Physical and Chemical Properties of Purified Tau Factor and the Role of Tau in Microtubule Assembly

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This paper describes the physical and chemical properties of purified tau, a protein which is associated with brain microtubules and which induces assembly of microtubules from tubulin. Purified tau is composed of four polypeptides which migrate at positions equivalent to molecular weights between 55,000 and 62,000 during electrophoresis on sodium dodecyl sulfate/polyacrylamide gels. These polypeptides are shown to be closely related by peptide mapping and by amino acid analysis. A comparison by various techniques of the high molecular weight microtubule-associated proteins with the tau polypeptides indicates no apparent relationship. Tau is found by analytical ultracentrifugation and by sedimentation equilibrium to have a sedimentation coefficient of 2.6 S and a native molecular weight of 57,000. Tau, therefore, must be highly asymmetric (an axial ratio of 20:1 using a prolate ellipsoid model), and yet possess little α-helical structure as indicated by circular dichroism. Isoelectric focusing shows tau to be a neutral or slightly basic protein. Tau is also seen to be phosphorylated by a protein kinase which copurifies with microtubules.

In the assembly process, tau apparently regulates the formation of longitudinal oligomers from tubulin dimers, and hence promotes ring formation under depolymerizing conditions and microtubule formation under polymerizing conditions. The known asymmetry of the tau molecule suggests that tau induces assembly by binding to several tubulin molecules per tau molecule, thereby effectively increasing the local concentration of tubulin and inducing the formation of longitudinal filaments. The role of tau is discussed in light of reports of polymerization induced by particular non-physiological conditions and by various polycations. The formation of normal microtubules over a wide range of tubulin and tau concentrations under mild buffer conditions suggests that tau and tubulin define a complete in vitro assembly system under conditions which approach physiological.

1. Introduction

Microtubules purified by successive cycles of polymerization and depolymerization have been reported to contain, in addition to tubulin, a variety of accessory proteins (Gaskin et al., 1974; Dentler et al., 1975; Murphy & Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1976a, b). The nature and amount of such accessory proteins depends apparently on the conditions of purification. In our laboratory, microtubule protein purified by cycles of assembly in glycerol at pH 6.4 has been found to consist of 90% tubulin, with the remaining 10% distributed among proteins of both high and low molecular weight (Weingarten et al., 1975; Cleveland et al., 1977b). Murphy & Borisy (1975), using a purification procedure without glycerol at pH 6.9, have
reported their preparations to consist of approximately 75% tubulin by weight, 12 to 20% high molecular weight components (greater than 250,000) and the rest in low molecular weight proteins. Rosenbaum and co-workers have reported (Dentler et al., 1975; Sloboda et al., 1976a,b) the accessory proteins from two-cycle microtubule preparations from a protocol using glycerol at pH 6.9 to consist of 35% of two high molecular weight components and 65% of a variety of other proteins. Working at pH 6.6 with glycerol-containing buffers, Keates & Hall (1975) have identified a 360,000 molecular weight protein (15% by weight) as the major accessory protein in their preparations. Despite these reported differences, all investigators have demonstrated the incompetence of tubulin, when separated from accessory proteins, for self-assembly under standard polymerizing conditions. Microtubule assembly has been reported in each case to be restorable by addition of the non-tubulin proteins. This raises several questions. Which of these proteins actually stimulates tubulin polymerization? What is the relationship among these proteins? What is the mechanism by which these proteins act?

Our previous paper (Cleveland et al., 1977b) introduced the purification of tau (all references to tau in this paper will refer to tau-I as purified in the previous paper), a heat-stable accessory protein which is present in our preparations of microtubules and which contains the majority of microtubule-inducing activity recoverable following removal of tubulin by phosphocellulose chromatography. In the present investigation we report the physical characterization of the purified tau protein. The molecular weight and sedimentation coefficient of tau have been determined by analytical centrifugation. Amino acid analysis and peptide mapping by a convenient, new technique have been used to demonstrate the close relationship of the four polypeptide chains visible on SDS/polyacrylamide gels. Circular dichroism has been used to suggest that, as is implied by the amino acid composition, little z-helical structure is present in tau. The neutral to slightly basic character of tau has been demonstrated on isoelectric focusing gels. In addition, the chemical relationship between tau and the high molecular weight protein in our preparations has been investigated by amino acid analysis and by peptide mapping. Finally, the possible role of tau and other accessory proteins in microtubule assembly is discussed in light of tau's physical properties and the apparent heterogeneity of microtubule-associated proteins as obtained from different procedures of microtubule purification. The relationship between assembly induced by tau protein and that induced by cations and polycations (Erickson, 1976; Erickson & Voter, 1976; Lee & Timasheff, 1975) or by dimethyl sulfoxide (Himes et al., 1976) is also discussed.

2. Materials and Methods

(a) Preparation of microtubule protein, purified tubulin and tau

Microtubule protein was prepared from porcine brain by the Weingarten et al. (1974) modification of the method of Shelanski et al. (1973). Purified tubulin, to be referred to as PC-tubulin, was prepared by phosphocellulose chromatography of microtubule protein as described by Weingarten et al. (1975). Tau was purified as indicated in the previous paper (Cleveland et al., 1977b) by a combination of phosphocellulose chromatography, ammonium sulfate precipitation and hydroxyapatite chromatography. Protein concentrations were determined by the method of Lowry et al. (1961), using bovine serum albumin as a standard.

† Abbreviations used: SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid.
Polyacrylamide gel electrophoresis was performed in a slab gel apparatus (Studier, 1973) according to the method of Laemmli (1970). Gels were stained immediately after electrophoresis in a solution of 0.25% Coomassie blue R250, 45% methanol, 9% acetic acid. Destaining was achieved by diffusion in a solution of 5% methanol, 7.5% acetic acid.

