Tau filaments from human brain and from *in vitro* assembly of recombinant protein show cross- β structure

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Abnormal filaments consisting of hyperphosphorylated microtubule-associated protein tau form in the brains of patients with Alzheimer's disease, Down's syndrome, and various dementing tauopathies. In Alzheimer's disease and Down's syndrome, the filaments have two characteristic morphologies referred to as paired helical and straight filaments, whereas in tauopathies, there is a wider range of morphologies. There has been controversy in the literature concerning the internal molecular fine structure of these filaments, with arguments for and against the cross- β structure demonstrated in many other amyloid fibers. The difficulty is to produce from brain pure preparations of filaments for analysis. One approach to avoid the need for a pure preparation is to use selected area electron diffraction from small groups of filaments of defined morphology. Alternatively, it is possible to assemble filaments *in vitro* from expressed tau protein to produce a homogeneous specimen suitable for analysis by electron diffraction, x-ray diffraction, and Fourier transform infrared spectroscopy. Using both these approaches, we show here that native filaments from brain and filaments assembled *in vitro* from expressed tau protein have a clear cross- β structure.

Alzheimer's disease is characterized by the presence of two kinds of fibrous aggregates in the brain, known as neuritic plaques and neurofibrillary tangles (1). Identical deposits also occur in later life in the brains of patients with Down's syndrome. The extracellular plaque deposits contain β -amyloid, a 40- to 43-aa proteolytic fragment of the much larger amyloid precursor protein. The intracellular neurofibrillary aggregates consist of paired helical filaments (PHFs) and straight filaments (SFs), which are made of microtubule-associated protein tau. Tau filaments with various morphologies are also found, in the absence of β -amyloid, in a range of dementing human tauopathies, some of which result from mutations in the tau gene itself (2).

Six tau isoforms are produced in adult human brain by alternative mRNA splicing from a single gene (3–5). They differ from each other by the presence or absence of 29- or 58-aa inserts in the N-terminal half and an additional 31-aa repeat located in the C-terminal half. Inclusion of the latter gives rise to three isoforms with four repeats each, whereas the other three isoforms have three repeats each. The repeats constitute the microtubule-binding domains of tau (6, 7). Similar levels of three- and four-repeat tau are found in normal cerebral cortex (5), and the tau filaments from Alzheimer's and Down's brain contain all six isoforms in a hyperphosphorylated state (8, 9). The repeat region of the tau molecule forms the dense core of PHFs and SFs, with the N- and C-terminal regions forming a fuzzy coat around the filament (10).

From their original isolation (11), the β -amyloid filaments were known to display a prototypical cross- β structure, yielding an x-ray diffraction pattern with a strong 0.47-nm meridional spacing characteristic of amyloid (12). The fibrils consist of a hydrogen-bonded β -sheet structure, in which the β -strands run

perpendicular to the long filament axis. The tau-containing filaments have not been so well characterized in detailed structural terms as β -amyloid, and there are conflicting reports in the literature about the nature of their internal structure. An early study by x-ray diffraction of bulk fibers made from preparations of PHFs showed evidence for a cross- β structure (13). However, this study was open to the objection that the PHF preparation from Alzheimer brain might have been contaminated by the strongly scattering β -amyloid. A later study by x-ray diffraction and spectroscopy found no evidence for β -structure in PHFs (14). Subsequently, the same group published evidence that the *in vitro* assembly of tau protein into filaments depended on a local sequence motif in the repeat region forming β -structure (15) and showed further that the effect of several mutations in tau protein that cause frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) was to enhance the local β -structure (16). There was also a claim that fibers made *in vitro* from fragments of tau but not those made from full-length tau exhibited a cross- β structure, although the morphology of these fibers did not resemble that of native PHFs (17). Finally, a recent investigation by spectroscopic techniques claimed there was evidence for native PHFs being comprised of α -helices (18).

