Understanding the Kinetic Roles of the Inducer Heparin and of Rod-like Protofibrils during Amyloid Fibril Formation by Tau Protein

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Background: The kinetic role of heparin and of intermediates populated during Tau protein aggregation is not fully understood. Results: Heparin contributes to the initial steps of fibrillation, whereas protofibrillar intermediates accumulate transiently. Conclusion: Heparin is involved in nucleation, and the transient rod-like protofibrils are off-pathway species. Significance: Understanding the kinetic role of heparin and the protofibrils has implications for the development of therapies for tauopathies.

The aggregation of the natively disordered protein, Tau, to form lesions called neurofibrillary tangles is a characteristic feature of several neurodegenerative tauopathies. The polyanion, heparin, is commonly used as an inducer in studies of Tau aggregation in vitro, but there is surprisingly no comprehensive model describing, quantitatively, all aspects of the heparin-induced aggregation reaction. In this study, rate constants and extents of fibril formation by the four repeat domain of Tau (Tau4RD) have been reproducibly determined over a full range of heparin and protein concentrations. The kinetic role of heparin in the nucleation-dependent fibril formation reaction is shown to be limited to participation in the initial rate-limiting steps; a single heparin molecule binds two Tau4RD molecules, forming an aggregation-competent protein dimer, which then serves as a building block for further fibril growth. Importantly, the minimal kinetic model that is proposed can quantitatively account for the characteristic bell-shaped dependence of the aggregation kinetics on the stoichiometry of protein to heparin. Very importantly, this study also identifies for the first time the transient rod-like protofibrils that are populated during the time course of fibril formation. The identification of these protofibrils as bona fide off-pathway species has implications for the development of therapies for tauopathies based on driving fibril formation as a means of protecting the cell from smaller, putatively toxic aggregates.

Tau is a microtubule-associated protein that is involved in microtubule stabilization and neurite outgrowth (1). In the adult human central nervous system, Tau exists in the form of six alternatively spliced isoforms; these isoforms differ by having three or four repeats in the C-terminal region, and zero, one, or two inserts in the N-terminal region (2, 3). The 31–32-residue-long pseudo-repeats in the C-terminal region together with the flanking proline-rich regions constitute the microtubule binding domain (4). In solution, Tau behaves as a random coil as judged by several tools, spectroscopic and otherwise, and hence belongs to the class of intrinsically unordered proteins (5–7).

The abnormal aggregation of Tau into paired helical filaments (PHFs) 3 and straight filaments is a hallmark of Alzheimer disease (AD) and other neurodegenerative tauopathies (8, 9). Interestingly, in AD it has been seen that the degree of cognitive impairment correlates better with neurofibrillary tangles (NFTs) composed of Tau than with senile plaques composed of the Aβ peptide (10, 11). Nevertheless, although the familial forms of AD are found to be associated with mutations that affect Aβ levels, which therefore implicate it in the causation of disease (12), no such mutations have been seen in the Tau gene. Tau is found to be hyper-phosphorylated and aggregated into NFTs in AD (13). In all the other tauopathies, however, mutations in the Tau gene link it to the disease context (14).

Tau fibrils formed in vitro from recombinant protein closely resemble those isolated from AD-diseased brain (15). They also possess the characteristic β cross motif that is a feature of all amyloid fibrils (16–18). It has also been shown that the repeat domain of Tau, which constitutes the protease-resistant core of the PHFs (19) and which forms bona fide PHFs in vitro, serves as an equally good model system for investigating the aggregation of Tau, and its faster aggregation time scales make it more amenable for study (15, 20).

Although Tau dysfunction is implicated in neurodegeneration, the identity of the toxic aggregate remains unknown. For several other amyloid-related disorders, soluble oligomers and

3 The abbreviations used are: PHF, paired helical filament; NFT, neurofibrillary tangle; AD, Alzheimer disease; ThT, thioflavin T; AFM, atomic force microscopy; TEM, transmission electron microscopy; NDP, nucleation-dependent polymerization; P, protein; H, heparin.
Heparin-induced Aggregation of Tau Follows an NDP Mechanism

protofibrils have been implicated as the toxic entities (21, 22). For Tau, the formation of canonical protofibrils has not been observed so far, although oligomers have been observed in vitro (23–25). Furthermore, except for one study (25), it is also not known whether these oligomers lie on or off the pathway of fibrillation. For other proteins, the evidence for on-pathway roles for oligomers and protofibrils is largely indirect (26, 27).

An important goal of in vitro studies of the aggregation of Tau is to identify whether putatively toxic amyloid protofibrils are populated during the course of fibril formation and to determine whether they are on-pathway aggregates directly involved in fibril formation.

