Stabilization of Tubulin by Deuterium Oxide[†]

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ABSTRACT: Tubulin is an unstable protein when stored in solution and loses its ability to form microtubules rapidly. We have found that D_2O stabilizes the protein against inactivation at both 4 and 37 °C. In H_2O-based buffer, tubulin was completely inactivated after 40 h at 4 °C, but in buffer prepared in D_2O, no activity was lost after 54 h. Tubulin was completely inactivated at 37 °C in 8 h in H_2O buffer, but only 20% of the activity was lost in D_2O buffer. Tubulin also lost its colchicine binding activity at a slower rate in D_2O. The deuterated solvent retarded an aggregation process that occurs during incubation at both temperatures. Inactivation in H_2O buffer was partially reversed by transferring the protein to D_2O buffer; however, aggregation was not reversed. The level of binding of BisANS, a probe of exposed hydrophobic sites in proteins, increases during the inactivation of tubulin. In D_2O, the rate of this increase is slowed somewhat. We propose that D_2O has its stabilizing effect on a conformational step or steps that involve the disruption of hydrophobic forces. The conformational change is followed by an aggregation process that cannot be reversed by D_2O. As reported previously [Ito, T., and Sato, H. (1984) *Biochim. Biophys. Acta 800*, 21−27], we found that D_2O stimulates the formation of microtubules from tubulin. We also observed that the products of assembly in D_2O/8% DMSO consisted of a high percentage of ribbon structures and incompletely folded microtubules. When these polymers were disassembled and reassembled in H_2O/8% DMSO, the products were microtubules. We suggest that the combination of D_2O and DMSO, both stimulators of tubulin assembly, leads to the rapid production of nuclei that lead to the formation of ribbon structures rather than microtubules.

Microtubules are cytoskeletal structures in eukaryotic cells that are involved in various types of motility processes, including chromosome positioning and separation during mitosis and meiosis, and vesicle transport. The protein subunit of microtubules, tubulin, is an interesting protein that binds nucleotides and a variety of antimitotic compounds, some of which are used as antitumor agents. Tubulin is known to be an unstable protein, losing its microtubule-forming activity over a period of hours when stored in solution at low temperatures. The loss in activity is accompanied by an irreversible aggregation process that involves structural changes resulting in changes in the fluorescence and circular dichroism spectra of the protein (*1*). This instability of tubulin has hindered biochemical and biophysical studies that involve long time periods. In the course of conducting long-term NMR experiments with tubulin, we found that D_2O has a dramatic stabilizing effect on the protein. This effect is examined in more detail in this report. In addition, we report the partial reversal of the inactivation of tubulin by D_2O.

EXPERIMENTAL PROCEDURES

Purification of Tubulin. Microtubule protein (tubulin containing microtubule-associated proteins) was isolated from bovine brain by two cycles of polymerization and depolymerization (*2*). Phosphocellulose chromatography was used to remove the associated proteins (*3*), and the protein was stored in PEM buffer[1] as frozen pellets at −70 °C.

For the stability and aggregation experiments described below, it was necessary to obtain tubulin in D_2O buffer. To accomplish this, tubulin was thawed and centrifuged at 40000*g* for 10 min to remove any aggregated material. The supernatant was made 8% in DMSO (*4*), 1 mM in DTT, and 0.5 mM in GTP and was incubated at 37 °C for 15 min to form microtubules. The suspension was divided into two portions that were centrifuged in a Beckman TL-100 ultracentrifuge at 100000*g* for 4 min at 37 °C. The pellets were suspended in PEM buffer containing 1 mM DTT that had been prepared with either H_2O or D_2O. The D_2O concentration in the PEM buffer prepared in D_2O was 89%. After 30 min on ice, the solutions were centrifuged at 40000*g* for 5 min. The protein concentrations of the supernatants were determined by the Bradford method (*5*), and the concentrations were adjusted with buffer, if necessary, so that they were equivalent.

