^{1}H NMR EVIDENCE FOR FLEXIBILITY IN MICROTUBULE-ASSOCIATED PROTEINS AND MICROTUBULE PROTEIN OLIGOMERS

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1. Introduction

The ability to perform reversible cycles of assembly and disassembly in preparations of mammalian brain microtubule protein [1,2] allows detailed molecular investigation in vitro of the mechanism of an important biological self-assembly system [3]. This process has a central role in the ordered function of the cytoskeleton, as for example in mitosis [4]. Cold-disassembled microtubule protein is composed of tubulin dimer (M_r 100 000) and oligomeric complexes of tubulin dimer plus microtubule-associated proteins (MAPs), principally the high-M_r group (HM_r1 and 2, M_r 270 000–350 000) and tau-group proteins (M_r 55 000–75 000) [3].

We now report a ^{1}H NMR study of microtubule proteins in which we find, in spite of the high molecular masses involved, that a substantial number of relatively sharp resonances are observed. These resonances are absent from preparations of tubulin dimer, but are present in preparations of microtubule-associated proteins which must therefore possess significant flexibility. We infer that the sharp resonances observed with the oligomeric complexes of microtubule protein derive, to a major extent, from the HM_r2 molecule.

Abbreviations: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; buffer A, 20 mM sodium phosphate, 0.5 mM $MgCl_2$ in D_2O (pD 6.5); buffer B, 0.1 M 2[*N*-morpholino]ethanesulfonic acid, 0.3 M NaCl, 0.5 mM $MgCl_2$, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethyl sulfonyl fluoride (pH 6.5)

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2. Materials and methods

Microtubule protein was prepared from fresh bovine brain using glycerol-free buffers, by the method in [5]. Pelletted samples of 3-times cycled microtubule protein were stored at −70°C. For NMR experiments, the protein was thawed, and cycled once with the ^2H_2O-containing buffer A, plus 1 mM GTP. The cold-dissociated protein was resuspended in buffer A at ~5 mg/ml, dialysed against several changes of this buffer over 4–5 h at 5°C, and used directly.

MAP-free tubulin dimer was prepared by chromatography on phosphocellulose pre-equilibrated with excess Mg^{2+} as in [6,7]. Peak fractions were dialysed immediately against several changes of buffer A.

MAPs were prepared by a procedure employing a heat treatment [8–10]. Bovine microtubule protein was prepared by a single cycle of assembly–disassembly in the presence of 4 M glycerol, and the microtubule-containing pellets were resuspended in buffer B. The clarified supernatant was placed in a boiling waterbath for 5 min, cooled to 0°C, clarified and treated with DEAE–Sephadex A-50. The solution containing MAPs was precipitated at 50% ammonium sulphate saturation, and the protein stored at 4°C, and clarified by centrifugation before use.

^{1}H NMR spectra were recorded at 270 MHz with a Bruker WH270 spectrometer in the Fourier transform mode using quadrature detection. A spectral width of 4200 Hz was used and 4096 or 8192 data points were collected. The free-induction decay was multiplied by an exponential function producing a line broadening of 2 Hz. For quantitative intensity measurements a 5 s delay between pulses was used to allow full relaxation of the chemical shift and intensity standard DSS.

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Protein concentrations were estimated as in [11] using bovine serum albumin as standard. SDS–Polyacrylamide gel electrophoresis was done as in [7].

3. Results

The 270 MHz ^{1}H NMR spectrum of microtubule protein in buffer A (fig.1) consists of a very broad component on which are superimposed a substantial number of relatively sharp lines. Under these conditions, the microtubule protein preparation exists as ~50% tubulin dimer (M_r 100 000) and 50% 30 S oligomer ($M_r \sim 5 \times 10^6$ [3]) [12,13]. The measured linewidths, which range from 20–40 Hz (after correction for exponential multiplication, but without correction for any contribution from spin–spin splitting), are clearly much too small to arise from a rigid body of M_r 5×10^6, for which linewidths of the order of 8 kHz would be expected, and are somewhat smaller even than expected for methyl groups in a protein of M_r 100 000 [14]. Addition of NaCl to 0.1 or 0.5 M to dissociate the 30 S oligomers [12] had no effect on the positions, linewidths or intensities of the sharp signals in the spectrum. These sharp resonances are thus independent of the state of association of the protein, and this, together with the magnitude of the linewidths observed, strongly suggests that they arise from a region or regions of polypeptide chain having substantial mobility with respect to the particle as a whole [14].