In vitro, amyloid fibril formation by Tau is extremely slow in the absence of polyanions, and the study of Tau aggregation becomes amenable only in the presence of polyanionic inducers that accelerate the reaction (28–35). In particular, the glycosaminoglycan heparin, whose structure closely mimics the highly sulfated regions of heparan sulfate, is commonly used to induce Tau aggregation in vitro (16, 17, 20, 23–25, 30–32, 35–47). Although Tau tangles are intracellular aggregates, they have been consistently found to contain components of the basement membrane such as heparan sulfate (48–51); it appears that this becomes possible during disease by mechanisms yet to be understood. Indeed, glycosaminoglycans such as heparan sulfate and other sulfated proteoglycans are also found in the amyloid deposits of many other proteins (52–54) even when the deposit is intracellular (55). Moreover, even though heparin is typically found outside the cell, the discovery that it, like other polyelectrolytes, can modulate amyloid fibril formation by many proteins (56–60) has led to its extensive use as a model for studying how other polyelectrolytes present within the cell, such as mRNA (61), might similarly affect amyloid fibril formation.

Nevertheless, the kinetic role of heparin in inducing the aggregation of Tau is not fully understood partly because there is still debate about whether polyanion-induced Tau aggregation into PHFs occurs by a nucleation-dependent polymerization (NDP) mechanism or not. An early pioneering study (20) utilizing artificially created Tau dimers suggested that an NDP mechanism operates and that heparin plays a role in the activation of Tau molecules leading to the formation of nucleation-competent oligomeric subunits. Similarly, studies with other inducers have also suggested an NDP mechanism for Tau aggregation (28, 29, 62). But a more recent study has suggested that polyanion-induced (including heparin-induced) Tau aggregation occurs not through a NDP mechanism but through essentially a downhill polymerization mechanism (30) in which heparin allosterically regulates conformational change into aggregation-competent subunits. Clearly, fundamental aspects of the heparin-induced aggregation reaction remain unresolved.

In this study the kinetics of amyloid fibril formation by the four repeat domain of Tau (Tau4RD) has been investigated in the presence of heparin at pH 7 and at 37 °C using thioflavin T (ThT) fluorescence as the probe. The kinetics is highly reproducible in the buffer conditions chosen, and AFM and TEM studies show that paired helical filaments as well as straight filaments are formed. In the minimal kinetic model proposed, which quantitatively describes all aspects of the aggregation reaction, heparin participates in the rate-limiting step of aggregation, with each heparin molecule binding sequentially to two Tau4RD molecules, leading to the formation of an aggregation-competent Tau dimer. Subsequent growth of fibrils appears to occur by stepwise addition of monomer protein. The minimal model quantitatively explains all aspects of the unusual dependence of aggregation kinetics on protein and heparin concentrations. Very importantly, this study also identifies for the first time rod-like protofibrillar forms around ~50 nm in length and ~2.6 nm in diameter, as measured by AFM, that are populated transiently during the aggregation reaction.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Characterization**—The pET22b plasmid containing the Tau4RD gene (pETTau4RPH) was a kind gift from Prof. Takashi Konno (63). The protein is 144 amino acids long, spans the four repeats (Gln-244—Phe-378) of the longest isoform hTau40, and has a His tag at the C terminus and an isoelectric point of 9.68. The full amino acid sequence of the protein is as follows: MQTAIVPMPLKNVKSXIGSTNELKHKPQGKGKVQINNKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVLSKTVSKGSLGNIHHKPGGGQVVEKSKLDKDRVQSKIGSLDHTHVPGGGKNIET-HKLTFLHHHHHH. It is to be noted that His-tagged constructs of the protein have also been used in previous studies of the aggregation of the repeat domain as well as full-length Tau (33, 34, 63) and in a previous comparison of the aggregation of Tau constructs with and without a His tag, when induced by arachidonic acid, it was reported that the aggregation mechanism is not affected by the presence of the His tag (33).

The protocol that was standardized for Tau4RD purification is a slight modification of published protocols (64, 65) and makes use of cation-exchange chromatography followed by size-exclusion chromatography. The purity of the protein was confirmed by SDS-PAGE and electrospray ionization-MS. The mass of the protein was the expected 15,607 Da when determined using a Waters Q-TOF Ultima mass spectrometer.

Absorbance, fluorescence, and CD spectra were acquired to confirm spectroscopic signatures of the purified protein. The molar extinction coefficient of the protein at 280 nm was determined by the BCA assay (kit from Thermo Scientific) using bovine serum albumin as a standard and was found to be 1221 M⁻¹ cm⁻¹. A dynamic light scattering experiment was performed to check that the purified protein was monomeric with the expected hydrodynamic radius (R_h) (40). The activity of the protein was determined using a tubulin polymerization assay monitored by turbidity measurements at 340 nm and at 37 °C as described earlier (4, 66). The activity was found to be reproducible across different protein preparations and better than that reported in the literature for proteins purified using a boiling lysis method (4).

**Buffers, Solutions, and Experimental Conditions**—All reagents used for experiments were of the highest purity grade available from Sigma unless otherwise specified. For the studies
Heparin-induced Aggregation of Tau Follows an NDP Mechanism

involving aggregation in Tris buffer at pH 7, the protein stored in 25 mM Tris buffer, pH 7, was diluted into the aggregation buffer (25 mM Tris buffer, 1 mM DTT, pH 7) to the desired concentration. The desired concentration of NaCl (0–150 mM) was added to the aggregation buffer from a concentrated stock solution (2 M NaCl). For the experiment performed in Tris buffer at pH 7.5, a desired volume of a 1 M Tris buffer, pH 8.0, stock was added to the aggregation buffer described above, such that the final pH was 7.5, whereas the buffer strength remained unchanged. After dilution into the aggregation buffer, the protein was incubated for 2 h at 37 °C to allow for the reduction of any covalent Tau dimers to monomers. The reaction was then induced by the addition of heparin (M₄, 12,000, Himedia Laboratories) of the desired concentration. The final pH remained unchanged, and the aggregation reaction continued to be maintained at 37 °C. The same heating block was used for all the experiments to reduce variability. Aggregation was also carried out in PBS buffer (pH 7.4) and this is described in the supplemental methods.

