Refolding of Urea-Denatured Tubulin: Recovery of Nativelike Structure and Colchicine Binding Activity from Partly Unfolded States

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ABSTRACT: Tubulin unfolding in urea proceeds via the formation of a partially unfolded intermediate state, stable in 2 M urea, that unfolds further in higher urea concentrations. The intermediate state had spectroscopic properties reminiscent of a molten globule and negligible colchicine binding activity. Refolding of totally unfolded tubulin in 8 M urea yielded an intermediatelike state characterized by partial burial of tryptophans and partial recovery of secondary and tertiary structures, although colchicine-binding activity of the protein was not regained. Further folding of this intermediatelike state, toward the native conformation, with respect to both structural and functional parameters did not occur. However, a significant percentage of colchicine binding activity and nativelike tertiary structure was recovered when refolding was initiated from partially denatured protein samples, viz., from <1.2 M urea. Thus, although high concentration of urea induced loss of structure and activity was irreversible, the conformational changes induced in restricted regions of tubulin by lower concentrations of urea, which are probably crucial for its various functional properties, could be reversed.

Refolding of denatured polypeptide chains in vitro has provided major insight toward understanding the problem of protein folding. Current studies indicate that refolding is a fast and cooperative process whereby a "random coil" structure initially folds to one or more intermediate states, possessing varied degree of structure prior to formation of native structure (*1*−*6*). The folding problem adds another dimension when the protein is large and multimeric. In such cases, both chain folding and chain association steps will be required for formation of native structure (*7*−*11*). Early stages in the reconstitution pathway of such systems are presumably similar to those involved in refolding of monomeric proteins, viz., independent folding of domains and subunits occur yielding "structured monomers", which finally undergo association and further folding to the native state (*8*, *12*). These two processes must be properly coordinated, otherwise unique specificity of a folded protein may not be achieved (*13*). Like unfolding of monomeric proteins, the dissociation and unfolding of multidomain and multimeric proteins has been shown to be reversible under optimum conditions of denaturation and renaturation (*8*, *12*, *14*−*19*). However, in some cases, reassociation competes with abortive side reactions like aggregation of partly folded intermediates, resulting in "irreversibly denatured", high molecular weight aggregates and only a partial recovery of structure and activity (*13*, *20*−*30*). Concentration-dependent reactivation of such systems is often seen.

The 100-kDa cytoskeletal protein tubulin is a noncovalent dimer of two related but nonidentical subunits. Previously it was reported from this laboratory (*31*) that the unfolding of tubulin involves the formation of a molten globule-like intermediate state near 2 M urea that underwent further unfolding in stronger denaturing conditions. This intermediate state had partially hydrated tryptophan residues, a significant amount of secondary structure, and negligible tertiary structure compared to the native state. It was of interest to find out whether this intermediate possesses any biological activity and whether the native state of tubulin can be reattained by in vitro refolding of its unfolded states. Our present observations aim at answering the above queries.

EXPERIMENTAL PROCEDURES

Materials. PIPES,[1] EGTA, and GTP for purification of tubulin were obtained from Sigma Chemical Co. Urea was from Aldrich Chemical Co., and stock solutions of urea were prepared freshly on the day of use. Acrylodan was obtained from Molecular Probes. Its concentration was checked spectrophotometrically using an extinction coefficient of 20 000 M^{-1} cm^{-1} at 391 nm in methanol. Radioactive colchicine ([^{3}H] ring C methoxy) with specific activity of 70 Ci/mmol was a product of New England Nuclear Corp., while DE 81 filter papers were obtained from Whatman. All other chemicals used were of reagent grade.

Purification of Tubulin. Tubulin was isolated from goat brain by two cycles of temperature- and GTP-dependent assembly and disassembly in buffer containing 50 mM PIPES, 1 mM EGTA, and 0.5 mM $MgCl_2$ (pH 7), followed by two further cycles in 1 M glutamate buffer (*32*). The purified tubulin, free from microtubule-associated proteins, was stored in aliquots at −70 °C. Protein concentrations were estimated by the method of Lowry et al. (*33*).

Sample Preparations. All spectroscopic measurements were carried out in buffer containing 20 mM sodium phosphate, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 7. For

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[1] Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine triphosphate; CD, circular dichroism; [θ], mean residue ellipticity; λ_{max}, wavelength of maximum fluorescence emission.