(c) Peptide mapping

Peptide mapping of protein bands isolated from polyacrylamide gels was performed by the newly developed technique of Cleveland et al. (1977a), which uses limited proteolysis in an SDS-containing buffer followed by resolution of the fragments so produced on high percentage polyacrylamide gels. Protein samples were initially radioactively labeled in vitro by the method of Rice & Means (1971) and then isolated from acrylamide gels. Proteolytic digestions were carried out according to Cleveland et al. (1977a) for 30 min at 37°C. Stained gels were fluorographed according to Bonner & Laskey (1974). Protease Staphylococcus aureus V8 (36-900-1) was obtained from Miles Laboratories. Chymotrypsin (CDI-1450) was purchased from Worthington.

(d) Amino acid analysis

For amino acid analysis, approx. 200 to 300 μg of each sample in purification buffer (0.1 N morpholinoethane sulfonic acid (pH 6.4 with NaOH), 1 mM 2 mercaptoethanol, 0.1 mM-EDTA, 0.5 mM-MgCl₂, 1 mM-EGTA) was hydrolysed in 6 M-HCl by incubation at 108°C for from 24 to 72 h and then applied to a Beckman 120B amino acid analyser equipped with a numerical integrator for the measurement of peak areas. Areas were converted into the appropriate nmol amounts by comparison with the areas obtained from application of a mixture of amino acids of known concentrations (from Beckman).

(e) Isoelectric focusing

Isoelectric focusing in cylindrical polyacrylamide gels followed by resolution of focused bands into the corresponding bands observed by standard SDS/acrylamide gel electrophoresis was performed using the 2-dimensional method of O'Farrell (1975), with the exception that the gels were cast with 2% (w/v) in 3 to 10 ampholytes purchased from Bio-rad.

(f) Nucleotide triphosphatase and protein kinase assays

To assay for the presence of ATPase or GTPase activity, protein samples at 0.5 mg/ml were mixed with a final concn of 1 mM of the appropriate nucleotide triphosphate containing 2 x 10⁶ disnts/min of tritiated nucleotide and the samples were incubated at 37°C. At various times portions were removed, spotted directly onto Whatman 3 MM paper and analysed by high-voltage electrophoresis (Smith, 1967) and liquid scintillation counting.

For measurement of protein kinase activity, assays were performed in purification buffer containing 0.52 mM-[γ-³²P]ATP (40 μCi/μmol), 10 mM-MgCl₂, 10 mM-NaF, and ± 20 μm-cyclic AMP. After 30 min incubation, samples were precipitated and washed with 20% trichloroacetic acid and counted. Alternatively, precipitates were resuspended in gel sample buffer (Laemmli, 1970), applied to an SDS/polyacrylamide gel, and autoradiographed using Ilford X-ray film.

3. Results

(a) The four polypeptides of tau are very closely related

Tau, as purified from microtubule protein by a combination of phosphocellulose chromatography, ammonium sulfate precipitation, and hydroxyapatite chromatography (Cleveland et al., 1977b), is seen by SDS/polyacrylamide gel electrophoresis to consist of four closely spaced bands of apparent molecular weights between 55,000 and 62,000.

To test whether these four bands are related proteins, we chose to use a newly developed peptide mapping technique which is especially suited to analysis of proteins
that have been isolated from SDS/acrylamide gels. The technique, which involves limited proteolysis in an SDS-containing buffer and separation of the peptide fragments on high percentage polyacrylamide gels, has been shown (Cleveland et al., 1977a) to produce patterns of peptide fragments which are both highly reproducible and characteristic of the protein being analysed. Either chymotrypsin or protease S. aureus was mixed with samples from each of the isolated bands and from whole purified tau. After incubation, the samples were applied to a 15% polyacrylamide gel. The proteolytic fragments produced are shown in Figure 1, along with the four isolated bands which were rerun on a low percentage gel to verify their purity. The digestions with protease S. aureus (Fig. 1(a) to (d), and (f)) show numerous identical peptides for each of the four tau bands, though some bands are clearly unique to the tau polypeptides of higher apparent molecular weights and some unique to those of lower molecular weights. Similar results showing many common peptides, along with some differences, are also seen in the chymotryptic digestions (Fig. 1(g) to (j), and (l)).

Further evidence for the close relationship among the four individual tau bands was obtained by subjecting each band (isolated as in the peptide mapping experiments) to amino acid analysis. The amino acid compositions so obtained are given in Table 1, as well as the composition obtained from hydrolysis of the initial purified tau, which contained all four bands. As is seen in the Table, the mol % values differ between the individual tau bands in most cases by less than 0.3% for each amino acid, with the largest discrepancies occurring in glycine, lysine and proline. Glycine values are probably higher than the actual content in the protein due to contamination from residual glycine in the running buffer of the preparative SDS/acrylamide gel from which the individual bands were isolated. The lysine values may also be less reliable, because of the presence of a leading shoulder on the lysine peak as obtained from the amino acid analyser, probably the result of a hydrolysis product of residual Coomassie blue. The amino acid composition for the initial purified tau (also in Table 1) matches quite well with the individual isolated tau bands, although glutamic acid and proline values appear somewhat higher in the purified tau than in the eluted bands.

Thus it would seem that the individual tau bands, as judged by peptide mapping and amino acid composition, are closely related.

(b) Is tau related to the high molecular weight accessory protein?

It has been shown recently that high molecular weight accessory proteins are not stable to prolonged storage at 4°C (Sloboda et al., 1976a,b). This observation has provoked the suggestion that tau may be a proteolytic fragment of these high molecular weight proteins.