Typically, the volume of the aggregating protein solution was 1.5 ml. At different time points of aggregation, 5–100-μl aliquots of the protein sample were withdrawn, depending on the protein concentration being studied, for analysis by ThT fluorescence and AFM. Before removing a sample aliquot, the aggregating mixture was mixed three times with a P200 pipette. Great care was taken to ensure that there was no variation in the mixing technique every time an aliquot had to be removed so that the kinetics were fully reproducible, as the addition of heparin to the protein resulted in immediate turbidity and the formation of insoluble material that settled at the bottom of the tubes.

ThT Fluorescence Assay—The ThT assay was performed at pH 7 in 25 mM Tris buffer for the Tris aggregation reactions and at pH 7.4 in PBS for the PBS aggregation reactions. For the assay, a final concentration of 1 μM protein and 10 μM ThT was used. For every reading a calculated amount of the protein according to the concentration used in the aggregation experiment was withdrawn from the sample and added to the ThT assay solution. Measurements were made using a Fluoromax-3 spectrofluorimeter (Jobin Yvon) as described earlier (67).

Sedimentation Assay—The extent of amyloid fibril formation at the end of the aggregation reaction at a time corresponding to five time constants of the ThT fluorescence-monitored kinetics was determined by centrifugation of the solution at 20,000 × g for 45 min upon which the fibrils formed were found to have sedimented down. The sediment was insoluble in water but soluble in 6 M guanidine HCl. The amount of protein present in the sediment was determined by measurement of the Tyr fluorescence at 303 nm of sediment dissolved in 6 M guanidine HCl. To determine equilibrium monomer concentrations, the supernatant was passed through an YM100 Centricon (Millipore) to remove residual, soluble fibrils, and the Tyr fluorescence of the monomeric filtrate was measured.

Atomic Force Microscopy (AFM)—AFM samples of fibrils and protofibrils were made at specified times of aggregation and for fibrils, also at times corresponding to five time constants (5τ) of the ThT fluorescence-monitored kinetics.

For preparing samples, a 100-μl neat aliquot of the sample was withdrawn at the designated time, applied onto freshly cleaved mica (Grade V1, SPI Supplies), and allowed to incubate for 2 min in the case of fibril samples and 5 min in the case of protofibril samples. The mica surface was then rinsed with 25 drops of filtered MilliQ water and dried under vacuum for 1 h. AFM images were obtained on a PicoPlus AFM instrument (Molecular Imaging, Inc.) operating in the non-contact mode using 75 kHz, 2.8 newton/m cantilevers with a rounding tip radius of <10 nm (Nanoworld AG). Details of acquisition parameters are described in the supplemental methods.

The diameters of the fibrils and protofibrils were determined by measuring the Z-heights of the structures in the AFM images (assuming them to be spherical or cylindrical) using the profile option of the program WSxM (68). Details of the analysis are described in the supplemental methods.

TEM—A protein solution diluted to 5 μM was placed on a freshly glow-discharged, 400 mesh carbon-coated copper grid (Ted Pella) for 1 min, washed twice with filtered MilliQ water for 45 s, and then stained with 2% uranyl acetate for 30 s. After air-drying, the grids were examined on a Tecnai G² 12 BioTwin electron microscope (FEI Co.) operating at 100 kV and equipped with a Gatan side mount 4k camera. Images were analyzed using the Gatan Digital Image micrograph software (Version 1.8).

Data Analysis—For the aggregation reactions in Tris buffer, the kinetic curves measured by ThT fluorescence were fitted to the single exponential equation,

\[ S = S_0 + a [1 - e^{-t/\tau}] \]  

(Eq. 1)

where \( S_0 \) is the signal at \( t = 0 \), \( t \) is the time, \( a \) is the amplitude of the signal change, and \( \tau \) represents the time constant of aggregation.

