Tau Protein Becomes Long and Stiff Upon Phosphorylation: Correlation between Paracrystalline Structure and Degree of Phosphorylation

T. Hagestedt, B. Lichtenberg, H. Wille, E.-M. Mandelkow, and E. Mandelkow
Max Planck Unit for Structural Molecular Biology, D-2000 Hamburg 52, Federal Republic of Germany

Abstract. In a previous report we have shown that microtubule-associated protein tau can be induced to form paracrystals (Lichtenberg, B., E.-M. Mandelkow, T. Hagestedt, and E. Mandelkow. 1988. Nature [Lond.]. 334:359-362). A striking feature was the high degree of elasticity of the molecules. We now report that this property is related to the state of phosphorylation. When tau is dephosphorylated by alkaline phosphatase, it becomes shorter and more elastic; when it is phosphorylated by Ca++/calmodulin-dependent kinase, it becomes longer and stiffer. This may provide a model for the control of structural properties of tau-like molecules by phosphorylation.

TAU is one of the microtubule-associated proteins (MAPs) in mammalian brain (Weingarten et al., 1975). When isolated from brain tissue it is a mixture of several polypeptides of apparent molecule mass values between 50 and 70 kD. The sequence of tau protein from mouse containing 364 amino acid residues has been reported (Lee et al., 1988a). A highly homologous human form of tau has been found in the neurofibrillary tangles of Alzheimer's disease (Goedert et al., 1988). The COOH-terminal part of tau contains three internal repeats and seems to be responsible for the binding to microtubules (Aizawa et al., 1988). This part is also homologous to the COOH-terminal region of microtubule-associated protein 2 (MAP2), another brain MAP (Lewis et al., 1988), suggesting that it might serve as a microtubule-binding domain for a family of proteins.

Tau is heat stable and soluble in perchloric acid (Cleveland et al., 1977; Lindwall and Cole, 1984b); this forms the basis for the isolation procedure (see Materials and Methods). Cole and co-workers showed that the apparent heterogeneity of tau can be reduced by controlling the state of phosphorylation (Lindwall and Cole, 1984b; Baudier and Cole, 1987a). Tau treated by alkaline phosphatase shows four main isoforms on SDS gels, termed tau-4. All of them can be phosphorylated by several kinases. In particular, the Ca++/calmodulin-dependent kinase (CaMK) induces a conformational change that results in an upward shift of the bands in the gel. Thus, when the protein is isolated in a mixed state of phosphorylation the number of observed bands is greater than four. The change in electrophoretic mobility is a convenient assay to monitor phosphorylation by CaMK; it is not observed after phosphorylation with other kinases such as protein kinase C.

Structural information on tau or other MAPs is limited so far. Hydrodynamic data (Cleveland et al., 1977) and metal shadowing (Hirokawa et al., 1988) suggest a rod-like shape. In our attempts to crystallize tau we have recently obtained paracrystals suitable for electron microscopy and image processing (Lichtenberg et al., 1988). They show distinct transverse banding and polarity, indicating that the protein subunits are aligned with the same orientations. A striking feature is that tau is highly elastic, in contrast to other paracrystalline proteins. The protein can stretch or contract by >300%, with a range of axial repeats between 22 and 68 nm. In this earlier study, the reasons for the heterogeneity in spacings were unclear. However, we noted that paracrystals of tau that appeared more highly phosphorylated also had a tendency towards longer spacings. This observation was the starting point for the present study. We have now prepared tau protein in different states of phosphorylation and analyzed the paracrystals obtained from it. The results show that phosphorylated tau becomes long and stiff. This provides a framework for discussing possible roles of tau in living cells and its regulation by phosphorylation.

Materials and Methods

Microtubule protein from porcine brain was prepared by a modified temperature cycle method (Mandelkow et al., 1985) and boiled for 15 min (Fellous et al., 1977), followed by a clearing spin. The supernatant containing MAP2 and tau was then applied to a Pharmacia Fine Chemicals (Uppsala, Sweden) FPLC Mono-S column and eluted with a NaCl gradient (buffer: 20 mM Pipes, pH 6.9, 0.05-1.0 M NaCl, 2 mM DTT, 1 mM EGTA, 1 mM MgSO4). The elution profile shows three main peaks (Fig. 1 a). SDS-PAGE of peak 1 shows the usual four to six bands of tau protein in the range of 50-70 kD, depending on phosphorylation (Fig. 1 b). Peaks 2 and 3 con-
tain mostly MAP2 and a small contamination from tau (Fig. 1 b). The tau present in peak 2 was separated from the non-tau proteins (mainly MAP2) by precipitation in 2.5% perchloric acid (Lindwall and Cole, 1984b). The tau-containing supernatant was dialyzed against 0.1 M Pipes, pH 6.9, and concentrated by precipitation in 45% ammonium sulfate.

