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Role of Cysteine-291 and Cysteine-322 in the Polymerization of Human Tau into Alzheimer-like Filaments

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Filamentous tau pathology is central to a large number of dementing disorders, including Alzheimer's disease in which polymerized tau is hyperphosphorylated. Previous studies on heparin-dependent tau polymerization, using recombinant tau isoforms lacking Cys-291, suggest that tau dimerization via Cys-322 is critical for initiation of assembly of soluble tau into filaments. We report heparin-dependent *in vitro* polymerization of human recombinant tau (1–383 isoform), containing both Cys-291 and Cys-322, into paired helical filaments as characterized by electron microscopy. Tau polymerization, under physiological tau concentrations in the presence of dithiothreitol (DTT), was followed by a Thioflavine S fluorescence assay. To understand the molecular basis for heparin-induced tau polymerization, we expressed and purified C291A, C322A, and C291A/C322A tau mutants. The DTT requirement for tau polymerization was abolished using either the C291A or C322A tau mutant and polymerization was not observed with the C291A/C322A tau double mutant. Analysis by sodium dodecyl sulfate gel electrophoresis showed that, unlike wild type tau, a significant amount of the C291A mutant and the C322A mutant is present as a disulfide bonded dimer. Taken together these results suggest that, in isoforms containing both Cys-291 and Cys-322, a dimeric tau with an intermolecular disulfide bond through either Cys-291 or Cys-322 is presumably acting as a seed for initiation of tau polymerization.

Key Words: **tau polymerization; heparin; Alzheimer's disease; thioflavine S fluorescence; tau filaments.**

Abbreviations used: AD, Alzheimer's disease; PHF, paired helical filaments; DTT, dithiothreitol; TPK II, tau protein kinase II; IMAC, immobilized metal affinity chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Thio S, thioflavine S; IPTG, isopropyl thio-β-D-galactoside; PCR, polymerase chain reaction; WT, wild type.

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Abnormal protein deposition in neurons is a hallmark of several incurable brain diseases (1–5). These brain diseases include Alzheimer's (Aβ and tau), Huntington's (huntingtin protein), spinocerebellar ataxia (ataxin protein), Parkinson's disease (α-synuclein), Prion diseases (prion protein) and a long list of other tau-based diseases collectively known as tauopathies (6). The underlying *in vivo* biochemical mechanisms in these neurodegenerative diseases remain elusive and are extremely difficult to study *in vivo*. *In vitro* studies in some cases support the hypothesis that protein aggregation proceeds via a nucleus-dependent (or seed) ordered protein polymerization (3). Examples of seeded protein polymerization are sickle-cell hemoglobin fibrillization (7), Aβ (8), and tau (9). Accordingly, the precursor of this seed or nucleation, initial protein:protein interaction between two soluble monomeric molecules, could be a target for therapeutic intervention.

Filamentous tau pathology in AD and other dementing disorders has led to the view that these filaments produce nerve cell degeneration. Moreover, abnormal tau, is an indicator of cytoskeleton disorganization and tau dysfunction, and is not unique to AD (6). If filament formation is responsible for neuron dysfunction within AD brain, then tau:tau interaction may provide a useful therapeutic intervention for inhibiting tau filament assembly. Ideally, the normal function of tau would not be affected by inhibiting this assembly paradigm. Thus, targeting an important early step in the polymerization process of tau, such as inhibiting tau:tau interaction, could prevent formation of tau polymers via a seeded intermediate. Such a strategy may pave the way for a broad-based approach for more effective and specific therapies for incurable tau-based dementias. Recent evidence suggests that in several neurodegenerative diseases with dementia, heparan sulfate may be involved in the assembly of tau protein into filaments (10).

In the previous studies (9, 11–13), several factors have been used to induce *in vitro* tau polymerization

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and these include heparin, heparan sulfate, or RNA. These studies have focused largely on tau constructs containing only microtubule binding repeat domains (11, 12, 14). Assembly of tau into filaments in the presence of fatty acids has also been reported (15). These reported *in vitro* protocols differ in incubation time, protein concentration, and in the filament morphology. Most of these studies utilize tau concentrations well above the intracellular concentration of 2–4 μM (16). Previous heparin-dependent tau polymerization studies (9, 11, 14) suggest that intermolecular disulfide-linked dimers through Cys-322 is critical for initiation of tau filament assembly, based on the numbering used in the longest form (1–441) of human tau (17). Notably, these studies were performed with truncated versions of recombinant tau lacking Cys-291. It was also reported that DTT inhibits PHF formation (14), while poor assembly of four repeat constructs was attributed to the formation of an intramolecular disulfide bond between Cys-291 and Cys-322 (9). Thus, the basis for PHF formation from tau isoforms 1–383, 1–441 and 1–412 containing both Cys-291 and Cys-322 and all the four repeat domains remains elusive from these previous studies (9, 11, 14).

