Detection of Disulfide Bonds in Bovine Brain Tubulin and Their Role in Protein Folding and Microtubule Assembly in Vitro: A Novel Disulfide Detection Approach†

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ABSTRACT: Cysteine residues in tubulin are actively involved in regulating ligand interactions and microtubule formation both in vivo and in vitro. These cysteine residues are sensitive reporters in determining the conformation of tubulin. Although some of the cysteines are critical in modulating drug binding and microtubule assembly, it is not clear how many of these normally exist as disulfides. The controversy regarding the disulfide bonds led us to develop a disulfide detection assay to reexamine the presence of the disulfide linkages in purified $\alpha\beta$ tubulin and explore their possible biological functions in vitro. The accessible cysteine residues in $\alpha\beta$ tubulin were alkylated with an excess of iodoacetamide to prevent artifactual generation of disulfide linkages in tubulin. After removal of excess iodoacetamide, tubulin was unfolded in 8 M urea. Half of the unfolded tubulin was treated with dithiothreitol to reduce any disulfide bonds present. The aliquots were then treated with iodo[^{14}C]acetamide and the incorporation of radioactivity was measured. We also used the same approach to detect the disulfide linkages in the tubulin in a whole-cell extract. We found in both cases that the samples which were not treated with dithiothreitol had little or no incorporation of iodo[^{14}C]acetamide, while the others that were treated with dithiothreitol had significant amounts of ^{14}C incorporation into tubulin. Moreover, the reduction of the disulfide linkages in tubulin resulted in inhibition of microtubule assembly (29−54%) and markedly affected refolding of the tubulin from both an intermediate and a completely unfolded state. All these data therefore suggest that tubulin has intrachain disulfide bonds in the α- and β-subunits and that these disulfides assist in correct refolding of tubulin from the intermediate unfolded state or help to recover the hydrophobic domains from the completely unfolded state. These disulfides also regulate microtubule assembly and the stability of tubulin in vitro. Our results suggest that tubulin disulfides may play a role in tubulin folding and that thiol−disulfide exchange in tubulin could be a key regulator in microtubule assembly and dynamics of tubulin in vivo.

Microtubules are organelles present in almost all eukaryotic cells. They are involved in a broad range of cellular functions such as cell shape maintenance, organelle transport, and cell division (*1*). Tubulin, the major structural component of microtubule, is a heterodimeric protein consisting of α- and β-subunits (*2*). Tubulin has 20 cysteine residues unequally distributed between the two subunits: 12 in α (*3*) and 8 in β (*4, 5*). These cysteine residues have proven to be remarkably sensitive markers for studying the interactions of different antimitotic drugs with tubulin (*6*). Certain compounds inhibit microtubule assembly and drug binding by interacting with particular cysteine residues in tubulin, and those sulfhydryl groups are considered as critical sulfhdryls for drug binding and microtubule poisoning (*6*). There has been a great deal of controversy regarding the disulfide bonds in tubulin. Lee et al. (*7*) detected two disulfide bonds in calf brain tubulin, while Eipper (*8*) found no disulfide linkage in rat brain tubulin. Ikeda and Steiner (*9*) reported two disulfide linkages per dimer in platelet tubulin prepared under strict anaerobic condition. Khan and Ludueña (*10*) proposed the presence of disulfides in tubulin on the basis of the ability of thioredoxin to regulate microtubule assembly in vitro. Correia et al. (*11*) reported that decay or denaturation caused artifactual formation of disulfides in tubulin. Other groups (*12*−*14*) also reported that the number of titratable sulfhydryl groups in tubulin varied in the presence of glycerol, suggesting that glycerol might initiate the formation of disulfide linkages in tubulin. Since the disulfide bonds in bovine brain tubulin purified by cycles of assembly and disassembly have never been detected or measured, we decided to search for intrinsic disulfide bonds in bovine brain tubulin using a novel disulfide detection approach; we also explored their possible functions in vitro. As there was always a possibility of forming artifactual disulfide linkages in tubulin during the unfolding process, we homogenized the bovine brain cerebra with excess iodoacetamide to modify the free cysteine residues of proteins so that the cysteines of all proteins, including tubulin, could not be involved in disulfide bonds during

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unfolding. Following ammonium sulfate fractionations, ion-exchange chromatography, and denaturation, half of the aliquots of tubulin containing tubulin-rich fractions were treated with DTT[1] to reduce any disulfide bonds. Excess iodo[^{14}C]acetamide was then added to alkylate the cysteine residues that were involved in disulfide linkages. With this disulfide detection approach, we found that all the fractions that were not treated with DTT had little incorporation of ^{14}C, while the fractions treated with DTT had significant amounts. These data therefore, suggested that tubulin possesses at least one intrachain disulfide linkage in both the α- and β-subunits. Using the same detection approach, we also measured the disulfides in purified $\alpha\beta$ tubulin and found 1−2 disulfide bonds; we observed that these disulfides regulate microtubule assembly markedly. These disulfides were also partially responsible for correct refolding from the unfolding state. Our results therefore, suggest that the oxidized state of tubulin could be the right candidate for specific chaperone-assisted refolding and that the oxidation/reduction status of the cell also could be a critical factor in regulating microtubule assembly and dynamics in vivo.

EXPERIMENTAL PROCEDURES (MATERIALS AND METHODS)

Materials. Iodoacetamide and dithiothreitol were purchased from Sigma (St. Louis, MO). BisANS was from Molecular Probes (Junction City, OR). Iodo[^{14}C]acetamide and [^{3}H]colchicine were purchased from American Radiochemicals (St. Louis, MO). Amplify reagent and Hyperfilm MP were from Amersham Life Science (Piscataway, NJ). Sources of all other materials were as previously described (*15*).

