

Microsecond Dynamics During the Binding-induced Folding of an Intrinsically Disordered Protein

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Abstract

Tau is an intrinsically disordered protein implicated in many neurodegenerative diseases. The repeat domain fragment of tau, tau-K18, is known to undergo a disorder to order transition in the presence of lipid micelles and vesicles, in which helices form in each of the repeat domains. Here, the mechanism of helical structure formation, induced by a phospholipid mimetic, sodium dodecyl sulfate (SDS) at sub-micellar concentrations, has been studied using multiple biophysical probes. A study of the conformational dynamics of the disordered state, using photoinduced electron transfer coupled to fluorescence correlation spectroscopy (PET-FCS) has indicated the presence of an intermediate state, I, in equilibrium with the unfolded state, U. The cooperative binding of the ligand (L), SDS, to I has been shown to induce the formation of a compact, helical intermediate (IL₅) within the dead time (~37 μ s) of a continuous flow mixer. Quantitative analysis of the PET-FCS data and the ensemble microsecond kinetic data, suggests that the mechanism of induction of helical structure can be described by a U \leftrightarrow I \leftrightarrow IL₅ \leftrightarrow FL₅ mechanism, in which the final helical state, FL₅, forms from IL₅ with a time constant of 50–200 μ s. Finally, it has been shown that the helical conformation is an aggregation-competent state that can directly form amyloid fibrils.

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Introduction

Intrinsically disordered proteins (IDPs) constitute a significant fraction of the human genome.¹ Even though they are by themselves devoid of any three-dimensional structure, IDPs have survived the course of evolution, which attests to their importance in cellular processes. IDPs invariably acquire structure in the presence of binding partners,^{2–11} although they may also form fuzzy protein complexes.^{12,13} Thus, the function of an IDP appears to be linked to structure acquisition induced upon ligand binding. The fact that IDPs can acquire structure when bound to a ligand not only suggests that they have functional roles, but also suggests a mode of regulation of their function. Tau is an IDP which binds to the microtubule assembly, and is involved in modulating microtubule growth in axonal cells.^{14,15} The protein has been well studied also because of its link with neurodegenerative diseases including Alzheimer's disease¹⁶ and various tauopathies.¹⁷ The primary sequence of tau can be divided into a N- terminal domain and a C-terminal domain (Figure 1(a)). The N-terminal domain consists of a projection domain that protrudes from the microtubule in the bound form, and a Pro-rich region that enhances microtubule binding.¹⁸ The C-terminal domain consists of four repeat regions (Figure 1(a)) which are directly involved in binding to microtubules, and which also form the structural core of the tau fibrils associated with various tauopathies.^{19,20} Repeat 2



Figure 1. (a) Cartoon representation of full-length human tau protein showing different domains. (b) Helix forming regions of tau-K18. The sequence of tau-K18 showing the four repeats. The helix forming residues in the four repeats, are highlighted in red. The two Cys residues are shown in green, Ser 316 (R3), Ile 328 (R3) and Ile 354 (R4) are shown in blue. Far UV-CD spectra of tau-K18 (c) and hTau40 (d) in the absence (solid line) and presence (dashed line) of 400 μ M SDS. The insets show the change in the relative ellipticity at 222 nm, with SDS concentration. The solid lines through the data are fits to equation 1. (e) The equilibrium SDS binding-induced folding transitions of hTau40 (•) and wt tau-K18 (•), monitored by far-UV CD at 222 nm, were converted into fraction bound (fb) and plotted against SDS concentration. The solid line through the data is a fit to equation 2. The data was obtained by equilibrium SDS binding-induced folding curves are shown in the insets. Each curve was repeated at least three times in independent experiments, to determine the standard deviations for the parameters obtained by the fits to equation 1 or 2.