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denaturation, the protein was incubated in appropriate urea concentrations for at least 30 min, during which time unfolding was complete. To initiate refolding, stock solutions of denatured protein was diluted to a final protein concentration of 1 μM (unless mentioned otherwise) in buffer containing the indicated final concentration of urea, mixed thoroughly and incubated for 2 h at room temperature. This incubation period was sufficient for refolding to occur as the properties we have monitored here attained a constant value within 30 min and showed no further change even after overnight incubation at 4 °C, followed by 30 min at room temperature.

Spectroscopic Measurements. Fluorescence spectroscopic studies were done on a Hitachi F3000 fluorescence spectrophotometer. To detect changes in intrinsic protein fluorescence, excitation was done at 280 nm and emission intensity was monitored at 333 nm. For experiments involving binding of acrylodan to tubulin, excitation of the drug was done at 390 nm and relative emission intensity was monitored at 470 nm. The excitation and emission bandwidths were fixed at 5 nm each and a square quartz cuvette of path length 1 cm was used for all measurements. The spectrum of each sample was corrected by subtraction of the buffer alone.

Circular dichroism studies were done on a Jasco J600 spectropolarimeter. Secondary structure was monitored by measuring negative ellipticity at 220 nm, using a cell of path length 0.1 cm. A spectral bandwidth of 1 nm and a time constant of 2 s was used for these measurements. Each spectrum was recorded as an average of 6 scans. Mean residue ellipticities at 220 nm were calculated from observed values of ellipticity using a mean residue weight of 111.

All spectroscopic measurements were made at 25 °C.

Assay for Colchicine Binding Activity. Biological activity of tubulin at different urea concentrations was estimated using radioactive colchicine. Tubulin (1 μM) at the appropriate urea concentration, was incubated for 45 min with [^{3}H]colchicine, in buffer containing 10 mM sodium phosphate and 10 mM $MgCl_2$, pH 7, at room temperature. The amount of radioactivity incorporated, which reflects the activity of the protein, was assayed by the DEAE-cellulose filter disc method (*34*, *35*). All radioactive measurements were repeated four times, with the statistical mean and error bar corresponding to each point calculated.

RESULTS

The ability to bind colchicine is one of the most important and best-characterized biological properties of tubulin. We observed that the colchicine binding activity of tubulin decreased as the protein was unfolded by gradually increasing concentrations of urea. Figure 1 shows the percent colchicine binding activity of tubulin remaining after urea treatment. Low concentrations of urea inhibited colchicine binding and a slight drop in activity could be seen even in 0.2 M urea. In 2 M urea, colchicine binding was totally inhibited, and only 50% activity remained in 0.6 M urea. Thus the partially folded intermediate state of tubulin formed at around 2 M urea does not possess any colchicine binding activity. After unfolding of tubulin in 8 M urea, refolding was initiated by appropriate dilution of the denatured protein in buffer and the recovery of colchicine binding activity was monitored. It can be seen from Figure 1 that the colchicine binding activity of tubulin could not be recovered upon lowering of

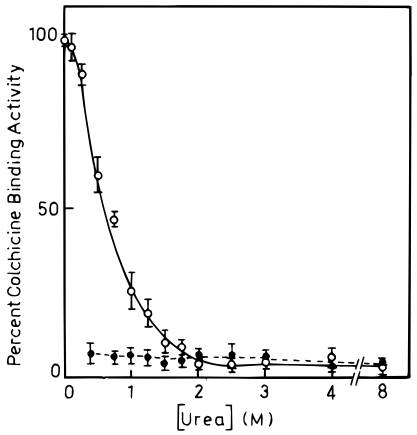

FIGURE 1: Changes in colchicine binding activity of tubulin during unfolding (○) in increasing urea concentrations and refolding (●) after denaturation in 8 M urea. Refolding was initiated from 8 M urea, by a 20-fold dilution of the denatured protein in buffer, and the final urea concentration was adjusted to those indicated in the figure. Tubulin concentration was 1 μM in all cases.

