Structural Principles of Tau and the Paired Helical Filaments of Alzheimer’s Disease

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Tau, a major microtubule-associated protein in brain, forms abnormal fibers in Alzheimer’s disease and several other neurodegenerative diseases. Tau is highly soluble and adopts a natively unfolded structure in solution. In the paired helical filaments of Alzheimer’s disease, small segments of tau adopt a β-conformation and interact with other tau molecules. In the filament core, the microtubule-binding repeat region of tau has a cross-β structure, while the rest of the protein retains its largely unfolded structure and gives rise to the fuzzy coat of the filaments.

TAU—A MICROTUBULE-ASSOCIATED PROTEIN

The identification of tau protein was closely linked to the discovery of microtubule self-assembly in the early 1970s. Microtubules represent one of the major cytoskeletal networks responsible for cell shape, mitosis, intracellular transport and other functions. They assemble from the protein tubulin (α-β-heterodimers of ∼100 kDa). Previously, tubulin was known as a major “colchicine-binding protein” in brain (14), but the conditions for assembling this protein into microtubules remained elusive until the work of Weisberg et al (122). A key observation was that microtubule assembly is regulated by microtubule-associated proteins (MAPs) and their state of phosphorylation (111); one of these proteins is the “tau factor”, which was first isolated from brain by Kirschner’s group (121). Its major function is the stabilization of microtubules, particularly in axons, and hence its expression is strongly up-regulated during neuronal development (37) [for reviews, see (20, 30, 43)].

Tau protein is unusual in that it is heat-stable and acid-stable, that is, tau is exceptionally well soluble, so that it does not precipitate during boiling and treatment with acids. Its spectral properties are characteristic of a “random coil” protein (25). Electron microscopy of tau gave ambiguous results due to its low contrast (129), but certain techniques of specimen preparation, such as quick-freeze deep-etching, revealed a microtubule-bound “assembly domain” (so-called, because this domain promotes the assembly of microtubules) and a “projection domain” which protrudes away from the microtubule wall (57). The projection domain represents roughly the N-terminal half of the molecule and the assembly domain the C-terminal half (114). High-resolution shadowing and image reconstructions from unstained microtubules decorated with tau molecules confirmed the largely disordered nature of tau, even when it is bound to the surface of microtubules (105).

Cloning of tau from mouse, cow and human (45, 56, 78) revealed primary sequences and domain compositions (Figure 1). Tau is unusually rich in polar and charged amino acids and has a basic character, except for the initial ∼120 residues, where negative charges predominate. Only five types of residues (G, K, P, S, T) make up half of the sequence. This explains the high solubility and unfolded nature of the protein; however, it renders its abnormal aggregation in Alzheimer’s disease (AD) even more enigmatic. In particular, at first glance, the sequence contains no elements that are particularly amyloidogenic, such as stretches of hydrophobic residues (as in the Aβ peptide) or glutamines (as in the poly-Q stretches of huntingtin), whose interactions across and along peptide strands favor the formation of stable β-sheets [for reviews, see (90, 119)]. Tau occurs in a number of isoforms, including “big tau” in the peripheral nervous system (4, 26, 47). There are six major isoforms in the human brain containing 352–441 amino acid residues, which arise from alternative mRNA splicing of exons 2, 3, and 10 of the Tau gene (46). The projection and assembly domains can be separated by chymotryptic cleavage behind Y197 (114), the C-terminal tail behind D421 can be removed by caspase 3 or 9 (40, 42, 102), a major N-terminal 17 kDa fragment can be generated by calpain (95), several cleavage sites can be generated by a thrombin-like activity (5, 71) and cleavage behind E391 in the C-terminal tail is observed in paired helical filaments (PHFs) (91). Such fragmentation reactions are notable, because smaller fragments of tau have an enhanced tendency to aggregate and could therefore nucleate aggregation within cells.