RESULTS

Formation of Amyloid Fibrils by Tau in the Presence of Heparin—In this study amyloid fibril formation by the four repeat domain of Tau (Tau4RD) in the presence of heparin (M₄, 12,000) has been studied in 25 mM Tris buffer at pH 7. Evidence for the formation of fibrils is provided by ThT binding (Fig. 1a, inset) and both in the absence of NaCl and in the presence of 25–150 mM NaCl (data not shown). Importantly, the kinetics of aggregation in Tris buffer appears to follow single-exponential kinetics with no lag phase at pH 7 (Fig. 1a) as well as at pH 7.5 (Fig. 1a, inset) and both in the absence of NaCl and in the presence of 25–150 mM NaCl (data not shown). The high reproducibility of the data is because of the care taken to ensure that the starting preparation was free of seeds and was as homogeneous as possible. Before the addition of heparin, Tau4RD exists as a monomer with the expected (6, 40) hydrodynamic radius (Rₑ) of ~3 nm, as determined by dynamic light scattering (data not shown), and exists as a random coil as evident from its CD spectrum (data not shown). The high reproducibility of the data in Tris buffer made it possible to carry out a rigorous study of the kinetics of fibril formation in that buffer.
In contrast, the aggregation kinetics in PBS buffer at pH 7.4 was found to be sigmoidal in nature in accordance with previous observations (32), displayed a final amplitude of ThT fluorescence at saturation that was almost 10-fold lower than that seen for aggregation in the Tris buffer, and was found to be not reproducible in terms of displaying a variable amplitude across protein preparations (supplemental Fig. 1a). Interestingly, the fibrils formed in the PBS and Tris buffers appear to have similar external morphologies as seen in AFM even though they appear to have different internal structures as monitored by FTIR spectroscopy (supplemental text and Fig. 2). In the future it will be important to determine by using high resolution probes such as hydrogen exchange NMR and solid-state NMR (69, 70) whether the fibrils formed in the two buffers do indeed differ in their internal structures (71).

In this study aggregation kinetics were studied over a range of protein and heparin concentrations using ThT fluorescence as the quantitative measure of the formation of structured, amyloid aggregates (72–76). The extent of fibril formation at any protein concentration was found to be the same whether monitored by ThT fluorescence, light scattering at 800 nm, or by direct measurement of the amount of fibrils formed using a sedimentation assay (Fig. 1b).

The Amyloid Fibrils Formed Include Characteristic PHFs—Because this is the first study of Tau aggregation in Tris pH 7 buffer, it was important to verify that the fibrils formed have the morphologies expected for Tau fibrils. AFM images show two kinds of structures, a twisted ribbon-like fibril previously described as a PHF and a flat ribbon-like fibril previously described as a straight filament. The PHFs appear to have a cross-over repeat of 80–100 nm and a maximal height of 10 nm, in accordance with previous observations (77). As also observed earlier (77, 78), two types of PHFs are seen; one in which two filaments appear to be wound around each other (Fig. 2a) and another that appears to be a single twisted ribbon (Fig. 2b). Other kinds of fibril morphologies are also seen (79). The twisted ribbon in Fig. 2c has a height of ~6 nm and a cross-over repeat of ~80–100 nm, whereas in the same field of view, a straight filament with a height of ~5 nm is also seen (Fig. 2c, green arrow).

The straight filaments and PHFs are also seen in a negatively stained TEM image of the fibrils (Fig. 2d). The average width of the straight filaments calculated from the TEM image (~10 nm) matches the average height of fibrils calculated from the AFM images, whereas the PHFs appear to have widths alternat-
Heparin-induced Aggregation of Tau Follows an NDP Mechanism

**FIGURE 3.** Formation of protofibrils and fibrils by 50 μM Tau4RD in the presence of 37.5 μM heparin in Tris buffer, pH 7, at 37 °C. **a,** an AFM image demonstrates the presence of short, rod-like protofibrils at 1 h of aggregation. The inset shows the beaded appearance of the protofibrils. The scale bar for the image corresponds to 600 nm, whereas that for the inset corresponds to 200 nm. **b,** distribution of heights of protofibrils is shown. The mean height calculated from the fit is 2.6 ± 0.5 nm. The inset shows the length distribution of the protofibrils is shown. The mean length calculated from the fit is 51 ± 16 nm. The solid line in both the image and the inset represents a fit to a Gaussian equation. **c,** an AFM image demonstrates the presence of fibrils at 1 h of aggregation. The scale bar for the image corresponds to 600 nm. **d,** distribution of heights of fibrils is shown. The mean height calculated from the fit is 10.2 ± 3.9 nm. The solid line represents a fit to a Gaussian equation.

**FIGURE 4.** The appearance and disappearance of protofibrils when 50 μM Tau4RD is aggregated in the presence of 37.5 μM heparin in Tris buffer, pH 7, at 37 °C. **a–c,** AFM images of protofibrils at 20 min, 40 min, and 1 h of aggregation are shown. **d,** an AFM image demonstrates the absence of protofibrils at 2 h of aggregation. The inset is an image demonstrating the presence of fibrils at the same time point. The scale bar in all images as well as the inset corresponds to 600 nm. The Z scale for the main images corresponds to 8 nm, and the color scale applies to the main images. The Z scale for the inset corresponds to 50 nm.

When AFM images are acquired at very early times of aggregation (<20 min), large non-fibrillar aggregates are seen. These non-fibrillar aggregates sediment down upon centrifugation, as do the fibrillar aggregates. Unlike the fibrillar aggregates, the non-fibrillar aggregates appear to be water-soluble, and the relative fraction of sediment that is water-soluble decreases with time of aggregation (data not shown). Such nonspecific aggregates have also been found to accumulate transiently during the course of fibril formation by other proteins (80–82). The non-fibrillar aggregates are not seen in AFM images acquired at later times of aggregation (Figs. 2 and 3, supplemental Figs. 2–4). At the end of the fibril formation reaction, the sedimentation assay indicates that 95 ± 3% of the protein is present as water-insoluble, but denaturant-soluble aggregate and only fibrillar aggregates are observed by AFM (Fig. 2, supplemental Figs. 2–4). It, therefore, appears that the nonspecific aggregates formed at early times break down to monomer and transform into amyloid aggregates.

