We have studied the conformation of tau protein and Alzheimer paired helical filaments (PHF) by several spectroscopic, scattering, and imaging methods revealing the overall shape and the conformation of the polypeptide backbone. Tau protein behaves in solution as if it were denatured; no evidence for compact folding was detected. The protein is highly extended, there is no defined radius of gyration, and the scattering is best described by that of a random (“Gaussian”) polymer. CD and Fourier transform infrared spectroscopy show only a minimal content of ordered secondary structure (α-helix or β-sheet). Similarly, PHFs from Alzheimer brain tissue show no detectable secondary structure by x-ray diffraction or spectroscopy. It is thus unlikely that the aggregation of tau into Alzheimer PHFs is based on interactions between strands of β-sheets (a model currently favored for other disease-related polymers such as β-amyloid fibers of Alzheimer’s disease).

Alzheimer’s disease, an age-related dementia, is characterized by amyloid plaques and neurofibrillary tangles. Both are formed from abnormally aggregated protein; the plaques contain the Aβ peptide (a breakdown product of a membrane protein) aggregated into fibers, the tangles contain tau protein aggregated into paired helical filaments (PHFs) (reviews, see Kosik (1992) and Lee and Trojanowski (1992)). In order to understand the origin of the disease it would be important to understand the abnormal state of these proteins (resulting from proteolysis, phosphorylation, or other modifications) and the subsequent aggregation. Abnormal fibers occur in other disease states as well; examples are the fibers of prion protein in scrapie or Creutzfeld-Jakob Disease (Prusiner et al., 1986), or the fibers associated with systemic amyloidosis consisting of transthyretin (Jarvis et al., 1993).

A view favored currently is that the assembly of these fibers is mediated by interactions between strands of β-sheets. This would be consistent with several observations. For example, the β-amyloid fibers and the PHFs are very stable, as expected of the many interactions between interlocking β-strands. Second, aggregation in vitro tends to occur in conditions where the protein adopts a β-conformation in solution, as judged by CD or FTIR spectroscopy (Hilbich et al., 1992; Fraser et al., 1991). Third, the β-structure can be detected directly in the abnormal fibers by x-ray diffraction or FTIR (Kirschner et al., 1986; Caughey et al., 1991; Gasset et al., 1993; Jarvis et al., 1993). For example, Alzheimer β-amyloid fibers have a cross-β-structure where the strands meander across the width of the fiber so that a 0.47-nm reflection (corresponding to the separation of strands) occurs on the meridian of the diffraction pattern (Kirschner et al., 1986; Inouye et al., 1993). Zhang et al. (1993) expanded this into a general principle of self-complementary peptides capable of locking subunits almost irreversibly into higher order aggregates such as “membranes” or fibers. It has been postulated that the aggregation follows a nucleation-condensation mechanism, and that a key step is the initial priming of nuclei which assume the β-conformation and then communicate to the next incoming subunits during elongation (Jarrett et al., 1993).

On the other hand, even when fibrous aggregates show common structural elements it may be premature to assume similar mechanisms of formation. This caveat is well illustrated by the recent x-ray crystallographic analysis of transthyretin (Terry et al., 1993). It is mainly composed of β-sheet; however, the interaction with other subunits is mediated by SH groups situated at the outer edges of the protein, capable of coupling to the neighboring monomer via S-S-bridges. In other words, this fiber is not formed by complementary β-strands, even though it has a predominant β-structure.

With this problem in mind we have studied the relationship between tau protein in solution and the PHFs formed from tau subunits in Alzheimer’s disease. A curious puzzle exists in this field: when tau was characterized it was clear from the start that it had only a negligible fraction of α- or β-conformation, if any (Cleveland et al., 1977). In this regard it was similar to other structural microtubule-associated proteins such as MAP2 (Voter and Erickson, 1982) This finding was independent of whether tau was from brain or expressed bacterially, and whether it was in its endogenous state of phosphorylation or free of phosphate (Wille et al., 1992). On the other hand Kirschner et al. (1986) found a cross-β-structure for filaments derived from Alzheimer tangles, similar to the β-amyloid fibers. This would mean that tau would have to acquire its β-conformation concomitantly with aggregation, and this in turn would provide an interesting mechanism for PHF assembly. However, since amyloid fibers and PHFs often coexist in senile plaques, there was an alternative explanation: the cross-β pattern of the tangle preparation could have arisen from the β-amyloid component, especially considering that x-rays are capable of picking out periodic structures even when they are present only as contaminants. We have therefore re-investigated the problem using new procedures for isolating PHFs and several methods sensitive to secondary structure. We now find that neither tau nor PHFs have a significant content of ordered secondary structure, and that tau behaves as if it were in a naturally “denatured” state.
Structure of Tau and Paired Helical Filaments

