in presence of D_2O was less than 15%, whereas it was more than 40% in H_2O-buffer under same condition.

Estimation of reactive cysteine by DTNB reaction

Tubulin has 20 cysteines and 18-20 cysteines are available for reaction with DTNB (11-13). Among 20 cysteines, 5-6 are fast reacting and rests are slow reacting with DTNB (11, 12) indicating that they are present in different environment of tubulin. Since these cysteines are located all over the primary sequence of both α and β subunits, it is expected that any structural change (local and global) occurring in tubulin upon ageing may be reflected in DTNB kinetics. Fig. 3 shows the DTNB kinetics of tubulin aged at 4°C and 37°C in the pres-

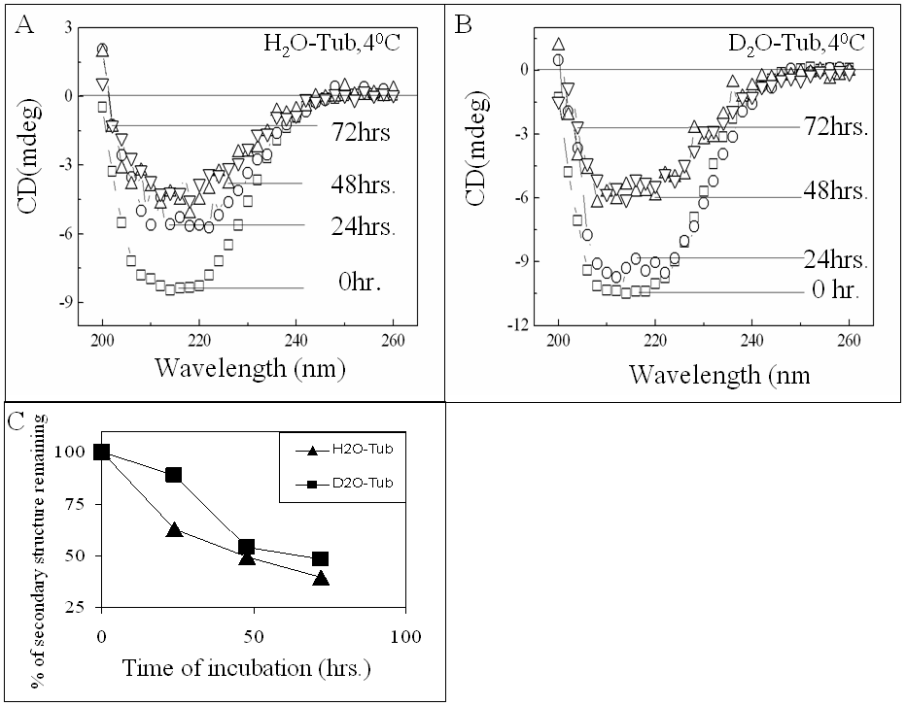

Fig. 2. Stabilization of the secondary structure of Tubulin by D_2O. Tubulin (1 μM) was incubated in 20 mM H_2O-NaPi and 20 mM D_2O-NaPi at 4°C for 72 h and far-UV CD spectra was monitored 24 h intervals. (A) CD spectra for Tubulin (1 μM) in 20 mM H_2O-NaPi, taken at time intervals 0 h (□), 24 h (○), 48 h (△), 72 h (▽) and. (B) CD spectra for Tubulin (1 μM) in 20 mM D_2O-NaPi, after 0 h (□), 24 h (○), 48 h (△), 72 h (▽) and. (C) The CD values at 220 nm at different time intervals were normalized with respect to the CD value of tubulin (1 μM) in 20 mM H_2O-NaPi (▲) and 20 mM D_2O-NaPi (■).

