Molecular Flexibility in Microtubule Proteins: Proton Nuclear Magnetic Resonance Characterization†

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ABSTRACT: Bovine microtubule protein preparations have been examined by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy at 270 MHz. Sharp resonances have been identified as deriving from microtubule-associated proteins. These resonances persist after self-assembly of microtubule protein. Brief tryptic treatment of assembled microtubules, specifically cleaving the microtubule-associated protein HMW2 (M_r = 270000), releases the pendant portion of HMW2 (M_r = 240000), three-quarters of which is in a flexible conformation. Isolated τ protein and HMW2 protein both show substantial flexibility; on recombination with tubulin dimer, τ shows considerable decrease in flexibility whereas HMW2 is unaffected. The observations may have important implications for the interactions between microtubules and other cytoskeletal structures.

The discovery of conditions for the reversible assembly and disassembly of preparations of mammalian brain microtubule protein (MT protein) (Weisenberg, 1972; Borisy & Olmsted, 1972) has stimulated intense study of the mechanism of the in vitro assembly process [for a review, see Scheele & Borisy (1979)]. It has been demonstrated that assembly can be performed with tubulin dimer under appropriate solution conditions (Frigon & Timasheff, 1975; Herzog & Weber, 1977, 1978; Himes et al., 1977), indicating that the capacity for microtubule formation resides in the tubulin dimer itself (Timasheff & Grisham, 1980). However, several lines of evidence indicate the importance of the microtubule-associated proteins (MAPs)[1] in promoting efficient assembly in vitro and their involvement in the structure of microtubules in vivo. Thus, we have the following: (i) The product of repeated cycles of assembly–disassembly is a mixture of tubulin and MAPs, including both τ-group proteins and high molecular weight proteins (HMWs) (Scheele & Borisy, 1979). (ii) The presence of MAPs reduces the critical concentration and the length of the lag phase of assembly of MT protein, implying the involvement of complexes of tubulin and MAPs as nucleating species in the assembly process (Sloboda et al., 1976). (iii) The assembly of microtubule protein in vitro generally proceeds by biphasic kinetics (Barton & Riazi, 1980). The fast phase correlates with the presence and kinetic involvement of oligomeric species composed of tubulin and MAPs (Bayley et al., 1983), which are relatively stable under assembly conditions (Martin et al., 1982; Palmer et al., 1982). (iv) Assembly of MT protein in vitro produces rough-walled microtubules that show peripheral material in electron microscopy (Zingsheim et al., 1979), also identified by immunological techniques as HMW-group MAPs. (v) In vivo bridges visible in cross section by negative staining have been identified with MAPs (Dentler et al., 1975), and the regular distribution of HMW proteins along the microtubule has been incorporated into a structural model (Amos, 1977, 1979). (vi) In vivo microtubules are labeled with either anti-τ or anti-HMW antibody, indicating close spatial relationship between MAPs and microtubules (Connolly et al., 1978; Lockwood, 1978; Sherline & Schiavone, 1978; Sheterline, 1978).

The two main categories of MAPs differ substantially in molecular size (τ group M_r = 50000–75000; high molecular weight group: HMW1 M_r = 300000; HMW2 M_r = 270000). The physical properties indicate an expanded structure with $s^0_{20,w}$ = 3–4 S for HMW2 (Vallee & Borisy, 1978). Similarly for τ, $s^0_{20,w}$ = 2.6 S, and its CD shows no evidence of organized secondary structure (Cleveland et al., 1977). Thus these proteins clearly do not possess the normal conformational characteristics of globular proteins, but the relationship of their physical properties to their function in microtubule assembly is so far unexplored.

Cold-dissociated MT protein, obtained by reversible assembly–disassembly procedures, contains tubulin dimer and oligomeric species of the dimer plus MAPs. The method of the preparation and the solution conditions determine the protein composition with respect to τ- and HMW-group MAPs, and these in turn affect the nature and properties of the oligomeric species that are present (Scheele & Borisy, 1979). Central to the understanding of the mechanism of microtubule assembly is the characterization of the conformational properties of individual components of MT protein preparations, their stoichiometry, affinity, and stability in the formation of oligomeric species, and the relationship between these multiprotein complexes and the assembled microtubules. In our previous studies of the MT protein system, evidence from CD spectroscopy (Martin et al., 1982) and hydrodynamics [analytical ultracentrifugation (Bayley et al., 1982) and quasi-elastic light scattering (Palmer et al., 1982)] has indicated the importance of these oligomers of tubulin plus MAPs in the behavior of MT protein in vitro under assembly as well as nonassembly conditions. It is the purpose of this paper to clarify further the properties of these oligomers and their components, with particular emphasis on the flexibility of these proteins as determined by NMR.