![Fig. 1. Peptide mapping of the isolated tau bands and of the high molecular weight polypeptides. (a) to (f) Mapping with protease S. aureus. (a) Tau25,000, (b) tau28,000, (c) tau26,000, (d) tau45,000; (e) the isolated high molecular weight component, and (f) purified whole tau were mapped with increasing amounts of protease S. aureus. Slots 1 to 4 of each quartet were incubated, respectively, with 0, 25 μg, 100 μg, and 200 μg of the protease/ml. All slots in (a) to (f) were electrophoresed on the same 15% gel. (g) Tau25,000, (h) tau58,000, (i) tau54,000, (j) tau54,000, (k) the high molecular weight component, and (l) purified whole tau mapped with chymotrypsin. Slots 1 to 4 of each quartet were incubated, respectively, with 0, 10 μg, 50 μg, and 200 μg of the protease/ml. All slots in (g) to (l) were electrophoresed on the same 15% gel. (m) to (q) Purified tau and the isolated tau bands were rerun on an 8.5% gel to verify their purity. (r) The isolated high molecular weight component rerun on a 5% gel.](image-url)
We have looked for such a direct relationship between tau and our high molecular weight protein, utilizing the techniques of peptide mapping, amino acid analysis, and cyanogen bromide fragment analysis. The major high molecular weight component of our preparations, a doublet of approximately 300,000 molecular weight (Fig. 1(r)), was prepared by isolation from a preparative SDS/acrylamide gel and then subjected to peptide mapping with protease S. aureus (Fig. 1(e)) and with chymotrypsin (Fig. 1(k)). When compared with the appropriate peptide patterns produced from tau (Fig. 1(f) and (l)), no obvious similarities can be observed. If tau were a fragment of this high molecular weight protein, although identity of the peptide maps would not be expected, some common bands should be seen. That this is not observed suggests that no precursor-product relationship is present.

The high molecular weight band as isolated from a preparative SDS/acrylamide gel was also subjected to amino acid analysis. Results of that analysis are tabulated along with the corresponding data for tau in Table 1. Though clear differences in amino acid compositions of tau and of the high molecular weight protein are seen, especially for proline, results conclusively showing tau not to be a fragment of the larger protein would have been obtained only (in view of the 5-fold difference in the molecular weights) if a particular amino acid had been five times more prevalent in tau than in the high molecular weight protein. Such a striking difference was not

Table I

*Amino acid compositions (mol %)* of tau, of each individual tau band and of the high molecular weight accessory protein in our preparations

<table>
<thead>
<tr>
<th>Tau52,000</th>
<th>Tau58,000</th>
<th>Tau56,000</th>
<th>Tau55,000</th>
<th>Tau whole</th>
<th>High molecular weight component</th>
</tr>
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<tr>
<td>Lys 9.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.7</td>
<td>10.1</td>
</tr>
<tr>
<td>His 2.3</td>
<td>2.5</td>
<td>2.2</td>
<td>2.2</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Arg 3.6</td>
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<td>3.7</td>
<td>3.8</td>
<td>3.6</td>
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<tr>
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<td>12.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.7&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>7.5</td>
<td>7.4</td>
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<td>1.1</td>
<td>1.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.5</td>
</tr>
<tr>
<td>Ile 2.7</td>
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<td>2.6</td>
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<td>2.8</td>
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<tr>
<td>Leu 5.6</td>
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<td>5.7</td>
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<td>7.1</td>
</tr>
<tr>
<td>Tyr 1.2</td>
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<td>1.4</td>
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</tr>
<tr>
<td>Phe 1.3</td>
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<td>2.1</td>
</tr>
<tr>
<td>Trp</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Cys</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hydroxyapatite-purified tau. Values are the average of 6 determinations.

<sup>b</sup> The lysine peak has a noticeable shoulder for samples eluted from gels and hence may be overestimated for those samples.

<sup>c</sup> The glycine peak is likely to be overestimated due to residual glycine from the running buffer for samples isolated from gels.

<sup>d</sup> Methionine values were extrapolated from data taken from 24, 48 and 72 h of hydrolysis.

<sup>e</sup> Estimated from the spectrophotometric procedure of Goodwin & Morton (1946).

<sup>f</sup> Estimated from the spectrophotometric procedure of Ellman (1959).
observed and hence the results of amino acid analysis cannot be used to determine whether the two proteins are related.

Finally, cyanogen bromide peptides were produced from the high molecular weight protein in our preparations (not shown), using the procedure described by King & Spencer (1970). Although even at a 600-fold excess of cyanogen bromide, quantitative cleavage of the methionine residues present in the proteins was not achieved (as judged by the partial reaction products visible in bovine serum albumin and ribonuclease cleavages which were performed in parallel as references), no similar fragments were observed between tau and the high molecular weight sample.

(c) Sedimentation coefficient and molecular weight of tau by analytical centrifugation

The sedimentation pattern of purified tau as obtained from analytical ultracentrifugation with Schlieren optics is given in Figure 2. A single, homogeneous component is observed. Extrapolation of data collected at different concentrations (between 1.75 and 0.6 mg/ml) yields a sedimentation coefficient $s_{20,w}$ of 2.6 S, which is virtually identical to the 2.7 S value indicated from the sedimentation of tau activity in a sucrose gradient (Cleveland et al., 1977b). The $s$ versus $C$ plot (not shown) can be described by an equation of the form $s = s_0 (1 - kC)$, where $k$ is a factor which depends largely on the size and asymmetry of the sedimenting species. The $k$ factor for tau is in the range of 0.05 to 0.06 ml/mg, which is consistent with it being a large and asymmetric structure (Creeth & Knight, 1965).