The chemical shifts of these resonances correspond closely to those observed in simple peptides (e.g., 0.9 ppm for Val, Leu, Ile methyl protons, 1.2 ppm for Thr methyl and 1.4 ppm for Ala methyl protons) and are thus consistent with a random coil structure for the flexible region(s). In the part of the spectrum due to aromatic residues, resonances from Tyr (6.9 and 7.2 ppm) and Phe (7.3 ppm) are observed, their intensities suggesting that those 2 residues are present in approximately equal numbers, but no resonances from Trp are detectable.

The possibility that the sharp resonances in the spectrum are due to the presence of some denatured protein is ruled out by several observations:

(i) After the NMR experiment, the sample was at least 90% competent in an in vitro assembly assay, and assembly was completely reversible;

(ii) The absence of sharp signals from Trp implicates a specific part of the protein sequence;

(iii) The relative intensities of the various sharp signals are reproducible in several independently prepared samples.

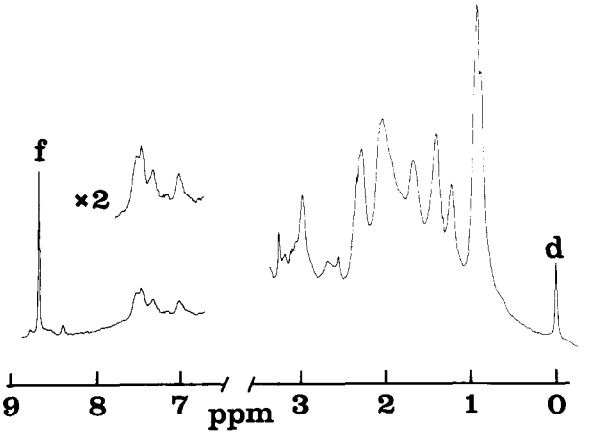

Fig.1. 270 MHz ^{1}H NMR spectrum of microtubule protein (7.3 mg/ml in buffer A). The resonances marked d and f arise from the internal standards DSS and formate, respectively. The aromatic region of the spectrum is shown both at the same vertical gain as the aliphatic region and at twice that gain.

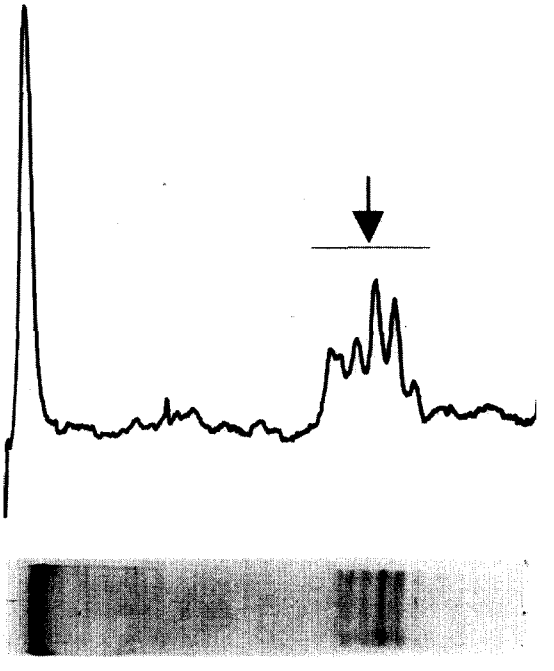

Fig.2. A 7.5% polyacrylamide gel of the MAP preparation stained with Coomassie blue, and the corresponding densitometer scan. The gel shows a single high M_r band corresponding to HM_r2. The tau group, indicated by the arrow is resolved into several bands.

