PURIFICATION OF A MICROTUBULE-ASSOCIATED PROTEIN BASED ON ITS PREFERENTIAL ASSOCIATION WITH TUBULIN DURING MICROTUBULE INITIATION

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1. Introduction

Microtubules prepared from brain tissue by successive cycles of assembly and disassembly contain, in addition to tubulin, several nontubulin proteins which will promote in vitro microtubule assembly [1–3]. The nontubulin proteins have been fractionated, and the principal assembly-promoting component identified as high molecular weight proteins (HMW [1] or MAPs [3]) and lower molecular weight tau proteins [2,4]. Although either of these fractions can independently promote the assembly of purified tubulin, microtubules assembled from unfractionated preparations of microtubule proteins generally contain both HMW-MAPs and tau, as well as several other less prominent nontubulin proteins (e.g., [5]).

We have examined the sequence of association of the various nontubulin proteins with tubulin by SDS–gel electrophoresis of the assembled polymers sedimented at time intervals during the assembly process. In the initial stage(s) of assembly, corresponding to protofilament formation, we find an enrichment for one of the HMW-MAPs (tentatively identified as MAP-2 [3]). Subsequently, the other nontubulin proteins are associated with the polymer (microtubules) sedimented. We have purified the MAP-2 from the protofilament pellet and have examined its capacity to promote the assembly of purified tubulin as well as the structure of the intermediates and microtubules assembled.

Abbreviations: MAPs, microtubule-associated proteins; HMW-MAPs, high molecular weight components; Pipes, piperazine-*N*, *N'*-bis[2-ethane-sulfonic acid]; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*, *N'*-tetraacetic acid: GTP, guanosine triphosphate: MAB, microtubule assembly buffer; PC, phosphocellulose; SDS, sodium dodecyl sulfate

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2. Materials and methods

Microtubule proteins were obtained from bovine brain by two cycles of assembly and disassembly utilizing a microtubule assembly buffer (MAB) containing 100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM GTP at pH 6.4. The cycled microtubule proteins were fractionated by phosphocellulose (Whatman P11) chromatography [2] to yield a PC-tubulin fraction and a PC-MAPs fraction. Both fractions were dialyzed against MAB at 4°C prior to use in assembly experiments.

In the sedimentation experiments, PC-MAPs fraction (0.4 mg/ml) was mixed with PC-tubulin (2 mg/ml) at 4°C; alternatively, the twice cycled unfractionated microtubule protein preparation (3 mg/ml) was used. The samples were incubated at 37°C with increasing time. In all of these experiments the Sorvall SS-34 rotor was at 37°C and the incubation intervals stated (fig.1) include the acceleration time of the rotor. The polymer formed was sedimented at 37°C by a 15 min centrifugation at 39 000 × *g*. This was resuspended in warm MAB and prepared for negative staining EM, SDS–gel electrophoresis, or for subsequent fractionation.