The molecular weight of tau was obtained by analytical ultracentrifugation using the high-speed equilibrium method with Raleigh optics (Yphantis, 1964). A plot of $\omega^2$ times the log of the fringe displacement versus $r^2$ yields a set of nearly parallel lines for the two different speeds of centrifugation used (see Fig. 3). The slopes of these lines indicate a molecular weight of 56,500, which compares favorably with the native molecular weight estimate of 61,000 obtained from combining activity measurements of sedimentation and diffusion coefficients of tau. In addition, in view of the banding pattern on SDS/polyacrylamide gels (four closely spaced bands between 55,000 and 62,000) and the recovery of activity from gel filtration under
FIG. 3. Equilibrium centrifugation of tau. The molecular weight of tau was determined with the short column, high-speed equilibrium method of Yphantis (1964). Tau at 1.75 mg/ml in purification buffer was centrifuged at 4°C for 22 h at 27,600 revs/min. Approach of equilibrium was monitored by the absence of change in the Raleigh fringe pattern over 4 h. The speed of the centrifuge was then increased to 31,400 revs/min. Equilibrium at this higher speed was reached within an additional 10 h of centrifugation. Approx. 4 fringes were crossed from the top to the bottom of the column. --○--○--, fringe displacement after 22 h at 27,600 revs/min; - - - - - - - - , fringe displacement after an additional 10 h at 31,400 revs/min.

Denaturing conditions, which indicated a denatured molecular weight of 70,000 (Weingarten, 1975), the sum of the evidence strongly implies that the native tau is a monomer.

(d) The ultraviolet spectrum of tau

The ultraviolet adsorption spectrum of tau in purification buffer was determined in a Cary 14 recording spectrophotometer. Two different tau preparations were analysed and a typical spectrum is given in Figure 4. An absorption maximum at 275 nm (0.29 at 1 mg/ml) was observed and an $E_{280\text{nm}}$ value of 2.8 was calculated.
This is a decidedly low value for a protein, with most yielding values two to five times higher. With the molecular weight taken as 60,000, this corresponds to a molar extinction coefficient at 280 nm of $1.68 \times 10^4$ M$^{-1}$ cm$^{-1}$.

(e) Determination of approximate $\alpha$-helical content of tau by circular dichroism

The circular dichroism spectrum of tau was obtained using a Cary 60 recording spectrophotometer and is shown in Figure 5, along with the spectrum of myoglobin, which was obtained as a control immediately prior to recording the tau spectrum.

![Circular dichroism spectrum of tau and myoglobin](image)

FIG. 5. Circular dichroism spectrum of tau. The circular dichroism of tau (0.32 mg/ml) in 0.1 M sodium phosphate (pH 6.4) was recorded between 200 nm and 250 nm with a Cary 60 recording spectrophotometer in a 1 mm strain-free quartz cuvet. The myoglobin spectrum was recorded immediately prior to the tau analysis to verify the proper functioning of the spectrophotometer. (---) Tau; (-----) myoglobin.

Greenfield & Fasman (1969) have shown that by least-squares fitting the circular dichroism spectrum of a protein with the spectra from model $\alpha$-helical, $\beta$-sheet and random coil polypeptides, it is possible to predict the percentages of helix, sheet and coil of the protein in question. Such predictions have been shown to be quite accurate for highly helical molecules, though the accuracy of prediction falls considerably for less helical proteins. Using this procedure, we have obtained an estimate of helical composition of 12% and a $\beta$-sheet composition of 20% for tau. Because the predictive value of the method declines with lesser amounts of secondary structure, the absolute levels of helix and sheet which are indicated for tau should not be taken too seriously. Nonetheless, the results do indicate that it is highly unlikely that tau possesses large amounts of helical structure.

(f) Tau does not contain carbohydrate

Tau was negative for carbohydrate as stained by the periodic acid–Schiff base technique given by Segrest & Jackson (1972). Easily observable staining developed for human transferrin (5.8% carbohydrate) in gel slots loaded with only 10 µg of protein, with very strong staining visible in severely overloaded slots (100 µg/slot). Hence, if tau contains carbohydrate, such carbohydrate is not likely to be present at levels of more than a few tenths of 1%.
The isoelectric points of the four tau bands were determined by isoelectric focusing in a first dimension, followed by resolution in a second dimension on a standard SDS/acylamide slab gel by the method of O'Farrell (1975). The pH gradient established in the isoelectric dimension, measured by insertion of a micro pH electrode directly into the focusing gel, was nearly linear between pH 4.0 and pH 8.25. Figure 6 shows the banding pattern obtained after the SDS dimension. Each of the four tau bands focuses in the isoelectric dimension as a series of discrete bands, corresponding to pI values of 8.0 for the most basic species (tau_{80k}) to 6.6 for the most acidic (tau_{62k}). A listing of the apparent pI values of the various spots which coelectrophorese in the SDS dimension with each of the four tau bands is given in Table 2. The two lower molecular weight tau species are seen to be the most basic (pI between 8.0 and 7.4), whereas the two higher molecular weight tau bands are nearly neutral (pI between 7.7 and 6.4). A division in this manner is consistent with the elution at slightly lower ionic strength of the higher molecular weight tau bands on the cation-exchangers hydroxyapatite and phosphocellulose (D. Cleveland & M. Kirschner, unpublished results) and the apparent increase in the lysine content with decreasing apparent molecular weight (see Table 1). Whether such heterogeneity in the isoelectric points of each of the tau bands is artifactually produced by hydrolysis.
PHYSICAL AND CHEMICAL PROPERTIES OF TAU

Table 2

Apparent pH values from Figure 9 of the various polypeptides which coelectrophorese in the SDS dimension with each of the tau bands after an initial isoelectric focusing of tau

<table>
<thead>
<tr>
<th>Apparent pH values of the coelectrophoresing polypeptides</th>
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<tbody>
<tr>
<td>Tau42,000</td>
</tr>
<tr>
<td>Tau38,000</td>
</tr>
<tr>
<td>Tau36,000</td>
</tr>
<tr>
<td>Tau58,000</td>
</tr>
</tbody>
</table>

or by proteolysis during purification, by differential phosphorylation either in vivo or in vitro (see below), or by genuine differences in the polypeptide chains is unknown.