Tau was dephosphorylated by incubation with alkaline phosphatase (80 U/ml, calf intestine; Boehringer Mannheim GmbH, Mannheim, FRG) in 50 mM Tris-HCl, pH 8.2, 50 mM NaCl, 1 mM MgCl$_2$, 2 mM PMSF for 5 h at 37°C. The reaction was stopped by precipitating the enzyme with 2.5% perchloric acid, followed by dialysis against 0.1 M Pipes, pH 6.9.

Dephosphorylated tau was rephosphorylated by incubation with CaMK (0.01 U from rabbit muscle, kindly provided by D. Söling, Göttingen, FRG; or prepared from porcine brain following Woodgett et al., 1983) in 50 mM Tris-HCl, pH 7.2, 10 µg/ml calmodulin, 5 mM MgCl$_2$, 0.15 mM CaCl$_2$, 0.1 mM ATP, for 1 h at 37°C. The reaction was stopped by boiling for 5 min. Tau protein was radioactively labeled using $\gamma$-32P[ATP (Amerham Buchler GmbH, Brauschweig, FRG; 1 mCi/ml, 5000 Ci/mmol), with 1 µCi in 0.1 mM ATP. Tau protein labeled with 32P was detected by autoradiography from destained dried gels, using Eastman Kodak Co. (Rochester, NY) X-Omat AR-5 film (exposure time 1 wk at room temperature).

Results

Isolation of Tau in Different States of Phosphorylation

The first step in the purification of MAPs consists of the preparation of microtubule protein by three cycles of assembly and disassembly, using either ATP or GTP as a cofactor. Microtubule assembly requires GTP but ATP can be used because of a copurifying nucleoside diphosphate kinase which converts ATP to GTP (Jacobs and Huitorel, 1979). An additional effect of ATP is that microtubule proteins become increasingly phosphorylated by copurifying protein kinases (Jameson et al., 1980). One consequence is that the interaction between microtubules and MAPs is reduced and microtubules become less stable (Lindwall and Cole, 1984a).

The second step in the preparation of MAP2 and tau makes use of the fact that these two proteins are heat stable so that the other proteins can be precipitated by boiling (Pelloux et al., 1977). The phosphorylation of MAP2 and tau thus obtained depends on whether ATP or GTP was used in the preceding assembly cycles, but in general the proteins are in a mixed state of phosphorylation. In our previous study (Lichtenberg et al., 1988) we had used this mixture, separated MAP2 and tau by ion-exchange chromatography, and grew paracrystals whose repeat turned out to be quite variable. The aim of the present study was to elucidate the reason for the variability, and we therefore sought for ways to obtain paracrystals in defined states of phosphorylation.

Fig. 1 a shows the elution profile of the boiled MAP2/tau mixture after passage through a cation-exchange column (Mono S). There are three major peaks at roughly 0.2, 0.3, and 1 M NaCl. SDS-PAGE shows that the first contains mostly tau, the second and third mostly MAP2. Up to six bands of tau can be discerned. As shown by Baudier and Cole (1987a) there are four major tau isoforms, but their position on the gel is shifted up upon phosphorylation by CaMK. Thus, when tau is in a mixed state of phosphorylation one observes more than four bands, as is the case here. The state of phosphorylation does not appreciably affect the elution profile of tau (i.e., peak 1 in Fig. 1 a). By contrast, the phosphorylation of MAP2 cannot simply be assessed from the SDS gels, but it can be correlated with the elution profile. Phosphorylated MAP2 elutes at lower ionic strength (coinciding roughly with peak 2 of Fig. 1 a), while dephosphorylated MAP2 elutes mainly at the position of peak 3 (data not shown).

In our earlier crystallization experiments, we had used not only protein from the first peak (tau), but also from the second peak, hoping to obtain paracrystals of MAP2. Well ordered paracrystals were indeed obtained (Fig. 3), but surprisingly their structure appeared indistinguishable from that of tau paracrystals. They always had near-maximal repeats (>60 nm), and when stained with ammonium molybdate showed the characteristic longitudinal striations (see following section). Considering the much larger size of MAP2, this coincidence seemed hard to accept. It turns out that the second peak contains a minor contamination of tau (clearly identified in the blots of Fig. 2 c, lane 2) with decreased electrophoretic mobility, suggesting that this tau was phosphorylated. Indeed it is this fraction which forms the paracrystals in spite of the excess of MAP2, as shown by the following experiments.