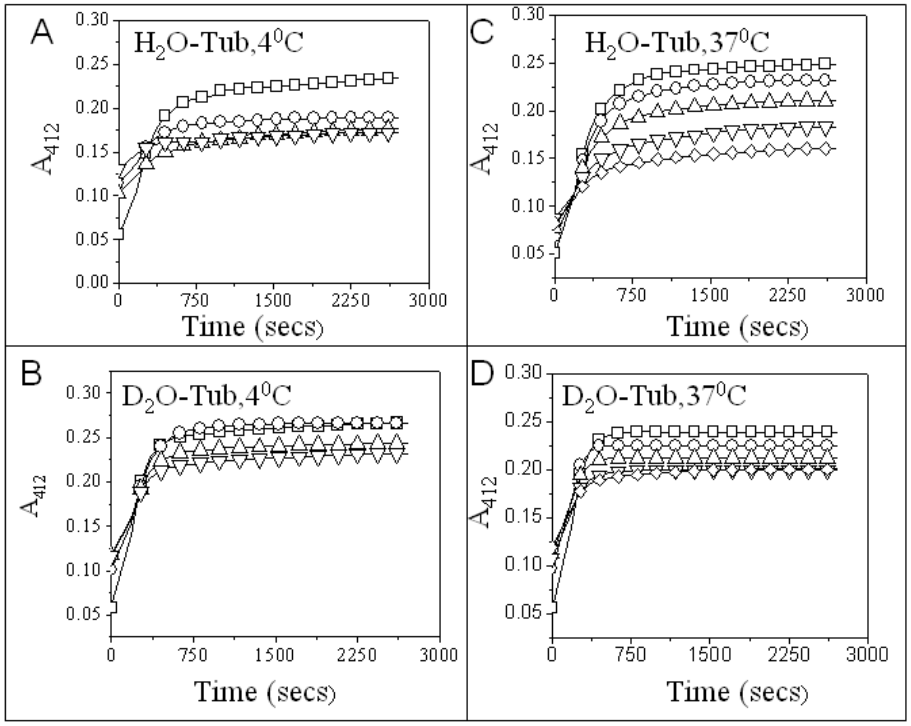

Fig. 3. Reduction in the loss of reactive cysteines of tubulin by D_2O. Tubulin (1 μM) was incubated in 50 mM H_2O-PEM and 50 mM D_2O-PEM at 4°C for 72 h (A and B) and also at 37°C for 8 h (C and D). Number of reactive cysteines were estimated every 24 h intervals, when incubated at 4°C and 1 h intervals, when incubated at 37°C. (A) Number of reactive cysteines of tubulin (1 μM) in 50 mM H_2O-PEM after 0 h (□), 24 h (○), 48 h (△), and 72 h (▽). (B) Number of reactive cysteines of tubulin (1 μM), in 50 mM D_2O-PEM after 0 h (□), 24 h (○), 48 h (△), and 72 h (▽). (C) Number of reactive cysteines of tubulin (1 μM) in 50 mM H_2O-PEM after 0 h (□), 2 h (○), 4 h (△), 6 h (▽), and 8 h (◇). (D) Number of reactive cysteines of tubulin (1 μM) in 50 mM D_2O-PEM after incubation in 0 h (□), 2 h (○), 4 h (△), 6 h (▽), and 8 h (◇).

Table 1. Estimation of cysteine of tubulin upon aging in H_2O-buffer and D_2O-buffer

Tubulin	4°C				37°C		
	0 h	24 h	48 h	72 h	0 h	4 h	8 h
H_2O-buffer	20 (5)	18 (9)	16 (11)	15 (12)	20 (5)	16 (9)	11 (11)
D_2O-buffer	20 (5)	20 (7)	19 (9)	18 (9)	20 (5)	18 (8)	16 (9)

The number of fast reacting cysteine is showing in parenthesis.
All data average of three experiments.

ence and absence of D_2O. Results of such experiments are summarized in Table 1. Ageing at both temperatures exposed more cysteines to solvents as number of fast reacting cysteines increased. After 24 h of incubation at 4°C, the number of fast reacting cysteines increased from 5 to 9 (increased by 80%) in H_2O-buffer and total number of cysteine decreased from 20 to 18 (decreased by 10%) (Fig. 3A), whereas in D_2O-buffer, the number of fast reacting cysteines increased from 5 to 7 (increased by 40%) and total number of cysteines remained 20 (no changed) (Fig. 3B). After 72 h of incubation, the number of fast reacting cysteines were 12 (increased by 140%) and 9 (increased by 80%) in H_2O-buffer and in D_2O-buffer, respectively, whereas the total number of cysteines were 15 (decreased by 25%) and 18 (decreased by 10%) in H_2O-buffer and D_2O-buffer respectively.

Similar trends were also observed when tubulin was incubated at 37°C in H_2O-buffer and D_2O-buffer respectively. Fig. 3 (C and D) shows the number of fast reacting cysteines and total cysteines with time of incubation at 37°C in H_2O-buffer and D_2O-buffer. After 4 h of incubation, the number of fast reacting cysteines in both H_2O-buffer and D_2O-buffer increased similarly but the total cysteine content decreased differently. The total cysteines content were 16 and 18 in H_2O-buffer and D_2O-buffer, respectively. After 8 h of incubation H_2O-buffer, the total number of available cysteine was 11 (decreased by 45%) and all were fast reacting cysteines indicating that the available free cysteines were in the surface. But in D_2O-buffer the number of fast reacting cysteines was 9 and total number of cysteines was 16 (decreased by 20%).