MATERIALS AND METHODS
Preparation of Tau Protein—Con structs of tau protein were designed and expressed in Escherichia coli as described previously (Biernat et al., 1992). They were based on the human tau isoforms provided by M. Goedert (see Goedert et al., 1989); the numbering of amino acids is that of the isoforms having 441 amino acids. E. coli cells were harvested by centrifugation 2.5 h after induction with isopropyl β-D-thiogalactoside, resuspended in 50 mM PIPES, pH 6.9, 5 mM EGTA, 1 mM MgCl₂, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride plus 10 μg/ml leupeptin, 2 mM benzamidin and lysed with lysozyme. Triton X-100 (1%), MgCl₂ (20 mM) and DNase (50 μg/ml) were added, the suspension was centrifuged, NaCl was added to the supernatant to 500 mM and the pellet was resuspended in 50 mM PIPES, pH 6.9, 500 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 5 mM dithiothreitol. The supernatant and the resuspended pellet were then boiled for 20 min. Both solutions were centrifuged and the supernatants dialyzed overnight against 2 liters of buffer MonoS A 120 mM PIPES, pH 6.9, 0.25 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 50 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, using dialysis bags with a 3.5-kDa exclusion limit. The proteins were purified by MonoS fast protein liquid ion-exchange chromatography with NaCl gradient. Most tau constructs eluted from the column between 0.3 and 0.4 mM NaCl. The protein purity was checked by SDS-polyacrylamide gel electrophoresis.

Chemical Cross-linking—The chemical cross-linking of tau was done as described (Wille et al., 1992), using N,N'-phenylene dimaleimide which cross-links the SH groups of cysteines. Because of its low solubility in water N,N'-phenylene dimaleimide was first dissolved in Me₂SO. The cross-linking reaction was done in PBS, pH 7, and room temperature, by adding aliquots of N,N'-phenylene dimaleimide to the protein solution over a period of 10 min with constant stirring (final concentration of N,N'-phenylene dimaleimide was 5-fold in molar excess over protein). After a reaction time of 4 h the covalently linked tau dimers were separated from other species by gel filtration (G75 column), using PBS, pH 7, as elution buffer. The proteins were centrifuged using Centricon prep (exclusion limit 10 kDa, Amicon).

Protein Denaturation—Since tau has only minimal secondary structure the thermal denaturation could not be monitored by CD. Thus the effect of denaturation had to be measured by other methods. We used urea up to 8 M, using a protein concentration with an A₂₈₀ value of 0.35 (roughly 0.135 mM, as determined with the Bradford assay, Bio-Rad). The absorption was measured against a background of the same urea concentration. As a control the absorption spectra between 240 and 320 nm of the specimens with or without urea were recorded.

Preparation of PHFs—PHFs were isolated from brain tissues provided by the Kathleen Price Bryan Alzheimer Research Center (Duke University Medical School, Durham, NC) and characterized for Alzheimer's disease according to standard procedures. We employed the procedure of Greenberg and Davies (1990) which results in the "soluble PHFs." Briefly, brain tissue was homogenized in a 10-fold excess of buffer H (10 mM Tris, pH 7.4, 1 mM EGTA, 800 mM NaCl, 10% sucrose), followed by centrifugation at 26,000 × g for 20 min at 4 °C. The pellet was resuspended again in 10-fold excess of buffer H and pelleted once more. The supernatants of both steps were adjusted to 1% (v/v) N-lauryl sarcosine and 1% (v/v) β-mercaptoethanol and incubated at 37 °C for 2.5 h on a shaker. After that both solutions were centrifuged at 82,000 × g for 35 min at 20 °C. The pellet was homogenized in 80 ml of buffer H, 1% (w/v) CHAPS, 1% (v/v) β-mercaptoethanol using a Potter homogenizer. The suspension was filtered through a 0.45-μm membrane (Schleicher & Schuell) and centrifuged at 82,000 × g for 1 h at 20 °C. The pellet was resuspended in 40 ml of buffer H, 0.1% β-mercaptoethanol using a Potter homogenizer, followed by discontinuous sucrose gradient centrifugation at 36,000 rpm in a Beckman TST41.14 rotor for 2 h at 20 °C. Only the interphase between 50 and 35% sucrose was collected.