We have previously reported (Woody et al., 1982) initial studies of MT protein with ^{1}H NMR, demonstrating the localization within MAPs of a substantial region of molecular

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[1] Abbreviations: MT protein, microtubule protein; MAPs, microtubule-associated proteins; HMW, high molecular weight group proteins; MgPCT, magnesium–phosphocellulose–tubulin dimer; Mes, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; CD, circular dichroism; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; TFE, trifluoroethanol.

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flexibility. In this work, we investigate the effects of assembly and identify the origins of the flexibility with respect to HMW2- and τ-group proteins, studying the effects of recombination of each one with tubulin dimer.

The results indicate that individually both τ and HMW2 possess flexibility but that in oligomeric complexes with tubulin, HMW2 retains this property fully whereas τ does not. In assembled microtubules, the flexibility resides in the large pendant portion of the HMW2 molecule, and this structural feature may be important in determining dynamic properties of the cytoskeletal microtubule network.

Materials and Methods

Protein Preparation. MT protein was prepared from fresh bovine brain either in the absence of glycerol by the method of Asnes & Wilson (1979) or in the presence of glycerol by a modification of the method of Shelanski et al. (1973) (Clark et al., 1981). For NMR experiments, MT protein was prepared by resuspension of the protein pellet in buffer A (D_2O, 20 mM phosphate, 0.5 mM $MgCl_2$, pD 6.5) followed by one cycle of assembly–disassembly, and subsequent dialysis as described previously (Woody et al., 1982).

MAP-free tubulin dimer was freshly prepared by chromatography over phosphocellulose pretreated with excess Mg^{2+} (Williams & Detrich, 1979; Clark et al., 1981). The peak fraction was transferred from the column buffer (25 mM Mes, 0.5 mM $MgCl_2$, 0.1 mM EGTA, pH 6.5) into buffer A by extensive dialysis against four changes of buffer and used immediately.

MAPs were prepared by a procedure employing a heat-treatment step that was based on the method of Sandoval & Weber (1980). The procedure has been modified from that reported previously (Woody et al., 1982). All solutions except buffer A contain 0.1 mM PMSF. Bovine MT protein was prepared from brain by a single cycle of assembly–disassembly in the presence of 4 M glycerol (Clark et al., 1981). GTP was not substituted or supplemented with ATP in this preparation. The microtubule-containing pellets were resuspended in buffer B (0.1 M Mes, 0.5 mM $MgCl_2$, 0.1 mM EGTA, 1 M NaCl, 3 mM β-mercaptoethanol, 0.1 mM PMSF, pH 6.75) containing 3.4 M glycerol, incubated on ice, and clarified by centrifugation (30000*g*, 4 °C, 30 min). The supernatant was placed in a boiling water bath for 5 min, cooled at 4 °C, and clarified by centrifugation (30000*g*, 4 °C, 30 min). The "whole MAP fraction" was precipitated from the supernatant at 50% ammonium sulfate saturation. The precipitate was resuspended in buffer A for NMR experiments or buffer B for chromatography or storage at −70 °C, after being frozen in liquid N_2. This whole MAP fraction was fully effective at 0.05–0.1 mg/mL in stimulating the assembly of tubulin dimer (0.95 mg/mL) in 1 M glycerol, pH 6.5 at 37 °C.

HMW2 and τ proteins were separated by chromatography of whole MAP fraction on Sepharose 4B (Pharmacia). Typically, 3–4 mL of clarified protein (50000*g*, 4 °C, 40 min) was applied to a 60 × 1.6 cm column of Sepharose 4B preequilibrated with buffer B (without glycerol). The column was eluted at 4 °C at 20 mL/h, and 3-mL fractions were collected. The fractions containing purified HMW2 or τ were identified by $NaDodSO_4$–polyacrylamide gel electrophoresis, pooled, and precipitated at 50% ammonium sulfate saturation. The precipitates were resuspended in buffer A and dialyzed overnight against several changes of buffer A.

Tryptic Digestion. Limited tryptic digestion of assembled microtubules, resulting in the release of the portion of HMW2 that projects from the microtubule surface, was performed as described by Vallee & Borisy (1977). Trypsin (Worthington, type TRTPCK; 0.5 μg/mg of MT protein) was added to microtubules assembled at 30 °C in buffer A containing 1 mM GTP. The digestion was quenched after 5 min by addition of 15-fold excess (by weight) soy bean trypsin inhibitor (Sigma, type I-S). Trypsin-treated microtubules were collected by centrifugation (50000*g*, 30 °C, 40 min) and resuspended in buffer A.

Assembly Kinetics. The kinetics of microtubule assembly were studied by turbidimetric assay at λ = 330 nm in a jacketed 1-cm cylindrical cell. MT protein (~1 mg/mL) at 15 °C in the appropriate buffer containing 1 mM GTP was induced to assemble by raising the temperature to 37 °C ($t_{1/2}$ for temperature jump was 12 s). Data recorded on the Cary 118 spectrophotometer were digitized and analyzed by nonlinear least-squares routines (Bevington, 1969) with a PDP11/23 computer.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J-41C spectropolarimeter with data transfer to the PDP11/23 for processing and graphics output, as described previously (Clark et al., 1981; Martin et al., 1982).