In view of these conflicting claims, we decided to reinvestigate the fine structure of tau-containing filaments using two approaches that unequivocally link the experimental observations to a well defined specimen. In the first place, we carried out selected area electron diffraction, as we had previously used for α -synuclein-containing filaments (19), on a frozen unstained preparation of PHFs and SFs isolated from the cerebral cortex of an individual with Down's syndrome. In this way, diffraction patterns could be recorded from small identifiable clumps of filaments of recognizable morphology, even down to the level of single filaments. In the second approach, we used the ability to assemble pure bacterially expressed full-length human tau protein in the presence of heparin (20) to create *in vitro* a uniform population of tau-containing filaments resembling those found in Alzheimer's disease and other tauopathies. We used wild-type tau and tau with the FTDP-17 mutations P301S (21) and K257T (22, 23). These *in vitro* assembled tau filaments were then analyzed by selected area electron diffraction, x-ray diffraction from macroscopic fibers, and Fourier transform infrared (FTIR) spectroscopy. For both the native filaments from brain and the *in vitro* assembled filaments, there was strong evidence for cross- β structure.

Materials and Methods

Preparation of Tau Filaments. Sarkosyl-insoluble material containing tau filaments was extracted from the frontal cortex of a

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Abbreviations: FTIR, Fourier transform infrared; PHF, paired helical filament; SF, straight filament.

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Fig. 1. Electron micrograph of a negatively stained preparation of abnormal tau-containing filaments from the frontal cortex of a patient with Down's syndrome. Examples of the characteristic PHF (P) and SF (S) are indicated. (Bar = 100 nm.)

patient with Down's syndrome, as described (9). Wild-type and mutant tau proteins were expressed in *Escherichia coli* BL21(DE3) and purified as described (5, 24). The tau isoforms used were: four-repeat wild-type (hT46, encoding the 412-aa isoform of human tau), four-repeat P301S, and three-repeat K257T (hT37 encoding the 381-aa isoform of human tau). Tau filaments were assembled in the presence of heparin as described (20).

Electron Microscopy and Diffraction. PHF preparations were assessed by negative staining for the best yield of filaments by using a Philips EM208S (Philips, Eindhoven, The Netherlands) microscope. For preparing frozen unstained specimens, 3- μ l samples were absorbed for 2 min onto thin carbon films that had been air-glow-discharged to capture the rather dilute filaments. The surface was then washed dropwise with 50 μ l of water, blotted, and rapidly frozen in liquid ethane. Synthetic filaments of tau were gently pelleted and the resuspended gels slowly tumbled to induce flow orientation. For cryomicroscopy of these much more concentrated specimens, 1 μ l of sample was placed on a holey carbon film, blotted, and plunged into liquid ethane. Grids were examined at 120-keV with a Gatan coldstage on an FEI Tecnai T12 (FEI, Eindhoven, The Netherlands) (for PHFs from brain) or on a Philips EM420 (for synthetic tau filaments) electron microscope. Low-dose diffraction patterns were taken from areas $\approx 1 \mu$ m in diameter with doses of 100–300 e/nm². A subsequent image was taken in defocused diffraction mode of the area used for diffraction to identify filament morphology and orientation. Camera calibration and rotational corrections were determined as described (19).

The images and diffraction patterns were manipulated for alignment with programs from the MRC image-processing suite (25). For the weak diffraction patterns obtained from native filaments (Fig. 1), the 0.47-nm region was selectively enhanced and the central inelastic scattering region reduced by a simple Gaussian radial filter to allow clear visualization of the weak signal.

X-Ray Diffraction. Synthetic tau filaments were aligned by using a stretch frame, as described (26). X-ray diffraction images were collected by using a Rigaku (Tokyo) rotating anode source (CuK α) and a MAR Research (Hamburg) image plate scanner (345 mm). Exposure times were 15–30 min at a specimen-to-

detector distance of 175 mm. The images were displayed and examined by using MOSFLM (27) and the positions of reflections recorded.

FTIR Spectroscopy. Attenuated total reflectance (ATR) FTIR was used to study the conformation of soluble and fibrillar tau. In ATR FTIR, a sample is applied to the surface of a high refractive index material, the internal reflectance element. By analysis of the amide I band in the 1,600- to 1,700-cm⁻¹ region (predominantly corresponding to amide C=O stretch), it is possible to determine the secondary structure composition of the sample (28).