The C-terminal half of tau contains three or four semi-conserved repeats, a feature shared with other MAPs. The general domain structure of tau is similar to that of the neuronal MAP2 and the ubiquitous MAP4; however, they possess a much larger N-terminal projection domain (21, 82, 94). The size of the projection domain appears to determine the spacing between microtubules in cells (23). Depending on developmentally controlled alternative mRNA splicing, the repeat domain of tau consists of three or four sequences of 31 or 32 residues each: R1 = Q244 – K274, R2 = V275 – S305, R3 = V306 – Q336, R4 = Q336 – Q366.
of alternating subunits of tubule assembly domain. The repeats constitute the core of the microtubule-binding domain, as well as the core of the paired helical filaments. Two hexapeptide motifs at the beginning of R2 and R3 promote paired helical filament (PHF) aggregation by inducing β-structure. ΔK280 and P301L are two FTDP-17 (frontotemporal dementia and parkinsonism linked to chromosome 17) mutations that strongly enhance the rate of PHF aggregation by increasing the propensity for β-structure.

Figure 1. Diagram of tau441, the longest isoform in human brain. The four repeats of 31 or 32 residues each are numbered. The inserts near the N-terminus and the second repeat can be alternatively spliced, giving rise to six isoforms. The N-terminal domain up to ~G120 has an acidic character, the other domains are basic. The left half (residues 1 to ~200) represents the "projection domain"; the right half the "microtubule assembly domain". The repeats constitute the core of the microtubule-binding domain, as well as the core of the paired helical filaments. Two hexapeptide motifs at the beginning of R2 and R3 promote paired helical filament (PHF) aggregation by inducing β-structure. ΔK280 and P301L are two FTDP-17 (frontotemporal dementia and parkinsonism linked to chromosome 17) mutations that strongly enhance the rate of PHF aggregation by increasing the propensity for β-structure.

Figure 2. Model of microtubule protofilament with bound kinesin and tau. The protofilament consists of alternating subunits of α- and β-tubulin (~450 residues each) arranged in a polar fashion. The head domain of kinesin, a microtubule-dependent motor protein (~350 residues), has the compact folding typical of most cytoplasmic proteins. By contrast, tau is natively unfolded, its structure is unknown in detail and modeled here as a random chain. Note that tau occupies a much larger volume than kinesin or tubulin.

| R4 = V337 – N368. R2 is encoded by exon 10 of Tau and is absent in the 3-repeat isoforms. The isoforms can be designated as 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, 2N4R, depending on the number of N-terminal inserts and C-terminal repeats. Fetal tau comprises only the shortest form (0N3R), with the other isoforms being added during brain development. In adult human brain, there is a balance of 3R and 4R isoforms, all of which assemble into PHFs (44, 66). This ratio can be perturbed in disease, for example, towards 4R isoforms in some cases of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (38, 60). Unlike humans, rodents express exclusively 4R tau in adult brain (76). An important property of tau is the large number of potential phosphorylation sites [for reviews, see (22, 115)]. This is due to the high frequency of phosphorylatable residues (45 S, 35 T and 5 Y in the largest isoform), combined with the open structure of tau which renders it accessible to many kinases. Many of the sites (up to 17, depending on the isoform) are part of serine-proline (SP) or threonine-proline (TP) motifs and represent targets of proline-directed S/T protein kinases, eg, MAP kinase, glycogen synthase kinase-3 (GSK-3β), cyclin-dependent kinase (cdk) 5, cdc2 and others. Additional sites are targeted by different kinases, including protein kinase A (PKA), protein kinase C, calcium-calmodulin-dependent kinase II (CaMKII), serum and glucocorticoid-dependent kinase (SGK), protein kinase B (PKB), MAP-microtubule-dependent kinase (MARK) and SAD kinases. Tyrosine kinases target Y18 [fyn, (79)] and Y394 [abl, (31)]. The lysine-isoleucine-glycine-serine motif (KIGS) or lysine-cysteine-glycine-serine motif (KCGS) motifs in the repeat domain (S262, S293, S324, S356) can be phosphorylated by MARK, PKA, SAD kinases, CaMKII and p70S6K, which strongly reduces the tau-microtubule interactions (36, 74, 96), [note that phosphorylation at these sites also inhibits tau aggregation, illustrating an analogous role for the repeat domain in the physiological and pathological functions of tau (106)]. A further potent detaching site is phosphoS214, which can be phosphorylated by PKA and other kinases of the AGC group (PKA/PKG/PKC group of protein kinases), and is up-regulated during mitosis (16, 63). Tau contains one or two cysteines in the repeat domain (C291 in R2, present in 4R isoforms, and C322 in R3), which can be engaged in intramolecular cross-linking affecting conformation, dimerization and aggregation (108).