Tubulin Preparation. Microtubules were purified from bovine cerebra by a cycle of assembly and disassembly, and the tubulin was purified therefrom by phosphocellulose chromatography (*16*). Experiments were performed in Mes buffer (100 mM Mes, pH 6.4, 0.5 mM $MgCl_2$ and 1 mM EGTA).

Sulfhydryl Group Modifications. The phosphocellulose-purified tubulin was reacted at 37 °C for 30 min with excess cold iodoacetamide to prevent artificial formation of disulfide linkages. Samples were then dialyzed against buffer containing 8 M urea. Half of the sample was treated with DTT to reduce the disulfide bonds, if present. Then the samples were treated with iodo[^{14}C]acetamide and incubated at 37 °C for another 30 min in the dark. After the reactions, the samples were precipitated with 10% trichloroacetic acid, the precipitates were collected by filtration, and the radioactivity of the filters was determined (*17*).

For autoradiography, the samples were run on a 6% polyacrylamide gel containing 0.1% SDS (*18*). After that, the gel was stained with Coomassie blue and destained with 5% methanol and 7.5% acetic acid. The same gel was also treated with amplifying solution for 20 min at room temperature with constant stirring. The gel was then dried in a Bio-Rad gel drier, model 583, followed by exposure to high-performance autoradiography film at −80 °C for 10−15 days.

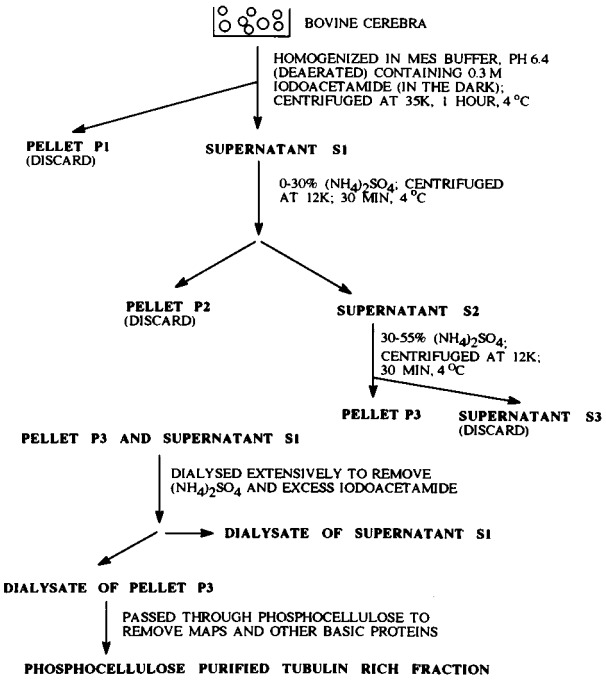

FIGURE 1: Schematic diagram of the disulfide detection assay. This diagram indicates how the disulfide detection method could be applied to determine the intrinsic disulfide bonds in a protein such as tubulin in a whole-cell extract.

Disulfide Detection Assay. To detect the intrinsic disulfide linkages in the tubulin of whole-cell extracts, we treated the bovine cerebra as shown in Figure 1. Cerebra were homogenized in deaerated Mes buffer containing 300 mM cold iodoacetamide and incubated in ice for 30 min in the dark. After that, the homogenate was centrifuged at 4 °C at 35K rpm for 1 h. The pellet P1 was discarded. The supernatant S1 was carefully removed and was subjected to 0−30% ammonium sulfate precipitation followed by centrifugation at 12K rpm for 30 min at 4 °C. The pellet P2 was discarded and the supernatant S2 was again subjected to 30−55% ammonium sulfate precipitation and was centrifuged at 12K rpm for 30 min at 4 °C. The supernatant S3 was discarded. The pellet P3 (obtained from the 30−55% ammonium sulfate precipitation) and the supernatant S1 were dialyzed extensively to remove ammonium sulfate and excess iodoacetamide. After that, the dialysate of pellet P3 was passed through a phosphocellulose column to remove microtubule-associated proteins and other basic proteins. The unbound fraction, which contains mainly tubulin, was termed phophocellulose-purified tubulin-rich fraction. Then, the dialyzed supernatant S1, the dialysate of the pellet P3, and the phophocellulose-purified tubulin-rich fraction were precipitated with chilled acetone [tubulin:acetone = 1:5 (v/v)] and the precipitates were centrifuged at 12K rpm for 30 min. The precipitates of the dialyzed supernatant S1, the dialyzed pellet P3, and the phophocellulose-purified tubulin-rich fraction were dissolved in Mes buffer, pH 8.6, containing 8 M urea and the

[1] Abbreviations: bisANS, bis-5,5′-[8-(*N*-phenyl)aminonaphthalene-1-sulfonic acid]; DMSO, dimethyl sulfoxide; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

protein concentrations of all of the samples were measured. Each of the samples was divided into two aliquots, one of which was treated with 0.1 mM DTT. All of the samples were then treated with iodo[^{14}C]acetamide (11.94 dpm/pmol) to a final concentration of 0.2 mM and incubated in the dark at 37 °C for 30 min. The samples were subjected to electrophoresis on a 10% polyacrylamide gel in the presence of 0.1% SDS. After that, the gel was soaked in amplifying solution for 20 min at room temperature with constant stirring. The gel was then dried and autoradiographed.