For the purification of MAP-2, the samples were

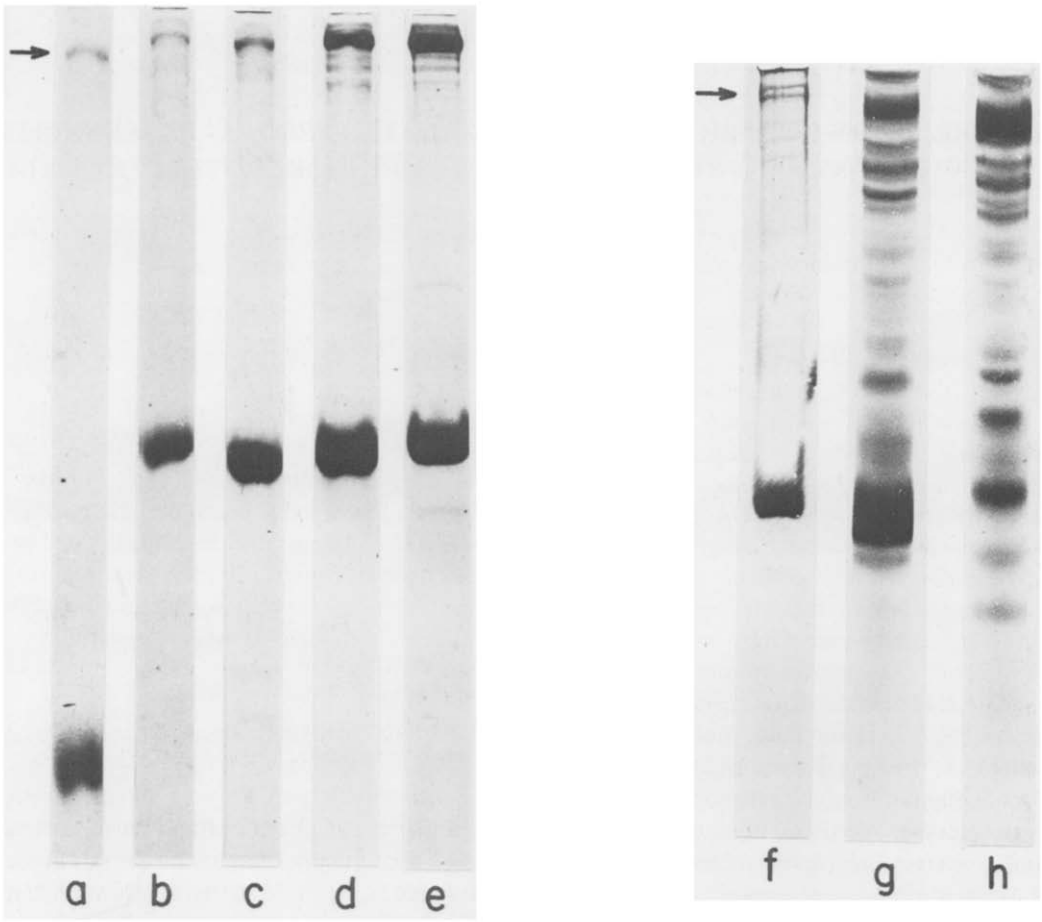

Fig.1. SDS–gels showing sequence of association of PC-MAPs (fig.1h, 0.4 mg/ml) with PC-tubulin (2 mg/ml) in sedimentable polymer formed at 37°C. Incubation for (a) 10 s, (b) 15 s, (c) 20 s, (d) 30 s, (e) 45 s, (f,g) 30 min, with low and high loading of gels. (a) had been electrophoresed further into the gel to show that only one HMW-MAP (arrow) was present in the 10 s pellet. Both MAP-1 and MAP-2 were present in microtubule pellets (arrow, f).

incubated briefly (10 s, 37°C), the polymer was sedimented as above, suspended in cold 4°C MAB, and fractionated by phosphocellulose chromatography [2].

Protein concentrations were determined by the Hartree modification [6] of the Lowry procedure. SDS–polyacrylamide gel electrophoresis was carried out using either 7.5% disc gels [7] or 10% slab gels with a 3% stacker [8]. Gels were stained with 0.5% aqueous Coomassie brilliant blue 250R and scanned at 546 nm with a Vitatron LD100 densitometer. The proportions of the various proteins were estimated by cutting out and weighing the areas under the densitometric scan peaks.

For negative staining EM samples were fixed for about 1 min by the addition of an equal volume of 4% glytaraldehyde in MAB at the appropriate temperature. One drop was applied to a formvar–carbon

coated grid for 1 min, washed with 5 drops of 0.4% photoflo, and stained with a drop of 1% aqueous uranyl acetate. For thin-sectioning studies, microtubules pelleted at 39 000 X *g* for 30 min were fixed with 4% glutaraldehyde (in MAB at 37°C) for 60 min, post-fixed in 1% osmium tetroxide at 4°C for 60 min, and prepared for thin-sectioning by standard methods (e.g. [9]).

Turbidometric measurements of microtubule assembly were made at 360 nm with a spectrophotometer (Gilford Model 2400) equipped with a temperature controlled cuvette chamber.