We report here heparin-dependent polymerization of the four repeat containing 1–383 tau isoform at physiological concentrations and characterization of tau filaments by electron microscopy. By using single (C291A and C322A) and double (C291A/C322A) tau mutants and Thio S fluorescence assay, we show that intermolecular disulfide formation through either Cys-291 or Cys-322 is also sufficient for initiating heparin-based tau polymerization, as monitored by Thio S fluorescence. The disulphide-bonded dimeric tau, either through Cys-291 or Cys-322, is presumably acting as a seed for tau polymerization and filament formation. Implication of these results with regard to inhibiting tau polymerization are discussed.

MATERIALS AND METHODS

Nickel sulfate, Mops buffer, heparin, and thioflavine S were purchased from Sigma. The inducer IPTG from GIBCO BRL and the BL21(DE3) cells were from Stratagene. Chelating Sepharose (fast flow) was purchased from Amersham Pharmacia Biotech. French Press (SLM Instruments, Inc.) was used to break the cells. Spectra Max Gemini fluorometer (Molecular Devices) was used to monitor tau aggregation using opaque 96-well plates from Costar. SDS–PAGE was carried out according to Laemmlli (18). Standard molecular weight markers were purchased from Gibco-BRL. The PCR primers were obtained from Sigma Genosys. For PCR, the PCR Express Thermal Cycler from Hybaid Limited was used.

Electron microscopy. Electron microscope studies were done at Western Michigan University with the help of Dr. Rob Eversole. Samples were prepared as indicated in the Thio S assay with the exception of Thio S. Samples were applied to formvar coated grids, dried, negatively stained with 1% uranyl acetate and examined using JEOL transmission electron microscope.

Cloning of tau mutants. DNA coding for WT tau (1–383 isoform) was used as the template DNA for generating tau mutants. QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to create single and double mutations following manufacturer's instruction. Purified DNA was transformed into INVαF′ cells from Invitrogen. Several colonies were picked and DNA was isolated using Concert nucleic acid purification system from GIBCO BRL. The desired clone was then retransformed into BL21(DE3) cells. One colony from each construct was picked for large scale DNA isolation using NucleoBond Nucleic Acid Purification tools from Clontech. Clones WT-11,1 (C291A) and WT-16,6 (C322A) were found to contain the correct DNA sequence. Upon verification of the correct sequence, WT-16,6 C322A DNA was used as template DNA using C291A primers to create a double mutant C291A/C322A. Clone WT-6,7dm (C291A/C322A) was confirmed by complete DNA sequencing.

Expression and purification of tau mutants. The WT tau (DE-105,2,3) and mutant tau clones (C291A, C322A and C291/C32A) were grown overnight in 200 ml Luria Broth containing 100 μg/ml ampicillin at 37°C with shaking. Thirty-five milliliter of overnight culture was used to inoculate 2 L of NS-85 media (19) containing 100 μg/ml ampicillin. The cells were grown to 0.4 A_{600} and induced with 1 mM IPTG for 4 h. Cells were then collected by centrifugation and the cell pellets were stored at −80°C until needed. Cell pellets from 2 L of culture were resuspended in 5 ml/g of 50 mM Tris–HCl, pH 8.0 containing protease inhibitors and were lysed in a French Press. The pH of the sample was adjusted to pH 8.0 using 2 M Tris and the sample was spun at 14,740*g* for 40 min. The pellet was discarded and NaCl (0.5 M, final) was added to the supernatant and mixed. The sample was boiled in a water bath for 15 min (20) and centrifuged at 14,740*g* for 30 min. The supernatant was removed and purified by IMAC (21). Purified tau proteins were dialyzed against 20 mM Mops buffer, pH 7.0, overnight at 4°C.

