Tau Proteins: The Molecular Structure and Mode of Binding on Microtubules

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Abstract. Tau is a family of closely related proteins (55,000–62,000 mol wt) which are contained in the nerve cells and copolymerize with tubulin to induce the formation of microtubules in vitro. All information so far has indicated that tau is closely apposed to the microtubule lattice, and there was no indication of domains projecting from the microtubule polymer lattice. We have studied the molecular structure of the tau factor and its mode of binding on microtubules using the quick-freeze, deep-etch method (QF·DE) and low angle rotary shadowing technique. Phosphocellulose column–purified tubulin from porcine brain was polymerized with tau and the centrifuged pellets were processed by QF·DE. We observed periodic armlike elements (18.7 ± 4.8 nm long) projecting from the microtubule surface. Most of the projections appeared to cross-link adjacent microtubules. We measured the longitudinal periodicity of tau projections on the microtubules and found it to match the 6-dimer pattern better than the 12-dimer pattern. The stoichiometry of tau versus tubulin in preparations of tau saturated microtubules was 1:~5.0 (molar ratio). Tau molecules adsorbed on mica took on rodlike forms (56.1 ± 14.1 nm long). Although both tau and MAP1 are contained in axons, competitive binding studies demonstrated that the binding sites of tau and MAP1A on the microtubule surfaces are mostly distinct, although they may partially overlap.

MICROTUBULES are one of the main cytoskeletal elements in eukaryotic cells and are particularly abundant in nerve cells. It is well known that there are several microtubule-associated proteins (MAPs)[1] which copurify with brain tubulin during repetitive cycles of temperature-dependent assembly and disassembly. Among these proteins in neuronal tissues, high molecular weight proteins (MAP1 and MAP2), and tau factor are major species, and recently several minor proteins have also been identified (6, 7, 17, 27, 28, 33, 41).

The high molecular weight microtubule-associated proteins (MAP1, MAP2) are flexible, rodlike structures ~100–200 nm long and form armlike projections when attached to microtubule surfaces (22, 31, 39, 40, 42). Recent structural studies demonstrated that microtubule domains in nerve cells are composed of microtubules, associated cross bridges, and granular materials (11, 14, 31). High molecular weight MAPs (MAP1, MAP2, 270,000-mol-wt MAP) were proven to be components of these cross bridges associated with microtubules in vivo (12, 14, 32).

Tau factor is composed of four to five polypeptides (55,000–62,000 mol wt) which were shown to be closely related by both peptide mapping and amino acid analysis (6), and were assumed to represent a highly asymmetric molecule by hydrodynamic data (7). This protein promotes polymerization of tubulin, is heat stable (41), and is able to bind to calmodulin in the presence of calcium (34). Recent immunocytochemical studies have shown that tau is mainly localized in axons (3). It has also been revealed recently that highly phosphorylated tau is a major element of paired helical filaments in Alzheimer's disease (8, 18, 23).

However, the molecular structure of tau and the mode of binding of tau to microtubules has remained unclear. This study was designed to disclose the molecular structure of tau by the quick-freeze, deep-etch method and low angle rotary shadowing technique, and has allowed us to reveal for the first time that tau is a rodlike structure (56.1 ± 14.1 nm long) and associates with microtubules with armlike projections (18.7 ± 4.8 nm long).

Materials and Methods

Isolation of Tau

Tau was prepared from porcine brain microtubules by the modification of a method described by Grundke-Iqbal et al. (8). Pellets of microtubule proteins obtained by cycles of temperature-dependent assembly and disassembly (30) were suspended in 3 vol of buffer containing 100 mM 2-(*N*-morpholino)ethane sulfonic acid MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 0.75 M NaCl, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 2.7. The suspension was then heated at 95°C in boiling water for 5 min and centrifuged at 25,000 *g* at 4°C for 30 min. The pH of the resulting supernatant was adjusted to 6.8, and it was then subjected to Bio gel A 1.5 m (Bio-Rad Laboratories, Richmond, CA) column chromatography and concentrated in order to prepare purified tau fractions (1.89

1. *Abbreviations used in this paper*: MAP(s), microtubule-associated protein(s); PC, phosphocellulose column purified; PEM, 0.1 M Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8.