NMR. ^1H NMR spectra were obtained at 270 MHz on a Bruker WH270 spectrometer in the Fourier-transform mode. The general procedures were described in the previous paper (Woody et al., 1982). Samples contained approximately 1–4 mg/mL protein in 0.4 mL of D_2O containing buffer A and 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as a chemical shift and intensity reference. Peak areas were determined by cutting and weighing peaks or by integration with computer graphics. The base line for peak areas was assumed to be a set of line segments connecting the minima between the following pairs of peaks (in ppm): (a) 0.62 (DSS) and 0.93; (b) 0.93 and 1.21; (c) 1.39 and 1.66; (d) 2.03 and 2.30; (e) 2.30 and ca. 2.6. The combined area of the 1.21 and 1.39 ppm peaks was resolved into two peaks by dropping a vertical line from the intervening minimum to the base line, as was that of the 1.66 and 2.03 ppm peaks. Intensities were measured relative to a known concentration of DSS. Where indicated, spectral resolution was improved by Gaussian multiplication (Lindon & Ferrige, 1980).

Miscellaneous. Protein concentration was estimated by the method of Bradford (1976) with bovine serum albumin as standard. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) as described previously. Since HMW2 and τ fractions may bind Coomassie blue dye anomalously, an independent concentration determination was performed by amino acid analysis with norleucine as a standard. Analytical ultracentrifugation was performed on the Beckman Model E ultracentrifuge with schlieren optics as previously described (Bayley et al., 1982). Electron microscopy was performed in parallel with the assembly assay. Rough-walled microtubules of normal morphology were observed in samples negatively stained with uranyl acetate.

Results and Discussion

Evidence for MT Protein Stability in D_2O. In view of the length of time (0.5–4 h) for accumulation of the NMR spectra, the known instability of MT protein preparations, and the possible effects of D_2O on assembly kinetics and oligomer distribution, it was necessary to carry out several types of control studies. First, it was important to establish the possible extent of denaturation occurring during these measurements. Near-UV CD spectra were taken for the samples in D_2O over a 24-h period and compared with those of controls in H_2O-containing buffers. Figure 1 shows that the D_2O buffer has a marked stabilizing effect on the protein conformation, there

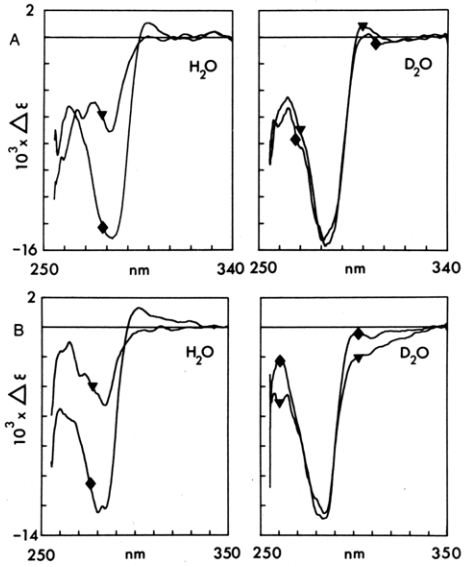

FIGURE 1: Near-UV circular dichroism spectra of (A) MT protein and (B) MgPCT in water and D_2O, showing effect of storage for 24 h at 5 °C. Protein concentration: (A) 1.2 mg/mL; (B) 1.3 mg/mL. Spectra recorded at 15 °C; (♦) $t = 0$; (▼) $t = 24$ h. 0.1 M Mes, pH 6.5.

being effectively zero change after 24 h at 5 °C, whereas the control shows substantial changes. Comparable experiments using Mg–phosphocellulose–tubulin dimer, known to be somewhat less stable than the MT protein preparation, show that D_2O also effectively stabilizes the dimer over a period of at least 24 h.

It was also necessary to establish assembly competence in D_2O after completion of the NMR experiments. Typically, the protein sample was made 1 mM in GTP and was warmed to 37 °C. Assembly was observed to proceed to >90% of the value expected for standard buffer conditions.

The kinetics of microtubule assembly in D_2O buffers have also been studied. Experiments in which the full-time course of assembly is analyzed show that the characteristic process of assembly of MT protein is biphasic (Barton & Riazi, 1980) and this is known to be a function of solution conditions, particularly pH (Bayley et al., 1983). In D_2O buffers, the same biphasic character persists with both processes being approximately 3-fold slower. The faster process correlates with the presence of oligomeric species containing tubulin plus MAPs, and the pH dependence of the amplitudes of the two phases parallels the known stability of oligomeric forms as a function of pH as shown by analytical ultracentrifugation (Bayley et al., 1982) and CD spectroscopy (Martin et al., 1982).