Determination of protein concentration. Protein concentration was determined using BCA Protein Assay Reagent (Pierce). IMAC purified tau and mutant proteins were electrophoresed on 12% SDS–PAGE and stained with Coomassie blue R-250. Purified protein was stored at −80°C.

Thioflavine S assay. This assay was carried out, with some modifications, as reported elsewhere (14, 22). Heparin was dissolved in deionized water at 5 mg/ml and stored at −20°C. Thio S was dissolved in 20 mM Mops, pH 6.5 at 3.2 mg/ml concentration, filtered using 0.2 μM Acrodisc, aliquotted in small volumes and stored at −20°C until needed. At the time of use, Thio S was diluted 50-fold using 20 mM Mops, pH 6.5 and kept in the dark. Heparin was diluted to 200 μg/ml in deionized water and freshly prepared DTT solution was used in all experiments. Polymerization reaction was set up containing 5 μM tau, 20 mM Mops, pH 7.0, 0.5 mM DTT, 64 μg/ml Thio S in 200 μl total volume using opaque 96-well plates. The polymerization reaction was initiated by adding 20 μg/ml heparin at the final concentration. The reaction components were mixed by tapping the plate quickly and immediately read for 2 h at 37°C in Spectra Max Gemini using excitation at 440 nm, emission at 520 nm with a wavelength cut off at 452 nm.

Tubulin polymerization assay. The tubulin polymerization assay was carried out essentially as described by Cytoskeleton. Tubulin (10 mg/ml) from Cytoskeleton (Cat. T238), was thawed on ice and diluted to 2 mg/ml with cold G-PEM buffer (80 mM Pipes, pH 6.8, 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM freshly made GTP). The assay was done in 96-well microtiter plates by incubating 75 μl of 2 mg/ml tubulin (1 mg/ml final) in a total of 150 μl G-PEM buffer containing tau at a final concentration of 7 μM. The reaction was initiated with the addition of tubulin and the plate was incubated at 27°C in a Molecular Devices microplate reader and the A_{340} was determined every 1 min for 30 min.

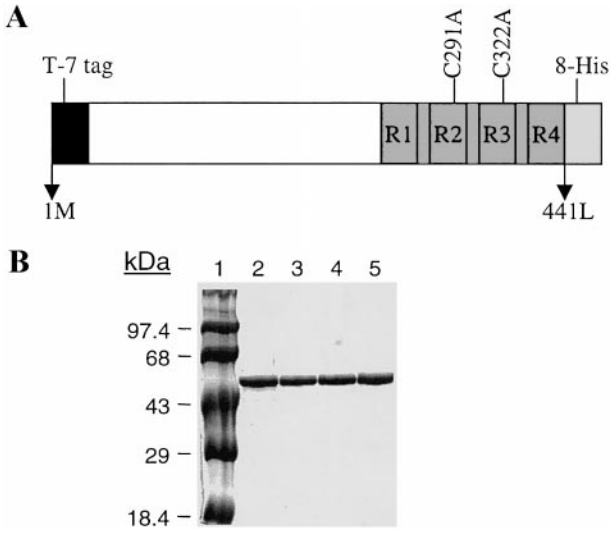

FIG. 1. (A) A schematic diagram of WT human recombinant tau (1–383 isoform) and its mutants (C291A, C322A, and C291A/C322A) containing all four microtubule binding domains (R1, R2, R3 and R4) used for tau polymerization studies. The 1–383 tau isoform lacks the two N-terminal inserts of 29 amino acids each from the longest (1–441) tau isoform (Goedert 1989). All the constructs contain a T7 tag at the N-terminus and an octa His tag at the C-terminus. (B) SDS–PAGE of IMAC purified WT recombinant tau and its mutants. Five micrograms of each purified tau protein was run on 12% SDS–PAGE gel under reducing conditions. Lane 1, molecular weight marker; lane 2, WT tau; lane 3, C291A mutant; lane 4, C322A mutant; lane 5, C291A/C322A mutant.

RESULTS AND DISCUSSION

Figure 1A shows WT tau and its mutants used in the current studies. The 1–383 isoform used lacks the two, 29 amino acid N-terminal repeat domains, based on numbering of the longest form (1–441) of human tau (17). Figure 1B shows an SDS–PAGE, under reducing conditions, of WT tau and its mutants purified by a combination boiling method (20) and IMAC (21). Using this two-step purification strategy, we routinely recovered 10–12 mg of purified recombinant tau from one liter of cell culture.