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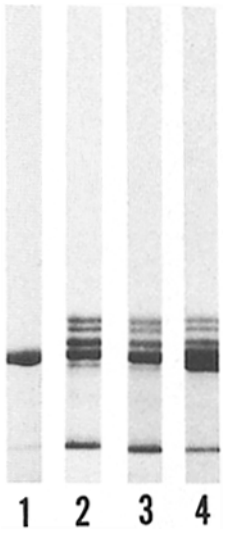

Figure 1. SDS-PAGE (7.5% running gel) of PC tubulin (lane *1*), purified tau factor (lane *2*), supernatant (lane *3*), and pellet (lane *4*) of suspensions of PC tubulin (1.0 mg/ml) and tau (0.4 mg/ml). The pellet was resuspended with PEM in a volume equal to the original suspension, and the same volume of the sample was applied (lanes *3* and *4*) on the gel.

mg/ml). Tau proteins were dialysed against PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8).

Isolation of Tubulin

Tubulin was prepared from porcine brain by phosphocellulose chromatography (41).

Protein Determination

Protein concentrations were estimated by the assays of Bradford et al. (5) and Itzhaki and Gill (19) using BSA as a standard.

Quick-Freeze, Deep-Etch Electron Microscopy of Tubulins Polymerized with Tau

Phosphocellulose column-purified (PC) tubulin was mixed with tau fraction (final concentration: tau 0.4 mg/ml, tubulin 1.0 mg/ml) and incubated at 37°C in the presence of 1 mM GTP for 30 min. As a control, PC tubulin without tau was incubated at 37°C in the presence of 20 μM taxol and 1 mM GTP (38). The resulting polymers were centrifuged at 19,000 rpm at 30°C for 30 min, and pellets were quick frozen and deep etched as described previously (9–11, 14). In some cases pellets were resuspended with a small amount of PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) (four times the pellet volume), chilled on ice for 30 min, supplemented with taxol to 20 μM, and rewarmed at 37°C for 15 min. The suspension was quick frozen without centrifugation. The samples were dissolved in Purelox (Ohylox Corp., Tokyo, Japan), and replicas were washed with distilled water, and put on Formvar-carbon-coated grids.

Quick-Freeze, Deep-Etch Electron Microscopy of Axons

Sciatic nerves were dissected out of rats and incubated for 30 min at room temperature in 0.1% saponin, 70 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 30 mM Hepes, pH 7.4, 10 μM taxol, and 0.1 mM phenylmethylsulfonyl fluoride as described previously (14). They were quick frozen as described previously (9–11, 14).

Low Angle Rotary Shadowing of Tau Molecules

Tau fractions were suspended in glycerol (50% glycerol PEM) at 50 μg/ml protein concentration and sprayed on freshly cleaved mica flakes as described by Tylor and Branton (36) and dried by vacuum evaporation. The samples were rotary shadowed with platinum in a freeze-fracture machine (model 301; Balzers, Hudson, NH) at an angle of 6°. The replicas were detached from mica with hydrofluoric acid, washed with distilled water, and collected on Formvar-carbon-coated grids.

Electron Microscopy and Measurement

Replicas were examined with a JEOL 2000EX electron microscope at 100 kV and photographed. Micrographs were printed with their contrast reversed. The length of tau projections in the tau-saturated microtubule pellets and the length of tau molecules on mica were measured under a magnifying glass. The center to center distances between adjacent arms in replicas of tau-saturated microtubules were measured in the same way.

Stoichiometry of Tubulin and Tau

PC tubulin was mixed with an excess amount of tau fraction and incubated at 37°C in the presence of 1 mM GTP with or without 20 μM taxol for 30 min. As a control, PC tubulin without tau was incubated at 37°C in the presence of 20 μM taxol and 1 mM GTP. The suspensions were centrifuged at 19,000 rpm at 30°C for 30 min. The resulting pellets were resuspended in PEM, chilled thoroughly on ice, and homogenized thoroughly. Protein concentration of the pellets and supernatants was determined by the assays of Bradford et al. (5) and Itzhaki and Gill (19). SDS-PAGE of the pellets and supernatants was performed according to the method of Laemmli using 7.5% acrylamide (25). Gels were stained with Coomassie Brilliant Blue, scanned, and the areas of peaks were measured by densitometry (model CS 9000; Shimadzu Corp., Kyoto, Japan).