The mixture of MAPs was dephosphorylated using alkaline phosphatase and run over a Mono S column, resulting in a shift of the elution peak 2 (containing MAP2) to the position of peak 3 of Fig. 1 a, whereas tau remained roughly in the 0.2 M salt fraction (Fig. 1 a, peak l). This means that a clean separation of tau and MAP2 on the Mono S column is best achieved in the dephosphorylated state. On SDS-PAGE the dephosphorylated tau protein showed only four bands and at somewhat lower positions, the second and fourth (tau2 and tau4) being more pronounced than the first and third (tau and tau3; compare Fig. 2 a, lanes 1 and 2). This protein was rephosphorylated using CaMK, leading to an upward shift of the four isoforms, with the same relative density distribution (Fig. 2 a, lane 3). The autoradiograph shows that phosphate has been incorporated into all four isoforms (Fig. 2 a, lane 4 and 5). Thus, with regard to phosphorylation and dephosphorylation our tau protein behaves as described by Baudier and Cole (1987a). This confirms that the tau protein used for the earlier crystallization experiments contained a mixture of more or less phosphorylated isoforms (as in Fig. 2 a, lanes 2 and 3). The properties of the paracrystals obtained from defined states of phosphorylation (as in Fig. 2 a, lanes 2 and 3) will be described in the next section. It should be kept in mind, however, that the test of phosphorylation by the shift in SDS gels detects only the type of phosphorylation which can be mimicked by CaMK; the cycled microtubule protein probably contains other kinases as well (e.g., cAMP-dependent kinase, protein kinase C, or others; see Vallee, 1980; Baudier and Cole, 1987b) which do not change the gel pattern yet might have an effect on the structure. This would explain why the range of variation in

The Journal of Cell Biology, Volume 109, 1989 164.4
the paracrystals with defined states of phosphorylation is less than what we had observed previously.

The fact that peak 2 of Fig. 1a (mainly MAP2) contained a small fraction of tau suggested that this protein was also in a different state of phosphorylation from that of the main tau peak 1 (compare Fig. 2b, lanes 1 and 2). To demonstrate this directly, the protein of the second peak was treated with perchloric acid which precipitates MAP2 and leaves tau in solution (Lindwall and Cole, 1984b). As shown in Fig. 2b, lane 3, this tau is largely phosphorylated (upward shift in the gel). Western blots with TAU1 antibody (Binder et al., 1985) identified all bands as tau (Fig. 2c). Paracrystals grown from this protein (Fig. 3) had long repeats and otherwise a similar appearance to those of the main tau peak, confirming that the paracrystals from the MAP2 peak (Fig. 1a, peak 2) indeed formed from the tau contamination. This highlights the strong tendency of tau molecules to adhere to one another even when there is a vast excess of other proteins.

### Structure of Tau Paracrystals

Fig. 3 shows two paracrystals of tau treated with different stains, 2% ammonium molybdate (Fig. 3a) or 2% uranyl acetate (Fig. 3b). They show periodic transverse striations with repeats of 66–67 nm. One observes two bright striations per repeat, termed bands 0 and 2, separated by 28 nm; i.e., roughly 40% of the repeat. Up to five other weak striations can be discerned, termed la, lb, 3, 4, and 5 (for details compare Fig. 6b below). The striations are best seen in uranyl acetate stain (Fig. 3b) and are largely buried in ammonium molybdate (Fig. 3a). In the latter stain only band 4 can be discerned by eye routinely. Its position between the bright striations is slightly asymmetric; it is closer to band 2 than to band 0. This provides an intrinsic marker of polarity (defined to be positive in the direction from band 0 to 2, 4, and the next 0). In Fig. 3 the minus end is at the bottom, the plus end is at the top. We will denote the region containing...
groups (Steven and Navia, 1982) this suggests that this stain represents largely the charge distribution, even when used as a negative stain.

In addition to the transverse striations, those ammonium molybdate paracrystals which have large spacings also show longitudinal striations, visible most clearly in the region

![Figure 2](image-url)

**Figure 2.** Phosphorylation of tau proteins. (a) SDS gel of tau protein isolated as in Fig. 1 a (peak 1). Lane 1, tau protein from the Mono S column without further treatment (i.e., in a mixed state of phosphorylation). Five to six bands can be discerned. Lane 2, tau eluted from the column and then treated with alkaline phosphatase for 5 h and then purified by perchloric acid in order to remove the enzyme. There are four distinct bands (from bottom to top, tau-4, following the nomenclature of Baudier and Cole (1987a) with M, of 54, 58, 61, and 64 kD, respectively). TAU2 and tau4 are more prominent than tau/ and tau3. Lane 3, the tau protein was first dephosphorylated (as in lane 3) and then rephosphorylated by CaMK. The four tau bands are shifted upwards, equivalent to an apparent increase of ~4 kD. Lane 4, SDS gel of tau protein dephosphorylated and then rephosphorylated by CaMK using γ-[32P]ATP. Lane 5, autoradiogram of the SDS gel of lane 4. (b) SDS gel of tau protein isolated as in Fig. 1 a. Lane 1, dephosphorylated tau from peak 1, similar to a, lane 2. Lane 2, MAP2 and the minor fraction of tau from peak 2 without further treatment. Comparison with lane 1 shows that most of this tau is phosphorylated. The MAP2 eluting in this peak is probably also largely phosphorylated. Lane 3, the protein of lane 2 was precipitated with perchloric acid in order to remove the non-tau components, tau was concentrated and then electrophoresed. The positions of the tau bands are similar to lane 2 (i.e., largely phosphorylated). (c) Immunoblot of the gel in b with the TAU1 monoclonal antibody (Binder et al., 1985), identifying tau both in the phosphorylated and in the dephosphorylated state.