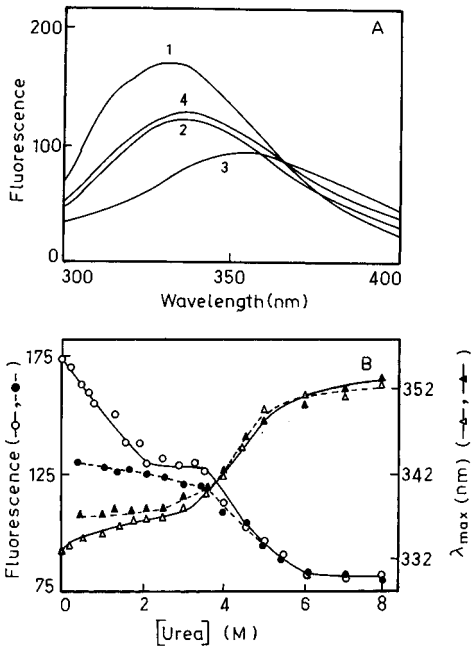

FIGURE 2: (A) Fluorescence emission spectra of native tubulin (1), tubulin denatured in 2 M (2) and 8 M (3) urea, and tubulin refolded from its totally unfolded state in 8 M urea (4). (B) Variation of intrinsic protein fluorescence at 333 nm (○, ●) and emission maximum (△, ▲) during tubulin unfolding and refolding. The open symbols refer to denaturation experiments, and filled symbols are for renaturation of the protein from 8 M urea. Refolding was carried out as described in Figure 1. Excitation wavelength was 280 nm and final tubulin concentration was 1 μM.

denaturant concentration from 8 M; even in urea concentrations as low as 0.4 M, the renatured protein's ability to bind colchicine remained negligible. Thus, the urea-induced loss of colchicine binding activity of the protein appears to be irreversible.

Changes in intrinsic protein fluorescence have often been used as a sensitive probe to monitor conformational transitions during unfolding and refolding of proteins. Figure 2A shows the fluorescence emission spectra of tubulin in its native, denatured, and refolded forms. The variation in tryptophan fluorescence intensity at 333 nm and emission maximum (λ_{max}) of 1 μM tubulin when the protein was first unfolded in urea and then refolded by dilution in buffer

containing appropriate concentrations of urea (as in Experimental Procedures) are shown in Figure 2B. We have described in our earlier paper (*31*) the changes in tryptophan fluorescence and λ_{max} that occurred when tubulin was denatured by increasing urea concentrations. The progressive decrease of tryptophan fluorescence and increase in λ_{max} occurred in two transitions, the first one resulting in the formation of an intermediate (I) state at around 2 M urea where the fluorescence intensity was almost 25% quenched compared to the native state and λ_{max} was 336 nm. This intermediate unfolded at higher urea concentrations by a second transition, until the fluorescence intensity decreased to approximately 55% that of the native state and λ_{max} was 353 nm in 8 M urea, corresponding to the totally unfolded state of tubulin. When refolding of tubulin was initiated from its totally unfolded state in 8 M urea, only partial recovery of tryptophan fluorescence and λ_{max} occurred. The fluorescence intensity at 333 nm increased gradually and λ_{max} shifted concomitantly toward blue as the urea concentration was decreased from 8 M urea. In urea concentrations near 2 M, almost 70% of the tryptophan fluorescence intensity of the native state could be recovered and λ_{max} was 337 nm. The midpoint of this refolding transition was at 4.5 M urea, and it followed closely the second transition of tubulin unfolding. The latter thus appears to be reversible and yields a state with partially hydrated tryptophan residues, which is very similar to the intermediate state formed by denaturation of tubulin in 2 M urea. We shall refer to this intermediatelike, partly refolded state of tubulin as I′ in the future, for convenience. When the urea concentration was lowered further, the tryptophan fluorescence and λ_{max} did not show much change; in 0.4 M urea only 73% fluorescence of the native state could be recovered while the emission maximum remained at 337 nm. This result indicates that the I′ state that is formed by refolding of tubulin from 8 M urea cannot undergo further folding toward the native state; i.e., the first urea denaturation transition probably is irreversible in these conditions. No significant change in this refolding pattern was observed with 0.2 μM tubulin (data not shown), suggesting that tubulin refolding probably does not depend on its state of association, i.e., whether the protein is in its monomeric or dimeric form.

