The Purification of Tau Protein and the Occurrence of Two Phosphorylation States of Tau in Brain*

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Two newly discovered properties of tau protein are reported; it is soluble in 2.5% perchloric acid and insoluble in 25% glycerol. These properties were exploited in the development of improved methods for the purification of tau. Treatment with perchloric acid did not alter the electrophoretic behavior of tau, and the products of the new isolation method were fully competent in the promotion of microtubule assembly. The application of the new purification techniques to bovine brain tissue demonstrated that tau exists endogenously in the dephosphorylated as well as in a phosphorylated state.

Microtubules are ubiquitous among eukaryotes and are involved in a wide variety of cellular functions involving structure and motility. Microtubule protein is composed largely of tubulin, but other proteins are included. After initial unsuccessful attempts to polymerize highly purified tubulin (1), a crude microtubule preparation (2) was found to polymerize in the presence of GTP, Mg²⁺, and the near absence of free Ca²⁺. Several families of proteins that significantly promote microtubule polymerization have since been isolated from crude microtubule preparations. One of these is the tau family, first described by Weingarten et al. (3). Methods for the purification of tau generally use either conventional chromatography (4) or a somewhat simpler procedure that takes advantage of the heat resistance of tau (5-8). These techniques have some disadvantages in that they are relatively slow and that they require microtubule protein to be partially purified by passage through cycles of polymerization at warm temperatures and depolymerization at cool temperatures. These problems limit many studies of tau to sources with a sufficiently high concentration of tubulin to allow the cycling procedure to be performed. We report here the discovery of two new properties of tau that can be exploited to simplify the purification and other manipulations of tau; tau is soluble in 2.5% perchloric acid and precipitates from solutions containing 25% glycerol. Taking advantage of these properties we have been able to extend previous studies which showed that tau can be interconverted in vitro between two distinct phosphorylation states which significantly affect the ability of tau to promote microtubule assembly (9). The present report demonstrates that both states of tau exist in brain tissue even though only the phosphorylated state had been recognized previously. The presence of both of these states of tau in vivo raises the possibility that the phosphorylation of tau may play a dynamic role in the control and regulation of microtubules and other molecules such as actin with which tau interacts in a phosphorylation-dependent manner (9-11).

EXPERIMENTAL PROCEDURES

Protein Purification—Microtubule protein was prepared from beef brain by a modification of the method of Shelanski et al. (12) as described previously (9). Both tubulin and the tau which was not subjected to perchloric acid were purified from microtubule protein as described (9). High molecular weight MAPs¹ were prepared according to the method of Borisy et al. (13). Dephosphorylated tau was prepared by treatment with alkaline phosphatase as described previously (9).

Several methods were used for purification of tau using perchloric acid. Two methods begin with crude beef brain extract prepared as described (9); in Method I the crude extract was taken to 35% ammonium sulfate saturation, and the insoluble fraction was removed by centrifugation. The supernatant was taken to 45% saturation, centrifuged, and the resulting pellet was resuspended in 2.5% perchloric acid. The slurry was incubated for 15 min at 15,000 x g. No tau was detected above the background when heavy loads of the pellet were analyzed electrophoretically. The supernatant made to 20% trichloroacetic acid was incubated for 15 min on ice and centrifuged for 15 min at 15,000 x g. The pellet was washed twice with 95% ethanol, dried under vacuum, and resuspended in buffer for analysis. Method II was as Method I, but ammonium sulfate fractionation was omitted, and trichloroacetic acid precipitation was replaced by precipitation with 25% glycero (v/v). When the supernatant from glycero precipita tion was dialyzed and analyzed electrophoretically only traces of tau were detected. Typically, 1 kg of brain tissue yielded about 8-10 mg of tau with either method.

Method III for purifying tau begins with a single cycle of the cold/warm procedure described previously (9) except that 1 mM ATP was added to the brain crude extract, and in the warm centrifugation the microtubule protein was pelleted through a layer of buffer containing 50% by volume (6.8 M) glycerol and 0.1 mM GTP. The resulting pellets can, optionally, be quick frozen in liquid N₂ and stored at -70°C. The (thawed) pellets were resuspended in 10-15% of the original volume of buffer on ice for 30 min with occasional passage in a Dounce homogenizer and then centrifuged to remove insoluble material. The tau was then purified as described in Method I beginning with the ammonium sulfate fractionation except that the perchloric acid was removed by dialysis.

Electron microscopy was performed as described previously (9) except that samples were not fixed prior to examination. Protein concentrations were estimated by the method of Lowry et al. (14). Gel electrophoresis was done as described (9) except that the dye front was not run off the gel.