Assembly Assays. Assembly reactions were carried out in 100 μL volumes containing 1.5 mg/mL tubulin, PEM buffer prepared in H_2O or D_2O, 1 mM DTT, 8% DMSO, and 0.5 mM GTP. After 15 min at 37 °C, the samples were

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[1] Abbreviations: PEM buffer, 0.1 M 1,4-piperazinediethanesulfonate, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid, and 1 mM $MgSO_4$; H_2O buffer, PEM buffer prepared in H_2O; D_2O buffer, PEM buffer prepared in D_2O; BisANS, 5,5′-bis(8-anilino-1-naphthalenesulfonate); DTT, dithiothreitol.

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centrifuged at 100000g for 4 min at 37 °C . The pellet was suspended in cold buffer and centrifuged at 40000g for 5 min at 4 °C. After the supernatant was removed, 100 μL of 0.1 M NaOH was added to dissolve any cold-insoluble pellets. Protein concentrations of both supernatants and the cold-insoluble pellets were determined. In some cases, the assembly was monitored by the increase in turbidity at 350 nm in reactions carried out in 400 μL volumes.

Colchicine Binding Assay. The increase in colchicine fluorescence that occurs when the drug binds to tubulin (*6*) was used to follow the loss in colchicine binding activity. Samples of tubulin were diluted with PEM buffer to a concentration of 5 μM, and colchicine was added to a concentration of 10 μM. The samples were incubated at 37 °C for 1 h, and the fluorescence at 430 nm was read against 10 μM colchicine using an excitation wavelength of 350 nm.

Measurement of Aggregation. The formation of aggregates was monitored by size-exclusion HPLC using a 600 mm × 7.8 mm Phenomenex Biosep-Sec-2000 column. Tubulin samples were incubated at either 4 or 37 °C at a concentration of 1 mg/mL. At different times, 250 μL aliquots were removed and applied to the column. In the case of the 37 °C incubation, the samples were placed on ice for 15 min to depolymerize any microtubules that may have formed and centrifuged to remove cold-insoluble polymers before injection. PEM buffer was used as the elution solution at a flow rate of 1.0 mL/min. Detection was at 280 nm. Blue dextran, tryptophan, and a Bio-Rad protein gel filtration standard mixture consisting of protein aggregates, thyroglobin (670 kDa), IgG (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa) were used as standards. Areas under the aggregate and tubulin dimer peaks were calculated using the NIH Image 1.60 program.

Electron Microscopy. Negative staining was used to observe the structures of the polymerized products in the assembly reactions. Samples were diluted 10- or 20-fold into PEM buffer containing 0.25% glutaraldehyde, applied to 300-mesh carbon-coated copper grids, stained with 2% uranyl acetate, and viewed with a JEOL JEM-1200 EX11 electron microscope.

NMR. The H_2O concentration of a solution after dialysis against a D_2O solution was determined by ^{1}H NMR using a Brucker Advance DRX 400 spectrometer operating at 400.13 MHz. The isotopic composition of the dialysate was determined by integration of the H_2O peak compared with the peak resulting from the addition of a known quantity of acetone to the dialysate.

Materials. D_2O (99.9%) was purchased from Cambridge Isotope Laboratories, Inc. BisANS was from Molecular Probes, Inc. GTP was obtained from Boehringer Mannheim. DTT and EGTA were from Sigma. Pipes was from Research Organics.

RESULTS

Assembly in D_2O Buffer. In experiments to be described later, we compare the assembly activity of tubulin after incubation in buffer containing either H_2O or D_2O. To reduce the number of variables, the assembly reactions were carried out under identical conditions, except for the presence or absence of D_2O. Therefore, the reaction solutions contained 8% DMSO. When the reactions were followed by turbidity