Analytical ultracentrifugation directly confirms the persistence of the 30S oligomer forms of MT protein when D_2O is substituted for H_2O in the buffer (Figure 2). The oligomeric form appears with a characteristic hypersharp schlieren peak, the sedimentation coefficient of which is strongly dependent on protein concentration, but which extrapolates close to $s^0_{20,w} = 30$ S. Thus the preparation behaves in a closely comparable manner in both D_2O and H_2O buffers, and the enhanced hypersharp characteristics of the microtubule protein preparation in D_2O could indicate even greater stability and uniformity of the 30S species in this medium.

In view of these results, we conclude that the MT protein retains its conformation, assembly competence, and oligomeric distribution during the course of the NMR measurements. In fact, D_2O has a marked stabilizing effect on the conformation of MT protein; an identical effect is found with MgPCT. The

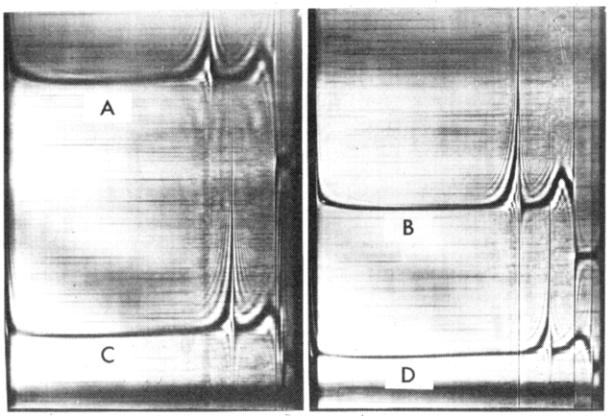

FIGURE 2: Analytical ultracentrifugation of microtubule protein at 13 °C in 20 mM phosphate in 60% D_2O (pD 6.5) at concentrations of (A) 1.9, (B) 3.8, (C) 5.7, and (D) 7.6 mg/mL. Photographs at 20 min at 50 726 rpm (A and C) and 50 703 rpm (B and D).

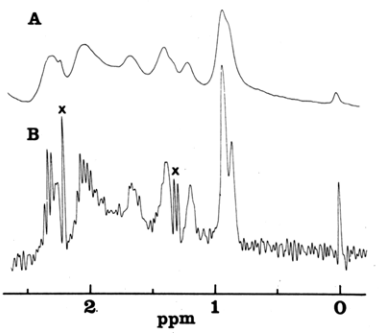

FIGURE 3: (A) ^{1}H NMR spectrum of cold-disassembled MT protein. Protein concentration was 4.7 mg/mL in buffer A plus 1 mM GTP, 10 °C. No pulse delay was used in this experiment, so the DSS peaks at 0 and 0.6 ppm are suppressed relative to the protein peak. (B) Resolution-enhanced spectrum of cold-disassembled MT protein. The sharp features marked x are due to traces of low molecular weight impurities, introduced during handling.

stabilizing effects of D_2O on microtubules in vivo were recognized from birefringence studies (Inoue & Sato, 1967).

^{1}H NMR of Cold-Dissociated MT Protein. The aliphatic region of the ^{1}H NMR spectrum of microtubule protein is shown in Figure 3, together with a resolution-enhanced spectrum, which reveals some finer details. As discussed in our previous paper (Woody et al., 1982), this spectrum clearly indicates the presence of a component in cold-dissociated microtubule protein with high mobility on the NMR time scale. Table I gives the chemical shifts and approximate intensities of the various protein resonances observed upfield from 2.5 ppm. The positions of the aromatic proton resonances and their total intensity are also given in Table I, though not shown in Figure 3 [cf. Figure 1 of the previous paper (Woody et al., 1982)]. The estimates of the number of protons and of residues are evaluated for a protein of M_r 100 000.

The resonances in the methyl region provide evidence that the large hydrophobic residues, Ile, Leu, and Val (0.9–0.95 ppm) and also Ala and Thr (1.4 and 1.2 ppm), are present in the highly flexible region. The methylene portion of the spectrum is more difficult to interpret unambiguously, but the resolution-enhanced spectrum (Figure 3B) provides evidence for a substantial contribution from Glu and Gln residues, in the form of the sharp multiplets centered at 2.3 and 2.0 ppm, characteristic of the β- and γ-proton resonances of these residues. The 2.0 ppm band may also contain signals from Met. The 1.7 ppm region probably contains contributions from the β- and δ-methylene protons of Lys residues and the β and δ protons of Arg.