Fluorescence. Aliquots of tubulin containing different concentrations of urea were incubated at 37 °C for 30 min in the presence or absence of DTT. The samples were then made 10 μM in BisANS and the fluorescence was measured in a Hitachi F-2000 spectrofluorometer. Excitation and emission were at 385 and 490 nm, respectively. For the tryptophan fluorescence assay, the fluorescence of all the samples was measured at 335 nm. Excitation was at 296 nm. All the fluorescence values were corrected for the inner-filter effect according to Lakowicz (*19*).

Sedimentation. Tubulin samples in assembly buffer containing 4 M glycerol, 6 mM $MgCl_2$, and 1 mM GTP were incubated with or without 1 mM DTT for 40 min at 37 °C. The samples were then centrifuged at room temperature for 5 min at 110000*g* in a Beckman airfuge. The pellets were resuspended in 0.1 N NaOH and the concentration of protein was determined.

Other Methods. The binding of [^{3}H]colchicine to tubulin was measured by filtration through DEAE-cellulose filters. The temperature and the time of incubation were, respectively, 37 °C and 1 h. Protein concentrations were determined by a modified procedure of Lowry et al. (*20*) with bovine serum albumin as a standard (*21*).

Circular dichroism studies were done on an Olis DSM 16 UV/vis CD spectrophotometer (On Line Instrument Systems, Inc.). The near-UV (250−310 nm) and far-UV (215−250 nm) regions were scanned in a 10-mm path (3 mL volume) cell. The protein concentrations were 1 μM. The DTT concentration was 0.1 mM. Appropriate blanks were subtracted from the observed spectra.

RESULTS

As we were interested in tracking natural disulfide linkages in the tubulin molecule and determining their possible functions in vitro, we first studied urea-induced unfolding of tubulin in the presence or absence of DTT, a reducing agent that cleaves disulfide linkages in proteins. Since the tryptophan residues and the hydrophobic domains are sensitive reporters in determining the conformation of proteins, we used these probes to study the role of disulfides in tubulin. Figure 2A showed that the fluorescence intensity ratio (I_{350}/I_{335}) increased with increasing concentrations of urea (0−4 M). Interestingly, that ratio (I_{350}/I_{335}) increased more when the same samples were treated with 0.1 mM DTT. Moreover, the effect of DTT on the ratio was more pronounced when the urea concentration was >2 M. The same observation was made when the λ_{max} of tubulin at different urea concentrations was measured in the presence or absence of 0.1 mM DTT (Figure 2B). The λ_{max} of tubulin was red-shifted (from 336 to 339−340 nm) with increasing concentrations of urea; the change in wavelength was about 3−4 nm. However, in the presence of DTT, the change in λ_{max} was about 7−12 nm (from 335 to 342−347 nm). As urea exposes the hydrophobic pockets of protein during the unfolding process and BisANS binds to the hydrophobic regions, giving a fluorescence maximum at 490 nm, we used BisANS as a probe to study the effect of DTT on urea-induced unfolding of tubulin (Figure 2C). We found that the fluorescence of the tubulin samples not treated with urea but treated with DTT was higher than the fluorescence of samples not treated with DTT.

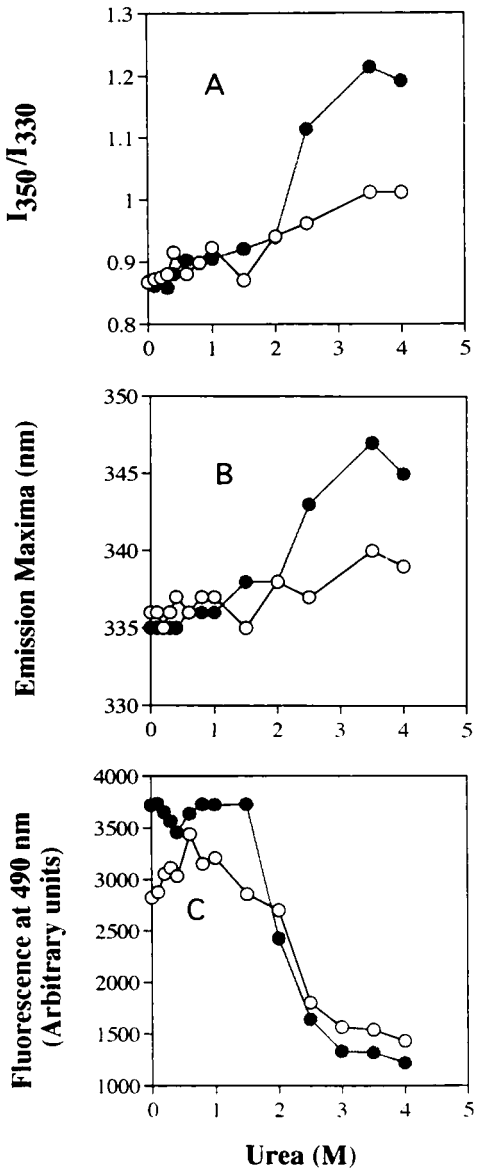

FIGURE 2: Effect of DTT on tryptophan and BisANS fluorescence in urea-induced unfolding of tubulin. (A) Aliquots of tubulin (1 mL of 1 μM) were treated with different concentrations of urea (0−4 M) and incubated at room temperature for 30 min. After that, each sample was divided into two aliquots; one was treated with DTT to a final concentration of 100 μM (●) and the other was treated with an equal volume of buffer (○). All the samples were then incubated at 37 °C for another 30 min. After the incubation, the fluorescence of all the samples was measured at 335 and 350 nm. The excitation wavelength was 296 nm. For panel B, everything was the same as for panel A except that the wavelength that gave maximum fluorescence at different unfolding conditions was measured. For panel C, everything was the same as for panel A except that each sample was mixed with BisANS (10 μM) and the fluorescence was measured at 490 nm. The excitation wavelength was 385 nm.