Tau-MAP1 and MAP1-MAP2 Displacement Experiments

MAP1A was purified by affinity chromatography on a cyanogen bromide–activated Sepharose 4B column containing our monoclonal antibody against MAP1A as described previously (31). After the application of crude extracts of rat brain, the column was washed with PEM containing 0.75 M NaCl and 0.5% NP-40. Bound polypeptides were eluted with 3 M $MgCl_2$ in 0.1 M PEM buffer, and peak fractions were dialyzed against 20 mM PEM buffer, and subjected to PAGE to check their purity.

PC tubulin was mixed with MAP1A at a weight ratio of 1:2.4 in the presence of 20 μM taxol and 1 mM GTP. Suspensions were incubated at 37°C for 30 min and were then layered onto 20% sucrose cushions containing tau at a weight ratio of 1 tubulin (in suspension) per 2 tau (in the cushion) or at a ratio of 1:0.2, 20 μM taxol and 1 mM GTP. Microtubules were centrifuged through the sucrose cushions at 30,000 *g* for 1 h at 30°C. Sucrose cushions lacking tau proteins were used as control.

In reciprocal experiments, PC tubulin was mixed with tau at a weight ratio of 1:0.4 in the presence of 20 μM taxol and 1 mM GTP. After incubation at 37°C for 30 min, the suspensions were centrifuged through 20% sucrose cushions containing MAP1A at a weight ratio of 1 tubulin (in suspension) per 2 MAP1A (in the cushion) or at a ratio of 1:1, 20 μM taxol, and 1 mM GTP. In these cases, the sucrose cushions of control samples contained no MAP1A.

In addition PC tubulin was mixed with tau and MAP1A at weight ratios of 1:1:1.2 in the presence of 20 μM taxol and 1 mM GTP. PC tubulin plus tau (1:1), PC tubulin plus MAP1A (1:1.2), and PC tubulin alone were also examined. After incubation at 37°C for 30 min, the suspensions were centrifuged through 20% sucrose cushions containing 20 μM taxol and 1 mM GTP. After their surfaces were washed with PEM, the pellets were resuspended in PEM. SDS-PAGE of the pellets and supernatants was performed according to the method of Laemmli using 7.5% acrylamide gels (25). Gels were stained with Coomassie Brilliant Blue, scanned with a densitometer (model CS 9000; Shimadzu Corp.), and the areas of peaks were measured.

MAP1 and MAP2 displacement experiments were performed similarly using MAP2 instead of tau proteins. MAP2 was purified from rat brains using a Superose 6 prepgrade gel filtration column (Pharmacia Fine Chemicals, Uppsala, Sweden) as described previously (15). PC tubulin was mixed with MAP1A at a weight ratio of 1:2.4 in the presence of 20 μM taxol and 1 mM GTP. Suspensions were incubated at 37°C for 30 min and then layered onto 20% sucrose cushions containing MAP2 at a weight ratio of 1 tubulin (in suspension) per 2.4 MAP2 (in the cushion), 20 μM taxol and 1 mM GTP. Microtubules were centrifuged through the sucrose cushions at 30,000 *g* for 1 h at 30°C. Sucrose cushions without MAP2 were used as controls. Reciprocal experiments were also carried out. In additional experiments PC tubulin was mixed with MAP1A and MAP2 at a weight ratio of 1:2:2 in the presence of 20 μM taxol and 1 mM GTP. Tubulin plus MAP1A (1:2), tubulin plus MAP2 (1:2), and tubulin alone were also examined. After incubation at 37°C for 30 min the suspensions were centrifuged through 20% sucrose cushions containing 20 μM taxol and 1 mM GTP. Microtubules were cen-