Table I: ^{1}H NMR Peak Positions and Intensities for Microtubule Protein

chemical shift (ppm)	protons/ 100 kdaltons[a]	assignment	residues/ 100 kdaltons
0.92	75 ± 11	Ile (γ, δ), Leu (δ) Val (γ)	13
1.21	13 ± 2	Thr (γ)	4
1.39	27 ± 2	Ala (β)	9
1.66	20 ± 2	Lys (β, δ), Arg (β, δ)	5
2.03	47 ± 5	Glu (β), Gln (β)	15
		Met (β, ϵ) (?)	(4)[b]
2.30	29 ± 5	Glu (γ), Gln (γ)	15
6.79	20 ± 7	Tyr (ϵ)	2
7.10		Tyr (δ)	
7.30		Phe (δ, ϵ, ζ)	2

[a] Mean of five independent experiments with the standard deviation calculated with Student's formula. The proton count and approximate residue count are evaluated per 100-kdalton protein. [b] The count of Met residues is very tentative and is based upon subtracting from the 2.0 ppm peak intensity the contribution expected from the β protons of Glu + Gln, on the basis of the 2.30 ppm intensity. The aromatic residue count is also uncertain but is based upon the observation that the tyrosine and phenylalanine peak areas are comparable.

Thus, the ^{1}H NMR spectrum of cold-dissociated MT protein indicates the presence of a substantial proportion of residues in flexible regions of the polypeptide chain. In addition to the residues assigned in Table I, residues such as Gly, Pro, Ser, Cys, Asn, and Asp may also occur in the flexible portion of the protein, but their resonances are not observed, being obscured by the strong signals due to residual HDO and buffer components. There is no evidence for either His or Trp protons, which implies that if these residues occur in the flexible portion of the chain, they must be considerably less abundant than Phe or Tyr. The total intensity of the resonances corresponding to the residues listed in Table I amounts to approximately 5–6% of the total protein in the sample. Allowing for the residues whose resonances would be obscured, it appears reasonable to estimate that no more than 10% of the residues of the microtubule protein have sufficient mobility (on the 10^{-8}-s time scale) to give rise to sharp NMR signals.

The demonstration of assembly competence and of enhanced stability in D_2O-containing buffers eliminates the possibility that the resonances derived from denatured protein. The reproducibility of the intensities, as indicated by the relatively small standard deviations, provides further support, as does the absence of any detectable resonance signals from two amino acids (Trp and His) known to be present in microtuble protein.

It was demonstrated in the previous paper (Woody et al., 1982) that the flexible portions of the polypeptide chain are not associated with tubulin. This conclusion was based upon (a) the absence of the sharp signals from the NMR spectrum of purified tubulin and (b) the presence of such signals in the spectrum of a partially purified MAP preparation. In a preliminary paper, Ringel & Sternlicht (1981) have reported the presence of narrow resonances in the ^{13}C NMR spectrum of bovine microtubule protein. The observation of sharp ^{13}C NMR signals for microtubule protein is qualitatively consistent with our results. However, their suggestion that tubulin itself is highly flexible, with up to 50% of the protein in a random-coil conformation, is inconsistent with our quantitation of the ^{1}H NMR for MT protein; it may reflect the difficulties in making quantitative intensity measurements in ^{13}C NMR. The assignment of the sharp signals to tubulin itself is also in conflict with our previous interpretation of the ^{1}H NMR results, which is further substantiated in this paper by additional experiments. Also, CD spectroscopy shows that the native protein contains extensive secondary structure (α 23%, β 31%, β-turn 16%; Bayley et al., 1983), and while this structure can be enhanced to 66% α-helix in 85% TFE, the transition involves a β to α transformation with the aperiodic component approximately constant at 30%. Thus there is no evidence in native tubulin for extensive random-coil conformation.

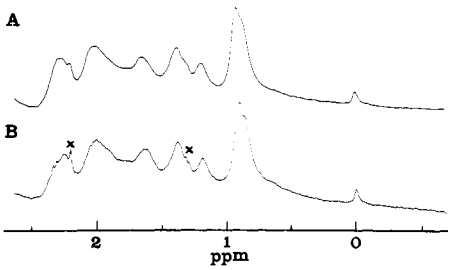

FIGURE 4: Effect of assembly on ^{1}H NMR spectrum of MT protein: (A) same as Figure 3A; (B) spectrum of assembled MT protein. Conditions identical with (A) except that sample was warmed to 30 °C and allowed to stand for 30 min before the spectrum was recorded. The features denoted by an x are due to trace low molecular weight impurities.

Effect of Assembly on ^{1}H NMR Spectrum of MT Protein. The aliphatic region of the ^{1}H NMR spectrum of the microtubule protein before and after assembly is shown in Figure 4. The spectra were initially recorded on the sample of microtubule protein in the disassembled form at 10 °C, and the sample was then warmed to 30 °C in the spectrometer. An additional 30 min was allowed before data were collected, to permit complete assembly. That assembly had occurred was indicated by visual inspection of the sample, and by comparison with the assembly kinetic experiments, performed in parallel under very similar conditions. Comparison of the spectra in Figure 4 shows that microtubule assembly has no significant effect on the intensity or positions of the sharp features in the NMR spectrum. The lines are sharper and better resolved in the spectrum of the assembled form, contrary to what might have been expected on the basis of the relative sizes of the species present. This sharpening effect presumably reflects a shorter rotational correlation time for the mobile regions due to the increased temperature and decreased local viscosity. Quantitative determination of the peak areas in the aliphatic region shows small increases in some peaks and decreases in others. Of the six peaks considered, only the 1.66 ppm peak showed a change in excess of the standard deviation for the corresponding peak given in Table I (Δn = 2.9 protons vs. a standard deviation of 2.2). Thus, within our experimental error, microtubule assembly leads to no significant changes in the number of mobile side-chain groups.