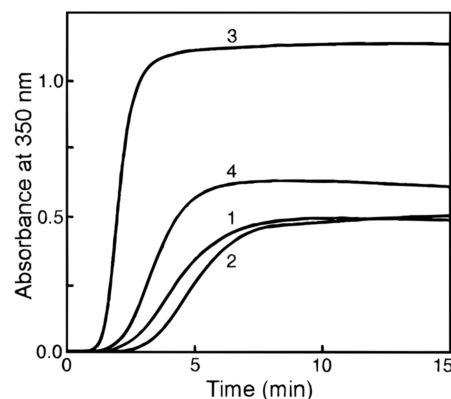

FIGURE 1: Assembly of tubulin in D_2O buffer and H_2O buffer. Tubulin at 1.5 mg/mL was assembled at 37 °C in the presence of 0.5 mM GTP. (1) Assembled in H_2O buffer with 8% DMSO. (2) Assembled in D_2O buffer without DMSO. (3) Assembled in D_2O buffer with 8% DMSO. (4) Tubulin was assembled in D_2O buffer containing 8% DMSO; microtubules were collected by centrifugation, suspended in H_2O buffer, and repolymerized at 1.5 mg/mL in the presence of 8% DMSO.

development, the increase observed in buffer containing D_2O was consistently about twice that which developed in buffer containing H_2O (Figure 1). This result suggested that a greater degree of assembly occurred in the D_2O buffer. However, when samples were centrifuged, there was no significant difference in the supernatant or cold-soluble pellet protein concentrations. In both cases, about 75% of the protein had assembled into cold-soluble products. When DMSO was omitted from the assembly reaction solution containing D_2O, the absorbance was reduced to the level seen for the H_2O/8% DMSO solution (Figure 1).

Since the degree of turbidity depends on the nature of the structure present, the results suggest a difference in the structure of the polymer formed in D_2O buffer containing DMSO. Examination of negatively stained samples showed that this was indeed the case. In the case of assembly in either H_2O/8% DMSO or D_2O without DMSO, the polymers were essentially all microtubules (Figure 2A,B), but a high percentage of structures formed in D_2O/8% DMSO were open ribbons and incompletely closed microtubules (Figure 2C).

Because there are differences between the acidity of D_2O and H_2O, the chemical activities of D^+ and H^+, and pK_a values of weak acids in D_2O and H_2O, the pH meter reading of a solution in D_2O must be corrected with the relationship pD = pH meter reading + 0.4 (*7*). The pH meter readings of the buffer solutions in the experiments described in Figures 1 and 2 were 6.9. To ensure that the difference in pH and pD did not contribute to the results, we also conducted assembly reactions in buffers with a pH meter reading of 7.3. This change did not alter the results shown in Figures 1 and 2.

The question of whether the effect of DMSO on the products of assembly in D_2O is reversible arose. To test this, tubulin was assembled in D_2O/8% DMSO, and the products were collected by centrifugation and suspended in H_2O buffer. This protein was reassembled in H_2O/8% DMSO at the same concentration as the original sample. Development of turbidity and the structure of the products were similar to those that had not been preassembled in D_2O/DMSO (Figures 1 and 2D).

