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A STUDY OF TUBULIN DIMER CONFORMATION BY NEAR-UV CIRCULAR DICHROISM

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Summary: The near-UV circular dichroism properties of tubulin dimer have been measured for different preparative methods. Tubulin dimer was obtained from assembly competent microtubule protein by gel filtration, or phosphocellulose ion-exchange chromatography in the presence of magnesium. Tubulin dimer prepared by the protocol of Weisenberg R.C. and Timasheff, S.N. (1970) Biochemistry 9, 4110-4116, was found to be markedly different due to some apparently irreversible change in conformation. We conclude that the removal of microtubule-associated proteins by phosphocellulose ion-exchange chromatography in the presence of magnesium can be performed without affecting the conformation of native tubulin dimer as judged by near-UV circular dichroism.

Pure tubulin dimer (WT-dimer) prepared by the "classical" scheme of Weisenberg and coworkers (1,2) has been well characterised by circular dichroism (3,4). The *in vitro* tubulin polymerisation system (5) using crude soluble brain extracts gave rise to a widely used method of preparation of brain tubulin: the assembly-disassembly method (6-7). This produces a mixture of proteins, (the MT-protein complex) of which tubulin forms the major constituent (>70%), the remainder being a group of proteins known collectively as microtubule-associated proteins (MAPs). MAP-free tubulin dimer of purity similar to WT-dimer may be prepared from MT-protein complex by ion exchange chromatography on phosphocellulose (MgPCT-dimer (8)) or gel filtration on Sepharose 6B (6BT-dimer (9)). Both WT-dimer (3) and dimer isolated from MT-protein complex (10) have been

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Abbreviations used are:-
MT - microtubule; MAP - microtubule associated protein; HMW - high molecular weight MAP; CD - circular dichroism; MES - 2[N-morpholino] ethane sulphonic acid; EGTA - Ethylene glycol bis(β-aminoethyl ether)-N, N'-tetraacetic acid; GTP - Guanosine triphosphate.

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shown to be capable of assembly into microtubules at high protein concentration (>8mg/ml)

The aim of our studies has been to compare the conformational properties and assembly characteristics of WT-dimer and MT-protein by circular dichroism (CD) and a turbidimetric assembly assay respectively. We have recently reported (11) that the near-UV CD spectrum spectra of MT-protein complex and MgPCT-dimer differ significantly from that of WT-dimer. In this communication we present further evidence, based on the observation that 6BT-dimer has a near-UV spectrum indistinguishable from MgPCT, that the tubulin dimer present in MT-protein complex and isolated from the latter represents tubulin in a conformation different from that displayed by WT-dimer.

Experimental Procedures

MT protein complex was prepared from fresh bovine brains by two cycles of a modification of the assembly-disassembly method of Shelanski (4) described elsewhere in detail (11). Immediately prior to experimentation, a suitable aliquot of MT-protein complex was removed from storage at -70°C, and subjected to one further cycle of assembly-disassembly. MAP-free tubulin dimer was prepared from MT-protein complex by chromatography on phosphocellulose that had been presaturated with Mg^{2+} (6,11). This material is MgPCT-dimer.

For gel filtration on Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden), typically, 3-4ml of MT-protein complex (4-8mg/ml) was applied to a 30 x 2.5cm column of Sepharose 6B (7) equilibrated with MEM100 buffer (100mM MES, 0.1mM EGTA, 0.5mM $MgCl_2$, pH 6.5) containing 4M Glycerol. The column was run at 25°C at a rate of 60ml/hr and 1.5ml fractions were collected.

Weisenberg tubulin (WT-dimer) was prepared by the method of Weisenberg and Timasheff (2), omitting the $MgCl_2$ precipitation step. Before each experiment a suitable aliquot of WT-dimer was removed from -70°C storage, thawed and dialysed against PMG buffer or reassembly buffer (0.01M MES, 1mM EGTA, 16mM $MgCl_2$, 10^{-4} GTP, 3.4M Glycerol, pH 7.0) to remove sucrose and clarified by centrifugation (100,000g, 20 minutes, 4°C).

Circular Dichroism (CD) spectra were recorded digitally from 340-250nm using a Jasco J41-C spectropolarimeter equipped with a model J-DPY Data Processor at a sensitivity of 50 x 10^{-2}m°/cm with an instrumental time constant of 16 seconds. Fused silica cells of path length 0.1, 0.2, 1, 2 and 4cm were used as required for near and far UV. The reported spectra are the average of multiple scans recorded from several samples from at least two independent protein preparations. T = 15°C unless otherwise specified. All spectra are expressed as molar circular dichroism $\Delta\epsilon$, based upon a mean residue weight of 110; molar ellipticity $[\theta]_{MRW} = 3300 \times \Delta\epsilon$

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (12), with the pH of the electrode buffer

increased to 9.1 (13) and using 7.5% gels. Protein concentration was determined by the method of Lowry (14) using bovine serum albumin as the standard.