RESULTS

Rapid Preparation of Semipure Tau Protein.—The principal factor used to simplify the purification of tau arose from the

¹ The abbreviations used are: MAPs, microtubule-associated proteins; MES, 2-(N-morpholino)ethanesulfonic acid; EDTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N"-tetraacetic acid.
Purification of Tau Protein

Ammonium sulfate fractionation was used rather than glycerol precipitation, since tau from the microtubule pellet has low solubility when treated with perchloric acid directly after resuspension; the reason for this is probably related to the glycerol used in the assembly of microtubes. Tau purified by this method produces typical microtubules when copolymerized with purified tubulin (Fig. 3).

Of all the constituents of the semipure tau seen in Fig. 1, A and B, only the tau proteins copolymerize with tubulin. This observation led to the development of a fourth method for the isolation of pure tau from semipure tau. As seen in Fig. 4, a highly purified tau fraction was obtained by copolymerization of semipure tau with tubulin, pelleting the resulting microtubules, dissolving the microtubules in fresh buffer on ice, then precipitating the tubulin by treatment with perchloric acid.

The Nature of the Product of These Isolation Procedures—
The purification based on the solubility of tau in 2.5% perchloric acid yields material that is more complex electrophoretically than products reported for earlier methods. There are eight components represented by seven electrophoretic bands, and they are the same tau proteins as those studied by Cleveland et al. (4), Sandoval and Weber (8) and others, except that they exist in two phosphorylation states. We previously described (9) two different phosphorylation states of tau in which conventionally isolated tau, stripped of phosphate by alkaline phosphatase (state I) appeared as a four-band pattern in gel electrophoresis, while phosphorylated tau (state II) appeared as a retarded but otherwise similar set of bands. Since this is such an unusual property it can be used to separate tau from other proteins in brain crude extract, but purification was further enhanced when it was combined with ammonium sulfate fractionation or with glycerol fractionation. Method I is represented by Fig. 1A, which shows an electrophoretic analysis of tau that was derived from brain crude extract by ammonium sulfate fractionation followed by perchloric acid treatment. The group of dark bands in the upper part of the gel are the tau bands. Method II depends on the discovery that tau has a low solubility in 25% glycerol.

Preparation of Pure Tau Protein—Another method (Method III) for the preparation of pure tau incorporates copolymerization of tau into microtubules by the passage of crude brain extract through a cycle of cool-warm polymerization before ammonium sulfate fractionation and perchloric acid treatment. Fig. 2 shows the products of this purification.

![Fig. 1. Purification of tau using perchloric acid.](image1)

Tau was purified from crude brain extract using perchloric acid fractionation: lane A, method I; lane B, method II; lane C, total perchloric acid-soluble fraction prepared as for method I but without ammonium sulfate fractionation.

![Fig. 2. Purification of tau protein.](image2)

Tau purified by method III: lane A, crude extract; lane B, microtubule protein after one cycle of polymerization; lane C, precipitate between 35 and 46% saturated ammonium sulfate; lane D, perchloric acid-soluble fraction.

observation that tau is soluble in 2.5% perchloric acid. This observation led to the development of a fourth method for the isolation of pure tau from semipure tau. As seen in Fig. 4, a highly purified tau fraction was obtained by copolymerization of semipure tau with tubulin, pelleting the resulting microtubules, dissolving the microtubules in fresh buffer on ice, then precipitating the tubulin by treatment with perchloric acid.

![Fig. 3. Polymerization of tubulin with tau purified using perchloric acid treatment.](image3)

Tubulin (0.18 mg/ml), 1 mM GTP, 0.1 M MES, 1 mM mercaptoethanol, 0.5 mM MgCl₂, 0.1 mM EDTA, 1.0 mM EGTA, pH 6.4, were mixed at 0 °C, and tau (0.03 mg/ml) was added to initiate the reaction. After rapid mixing, the samples were immediately transferred to a thermostatically controlled spectrophotometer for recording the absorbance at 350 nm at 30 °C. Inset shows an electronmicrograph of microtubules prepared under similar conditions (except tubulin concentration was 0.55 mg/ml; tau was 0.1 mg/ml). The unfixed samples were stained with uranyl acetate. Bar = 0.1 μm.
The products of our new isolation procedure are considered to be tau proteins, not only because as a group they promote microtubule assembly and copolymerize with tubulin, but also because state II tau was further phosphorylated without substantial additional change in electrophoretic mobility (9). The new purification methods produce a mixture of these two states, which appear as five bands in low resolution electrophoresis and as seven (or occasionally eight) bands at higher resolution. The new purification methods produce a mixture of these two states, which appear  as five bands in low resolution electrophoresis and as seven (or occasionally eight) bands at higher resolution. The new purification methods produce a mixture of these two states, which appear  as five bands in low resolution electrophoresis and as seven (or occasionally eight) bands at higher resolution.