Our first goal was to determine if synthetic tau filaments could be produced *in vitro* at physiological tau concentrations. Filament formation was initiated by heparin in the presence of DTT and was monitored by electron microscopy as a function of time. Formation of tau filaments from tau aggregates was time-dependent (data not shown). Figure 2 shows an electron micrograph of WT tau after incubation for 24 h in the presence of heparin. The average filament width was 18 nm and average crossover repeat was 90 nm. This is consistent with PHFs reported by others (12). Control experiments without tau and without heparin did not show any filament formation (data not shown).

The Thio S fluorescent probe is routinely used to identify fibril pathology (NFTs) in AD brain and for *in vitro* staining of PHFs (11, 23, 24). Thio S was selected for developing the assay because it is known to decorate NFTs in AD brain and can be used to follow tau filament formation in the presence of inducers (14). Therefore, we followed heparin-induced tau polymerization at 5 μM by Thio S binding assay in a 96-well microtiter format. Tau polymerization was not observed in the absence of heparin (Fig. 3). For WT tau, there is a DTT requirement for both the rate of tau polymerization and the extent of tau polymerization. The optimal concentration is 0.25–0.5 mM DTT under defined conditions of temperature, pH, and heparin concentration described under Materials and Methods. As shown in Fig. 3, higher concentrations of DTT are inhibitory for tau polymerization. These results suggest that in the absence of DTT, tau polymerization was blocked presumably due to the presence of an intramolecular disulfide link between Cys-291 and Cys-322 of repeat domains R2 and R3, respectively. Higher concentrations of DTT ($>$0.50 mM) seem to be inhibitory, suggesting that an intermolecular disulfide bond in dimeric tau between either two Cys-291 or Cys-322 is essential for tau aggregation and is sensitive to cleavage at higher DTT concentrations ($>$0.50 mM). In the WT tau, the lower DTT concentrations ($<$0.5 mM) seem to facilitate cleavage of intramolecular disulfide bonds between Cys-291 and Cys-322 and this appears to shift the equilibrium toward the forma-

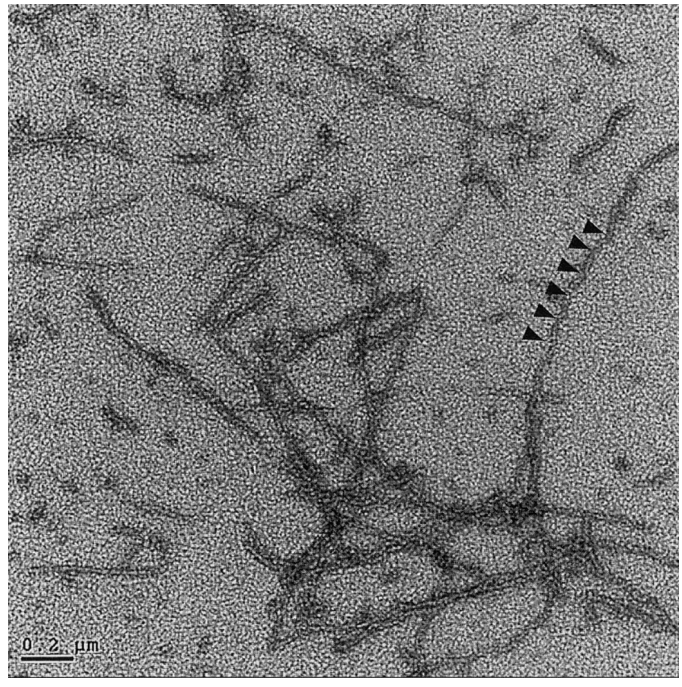

FIG. 2. Transmission electron microscopy of WT tau (5 μM) polymerized in the presence of heparin. Samples were applied to formvar coated grids, dried, negatively stained with aqueous 1% uranyl acetate and examined using a JEOL transmission electron microscope. The bar in the micrograph corresponds to 0.2 micron. The arrows indicate crossover repeats.