Protofibrils Are Transiently Populated during the Aggregation of Tau4RD in the Presence of Heparin—Fig. 3a demonstrates the existence of a distinct population of protofibrils cap-

ning between 9 and 16 nm, again with a cross-over repeat of ~80 nm (78).

At 1 h of aggregation, protofibrils and fibrils are seen in AFM images (Fig. 3); at 2 h, no protofibrils and only fibrils (Fig. 4d) are seen. Fibrils become longer from 1 h when protofibrils were last seen (Fig. 3c) to the end of the reaction (supplemental Figs. 2b and 3b; see the insets also), and this difference is visible by AFM. The height distribution of the few fibrils seen at the 1 h time point, when protofibrils are last seen, spans the entire range of heights seen at the end of the aggregation process (Fig. 3d and supplemental Fig. 3e). Thus, the fibrils do not signifi-
Heparin-induced Aggregation of Tau Follows an NDP Mechanism

Fibrillation of Tau4RD Is Sensitive to the Concentration of Heparin Present in the Buffer—The single exponential nature of the kinetics of aggregation is observed at all heparin concentrations (Fig. 5a). Heparin accelerates the apparent rate of fibril formation at low concentrations; this result is consistent with previous results (20, 30–32, 35, 36, 45, 47). Above ~25 μM heparin, the apparent rate decreases with a further increase in heparin concentration. Hence, the apparent rate of fibril formation displays a bell-shaped dependence on heparin concentration (Fig. 5b).

Interestingly, the amplitude of the change in ThT fluorescence displays a hyperbolic dependence on heparin concentration (Fig. 5c). Because the binding of heparin to Tau is tight, with a dissociation constant in the sub-micromolar region (44, 45), the hyperbolic dependence of the extent of fibril formation on heparin concentration (Fig. 5c) can be used to estimate reliably the stoichiometry of protein bound to heparin in the rate-limiting steps of aggregation; one heparin molecule appears to bind only 2–3 protein molecules.

Fibril Formation by Tau4RD in the Presence of Heparin Is Sensitive to Protein Concentration—The ThT fluorescence-monitored aggregation kinetics in the presence of 37.5 μM heparin are single-exponential at all protein concentrations (Fig. 6a), and the apparent rate constant shows a bell-shaped dependence on protein concentration (Figs. 6b and 5b) that peaks at a protein concentration of ~75–80 μM.

The amplitude of the change in the ThT signal has a linear dependence on protein concentration in the range of protein concentrations where the apparent rate constant increases and then decreases (Figs. 1b and 6c), but at very high protein concentrations the ThT fluorescence signal does reach a limiting value (Fig. 6c). The dissociation constant for Tau bound to heparin is less than 1 μM (44, 45). Hence, when the binding of protein to heparin, as monitored by the amplitude of ThT fluorescence change accompanying the aggregation reaction, is carried out at protein concentrations greatly exceeding the dissociation constant, the binding curve yields not the dissociation constant but only the stoichiometry of protein bound to heparin (83). Fig. 6c indicates, therefore, that only 1 heparin molecule is bound to 20 protein molecules when fibril formation is complete.

Fig. 6c also reveals that there exists a critical protein concentration below which Tau does not form fibrils. Linear regression analysis of the data in Fig. 6c provides a value of 5.0 ± 2.1 μM for the critical concentration; similar regression analysis of the rate constant dependence on Tau concentration (for the four lowest protein concentrations) (Fig. 6d) provides a value of 2.4 ± 0.5 μM. In confirmation of a critical concentration below which Tau does not aggregate, it was found that at protein concentrations of 2 and 3 μM, no increase in ThT fluorescence was seen even at 100 h, indicating that aggregation does not occur at these protein concentrations (data not shown). To further confirm the existence of a critical concentration, the solution at the end (five time constants) of the fibrillation reactions of 25, 50, and 100 μM protein was centrifuged at 20,000 × g for 45 min. It was found (data not shown, see “Experimental Procedures”), that the supernatant solutions in each case still contained 1 ± 0.5 μM protein, as expected if the critical concentration for aggregation is in the range of 1–3 μM.

Fibril Formation by Tau4RD in the Presence of Heparin Depends on the Stoichiometry of the Two Partners—The bell-shaped dependence of the fibrillation rate constants on both
Heparin and protein concentration are coincident when the rate constants are plotted against the molar ratio of protein to heparin, and the peak of this bell-shaped pattern lies at a ratio of $\frac{1}{10}$ (Fig. 7). When the molar ratio is kept fixed and the protein and heparin concentrations are varied accordingly, the rate remains the same, proving that the role of stoichiometry is not incidental but rather crucial to the aggregation process (data not shown).