To confirm the above observations, we also used acrylodan as a probe to study changes in tertiary structure of tubulin while unfolding and refolding. Acrylodan is a polarity-sensitive probe that can link covalently with sulfhydryl groups in proteins. It shows very little fluorescence when free in solution but its thiol adduct is highly fluorescent (*36*) and shows large changes in emission λ_{max} that can be correlated to polarity changes in its environment. Figure 3 shows the changes in fluorescence intensity and λ_{max} of tubulin-bound acrylodan as the protein was first unfolded by urea and then refolded by diluting out the urea. It was observed that as tubulin was unfolded in gradually increasing urea concentrations, the acrylodan fluorescence intensity at 470 nm increased markedly and λ_{max} shifted progressively toward blue. In 2−3 M urea, the fluorescence was almost 40% increased with respect to that in absence of urea, while λ_{max} shifted from 485 to 460 nm. As urea was increased beyond 3 M, the acrylodan fluorescence decreased drastically and λ_{max} was red-shifted. In 8 M urea the fluorescence intensity at 470 nm became practically negligible and λ_{max} was 520 nm. These observations suggest that the environment of the labeled cysteine residues probably become more hydrophobic in the intermediate state; i.e., these concentrations of urea probably induce a compactness of structure of some parts of the protein which possibly contain sulfhydryl groups in their vicinity. Acrylodan fluorescence intensity increased and λ_{max} gradually shifted toward blue when refolding of the protein initiated from its unfolded state. Near 2 M urea, a large blue shift in λ_{max} to 460 nm had occurred, which is identical to that noted for the intermediate state. However, acrylodan fluorescence intensity in these conditions was much less compared to that in the intermediate. Further lowering of urea concentration up to 0.4 M caused very little change in fluorescence intensity or λ_{max}. These results thus confirm our previous observations and suggest that refolding of tubulin in vitro from its totally unfolded state results in formation of an I′ state where only partial recovery of tertiary structure had occurred. Further restoration of tertiary structure of this I′ state toward the original, native form could not be achieved.

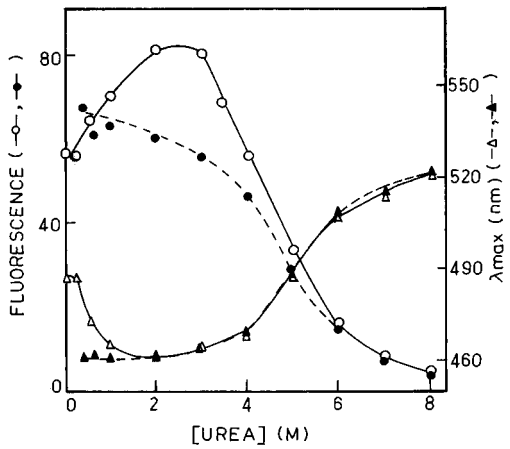

FIGURE 3: Variation of tubulin-bound acrylodan fluorescence intensity at 470 nm (○, ●) and emission maximum (△, ▲) during unfolding and refolding of the protein. The open symbols refer to denaturation experiments and filled symbols are for renaturation of the protein from 8 M urea. Refolding was carried out as described in Figure 1. Excitation wavelength was 390 nm and final concentration of the labeled protein was 1 μM.

To see whether secondary structure of the protein could be recovered under the renaturation conditions used, changes in negative ellipticity in the far-UV region (220 nm) of the protein was monitored at different urea concentrations, during both unfolding and refolding. Figure 4A shows the far-UV CD spectra of tubulin in its native, intermediate (I), unfolded, and refolded (I′) forms. It can be seen from the figure that the CD spectra of the I and I′ forms of tubulin are nearly similar, and considerably less pronounced than that of the native state. The variation of mean residue ellipticity at 220 nm ($[\theta]_{220}$) with increasing urea concentration (Figure 4B) followed a biphasic pattern, with a plateaulike region near 2 M urea, where the I state is formed. The formation of the I state was accompanied by almost 40−45% loss in negative ellipticity at 220 nm. As the I state unfolded at higher urea concentrations, negative ellipticity in this region decreased until there was almost total loss of structure of the CD band in 8 M urea. When the urea concentration was lowered by dilution of the unfolded (in 8 M urea) protein sample in buffer, the $[\theta]_{220}$ values increased gradually. The increase in ellipticity occurred almost quantitatively, resembling closely the second phase of denaturation. At 2 M urea,