The samples described in Fig. 5 were copolymerized with purified tubulin as described in Fig. 3. The final extent of polymerization (ΔA305) was obtained after at least 1 h of incubation at 30 °C.

<table>
<thead>
<tr>
<th>Type of tau</th>
<th>Assembly conditions</th>
<th>Extent of assembly (ΔA305)</th>
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<tbody>
<tr>
<td></td>
<td>Tubulin Tau</td>
<td>Tau exposed to perchloric acid</td>
</tr>
<tr>
<td>State I (dephosphorylated)</td>
<td>0.18</td>
<td>0.03</td>
</tr>
<tr>
<td>State II (untreated)</td>
<td>0.55</td>
<td>0.10</td>
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**Fig. 4** (left). Highly purified tau prepared from partially purified tau by copolymerization with tubulin. Tau (0.7 mg/ml) purified by method I was mixed with tubulin (0.9 mg/ml) and 1 mM GTP in assembly buffer (see Fig. 3); after 60 min of incubation at 37 °C the resulting microtubules were pelleted, and, after resuspension at 0 °C, the tubulin was removed by precipitation in 2.5% perchloric acid. Lane A, initial semi-pure tau; lane B, tau recovered after copolymerization.

**Fig. 5** (right). Electrophoretic pattern of tau exposed to perchloric acid. Tau (0.9 mg/ml) prepared without the use of perchloric acid treatment was the starting material. Lane A, starting material (state II tau); lane B, starting material was treated with 2.5% perchloric acid and precipitated with 20% trichloroacetic acid as in Fig. 1C; lane C, starting material treated with perchloric acid and precipitated with 25% glycerol as in Fig. 1B; lane D, starting material that had been dephosphorylated (state I tau); lane E, tau as in lane D treated with perchloric acid and precipitated with trichloroacetic acid. All lanes were from the same gel, but irrelevant lanes were removed between C and D.

**Fig. 6.** Microtubules assembled with perchloric acid-treated tau. Microtubules polymerized as described for Table I were stained with uranyl acetate and examined unfixed by electron microscopy. Top, microtubules polymerized using untreated tau; middle, microtubules polymerized using tau treated as in Fig. 5B; bottom, microtubules polymerized using tau treated as in Fig. 5C. Bar = 0.1 μm.

It was necessary to prove that the electrophoretic complexity in our preparation was not created by structural alterations of tau caused by perchloric acid. Tau was purified by the method of Sandoval and Weber (8) and then either dephosphorylated by alkaline phosphatase or left untreated. Both the untreated (e.g., Fig. 5, A–C) and the dephosphorylated (e.g., Fig. 5, D and E) samples were exposed to perchloric acid, and neither sample showed any alteration in the properties of tau. Fig. 5 demonstrates that there was no change in the sodium dodecyl sulfate electrophoretic patterns after perchloric acid exposure. Table I shows that the ability of tau to promote the development of turbidity of a warmed tau-tubulin mixture was unchanged by prior perchloric acid treatment, while Fig. 6 confirms that the resulting microtubules were identical whether the tau was treated by perchloric acid or not. The microtubules formed using dephosphorylated tau, either perchloric acid treated or not, were indistinguishable from those using untreated tau (not shown). Since perchloric acid does not alter either phosphorylated or dephosphorylated tau, it is evident that the four tau proteins occur in both phosphorylation states in brain and give rise to the complex electrophoretic patterns characteristic of the tau purified by the methods described in this report. Although the mixes of states I and II, therefore, are seen to be endogenous, method III produced tau in state II. The absence of state I tau was not due to a loss in the various waste fractions as shown by analysis of perchloric acid extracts of those fractions. The absence of state I tau in the product of method III was probably due to phosphorylation during the warm step in cycle purifications when sufficient ATP was present (see Figs. 7 and 8).
incubated with microtubule protein and ATP for 1 or 4 days, the tau was converted to state II; note the difference in mobilities between the proteins in the two left lanes of Fig. 7A and those of the bands in Fig. 5, D and E.

The complexity of tau isolated from brain tissue might be somewhat variable because of kinases in crude extracts, especially if exogenous ATP is added. Indeed, as reported previously (9), a kinase is present in brain which is capable of phosphorylating tau from state I (unphosphorylated) to state II. Although conventionally purified tau commonly gives an electrophoretic pattern largely representing state II, the tau purified from crude brain extract using perchloric acid gives a seven-band pattern which is indistinguishable from the seven-band pattern obtained by mixing tau from the two phosphorylation states. Fig. 7 demonstrates that material represented by this seven-band pattern was readily converted to the four fully phosphorylated components of state II tau, but only when ATP and a source of the kinase were present (Fig. 7A, two left lanes). This transformation was not observed if GTP was substituted for ATP (Fig. 7A, third and fourth lanes) and did not occur in the absence of crude (cycled) microtubule protein which served as the source of kinase (Fig. 7B). The kinase did not copurify with tubulin or high molecular weight MAPs (Fig. 8). The conversion was very slow when thrice cycled microtubule protein was used as the source of kinase; crude brain protein would most likely catalyze phosphorylation much more rapidly, but its use is impractical due to the presence of proteases.