Hydrated (H₂O) thin-film spectra were collected by using a Nicolet 800 FTIR spectrometer equipped with an MCT detector and purged with dry air. Samples were scanned in an out-of-compartment horizontal attenuated total reflectance accessory with a high-throughput 73 $\times$ 10 $\times$ 6-mm 45° trapezoidal germanium internal reflectance element. The samples were applied (50 μ l of 1–2 mg/ml solutions) and dried while being spread constantly with a spatula in a stream of dry nitrogen. Water vapor spectra were collected by using a clean internal reflectance element with reduced purge and subtracted from the sample spectrum until the region between 1,700 and 1,800 wavenumbers was featureless. Data processing was done with GRAMS32 (Galactic Industries, Salem, NH), as described (29, 30). Raw spectra were further processed to enhance the component bands by second derivative analysis and Fourier self-deconvolution. Secondary-structure analysis was performed by using curve-fit spectra, as described (28–32). This approach has been shown to provide accurate secondary-structure analysis for different kinds of proteins of known structure (31, 33, 34).

Results

Electron Diffraction from Native Tau Filaments. Examination of a negatively stained preparation of sarkosyl insoluble material from a Down's syndrome brain showed that the filamentous material consisted mainly of PHFs with a few SFs (Fig. 1), both clearly recognizable by their distinctive morphology, together with small amounts of much thinner filaments likely to be β -amyloid. In low-magnification search mode on the electron microscope, it was possible to search unstained frozen grids for small clumps of filaments of clear morphology, to then take a selected area diffraction pattern, followed by an image of the exact area from which the diffraction pattern had been generated. In areas where the grid had little amorphous ice (Fig. 2A), the morphology of the filaments was particularly clear, with the PHFs having a barley-sugar twist appearance. This appearance must arise from a partial one-sidedness of the imaging, caused probably by adhesion of the filaments to the carbon coating of the grid, and the images are reproduced so the twist appears left-handed, as established earlier by metal shadowing (35). The corresponding diffraction pattern (Fig. 2B) showed a clear arc with partial orientation at a spacing of ≈ 0.47 nm and in a direction corresponding to the axes of the roughly oriented group of filaments, indicating a cross- β -fold. On further electron exposure, the arcs at 0.47-nm spacing rapidly disappeared, indicating they arose from proteinaceous material. In places where there was more amorphous ice, the fully hydrated filaments had a less distinct morphology (Fig. 2C) but still gave rise to a diffraction pattern with a clear 0.47-nm arc (Fig. 2D). The diameters of the filaments in ice (Fig. 2C) appeared smaller than those of the partially dried ones (Fig. 2A). This is to be expected, because the filaments are believed to have a dense core consisting of the ordered repeat region of tau, surrounded by less-dense disordered parts corresponding to the N- and C-terminal regions of tau (10). If the scattering density of the diffuse outer parts is roughly equal to that of water, only the dense core will be visualized, leading to a smaller apparent

Fig. 2. Selected area electron diffraction patterns from an unstained frozen preparation of tau filaments from a patient with Down's syndrome. *A*, *C*, *E*, and *G* show the areas from which the corresponding diffraction patterns *B*, *D*, *F*, and *H* were recorded. In *A*, the clump of filaments is partially dehydrated but shows clearly the morphology of PHFs. In *C*, the filaments are fully embedded in ice. In *E*, the scattering arises predominantly from one PHF and in *G* from one SF. The diffraction patterns (*B*, *D*, *F*, and *H*), which have been aligned relative to the images (*A*, *C*, *E*, and *G*) and which have had the strong central scattering removed, all show a clear arc at 0.47-nm spacing (arrowed) in the axial direction of the filaments, indicative of cross- β structure.

diameter. By optimizing the electron dose and camera length, it eventually became possible to record diffraction patterns that arose predominantly from a single filament. Fig. 2*F* shows a diffraction pattern from a PHF (Fig. 2*E*), and Fig. 2*H* shows a

Fig. 3. Selected area electron diffraction patterns from tau filaments assembled *in vitro*. *A*, *C*, and *E* show images of areas from which diffraction patterns *B*, *D*, and *F* were recorded. (*A* and *B*) Wild-type four-repeat human tau. (*C* and *D*) P301S mutant 4-repeat tau. (*E* and *F*) K257T mutant three-repeat tau. The diffraction patterns show strong scattering in the 0.47-nm region with partial orientation indicative of cross- β structure.

diffraction pattern from an SF (Fig. 2*G*). Although there are small fragments of filaments in the background of the areas from which the patterns came, the sharper orientation of the 0.47-nm arcs, compared with the arcs from clumps of filaments (Fig. 2*A–D*), indicates the patterns are arising predominantly from the long filament in each case. The patterns are, of course, quite noisy, because they come from such a small amount of scattering material, but the arc-like feature at 0.47-nm spacing in an axial direction is clear. The radial width of the arc is of the order of $1/40 \text{ nm}^{-1}$, indicating a lower limit for the extended axial spatial coherence of scattering from the cross- β -fold. The limited angular extent of the arc from the single filaments implies that the component β -strands can diverge at most by $\approx 5^\circ$ from perpendicular to the filament axis.