(h) Enzymatic activities of tau

Tau was assayed for ability to bind ATP and GTP. A portion was mixed in each case with a final concn of 1 mM of the nucleotide triphosphate containing $2 \times 10^6$ disintegrations/min of tritiated nucleotide, and the samples were then incubated for up to 60 min at 37°C. To detect tightly bound nucleotide, the samples were then desalted on Bio-Gel P10 into nucleotide-free purification buffer. Although binding of as little as 0.01 mol per mol of tau would have produced a signal five times larger than the background, no binding was detectable for either GTP or ATP.

The presence of ATPase and GTPase activity in tau was also investigated. Samples of tau were mixed with the appropriate tritiated nucleotide triphosphate as before and incubated at 37°C. At various times up to 60 minutes, portions were removed, subjected to high-voltage paper electrophoresis, and counted in a liquid scintillation counter in order to determine the nucleotide mono-, di- and triphosphate levels. No GTPase activity and no ATPase above that accountable to residual protein kinase activity (see below) was observed, even after 60 minutes of incubation.

In view of the reports of a protein kinase activity which copurifies with microtubules (Eipper, 1974; Sloboda et al., 1975; Rappaport et al., 1976), a preliminary investigation of protein kinase present in purified tau was undertaken. The level of kinase activity found in purified tau is given in Table 3, along with similar data for microtubule protein (purified by two cycles of assembly), for PC-tubulin, and for crude tau (all non-tubulin proteins recoverable from phosphocellulose chromatography of microtubule protein). A substantial level of kinase activity is found in crude tau, with a very low level of activity fractionating with PC-tubulin. We believe, as Eipper (1974) originally suggested, that this residual level of kinase activity represents a contaminating enzyme and is unlikely to be intrinsic to tubulin. In addition, during purification of tau from crude tau, as specific activity for microtubule formation in the reconstituted system of tau + tubulin increases three- to fourfold, the specific activity of the protein kinase declines threefold as assayed with both histone and tau or crude tau as substrate. We believe, therefore, that tau probably contains no intrinsic kinase.
Nonetheless, when mixed with PC-tubulin, phosphate is preferentially incorporated into the tau fraction rather than the tubulin. This is true when purified tau or crude tau is used as the phosphate acceptor. As demonstrated in Figure 7, roughly 20 times as much $^{32}$P is incorporated per mg of protein into the tau bands as into the tubulin. An earlier report by Sloboda et al. (1975) indicated preferential incorporation of phosphate into a high molecular weight accessory protein. In our experiments we have found that a reconstituted crude tau/PC-tubulin system significantly phosphorylates a number of associated proteins, including a high molecular weight component, in which is incorporated about 10% of the total protein bound phosphate (Fig. 7(c) and (d)).

Figure 7 also demonstrates the care which needs to be exercised when analysing the purity of a preparation of tubulin. In Figure 7(a), purified tau and PC-tubulin were mixed in a weight ratio of 1:7. This level of tau is more than sufficient to promote complete assembly of the tubulin, yet the tau bands are barely visible on stained gels. Higher protein loadings of the gels do not result in appreciable improvement in the detection of the tau bands, since staining from the increasingly diffuse tubulin band(s) tends to obscure any increase in the intensity of the tau staining.

(i) Properties of tau-induced microtubules

As reported earlier, in the reconstituted system with PC-tubulin, tau stimulates the formation of microtubules of normal morphology (Fig. 8(a)). After incubation for 15 minutes at 37°C, sheets, ribbons, and other tubulin oligomers are not observed. The structures of abnormal morphology described by Erickson (Erickson, 1976; Erickson & Voter, 1976) which are stimulated from PC-tubulin by a variety of basic or polycationic agents are not seen. When tau is added to PC-tubulin at 4°C in the
absence of added GTP, tubulin oligomers which may be identified by electron microscopy as double rings and which have been strongly implicated as intermediates in the assembly of microtubules are formed. As reported earlier (Cleveland et al., 1977b), as much as 50% of the polymerizable PC-tubulin at 0.95 mg/ml may be found in these ring structures. The addition of GTP to this ring-containing mixture, however, even when the sample is maintained on ice throughout, results in the loss of the ring structures and the formation of sheets and curled structures as shown in Figure 8(b). This finding contrasts with the results obtained with unfractionated microtubule protein (purified by two cycles of assembly and disassembly) which, when maintained at low temperature in the presence or absence of GTP, consists of ring oligomers and tubulin dimers with no larger aggregates observed. Microtubules formed from the reconstituted system of tau and PC-tubulin also do not fully depolymerize into rings and dimers when cooled to 4°C; instead, these microtubules disassemble only into sheets and other fragments, as given in Figure 8(c). A few rings and spirals may be found, but the majority of tubulin is in higher order structures. Again, unfractionated microtubule protein, purified on the basis of its ability to disassemble when incubated at low temperatures, contains only rings and dimers under these conditions.
may indicate that the tau-induced microtubules are more stable than their unfractionated microtubule protein counterparts, with tau driving the dimer-microtubule equilibrium significantly in the direction of polymerization. Alternatively it is possible that the reconstituted system is comprised of one or more components which are less functional than their counterparts in the unfractionated system and which are no longer able to respond to the standard warm/cold control mechanism.

Microtubules formed from tau and PC-tubulin do retain calcium sensitivity. When polymerization is attempted in the presence of millimolar concentrations of calcium, the only large tubulin oligomers which can be observed are rings. Similarly, tau-stimulated microtubules are fully depolymerizable into rings and dimers by addition of calcium, as are microtubules formed from the initial unfractionated microtubule protein.
4. Discussion

This report represents the first physical and chemical characterization of a microtubule-associated protein which has been purified for its ability to induce polymerization of purified tubulin into normal microtubules under physiological conditions. As reported earlier (Weingarten et al., 1975), the removal of accessory proteins from tubulin by chromatography on phosphocellulose renders the tubulin unpolymerizable (as assayed at concentrations up to 10 mg/ml). The majority of microtubule-inducing activity recoverable in the associated proteins following phosphocellulose chromatography has been found to fractionate with a set of polypeptides which migrate during electrophoresis with apparent molecular weights between 55,000 and 62,000 (Cleveland et al., 1977b).