Analytical ultracentrifugation was carried out using a Beckman Model E with a single sector cell and Schlieren optics. Samples (at several protein concentrations) were centrifuged at a rotor speed of 48 000 rev./min at 5°C and photographs were taken at 4 min intervals. $s_{20,w}$ values were obtained by extrapolation to infinite dilution.

3. Results

The composition of the twice cycled unfractionated microtubules (similar to fig.1g) was ~60% tubulin, 16% HMW-MAPs, 8% tau proteins and 16% of other bands. In a less heavily loaded gel (fig.1f), it was clear that the two HMW-MAPs (MAP-1 and MAP-2, arrow) were the major nontubulin species. The PC-MAPs fraction contained most of the nontubulin proteins (fig.1h). The PC-tubulin fraction showed a single band on SDS–gels (as in fig.2c) and was incapable of self-assembly at the highest concentration (6 mg/ml) tested.

The order of appearance in time of the nontubulin proteins in the sedimentable polymers is shown in fig.1a–e. The first of these proteins associated with tubulin was the faster migrating of the HMW components, MAP-2 (fig.1a, arrow). With increased incubation times, there was a sequential appearance of MAP-1 (fig.1b,c), the other nontubulin proteins (fig.1c–e), and at still later times, the tau proteins (fig.1g). Densitometric scans of these gels showed that the proportion of MAP-2 to total protein in the polymer increased from about 10% (fig.1a), where it represented the only nontubulin protein, to 40% (fig.1e) in the first 45 s incubation. When the incubation times exceeded 60 s, there was an increase in the other nontubulin proteins and of

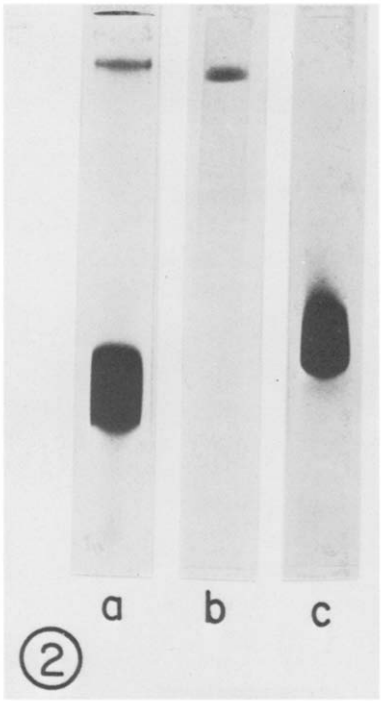

Fig.2. SDS–gels of 10 s (a) assembly product (conditions as in fig.1), (b) PC purified MAP-2, (c) PC-tubulin.

tubulin (fig.1f,g), and MAP-2 represented only ~7.5% of the total protein. These results were obtained from experiments in which PC-MAPs and PC-tubulin were mixed and incubated immediately at 37°C to promote assembly. Similar results (not shown) were obtained from experiments using the unfractionated microtubule proteins.

The early assembly product(s) consisting of tubulin and MAP-2 (fig.2a), was again fractionated on a phosphocellulose column to yield purified MAP-2 (fig.2b) and tubulin (fig.2c). Negative staining of samples of purified MAP-2 ($\leq$0.4 mg/ml) and tubulin (2–6 mg/ml) alone or immediately following mixing showed no identifiable polymer structures. Incubation of the mixture at 4°C resulted in the formation of ring structures (fig.3,4) exhibiting a sedimentation coefficient ($s_{20,w}$) of 33 ± 1 S. Similar structures were formed with the unfractionated microtubule proteins under depolymerizing conditions, with mixtures of total MAPs and tubulin at 4°C, and as cold disassembly products of MAP-2 initiated microtubules. The structure(s) of the polymer formed by the

unfractionated microtubule proteins, mixtures of PC-MAPs and PC-tubulin, or mixtures of purified MAP-2 and tubulin (with or without a pre-incubation period at 4°C), was examined. Figures 3–8 are from a typical experiment in which MAP-2 and tubulin were mixed, incubated at 4°C, then warmed to 37°C and sampled at intervals. Rings were the predominant structure at 4°C (fig.3) and after 20 s at 37°C (fig.4).