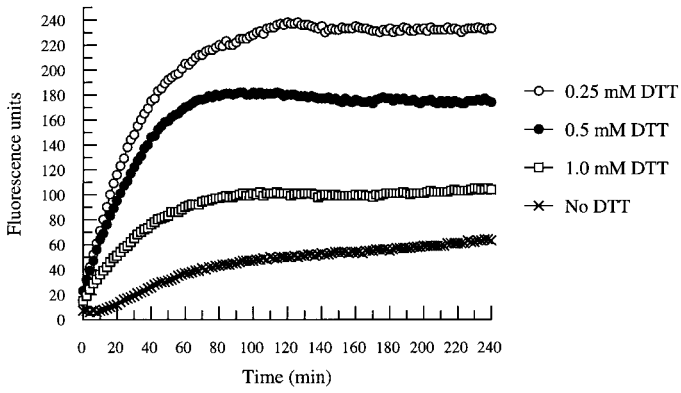

FIG. 3. The effects of varied concentrations of DTT on the WT tau polymerization measured by Thio S fluorescence assay. The reaction was set up in 200 μl of total volume at a final concentration of 20 mM Mops, pH 7.0; 5 μM tau; 62 μg/ml Thio S and the reaction was initiated with 20 μg/ml heparin. The concentration of DTT was varied as indicated in the legend.

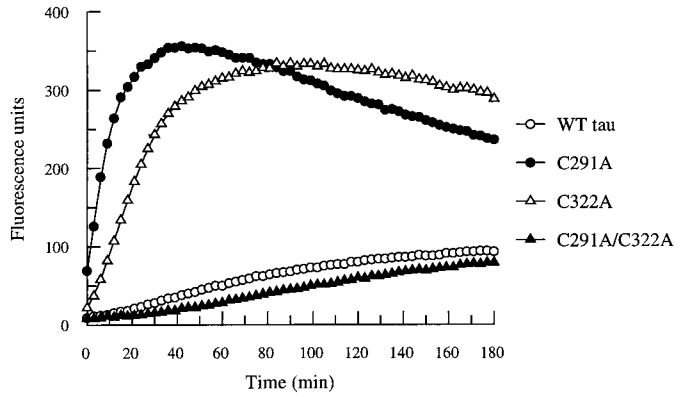

FIG. 5. Polymerization assay of WT tau and its mutants in the absence of DTT measured by Thio S as described in the legend to Fig. 3.

tion of intermolecular disulfide bond either between Cys-291 or Cys-322. At higher DTT concentrations (1 mM and above), presumably these intermolecular disulfide bonds are disrupted and aggregation is inhibited (Fig. 3).

To understand the molecular basis for heparin-based tau polymerization, we expressed and purified C291A, C322A, and C291A/C322A tau mutants (Fig. 1B). Biochemical characterization of these mutants, based on heparin-induced TPK II phosphorylation (data not shown) and tubulin polymerization revealed that these mutants are functionally comparable to wild type tau (Fig. 4). These well characterized tau mutants were studied in the Thio S fluorescence assay. Figure 5 shows heparin-induced polymerization of these tau mutants in the absence of DTT. As shown, the requirement for DTT was abolished when either Cys-291 or Cys-322 was mutated to Ala-291 or Ala-322, respectively. The double mutant tau C291A/C322A failed to polymerize under similar conditions. Moreover, in contrast to wild type tau, the double mutant tau did not polymerize in the presence of 0.5 mM DTT. These results suggest that one of the cysteines (Cys-291 or Cys-322) is necessary and sufficient to promote heparin-induced tau polymerization. Accordingly, in contrast to WT tau, the presence of an intramolecular disulfide bond between Cys-291 and Cys-322 seems to be eliminated and the formation of an intermolecular bond appears to be facilitated either through Cys-291 or Cys-322 in these mutants. With the C291A mutant (Fig. 5), we observed that after 60 min there is a slow time-dependent decrease in Thio S fluorescence. This phenomenon was not observed when DTT (>0.1 mM) was present (Fig. 6A). At this time we don't understand the basis for this apparent loss of Thio S fluorescence with the C291A mutant in the absence of DTT (Fig. 5).

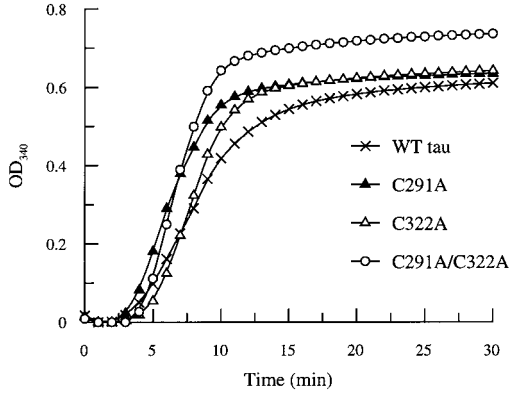

FIG. 4. Comparison of tubulin polymerization of WT tau with its mutants. For assay details, see Materials and Methods.