The binding of tau to microtubules is mediated through the repeat domain (which binds only weakly by itself but provides specificity for microtubule assembly), in combination with the adjacent proline-rich flanking domains (which provide efficient targeting to the microtubule surface, according to the "jaws" model (100). In general, 4R tau binds to microtubules more tightly than 3R tau (18, 50, 85). Phosphorylation, especially of the repeat domain, tends to decrease the affinity of tau for microtubules (13, 15). Like soluble tau, microtubule-bound tau is mostly in a natively unfolded state (Figure 2) and is therefore poorly visible by X-ray fiber diffraction or (cryo-) electron microscopy (1, 105). Tau appears to bind in an extended fashion to the outer tips of the microtubule protofilaments; consequently, when microtubules are disassembled by low temperature, tau stabilizes the ring-like disassembly products consisting of tubulin oligomers. Nevertheless, the binding of tau to microtubules is dynamic, as nuclear magnetic resonance (NMR) studies have revealed a high mobility of most residues, even in the bound state (128), and as cellular microtubule-bound tau is in rapid equilibrium with unbound tau (83, 104). Tau and motor proteins bind to overlapping sites on the outer surface of microtubules and bind in a competitive fashion. This explains why tau can interfere with transport along microtubules, which leads
The conformation of soluble tau is unknown in detail, but is presumably highly variable, as expected for a natively unfolded protein. NMR studies of the repeat domain have revealed little secondary structure, but there are notable motifs of nascent $\beta$-structure near the beginning of R2 and R3 (39, 88). These motifs coincide with the regions involved in PHF assembly (see below). Furthermore, stretches of ~10 residues after the hexapeptide motifs tend to become helical upon interaction with phospholipid micelles (8), which may mimic the negatively charged microtubule surface. Internal compaction of the repeat domain is evidenced by the relative proximity of fluorescence-labeled residues, as judged by fluorescence energy transfer. Regarding global folding, fluorescence resonance energy transfer (FRET) studies have pointed to a paperclip-like folding, resulting in the juxtaposition of the repeat domain with the C-terminal and N-terminal ends of the molecule (67, 108) (Figure 3). This doubly folded conformation is reminiscent of the discontinuous epitopes of certain anti-tau antibodies (Alz50, MC1, TG3), which recognize early stages of AD pathology and are generated by folding of the N-terminus over the repeat domain (19, 68, 69).

Mutations in the Tau gene cause FTDP-17 (61, 99, 112). At the level of the expressed protein, some mutations perturb the balance of splicing isoforms, mostly in favor of 4R tau (38, 61, 112). These forms bind more tightly to microtubules than 3R forms, which may lead to overstabilization of microtubules and suppression of microtubule dynamics (41). Other mutations alter the protein sequence, mostly in the repeat domain. With regard to tau structure in solution, all mutants investigated so far are similar, in that they have shown the spectroscopic signature of natively unfolded proteins (6). The mutations decrease binding of tau to microtubules, which may lead to microtubule destabilization, although the effects are only moderate (55, 59, 83). In addition, some mutations also increase the propensity of tau to aggregate (49, 89). This is particularly pronounced for mutations that enhance the $\beta$-sheet propensity in the regions of the hexapeptide motifs, for example, $\Delta$K280 and P301L (10).