The absence of significant changes in the NMR upon assembly indicates that the flexibility observed in oligomeric species present in cold-disassembled microtubule protein persists undiminished even in the macroscopic structure of assembled microtubules. Estimation of the relative molecular mass of a microtubule is necessarily approximate but, for a microtubule of length 10 μm, may be estimated as $M_r \sim 10^9$. The mobile portions must therefore be able to undergo relatively free and rapid reorientation, largely independent of the supramolecular assembly as a whole. The most reasonable explanation for this result is that the flexible region is associated with the pendant portion of the HMW-group proteins. These have been identified as the diffuse-staining structures located at regular intervals along the length of the microtubule (Amos, 1979) and extending laterally in processes that appear

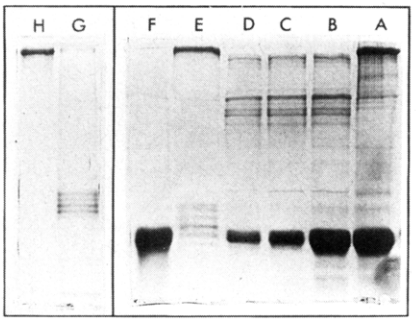

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis on a 7.5% gel showing products of trypsin treatment of assembled microtubules and preparation of pure τ and HMW2 fractions. The tracks are as follows: (A) MT protein; (B) total trypsin digest (t = 5 min); (C) supernatant of trypsin digest; (D) same as (C) after boiling for 5 min; (E) whole MAP fraction from MT protein; (F) resuspended microtubules from trypsin treatment; (G) purified τ fraction; (H) purified HMW2 fraction.

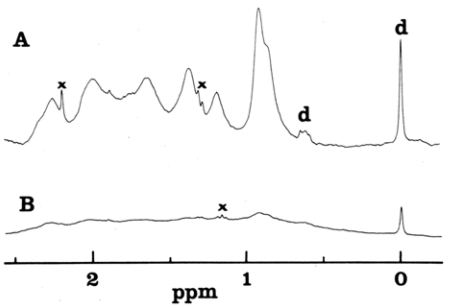

FIGURE 6: (A) ^1H NMR spectrum of supernatant fraction from tryptic digestion of assembled microtubules. Protein concentration was 1.5 mg/mL in buffer A, 10 °C. A 5-s pulse delay was used. (B) ^1H NMR spectrum of resuspended pellet from tryptic-digestion experiment. Protein concentration was 1.3 mg/mL in buffer A, 10 °C. No pulse delay was used in this experiment. The spectra are presented at approximately equal vertical gains. The features marked x are due to low molecular weight impurities, while those marked d are due to DSS.

unstructured in electron microscopic preparations (Zingsheim et al., 1979). This hypothesis may be tested directly with selective proteolytic cleavage of assembled microtubules.

Effect of Tryptic Digestion on ^1H NMR of Microtubules. In the experimental approach of Vallee & Borisy (1977), mild tryptic digestion of microtubules assembled from microtubule protein causes specific cleavage of the HMW2 protein into a large fragment (M_r ~240 000), which is released, and a small fragment (M_r 30 000–40 000), which remains associated with the assembled microtubule. This procedure was followed for the MT protein in D_2O-containing buffers, and the progress of the modification was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5). After tryptic digestion for 5 min, the intense HMW2 band disappeared, giving rise to bands at M_r 240 000, 215 000, and 185 000 and at M_r 32 000–39 000 (Vallee, 1980). Sedimentation produced a supernatant (track C) containing these components plus some tubulin dimer, the content of which was selectively reduced by heat treatment. By comparison, the pellet collected by centrifugation is essentially devoid of high molecular weight components (track F). Vallee & Borisy (1977) have shown by electron microscopy that the pellet from such a procedure consists of smooth-walled microtubules.