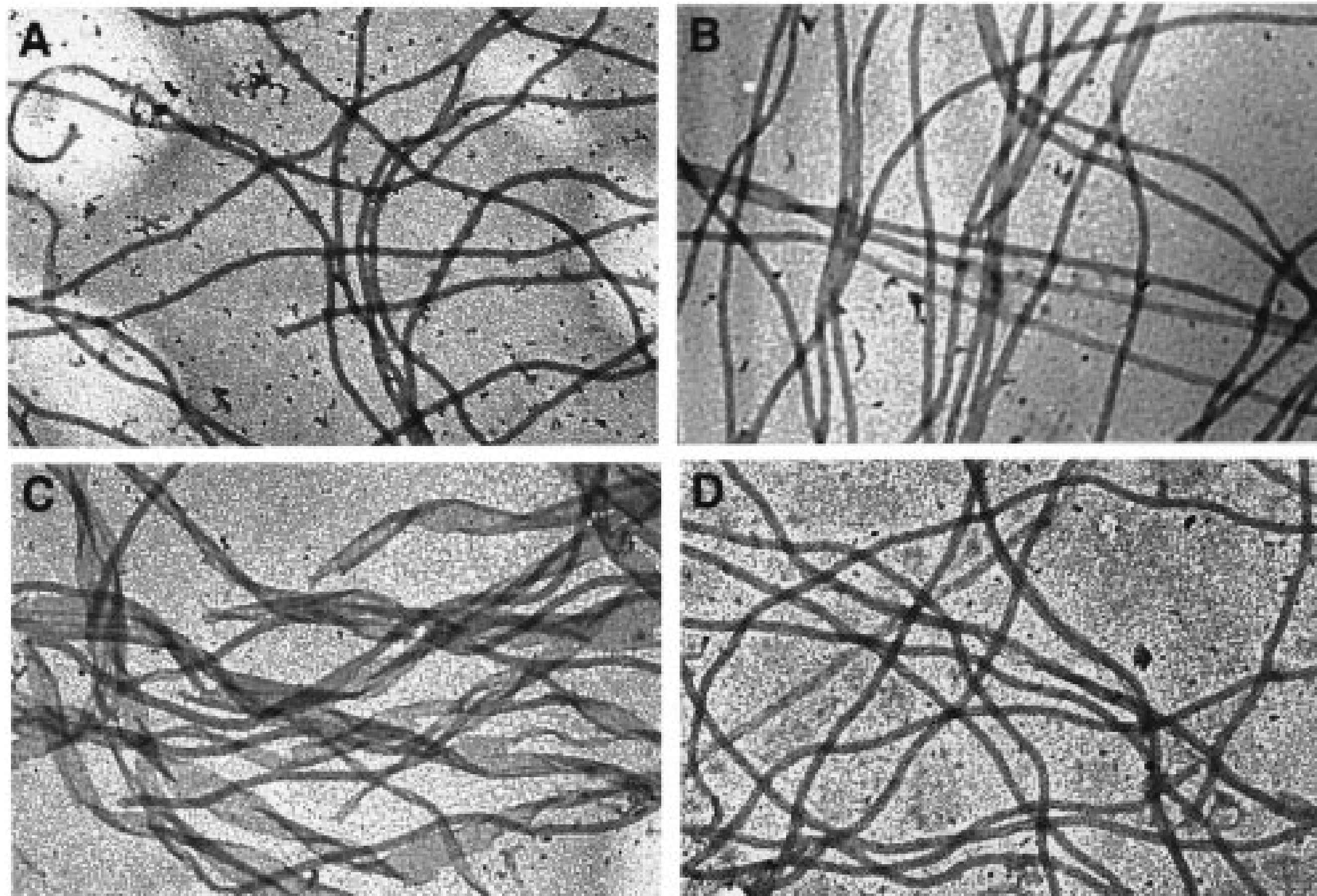

FIGURE 2: Structure of assembly products. Samples from the assembly reactions described in the legend of Figure 1 were negatively stained and examined by electron microscopy (40000× magnification) as described in Experimental Procedures. (A) Assembly in H_2O buffer with 8% DMSO. (B) Assembly in D_2O buffer without DMSO. (C) Assembly in D_2O buffer with 8% DMSO. (D) Assembly in D_2O buffer and then in H_2O buffer, both containing 8% DMSO.

Loss of Assembly Activity. Tubulin was incubated in buffer containing either H_2O or D_2O at 4 °C for a total period of 54 h. Samples were removed at different times, diluted to 1.5 mg/mL, and tested for assembly competence. The amount of polymerized tubulin was calculated from the protein concentration in the cold-soluble pellet following centrifugation and suspension of the pellet in cold PEM buffer. Figure 3A depicts the results of these experiments. In H_2O buffer, tubulin had lost the ability to form microtubules within 40 h. On the other hand, D_2O afforded complete stability for 54 h. There was no significant amount of a cold-insoluble pellet formed during the assembly reaction in either H_2O buffer or D_2O buffer.

The stability was also determined at 37 °C. In this case, tubulin was incubated for a period of 8 h. During this time, the sample in H_2O buffer became turbid but the sample in D_2O buffer did not. After 8 h, the sample in H_2O buffer had completely lost the ability to assemble, whereas the sample in D_2O buffer had retained 80% of its initial assembly activity (Figure 3B).