(R2) is either present or absent in the protein, depending on how splicing of exon 10 of the tau gene has occurred.²¹ Mutations linked to various diseases are predominantly found in the Cterminal repeat domain²²; hence, the aggregation mechanisms of the repeat domain fragments of the protein, namely tau-K18 (R2 present) and tau-K19 (R2 absent) have been studied extensively.^{23,24}

Sodium dodecyl sulfate (SDS) is a phospholipid mimetic, and is often used to simulate the environment at the plasma membrane.²⁵ SDS is

generally used for effecting the unfolding of proteins, at concentrations above its critical micellar concentration (CMC).²⁶ It has been suggested that the binding of SDS micelles to a protein leads to repulsion between the anionic headgroup of SDS and anionic amino acid side-chains.²⁷ Nevertheless, it has been shown that positively charged, but not negatively charged, peptides can acquire helical structure in the presence of SDS.²⁸ It appeared therefore that the negatively charged head group of SDS bound strongly to positively charged amino acid residues (Lys and Arg) in the

peptide, and that the hydrophobic interactions of the fatty acyl chain with the peptide ultimately drove folding. SDS has also been shown to induce the folding of the α -synuclein, an IDP, wherein two helical forms were found to be populated at both micellar and sub-micellar SDS concentrations.^{2,} Both tau-K18 and tau-K19 were found to acquire helical structure in the presence of micelles formed by lipids and SDS.^{30,31} A study of this helical form by NMR revealed the regions which acquired helical structure (Figure 1(b)).^{30,31} Short, ordered helices were shown to form in each of the repeats at the microtubule binding site, but the mechanism of helix formation was not delineated. Mutations in the R2, repeat 3 (R3) and PHF6 region of tau-K18 have been shown to abolish the formation of complexes with phospholipids³²; signifying the importance of these sequence segments. Other IDPs, including $A\beta^{33}$ and amylin³⁴ are also known to acquire helical structure upon binding to SDS micelles.

In this study, the mechanism of acquisition of helical structure by tau-K18 upon binding SDS, has been studied using multiple probes. Characterization of the structural transition at equilibrium, using circular dichroism (CD) and fluorescence spectroscopy, has suggested that it is an all-or-none, two-state transition, in which unfolded protein binds to SDS in one step to form penta-liganded protein that has helical structure. Photoinduced electron transfer coupled to fluorescence correlation spectroscopy (PET-FCS) studies have shown, however, that in the absence of SDS, the native conformation exists in a very rapid (sub-microsecond) equilibrium with a compact intermediate conformation. Microsecond mixing kinetic experiments have revealed a compact helical intermediate on the way to the formation of the final helical conformation. Overall folding to the final helical conformation has been described quantitatively by a model in which the fully ligand-bound intermediate conformation is formed by a conformational selection (CS) mechanism. Finally, it has been shown that the SDS-bound, helical conformation can aggregate directly to form amyloid fibrils.

Results

Constructs used in the study. This study focuses on the mechanism of formation of a helical conformation of tau, induced by SDS binding. Tau-K18 is a fragment (residues from 244 to 372) of, tau, having the four microtubule binding repeat (MTBR) domains. It does not have any Trp residue. Hence, two mutant variants were generated in order to study the folding and dynamics of tau-K18 using fluorescence, FRET and PET-FCS as the probes. Ile 328 and Ile 354 were mutated to Trp and Cys, respectively (Figure 1(b)) in Cys354 tau-K18. Similarly, Ile 328 and Ser 316 were mutated to Trp and Cys, respectively (Figure 1(b)) in Cys316 tau-K18. Cys354 tau-K18 was used to monitor the structural transition across R3 and repeat 4 (R4), and Cys316 tau-K18 was used to monitor helix formation in R3 (Figure 1(b)) in FRET measurements. Both the mutant proteins were generated in the background of pseudo wt tau-K18, in which the two native Cys residues (Figure 1(b)) had been mutated to Ser.