Results and Discussion

The MT-protein complex obtained by assembly at 37°C is highly polymerised material with structure in the electron microscope characteristic of native microtubules. On cold disassembly, after clarification to remove a small amount of aggregated material, the solution contains a mixture of 'rings' and tubulin dimer (9). The exact nature of these rings depends upon various physical parameters (pH, temperature) and the presence of glycerol in the preparative method (15). However, these rings are always composed of tubulin dimer and MAPs.

The separation of rings and dimer in the cold dissociated material by gel filtration at 20°C on Sepharose 6B is shown in Fig. 1. If gel filtration is performed at lower temperature, the proportion of dimer is somewhat enhanced. The ring component elutes close to the exclusion limit of Sepharose 6B (MW 4×10^6) and the dimer component clearly undergoes gel permeation. For spectroscopy, the peak dimer fraction and the subsequent one or two tubes are pooled.

Polyacrylamide electrophoresis of these pooled fractions (Fig 2) shows the clear distinction between rings and dimer. In the ring fraction several MAPs are identifiable (HMW1 & 2, tau group, etc.). By contrast,

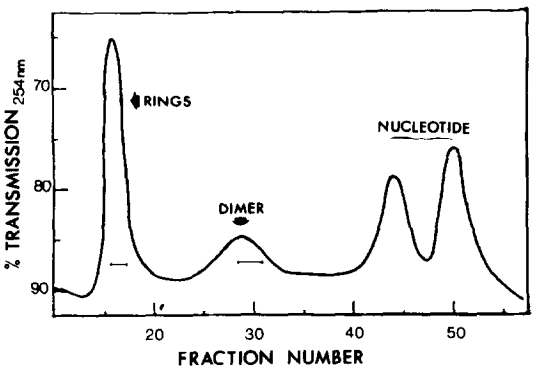

Fig 1 Gel filtration of microtubule protein complex on Sepharose 6B at 20°C showing separation of ring and dimer fractions.

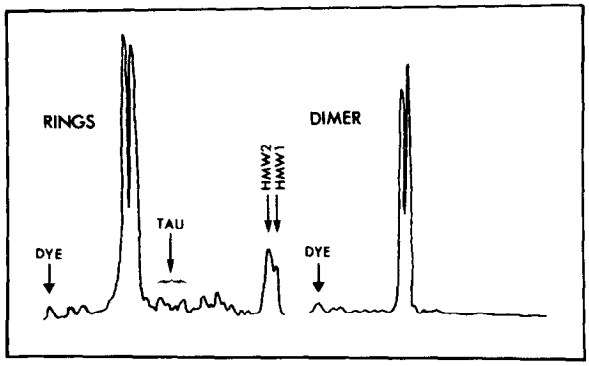

Fig 2 Polyacrylamide gel electrophoresis of Sepharose 6B ring and dimer fraction; densitometer traces of gels stained with Coomassie blue.

dimer fraction is estimated to be >99% dimer protein (i.e. the tubulin αβ heterodimer MW110,000); even heavy overloading (~200μg protein) of the gels indicates only very minor contamination with other proteins.

Fig 3 shows the near-UV absorption spectra of the two main components. The spectrum of rings shows clear evidence of light scattering even at 350nm and this contributes to the lower A_{277}/A_{250} ratio. The spectrum of dimer is much less affected by light scattering and the ratio A_{277}/A_{250} is 1.924 with A_{277} =0.969 for dimer 1mg/ml and 1cm path length cell, uncorrected for any scattering contribution. The region 250-270nm shows some fine structure indicative of the phenylalanine content;

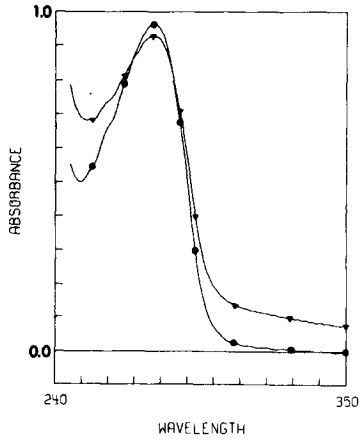

Fig 3 Near UV Absorption Spectra of (●) dimer and (▼) ring fraction from Sepharose 6B gel filtration of microtubule protein complex, normalised to 1.0 mg/ml and 1 cm pathlength, uncorrected for scatter.

the main peak corresponds to tyrosine and tryptophan absorption. Guanine nucleotide content, estimated by UV absorption following precipitation of the protein with 5% perchloric acid corresponds to 2 moles per tubulin dimer Preliminary results of coherent light intensity fluctuation measurements (Palmer, Satelle, Clark, Martin and Bayley, unpublished) show that the peak and subsequent fractions of dimer contain less than 0.2% high MW components.