Samples incubated in both H_2O buffer and D_2O buffer for 8 h at 37 °C and 54 h at 4 °C were analyzed by SDS-PAGE and showed no signs of degradation (data not shown).

Loss of Colchicine Binding. The colchicine binding activity of tubulin is lost at a slower rate than assembly activity (*8*). To measure the decrease in the extent of colchicine binding over a reasonable time period, tubulin was incubated at 37 °C. The results are presented in Figure 4. Although under these conditions the protein lost activity in D_2O buffer, the rate was much slower than in H_2O buffer. In H_2O buffer, 50% of the activity was lost in about 7 h, whereas in D_2O buffer, the activity had not decreased by 50% after 24 h.

Exposure of Nonpolar Residues. BisANS is a probe for nonpolar regions of proteins. Upon BisANS interacting with hydrophobic regions, its fluorescence is greatly enhanced. During the process of tubulin inactivation, the extent of binding of BisANS increases (*9*), indicating that hydrophobic regions become exposed. In Figure 5, data for the binding of BisANS after various incubation periods in H_2O buffer and D_2O buffer are presented. D_2O did decrease the rate at which the hydrophobic regions are exposed to a small extent, but the final percent change in fluorescence was equivalent in both solvents. Interestingly, the fluorescence value of BisANS and tubulin at zero time was 28–45% lower in D_2O buffer than in H_2O buffer, suggesting a lower degree of binding in D_2O. D_2O had no effect on the fluorescence of BisANS in the absence of tubulin.

Aggregation of Tubulin. The observation that at 37 °C in H_2O buffer the tubulin solution became turbid indicates that aggregation occurred. To determine whether aggregation also occurred at 4 °C, size-exclusion HPLC was used. Freshly prepared tubulin showed one major elution peak at the elution

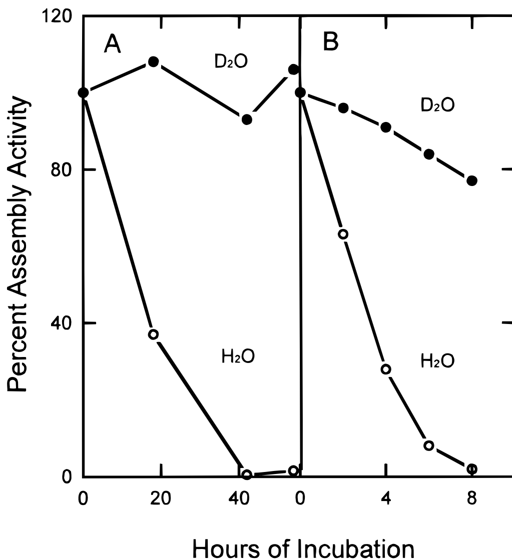

FIGURE 3: Loss of assembly activity with time. (A) Tubulin at 2.2 mg/mL in H_2O buffer or D_2O buffer was stored at 4 °C. At the times indicated, samples were removed, diluted to 1.5 mg/mL, and made 8% in DMSO and 0.5 mM in GTP. The samples were assembled as described in Experimental Procedures. The concentration of the cold-soluble pellet after centrifugation and suspension in the original volume represents the concentration of polymerized tubulin formed. At time zero, this concentration was 1.1 mg/mL. (B) Tubulin at 2 mg/mL was stored at 37 °C in H_2O buffer and D_2O buffer and treated as described for panel A. The concentration of the cold-soluble pellet at time zero after suspension was 1.0 mg/mL.