bands 0, 1a, 1b, and 2 as 0>>2, and the region including bands 2-5 and the next band 0 as 2>>0.

A clear distinction between the paracrystals of Fig. 3 is that with ammonium molybdate the region 2>>0 is stained much more darkly than the region 0>>2, in contrast to uranyl acetate where the stain has a more even distribution. The differences in staining are most pronounced when the paracrystals have near-maximal repeats, and they tend to even out with shorter spacings. The effect is likely to be related to the structure of tau (see Discussion). Positive staining with uranyl acetate (i.e., staining followed by extensive rinsing) produces a pattern nearly indistinguishable from negative staining. Since uranyl acetate has a high affinity for charged

![Figure 3](image-url)

**Figure 3.** Electron micrographs of tau paracrystals observed with different staining procedures. These paracrystals were grown from a preparation of MAP2 containing traces of tau (see Fig. 2 b, lane 2). The paracrystal was negatively stained with 2% ammonium molybdate (a) or with 2% uranyl acetate (b). The periodicities are 66-67 nm, close to the maximum repeat value observed (68 nm). This is typical of phosphorylated tau. In a, the region between the bright bands 0 (small arrows) and 2 is light and the region from 2 to 0 is dark (these regions are termed 0 >>2 and 2 >>0, respectively). In b, the staining difference between these two regions is less pronounced, and the specimen shows a more detailed banding pattern. In the dark region of a one observes longitudinal striations which are typical of the ammonium molybdate staining of paracrystals with large spacings. Polarity is from bottom to top (large arrows).
2>>0 (Fig. 3 a, see also Fig. 5 a). Their lateral spacing is irregular. The finest visible fibrils have spacings down to 3-6 nm, but they also tend to group together into wider bundles spaced >10 nm apart.

The repeats in Fig. 3 are near the maximum value of 68 nm. The paracrystals were obtained from the second protein peak of the Mono S column which is largely phosphorylated (Fig. 2 c, lane 2), and the paracrystals obtained from it have near-maximal repeats. These results prompted us to check if phosphorylation had any influence on the structure of the paracrystals. Tau protein was therefore dephosphorylated by alkaline phosphatase and paracrystals were grown. There was a marked reduction of periodicity (55 nm and less). Fig. 4 a shows a typical example of 53-nm periodicity. When tau was first dephosphorylated and then rephosphorylated with CaMK the repeats of the paracrystals increase again to near-maximal values (Fig. 4 b, 67-nm repeat).

Apart from the effect on periodicities we noted that phosphorylation also influences the apparent flexibility of the paracrystals. This property can be deduced from regions where paracrystals are bent over (Fig. 5). With phosphorylated specimens (having long repeats) the order around the bends is lost, as if the interaction between the tau molecules was broken (Fig. 5 a, 66-nm repeat). By contrast, dephosphorylated samples with shorter repeats bend in an ordered fashion, reminiscent of an accordion. A striking example is the massive aggregate, >6 µm in diameter, of coiled-up paracrystals in Fig. 5 b. A detail is shown in Fig. 5 c; the repeat in the straight sections is ~50 nm, in the bend region it depends on the radius of curvature; i.e., it is 54 nm on the outside but only 22 nm on the inside. In other words, phosphorylated samples become not only long but also stiff, dephosphorylated ones are shorter and elastic.

The structural resolution of well-ordered paracrystals extends to ~5-6 nm, as judged from the optical or computed diffraction patterns (Fig. 6 a). Up to 15 orders of the repeat have been observed. Images of selected specimens were densitometered and computer processed to obtain the density distribution. Fig. 6 b is a high magnification view of a paracrystal, printed on the same length scale as the reconstructed densities of Fig. 6 c. They show the average density variations along the long axis of two particles. The x-axis was scaled to the same repeat in order to make the particles comparable. As shown previously, with this scaling the peak positions from particles with very different periodicities can be nearly superimposed. This accordion effect arises from the elasticity of tau and distinguishes it from other paracrystalline structures. After image processing, the seven density peaks per repeat can be seen not only with uranyl acetate–stained specimens (Fig. 6 b, bottom trace), but also with ammonium molybdate (Fig. 6 b, top, and Fig. 5 a). As mentioned above, peak 4 is the highest one in the region 2>>0, and it is offset towards peak 2. Thus the polarity is from left to right in Fig. 6 b. Note also the low mean density in region 2>>0 of Fig. 6 b (top, ammonium molybdate stain) which corresponds to the dark regions in Figs. 3 a or 5 a.