Figures 6A and 6B show the effect of DTT on polymerization of C291A and C322A tau mutants, respectively. As shown, as the concentration of DTT is increased, both the rate and extent of tau polymerization are decreased. An SDS-PAGE analysis for tau and its mutants at time zero is shown in Fig. 7. As shown, a significant amount of C291A or C322A mutant is present as a disulfide bonded dimer under nonreducing gel conditions (lanes 3 and 4). In contrast, WT tau or double mutant (C291A/C322A) was predominantly monomeric under these conditions (lanes 2 and 5). Under reducing gel conditions, as expected, wild type tau and its mutants were monomeric and were indistinguishable from each other (Fig. 7, lanes 7 to 10). An analysis of tau species by gel scanning showed that under these conditions, wild type tau and its double mutant were 91% (lane 2) and 98% (lane 5) monomeric, respectively. The single mutant C291A was 40% monomeric (lane 3), while the single mutant C322A mutant was 47% monomeric (lane 4). Similar SDS–PAGE analysis was obtained when samples were incubated under identical assay condition for 1 or 4 h at 37°C (data not shown). These results when taken together with Thio S aggre-

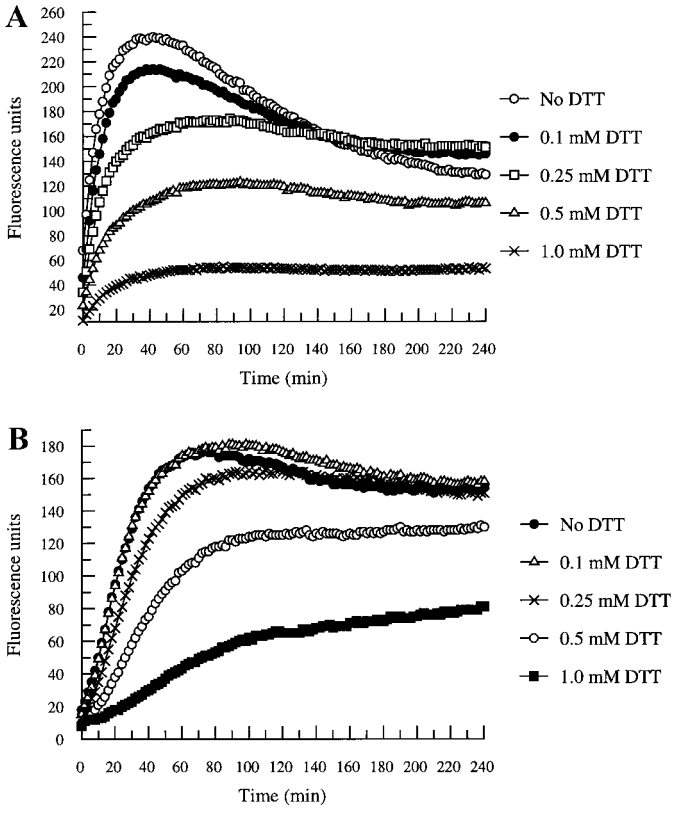

FIG. 6. (A) The effects of varied concentrations of DTT on the C291A mutant tau polymerization measured by Thio S fluorescence assay as described in the legend to Fig. 3. The DTT concentration was varied as indicated in the legend. (B) The effects of varied concentrations of DTT on the C322A mutant tau polymerization measured by Thio S fluorescence assay as described in the legend to Fig. 3. The DTT concentration was varied as indicated in the legend.

gation assay (Fig. 5) suggest that an initial tau dimer with intermolecular disulfide bond between either two Cys-291 or two Cys-322 is required for seeding tau polymerization.

Figure 8 shows the effect of DTT addition on preformed WT tau polymers in the Thio S assay. These results show that once the tau polymers are formed, DTT had no effect. Presumably, tau:tau interaction followed by disulfide bonded tau dimerization initiates tau polymerization, and further nucleation and elongation are driven by monomeric tau species. This interpretation is consistent with a reducing SDS–PAGE of tau and its mutants at 1 and 4 h of incubation. These results show that tau and its mutants are predominantly monomeric at DTT concentrations of 1 mM and above (data not shown).