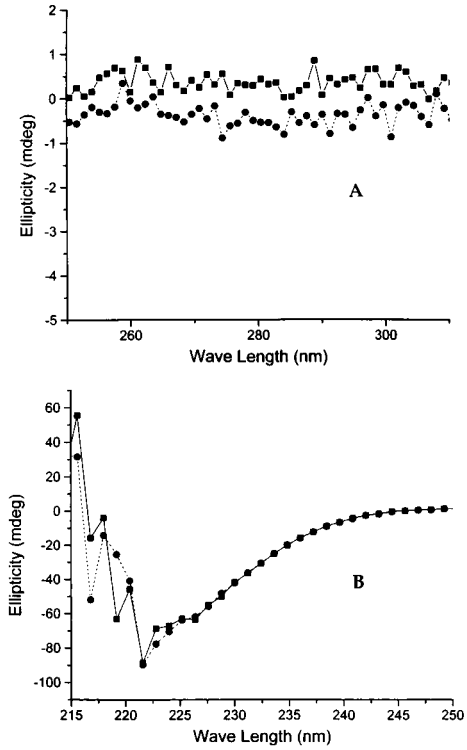

FIGURE 3: Effect of DTT on near- and far-UV CD spectra of tubulin. Tubulin (1 μM) samples (3 mL) were treated with or without DTT (500 μM) and incubated at 37 °C for 30 min followed by scanning of the CD spectra in the near-UV (250−310 nm) (A) and far-UV (215−250 nm) (B) regions. (●) DTT-treated samples; (■) untreated samples.

The significant differences in fluorescence between DTT-treated and untreated samples were observed up to 1.5 M urea concentration; at concentrations of 2 M or higher, the differences were markedly reduced.

To explore further the effect of DTT on the secondary and tertiary structures of the GTP-depleted native tubulin, we studied the negative ellipticity of tubulin in the near- and far-UV regions in the presence or absence of DTT (Figure 3). We found that the effect of DTT in the near-UV region was almost or entirely indistinguishable (Figure 3A) from the DTT-untreated samples. The typical U-shaped pattern of negative ellipticity of tubulin was not detected between 250 and 310 nm. A smaller effect was consistently noticed in the far-UV region (Figure 3B). The effect of DTT was greatest in the 219−220 nm region as the negative ellipticity of the DTT-treated tubulin was decreased by 10−60%; at 220.4 nm, the decrease in ellipticity by DTT was 10%. The data for far- and near-UV regions were the average of three separate experiments.

As DTT causes significant effects on the conformation of tubulin (Figures 2 and 3), it is conceivable that tubulin might have disulfide linkages that play a significant role in the structure of tubulin. However, to detect the intrinsic disulfide linkages in tubulin in the whole-cell extract, we developed a disulfide detection approach to find the disulfide bonds. The whole method is described under Materials and Methods and is schematically outlined in Figure 1.

Since tubulin contains 20 cysteine residues distributed between the α- and β-subunits, we added enough iodoacetamide to the deaerated buffer so that the accessible cysteines of all of the proteins, including tubulin, would be

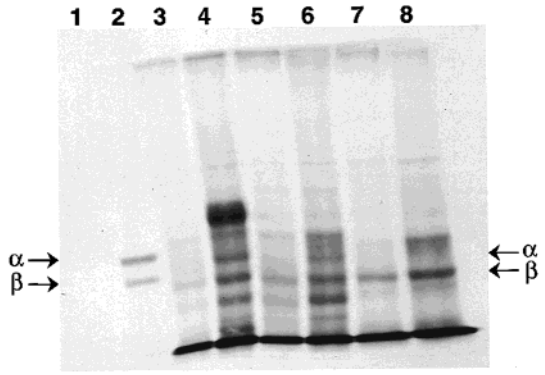

FIGURE 4: Detection of intrinsic disulfide linkages in the tubulin in whole-cell extract. Sample preparation was described under Materials and Methods and schematized in Figure 1. However, after acetone precipitation, the precipitates of all the fractions (cell extract, 30−55% ammonium sulfate fraction, and phosphocellulose-unbound fraction) were dissolved in Mes buffer, pH 8.6, containing 8 M urea, and the protein concentration was determined. Each of the fractions (300 μL; 1 mg/mL) was divided into two aliquots; one was treated with 0.1 mM DTT and the other was treated with an equal volume of buffer and incubated at 37 °C for 30 min. After that, all the fractions were made 500 μM in iodo[^{14}C]acetamide (11.94 dpm/pmol) and incubated at 37 °C for another 30 min. The samples were then subjected to electrophoresis on a 10% polyacrylamide gel in the presence of 0.1% SDS (*34*), followed by treatment of the gel in amplifying solution for fluorography. The exposure time to the autoradiography film was 15 days. The samples were treated as follows: lane 1, 15 μg of tubulin; lane 2, 15 μg of DTT-treated tubulin; lane 3, 100 μg of dialysate of supernatant S1; lane 4, 100 μg of dialysate of supernatant S1 + DTT; lane 5, 100 μg of the dialysate of pellet P3; lane 6, 100 μg of the dialysate of pellet P3 + DTT; lane 7, 50 μg of phosphocellulose purified tubulin-rich fraction; lane 8, 50 μg of phosphocellulose-purified tubulin-rich fraction + DTT.