We next turn to the question of the molecular length of tau protein. This is given by the repeat distance (which can be measured accurately) plus the overlap between successive sets of molecules (which is not known a priori). There are several arguments suggesting that the overlap largely coincides with region 0>>2. Firstly, this region contains more protein than the region 2>>0, judging from the stain distribution (Fig. 3). Secondly, the paracrystals appear to bulge outwards between bands 0 and 2, consistent with a greater number of adjacent molecules. Thirdly, in those cases where the striations can be followed to the very end of a paracrystal they appear to terminate either near a band 0 or a band 2; these correspond to the minus and plus ends, respectively, according to our definition of polarity. This is particularly clear at the minus end, terminating with band 0, an example of which is given in Fig. 7. These arguments imply that a tau molecule begins roughly at a band 0 and ends at the band 2 following the next band 0. In other words, the region 0>>2 represents the overlap between successive sets of tau molecules, whereas 2>>0 is a nonoverlap region (as indicated by

Hagestedt et al. Tau Protein Structure, Elasticity, and Phosphorylation

1647
Figure 5. Paracrystal flexibility. (a) Paracrystal (phosphorylated, stained with 2% ammonium molybdate) bends over several times. The order around the bends is lost, indicating that the interaction between the molecules is broken. The periodicity is 66 nm. b shows a large deposit of seemingly disordered filaments of dephosphorylated protein, but the blowup in c reveals that it consists of well-ordered paracrystals (stain 2% ammonium molybdate). The bend region is reminiscent of an accordion; i.e., the order is preserved. The periodicity outside the bend region is \( \approx 50 \) nm, within the bend it varies between 22 (inside) and 54 nm (outside). As a rule, ordered bending occurs only with particles whose periodicity is clearly lower than the maximum of 68 nm, indicating that these are the more flexible ones.

Figure 6. Image reconstruction of paracrystals. (a) Optical diffraction pattern of a paracrystal stained with ammonium molybdate. Reflections up to the 11th order are visible, up to 15 orders can be discerned in the computed Fourier transform. (b) Magnified view of a paracrystal aligned and scaled with the image reconstruction below. (c) Image reconstruction of paracrystals. Top, average phosphorylated specimen stained with ammonium molybdate. Bottom, dephosphorylated specimen stained with uranyl acetate. The reconstructions have been scaled to the same periodicity in order to facilitate the comparison. The positions of the protein peaks are similar but there are differences due to staining. Note the asymmetric position of peak 4 which serves as a marker for polarity (− end, left; + end, right).

The Journal of Cell Biology, Volume 109, 1989 1648
of the molecules at their plus ends, but the striations would also be accounted for by the filled densities at the minus ends of the molecules. The long axis of the paracrystal points in the direction of the x-axis. The observed transverse striations would be the result of many tau molecules aligned in register. Below the diagram is a schematic representation of the density along the paracrystal; note the higher density in region 0>>2 and the asymmetric position of band 4. The difference between this model and the previous one is that the overlap region is now defined in more detail; i.e., one tau molecule extends from one band 0 beyond the next one to the subsequent band 2. This increases the total length by 30–40%, giving a maximal extension in the 90–95-nm range.

Discussion

Influence of Phosphorylation on Length and Elasticity of Tau

One of the main results of our previous study was that tau was highly elastic, as seen by the more than threefold variation in the repeat of the paracrystals. This makes tau distinct from all other known proteins that form paracrystals. We have now tried to identify the source of this elasticity and conclude that it is, at least in part, related to the state of phosphorylation. Phosphorylated tau has a nearly maximal extension and is comparatively stiff. Dephosphorylated tau becomes shorter and more flexible.

Initially our starting material was tau protein phosphorylated to a variable degree by endogenous kinases present in microtubule protein (Sloboda et al., 1975; Vallee, 1980). This phosphorylation is more extensive when the protein is prepared in the presence of ATP instead of GTP, presumably because the kinases require ATP. One result of phosphorylation is that MAPs bind to microtubules less tightly, stabilize them less efficiently (Jameson et al., 1980; Murthy and Flavin, 1983; Burns et al., 1984; Lindwall and Cole, 1984a), and lead to a more dynamic behavior, as judged by microtubule oscillations (Mandelkow et al., 1988).