There are six isoforms of human tau that are expressed in adult human brain and all six isoforms form PHFs (17). These arise from alternate splicing of mRNA from a single gene located on the long arm of chromosome 17, leading to the various tau isoforms (17, 25–28). They differ from each other with respect to

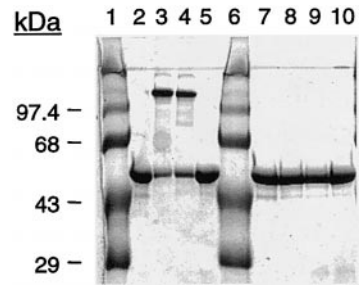

FIG. 7. SDS–PAGE analysis under nonreducing and reducing conditions for WT tau and its mutants at time zero. Reaction mix contained 5 μM tau, 20 mM Mops and 0.5 mM DTT in 200 μl total volume. At t = 0, 32 μl of sample was mixed with 8 μl of 5× loading buffer with or without β-mercaptoethanol. Samples were boiled for 2 min and were loaded onto a 12% SDS–PAGE gel. Gel was stained with Comassie blue R250. Lane 1, molecular weight marker; lane 2, WT tau; lane 3, C291A mutant; lane 4, C322A mutant; lane 5, C291A/C322A double mutant; lane 6, molecular weight marker; lane 7, WT tau; lane 8, C291A mutant; lane 9, C322A mutant; lane 10, C291A/C322A double mutant. Lanes 2 to 5 are under nonreducing and lanes 7 to 10 are under reducing conditions.

the presence of three or four tandem repeats of 31 amino acids each in combination with the two N-terminal inserts of 29 amino acids. Thus, the full length tau which contains four tandem repeats and the 58 amino acid N-terminal insert is the longest form of tau with 441 amino acids (17). For example, a tau isoform lacking the 58 amino acid N-terminal insert contains 383 amino acids. The four 31 amino acid repeat domains are known as microtubule binding domains; R1, R2, R3, and R4. In tau isoforms 1–352, 1–381, and 1–410, the R2 domain containing Cys-291 is missing and these are tau isoforms containing only three internal repeats (R1, R3 and R4). Studies with the shortest tau isoform (1–352) lacking Cys-291 and

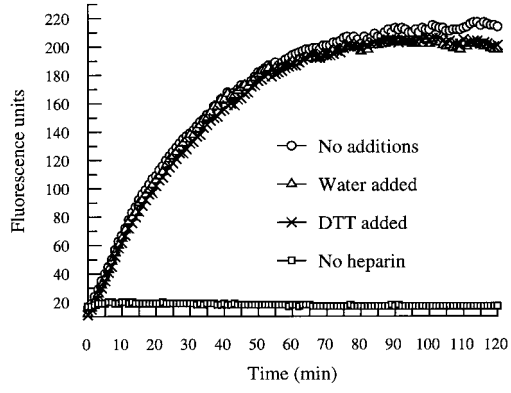

FIG. 8. The effects of addition of high concentration of DTT on the prepolymerized WT type tau initiated in the presence of 0.5 mM DTT. Polymerization assay was set up as described in the legend to Fig. 3. At 48 and 111 min after polymerization, DTT was added to a final concentration of 2.5 and 5 mM, respectively. Thio S fluorescence was read until 120 min. As a control, water was added at the same time point as DTT.

its truncated versions suggest that formation of synthetic PHFs depends on tau dimers generated by intermolecular disulfide bonds between the two Cys-322 molecules (11). These studies were carried out at protein concentrations (40 μM and above) that are much higher than intracellular tau concentrations of 2–4 μM (16). Moreover, in these studies, PHF assembly was not observed with constructs containing four repeat domains containing an additional cysteine (Cys-291). An intramolecular disulfide bonded dimer between Cys-291 and Cys-322 was proposed as the likely explanation. However, it has also been reported that DTT inhibits PHF assembly (14). Thus, the basis for PHF formation from tau isoforms 1–383, 1–441 and 1–412 containing both Cys-291 and Cys-322 and all the four repeat domains remains elusive.