In order to improve the purification further we applied the PHF-fraction resuspended in PBS, pH 7, to a sucrose gradient of 1.4 and 2 μM sucrose, as described by Kirschner et al. (1986). Lipofuscin (= aggregated lipids and amino acids, Taubold et al. (1975)) is known as a contaminant of PHF preparations. This pigment can be separated by density gradient centrifugation where lipofuscin can be collected at a density below 1.15 g/ml (Siskotos, 1974). The PHFs accumulate at the interphase between 50 and 35% sucrose was collected.

The PHF fraction was resuspended in PBS, pH 7, 900 mM NaCl and centrifuged at 30,000 rpm in a Beckman TST41.14 rotor for 1 h at 20 °C on a CsCl cushion (1.45 g/ml). This step was repeated three times. This procedure also improved the purification by removing soluble protein components other than PHFs. Furthermore, the concentration of the PHFs in a CsCl cushion allows one to resuspend them easily. (By contrast, PHFs pelleted in a test tube can be resuspended only with difficulty.)

X-ray Solution Scattering—This was done as described (Spann et al., 1987) using the instrument X33 of the EMBL Outstation at DESY (Koch and Bordes, 1983). Protein concentrations were around 10–20 mg/ml, the protein solution was contained in a thermostatted cell covered with 50-μm windows and 1-mm deep. The x-ray wavelength was ~0.15 nm, specimen to detector distance ~4 m, and scattering patterns were recorded on a position-sensitive detector averaging over a 90° quadrant (Boulin et al., 1988).

X-ray Fiber Diffraction—This was done on an Elliot GX-21 (Enraf-Nonius) rotating anode generator (35 kV and 34 mA), using double spaced CuKα radiation. Diffraction images were recorded on a Fuji Imaging Plate with exposure times of 15 min for microtubules and up to 64 h for hydrated and dried PHFs. The camera length varied between 100 and 400 mm. The PHF specimens were prepared by centrifuging the fibers onto a mylar foil. Microtubules were first polymerized at 37 °C and stabilized by 6 μl taxol. Partial orientation of microtubules of PHFs was ascertainment in a polarizing microscope under crossed polarizers.

Electron Microscopy—For negative staining we used 600-mesh carbon-coated copper grids which were glow discharged twice (CTA 010, Balzers Union). The grids were placed on a drop of protein solution, incubated for 5 s, placed on a drop of 2% uranyl acetate, pH 4.5, incubated for 20 s, and washed with 2 drops of water. The staining and washing solutions were first filtered through a 0.2-μm membrane.

For gileroy spraying the solution of tau protein was diluted 10 times with spraying buffer (1.5 mM NH₄Ac, 3 mM EGTA, 30 μM MgCl₂, 50 mM NaCl, 0.1% (v/v) glutaraldehyde, pH 7.4 (final control)). While rotating the specimen was shadowed with a 1.5-nm Na/Cu layer at an angle of 45°. The replica was stabilized by a 15–20-nm carbon film. The mica sheets were kept overnight in a humid chamber in order to facilitate the transfer of the replicas onto 600-mesh copper grids (made hydrophilic by glow discharge). The replicas were examined in a Philips CM12 electron microscope at 100 kV.