SDS induces a structural transition to a helical state. Far-UV CD measurements showed that helix formation is induced in wt tau-K18 upon addition of sub-micellar concentrations of SDS (Figure 1(c)). It was important to check if SDS could also induce helical structure in the full-length variant of human tau, hTau40. hTau40 not only showed induction of helical structure (Figure 1(d)). but its equilibrium folding transition was coincident with that of wt tau-K18 (Figure 1(e)). This suggested that the helix forming regions in tau-K18 are likely to be identical to those in hTau40. and thus the mechanism of helical structure acquisition is likely to be the same in full length tau and its fragment. Since all previous work on the helical transition of tau had been carried out on tau-K18 and tau-K19,^{30,31} it was decided to use tau-K18 to elucidate the mechanism of helix formation. The equilibrium SDS-induced folding transitions shown in Figures 1(b)-(d), were found to fit well to an all-or-none folding transition (Scheme 1) in which the unstructured state (U) is in equilibrium with the bound, folded state, FL_n. The fit indicated a value of $(4 \pm 0.6) \times 10^{10} \,\mu\text{M}^5$ for K, and a value of 5 for n.

The helical transition of tau-K18 is associated with structural changes across R3 and R4, and within R3. The far-UV CD spectra of Cys354 and Cvs316 tau-K18 showed the induction of helical structure upon SDS binding (Figures 2(a), (b)), similar in extent to that seen in the case of wt tau-K18 (Figure 1(c)). The equilibrium SDS-induced structure formation transitions for both Cys354 and Cys316 tau-K18 were sigmoidal, when measured by far-UV CD (insets in Figures 2(a), (b)). The fluorescence intensity was found to increase, with a concomitant blue shift, in the fluorescence emission spectrum, upon addition of SDS in the case of both Cys354 and Cys316 tau-K18 (Figures 2(c), (d)). It should be noted that Nacetyl tryptophan amide, a Trp analogue, did not show a change in fluorescence, upon the addition of 360 µM SDS (data not shown), indicating that SDS had no effect on Trp fluorescence, at the concentrations used. This indicated that the environment around Trp 328 was indeed different in the helical conformation, from that in the fully disordered state. The transition between the disordered and helical states was fully reversible (Figure S1(a)).

FRET was used to get more insights into the structures of the conformations populated during



Figure 2. Characterization of DANS-labelled and unlabelled Cys354 and Cys316 tau-K18 in the absence and presence of SDS. Far-UV CD spectra of (a) Cys354 and (b) Cys316 tau-K18 in the absence (solid black line) and in the presence of 400 μ M SDS (dashed black line). The insets show the change in the far-UV CD signal at 222 nm, with increasing SDS concentration. Trp fluorescence emission spectra of labelled (purple) and unlabelled (green) Cys354 (c) and Cys316 (d) tau-K18 in the absence (solid line) and in the presence of 400 μ M SDS (dashed line), collected using an excitation wavelength of 295 nm. The insets show the change in the fluorescence signal at 330 nm. The data was obtained by equilibrating ~ 5 μ M of protein with the indicated SDS concentrations, for ~ 30 min. The solid lines through the data in the insets are fits to Eq. (1). For clarity, representative equilibrium SDS binding-induced folding curves are shown in the insets. Each curve was repeated at least three times in independent experiments, to determine the standard deviations for the parameters obtained by the fits to Eq. (1).

the course of folding. FRET pairs were generated to report on the structural changes between repeats R3 and R4, and within R3. Cys354 and Cys316 labelled independently tau-K18 were with IAEDANS, which acted as the FRET acceptor and Trp 328 acted as the donor. Figures 2(a) and (b) compare the fluorescence spectra of unlabelled and DANS-labelled Cys354 and Cys316 tau-K18. The fluorescence of Trp 328 in U, as well as in FL₅ was guenched in the DANS-labelled protein variants, while the wavelength of the maximum fluorescence emission remained unchanged. The insets in Figures 2(c) and (d) show that the equilibrium SDS-induced folding transitions of the unlabelled and labelled protein variants matched each other. The equilibrium folding transitions monitored by fluorescence emission at 330 nm and by far-UV CD, for the labelled and unlabelled protein variants, were found to be coincident with

those monitored by far-UV CD for both unlabelled proteins (Figure S1(b)).