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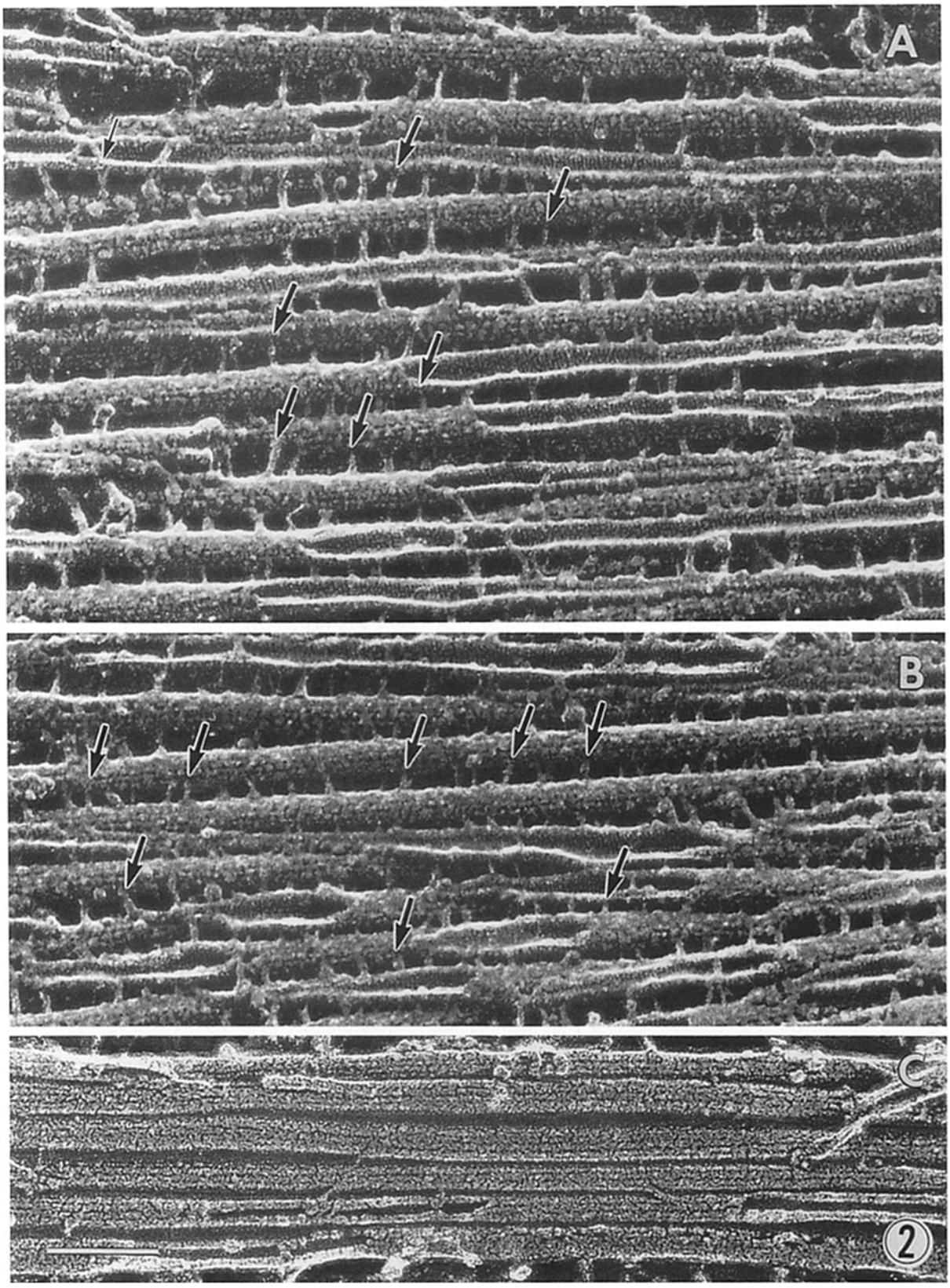

Figure 2. Higher magnification views of quick-frozen, deep-etched microtubule pellets polymerized with (*A* and *B*) and without tau (*C*). Although only tightly packed microtubules are observed in *C*, numerous projections (*arrows*) exist on the microtubules polymerized with tau (*A* and *B*). Bar, 100 nm.