CD Spectroscopy—CD spectra were obtained on a Jobin-Yvon Dichrograph III from 190 to 230 nm (made available by Dr. J. Alves, Institute of Biophysical Chemistry, Medical University Hannover). Proteins were examined in buffers MonoS A, Tris, pH 7, and PBS, pH 7, at temperatures of 4, 22, 37, and 54 °C (thermostatted by a Haake F3 thermostat). The temperature was varied between pH 2 and 10 in steps of 1 pH unit. The background level was determined using the same windows and buffers. The thickness of the protein solution was 0.1 mm, the protein concentration 0.1 mg/ml. The spectra were evaluated using the program CIRCULAR (Dr. F. Peters and J. Greipel, Hannover, Germany) which determines the relative contributions of α-helix, β-sheet, and random coil content of a set of histograms associated with the spectra.

FTIR Spectroscopy—Infra red spectra (1000 interferograms each) were obtained on a Bruker IFS 88 Spectrometer (made available by K. Gerwert, Max-Planck-Institute Dortmund, Germany). The temperature was 32 °C, spectral resolution 2 cm⁻¹, and CsF windows with 6-μm spacers were used. The buffer was changed to D₂O and 100 mM NaCl by incubation overnight for complete exchange of H₂O. 100 μl of the suspension were applied to one window and concentrated in a stream of N₂. The layer was homogenized by turning and pressing the two windows against one another.

RESULTS
Structure of Tau Protein—The domain structure of tau is diagrammed in Fig. 1. As an introduction we briefly summarize some known structural features. As mentioned above, bovine brain tau has very little secondary structure as judged by CD (Cleveland et al., 1992); tau can be boiled and dissolved in dilute acids, and yet it does not lose its microtubule-assembly promoting activity (Fellous et al., 1977). This argues that overall chain folding is not very important for the interaction of tau with microtubules (although other less obvious functions may be affected by this treatment). The sequences of all tau isoforms contain an unusually high propor-
Graphs derived from human tau (largest isoform htau40 with 441 residues, of the C-terminal tail included (details in Biernat et al., 2019)). The sequences contain 3 or 4 internal repeats (numbered boxes) in the C-terminal half and up to two inserts near the N terminus (shaded). The numbering follows that of htau40. Construct K12 (derived from htau23) contains repeats 1, 3, 4, with part of the C-terminal tail included (details in Biernat et al., 1992). Construct K0 (derived from bovine tau40) contains repeats 1–4 plus part of the C-terminal tail, K1 is similar, but the full tail included, K2 contains repeats 1, 3, 4, and the full tail (for details see Steiner et al., 1990). The graphs below show the secondary structure predictions according to Lupas et al., 1998; r = random (77%), α = α-helix (a few regions which coincide with 15%), β = β-sheet (5%). The α-helical coiled coil prediction according to Lupas et al., 1991 is negative (not shown).

The structure of hydrophilic residues and many prolines (Lee et al., 1988; Goedert et al., 1989; Himmler et al., 1989), again arguing against compact folding. This is borne out by structure predictions (Fig. 1b, following Gascuel and Golmard, 1988), or of α-helical coiled coil (following Lupas et al., 1991), none of which show significant secondary structure. Tau aggregates readily into dimers and higher aggregates which can be separated by gel filtration; the dimers are antiparallel and could represent an early step in PHF assembly (Wille et al., 1992). Tau is difficult to visualize in the electron microscope because of its low contrast (Zingsheim et al., 1979), but the glycerol-spray technique reveals the structure of an elongated and flexible rod, about 35 nm in length (Fig. 2, Wille et al., 1992).

Since electron microscopy suffers from potential artifacts we sought to determine the shape of tau in solution. This can be done by small angle x-ray scattering which is capable of sensing both the overall shape and internal structure of proteins. When plotting the x-ray scattering as a Guinier plot (Fig. 3a, bottom) a typical globular protein like lysozyme shows a straight line from which one can deduce a radius of gyration of 1.5 ± 0.05 nm. Surprisingly, tau shows no straight line in the Guinier plot but rather a continuous change in curvature (Fig. 3a, top) so that no defined Rg value can be assigned. This result holds for whole tau, as well as tau constructs containing different combinations of domains (K0, K1, K2, K12, for definitions see Fig. 1) and for the K12 dimer.