irreversibly modified during homogenization of bovine cerebra and would therefore be unable to form either intra- or intermolecular artificial disulfide bonds. The supernatant S1, obtained after centrifugation of the homogenate, was fractionated with 0−30% ammonium sulfate to discard the unnecessary proteins (P2) as well as to obtain a 30−55% ammonium sulfate pellet from the supernatant S2 (0−30% ammonium sulfate supernatant). Therefore, we fractionated the supernatant S2 with ammonium sulfate (30−55%) to prepare a tubulin-containing fraction (dialysate of pellet P3) that was later purified by phosphocellulose chromatography. All the fractions (supernatant S1, dialysate of pellet P3, and phosphocellulose-purified tubulin-rich fraction) were denatured in 8 M urea and were dialyzed extensively to remove excess ammonium sulfate and iodoacetamide. Half of each fraction was treated with 0.1 mM DTT to reduce the disulfide linkages. To all the samples, iodo[^{14}C]acetamide was added to modify the free cysteines that were involved in disulfide bonds. Since the free sulfhydryl groups were premodified with cold iodoacetamide, there was relatively little incorporation of ^{14}C into the protein samples not treated with DTT (Figure 4, lanes 1, 3, 5, and 7). The fractions treated with DTT had significant amounts of incorporation of ^{14}C (Figure 4, lanes 2, 4, 6, and 8).

We also used the same disulfide detection assay to determine the disulfide bonds in purified tubulin. We found that tubulin samples not treated with DTT had little incorporation of ^{14}C iodoacetamide and had faster mobility in SDS−polyacrylamide gel electrophoresis (Figure 5, lane

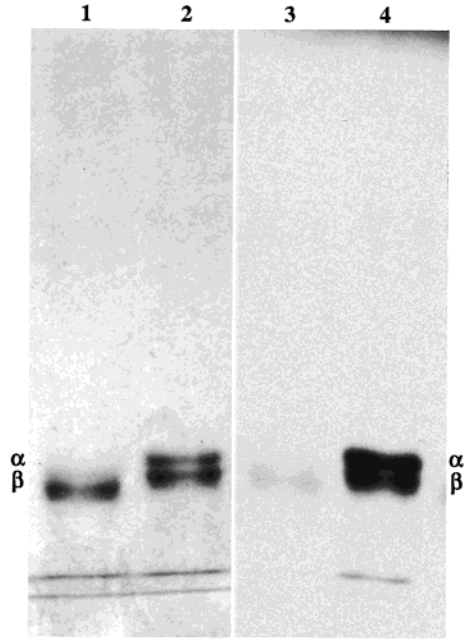

FIGURE 5: Detection of disulfide linkages in purified tubulin. Conditions were as in Figure 4 except that the sample was purified tubulin instead of whole-cell extract and the percentage of acrylamide used in gel electrophoresis was 6% instead of 10%. The gel was stained with Coomassie blue, destained with a mixture of methanol and acetic acid, and treated with amplifying solution for fluorography. Lanes 1 and 2 show the Coomassie blue-stained gel, and lanes 3 and 4 show the same gel that was fluorographed. The exposure time to autoradiography was 10 days. The samples used in gel electrophoresis were as follows: lanes 1 and 3, 15 μg of tubulin; lanes 2 and 4, 15 μg of tubulin + DTT.

Table 1: Effect of DTT on the Incorporation of Iodo[^{14}C]acetamide into Tubulin[a]

reactions	mol of ^{14}C/mol of tubulin
tubulin	0.036 ± 0.008
tubulin + 0.1 mM DTT	2.820 ± 0.042

[a] Duplicate sets (1 mL) of $\alpha\beta$ tubulin (5 μM) were incubated in the presence of 5 mM iodoacetamide at 37 °C for 30 min in the dark, followed by extensive dialysis in the presence of 8 M urea at room temperature. After that, half of each sample was treated with 0.1 mM DTT and the other half with an equal volume of buffer, and all the samples were incubated for another 30 min at 37 °C. After the incubation was over, iodo[^{14}C]acetamide (1.194 dpm/pmol) was added to each sample to a final concentration of 500 μM and the incubation at 37 °C was continued for another 30 min in the dark. Incorporation of ^{14}C was measured as described under Materials and Methods. Standard deviations are shown.

3) while the sample treated with DTT had significant amounts of incorporation of ^{14}C in both α- and β-tubulins (Figure 5, lane 4). There was no high molecular weight polymer or complex of tubulin detected in the DTT-untreated tubulin sample (Figure 5, lane 1). Moreover, Table 1 supported the idea that the tubulin had natural disulfide bonds as there were approximately three sulfhydryl groups per tubulin dimer detected by treatment with DTT.

As disulfides play an important role in the conformation of tubulin, it is conceivable that they could have significant effects on the formation of microtubules. This was found to be the case. We found that the reduction of the disulfide linkages of tubulin by DTT caused significant inhibition of microtubule assembly (29−53%) in vitro as determined by the sedimentation assay (Table 2).