Electron Diffraction from Synthetic Tau Filaments. The ability to assemble large quantities of tau filaments from pure expressed full-length tau protein in the presence of heparin gives an opportunity to examine bulk properties of the filaments in a way hard to do with material from brain preparations. Filaments assembled from four-repeat wild-type tau tend to be very short (Fig. 3*A*), so little if any ordering can be obtained on the microscope grid. The diffraction pattern therefore typically shows a 0.47-nm ring with little or no indication of orientation (Fig. 3*B*). The filaments assembled from four-repeat tau with the P301S mutation are considerably longer (Fig. 3*C*), although more flexible than native filaments from brain. Some degree of ordering can be achieved on the grid, as indicated by the arcing

Fig. 4. X-ray diffraction pattern from a partially aligned fiber of tau filaments assembled *in vitro*. The filaments were assembled from P301S mutant four-repeat human tau. There is strong meridional scattering at a spacing of 0.47-nm and a weak ring, shown with enhanced contrast in the central inset, at a spacing of 1.3 nm.

of the 0.47-nm ring in the diffraction pattern (Fig. 3D). Similar results were obtained with three-repeat tau with the K257T mutation (Fig. 3E and F). In control grids prepared with a frozen solution of heparin alone as the specimen, only diffuse scattering was observed with no sharp ring in the 0.47-nm region (data not shown).

X-Ray Diffraction from Synthetic Tau Filaments. The x-ray diffraction pattern from a partially aligned fiber made of filaments assembled from full-length P301S mutant four-repeat tau showed strong scattering in the 0.47-nm region, with most intensity lying in arcs in the meridional direction (Fig. 4). The area inside this ring showed only weak diffuse scattering, although a faint ring was observable at a spacing of 1.3 nm.

FTIR of Synthetic Tau Filaments. The FTIR spectrum of unassembled tau in solution shows negligible β -structure (Fig. 5).

Fig. 5. FTIR spectra of soluble and filamentous P301S mutant four-repeat human tau. The spectrum of soluble tau (dashed line) shows no indication of β -structure, but the filamentous sample (solid line) shows a strong peak at 1,628 cm^{-1} , indicating that the assembled filaments have significant β -structure.

However, the spectrum obtained from assembled filaments, in this case of P301S mutant four-repeat tau, showed a significant peak in the amide I region centered at 1,628 cm^{-1} (Fig. 5). This is a position typical for amyloid filaments of other proteins, and secondary structure analysis of the spectrum gave an estimate of $57 \pm 6\%$ β -sheet.

Discussion

Previous investigations of the molecular fine structure of tau filaments have given rise to conflicting results and interpretations, with claims for and against β -structure and also for an α -helical structure (13–18). In the case of filaments isolated from brain, part of the difficulty with any measurement on a bulk sample is to ensure the preparation is clean and homogeneous. This is particularly the case for tau filaments isolated from the brain of patients with Alzheimer's disease or Down's syndrome because of the likely contamination with small amounts of β -amyloid, as the latter shows such a strong β -structure signature with many physical probes. Two possible ways around this problem are either to use a probe sufficiently sensitive to work with small groups of filaments of demonstrably defined morphology or to assemble filaments *in vitro* from purified protein so the population studied by bulk methods is known to be homogeneous. In this study, we have used both these approaches by carrying out selected area electron diffraction on small groups of filaments isolated from brain and by assembling full-length tau protein in the presence of heparin for bulk studies.