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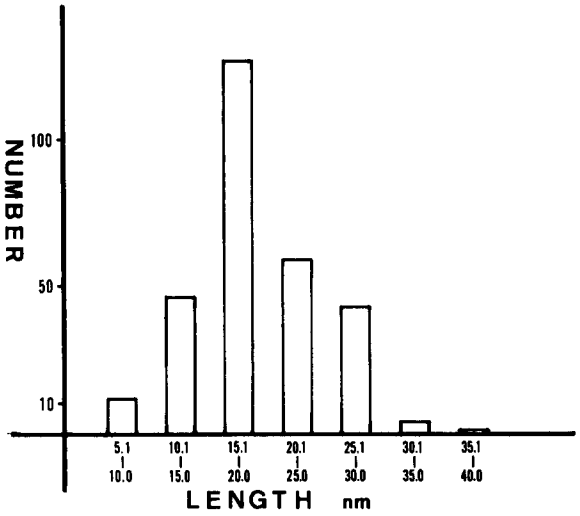

Figure 3. A histogram showing the length–frequency distribution of tau projections on microtubules. $n = 292$.

trifuged at 30,000 *g* for 1 h at 30°C. SDS-PAGE of the pellets and supernatants was performed.

Results

Fig. 1 shows SDS-PAGE analysis of PC tubulin (lane *1*), purified tau factor (lane *2*), the supernatant (lane *3*), and pellet (lane *4*) of a suspension of PC tubulin plus tau incubated at 37°C for 30 min in the presence of 1 mM GTP. The tau factor from porcine brain prepared by heat treatment at a low pH was composed of five bands (molecular weight of 50,000–65,000) (lane *2*). Boiling at a low pH removed the high molecular weight MAPs while tau remained soluble (8). The presence of tau promoted polymerization of PC tubulin, and thus, in the pellet we found tubulin plus tau.

Tau Forms Armlike Projections on Microtubule Surfaces

Fig. 2 displays high magnification views of the tau–microtubule pellet processed by the quick-freeze, deep-etch method. As can be seen, the pellet contained numerous parallel microtubules, each with numerous armlike projections (<20 nm long) attached to the microtubule surfaces. The projections were short and straight and appeared to cross-link adjacent microtubules. When PC tubulin was polymerized with tau in the presence of 10 μM taxol and 1 mM GTP, we observed similar projections on the microtubules. Because samples which contained only PC tubulin exhibited bare surfaces, (Fig. 2 *C*), we could conclude that tau forms straight, short, armlike projections on the surfaces of microtubules.

Fig. 3 shows a histogram of the length–frequency distribution of tau projections on microtubules. The average length was 18.7 ± 4.8 (SD) nm.

The projections appeared to cross-link adjacent microtubules. To check the possibility that centrifugation induced the patterns we observed, we resuspended the tau-saturated microtubules in a small volume of PEM, incubated them on ice for 30 min, and after homogenization rewarmed the suspension in the presence of 20 μM taxol and 1 mM GTP. The suspension was then quick frozen without centrifugation. In such preparations most of the microtubules tended to run