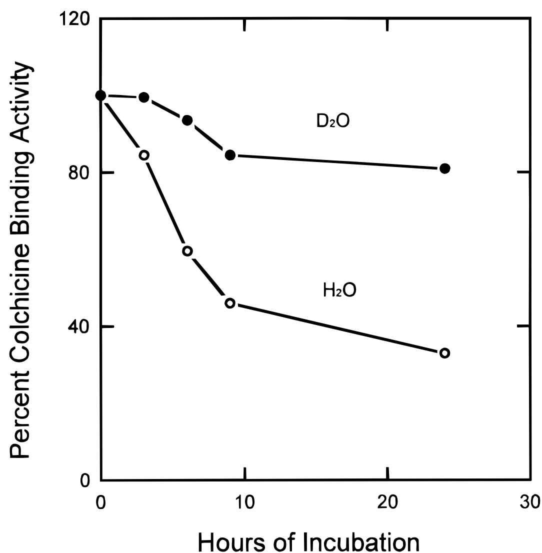

FIGURE 4: Loss of colchicine binding activity upon storage. Tubulin was incubated at 1.5 mg/mL and 37 °C in H_2O buffer and D_2O buffer. At the times shown, samples were diluted to 5 μM (0.5 mg/mL) and colchicine was added to a final concentration of 10 μM. The samples were incubated for 1 h at 37 °C, and the fluorescence was read at 430 nm with an excitation wavelength of 350 nm. The band-passes were 5 nm each. The values are normalized to 100% for the time zero value. The data shown represent the average of two experiments. Both experiments gave a similar half-life of colchicine binding decay in H_2O.

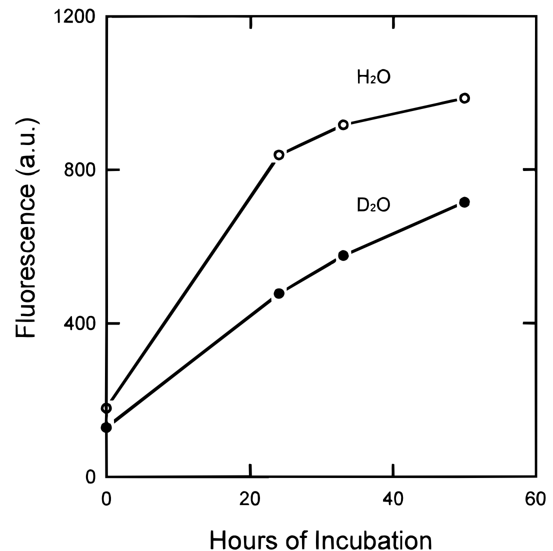

FIGURE 5: Increase in the extent of BisANS binding upon storage. Tubulin was incubated at 1.0 mg/mL in H_2O and D_2O buffer. At the times shown, samples were taken and diluted to 2 μM (0.2 mg/mL) and BisANS was added to a final concentration of 10 μM. The fluorescence was read immediately using an excitation wavelength of 385 nm and an emission wavelength of 490 nm. The band-passes were 2 nm each. The data shown came from a single experiment, and each data point is an average of duplicate readings. A second experiment gave similar results.

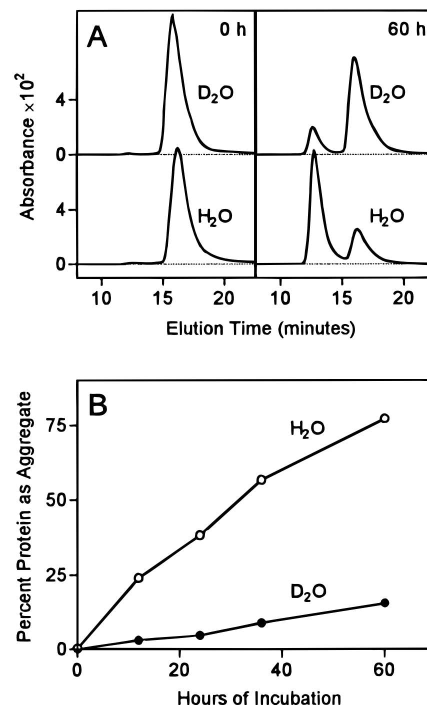

FIGURE 6: Increase in the level of tubulin aggregation upon storage. Tubulin at 1 mg/mL was incubated at 4 °C in H_2O buffer and D_2O buffer for the times shown. Samples were removed and injected into a size-exclusion column as described in Experimental Procedures. (A) Elution patterns at 0 and 60 h. (B) The combined area of the aggregate peak and the tubulin dimer peak was calculated, and the percentage of the aggregate peak is plotted against time of incubation.

volume expected for a protein with a mass of 100 kDa (Figure 6A). A very small peak at the void volume was also noticeable. After 60 h at 4 °C in H_2O buffer, the peak at the void volume had become the predominant one, representing 77% of the total area. In marked contrast, after 60 h in D_2O buffer, the aggregate peak represented only 16% of the total area (Figure 6A). The time course of the increase in the amount of aggregate is presented in Figure 6B.