The typical near-UV CD spectra of the dimer is shown in Fig 4a. There is a small leading positive CD lobe at 300nm and then negative CD representing the summed contribution of aromatic residues. The signal

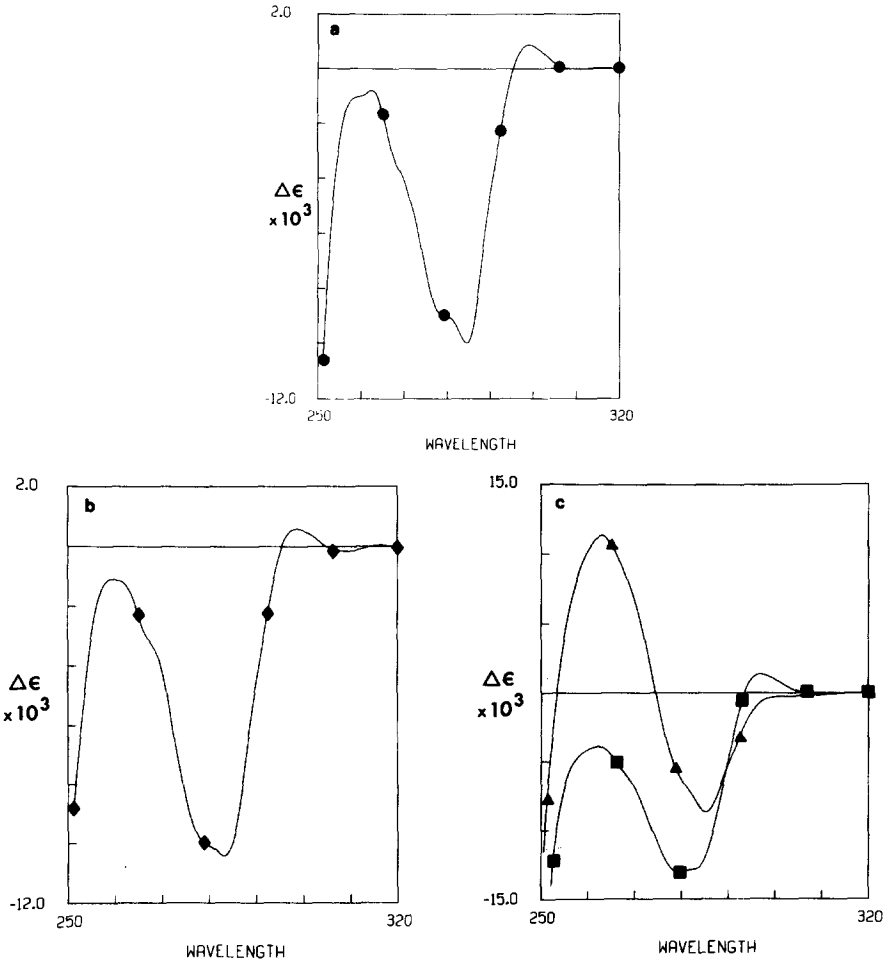

Fig 4 Near UV CD spectra of tubulin dimer and microtubule protein preparations; molar CD based on mean residue MW = 110.
a) Sepharose 6B dimer (●)
b) MgPCT-dimer (◆)
c) WT-dimer (▲) and MT protein complex (■).

comes close to zero at 260nm. The same CD spectrum is obtained when Sepharose 6B chromatography is performed at 20°C (plus glycerol) or 5°C (without glycerol) and it is independent of protein concentration. This spectrum is compared in Fig 4b with that of MgPCT-dimer. Also shown is the near-UV CD of WT-dimer and MT-protein complex (Fig 4c). Fig 4a and 4b show that MAP-free tubulin dimer has a characteristic near-UV CD spectrum which is the same whether it is prepared simply by gel filtration of cold-disassembled microtubule protein complex, or by ion-exchange chromatography on phosphocellulose in the presence of Mg^{2+} (8). Since gel filtration introduces no additional chemical treatment, and maintains the same buffer conditions as the starting material (MT-protein complex), we conclude this spectrum is typical of the tubulin dimer in the MT-protein complex. We have previously shown the similarity of the near-UV CD of MT-protein and MgPCT-dimer and the further identity of MgPCT-dimer with Sepharose 6B dimer confirms that this family of spectra are typical of the major component of MT-protein, namely the tubulin $\alpha\beta$ heterodimer. The spectrum of the WT-dimer in this region is significantly different, as discussed elsewhere (11).

The far UV-CD spectrum of Sepharose 6B dimer is found to be effectively the same as that of MT-protein complex, WT-dimer and MgPCT-dimer. Thus there is no large difference detectable in secondary structure between these various preparations.

These results indicate that tubulin dimer can be readily obtained free of MAPs, by gel filtration of cold-disassembled MT-protein obtained by the assembly-disassembly procedure. By gel electrophoresis and by laser light scattering this material corresponds to 99% pure tubulin dimer of MW 110,000. It has a near-UV CD spectrum identical with that of the phosphocellulose treated material (MgPCT-dimer), and thus authenticates this material as highly purified native tubulin dimer. From the similarity with the MT-protein complex we believe this material represents the conformation of tubulin dimer as present in the MT-protein

complex, which is the most efficient assembly-competent system as judged by the low values of critical concentration. The small but reproducible differences in CD properties between rings and dimers are presently under investigation.

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