Microsecond conformational dynamics occur in the disordered and helical state ensemble. In order to determine the mechanism of SDS-induced folding, it was first necessary to determine whether tau-K18 exists in only one conformation (the fully disordered U state) in the absence of SDS. To determine whether this is so indeed, or whether the U state exists in rapid equilibrium with one or more partially folded conformations (I), PET-FCS measurements were carried out. Such measurements report on intra-molecular dynamics which a single protein molecule undergoes while diffusing through a small confocal volume (1 fl), when such dynamics is faster than the diffusion time. A Trp residue will guench the fluorescence of a suitably placed oxazine dye adduct on the protein through PET, when a Trp-dye complex is formed transiently through microsecond fluctuations in the protein. Not only is the time scale of the dynamics measured, but so is the equilibrium constant between the unquenched and transiently quenched conformations.

For executing the PET-FCS experiment, Cys354 and Cys316 tau-K18 were labelled with an oxazine dye, Atto 655 maleimide. It was expected that if microsecond protein dynamics led to Trp 328 and Atto 655 coming into van der Waals contact (0.5 nm)³⁵ to form a transient complex, the fluorescence of the dye would be quenched because of PET.³⁶ The physical process of PET is very fast, having a rate constant in the picosecond time regime.³⁷ This implies that the quenching process will be limited by the rate constant of the formation of the complex.³⁸ Thus, PET-FCS allows the study of not only the diffusion process, but also of intra-molecular dynamics within the protein having time constants at least one order of magnitude faster than the diffusion time.

The autocorrelation functions (ACFs) obtained from the PET-FCS data for both the protein variants, Cvs354 and Cys316 tau-K18, showed two exponential components in addition to the diffusion component in the absence, as well as in the presence of 360 μ M SDS (Figures 3(a), (b), Eq. (8)). This suggested that the conformational fluctuations in both U and FL₅ led to the sampling of two dark states. PET-FCS was also carried out with the corresponding Trp-less variants, to determine whether the exponential components indeed originated from PET, or from any other photophysical process. The ACFs obtained from the corresponding Trp-less variants showed only the slow exponential component in addition to the diffusion component (Figures 3(a), (b), Eq. (9)), suggesting that this component arose from a fluctuation in the environment of the Atto moiety. The fast component was absent in the Trp-less variants, suggesting that it arose as a result of PET. For both Cys354 and Cys316 tau-K18, the constant of this fast conformational time fluctuation was found to increase in a sigmoidal manner with an increase in the concentration of SDS (Figure 3(c)). However, the amplitude had only marginal dependence SDS а on concentration (Figure 3(c), inset). The 200-400 ns fluctuation in U transitioned into an 800-1200 ns fluctuation in FL_5 in the presence of 360 μ M SDS. It seemed that similar fluctuations, between R3 and R4, and within R3 occur within the U state ensemble and in the structured FL₅, but are slowed down in the latter by structure acquisition. It is important to note that the sums of the amplitudes of the two exponential phases seen in the absence and in the presence of SDS, obtained from the PET-FCS measurements, were in very good agreement with those calculated from the emission spectra fluorescence (Table S1, Figure S2). Hence, the ensemble spectral

measurements validated the PET-FCS measurements, in the case of both U and FL₅.

The equilibrium SDS-induced folding data, both CD and fluorescence, suggested that 5 SDS molecules bound to each protein molecule, which should have increased the mass of each protein molecule by 1442 Da. It was therefore possible that the protein molecule would increase in size upon binding SDS, even though the acquisition of helical structure would tend to make it more compact. Analysis of the diffusion time obtained from the PET-FCS ACFs showed that the hydrodynamic radius, R_H, increased in a sigmoidal manner with an increase in SDS concentration (Figure 3(d)). The mid-point of the sigmoidal transition monitored by R_H was similar (~165 μ M) to that of the CD and fluorescence monitored transitions. Since a change in R_H is not proportional to the concentration of the SDSbound conformation, the R_H-monitored transition was not fitted to equation 1 describing an all-ornone folding transition. The observed increase in R_H upon SDS-induced folding also suggested that tau-K18 exists as a collapsed globule and not as a random coil in the absence of SDS. High-resolution probes such as NMR³⁹ and smFRET⁴⁰ have shown that under physiological buffer conditions, the properties of tau deviate from those of an ideal random-coil. Indeed, the R_H was also seen to increase upon the addition of 6 M urea (data not shown). It is possible that the increase in R_H with increasing SDS concentration might have arisen because of oligomerization of the protein, and not just an increase in size caused by the binding of SDS. This is, however, an unlikely possibility, as the midpoint of the R_H-monitored equilibrium folding transition, which was carried out at protein concentrations in range of 2 to 5 nM, matches those of the structural transitions monitored by far-UV CD and fluorescence, which were carried out at much higher protein concentrations in the range of 5 to 10 μM.