Table 2: Effect of DTT on the Polymerization of Tubulin[a]

set	condition	polymer mass (mg/mL)	% inhibition of assembly
1	−DTT	0.847	29
	+ DTT	0.599	
2	−DTT	1.455	38
	+ DTT	0.9023	
3	−DTT	2.204	54
	+ DTT	1.008	

[a] Tubulin in 250 μL of Mes buffer containing 1 mM GTP was incubated for 15 min at 37 °C in the presence or absence of 1 mM DTT; samples were then mixed with another 250 μL of Mes buffer containing 8 M glycerol, 12 mM $MgCl_2$, and 1 mM GTP. The mixture was incubated for an additional 40 min at 37 °C and then spun for 5 min at 110000*g* in a Beckman airfuge. The pellet was solubilized in 108 μL of 0.1 N NaOH and its protein content was measured by the method of Lowry et al. (*20*). The percent inhibition was calculated by considering the polymer mass of the untreated tubulin as 100%. Sets 1, 2, and 3 represent three independent experiments.

Guha and Bhattacharyya reported the formation during refolding of a stable intermediate state of tubulin by 2 M urea (*22*) and recovery of the biological activity of tubulin during refolding from the stable intermediate (*23*). We therefore examined the effect of DTT on the recovery of the nonfunctional higher-order structure or the hydrophobic domains of tubulin from the complete unfolded state in vitro (Figure 6) and also studied the effect of DTT on the recovery of the native structure of tubulin in vitro by refolding from the stable intermediate state (Figure 7). In the experiment shown in Figure 6, we used tryptophan and BisANS as markers to study the refolding process after denaturation in 8 M urea. Refolding was initiated from 8 M urea by 20-fold dilution of the denatured protein in buffer, and the final concentration of urea indicated in Figure 6 was adjusted. The tubulin concentration in all the samples was 1 μM. Figure 6A shows that the recovery of tryptophan fluorescence intensity increased as the urea concentrations decreased. The difference in tryptophan fluorescence between DTT-untreated and -treated tubulin at any particular urea concentration was significant. For example, in the case of 0.4 M urea, 14% less fluorescence was recovered when the tubulin was treated with 0.1 mM DTT. In the 0.4−4.0 M urea range, DTT inhibited recovery of tryptophan fluorescence by 14.0% ± 1.8%. However, the same observation was made when BisANS was used as a probe to study the recovery of the hydrophobic areas from the unfolded state (Figure 6B). At any particular concentration, for example at 0.4 M urea, 9% less fluorescence was recovered when the tubulin sample was treated with 0.1 mM DTT. In the 0.4−4.0 M urea range, DTT inhibited recovery of BisANS fluorescence by 8.6% ± 1.8%.

We also looked at the effects of DTT on the extent of recovery of functionally active tubulin structures from the stable intermediate state using tryptophan and [^{3}H]colchicine as probes (Figure 7). We found exactly the same phenomenon that we found in the recovery of the nonfunctional intermediate structures from the completely unfolded state (Figure 6). At any particular urea concentration, the recovery of tryptophan fluorescence by the DTT-treated sample was always less than the recovery of fluorescence gained by the DTT-untreated sample (Figure 7A). In the 0.4−2.0 M urea range, DTT inhibited recovery of tryptophan fluorescence by 8.0% ± 2.3%. This observation was also consistent with

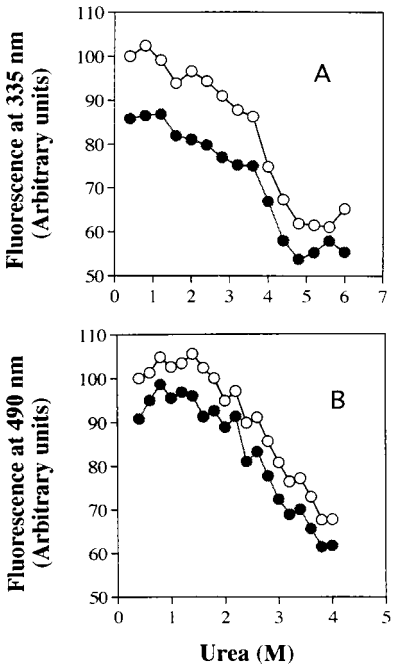

FIGURE 6: Effect of DTT on change in tryptophan and BisANS fluorescence of refolded tubulin after denaturation in 8 M urea. Refolding was initiated from 8 M urea by a 20-fold dilution of the denatured protein in buffer with (●) or without (○) DTT (100 μM), and the final concentration of urea was adjusted to the values indicated in the figure. The final concentration of tubulin was 1 μM in all cases. For panel A, the tryptophan fluorescence (335 nm) was measured. The excitation wavelength was 296 nm. For panel B, BisANS fluorescence (490 nm) was measured when the excitation wavelength was 385 nm.