The NMR spectra of the supernatant and the resuspended pellet are shown in Figure 6 and clearly indicate that the origin of the sharp resonances is in the supernatant, i.e., with material released by the tryptic digestion. This appears to be almost

Table II: Amino Acid Compositions (mol %) of Bovine HMW2 and τ Fractions Compared with Published Data[a] on Porcine MAP Fractions

amino acid	bovine whole τ fraction	porcine whole τ fraction[a]	bovine HMW2	porcine HMW proteins[a]
Lys	8.4	9.7	8.2	10.1
His	1.5	2.6	2.4	1.8
Arg	3.2	3.6	3.3	3.2
Asp		9.7		8.4
Asp + Asn	9.1		8.8	
Thr	7.2	7.6	6.6	6.7
Ser	9.8	10.1	9.4	10.4
Glu		10.8		13.6
Glu + Gln	13.7		17.5	
Pro	11.2	10.4	8.5	6.1
Gly	10.5	10.9	7.0	10.9
Ala	9.1	7.9	8.6	9.1
Val	4.9	5.1	5.3	4.6
Met	1.0	1.2	0.6	1.5
Ile	2.3	2.6	2.7	2.8
Leu	5.8	5.5	6.7	7.1
Tyr	0.8	1.0	1.4	1.6
Phe	1.2	0.9	2.0	2.1
Cys	0.06	0.2	0.2	

[a] Data from Cleveland et al. (1977).

exclusively the pendant portion of HMW2 as characterized by Vallee & Borisy (1978), which has been implicated as being responsible for the anomalous concentration dependence of the sedimentation coefficient of the 30S species. We note that the HMW1 band also disappears upon tryptic digestion. However, HMW1 is only a minor component in this preparation.

^1H NMR of τ-Group Proteins and HMW2; Reconstitution with Tubulin Dimer. Although the tryptic digestion experiment provides strong evidence for the location of the flexible polypeptide chain in the pendant portion of HMW2, the role of τ protein needs to be clarified. It is not clear what the fate of the τ proteins is in the tryptic digestion experiments. Because of its low staining capacity, τ is not readily identified on gels in the presence of other proteins, particularly tubulin dimer. We previously showed (Woody et al., 1982) that an unfractionated mixture of MAPs (Figure 5, track E) shows the sharp resonances characteristic of MT protein. However, it is important to establish the relative importance of τ and HMW2 to the flexible regions observed in unfractionated MAPs and to investigate the effect of tubulin dimer on the flexibility of the MAP components.

Fractionation of a MAP preparation prepared by a slightly modified procedure gave purified τ proteins and HMW2 (Figure 5, tracks G and H). It may be noted that the τ bands show progressively intense staining, with the most intense at M_r 55 000, differing somewhat from the preparation of Sandoval & Weber (1980) (from porcine brain) in which an alternation of stained band intensity is observed. The HMW2 preparation is devoid of HMW1, which is known to be lost in the heat treatment (Kuznetsov et al., 1981). This preparation is evidently free from specific proteolytic digestion products.

The results of amino acid analysis for the preparations of τ protein and HMW2 protein are given in Table II and compared with the results of Cleveland et al. (1977) for τ and HMW protein (HMW1 and HMW2), respectively. In general the results for the τ preparation show good agreement with the published values for the whole τ fraction, with small deviations for residues Glu, Ala, Lys, and His. For HMW2, the results show close agreement for most residues, with significant

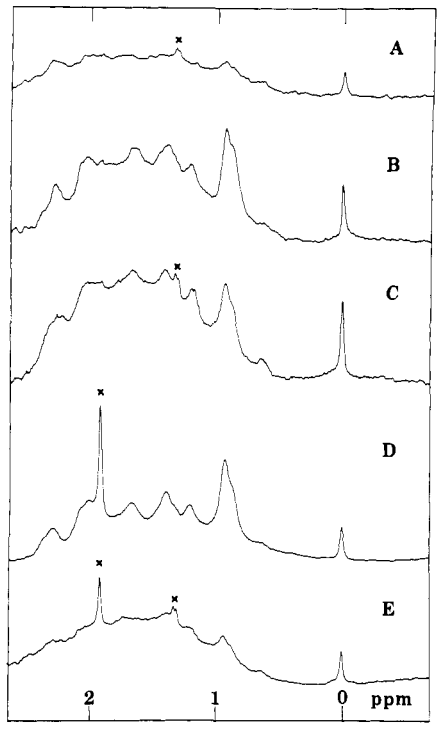

FIGURE 7: ^{1}H NMR spectra of τ protein and HMW2 protein before and after addition of tubulin dimer: (A) MgPCT (1.68 mg/mL), gain 0.5; (B) HMW2 protein (0.84 mg/mL), gain 1.0; (C) HMW2 protein (0.42 mg/mL) plus MgPCT (0.82 mg/mL), gain 2.0; (D) τ protein (0.70 mg/mL), gain 1.0; (E) τ protein (0.29 mg/mL) plus MgPCT (0.99 mg/mL), gain 2.0. Vertical gain is adjusted to provide comparisons; sharp features marked x are due to traces of low molecular weight impurities.

differences for Glu, Pro, Gly, and Lys. These may be due to the absence of HMW1 protein, which is removed in the heat step used for the HMW2 protein preparation.

The ^{1}H NMR spectra of τ and HMW2 are shown in Figure 7. The spectra of both proteins show evidence for highly flexible polypeptide regions. For τ protein (Figure 7A), the high-field methyl resonances of Ile, Val, and Leu have an intensity corresponding to 113 residues, which is comparable to the total number of such residues in τ [78 according to the amino acid composition data of Cleveland et al. (1977)]. Thus, our data indicate that purified τ protein is essentially a random coil. This is consistent with the anomalously low sedimentation coefficient, large Stokes radius, and weak far-UV CD (Cleveland et al., 1977).