The properties of purified tau, along with the properties of the microtubule-inducing activity as monitored by quantitative electron microscopy (Cleveland et al., 1977b), are summarized in Table 4.

### Table 4

**Summary of the physical properties of tau**

<table>
<thead>
<tr>
<th>Property</th>
<th>Method of measurement</th>
<th>Tau polypeptides</th>
<th>Tau activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\theta_{20,w}$</td>
<td>Analytical ultracentrifugation</td>
<td>2.6 S</td>
<td>—</td>
</tr>
<tr>
<td>$\theta_{20,w}$</td>
<td>Sucrose gradient centrifugation</td>
<td>2.7 S</td>
<td>2.7 S</td>
</tr>
<tr>
<td>$D_{20,w} (\text{cm}^2/\text{s})$</td>
<td>Gel filtration</td>
<td>$3.8 \times 10^{-7}$</td>
<td>$3.8 \times 10^{-7}$</td>
</tr>
<tr>
<td>$\bar{\theta} (\text{cm}^3/\text{g})$</td>
<td>Calculated from amino acid composition</td>
<td>0.722</td>
<td>—</td>
</tr>
<tr>
<td>Native molecular weight</td>
<td>From values of $\theta_{20,w}$ and $D_{20,w}$</td>
<td>61,000</td>
<td>61,000</td>
</tr>
<tr>
<td>Native molecular weight</td>
<td>Equilibrium centrifugation</td>
<td>57,000</td>
<td>—</td>
</tr>
<tr>
<td>Denatured molecular weight</td>
<td>Gel filtration in guanidine-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>weight</td>
<td>HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denatured molecular weight</td>
<td>SDS/acrylamide gel</td>
<td>55,000–62,000</td>
<td>—</td>
</tr>
<tr>
<td>$p_I$</td>
<td>Isoelectric focusing</td>
<td>6.4–8.0</td>
<td>—</td>
</tr>
<tr>
<td>$\alpha_{helix}$</td>
<td>Circular dichroism</td>
<td>12%</td>
<td>—</td>
</tr>
<tr>
<td>$E_{280} (1 \text{ cm})$</td>
<td>Ultraviolet absorption spectrum</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>$\epsilon_{280} \text{ M}^{-1} \text{ cm}^{-1}$</td>
<td>Ultraviolet absorption spectrum</td>
<td>$1.68 \times 10^4$</td>
<td>—</td>
</tr>
</tbody>
</table>

† Values taken from Cleveland et al. (1977b).
‡ From Weingarten (1975).

The failure to achieve separation of the four tau polypeptides from each other using several ion-exchanging resins, gel filtration, and sucrose gradient centrifugation, initially suggested to us that the four observable bands on SDS/acrylamide gels were close relatives of the same protein. Peptide maps which were clearly similar for each of the tau polypeptides (Fig. 1) were generated. Nearly identical amino acid compositions were also observed (Table 1) for the isolated bands confirming the peptide mapping results. The significant similarity in the polypeptide bands of purified tau is hence very convincing.

The chemical property by which the tau bands differ, and which is responsible for different electrophoretic mobilities and hence apparent different molecular weights on SDS/acrylamide gels, remains to be discovered. It is possible that differential
phosphorylation of the bands may be important in view of the finding of significant protein kinase activity in microtubule protein purified by two cycles of polymerization and depolymerization. Indeed, we have shown that when compared with tubulin or with histone, the associated proteins (crude tau or purified tau) are significantly better substrates for this kinase. High levels of phosphate have been seen to be incorporated into tau in a reconstituted system with PC-tubulin (Fig. 7 and Table 3). On the other hand, incubation of purified tau with *Escherichia coli* alkaline phosphatase or with potato acid phosphatase does not affect the banding pattern visible on SDS/acylamide gels stained with Coomassie blue, even though incubation with these phosphatases results in the removal of at least 80% of the phosphate which can be incorporated under *in vitro* conditions (D. Cleveland & M. Kirschner, unpublished observations).

The most obvious choice for the observed electrophoretic differences among the tau bands is, of course, molecular weight. Such differences could be the result of non-identical but similar gene products or the result of some post-translational modification event such as specific proteolysis *in vivo* or may represent artifactual proteolysis during purification. We have tried to minimize this latter possibility (see below) and have found no differences in the tau bands so obtained. Nonetheless, this is a difficult alternative to eliminate. Amino or carboxyl-terminal analyses of the isolated bands are probably necessary to demonstrate more definitively the presence or absence of such proteolysis.

Indeed, the recent suggestion of Sloboda *et al.* (1976b) that tau “may be a breakdown product of MAPS 1 and 2” (a high molecular weight doublet seen by those investigators to be major associated proteins in their preparations of microtubules) has motivated us to look for such a precursor-product relationship between tau and the high molecular weight component seen in our preparations. Peptide mapping of this high molecular weight doublet showed no similarities to tau (Fig. 1). Cyanogen bromide cleavage of tau and the high molecular weight protein also showed no similar products. Although the addition of any of three exogeneous proteases to the high molecular weight component results in the production of a major, semi-stable 61,000 molecular weight fragment and suggests that the tau bands may have arisen in a like manner, peptide mapping of this fragment indicates no relationship to tau (D. Cleveland & M. Kirschner, unpublished work). In addition, this fragment is devoid of microtubule-inducing activity. Moreover, tau has been purified in buffers containing *p*-phenylmethlysulfonyl fluoride, a potent inhibitor of serine proteases, with no apparent differences in the relative amounts of tau found in the associated proteins. A second method of purification of tau directly from brain extract which obviated the need for incubation of the protein (particularly in whole extracts) for extended periods at 37°C, as is necessary in the conventional procedure, was developed to minimize proteolysis (Cleveland *et al.*, 1977b). The characteristic tau bands were again observed. In all procedures attempted, no evidence was generated which would suggest or support a precursor-product relationship between tau and the high molecular weight components in our preparations. (See note added in proof.)