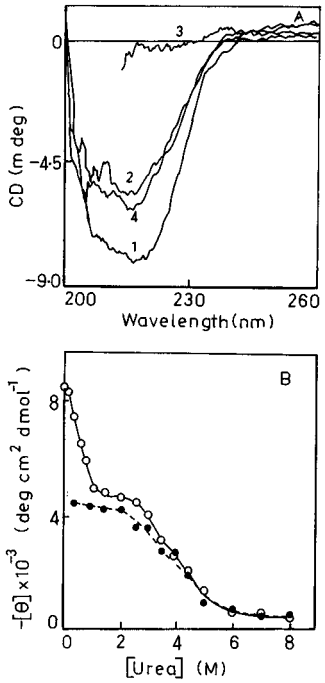

FIGURE 4: (A) Far-UV CD spectra of native tubulin (1), tubulin denatured in 2 M (2) and 8 M (3) urea, and tubulin refolded from its totally unfolded state in 8 M urea (4). (B) Urea dependence of mean residue ellipticity at 220 nm during unfolding of tubulin (○) and its refolding from 8 M urea (●). Refolding was done as in Figure 1. Final tubulin concentration was 1 μM and a 0.1 cm path length cell was used.

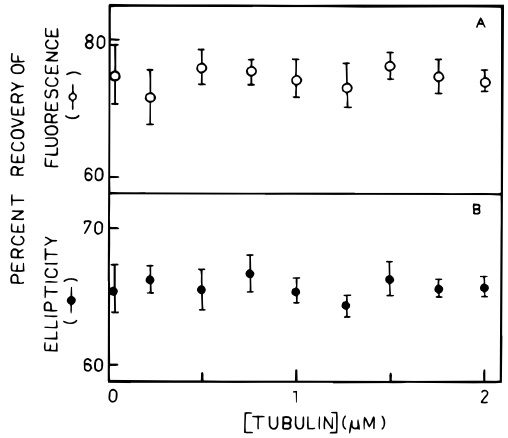

FIGURE 5: Effect of tubulin concentration on its extent of refolding measured with respect to tryptophan fluorescence intensity at 333 nm (○) and ellipticity at 220 nm (●). Different concentrations of tubulin were first denatured in 8 M urea and then refolded by a 20-fold dilution in urea-free buffer. Final tubulin concentrations are indicated in the figure. Percent recovery of fluorescence and ellipticity at each tubulin concentration was calculated with respect to those of an identical concentration of tubulin exposed to similar denaturing conditions.

almost 58% of nativelike secondary structure was regained. Further lowering of urea concentration up to 0.4 M urea caused practically no further increase in $[\theta]_{220}$ values. These data indicate that the second denaturation transition signifying formation of the unfolded state from the intermediate could be reversed while the first phase that manifests the formation of the intermediate could not. Hence, an intermediatelike I′ state could be formed in vitro from unfolded tubulin, but further recovery of secondary structure was not possible.

Our CD results thus confirm our fluorescence observations and indicate that only partial refolding of tubulin up to an I′ state could be achieved from its unfolded state. The I′ state resembled the partly unfolded intermediate state formed by denaturation of tubulin near 2 M urea. Both possess about 60% secondary structure and altered tertiary structure compared to the native state. Further refolding of this I′ state toward the native state could not be achieved under the conditions employed.

Refolding of many proteins, especially large ones, is often complicated by a kinetic competition between renaturation and aggregation of their partially folded states, which leads to very low yields of the refolded state. Lowering of protein concentration minimizes aggregation and recovery of the active state was observed at protein concentrations around 1−100 μg/mL (*8*, *10*, *20*, *37*). Tubulin also is very susceptible to self-association, and the effect is more pronounced in denaturant concentrations that induce formation of the intermediate state. However, the extent of aggregation in the presence of 2 M urea was found to be markedly reduced upon even a 2-fold lowering of tubulin concentration from 1 μM (S. Guha and B. Bhattacharyya, unpublished observations). To determine whether aggregation of the partially folded state interferes with the renaturation process in our case, refolding experiments were done at different protein concentrations (0.02−2 μM), after prior denaturation in 8 M urea. Refolding was monitored with respect to both tryptophan fluorescence intensity, a probe for tertiary structure (Figure 5A), and ellipticity at 220 nm, as an estimate of secondary structure (Figure 5B). The observed values of the spectroscopic parameters for the refolded protein were normalized with respect to those of another sample of tubulin denatured in identical conditions to obtain the percentage yield of renaturation. It can be seen from the figure that the percent recovery of both fluorescence and ellipticity showed no particular dependence on tubulin concentration in the range studied. This indicates that probably aggregation does not interfere significantly with the refolding phenomenon described here.