This behavior is reminiscent of polymers described as “worm-like chains” or “Gaussian coils” where the direction of the chain varies in a more-or-less random fashion and thus is not compacted into a globular form (Kratky, 1982; Kirste and Oberthür, 1982); with proteins, this type of structure is approximated in the denatured state (Damascun et al., 1993). An appropriate representation is the Kratky plot (Fig. 3b, top) which shows three segments, a rising part, a roughly horizontal one, and another rise; the intercept between segments 2 and 3 is a measure of the persistence length of the chain (1.97 nm in the example shown). Again tau is clearly different from a globular protein (Fig. 3b, bottom), it has the characteristics of a denatured protein. As a consequence the observation of rather straight particles in the electron microscopy requires interpretation; they probably appear straighter than they are in solution because they become oriented in the wake of the retracting glycerol droplet (cf. Nave et al., 1989).

Additional evidence for the apparent “denatured” state of tau comes from absorption spectroscopy (data not shown). As an example, construct K12 contains two Tyr (Try-310, Tyr-394) but no Trp. The absorption spectrum of Tyr generally depends on the hydrophobicity of the environment. However, when we increase the concentration of urea from 0 to 8 M there is no change in extinction coefficient, and the spectrum between 240 and 310 nm remains the same, as if both Tyr residues were fully exposed in all conditions.

**Fig. 1.** Diagram of tau isoforms and constructs. Those were derived from human tau (largest isoform htau40 with 441 residues, Goedert et al., 1989) or bovine tau (largest isoform btau40 with 430 residues, Himmler et al., 1989). The sequences contain 3 or 4 internal repeats (numbered boxes) in the C-terminal half and up to two inserts near the N terminus (shaded). The numbering follows that of htau40. Construct K12 (derived from htau23) contains repeats 1, 3, 4, with part of the C-terminal tail included (details in Biernat et al., 1992). Construct K0 (derived from bovine tau40) contains repeats 1–4 plus part of the C-terminal tail, K1 is similar, but the full tail included, K2 contains repeats 1, 3, 4, and the full tail (for details see Steiner et al., 1990). The graphs below show the secondary structure predictions according to Lupas et al., 1998; r = random (77%), α = α-helix (a few regions which coincide with 15%), β = β-sheet (5%). The α-helical coiled coil prediction according to Lupas et al., 1991 is negative (not shown).

**Fig. 2.** Panel of htau23 particles prepared by the glycerol spray rotary shadow technique. The molecules appear rod-like, the lengths are 35–40. Bar = 30 nm.

**Fig. 3.** X-ray solution scattering of btau4, the largest of the bovine tau isoforms (Himmler et al., 1989) and lysozyme (control) plotted according to Guinier (a) and Kratky (b). a, the Guinier plot of tau is curved indicating that no defined radius of gyration can be assigned, in contrast to lysozyme whose scattering follows a straight line with Rg = 1.5 nm ± 0.05 nm. b, the Kratky plot of tau shows the three segments typical of a Gaussian polymer. The transition point between the second and third segment (dashed line) is at S = 0.154 nm−1, indicating a persistence length of α = 1.97 nm (a = 1.91/(2πS)), for details see Kratky (1982). The bell-shaped curve below represents the scattering from lysozyme.
We probed several constructs by CD spectroscopy which is particularly sensitive to secondary structure (Johnson, 1988). For tau isoform htau23 and construct K12 the α-helix content was estimated at 0 and 10%, the β content at 12 and 14% (Fig. 4). These values are consistent with the earlier measurements of Cleveland et al. (1977); they are so low that the quantification becomes unreliable, i.e. the fraction of secondary structure is negligible both for whole tau and the microtubule binding region. The same result is obtained when we increase the ionic strength or the temperature (data not shown). However, the α-helix content can be increased artificially by adding TFE (Clare et al., 1986); in the case of tau it rises to 30% in the presence of 50% TFE (Fig. 4). Finally, there was no detectable difference in the CD spectrum between tau monomers and cross-linked dimers, so that dimerization is not visibly accompanied by an induction of secondary structure (data not shown).