Our studies in the absence of DTT with WT tau (1–383 isoform) containing both Cys-291 and Cys-322 indicate that heparin-induced tau polymerization at physiological concentrations is very poor (Fig. 3), presumably due to the formation of an intramolecular disulfide linkage between Cys-291 and Cys-322. However, addition of low DTT concentrations (Fig. 3) seem to be sufficient to break this intramolecular bond and formation of some tau dimers via intermolecular interaction between Cys-291, Cys-322 or both can occur. Accordingly, tau polymerization occurs rapidly under these conditions and eventually these polymers form tau filaments (Fig. 2).

In the previous studies (9, 11), the role of Cys-291 in *in vitro* tau polymerization was overlooked due to a lack of a systematic study on tau mutants containing all the four repeat domains. For the first time, the role of Cys 291 in promoting tau aggregation was established by studying polymerization of 1–383 tau isoforms mutated at Cys-291, Cys-322, or Cys-291 and Cys-322. These results show (Fig. 5) that the tau mutant C322A that contains only Cys-291 and this, like C291A mutant, is competent to undergo heparin induced polymerization in the absence of DTT. Thus, one of the two cysteines, Cys-291 or Cys-322, is essential for dimerization of tau. This dimer presumably acts as a seed for further polymerization resulting in PHFs. This interpretation is consistent with studies (Fig. 8) which show that high DTT concentrations had no effect on preformed tau polymers. However, if higher DTT concentrations ($>$1 mM) are included in the polymerization assay at the start of incubation, inhibition of polymerization results due to the inability of tau molecules to form intermolecular disulfide bonds either through Cys-291, Cys-322, or both.

Several factors have been used to induce *in vitro* tau polymerization and these include heparin, heparan sulfate, RNA, or arachidonic acid (9, 11–13, 15). The mechanism of *in vitro* tau assembly from tau monomers may depend upon the nature of the inducer used. The identity of pathological factors that trigger the formation of tau filaments from the highly soluble precursor *in vivo* remains unknown. In this regard, recent evidence suggests that in several neurodegenerative diseases with dementia, heparan sulfate may be involved in the assembly of tau protein into filaments (10). PHF composed of phosphorylated tau in inclusion-body myositis, a sporadic muscle disease, contain RNA and a RNA-binding protein (29). Available evidence also indicates (30) that there is Aβ within the cytoplasm of neurons affected by AD and accumulation of Aβ precedes tangle formation. Regardless of the nature of the pathological factor involved, it is clear that tau polymerization is a common event in most of the known tauopathies (Larner 6). In this regard, it is important to note that inhibition of this pathological event should not effect tau-mediated microtubule assembly.

Inclusions of tau protein and tau filamentous pathology seem to be associated with memory loss in a large number of neurodegenerative diseases (tauopathies) including AD (6). These neurodegenerative diseases develop tau pathology without amyloid plaques, suggesting that tau polymerization alone is toxic to cells and filamentous tau pathology may represent a pathobiological response of the neuron due to a variety of insults. In contrast to amyloid plaques, tau-based lesions do not appear to be a normal aspect of aging. It seems to indicate that something is wrong with the cytoskeleton, a characteristic of degenerating neurons. Moreover, there appears to be a link between tau mutation, cytoskeleton disorganization, and dementia (6, 31).

Some common features that appear in all of these tauopathies are tau hyperphosphorylation and tau polymerization. There are basically two strategies for targeting tau as a therapeutic target (6). The first approach is to inhibit kinases involved in tau hyperphosphorylation (6), while the second strategy is to interfere directly with the polymerization of tau (6). Our studies underscore the importance of the first step in tau polymerization, namely tau dimerization. Since tau polymerization can be initiated at low micromolar concentrations of tau protein, initial tau dimerization appears to be a low affinity interaction.

There is evidence which suggests that the third repeat (306V to 336Q) is primarily involved in tau aggregation (32). Recently, it has been reported that PHF assembly is initiated by a short sequence motif from third repeat (306-VQIVYK-311) in tau protein (22).

Accordingly, this evidence supports the notion that in a pathological state tau dimerization occurs presumably through the third repeat domain and this facilitates formation of disulfide cross-linking and perhaps PHF formation in the presence of yet unidentified inducers. Thus, it is possible to target a region on the third repeat domain of tau to inhibit non-covalent tau: tau interaction that presumably facilitates disulfide

linkages through either Cys-291 and/or Cys-322, tau filament nucleation, and elongation (polymerization).

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