Reversibility of Inactivation and Aggregation. The experiments described in Figures 1 and 2 demonstrated that the

effect of D_2O on the nature of the assembly product was reversible. We then raised the question of whether the inactivation could be reversed by D_2O. In an experiment in which tubulin was stored for 36 h in H_2O buffer at 4 °C, the assembly activity had fallen to 19% of the original value. At this time, the protein was dialyzed against D_2O buffer. After 4 h of dialysis, the activity increased to 50%. The H_2O concentration of the dialysate was determined by ^1H NMR spectroscopy and was found to be 11%, the same as that of the dialysis buffer. Further dialysis did not restore any more activity. The products of assembly after dialysis were examined in the electron microscope, and the structures were identical to those normally seen in assembly reactions carried out in D_2O. The distribution between aggregate and dimer, which was 52% aggregate after the 36 h period, did not change after dialysis in D_2O buffer. Thus, the amount of reactivation was equivalent to the amount of dimer present. Tubulin that had been incubated for 90 h and showed no residual activity and little dimer present could not be reactivated by dialysis in D_2O.

DISCUSSION

A number of studies of the effects of D_2O on protein aggregation have been reported. In many cases, D_2O stabilized the aggregated form of oligomeric proteins. For example, the deuterated solvent stabilized the oligomeric form of halophilic malate dehydrogenase (*10*), lactic dehydrogenase (*11*), and glutamate dehydrogenase (*11*). D_2O also increased the level of aggregation of phycocyanin (*12*), the level of association of the dimer form of β-lactoglobulin A to form the octamer (*13*), the rate of actin polymerization (*14*), the rate of polymerization of flagellin (*15*), and the rate as well as the extent of subunit association of N^{10}-formyltetrahydrofolate synthetase (*16*). Not all proteins are similarly affected by D_2O though. For example, Berns et al. (*12*) found that the aggregation of several proteins was not affected by the solvent. D_2O is also known to affect the stability of some proteins. The thermal stability of flagellin (*15*), ribonuclease (*17*), human factor XIII (*18*), and β-lactoglobulin (*19*) and the stability of ovalbumin in urea (*20*) are increased in D_2O. On the other hand, the thermal stability of val-tRNA^{val} synthetase was unaffected by D_2O (*21*).

Despite the many studies, it has not been possible to adequately describe the mechanism of the effects D_2O on protein structure (reviewed in ref *22*). In a number of cases, the effect of stabilizing the aggregated form of proteins has been attributed to the enhancement of hydrophobic interactions because such interactions are stronger in D_2O than in H_2O (*23*). It has also been proposed that deuterium bonds are stronger than hydrogen bonds (*24*), and several groups have suggested that D_2O may have its effect, at least in part, on hydrogen bonding in proteins (*13*, *15*, *20*). However, in many cases in which the solvent isotope effect on hydrogen bonding was studied, enthalpic and entropic effects canceled each other and, as a result, there was little effect of D_2O (*7*). Another factor that must be considered is the effect of the solvent on the pK_a values of amino acid residue side chains in the protein (*25*). The complexity of the solvent isotope effect can be seen in a study on the refolding of hen egg white lysozyme in which it was not possible to contribute the effect to any one factor (*26*).