The sequence of tau (Lee et al., 1988a) shows acidic NH₂- and COOH-terminal tails and a basic interior. Tau binds to the acidic COOH terminus of tubulin (Littauer et al., 1986) so that from charge complementarity one would expect the COOH-terminal part of tau to interact with tubulin; this is in fact borne out by studies on a microtubule-binding fragment of tau (Aizawa et al., 1988). It seems, therefore, reasonable to assume that the phosphorylation of tau that affects its interaction with microtubules should take...
place in the COOH-terminal half; it could work by reducing the charge–charge interactions.

This is a static view of the situation and does not take into account the possible role of tau's elasticity. One might speculate that this property is important for the elasticity of the cytoplasm. Microtubules have to be stabilized (presumably by tau and other MAPs) and are probably cross-linked to other structures, but this must not interfere with the transport of material along them (e.g., by translocator proteins). The stabilizing and/or cross-linking proteins would have to be flexible, a condition that is apparently met by tau protein. Thus, if phosphorylation regulates tau's elasticity one would expect to find it on the domain projecting away from microtubules rather than on the microtubule-binding domain. Electron microscopy evidence suggests that the distinction between such domains may be real (Hirokawa et al., 1988). Conceptually one should therefore distinguish between phosphorylation affecting microtubule assembly (located on the microtubule-binding domain) and phosphorylation affecting elasticity and interactions with other structures (located on a projection domain). In practice, the two effects appear to be closely related, at least in the case of phosphorylation by the CaMK since this affects both microtubule assembly (Lindwall and Cole, 1984a) and elasticity (as shown here).

The site(s) of phosphorylation are not known at present, but there is some evidence that they might be in the COOH-terminal part of tau. Phosphorylation by a kinase which acts similarly to CaMK (in terms of reducing the electrophoretic mobility) acts on an epitope which tau shares with the paired helical filaments of Alzheimer's disease (Ishiguro et al., 1988), and the common region is in the COOH-terminal third of tau (Kondo et al., 1988). It is noteworthy that this part contains the sequence lys-ser-pro (residues 295–297), a sequence that is phosphorylated in neurofilaments and binds antibodies which cross-react between neurofilaments, MAP2, tau, and Alzheimer's tangles (Kosik et al., 1986; Lee et al., 1988b). Such an epitope occurs just downstream from the internal repeats of tau which form part of the potential microtubule-binding domain (compare Lee et al., 1988a; and Aizawa et al., 1988).

Structural Features of Tau

The key features of the structure of tau are summarized in Fig. 8 b, showing the molecule in different states of extension but similar substructure. There are at least two major densities (0 and 2), three intermediate ones (3, 4, 5), and two minor ones (la, lb). A striking feature of the paracrystals is the difference in contrast obtained with different stains. Bands 0 and 2 always stand out brightly (Fig. 3, a and b). However, with ammonium molybdate the difference between regions 0>>2 and 2>>0 is much more pronounced than with uranyl acetate, particularly in the case of paracrystals with long repeats where the protein is phosphorylated. Moreover, there is little difference between negative and positive staining by uranyl acetate. This stain has a strong affinity for charged groups (positive and negative) so that even the images of negatively stained structures contain an appreciable contribution from positive staining (compare studies with immunoglobulins, Steven and Navia, 1982; or collagen, Tzaphlidou et al., 1982). Since tau has a high content of charged residues we assume that the images of uranyl-acetate–stained paracrystals largely reflect the charge distribution. In principle this should allow us to relate the staining to the amino acid sequence. In practice this is not yet possible because, unlike the case of collagen, we cannot assume that the structure corresponds linearly to the sequence. Since tau contains (at least) 364 residues (Lee et al., 1988a) the maximum length for the extended chain would be ~140 nm, 50% more than the observed maximum length. Tau has very little secondary structure (Cleveland et al., 1977) so that realistic predictions of the chain folding cannot be made at present. Studies using monoclonal antibodies with known epitopes are underway to determine the folding of tau in the paracrystals. This should yield constraints which would help us to relate the sequence with the stain distribution.

In contrast to uranyl acetate, ammonium molybdate is regarded as a more faithful negative stain. This would mean that bands 0 and 2 have not only the highest charge but also the highest mass. Moreover the brightness of 0>>2 supports this as the overlap region. Region 2>>0 accumulates more stain because there is less protein in the nonoverlapping part. In addition, it seems that the molecules are more loosely packed, allowing stain to penetrate more deeply. This would explain the contrasty appearance of the fibrils in this region (Fig. 3 a), each of which probably represents a projection of several tau molecules. Judging by the thinnest observed fibrils, a tau molecule would be ~3–4 nm wide. The ratio of length to width would agree well with the hydrodynamic axial ratio of 20 determined by Cleveland et al. (1977).