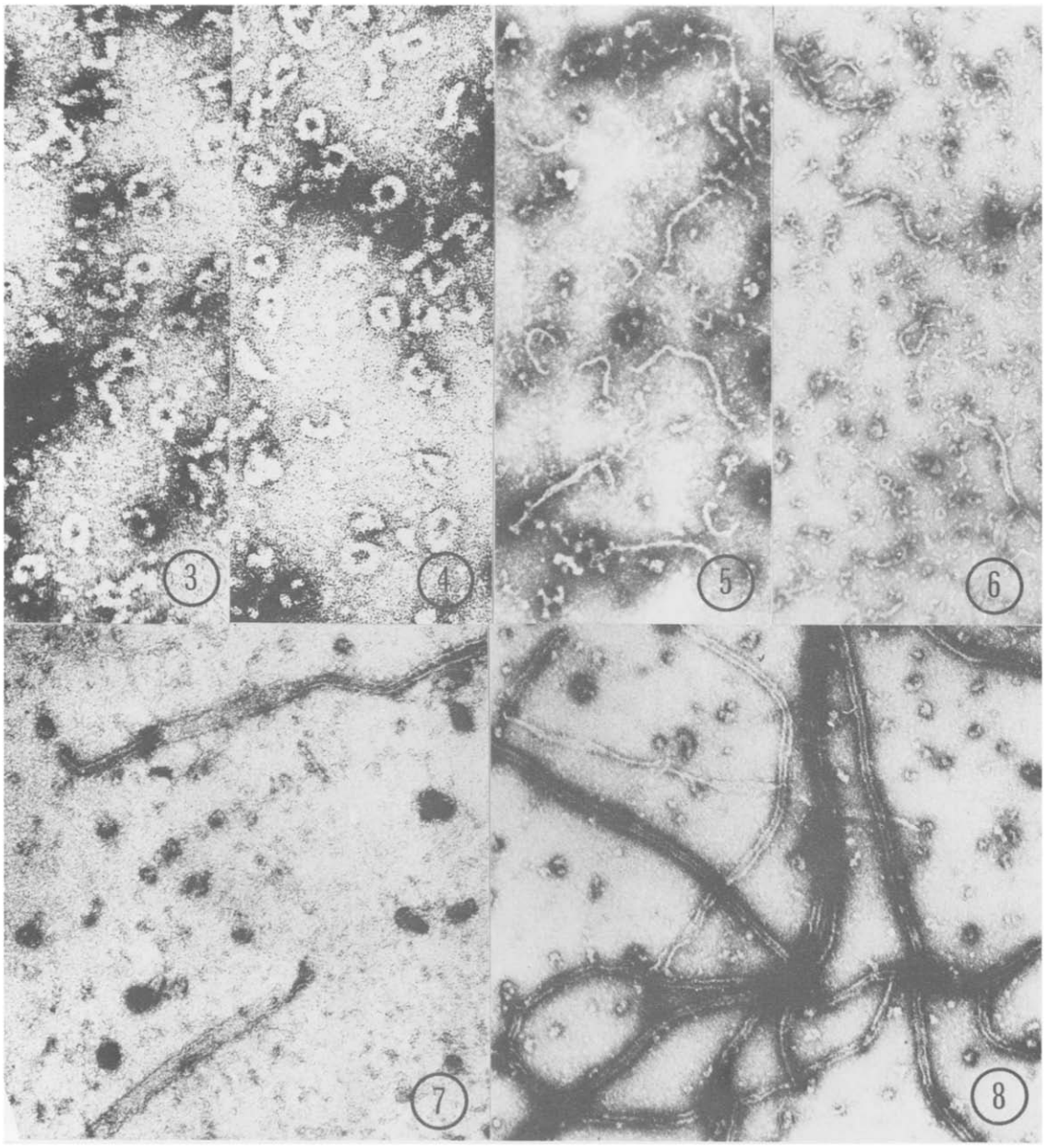

Fig.3–8. Negative staining of polymer assembled in mixture of PC-MAPs (0.4 mg/ml) and PC-tubulin (2 mg/ml) pre-incubated at 4°C to form rings (3), and after 20 s (4), 30 s (5), 60 s (6), 90 s (7) and 180 s (8) incubation at 37°C. (3, 4, × 120 000; 5, 6, × 40 000; 7, 8, × 60 000).