Studies of the effects of D_2O on microtubules in vivo have been reported since 1935 (*27*). These studies have demonstrated that D_2O increases the birefringence and volume of the mitotic spindle as well as the number and length of the spindle microtubules (reviewed in ref *28*), indicating an increase in the extent of tubulin polymerization caused by D_2O. D_2O is also an antimitotic agent (*29*) and stabilizes the spindle (*30*), suggesting that the deuterated solvent may affect the dynamics of mitotic microtubules. Stabilization of microtubules may explain the antagonistic effect of D_2O on such antimitotic agents as colchicine (*31*) and methyl benzimidazol-2-ylcarbamate (*32*). It has also been shown that D_2O stimulates tubulin assembly in vitro (*33*−*35*). The results presented in Figures 1 and 2 confirm these previous findings. In the absence of DMSO and under the conditions used, assembly occurred in D_2O but not in H_2O.

Stimulation of tubulin assembly by D_2O is most probably explained by the increase in the strength of hydrophobic interactions in this solvent as proposed by Itoh and Sato (*34*) because tubulin assembly is an entropically driven process (*36*). But D_2O has a second effect on tubulin, preventing inactivation and aggregation of the protein. Inhibition of the aggregation process is opposite to the effect of the solvent on tubulin polymerization and on the aggregation of the proteins described above. It has been proposed that the mechanism of tubulin inactivation involves a rate-limiting unimolecular conformational change before aggregation occurs because the rate of tubulin aggregation does not show a dependence on protein concentration (*1*). This two-step process, which has also been proposed for the inactivation of β-lactoglobulin (*22*), is represented by the equation

$$\mathrm{Tu} \rightleftharpoons \mathrm{Tu}^* \rightarrow (\mathrm{Tu}^*)_x$$

Undoubtedly, the process involves more than one intermediate step, and it is difficult to assign the step or steps that are affected by D_2O. However, D_2O would be expected to decrease the rate of steps that involve conformational changes and the disruption of hydrophobic interactions in the dimer because these interactions are stronger in D_2O than in H_2O. The experiments with BisANS showed that nonpolar regions are exposed during the inactivation process and the rate of exposure was decreased somewhat by D_2O. But this effect of D_2O was not nearly as large as the effect on assembly activity and aggregation. Thus, exposure of nonpolar groups still occurs in D_2O without the loss of activity or the production of aggregates. The BisANS binding data are difficult to assess, however, because it is not known what steps in the inactivation process contribute to the increase in the extent of BisANS binding and what effect aggregate formation has on the binding. It should also be pointed out that in measuring inactivation, the protein was incubated at 0 °C but assembly was measured at 37 °C. We do not know whether the temperature shift has different consequences on the protein in D_2O and H_2O. Very likely, D_2O does not affect the aggregation step because aggregation was not reversed by the solvent, whereas the inactivation was reversed in proportion to the amount of dimer present in solution. It is more probable that D_2O reversed a conformational(s) state to an active form(s) or perhaps prevented the inactivation of a metastable state upon the temperature shift to 37 °C. An interesting point is that D_2O appeared to decrease the

extent of BisANS binding to tubulin because the fluorescence value at time zero was lower in D_2O than in H_2O, suggesting that tubulin has a more compact structure in D_2O.

It has been proposed that the tubulin aggregation process involves the formation of disulfide bonds (*1*). Our studies were carried out in the presence of the reducing agent DTT which should have prevented disulfide formation. Incubation of tubulin at 37 °C for periods of up to 30 days also results in the formation of isoaspartyl bonds (*37*) and incubation at high pH and temperature in lysinoalanine cross-links (*38*), but these processes probably do not contribute to the changes we observed over a much shorter time period at 4 °C and neutral pH.

Why are the structures formed during assembly in D_2O/DMSO different from those formed in H_2O/DMSO? Both D_2O and DMSO are stimulators of tubulin assembly, lowering the critical protein concentration for polymerization. There is possibly more than one pathway to polymerization. One pathway leads to nuclei that form microtubules, and another pathway might lead to nuclei that are seeds for ribbon formation. The combination of D_2O and DMSO could increase the rate of formation of nuclei that lead to ribbons. This is similar to what happens when the DMSO concentration in H_2O is above 10% (*39*) or high concentrations of organic sulfonate salts are present (*40*).

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