Structure of PHFs—After several years of uncertainty there is now little doubt that PHFs consist mainly of tau. PHFs contain all six human tau isoforms, all of them in an abnormally phosphorylated state (Kiezak-Reding and Yen, 1991; Goedert et al., 1992). In the electron microscope (Fig. 5) PHFs appear as two strands twisted around one another (hence the name); the width varies between 10 and 22 nm, and the periodicity is around 75–80 nm (Crowther and Wischik, 1985), although “straight” filaments without twist exist as well (Crowther, 1991). The highly stable core of PHFs contains the microtubule-binding domains of tau while the “fuzzy coat” can be cleaved off by proteases (Wischik et al., 1988; Kondo et al., 1988). This domain is also particularly prone to form PHF-like filaments in vitro (Wille et al., 1992).

In order to study PHFs one must overcome two problems. One is the problem of quantity which one needs for biophysical studies, the second is that of purity. We therefore extended and scaled up the preparation of Greenberg and Davies (1990) and took care to remove non-PHF components such as lipofuscin, ferritin, and other material. In particular, since x-rays are exquisitely sensitive to periodic structure we attempted to prepare the PHFs free of β-amyloid fibers. Electron micrographs (Fig. 5) confirm that the preparation contains highly purified PHFs. This preparation was then used for the x-ray and FTIR spectroscopic studies.

X-ray fiber patterns are normally interpreted most easily when the fibers are as parallel as possible. Orientation can be induced in several ways, for example by drawing the concentric solution into thin quartz capillaries which can result in spontaneous orientation, or by centrifuging the fibers onto a mylar foil where some orientation occurs during sedimentation. In either case the degree of orientation was assessed under a polarizing microscope or directly in a fine focus x-ray camera (GX21) equipped with a position-sensitive detector (Fuji imaging plate). As a control we prepared microtubules in identical conditions since their x-ray pattern is known, and, in particular, since they contain both α- and β-secondary structure whose scattering can be detected in the pattern.

Fig. 6, a and b, shows the wide and small angle pattern of pelleted microtubules. These structures can be oriented quite well by either of the above methods, but here we have chosen an example of rather poor orientation in order to facilitate the comparison with the PHF pattern below (for an example of good orientation and its interpretation see Mandellkow et al. (1977) and Mandellkow (1986)). The dark ring around 0.35 nm (arrow w) arises from the scattering of water. The fibers have a preferred direction in the vertical direction (meridian of the pattern). The low angle pattern (h) shows equatorial reflections (arrows a-c) corresponding to \( J_3 \) and \( J_{13} \) Bessel terms, near-meridional reflections (arrows d-f) arising from \( J_3 \) and \( J_{10} \) Bessel terms on the 4-nm layer line. These low-resolution features arise from the contrast between the protein and the solvent. Vestiges of secondary structure show up at higher angles in Fig. 6a: peaks around 1 nm\(^{-1}\) (arrow g) arise from superpositions of off-meridional peaks on layer lines 1 and 2, and a near-meridional peak on layer line 4 and are indicative of juxtaposed α-helices which run at an angle to the microtubule

**Fig. 4.** CD spectra of htau23 in MonoS buffer A, construct K12 in Tris buffer, pH 7, and K12 in Tris buffer, pH 7, and 50% TFE. The estimated content of α-helix is <5% in the first two cases, with TFE it rises to ~30%.

**Fig. 5.** Electron micrographs of negatively stained paired helical filaments from Alzheimer brain tissue. Arrowheads mark longitudinal periodicity (~75–80 nm), the width varies typically between 10 and 22 nm. Top, overview (bar = 200 nm); bottom, detail (bar = 100 nm). This preparation was used for x-ray and FTIR experiments.
axis. Peaks $h \approx 2 \text{ nm}^{-1}$ and $i \approx 2.2 \text{ nm}^{-1}$ are near-meridional reflections from layer lines 8 and 9. They reveal the presence of strands of $\beta$-sheet running roughly perpendicularly to the microtubule axis, reminiscent of a cross-$\beta$-structure. These features are also prominent in electron diffraction patterns from glucose-embedded tubulin sheets (Downing and Jontes, 1992).