The specter that possible proteolytic activity was responsible for the appearance of tau was raised by Sloboda *et al.* (1976a,b) from their data, which showed that incubation at 4°C over a period of up to 300 hours of a preparation of total microtubule-associated proteins resulted in the loss of initial high molecular weight components. Because microtubule stimulatory activity remained essentially unchanged
after such prolonged incubations (though the rate of assembly was somewhat diminished), these authors concluded that "MAPS 1 and 2 are involved in both initiation and elongation processes but that after their breakdown they are less efficient at initiation, but equally effective at stimulating elongation" (Sloboda et al., 1976b). They did not discuss an alternative interpretation that, since their initial associated protein preparation was only 36% high molecular weight components, the microtubule-stimulatory assembly factor(s) might reside in the remaining 64% of the protein. Indeed, we believe that their data provides strong evidence for the presence of tau protein as a stimulatory factor in their preparations. The appearance of the tau peptides as a set of bands migrating faster than the 75,000 molecular weight marker on the SDS/acrylamide gels shown in Figure 21 of Sloboda et al. (1976a) and in Figure 7 of Sloboda et al. (1976b) is almost unmistakable†. By densitometry (Fig. 8 of Sloboda et al., 1976b), bands which appear to be the tau peptides comprise 28% by weight of the initial accessory fraction, compared with the 36% in high molecular weight components. After 130 hours at 4°C, when only 44% of the initial high molecular weight components remain, 82% of the original tau is still present and microtubule-stimulatory activity is undiminished. Although it is possible, as Sloboda et al. (1976b) have concluded, that stimulatory activity does reside in the high molecular weight components, and that the breakdown products of those components retain activity, a simpler and more direct interpretation is that microtubule-inducing activity is present in the tau fraction which remains reasonably intact during the period of incubation.

The combination of a native molecular weight with a sedimentation coefficient has indicated that tau is a highly asymmetric molecule which, when modeled with a prolate ellipsoid, corresponds to an axial ratio of 20:1 (Penningroth et al., 1976; Cleveland et al., 1977b). The decided asymmetry of tau is not particularly surprising, since such asymmetry would probably allow a single tau molecule to interact with several tubulin dimers. Ample precedent for such asymmetry in structural proteins is provided by the elongated tropomyosin molecule, which is known to bind to multiple actin molecules in the thin filaments of muscle and which, like tau, sediments at 2.6 S and has a native molecular weight of 70,000 (Woods, 1969). The circular dichroism spectrum, however, indicates little α-helical structure in tau. This is consistent with tau’s relatively high proline and glycine content. Therefore, the polypeptide structures which produce the observed asymmetries of tau and of tropomyosin (which is almost entirely helical) must be quite distinct.

The finding that tau seems to regulate longitudinal interactions in microtubules, i.e. the conversion of tubulin subunits into rings or protofilament lengths, when coupled with the finding that the tau molecule is elongated, suggests the possibility that tau functions in assembly of microtubules by binding several dimers per tau molecule, thereby effecting a local increase in the tubulin concentration. The result of such an increase could be the formation of longitudinal filaments of tubulin which curl into rings under depolymerizing conditions and assemble into microtubules.

† It is difficult to compare directly the less highly cross-linked gels of Sloboda et al. (1976a,b) with those of Cleveland et al. (1977b) particularly since Sloboda et al. included no molecular weight markers below 75,000. However, a major set of 3 or 4 bands running just below the 75,000 molecular weight marker with the general appearance of the tau peptides is visible on gels shown by Sloboda et al. which were loaded with associated proteins prepared by phosphocellulose chromatography in a manner similar to that used in our purification of tau.
under polymerizing conditions. Indeed, the reconstituted tau + tubulin system
demonstrates the conversion of 6 S tubulin dimers into rings when incubated in the
absence of GTP. The purified system, however, differs from the initial unfractionated
microtubule protein in that, even when maintained on ice throughout, the addition
of tau to tubulin in the presence of GTP results in the production of sheets or ribbons
of protofilaments. It thus appears that the reconstituted system is more heavily
primed toward microtubule formation, with lateral aggregation occurring even at
low temperature. A similar effect is seen when the reconstituted system is cooled
after polymerization. Depolymerization is only partial, with the same sheets and
ribbons again reappearing.

The existence of a class of proteins such as tau which are required for a specific
interaction with tubulin for the induction of polymerization offers an attractive
method for regulation in vivo. Microtubules will apparently not form spontaneously
from purified 6 S tubulin subunits nor will these 6 S subunits add onto existing
nucleation seeds such as flagellar axonemes (Witman et al., 1976). Tau, on the other
hand, has been shown to remain associated with tubulin through repeated cycles of
polymerization and depolymerization and to restore competence for rapid and
efficient polymerization to purified tubulin (Cleveland et al., 1977b). Growth onto
flagellar seeds is also restored. From these properties, and the demonstration that
the final level as well as the rate of assembly is dependent on the concentration of
added tau, it has been determined that tau is required for both microtubule nucleation
and for elongation and that it acts in a stoichiometric rather than a catalytic manner
(Witman et al., 1976).