Hirokawa et al. (1988) studied single tau molecules by metal shadowing. They observed a wide length distribution with a mean of 56 nm, of which 18 nm are considered to project away from the microtubule wall. In view of tau's elasticity, the variation in length is not surprising; the mean length corresponds to a state of intermediate contraction. The relationship between the assembly or projection domains and the paracrystalline structure of tau is not clear at present. We hope that this can be answered by experiments in which paracrystals are labeled with tubulin.

A number of authors have shown recently that tau is one of the components of the neurofibrillary tangles of Alzheimer's disease (e.g., Goedert et al., 1988). This raises the possibility that the interaction between the Alzheimer paired helical filaments is related to the interaction between tau molecules in the paracrystals. Image reconstruction of the paired helical filaments shows an axial subunit repeat of 3 nm and a cross-over repeat of the strands of 78 nm (Crowther and Wischik, 1985). By contrast, no helical structure is evident in the tau paracrystals, and the above repeats are also not observed. Thus, no obvious relationship between the two structures is recognizable at present.

Why Is Tau Elastic?

The short answer is that we do not know. The evidence for tau's elasticity is derived from structural changes, as described above and in more detail in our previous report (Lichtenberg et al., 1988). In this regard tau paracrystals behave very differently from other known paracrystals. However, the underlying physical principles are not understood. By way of comparison, a well known example of an elastic protein is that of elastin which has been studied for many years, and the usual explanation given in many textbooks is that of more or less randomly coiled molecules connected by specific cross-links. However, other views have also been
held (e.g., liquid drop elastomer; Weis-Fogh and Andersen, 1970), including that of a regular secondary structure stabilized by hydrophobic interactions (Urry, 1988). The sequence of elastin is known (Raju and Anwar, 1987); it resembles that of tau in that it has a high content of proline and glycine. However, its overall character is different because elastin is highly hydrophobic while tau is hydrophilic. Moreover, tau is not a random coil but a rod-like particle. It can stretch up to 2/3 of a maximally extended chain so that it is little room for coiling. It seems therefore unlikely that the theories put forward to explain the elasticity of elastin could hold for tau as well.

We are grateful to Dr. H. D. Soling (Göttingen, FRG) for providing CaMK, to Drs. L. Binder (Birmingham, AL) and A. Matus (Basel, Switzerland) for tau antibodies, and to C. Haas for expert technical assistance and photography.

This project was supported by the Bundesministerium für Forschung and Technologie and the Deutsche Forschungsgemeinschaft. This study contains part of the doctoral theses of T. Hagedstedt and B. Lichtensteig. Received for publication 13 March 1989 and in revised form 12 June 1989.

References

Aizawa, H., H. Kawasaki, H. Murofushi, S. Kotani, K. Suzuki, and H. Sakai. 1988. Microtubule-binding domain of tau proteins. J. Biol. Chem. 263: 7703–7707.

Baudier, J., and R. D. Cole. 1987a. Phosphorylation of tau protein to a state like that in Alzheimer’s brain is catalyzed by a calcium/calmodulin dependent kinase and modulated by phospholipids. J. Biol. Chem. 262:17577–17583.

Baudier, J., and R. D. Cole. 1987b. Separation of the different microtubule-associated tau protein species from bovine brain and their mode II phosphorylation by calcium/phospholipid dependent protein kinase C. J. Biol. Chem. 262:17584–17590.

Binder, L., A. Frankfurter, and L. Rehberg. 1985. The distribution of tau in the mammalian central nervous system. J. Cell Biol. 101:1371–1378.

Burns, R. G., K. Islam, and R. Chapman. 1984. The multiple phosphorylation of the microtubule-associated protein MAP-2 controls the MAP2-tubulin interaction. Eur. J. Biochem. 141:609–615.

Cleveland, D. W., S.-Y. Hwo, and M. W. Kirschner. 1977. Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. J. Mol. Biol. 116:227–247.

Crowther, R. A., and C. M. Wischik. 1985. Image reconstruction of the Alzheimer paired helical filament. EMBO (Eur. Mol. Biol. Organ.) J. 4:3661–3665.

Fellous, A., J. Francon, A. M. Lennon, and J. Nunez. 1977. Microtubule assembly in vitro: purification of assembly promoting factors. Eur. J. Biochem. 78:167–174.

Goedert, M., C. Wischik, R. Crowther, J. Walker, and A. Klug. 1988. Cloning and sequencing of the CDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. Proc. Natl. Acad. Sci. USA. 85:4051–4055.

Hirokawa, N., Y. Shiromura, and S. Okabe. 1988. Tau proteins: the molecular structure and mode of binding to microtubules. J. Cell Biol. 107:1449–1459.