Tubulin being a dimer, its refolding from the totally unfolded state will depend on several factors, including folding of various domains and subunits and finally correct association between them. To see whether recovery of the active form of tubulin occurs from lower denaturant concentrations that cause only partial unfolding, probably in localized areas of the protein, the following experiment was done. Tubulin was denatured in low concentrations of urea up to 2 M, and refolding was initiated by a 10-fold dilution. The final protein concentration in all cases was 1 μM and urea concentration was adjusted to 0.2 M. Recovery of native structure was monitored with respect to colchicine binding activity and tryptophan fluorescence emission maximum and compared with that of 1 μM tubulin denatured in 0.2 M urea under identical conditions (Figure 6). Renaturation of tubulin from 2 M urea resulted in very little enhancement of colchicine binding activity, while λ_{max} of tryptophan emission remained at 337 nm, similar to that obtained when an identical experiment was performed from 8 M urea. Thus, refolding initiated from 2 M urea resulted in very little recovery of nativelike activity and structure. The tryptophan emission maximum, however, shifted progressively toward blue when refolding was initiated from gradually decreasing urea concentrations below 2 M. Tubulin denatured in 1.2 M urea showed a λ_{max} of 335 nm upon

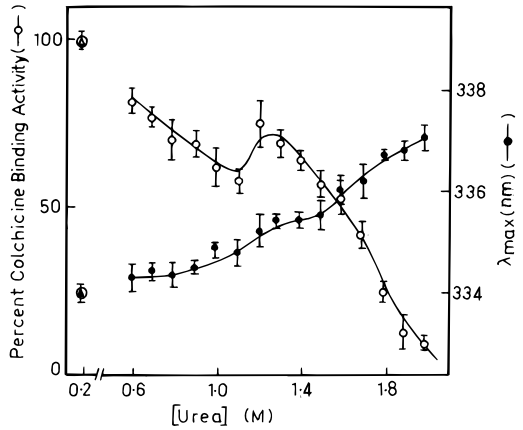

FIGURE 6: Change in colchicine binding activity (○) and tryptophan emission maximum (●) of refolded tubulin after denaturation in low urea concentrations. The urea concentrations used to denature tubulin are shown in the figure. Tubulin was refolded by a 10-fold dilution from the respective urea concentration and final urea concentration was adjusted to 0.2 M in each case. Final tubulin concentration was 1 μM in all cases. Colchicine binding activities at each urea concentration were normalized with respect to that of 1 μM tubulin, identically treated in 0.2 M urea (circled open triangle), and its corresponding λ_{max} value (circled solid triangle) is also shown.

refolding in 0.2 M urea. This value underwent little change in lower urea concentrations up to 0.6 M. The corresponding value of λ_{max} for 1 μM tubulin incubated in 0.2 M urea for identical times was 334 nm, comparable to that obtained for the refolded state. Percent recovery of colchicine binding activity of refolded tubulin also showed a steady increase when the concentration of urea used to denature it was lowered below 2 M. In denaturant concentrations around 1.2−1.4 M, almost 70% of original colchicine binding activity could be restored after refolding. Between urea concentrations of 1.2 and 1 M, colchicine binding activity showed a slight dip and again began to increase progressively at lower urea concentrations. In 0.6 M urea, 80% of original colchicine binding activity was recovered. It is evident from these data that although refolding of totally unfolded tubulin could not be achieved beyond the I′ state, almost nativelike structure with a considerable amount of biological activity could be regained when the protein was denatured in urea concentrations around 1.2 M. The reason behind the slight decrease in colchicine binding of refolded tubulin after denaturation in urea concentrations around 1 M was not clear.

DISCUSSION

The unfolding behavior of the dimeric protein tubulin has already been reported (*31*). The unfolding in urea was found to be a two-step process at pH 7. The first step led to the formation of an intermediate conformation, stable at around 2 M urea, followed by a second step that was due to unfolding of the intermediate. This intermediate had about 60% secondary structure, partially exposed aromatic residues, and less tertiary structure compared to the native state. An intermediate with similar spectroscopic characteristics has also been detected during thermal denaturation of tubulin (*38*).