The major question remaining unresolved by the purification and characterization
of tau is whether it is unique in its capacity to induce microtubule assembly and,
correspondingly, whether it functions in vivo to regulate the assembly process. Several
laboratories have now reported the presence of a number of other proteins which
copurify with tubulin through multiple cycles of assembly and disassembly, although
none of these has yet been purified and characterized. In each report, the separation
of non-tubulin proteins from tubulin has been shown to render the tubulin incom-
petent for self-assembly (though to varying degrees), with the readdition of the
associated components restoring polymerizability. Murphy & Borisy (1975) reported
the separation of tubulin from the major accessory components (two high molecular
weight proteins) in their preparations of microtubules using an anion-exchanging
resin. Though some of the isolated tubulin was apparently still able to polymerize
without added accessory protein, full polymerization (80%) could be restored by the
addition of an accessory protein fraction which was composed of 60% high molecular
weight proteins. A later report (Murphy et al., 1977) indicated that both high and
low molecular weight associated proteins could stimulate assembly to comparable
levels when compared on a mass basis. Keates & Hall (1975), using gel filtration
under high salt conditions, demonstrated the loss of polymerizability when tubulin
was removed from accessory proteins (the major component of which electrophoresed
at 360,000 molecular weight). More recently, Sloboda et al. (1976a,b) reported
fractionation of tubulin from non-tubulin proteins using the phosphocellulose
approach of Weingarten et al. (1975). The associated protein fraction, eluted in a single
step from phosphocellulose, was composed of 36% of two high molecular weight
proteins, which were concluded to contain microtubule-inducing activity. In our own
work, in addition to the major activity which has been characterized in this paper,
a second activity (called tau-II) which comprises one-third of the total recoverable activity, has been differentiated from the major (tau-I) activity (Cleveland et al., 1977b) by its stronger binding to hydroxyapatite, by its heat lability, and by the absence of the characteristic tau bands on SDS/acrylamide gels. This fraction does contain polypeptides of very high and very low molecular weight.

The sum of the evidence suggests that other proteins in addition to tau induce microtubule assembly. None of these has been purified, however, so it is not clear which components in each preparation actually have activity. In general, assays of activity in purified fractions have not been reported along with fractionation of mixtures of associated proteins. It is clearly possible that an individual cell may use more than one component to regulate assembly, and hence it is conceivable that there may be a number of associated proteins which stimulate polymerization in vivo. Until other accessory proteins are purified, assayed quantitatively for stimulatory activity and analyzed for possible relationship to each other, the number and relationships among these proteins will remain unclear.

Recently it has become clear that one cannot show an absolute requirement for accessory proteins in vitro. Two laboratories have reported the production of microtubules or of large aggregates of tubulin which resemble microtubules and which may be formed from purified tubulin by addition of various polycationic substances (Erickson, 1975, 1976; Lee & Timasheff, 1975; Erickson & Voter, 1976). Lee & Timasheff have shown that tubulin purified by ammonium sulfate fractionation and DEAE-Sephadex chromatography (Weisenberg et al., 1968) could be induced to form microtubules of apparently normal morphology in the presence of 3.4 M-glycerol and 16 mM-Mg$^{2+}$. Erickson (1975, 1976) has reported that phosphocellulose-purified tubulin is stimulated to form a mixture of normal and double-walled microtubules when high concentrations of ribonuclease A are added. Though these assembly conditions with polycations are clearly non-physiological and though assembly proceeds only near conditions in which tubulin is marginally soluble, the evidence does imply, as Erickson initially suggested, that microtubule-associated proteins may function in assembly primarily through electrostatic interactions with the acidic tubulin dimer, and that such interactions (particularly given the wide range of reported associated proteins) may be non-specific. This suggestion is further supported by the demonstration of ring assembly from purified tubulin in the presence of high levels (greater than 10 mM) of magnesium (Frigon & Timasheff, 1975a,b).

On the other hand, we have found purified tau protein to be neutral or only mildly basic (see Table 2). As judged by binding to two cation-exchanging resins (phosphocellulose and hydroxyapatite), tau is less basic than most of the associated microtubule proteins in our preparations, eluting in the first fractions of protein during gradient elutions from cationic resins. Yet, microtubule-stimulatory activity (assayed at comparable concentration of each accessory protein fraction) copurified with tau rather than with the presumptively more basic fractions, which eluted at higher ionic strengths. In addition, tau protein is not a common protein in cell extracts, existing in about the same ratio to tubulin in the cell as it is in the final microtubules (Cleveland et al., 1977b). Finally, the formation of normal microtubules over a wide range of tubulin and tau concentrations under mild buffer conditions and physiological concentrations of divalent cations suggests that the tau + tubulin system comes close to recreating in vivo conditions. Both polymerization with magnesium and with polycations occur under unusual buffer conditions, produce abnormal microtubules
or proceed over a narrow range of conditions close to the point of tubulin precipitation.

Though tau binds to microtubules with a fixed stoichiometry through cycles of assembly and disassembly, and the group of proteins having the principle activity are closely related and of rather unusual structure, the question of specificity remains to be addressed more directly. The report of Himes et al. (1976) that purified tubulin which is incompetent for self-assembly under standard buffer conditions may be induced to polymerize by addition of dimethyl sulfoxide to 10%, underscores the suggestion from the polycation data that sufficient structural information is present in the tubulin alone for the production of microtubules of normal morphology. The function of tau, therefore, seems to be to shift an unfavorable equilibrium. In the cell, tau may mobilize tubulin subunits which would not normally assemble under physiological conditions. We would like to know more directly whether tau functions in this manner in vivo or indeed whether tau and other microtubule-associated proteins interact with microtubules in the cell. It is hoped that a definitive answer to this question will come from comparative studies of microtubule-associated proteins and from fluorescent antibody studies correlating the intracellular distribution of these proteins with the corresponding distribution of microtubules. Recent immunofluorescent studies using anti-tau antibody by Connolly \& Kalnins in collaboration with our laboratory show that the distribution of tau protein in interphase and mitotic fibroblasts parallels almost exactly the tubulin distribution (Connolly et al., 1977).

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REFERENCES


Note added in proof: Recently, in collaboration with Connolly & Kalnins at the University of Toronto, we were able to obtain precipitating antiserum against purified tau. This antiserum reacted with all four tau bands but showed no reaction against the purified high molecular weight component, by Ochterlony double diffusion. This further supports the notion that tau and the high molecular weight components are unrelated.