### ASSEMBLY OF TAU INTO PAIRED HELICAL FILAMENTS

The molecular fine structure of PHFs (Figure 4) is unknown in detail, but represents one of the major goals in the field, as it may aid in the development of methods and drugs to prevent aggregation. There was a long gap between Alzheimer’s discovery of neurofibrillary tangles (2) and the identification of PHFs as their basic elements (72, 117). Further time elapsed with attempts to find procedures for the isolation of PHFs (62, 126). One important advance was the image reconstruction of PHFs from negatively stained electron micrographs, which showed each half of the PHF to be composed of three protein densities, with overall dimensions of ~8 nm × 20 nm (28); subsequent work showed an analogous doubly tripartite structure for “straight filaments”, a minor variant of filament preparations from AD brain (27). These variants depend on details of the charge distribution around the $\beta$-structure forming motifs in the repeat domain at the beginning of R3 and R4 (32). The search for the protein composition of PHFs revealed tau as the major component (17, 29, 45, 53, 75, 80, 92, 125, 127) and molecular cloning elucidated tau sequences from several mammalian species (45, 46, 56, 78). This set the stage for the expression of recombinant tau and the structural and biochemical analysis of tau and PHFs.

One of the difficulties in studying PHF assembly from soluble tau was the high intrinsic solubility resulting from its...
hydrophilic nature. A further problem was to identify criteria for the in vitro generation of bona fide PHFs. These problems were solved by searching for tau constructs and assembly conditions, including dimerization of the protein by disulfide crosslinking, which accelerates PHF assembly (123). A further important step was the discovery that polyamionic molecules, such as sulphated glycosaminoglycans, RNA, acidic peptides and fatty acid micelles, induce assembly of full-length tau (48, 70, 97, 124).

The question of whether PHFs can be regarded as “amyloid” was a matter of extended debate. Amyloid is defined structurally as a fibril whose backbone consists of β-sheets in a “cross-β” arrangement, recognized by a meridional 0.47 nm reflection in fiber diffraction patterns. On this basis, Kirschner et al (73) proposed a cross-β structure for both types of fibers found in the brains of AD patients, namely amyloid plaques (made of the Aβ peptide) and neurofibrillary tangles (made of tau). In the case of PHFs, the 0.47 nm reflection was weak, the purity of the preparation was somewhat uncertain and later studies failed to confirm the reflection (107). Instead, other types of reflections were reported, which suggested a non-amyloid packing of the protein. The puzzle was partially solved with the realization that the aggregation of tau is based on short hexapeptide motifs in the repeat domain (275VQINK280 and 306VQIVYK311 at the beginning of R2 and R3, (9, 10, 51, 64) (Figure 1). These “aggregation motifs” have a partially hydrophobic character and tend to interact with a cross-β structure, contributing to the core of PHFs, while the rest of the protein remains largely disordered and makes up the fuzzy coat. In recent years, as a result of improvements in specimen preparation and diffraction techniques, the amyloid nature of tau filaments purified from human brain or assembled from full-length recombinant protein has been conclusively demonstrated (7, 12). The hexapeptide aggregation motifs coincide with sequences where nascent β-structure can be detected in soluble tau by NMR spectroscopy, and indeed, this region reveals a very low mobility compared with the fuzzy coat (88, 110). The importance of the hexapeptide motifs is further underscored by proline-scanning mutagenesis, where single prolines can interrupt β-structure and inhibit aggregation (“anti-aggregation” mutants); or, conversely, by mutations that enhance the propensity for β-structure, such as AK280 and P301L, and thus promote aggregation (“pro-aggregation” mutants) (71).