**Kinetic Simulations**—The utility of kinetic simulations in determining protein aggregation mechanisms has been discussed (84, 85), and in this study kinetic simulations were carried out using the program Tenua, a variant of KINSIM (86), to determine the minimal model that describes all important features of the data. The model had to explain 1) the absence of a lag phase in the kinetics, 2) the bell-shaped dependence of the aggregation rate on heparin concentration, 3) the hyperbolic dependence of the extent of fibril formation on heparin concentration, 4) the bell-shaped dependence of the aggregation rate on protein concentration, 5) the linear dependence of the extent of fibril formation on protein concentration, and 6) the bell-shaped dependence of aggregation rate on the ratio of protein concentration to heparin concentration. Moreover the model needs to be consistent with the observation that nonspecific, non-fibrillar, and protofibrillar aggregates form transiently, early during aggregation.

![Graph 1](image1.png)

**FIGURE 6.** Dependence on protein concentration of ThT fluorescence-monitored kinetics in Tris buffer, pH 7. a, kinetics of fibril formation by 10 μM (○), 25 μM (●) and 50 μM Tau4RD (▲) in the presence of 37.5 μM heparin are shown. The continuous line through the data points represent least-squares fits to a single-exponential equation. a.u., arbitrary units. b, the apparent rate constant of ThT-monitored kinetics is plotted against protein concentration. The continuous line through the data points was drawn by inspection. c, the amplitude of the change in ThT fluorescence when protein is added to 5 μM heparin (●) and when protein is added to 37.5 μM heparin (□) as a function of the molar ratio of protein:heparin is shown. The straight line through the data points is a least-square fit. The continuous line through the data points was drawn by inspection to guide the eye. The error bars in a–c represent the spread in the data calculated from two or more independent experiments.

![Graph 2](image2.png)

**FIGURE 7.** Dependence of the apparent rate constant of ThT fluorescence-monitored kinetics on the molar ratio of protein to heparin. The apparent rate constant of fibril formation is plotted as a function of molar ratio from aggregation experiments wherein the protein concentration is held constant and the heparin concentration is varied (white symbols) and wherein the heparin concentration is held constant and the protein concentration is varied (black symbols).

Heparin-induced Aggregation of Tau Follows an NDI Mechanism
forming off-pathway to the main aggregation pathway. This off-pathway step accounts for the observation that fibril formation is slowed down at high protein concentrations.

Kinetic simulations (supplemental Figs. 6 and 7) show that this model satisfactorily explains all aspects of the experimental data described above. An important assumption of the simulations was that P in PH as well as in PHP is as capable of binding ThT as is P in the fibrils, which is consistent with previous results (87) that had indicated that initial monomeric intermediates formed during aggregation can bind ThT(S). It should be noted that the simulation has been carried out with the minimum number of aggregate growth steps necessary to account for the basic features of the data.

DISCUSSION

Mechanism of Heparin-induced Amyloid Fibril Formation by Tau—Heparin has been used extensively as an inducer in studies of Tau aggregation (16, 17, 20, 23–25, 30–32, 35–47), but only a few quantitative models (20, 30, 45) have been proposed to describe important aspects of amyloid fibril formation. However, even in these elegant models, the kinetic role of heparin either in activation, in nucleation, or in allosteric regulation was not quantitatively described as was not the unusual dependence of aggregation kinetics on both protein and inducer concentrations (20, 30, 45). An explicit description of the Tau fibrillation mechanism as NDP has been made only in studies that have examined the effect of inducers other than heparin (28, 29, 87) or used phosphorylated or truncated protein (88, 89), but even in these studies the unusual dependencies of aggregation kinetics on both protein and inducer concentrations were not quantitatively described.

Uncertainty about whether heparin-induced Tau aggregation follows a NDP mechanism had arisen because a recent study suggested that it follows instead an isodesmic (downhill) mechanism (30). However, in this as well as in other previous studies (20, 28, 29, 45, 62), both a dependence of aggregation rates on protein concentration and a critical concentration \( \left[ \text{H} \right] = 1–3 \text{ M} \) are observed (see “Results”), as expected for a NDP mechanism but not for an isodesmic mechanism (84, 90, 91). The latter also seems unlikely because the morphology of Tau fibrils (Figs. 2 and 3 and supplemental Figs. 2–4) is not consistent with that of an isodesmic polymer, and moreover, progressively larger, small pre-fibrillar oligomers are not observed.

The minimal model shown in Fig. 8 is based on a ligand-induced NDP mechanism. The steps leading to the formation of a dimer are the slowest steps, and hence, the heparin-bound dimer functions as would a nucleus in a NDP mechanism. No lag phase in the aggregation kinetics is observable only because of the specific values of the rate constants defining the two nucleation steps (supplemental Fig. 6) in the Tris buffer system utilized in this study. Sigmaoidal kinetics, typical of NDP (92, 93), are, however, seen in PBS buffer (supplemental Fig. 1a). Importantly, fibrils formed in the Tris buffer can function as seeds to eliminate the initial lag phase otherwise observed during aggregation in PBS buffer (data not shown).

The Aggregation Model Is Based on a NDP Mechanism—The mechanism of heparin-induced Tau aggregation has been conclusively described as NDP by only one previous study that made use of artificially created covalent Tau dimers (20). Other kinetic studies of heparin-induced Tau aggregation have data that are consistent with a NDP mechanism, but these data were not fitted to quantitative models of NDP (32, 45). An explicit description of the Tau fibrillation mechanism as NDP has been made only in studies that have examined the effect of inducers other than heparin (28, 29, 87) or used phosphorylated or truncated protein (88, 89), but even in these studies the unusual dependencies of aggregation kinetics on both protein and inducer concentrations were not quantitatively described.