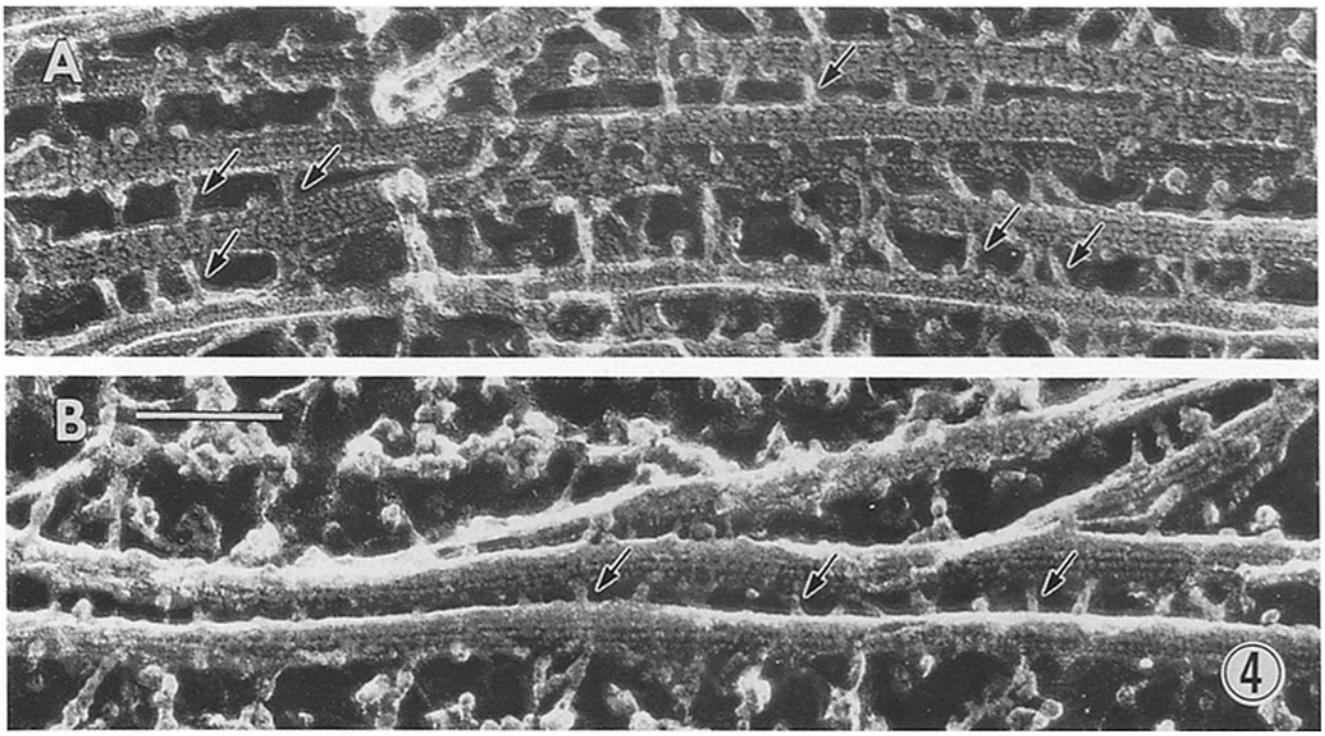

Figure 4. Quick-frozen, deep-etched suspension of microtubules saturated with tau proteins. Note the frequent cross bridges (*arrows*) between the microtubules. Bar, 100 nm.