When purified tubulin is added to τ protein, the intensity of the high-field methyl resonance in the mixture is only 59% of that expected, after the effect of dilution is taken into account. The 40% reduction observed here is almost certainly a lower limit for the following reasons. It is likely that only a portion of the τ protein combines with the tubulin because the mass ratio of τ:tubulin is in considerable excess over that characteristic of the microtubule protein preparation ($\sim$0.05). Also, recombination may be incomplete because the conditions of pH, temperature, and ionic strength are not necessarily optimal, and the ability of this τ fraction to recombine with tubulin has not been fully quantitated. Further, while purified tubulin does not give sharp peaks in the ^{1}H NMR spectrum, it may contribute to the intensity of the high-field methyl peak, and it is present in excess. Thus we observe a significant immobilization of the residues in τ upon recombination with tubulin, and as a minimum value, it appears that about half of the residues may have undergone essentially complete immobilization.

The high-field methyl resonances of Ile, Val, and Leu in HMW2 (Figure 7B) correspond to approximately 252 residues for a value of M_r = 270000 compared with a total of 360 such residues in HMW2 [Cleveland et al. (1977) and Table II], i.e., corresponding to approximately three-quarters of the molecule. Purified HMW2, therefore, shows evidence for both an extensive highly flexible region and, perhaps, one or more compact globular domains. The hydrodynamic properties (Vallee & Borisy, 1978) and CD spectrum of HMW2 (P. M. Bayley, D. C. Clark, and S. R. Martin, unpublished results) are consistent with substantial random-coil character.

The results of adding purified tubulin to HMW2 are also shown in Figure 7. The area of the high-field methyl resonance apparently shows a small increase (18%) over that expected, taking dilution into account. All of the factors mentioned in the case of τ would apply equally in the HMW2 reconstitution experiment, but only the tubulin contribution would actually lead to an increase in intensity. Although there might be a small increase in the extent of flexibility in HMW2 upon reconstitution with tubulin, the simplest interpretation of the present data is that no significant change in flexibility occurs in HMW2 upon combination with tubulin.

It thus appears that in its interactions with tubulin dimer, τ, which has an open and flexible structure, undergoes a substantial reduction in the degree of flexibility. Vallee & Borisy (1978) observed smooth 20S rings in mixtures of tubulin and purified τ, and we infer that a considerable conformational restriction is imposed on τ on forming this type of oligomeric complex. By contrast, HMW2, which also has an open flexible structure in a large part of the molecule, is able to form 30S oligomers without substantial impairment of its flexibility. The interaction between tubulin and HMW2 presumably occurs most strongly through the M_r 30000–40000 fragment, which remains associated with the microtubules after partial proteolysis of HMW2 (Vallee & Borisy, 1978; Vallee, 1980). This would leave the major portion of HMW2 unaffected by these interactions and allow it to retain a high degree of mobility even in combination with an assembled microtubule. We therefore infer that the flexible portion observed by NMR is located within the pendant part of HMW2, and this is confirmed by the tryptic-digestion experiments. Scheele & Borisy (1979) rationalized the anomalously low sedimentation coefficient of the 30S oligomer and its strong dependence on protein concentration in terms of a "viscous-drag" effect, produced by the pendant portion of HMW2. The flexible structure demonstrated by ^{1}H NMR to be a major component of this part of the HMW2 structure could indeed be responsible for the unusual hydrodynamic properties of 30S oligomers and also could account for the diffuse structure observed (in electron microscopic studies) peripheral to the surface of rough-walled microtubules assembled in vitro from MT protein.

With regard to assembled microtubules, the presence of a high degree of molecular flexibility in the HMW2 proteins could have important biological function. MAPs have been implicated in promoting the interactions of microtubules with other cytoskeletal components, e.g., interactions between microtubules and actin microfilaments in vitro (Griffith & Pollard, 1978) and between microtubules and intermediate filaments in vivo (Geiger & Singer, 1980) and in vitro (Runge et al., 1981). It may be postulated that in an interacting polymer network, the nature of the cross-linking structures will be an important factor in determining the physical properties of the subsequent three-dimensional matrix. The flexibility observed in HMW2 may thus be important in conferring

specific structural properties to the cytoskeletal network in regions of interaction between microtubules and other components. At the molecular level, this flexibility would confer structural stability together with the capacity to respond to time-dependent requirements for reorientation and translocation within the cytoplasm, which would not be available in a rigidly cross-linked cytoskeleton. Such a model would also allow a degree of control by modulation of the properties of the bridging structures by enzymic modifications such as phosphorylation, by specific ligand binding, or by attachment of additional protein or other soluble factors.

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