The reason for dealing with the microtubule pattern is to show that secondary structure can indeed be revealed by the method, yet none of the diagnostic reflections are detectable in the x-ray patterns from PHFs (Fig. 5, c and d). It was not possible to obtain a measurable degree of order in these specimens, indicating that they are too much tangled up. There is a ring from water around 3.5 nm$^{-1}$ in Fig. 6c which largely disappears upon drying. Significantly, there are no reflections around 1 nm$^{-1}$ or 2 nm$^{-1}$ (which would reveal packed $\alpha$-helices and their axial repeats), nor around 2.2 or 2.8 nm$^{-1}$ (which would reveal the spacing between $\beta$-strands or between residues along the strands), even after very long exposure times. In other words, the PHF pattern contains no traces of secondary structure. At low resolution (Fig. 6d) we can detect a strong central scatter with a superimposed peak at $S_1 = 0.11$ nm$^{-1}$, followed by a weaker peak at $S_2 = 0.27$ nm$^{-1}$. The first of these probably arises from the PHFs as a whole and indicates an effective diameter of $d = 15.5$ nm (calculated from $d = 1.7/S$ in the solid cylinder approximation), a reasonable value considering that the diameter of PHFs varies between 10 and 22 nm in electron micrographs (Fig. 5). The second peak is probably not simply a higher order of the first (because this would have to lie at a smaller $S$-value, about 1.6 $S_1$). Its spacing of 1/0.27 = 3.7 nm is reminiscent of the weak 3 nm periodicity described by Crowther and Wischik (1985) and could represent an axial repeat along the PHFs.

Finally, we wanted to substantiate the above results by a spectroscopic technique sensitive to secondary structure. Because PHFs are aggregates and scatter light CD was not applicable. However, FTIR provides an alternative since the infrared signals are less affected by aggregates. The amide I absorption maximum at 1658 cm$^{-1}$ (thick arrow) which indicates that there is no pronounced $\beta$-structure (if this were dominant one would expect the peak around 1620–1630 cm$^{-1}$, thin arrow). The spectra were obtained after exchanging H$_2$O for D$_2$O because this strongly reduces the background of water to the amide I region. Another way to evaluate the data is by using the ratio $A_{1658}/A_{1690}$. For PHFs this ratio is $\approx 0.75$; for comparison, a protein dominated by $\beta$-structure such as the prion protein has a ratio of 1.35.
peptides which could lead to almost indestructible aggregates (Zhang et al., 1993).

However, there are exceptions which disturb the attractive simplicity of the paradigm. In one fortunate case, transthyretin, the three-dimensional x-ray structure of the subunit is known (Terry et al., 1993). The protein is indeed almost all $\beta$, it assembles such that the $\beta$-strands run roughly across the fiber (cross-$\beta$-conformation), and yet the interaction between subunits is not based on $\beta$-strands but rather on exposed SH groups generating S-S cross-bridges. Second, peptides derived from proteins may adopt a $\beta$-structure in solution and aggregate as such, yet in the complete protein the same peptide could have a different conformation (one example being the peptides derived from the $\alpha$-helical region of intermediate filaments which in isolation form aggregates with $\beta$-structure, Geisler et al. (1993)). Third, there are examples where no secondary structure is visible on the subunit level or in the fiber, this is the case for PHFs described here.

Our experiments were originally prompted by the seeming contradiction between different reports in the literature. On one hand, tau in solution was considered to be very low in secondary structure, as judged by CD (Cleveland et al., 1977), and it had a flexibility reminiscent of denatured and unfolded proteins (as measured by proton NMR, Woody et al. (1983)). On the other hand, PHFs were thought to have a cross-$\beta$-structure, very similar to the amyloid fibers of Alzheimer’s disease, as observed by fiber x-ray diffraction (Kirschner et al., 1986). In those days the subunit composition of PHFs was still a matter of debate, but today we know that they consist almost exclusively of tau (Kosik et al., 1986; Kieszkzak-Reding and Yen, 1991; Goedert et al., 1992), and PHF-like fibers can even be reassembled in vitro from domains of tau protein (Wille et al., 1992). So where does the presumptive $\beta$-structure of PHFs come from?