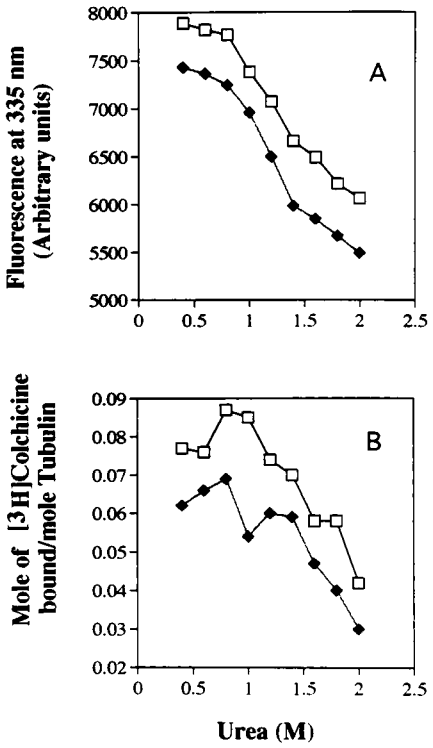

FIGURE 7: Effect of DTT on tryptophan fluorescence and colchicine binding activity of refolded tubulin after denaturation with urea. The urea concentrations used to unfold tubulin are shown in the figure. After unfolding, tubulin was refolded by a 10-fold dilution from the respective urea concentration in the presence (◆) and absence (□) of 0.1 mM DTT and incubated at 37 °C for 2 h. The final concentration of urea was adjusted to 0.2 M in each case. For tubulin, the final concentration was 1 μM. For panel A, the samples were excited at 296 nm and the fluorescence of each sample was measured at 335 nm. For panel B, the refolded tubulin samples were incubated with 10 μM [^{3}H]colchicine (44.4 dpm/pmol) at 37 °C for 1 h. The binding of colchicine to tubulin was measured by filtration on DEAE-cellulose.

the drug binding assay when [^{3}H]colchicine binding was used as a probe to study the role of the disulfide linkages in the recovery of the functional structure of tubulin from a stable intermediate unfolded state. We found that drug binding decreased by 20−30% in the presence of 0.1 mM DTT.

DISCUSSION

Cysteines in tubulin generally are critical residues in modulating conformation, ligand binding, and physiological functions both in vivo and in vitro (*6*). However, as tubulin is a multicysteine protein, it is conceivable that some of the critical cysteines in tubulin could be involved in disulfide bonds and engage in thiol−disulfide exchange: it has frequently been observed that oxidation of the sulfhydryl groups of tubulin affects the binding of drugs and microtubule assembly (*6*). There has been a great deal of controversy regarding the presence of intrinsic disulfide bonds in tubulin (*7*−*14*). On one hand, Eipper (*8*) searched for disulfide bonds in tubulin and did not find any, and there are no disulfides in the published three-dimensional structure of tubulin (*33*). On the other hand, Lee et al. (*7*) found two disulfides in calf brain tubulin. However, this tubulin was prepared in aerobic conditions, which could favor the artifactual formation of disulfides. Ikeda and Steiner (*9*) found that platelet tubulin prepared under anaerobic conditions contains two disulfide bonds. However, we now know that platelet β-tubulin consists of the unusual β_{VI} isoform, one apparently restricted to hematopoietic tissues, that has cysteines not present in the other β isoform (*24*). Nevertheless, the results of Lee et al. (*7*) and Ikeda and Steiner (*9*) raise the possibility that disulfides can exist in tubulin. In fact, Khan and Ludueña (*10*) demonstrated the likehood of disulfides in bovine brain tubulin. It is possible that the precise conditions of the tubulin preparation procedure used could affect the detectability of disulfide bridges. For example, the pH, temperature, and buffer components could all conceivably influence the likehood of formation of disulfide bonds in tubulin during the purification. The same argument could be made for the duration of the time intervals between the death of an animal, the removal of its brain, and the commencement of the tubulin isolation procedure, such time intervals are often not reported in the literature. Similarly, many procedures used in tubulin preparation could conceivably discriminate between populations of tubulin containing disulfide bonds and populations lacking them. This is much more likely to happen to tubulin than to many other proteins since tubulin has a highly labile conformation (*6*), and as we have shown here, the disulfides can significantly affect that conformation. It is quite reasonable to postulate, therefore, that the presence of a particular disulfide bond in a tubulin molecule may determine whether that molecule will bind to an ion exchanger such as DEAE-cellulose or phosphocellulose or whether it will polymerize with other tubulin molecules into a microtubule. In any isolation procedure, a considerable fraction of tubulin is likely to be lost. In certain procedures, therefore, that fraction could consist of those molecules

lacking disulfides; in others, it would consist of those molecules containing disulfides. Our objective in this work has been to search for "natural" disulfides and to examine the effect of these disulfides on the conformation of tubulin.

As disulfide bonds generally impart structural stability to proteins, we unfolded tubulin with different concentrations of urea to study the effect of DTT on the structure. From Figure 2, it appears that the tubulin has intrinsic disulfide linkages that maintain the secondary structure during unfolding and impart stability to the tubulin molecule. With the reduction of the disulfide bonds, tubulin loses its residual structure that was maintained by the disulfide bonds even in the presence of urea. For that reason, the ratio of fluorescence intensity (I_{350}/I_{335}) was increased, the λ_{max} was red-shifted, and the BisANS fluorescence was greater in the DTT-treated sample than in the untreated one, especially at low urea concentrations (below 1.5 M); unfolding with higher concentrations of urea (>2 M) perturbed the secondary structure of tubulin in such a way that most of the hydrophobic domains were exposed and disorganized. Because of that, disulfides in the tubulin could not protect the hydrophobic domains under those stringent conditions and thus, the BisANS fluorescence differences between DTT-treated and untreated samples at higher urea concentrations were small.

We also studied the effect of DTT on the negative ellipticity of tubulin in the near- and far-UV regions by CD-spectrum analysis (Figure 3). It appeared that the addition of DTT to the native tubulin made a subtle local conformational change by affecting the secondary structure of tubulin, as shown by the observation that the negative ellipticity decreased in the far-UV region (Figure 3B). This effect, however, was not reflected in the near-UV region, in which the change in ellipticity induced by DTT was not significant (Figure 3A). Moreover, the typical pattern of the negative ellipticity of tubulin (U-shaped) was not observed at 1 μM tubulin concentration. At higher concentrations of tubulin (20 μM), the typical pattern of ellipticity in the near-UV region was observed, but there was no significant effect of DTT (data not shown). However, it may be possible that the reduction of disulfide linkages affected a certain domain of tubulin in such a way that a change in magnitude was not detectable in the near-UV region by CD spectroscopy, although other data (Figure 2) and the far-UV data (Figure 3 B) suggested that the disulfide bonds in tubulin affected the conformation of tubulin.