The Minimal Model Describes the Dependence of the Aggregation Kinetics on Heparin Concentration—The observation that the ThT fluorescence saturates at a heparin concentration...
Heparin-induced Aggregation of Tau Follows an NDP Mechanism

approximately half that of the protein present (Fig. 5c) forms the basis of the model (Fig. 8) that a complex of one heparin molecule with two bound Tau molecules forms in the rate-limiting steps of aggregation. Heparin (H) brings two positively charged monomeric protein (P) molecules together, thus enabling them to interact and undergo the conformational change necessary to form the aggregation nucleus (PHP). Indeed, heparin has been shown to induce conformational changes in Tau (44, 46), and a role for heparin in regulating the conformation of Tau has been proposed earlier (30). It has also been postulated that heparin functions by binding sites of residual β structure and facilitating β-strand interactions across different Tau molecules (38). Indeed, two such hexapeptide motifs have been demonstrated to be minimal Tau-Tau interaction motifs that form PHFs in vitro (39).

An earlier study of the induction of Tau aggregation by the small molecule thiazine red had indicated that the rate-limiting step in fibril formation is dimerization (62), but in that study the aggregation rate was found to not depend on the stoichiometry of protein to inducer as it does, characteristically, for heparin-induced aggregation (31, 62). This is the first study to demonstrate, quantitatively, the kinetic role of an aggregation-competent non-covalent dimer in the mechanism of heparin-induced aggregation. Previous studies (20, 32, 47) of the aggregation of a covalently linked dimer of Tau had suggested, qualitatively, that covalent dimerization is important because of its effect in increasing the apparent rate of aggregation.

Thus, the minimal model (Fig. 8) is consistent with the observation that the apparent rate constant of fibril formation reaches its maximum value when the heparin concentration is half the protein concentration (Figs. 5 and 7). All protein molecules are then tightly bound to heparin molecules in PHP complexes, the concentration of PHP is at its maximum value, and the apparent rate of aggregation is at its maximum value. At higher concentrations of heparin, the apparent rate constant of fibril formation decreases because the PHP concentration begins to decrease from its maximum value when the free heparin concentration becomes much larger than the equilibrium constant for PH dissociation. A similar effect of polyanions has been observed on the folding of the dimeric Arc repressor (94).

Other explanations for the inhibitory action of heparin at high concentrations have been proposed. The explanation based on the existence of a low affinity binding site for heparin, binding to which at high heparin concentrations would reduce the concentration of the aggregation-competent PHP molecules, is plausible (31), especially as it is known that Tau has multiple heparin binding sites (44), but it requires a more complex model than the minimal model shown in Fig. 8. The explanation based on heparin exerting a Debye screening effect (45) is unlikely because even at the highest heparin concentration used in this study (75 μM), its contribution to the ionic strength would not exceed 7 mM, whereas the ionic strength effect on the apparent rate of fibril formation is seen at significantly higher NaCl concentrations (supplemental Fig. 1b).

The Minimal Model Describes the Dependence of the Aggregation Kinetics on Protein Concentration—The observation made in this study that the apparent rate constant for fibril formation increases with protein concentration and reaches its maximum value when the concentration of protein is twice that of the heparin present (Figs. 6 and 7) is accounted for by the minimal model (Fig. 8). Several previous studies had examined the effect of varying protein concentration and the effect of the molar ratio of Tau:heparin on Tau polymerization (20, 30, 31, 45). Although concluding that the stoichiometry of the two partners influences fibrillation, they could not explain the mechanistic basis for this dependence.

To account for the observation that the apparent rate of fibril formation then reduces with a further increase in protein concentration, the minimal model includes an additional process that is off-pathway to the pathway for the formation of the aggregation-competent dimer. In this off-pathway process, heparin binds to a tight binding site on each Tau molecule, much more tightly than it does to the site that leads to the formation of PH and PHP on the main aggregation pathway (Fig. 8 and supplemental Fig. 6). Binding of heparin to this tight binding site leads to the formation of the complex P*H, whose conformation is such that it is incapable of participating in the fibril formation reaction. This study, therefore, provides direct kinetic evidence in support of the insightful suggestion made several years ago that assembly-incompetent complexes of Tau and polyanion are also likely to form during the course of the aggregation reaction (20, 31).

An alternative off-pathway process that would also result in fibril formation becoming slower at high protein concentrations is one that would lead to the formation of an aggregation-incompetent dimer, PP, in a process that is not heparin-dependent and that is off the pathway for heparin-facilitated dimer formation. The binding constant for the formation of PP would have to be weak, so that it would form significantly only at high protein concentrations, thereby slowing down fibril formation. Indeed, it has been reported that Tau is capable of forming non-covalent dimers (32).