On the basis of these data, one can anticipate the analysis of PHF structure in several steps. The backbone of PHFs consists of cross-β structure, and therefore, by analogy with other amyloid fibers (eg, Aβ peptide, yeast prion peptide) (90), it is likely that tau fibers also consist of protofibrils made up of pairs of juxtaposed β-sheets, interacting axially by hydrogen bonding between their main chain strands and laterally through the sidechains across the sheets. As these residues are near one another their distances can probably be determined by spectroscopic methods. Examples are the recent studies employing site-directed electron paramagnetic resonance labeling of tau (86, 87), which have concluded that residues near the beginning of R2 (272–289) and R3 (303–320) must lie close to the corresponding residues in neighboring molecules, which could be achieved theoretically by arranging them in successive turns of a beta helix. A higher level of organization will be the arrangement of protofibrils within a PHF. Their number and interactions are currently unknown, but there are several constraints for possible arrangements: (1) The mass-per-length of the PHF core, determined by scanning transmission electron microscopy, is about 60–70 kDa/nm, equivalent to roughly 3.5–4.5 repeat domain molecules per nm (11, 125); for variations among PHFs, see (77). By comparison, successive molecules in a cross-β structure are spaced 0.47 nm apart. This is tantamount to ~2 molecules per nm, which would allow only ~2 protofibrils. (2) The cross-section of the PHF core is about 8 nm × 20 nm. This area is divided up into two halves, each containing three density peaks (and intervening valleys of lower density), so that the effective area is estimated at ~80 nm² (27). These features, combined with the typical density of compact protein domains of ~0.8 kDa/nm³, represent boundary conditions which models of tau folding in PHFs will have to meet. The least well-defined aspect of the PHF structure is the fuzzy coat (125). PHFs assembled from full-length tau or from its repeat domain have similar apparent dimensions by electron microscopy, suggesting that the non-repeat parts, which comprise ~70% of the protein (roughly residues 1–240, 370–441), make only a small contribution to the images, presumably because they retain their natively unfolded character (7). The extent of the fuzzy coat is best visualized by immunogold labeling where antibody-binding sites can extend away from the center of the PHF (33). Nevertheless, a substantial fraction of tau molecules in PHFs must have a folded conformation, because PHFs can be immunopurified with antibody MC-1, whose epitope comprises tau residues near the N-terminus and within the repeat domain (68).

Filamentous tau from AD brain is extensively phosphorylated and there is an ongoing discussion on how phosphorylation and aggregation are related. This issue has been addressed using tau phosphorylated by different kinases and/or pseudophosphorylated forms of tau, where certain residues were exchanged for glutamate [for reviews, see (65, 115)]. These studies have yielded mixed results, perhaps not surprisingly, considering the number of potential phosphorylation sites in tau, the limited specificity of kinase preparations and the heterogeneity of phosphorylation states. In the case of the KXGS motifs in the repeat region, phosphorylation inhibits aggregation rather than promoting it (106). In the present context the important point is that aggregation of tau into bona fide PHFs can be achieved without phosphorylation, demonstrating that phosphorylation does not have a major influence on PHF structure. In cells, phosphorylation of tau can change its properties on at least two levels, namely tau-microtubule interactions and tau–tau interactions. Phosphorylation tends to decrease the binding of tau to microtubules (35), with phosphorylation of the KXGS motifs and S214 having particularly pronounced effects (63). The result is a decrease in microtubule stability, but more importantly, an increase in the cytosolic pool of tau, which can contribute to aggregation into PHFs.

An important goal of studying PHF assembly is to use the information gained for preventing neuronal degeneration in AD and other neurodegenerative diseases.
One of the obstacles in generating cell or animal models has been the high solubility of tau, which means that overexpression of the protein is usually not sufficient to generate neurofibrillary tangles in a reasonable time. This problem can be overcome by using mutations that accelerate the formation of β-structure, for example, ΔK280 and P301L, by modifying the vicinity of the hexapeptide motifs to allow more extensive β-conformation (10), or by using multiple FTDP-17 mutations (120). When these mutations are combined with overexpression in transgenic mice, neurofibrillary tangles develop after ~1 year (81, 93, 101, 103). Another solution was a mouse model overexpressing wild type 3R and 4R human tau isoforms on a mouse tau knockout background with an excess of 3R over 4R isoforms (3). Such models allow one to test hypotheses on tau-induced neurodegeneration, such as the question of tau aggregation vs. toxicity, or the relationship between tau and Aβ toxicity. Our tau-inducible neuronal cell models have indicated that aggregation is toxic and that it can be prevented by switching off tau expression, or by using compounds that inhibit aggregation (71) (Figure 5). So far, a number of different mouse models have been described, resulting in a range of findings that are beyond the scope of this article [for reviews, see (34, 52, 84, 118)]. Furthermore, once the conditions for tau aggregation will be better defined, it will become easier to identify more potent aggregation inhibitors than those currently known (24, 98, 116). Low-molecular weight compounds have already been effective in preventing tau aggregation in some cell and animal models (54, 71). Thus, there is hope that this search will lead to the development of compounds that will keep the buildup of tau filaments under control in human brain.

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