The total number of cysteines and fast reacting cysteines available for DTNB reaction decreased with ageing and it was more pronounced in H_2O-buffer. This is probably due to aggregation of tubulin upon ageing not for cysteine modification as after complete denaturation of 72 h aged tubulin with 8 M urea, the number of cysteine was around 20 in both H_2O-buffer and D_2O-buffer. Here, we observed that the number of cysteine residues decreases less amount when ageing was carried out in D_2O-buffer compare to H_2O-buffer. We therefore concluded that D_2O preserved the native conformation of tubulin significantly when incubated at 4°C and as well as at 37°C.

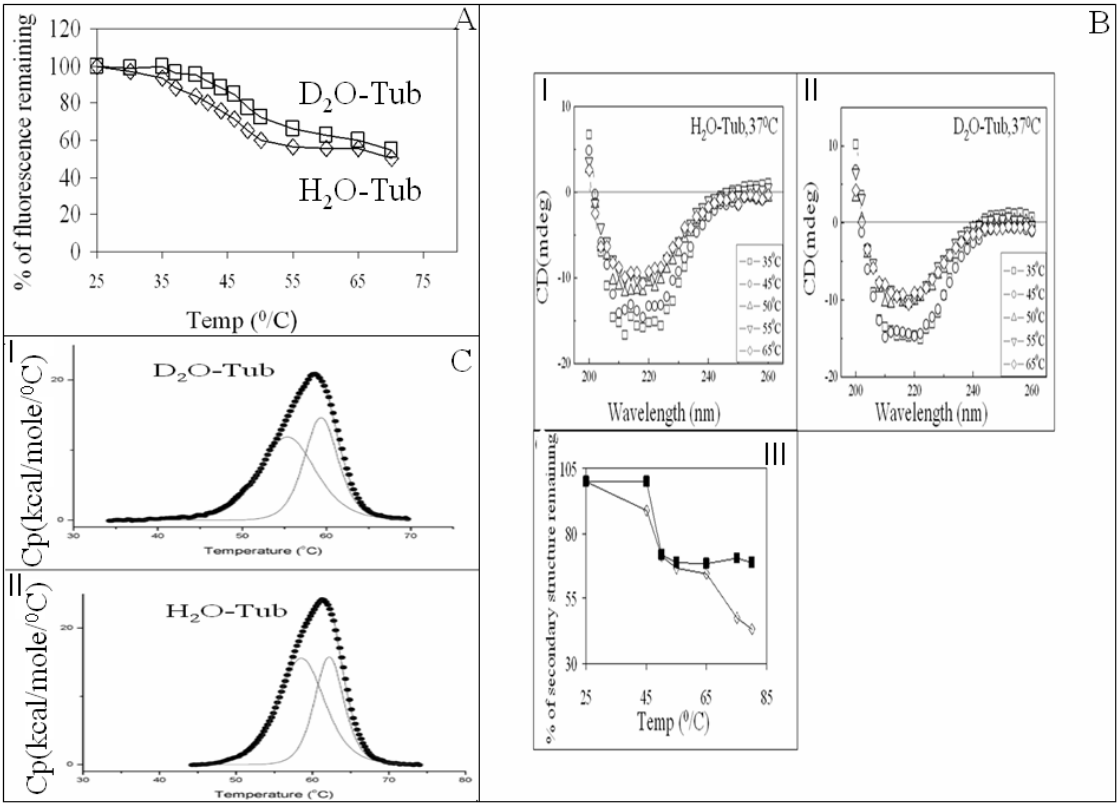

Fig. 4. Thermostability of tubulin in D_2O. (A) Plot of relative fluorescence intensity against temperature for tubulin (1 μM) in 50 mM H_2O-PEM (-○-) and 50 mM D_2O-PEM (-□-) (B). Thermostability of tubulin in D_2O were monitored by circular diachroism spectroscopy. Far-UV CD spectra of tubulin (1 μM) recorded at 25°C. (I) CD spectra of tubulin (1 μM) in 20 mM H_2O-NaPi after incubation at 30°C (-□-), 45°C (-○-), 50°C (-△-), 55°C (-▽-), 65°C (-◇-). (II) CD spectra of tubulin (1 μM) in 20 mM D_2O-NaPi after incubation at 30°C (-□-), 45°C (-○-), 50°C (-△-), 55°C (-▽-) and 65°C (-◇-). (III) Normalized CD values at 222 nm plotted as a function of temperature of tubulin (1 μM) in 20 mM H_2O-NaPi (-◇-) and 20 mM D_2O-NaPi (-□-). (C) Differential scanning calorimetric thermograms of tubulin (8 μM) in 50 mM H_2O-PEM (I) and 50 mM D_2O-PEM (II). Details in materials and methods.