DISCUSSION

The original purification of tau was done by Kirschner and co-workers (3) who capitalized on the copolymerization of tau proteins with tubulin when cell extracts were warmed in the presence of Mg, GTP, EGTA, and glycerol. Since cold temperatures depolymerize most microtubules, repeated cycles of warming and cooling can be used to purify tau from other proteins. A second, somewhat simpler approach to the purification of tau takes advantage of the heat resistance of tau. Several minor variations of this approach have been used (6, 8, 15), but all start with cycling of microtubule protein prior to heating.

The solubility of tau in the presence of perchloric acid and its low solubility in the presence of glycerol give two new tools for the purification and characterization of tau. The solubility in perchloric acid, in particular, is useful since it can be applied very rapidly and thus captures tau in its natural state of phosphorylation before tau can be subjected to significant enzymatic activity. Clearly (Table I, Fig. 6) short-term exposure to perchloric acid did not seem to affect the properties of tau. In our more extended experience, however, we found that after 2 to 4 h of exposure to 2.5% perchloric acid the yields of soluble tau began to decline. One possible explanation for this phenomenon is that perchloric acid denatures tau slowly; therefore, exposure to the acid should be held to a minimum.

By taking advantage of these properties and other previously known properties of tau such as heat stability and ability to copolymerize with tubulin many types of manipulation of tau become simple which were difficult or impossible previously. For instance, the purifications seen in Fig. 1, A and B, which yield 50–75% pure tau from crude brain in a few hours, are equally applicable to very large and very small samples and can be applied to frozen brain as well as fresh brain. Additionally, these unique solubility properties of tau allow it to be readily separated from other proteins such as kinases or phosphatases when phosphorylation states are to be studied or from tubulin or actin when their copolymerization with tau.

![Fig. 7. Conversion of perchloric acid-treated tau to state II tau.](image)

![Fig. 8. Incubating tau with various microtubule components: electrophoretic analysis.](image)
is to be studied. These separations can be achieved rapidly and efficiently even when small quantities of tau are involved, by taking the sample of 2.5% perchloric acid, then recovering the tau from the soluble fraction by dialysis to remove the acid, or by precipitation of the tau with 20% trichloroacetic acid, or by precipitation of the tau with 20% trichloroacetic acid or 25% glycerol. The glycerol precipitation has the advantage of being a purification step which may be useful in certain circumstances, but it is inefficient at low tau concentrations, e.g. below 0.1 mg/ml.

Both phosphorylation states of tau are present in brain in vivo, even though only the four components of state II seem to have been recognized. Other workers have probably isolated the mix of tau proteins in states I and II, observed as five electrophoretic bands on gels of low acrylamide concentration. For instance, Cleveland et al. (4) presented an alternate procedure for the purification of tau without cycles of polymerization which yielded five gel bands. Theurkauf and Vallee (16) reported proteolysis of tau on treatment with acid phosphatase, but the published picture of their polyacrylamide gel might be explained by partial conversion of state II tau to state I tau through dephosphorylation. In view of the observation (Fig. 2) that tau can be phosphorylated during the warm step of the cycling procedure to yield state II tau, it is not surprising that published gels of purified tau sometimes show four bands (e.g. Herzog and Weber (7)) and sometimes five bands (e.g. Selden and Pollard (10)).

When our source of bovine brains switched from steers (relatively young) to older bulls and cows, we noted that the endogenous ratio of phosphorylation states seemed to be altered. Moreover, the kinase activity of the brain crude extract seemed somewhat lower in the case of the older animals. Although more extended and systematic studies need to be done to establish the point, these preliminary observations suggest that physiological factors in aging or hormonal conditions might affect the phosphorylation state of tau and presumably its function. This phenomenon might be parallel to the one observed by Schroeder et al. (17) who report six times as much kinase activity in calf brains as was found in animals 11 to 13 years of age. The fact that tau exists in two different phosphorylation states in vivo implies some difference in function or control. For example, a preliminary experiment in which gray matter and white matter from a single brain were separated prior to perchloric acid purification of tau gave different ratios of the two states of tau. The two states of tau may be used for different purposes in the cell; additionally the cell may rely upon interconversion between the two states to regulate some dynamic process involving microtubules.

REFERENCES