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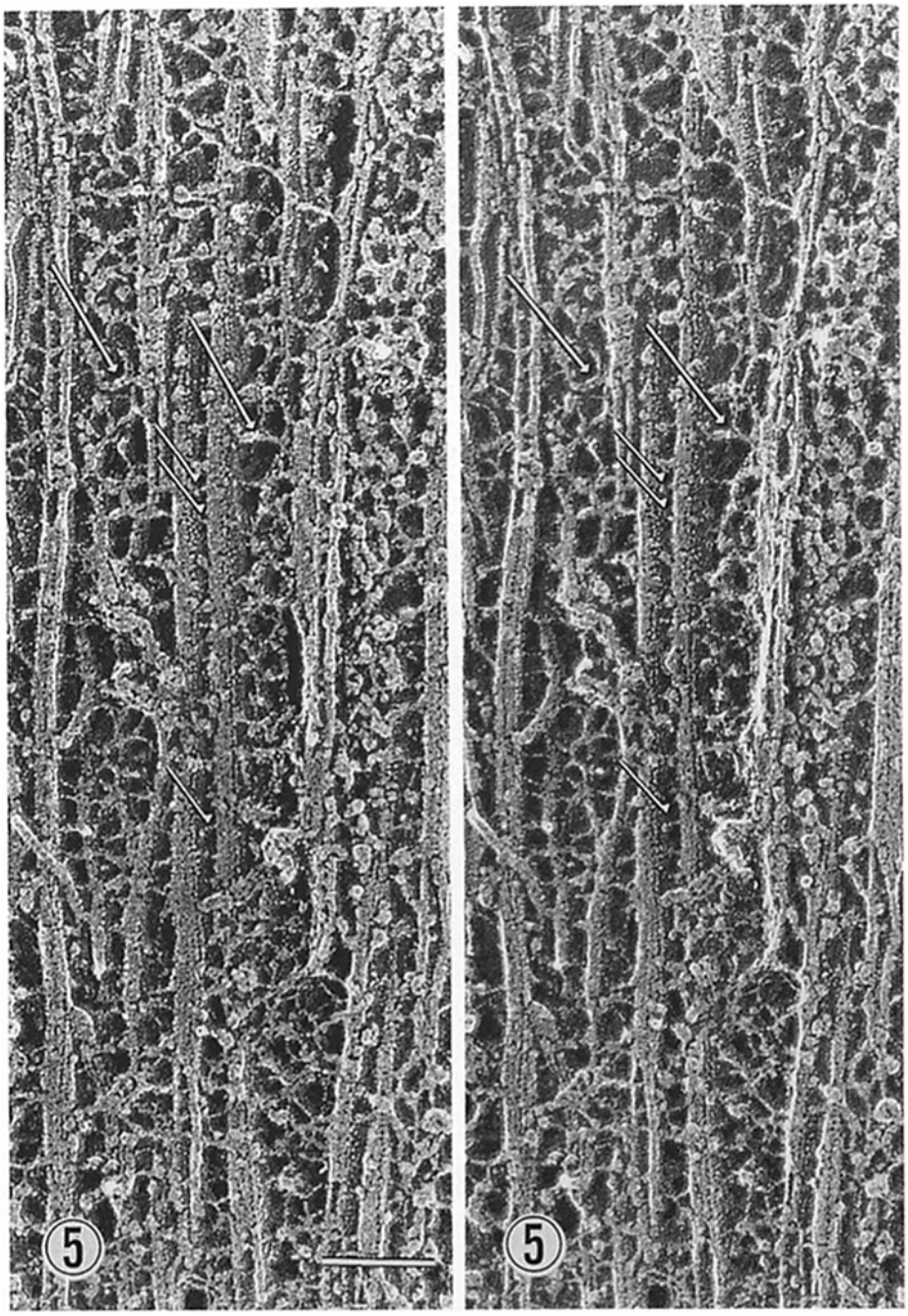

Figure 5. A stereopair of an axoplasm in a rat sciatic nerve quick-frozen and deep-etched after saponin extraction. Frequent short cross bridges (*short arrows*) are found between microtubules. Longer strands are also associated with microtubules (*long arrows*). Bar, 100 nm.

randomly and granular structures were attached on the microtubule surfaces; however, when microtubules ran parallel and close to each other we found frequent cross-links between adjacent microtubules as shown in Fig. 4. These results suggest that tau proteins cross-link microtubules when microtubules are in very close proximity to each other.

Furthermore, we examined microtubule domains in the axons of rat peripheral nerves after saponin extraction. As shown in a stereopair in Fig. 5, microtubules are linked with each other via fine short cross bridges exactly like those found in the microtubules saturated with tau in vitro (See Fig. 4 in reference 11). Longer strands tending to form networks were also associated with microtubules (Fig. 5).

Longitudinal Periodicity of Tau Projections

We measured distances between adjacent projections on individual microtubules. Fig. 6 is a histogram of longitudinal spacing of adjacent arms on the same microtubules. The dots and arrows indicate spacings predicted by a 12-dimer superlattice model and a 6-dimer superlattice model, respectively (1, 20). Fig. 7 shows a schematic diagram of the 6-dimer superlattice model. The numbers 11, 15, 22, 27, 33, 37, and 48 on Fig. 7 indicate predicted spacings by a 6-dimer superlattice model. As shown in Fig. 6, the longitudinal spacings observed in tau-saturated microtubule pellets match the 6-dimer pattern better than the 12-dimer pattern. The actual data fit with spacings of 11, 15, 22, 26, 33, and 48 nm, while there

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