Temperature dependent unfolding study

Temperature dependent unfolding process of tubulin was monitored by fluorescence spectroscopy, circular dichroism, and differential scanning calorimetry (DSC) to find out whether deuterium oxide prevented this process any significant extent.

Fluorescence study

Unfolding of a protein causes exposure of buried residues to solvent, and this process can be followed by measuring quenching of tryptophan fluorescence. Fig. 4A shows percentage of fluorescence intensity at 333 nm of tubulin in H_2O-buffer and D_2O-buffer. When the temperature was raised to 37°C, the fluorescence value decreased to 12% in H_2O-buffer and decreased to only 3% in D_2O-buffer. At 50°C, the decreasing amount was 40% in H_2O-buffer and 28% in D_2O- buffer. After that, the rate of increase of fluorescence intensity with temperature were similar in both H_2O-buffer and D_2O- buffer. These results are indicating that stabilization effect of deuterium oxide was more up to 60°C. During the initial phases of increase in temperature, breaking of hydrogen bond as well as hydrophobic bonds were occurred. D_2O prevented initial phases of temperature dependent unfolding through stabilizing the structure, and after that D_2O had no significant effect on tubulin conformation as the H-bonds and hydrophobic bonds were already broken up.

Circular dichroism study

The CD-spectra in far-UV region of tubulin were taken in H_2O-buffer and D_2O-buffer with increasing temperatures (Fig. 4B). The CD values at 222 nm with temperature are presenting in Fig. 4B (III). As temperature rises from 30°C to 45°C, the CD value at 222 nm decreased to 15% in H_2O-buffer with no change in CD values in D_2O-buffer. This indicates no loss of secondary structure of tubulin in D_2O-buffer during increase of temperature from 30°C to 45°C. The CD value decreased to 70% at 80°C in H_2O-buffer but in D_2O-buffer, the CD value

Table 2. DSC results

	T_m (1)	T_m (2)
Tubulin in H_2O-buffer	55.4 ± 0.5	59.35 ± 0.12
Tubulin in D_2O-buffer	58.61 ± 0.41	62.17 ± 0.13

decreased to only 35%. These results are indicating that deuterium oxide prevented temperature dependent loss of secondary structure significantly.

Differential scanning calorimetry study (DSC)

The DSC scans were executed with tubulin in both H_2O-buffer and D_2O-buffer to assess differences in their thermal unfolding behavior. Scans were accomplished in the temperature range 20 to 80°C. DSC thermograms (represented by the plot of heat capacity versus temperature) for tubulin in H_2O-buffer and D_2O-buffer are shown (Fig. 4C). Thermal unfolding reactions were found to irreversibly aggregate the protein at experimental pH values tested, thus we decided not to evaluate any thermodynamic parameters. The DSC profiles of tubulin were fitted using the non 2-state model. The raw data is represented by closed circles and the fit by lines in Fig. 4C. Each of the scans deconvoluted into two component peaks and the T_m for the component peaks were found to be on the higher side in case of the D_2O-buffer. Table 2 represents the T_{m1} and T_{m2} values for the individual scans. Even the first transition in the scans was also not reversible. In case of the aqueous system, scans executed till 56°C and in D_2O-buffer scans executed till 59°C, did not yield reversibility.

The melting temperature in D_2O-buffer means stronger affinity between α and β-tubulin subunits. This might be due to increased hydrophobic interaction between tubulin subunits and stronger deuterium bonding in tubulin in D_2O compare to hydrogen bonding in tubulin in H_2O. Stronger affinity between tubulin-subunits causes structural and conformational stability.

The mechanism of the effect of D_2O on protein stabilization is not well established. Some of the physical properties of D_2O and H_2O are different (2, 14). In the case of proteins, these difference in properties between H_2O and D_2O, leads to difference in hydration of the buried non-polar group due to solvent isotopic effect, causing a decrease in the enthalpy of unfolding (positive stabilization effect) and changes of entropy (negative stabilization effect) in D_2O (15). In the case of tubulin, it appears that the positive stabilization of the change of enthalpy is higher than the negative stabilization effect of the change of entropy resulting in net stabilization by D_2O. This may be attributed to solvent isotopic effect, which causes stronger hydrogen bond and greater hydrophobic interaction in D_2O. Deuterium oxide stabilizes microtubules from dilution-induced disassembly (16) and also promotes tubulin polymerization (9). Hydrophobic interactions that are more pronounced in D_2O may also play major role in stabilization of tubulin.