Selected area electron diffraction from unstained filaments isolated from brain showed a clear ring at 0.47-nm spacing. Where the groups of filaments were partially oriented on the grid, the diffraction pattern showed orientation with the arc of intensity in the axial direction of the filaments, characteristic of a cross- β structure and arising from the intermain-chain spacing. By refining the conditions for recording the diffraction patterns, it became possible to record patterns that arose predominantly from a long single filament with smaller contributions from other short fragments of filament in the illuminated area of the grid. These single-filament diffraction patterns, although noisy, clearly showed the same cross- β pattern. Because the exact area used for diffraction was also imaged, the morphology of the diffracting filament(s) could be identified. Thus, it could be seen that PHFs and SFs gave essentially the same cross- β pattern. The similarity of pattern is to be expected, because the PHF and SF are believed to arise from different relative arrangements of two protofilaments (36). Similar cross- β patterns were also obtained from assembled tau filaments made from three- or four-repeat isoforms. However, in this case, the degree of orientation that could be induced was less than for the native filaments, because the assembled filaments were not straight. X-ray diffraction from a partially oriented fiber of assembled filaments also gave a clear indication of cross- β structure with a strong arc at 0.47-nm spacing. A very weak ring was also visible at ≈ 1.3 -nm spacing, but the origin of this is not clear, because the reflection from stacked β -sheets would normally be in the 1.0- to 1.1-nm region. However, amino acids with bulky side chains (Gln, Lys, Leu, and Ile) make up 30% of the 95-aa core peptide (see below), whereas amino acids with small side chains (Gly and Ala) are under-represented, which could bias the sheet spacing to the larger value (37). FTIR spectroscopic measurements on the assembled filaments also gave a clear indication of β -structure, which was absent in unassembled soluble tau protein.

The images of PHFs when fully embedded in ice were noticeably narrower than those partially embedded. A possible explanation of this observation is that the filaments consist of a dense core of ordered protein surrounded by a diffuse coat of disordered protein (10). The diffuse coat, which consists of the very hydrophilic N- and C-terminal regions of the tau molecule, can be stripped by proteases to leave a protease-resistant core. The

stripped core filament has a similar width and morphology to the unstripped one when visualized by negative staining with a penetrating stain such as phosphotungstate, although unstripped filaments look thicker when stained with uranyl acetate, suggesting that under the latter conditions, the coat is visualized, albeit in a collapsed state. In an unstained state in amorphous ice, the diffuse hydrophilic coat will have a scattering density close to that of amorphous ice and will therefore not be readily visualized in micrographs, the image contrast arising mainly from the dense core. In filaments not fully embedded, the diffuse coat will collapse onto the core, giving an image that looks thicker and more akin to that seen with uranyl acetate staining. The dense core of the filament that contains the ordered cross- β structure is described here.

After protease digestion, the protease-resistant core of the PHF consists of various fragments of three- and four-repeat isoforms, but all of a length of ≈ 95 aa and all arising from the repeat region of the molecule (38, 39). This corresponds roughly to three of the 31- to 32-aa repeats. The mass per unit length of the pronase-stripped filaments was measured by scanning transmission electron microscopy to be 65,000 Da/nm (10). Assuming a protein-packing density of 0.00122 nm³/Da, this would give a cross-sectional area of ≈ 80 nm², a figure in good agreement with what was observed in the computed cross sections of PHFs and SFs (36). If the measured mass per unit length is expressed per 0.47-nm cross- β spacing, there would be 30.5 kDa per 0.47 nm, corresponding almost exactly to three of the 95-aa peptides. To express this in terms of the mass in one of the two protofilaments

that make up the PHF or SF, this means that there would be three 95-aa peptides per 2×0.47 -nm cross- β spacing along each protofilament. This number has to guide any detailed model put forward for the way in which the 95-aa core peptide is folded in a cross- β fashion to form the core of the filament. Models based on β -strands forming stacked sheets (40) or β -helices (41) would be compatible with our observations.

Detailed analysis of the molecular secondary structure of amyloid filaments has previously been carried out mostly with deposits that form extracellularly (42). Considerably less work has been done on filaments that form intracellularly in late-onset neurodegenerative diseases. Among these diseases are the α -synucleinopathies, such as Parkinson's disease, diseases arising from expanded glutamine repeats, such as Huntington's disease, and diseases with filamentous tau protein deposits, such as Alzheimer's disease (43). Filaments assembled from α -synuclein were shown by various techniques to possess cross- β structure (19), as were synthetic filaments formed from exon 1 of huntingtin with 51 glutamines (44). We show here that tau filaments, both those extracted from human brain and those assembled *in vitro* from recombinant protein, also have a clear cross- β structure. Thus, the three major kinds of intracellular amyloid filaments characteristic of neurodegenerative diseases share this structure with the extracellular deposits seen in other amyloid diseases.

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