Disulfide detection (Figures 4 and 5) assays confirmed that tubulin had at least two intrinsic disulfide bonds distributed between the α- and β-subunits. These were intrasubunit bonds as shown by the absence of high molecular weight complexes in DTT-untreated samples (Figure 5, lane 1). We detected some incorporation of ^{14}C into the DTT-untreated sample, especially into the tubulin (Figure 4, lanes 1, 3, 5, and 7; Figure 5, lane 3). As tubulin exists as numerous isoforms (*25*), differing in their amino acid sequences (*26*), and since the $\alpha\beta_{III}$ tubulin isoform is conformationally and structurally more rigid than $\alpha\beta_{II}$ (*27*), the small incorporation that was detected, especially in the dialysate of pellet P3, the phosphocellulose-purified tubulin-rich fractions (Figure 4, lanes 5 and 7) and in purified tubulin (Figure 5, lane 3) were probably due to partial modification of the cysteines of $\alpha\beta_{III}$ by iodoacetamide in unfractionated tubulin. As a result, when tubulin is unfolded, the inaccessible cysteines of $\alpha\beta_{III}$ are exposed and react with iodo[^{14}C]acetamide. Although it is highly probable that tubulin contains disulfide bonds in vivo, we cannot rule out the possibility that disulfide bonds form extremely rapidly when the brain is homogenized. Even assuming the latter, namely, that these disulfide bonds were artifactual, our data indicated that at least four cysteine residues in both α- and β-subunits were in close proximity to form disulfide bonds during tubulin isolation and implied that their presence must be considered when evaluating any results obtained with tubulin in vitro, including studies on ligand binding, GTPase activity, microtubule assembly, and microtubule dynamics.

As certain sulfhydryl groups were assembly-critical in vivo (*6*), it is possible that formation of disulfide bridges involving assembly-critical sulfhydryl groups could freeze the conformation of a part of the tubulin molecule in such a way as to affect microtubule assembly. Moreover, thioredoxin and thioredoxin reductase are associated with microtubules in vivo (*28*) and may regulate microtubule assembly in vitro (*10*). Also, the assembly/disassembly process has been linked with a rise in the levels of oxidized glutathione and protein−glutathione complex (*29*). Therefore, we might speculate that the extent of microtubule assembly could be modulated by changing the sulfhydryl/disulfide status of the cell via oxidation or reduction.

Table 1 shows that there are approximately three sulfhydryl groups per tubulin dimer exposed by treatment with DTT. This would correspond to between one and two disulfide bonds. At first glance, one might expect an even number of sulfhydryl groups, since each disulfide bond, when reduced by DTT, should yield two sulfhydryl groups. However, we must recall that brain tubulin consists of a mixture of isoforms and that these might differ in their susceptibility to oxidation both in vivo and during purification. Also, since tubulin is known to be subject to a large variety of posttranslational modifications (*30*), it is possible that only a fraction of the population of tubulin molecules contains disulfide bonds. Nevertheless, the results shown in Figures 4 and 5 clearly indicate that tubulin contains disulfide bonds. Hence, there must be at least one disulfide bond in each subunit, even if the yield is fractional.

As reduction of the disulfide linkages plays an important role in proper refolding (*31, 32*) and causes significant inhibition of microtubule assembly (29−53%) in vitro (Table 2), it is possible that cleavage of the disulfide bonds affects the structure of tubulin in such a way that the reduced or partially relaxed form of tubulin detected by CD and other techniques was not the right conformation to participate in normal microtubule assembly. It appeared that the compact form of the structure could be one of the key regulators in modulating microtubule assembly.

The refolding study (Figures 6 and 7) showed clearly that DTT affects the refolding process significantly (10−15%) and that at any urea concentration the recovery of higher-order structure was inhibited by DTT. These data, therefore, suggest that the cysteine residues involved in disulfide bonds are located at a certain region in the tubulin molecule that plays a critical role in forming a fully compact functional form. Although the effect of reduction of disulfide bonds is not so severe that tubulin cannot attain its higher-order structure, the effect is nevertheless sufficient to inhibit

significantly the refolding process and the formation of the microtubule lattice. Moreover, as each isoform differs structurally and functionally from the others, and since the tubulin samples that we used for our study were a mixture of isoforms, it is possible that the effect of DTT on the different isoforms was different because of unequal distribution of the disulfide bonds among the isoforms.

Although are reports that the GroEL−GroES complex can assist in the refolding of tubulin (*34*−*36*), recent work (*37*) indicates that there is a group of specific chaperonins and cofactors that are required for the proper folding of the $\alpha\beta$ tubulin dimer. These chaperonins and cofactors apparently form a quasi-complex with the α and β polypeptides and then release the $\alpha\beta$ heterodimer in its native conformation (*37*). Our results indicate that disulfides facilitate proper refolding of tubulin in vitro and suggest that disulfide bonds may exist in vivo. We can speculate, therefore, that disulfides play a role in chaperonin- and cofactor-assisted refolding in vivo and that pathological states in which the oxidation status of a cell is altered may seriously affect tubulin folding and therefore microtubule assembly and dynamics.

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