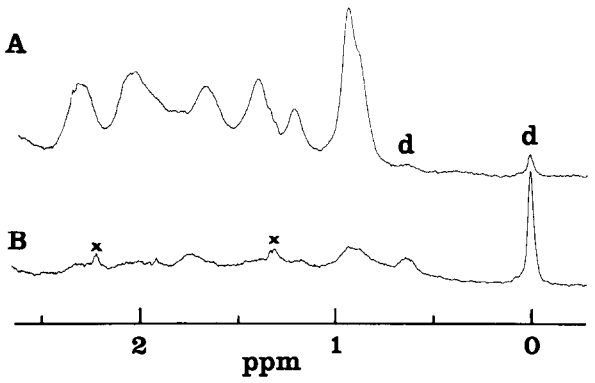

Fig.3. The aliphatic region of the 270 MHz ^{1}H NMR spectra of: (A) MAPs (0.65 mg/ml); (B) tubulin dimer (2.7 mg/ml). Allowing for the different protein concentration of the two samples, the spectra are presented at approximately equivalent vertical gain. The resonances marked d in the top spectrum (but present in both) arise from DSS, and those marked x from low-M_r impurities.

Accurate intensity measurements in spectra such as that in fig.1 are obviously difficult because of the uncertainty in drawing an appropriate baseline. However, at a conservative estimate (based on comparison with the intensity of the DSS signal and a knowledge of the protein concentration) the intensity of the sharp component of the spectrum corresponds to ~50 residues/100 000 M_r.

This microtubule protein preparation contains ~75% tubulin and ~25% MAPs [13]. Samples of purified tubulin and MAP fraction were examined to identify the origins of the resonances. SDS–Polyacrylamide gel electrophoresis (fig.2) shows that the MAP fraction contains approximately equal amounts of HM_r2 and tau-group proteins, assuming identical staining efficiency. HM_r1 is absent, as expected from its known heat-lability [15]. The purified tubulin dimer has an NMR spectrum (fig.3b) devoid of the strong sharp peaks observed in microtubule protein. Instead, the spectrum is consistent with that of a globular protein of M_r 100 000. By contrast, the NMR spectrum of the MAP fraction (fig.3a) shows the sharp peaks identical in position and relative intensity to those found for microtubule protein (fig.1).

4. Discussion

These experiments show clearly that some component of the microtubule protein preparation contains a highly mobile region of polypeptide chain, and that this mobility is retained in the 30 S oligomeric species. The striking differences between the NMR spectra of purified tubulin and of MAPs (fig.3) demonstrate that the observed sharp resonances are associated with MAPs and not with the tubulin molecule. It seems likely that both HM_r2 and tau could contribute to the spectrum of the free MAPs in fig.3a. Both proteins have anomalously low sedimentation coefficients [16,17] and low secondary structure ([17], unpublished), consistent with substantial regions of flexible polypeptide chain.

The extent of the mobile region of the polypeptide chain in the MAP component of the microtubule protein may be estimated as follows. The HM_r2 content of this preparation is ~20% by wt [13,15]. If all of the sharp resonances are attributed to HM_r2, this would require ~600 residues or ~25% of the protein to be highly flexible. Tau protein is much more difficult to estimate in SDS gels, but the content appears to be at most 5%. For tau to account for the observed intensities in microtubule protein, essentially the whole molecule would have to be flexible. Definitive evaluation of the relative contributions to the NMR spectra will require reconstitution experiments with purified individual MAP components.

Several lines of evidence indicate that HM_r2 may possess significant flexibility in oligomeric forms of microtubule protein. Microtubules assembled from whole microtubule protein show periodic projections [18–20] which have been shown by specific antibody labelling [21,22], reconstitution experiments [9,23,24] and protease digestion [16,25] to be due to a large portion of the HM_r2 molecule. (By contrast, microtubules assembled from tubulin and tau have relatively smooth walls [9,24].) The hydrodynamic properties of 30 S oligomers of microtubule protein have been attributed to the large pendant portion of HM_r2 associated with the ring species [3]. It is therefore reasonable to postulate that the resonances observed in microtubule protein derive to a major extent from the flexibility of this portion of the HM_r2 molecule. Preliminary experiments using tryptic digestion of microtubule protein are consistent with this hypothesis.

A role has been proposed for MAPs as bridging structures promoting the interactions of microtubules with other cytoskeletal components such as actin microfilaments and intermediate filaments [26,27]. The flexibility of microtubule-associated proteins could thus have considerable functional significance.

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