MATERIALS AND METHODS

Materials

Guanosine 5'-triphosphate (GTP), PIPES, $MgCl_2$, EGTA, D_2O, and DTNB, were purchased from SIGMA. Unless otherwise mentioned, the PEM (50 mM PIPES, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 6.9) was used as the experimental buffer. The pH was maintained at 6.9 in aqueous solvent and 7.3 [pD = pH + 0.4] in D_2O solvent (9). The concentration of D_2O was 95% in D_2O-buffer.

Purification of tubulin from goat brain

Tubulin was isolated from goat brain by two cycles of temperature-dependent assembly and disassembly in PEM buffer in the presence of 1 mM GTP, followed by two more cycles in 1 M glutamate buffer (17). The purified tubulin, free of MAPs was checked by 8% SDS-PAGE. Aliquots were flash-frozen in liquid nitrogen and stored at −70°C. The protein concentration was estimated by the method of Bradford (18) using bovine serum albumin as the standard.

Fluorescence spectroscopic studies

Fluorescence spectroscopic studies were done to detect the changes in the intrinsic protein fluorescence using a Hitachi F-3010 fluorescence spectrophotometer. Excitation was at 280 nm and wave length scan was done in at 300-450 nm, to monitor the emission maxima. The buffer systems used for the fluorescence spectroscopic studies were 20 mM H_2O-sodium phosphate (H_2O-NaPi) and 20 mM D_2O-sodium phosphate (D_2O-NaPi). Tubulin in both the buffers (final concentration 1 μM) was incubated at 4°C for 72 h. Spectroscopic data was collected at every 6 to 12 hours interval. This experiment was also performed at 37°C for 8 h and spectroscopic data were collected after 1 hour of time interval.

To study the effect of temperature, tubulin in both 20 mM H_2O-NaPi and 20 mM D_2O-NaPi (final concentration 1 μM), was incubated at various temperatures ranging from 25°C to 70°C for 30 minutes.

Circular dichroism studies

Circular dichroism studies were done on a Jasco J600 spectropolarimeter. Secondary structure was monitored in the 200-260 nm wavelength regions using a cell of path length 0.1 cm. A spectral bandwidth of 1 nm and a time constant of 2 s, with the scan speed of 50 nm/min, were used for these measurements. Tubulin in both H_2O-NaPi and 20 mM D_2O-NaPi (final concentration 1 μM) was incubated at 4°C for 72 hours and CD spectra was taken at an interval of 24 hours. All measurements were done at 25°C.

Change in the secondary structure of tubulin with the gradual increase of temperature, in both H_2O-NaPi and 20 mM 95% D_2O-NaPi (final concentration of tubulin 1 μM), were monitored in the 200-260 nm wavelength region using a cell of

path length 0.1 cm and the other instrumental parameters were kept unchanged. Tubulin, in both the buffers (final concentration 1 μM) was incubated at temperatures 25-80°C. Starting at 25°C, the temperature was increased by 5°C, and tubulin was incubated at each temperature for approximately 30 minutes.

Measurement of DTNB kinetics

Formation of thio-nitrobenzoate anion (TNB) by DTNB reaction with the free sulfhydryl groups was monitored by measuring absorbance at 412 nm (ε_{412} = 13,600 $M^{-1}cm^{-1}$ for TNB). All the experiments were carried out in 50 mM H_2O-PEM and 50 mM D_2O-PEM at 25°C. Absorbance was recorded continuously for 1 h, using a Perkins Elmer Spectrophotometer. Tubulin in both the buffers (final concentration 1 μM) was incubated at 37°C separately for 0-8 h, with intervals of 1 h. After the incubation, each protein sample was allowed to attain 25°C, and DTNB was mixed at a final concentration of 400 μM. In another set of experiment, tubulin in both 50 mM H_2O-PEM and 50 mM D_2O-PEM (final concentration 1 μM) were incubated at 4°C for 72 h and absorbance at 412 nm was monitored at an interval of 24 h.

Differential scanning calorimetry study

Thermal unfolding studies on the protein were made on the ultra-sensitive differential scanning calorimeter, VP-DSC (Microcal Inc.). The protein samples were dialyzed extensively with four changes of PEM buffer (1 : 500) at 4°C. The protein concentration used for all the experiments was 8 μM, unless otherwise mentioned. The protein sample and dialysates were filtered through 0.22-micron filter and degassed prior to loading the DSC cell. The sample cell and reference cell was introduced with the 0.5 ml of protein and dialysates buffer respectively. The calorimeter was up scanned at a constant rate of 60°C/hour. For the collection of data analysis the calorimeter is interfaced with microcomputer.

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