Ishiguro, K., Y. Ibara, T. Uchida, and K. Imahori. 1988. A novel tubulin-dependent protein kinase forming a paired helical filament epitope on tau. J. Biochem. (Tokyo). 104:319–321.

Jameson, L., T. Frey, B. Zeeberg, and M. Caplow. 1980. Inhibition of microtubule assembly by phosphorylation of microtubule-associated proteins. Biochemistry. 19:2472–2479.

Kosik, K., C. Iacoshim, and D. Selkoe. 1986. Microtubule-associated protein tau is a major antigenic component of paired helical filaments in Alzheimer disease. Proc. Natl. Acad. Sci. USA. 83:4044–4048.

Kondo, J., T. Honda, H. Morii, Y. Hamada, R. Miura, M. Ogawa, and Y. Ibara. 1988. The carboxyl third of tau is tightly bound to paired helical filaments. Neurosci. 1:927–934.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

Lee, G., N. Cowan, and M. Kirschnerr. 1988a. The primary structure and heterogeneity of tau protein from mouse brain. Science (Wash. DC). 239:285–288.

Lee, V., L. Otvos, M. Carden, M. Hollosi, B. Dietzschold, and R. Lazzarini. 1988b. Identification of the major multiphosphorylation site in mammalian neurofilaments. Proc. Natl. Acad. Sci. USA. 85:1998–2002.

Lewis, S. A., D. Wang, and N. W. Cowan. 1988. Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. Science (Wash. DC). 242:936–939.

Lichtenberg, B., E.-M. Mandelkow, T. Hagedstedt, and E. Mandelkow. 1988. Structure and elasticity of microtubule-associated protein tau. Nature (Lond.). 334:359–362.

Lindwall, G., and R. D. Cole. 1984a. Phosphorylation affects the ability of Tau protein to promote microtubule assembly. J. Biol. Chem. 259:5301–5305.

Lindwall, G., and R. D. Cole. 1984b. The purification of tau protein and the occurrence of two phosphorylation states of tau in brain. J. Biol. Chem. 259:12241–12245.

Littauer, U. Z., D. Gwoon, M. Tissera, I. Ginsburg, and H. Poonstingl. 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. Proc. Natl. Acad. Sci. USA. 83:7162–7166.

Mandelkow, E.-M., M. Herrmann, and U. Rühl. 1985. Tubulin domains probed by subunit-specific antibodies and limited proteolysis. J. Mol. Biol. 185:311–327.

Mandelkow, E.-M., G. Lange, A. Jagla, U. Spann, and E. Mandelkow. 1988. Dynamics of the microtubule oscillator: role of nucleotides and tubulin-MAP interactions. EMBO (Eur. Mol. Biol. Organ.) J. 7:357–365.

Matsumura, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10034–10038.

Murthy, A. S. N., and M. Flavin. 1983. Microtubule assembly using the microtubule-associated protein MAP-2 prepared in defined states of phosphorylation with protein kinase and phosphatase. Eur. J. Biochem. 137:37–46.

Raju, K., and R. A. Anwar. 1987. Primary structures of bovine elastin a, b, and c deduced from the sequences of cDNA clones. J. Biol. Chem. 262:5755–5762.

Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greenberg. 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. Proc. Natl. Acad. Sci. USA. 72:177–181.

Steven, A. C., and M. A. Navia. 1982. Specificity of stain distribution in electron micrographs of protein molecules contrasted with uranyl acetate. J. Microsc. (Oxford). 128:145–155.

Tzaphlidou, M., J. Chapman, and M. Al-Samman. 1982. A study of positive and negative staining for electron microscopy using collagen as a model system. II. Staining by uranyl ion. Micron. 13:133–145.

Urry, D. W. 1988. Entropic elastic processes in protein mechanisms. I. Elastic structure due to an inverse temperature transition and elasticity due to internal chain dynamics. J. Protein Chem. 7:1–34.

Vallee, R. B. 1980. Structure and phosphorylation of microtubule-associated protein 2 (MAP2). Proc. Natl. Acad. Sci. USA. 77:3206–3210.

Van Bruggen, E., J. van Breemen, W. Keegstra, E. Boekema, and M. van Heel. 1986. Two-dimensional crystallization experiments. J. Microsc. (Oxford). 141:11–20.

Van Weigart, M. D., A. H. Lockwood, S. Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. USA. 72:1858–1862.

Weis-Fogh, T., and S. O. Andersen. 1970. New molecular model for the long-range elasticity of elastin. Nature (Lond.). 227:718–721.

Woodgett, J., M. Davison, and P. Cohen. 1983. The calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle. Eur. J. Biochem. 136:481–487.