By 30–60 s, protofilaments were present (fig.5,6), either singly or in small aggregates. By about 90 s, protofilament sheets were formed (fig.7) and rings were rarely seen. By 180 s, microtubules were the most prevalent structures (fig.8) and at later times were the only structures seen. An identical sequence of structures was seen for unfractionated microtubule proteins or for PC-MAPs and PC-tubulin mixtures, pre-incubated at 4°C to form rings, and then incubated at 37°C to promote assembly. However, rings were not essential to this sequence, nor were they a requirement for microtubule initiation. When PC-MAPs or purified MAP-2 were mixed with tubulin and immediately warmed to 37°C rings were never seen. Protofilaments were the first polymer detected, followed by protofilament sheets and microtubules. The polymer structures in the sedimented pellets of fig.1a–e were also examined by negative staining. Figures 1a,b corresponded to protofilaments, fig.1c,d were mainly protofilaments and sheets and fig.1e was predominantly short microtubules.

Although MAP-2 was preferentially enriched in the initial stages of assembly and was competent to initiate the assembly of purified tubilin, it was not limited to an initiation function. Turbidometric analysis (fig.9) showed that the addition of increasing amounts of MAP-2 to a fixed concentration of tubulin resulted in both more rapid initial rates of assembly and higher equilibrium values. This is consistent with MAP-2 functioning in the initiation as well as in the elongation of microtubules [3,10]. Near maximal absorbance values at equilibrium were obtained when MAP-2 represented ~16% of the total protein assembled (fig.9e). SDS–gels of these microtubules resembled fig.2a and scans of the gels confirmed that MAP-2 was 12–15% of the total protein.

Finally, the microtubules assembled in the presence of MAP-2 alone were examined by thin-section EM. These microtubules, unlike those assembled using total PC-MAPs or the unfractionated microtubule proteins, lacked the obvious surface filaments, appeared relatively smooth and frequently were closely packed in the microtubule pellet (fig.10). Addition of a total PC-MAPs fraction (0.3 mg/ml) to those microtubules for 60 s prior to centrifugation produced microtubules which had projecting surface filaments and which were not so closely packed in the pellet (fig.11).

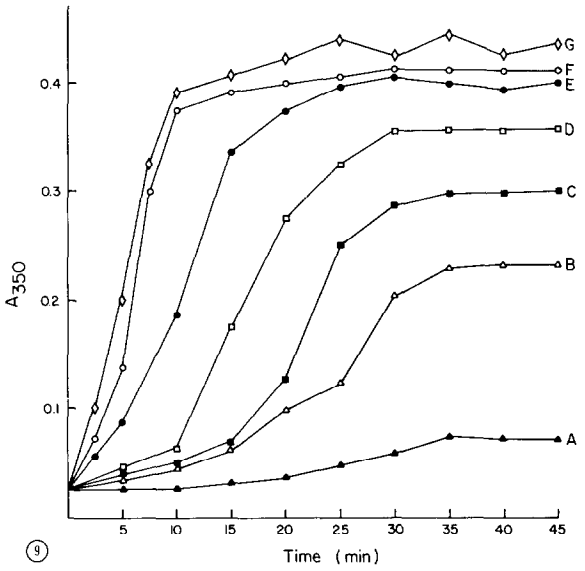

Fig.9. Stimulation of tubulin (2 mg/ml) assembly by increasing amounts of PC-purified MAP-2. (A) 0.05 mg/ml, (B) 0.10 mg/ml, (C) 0.15 mg/ml, (D) 0.25 mg/ml, (E) 0.35 mg/ml, (F) 0.45 mg/ml, (G) 0.55 mg/ml.