The Minimal Model and Fibril Growth—In the minimal model (Fig. 8), fibril growth occurs by monomer addition to the aggregation-competent dimer, and heparin is not assigned any role in fibril elongation. On the other hand, if elongation were to occur by linear addition of either PHP or PH, then the observations here (see below) and elsewhere (30, 43) that heparin is not an integral part of the fibril structure would require that heparin dissociate from the growing fibril once its role in elongation is over. This would happen, for example, if growth of the fibril is accompanied by conformational changes in the assembled Tau molecules, which lead to the dissociation of heparin from the fibrils. Such a possible role of heparin in fibril elongation cannot be ruled out in this study because it would be after the rate-limiting step.

There has been some debate about whether heparin is a major constituent of fibrils (30, 43, 44). The observation made in this study that the final fibrils contain less than one heparin molecule for every 20 protein molecules (see “Results”) is the strongest evidence so far that heparin is only a minor constituent of fibrils. Indeed, an earlier study using radio-labeled heparin had also estimated that fibrils contained less than 1 heparin molecule for every 20 protein molecules (30). Not surprisingly, then, neither the height distributions of fibrils, as determined by AFM (supplemental Figs. 3 and 4), nor their internal struc-
uences, as determined by FTIR spectroscopy (supplemental Fig. 5), are affected by the stoichiometry of protein to polyanion present during aggregation.

Protophilbils Are Transiently Populated during the Aggregation Process—A significant result of this study is the identification of short canonical protophilsibs, ~2.6 nm thick and ~50 nm long, that are very transiently populated during the course of amyloid fibril formation (Figs. 3 and 4). They are beaded structures (Fig. 3a, inset), and in that sense, they are similar to the linear arrays of spherical nucleation units seen earlier (25). The rod-like protophilsibs are, however, distinct from not only the spherical nucleation units and the granular Tau oligomers (23), both of which are large (>20 nm) structures formed by full-length recombinant hTau40 as fibrillation intermediates, but also from the soluble, heterogeneous oligomeric population formed by a C-terminal fragment of human Tau (24).

The observation that the rate of growth of fibrillar material as measured by ThT fluorescence (Fig. 1a) as well as the rate of increase in fibril length as observed by AFM (Fig. 3c and supplemental Figs. 2b and 3b; see insets also) is much slower than the rate of disappearance of the protophilsibs (Fig. 4) suggests that the protophilsibs are not direct precursors to fibril formation. Instead they accumulate off the main pathway of fibril formation but too transiently for either the extent or rate of their formation to be determined. The surprisingly fast rate at which they disappear suggests that they are unstable and, hence, are populated to a small extent. The minimal model (Fig. 8) can suggest how they may form in a process that is off the main pathway for fibril formation. The protophilsibs could form rapidly and reversibly from $\text{P}^\text{H}$ (Fig. 8, supplemental Fig. 6), which is formed much more rapidly than is $\text{PH}$ on the main aggregation pathway. As $\text{PH}$ undergoes further transformation into $\text{P}^\text{H}$ and then into larger aggregates, thermodynamic linkage (95) leads to, at first, a reduction in the concentration of $\text{R}$, which is then replenished at the cost of a reduction in the concentration of $\text{PH}$. Ultimately, linkage would lead to the dissociation of the protophilsibs into $\text{P}^\text{H}$, whose rate appears to be rapid.

To demonstrate that the rod-like protophilsibs also form in vivo is an important goal of future studies, made difficult both by their small (suboptical resolution) size and by their ephemeral existence. Nevertheless, their identification in vitro suggests new ways in which Tau might behave in neurons in the normal state or when the normal state is perturbed. Indeed, the discovery that Tau can form protophilsibs under near-physiological conditions in vitro highlights the importance of in vitro studies. Because in vivo conditions may be very different in the normal and diseased state, in vitro studies can suggest ways in which the biochemical system may adapt upon such change.

Physiological Relevance of This Study—This study is important from two perspectives. First, the quantitative demonstration that the glycosaminoglycan heparin plays a role in only the nucleation steps and not the elongation steps of Tau aggregation in vitro implies that very small amounts of heparin would be capable of triggering Tau fibrillation in vivo. This is important from the perspective of understanding tauopathies because, although glycosaminoglycans have been detected in NFTs in various tauopathies (48–51, 96) and have been detected in nerve cells at early stages of neurodegeneration (8, 35, 97), their role in Tau aggregation and disease progression has eluded proper understanding. Second and importantly, the observation made here for the first time that Tau can form protophilsibs similar in morphology to those formed by other proteins (98–100) has important implications for the control of disease progression in tauopathies. If Tau protophilsibs also turn out to be toxic like other protophilsibs (21, 101), then their identification in this study as off-pathway species has important implications for the development of therapies based on driving NFT formation as a means to remove the soluble, toxic protophilsibs from the cytoplasm. The identification of putatively toxic Tau protophilsibs is especially important in the context of in vivo studies that have indicated that cognitive deficits and cell death are unrelated to NFT accumulation (102–104) and that Tau oligomers, and not fibrils, impair memory and induce synaptic dysfunction (105). In future studies it will be important to study the toxicity of the protophilsibs and whether compounds that inhibit Tau fibril formation (106, 107) also affect the extent of protophilsib formation.

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Heparin-induced Aggregation of Tau Follows an NDP Mechanism

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