Our answer is that we find really no detectable $\beta$-structure in PHFs, as judged by x-ray diffraction and FTIR. Likewise, we find no detectable secondary structure in tau protein or various constructs, in a wide range of solvent conditions (varying pH, salt, or other parameters) as judged by x-ray scattering or CD. Tau can be induced to form dimers which are thought to be intermediate steps in PHF assembly (Kieszkzak-Reding and Yen, 1991; Wille et al., 1992), yet the dimers show no signs of secondary structure either. Thus the observations on tau monomers, dimers, and PHF polymers are mutually consistent. The only condition where secondary structure was observed was in the presence of TFE which is a strong inducer of $\alpha$-helix; in this case the CD spectrum indicated about 30% $\alpha$-helix.

If tau has little or no secondary structure, what are the alternatives? By all criteria available, tau may appropriately be regarded as a “natively denatured” protein. This view is supported by many observations. 1) Tau is resistant to heat and acid treatment without loosing its ability to promote microtubule assembly (Weingarten et al., 1975; Fellous et al., 1977). 2) Tau’s flexibility is similar to that of a denatured protein (Woody et al., 1983). 3) Tau has no detectable secondary structure, as discussed above. 4) Tau has no compact folding in solution, but rather resembles a “Gaussian polymer” with a persistence length of $\sim$2 nm (Fig. 3); in this regard it is similar to typical denatured proteins (Damaschun et al., 1983). 5) The binding of tau to microtubules is not defined by clearly identifiable residues, but rather distributed over many weakly interacting sites within the C-terminal half (Butner and Kirschner, 1991). 6) In the electron microscope, tau or tau constructs appear rod-like; their contrast is unusually low, and they are unusually long considering their molecular weight. Both features are explained by a largely extended conformation (Wille et al., 1992).

In fact the rod-like appearance could be the result of the glyc-
Structure of Tau and Paired Filaments

There is no unique folding (Tyler and Branton, 1980; Navet et al., 1989) of the regions that define the interactions with other proteins. The PHFs, or between tau and microtubules, are not considered in the study of particular importance for the interaction between tau molecules in PHFs. Thus, it is usually not visible at all (Zingsheim et al., 1983). The nature of this aggregation mechanism cannot simply be modeled on the basis of other known structures, in particular the secondary and nonperiodic structures such as p-turns that are not destroyed by drying. The peak at 15 nm for S = 0.11 nm⁻¹. This is in excellent agreement with electron microscopy and represents an independent x-ray estimate of the mean size of the PHF filaments. Assuming a molecular mass of 45,85 kDa (for htau40) and a partial specific volume of 0.73 ml/g, one molecule of tau would occupy a volume of V = 55.6 nm³ and thus a height h = 0.29 nm along the PHF cylinder. The second small angle peak at Sₕ corresponds to a periodicity >10 times larger, about 3.8 nm. It does not qualify as a second subsidiary maximum of the transform of a cylinder. However, it could come from a different type of periodicity, for example, an axial repeat along the fiber axis. A repeat repeat over this value (3 nm) was mentioned by Crowther and Wischik (1985), although it is usually not apparent in electron images, presumably because of the contrast visible by x-rays is masked by the negative stain. On the other hand we cannot at present rule out some other artifact; for example, remnants of membranes or stacks of membranes could possibly give rise to maxima at similar spacings.

Finally, one may ask why we did not observe the x-ray reflections diagnostic of a cross-β structure described by Kirschner et al. (1986). We think this is primarily due to the subsequent improvement in PHF preparation methods. Kirschner et al. prepared cortex samples following Ihara et al. (1983) which does not completely separate PHFs and amyloid fibers. These two structures generally occur in close proximity, and both can be observed by electron microscopy in earlier preparations (Wischik et al., 1988; Spillantini et al., 1990). In addition, these preparations also contain other contaminants such as lipofuscin, ferritin, or collagen (Kosik, 1989). Both the β-amyloid and the lipofuscin components could generate reflections in the 0.47-nm range (Kirschner et al., 1986). To circumvent these problems Greenberg and Davies (1990) devised a new procedure which results mostly in soluble PHFs and removal of the above contaminants. We applied an additional density centrifugation step in order to further eliminate lipofuscin (see "Materials and Methods"). These PHF fibers showed no evidence for β-structure, as described above. Thus we cannot invoke interactions between β-strands as a mechanism for tau-tau interactions in PHFs so that the nature of the aggregation mechanism cannot simply be modeled on the basis of other known structures.

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