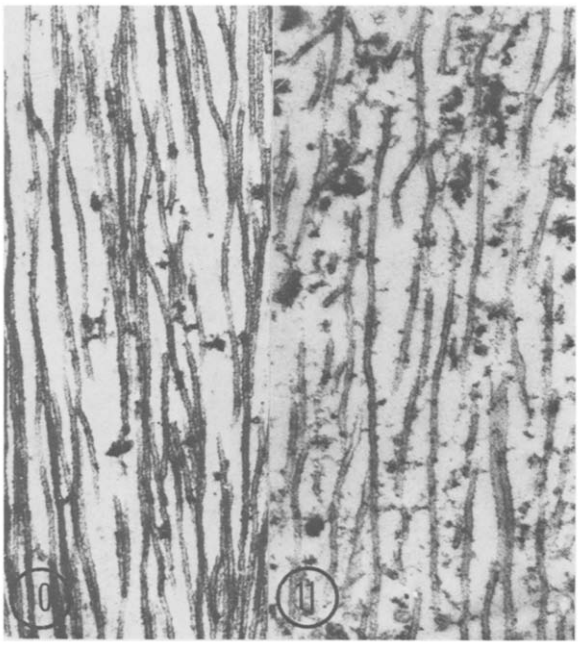

Fig.10–11. Thin sections of pelleted microtubules in longitudinal views, assembled using 2 mg/ml PC-tubulin and 0.4 mg/ml of purified MAP-2 (10), and after a 60 s incubation at 37°C with total PC-MAPs fraction (11). × 60 000.

4. Discussion

The results of the sedimentation studies and correlated negative staining indicate an assembly sequence for microtubules in which MAP-2 functions preferentially in the initiation of protofilament assembly. The other MAPs, including MAP-1 and tau proteins which associated at the later stages of assembly, may play a more prominent role in microtubule elongation. Although the HMW-MAPs [1,3,5], tau proteins [2,4] or MAP-2 (present paper, [10]) will independently stimulate both the initiation and elongation stages of tubulin assembly, our results show that when all of the MAPs are present there is some stage specificity of their association with tubulin. We are not suggesting that all of the MAP-2 in the total MAPs fraction is sequestered in the protofilaments first formed during assembly (see below).

Ring structures present in samples of depolymerized brain microtubules have been suggested to participate in the initiation and elongation of microtubules (e.g. [11]). However, more recently it has become clear that rings are not obligatory intermediates for microtubule elongation [12,13]. Our results indicate that rings are not necessary for the initiation of microtubule assembly either. In the mixtures of total PC-MAPs and tubulin or MAP-2 and tubulin, which were immediately raised to 37°C to promote assembly, rings were never detected as early (or late) assembly products. When rings were present, as for example in the unfractionated microtubule proteins, they also may not have a direct contribution to initiation. The rings formed at low temperatures are believed to consist of tubulin and the MAPs [2,3,14]. However, we find only MAP-2 as a component of the protofilaments first formed under assembly conditions. One interpretation of this result is that rings may rapidly dissociate at the assembly temperature, and that the MAPs released are then available to reassociate with tubulin at the different stages of the assembly sequence.

The surface filaments on in vitro assembled microtubules have been equated with the presence of the HMW-MAPs [1,15]. These filaments are distributed in a helical superlattice on the tubule wall with an approximate axial repeat distance of 32 nm between successive filament projections [16]. A thermostable MAP fraction has been prepared [10], enriched to ~80% for MAP-2, which will stoichiometrically promote microtubule assembly. The microtubules formed with saturating amounts of this MAP-2 fraction, when fixed in the presence of tannic acid and examined by thin-section electron microscopy, demonstrated the same axial periodicity seen on microtubules polymerized from unfractionated microtubule protein [10]. The microtubules we have assembled with MAP-2 and fixed without tannic acid do not have obvious surface filaments (fig.10), however, a brief incubation of these tubules with a total MAPs fraction results in the appearance of the surface filaments (fig.11). This result raises the possibility that MAP-2 may specify the sites of binding for the other microtubule associated proteins.

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