In this paper, we have focused attention on the refolding characteristics of tubulin from its totally unfolded as well as partially unfolded states. Intrinsic protein fluorescence, acrylodan fluorescence, and CD data all indicate that the second phase of tubulin denaturation in urea could be reversed; the first phase apparently was irreversible under the conditions used. Refolding of tubulin from 8 M urea thus yields an intermediatelike state I′, which is formed at around 2 M urea and cannot undergo further folding toward the native state. Formation of the I′ state was accompanied by almost 60% restoration of far-UV ellipticity and a partial burial of aromatic residues. The environment of the cysteine residues in I′ was also similar to that observed in the denaturation intermediate.

Experiments with acrylodan also pointed out another feature of the intermediate not noticed previously. While formation of the intermediate was characterized by partial hydration of tryptophans, some cysteine residues probably get more buried than in the absence of urea (Figure 3). That low concentrations of urea (1−2 M) induce a "tightening" or compactness in tubulin structure was also reported by Sackett et al. (*39*). They observed that the accessibility of tubulin to proteases like trypsin and chymotrypsin decreased at 1−2 M urea, while at higher urea concentrations proteolysis increased, corresponding to extensive unfolding. These results suggest that while some parts of the protein were unfolded in low urea conditions, other parts became more compact.

Colchicine binding activity of tubulin was found to be very sensitive to urea concentration; less than 2 M urea caused total inhibition of activity. Sackett et al. (*39*) reported a severalfold enhancement of fluorescence of the tubulin-bound colchicine analog 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone (MTPT) upon exposure to urea, at concentrations below 2 M. At higher urea concentrations or longer exposure times there occurred a quenching of fluorescence. A 1.5-fold enhancement of colchicine fluorescence was also observed by us when the tubulin−colchicine complex was treated with <1 M urea (data not shown). However, such an enhancement of colchicine binding in this range of urea concentration was not observed when the filter disc assay method, using [^{3}H]colchicine, was applied to assay colchicine binding activity (Figure 1).

Although refolding of totally denatured tubulin resulted in substantial recovery of both secondary and tertiary structure (I′ state), very little colchicine binding activity was regained. Thus, in the I′ state, probably the colchicine binding domain had not yet been folded. It was observed that substantial colchicine binding ability of tubulin could be recovered only upon refolding from urea concentrations near 1.2 M, which had caused near total inhibition of activity; i.e., low-urea-induced loss of activity was reversible.

In addition to colchicine binding activity, Sackett et al. (*39*) also performed an extensive study on differential urea sensitivities of other functional properties of tubulin. Both polymerization and GTPase activities of tubulin were found to be inhibited by low urea concentrations of <1 M; loss of polymerization activity at very low urea concentration could be reversed. Inhibition of in vivo polymerization by low urea has also been reported in amoeba *Chaos* (*40*) and *Echinosphaerum* (*41*). Removal of urea led to rapid repolymerization in the latter case.

These results can be interpreted as follows. The various functional properties of tubulin are distributed in different areas of the dimer and low urea concentrations probably affect the secondary and tertiary structure in these areas, thus disrupting the function attributed to that particular region.

Refolding from the totally unfolded state of tubulin cannot restore the original structure to these areas; these local changes are reversed only when milder denaturing conditions that cause only restricted unfolding are used.

The question of incorrect subunit association in the I′ state that hinders further folding of the dimer toward more native structure also cannot be ruled out. In that case, at low concentrations of tubulin, where the monomeric form predominates, an increase in renaturation yield is expected. Our data, however, proved to be contrary. Also, while very little recovery of native state, with reference to both structural and functional parameters, could be achieved from 2 M urea, considerable refolding from 1.2 M was observed. Thus, subtle changes must occur on the protein between 1.2 and 2 M urea, which are sufficient to bring about a marked change in its refolding behavior.

In conclusion, we have shown here that in vitro folding of tubulin from its unfolded state in 8 M urea can be achieved only up to a partly folded state that resembles the molten globule-like intermediate state of tubulin formed in 2 M urea. Renaturation to the active folded form could be achieved only when refolding was done from tubulin in urea concentrations less than 1.2 M. In vivo folding of a nascent, unfolded tubulin chains probably occurs cotranslationally and is mediated by chaperones. Evidence of cytosolic chaperonin-mediated folding of tubulin monomers has been reported (*42*, *43*) and involvement of "quasi-native" chaperonin-bound intermediates with a significant amount of structure and GTP binding ability during tubulin folding is also being studied by other groups (*44*, *45*).

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