Inter-molecular FRET experiments were also carried out to check for the presence of oligomers at 10 µM protein concentration in the absence and in the presence of 360 µM SDS. Two mutant proteins were generated, in the background of pseudo-wt tau-K18, for this purpose. In one construct, Ile 328 was mutated to Trp (I328W tau-K18, donor only protein); in the other construct, Ile 354 was mutated to Cys (I354C tau-K18) and labelled with IAEDANS (acceptor only protein). For executing the experiment, 5 µM I328W tau-K18 and 5 µM DANS labelled I354C tau-K18 were mixed (Trp acted as the FRET donor and DANS as the acceptor), and the fluorescence emission spectra were acquired in the absence and in the presence of 360 µM SDS. The fluorescence emission spectra of 5 µM I328W tau-K18 and labelled I354C tau-K18 DANS were also independently acquired. The fluorescence signals



Figure 3. Microsecond dynamics in tau-K18 monitored by PET-FCS. The autocorrelation curves of Atto 655labelled (a) Cys354 and (b) Cys316 tau-K18 obtained in the absence and in the presence (insets) of 360 μ M SDS. The data was fit to an autocorrelation function having one diffusion component and two exponential components (Eq. (8)). The autocorrelation curves of the corresponding Trp-less proteins are shown in red and were fit to equation 9. (c) The dependences of the time constant of the fast phase Cys316 (\blacktriangle) and Cys354 (\bullet , inset) tau-K18 of the fast phase, as obtained from fitting the autocorrelation curves at different SDS concentrations on SDS concentration. (d) The R_H values for Cys354 (\blacktriangle) and Cys316 (\bullet) tau-K18 were calculated from the diffusion time and are plotted against SDS concentration. The error bars represent the standard deviations obtained from three independent experiments. The lines through the data in panels c and d are drawn by inspection only. The parameters obtained from data fitting are shown in Table 1.

from Trp and DANS in the donor only sample and acceptor only sample, respectively, were found to be identical to those of the mixed sample. This suggested that inter-molecular FRET did not occur in the mixed sample (Figure S3). It should, however, be noted that the value of R_0 , the Forster distance (Eq. (6)), for the Trp-DANS FRET pair is 20.9 Å.³⁷ Hence, this FRET pair is sensitive to changes in distance only in the range of 10.5 to 31.5 Å. If the distance between the donor and the acceptor in the oligomer were larger than 31.5 Å, no change in FRET efficiency would be observed. The absence of oligomers was confirmed by the observation that the absorbance spectra, of both Cys354 tau-K18 and Cys316 tau-K18, were identical in the presence and absence of 400 μ M SDS (Figure S4). The absence of any contribution from

scattering to the spectra collected in the presence of SDS, confirms the absence of oligomers.

Kinetics of the structural transition induced by SDS binding. The equilibrium fluorescencemonitored and far-UV CD-monitored SDS-induced folding transitions appeared to be all-or-none, suggesting that any intermediates present were too sparsely populated at equilibrium, to be detected. The PET-FCS experiments showed that, in the absence of SDS, the U state exists in rapid equilibrium with the intermediate, I, and suggested that I might have a productive role in the formation of the helical conformation in the presence of SDS. To detect such unstable intermediates in the folding reaction, kinetic experiments were carried out. The folding reaction induced by SDS binding was found to be too fast to be monitored by stopped flow mixing with a dead time of 2 ms, but could be monitored using a home-built CFM system with a dead time of 37 μ s.⁴¹

The kinetics of the folding of unlabelled and DANS-labelled Cys354 and Cys316 tau-K18, induced by SDS-binding, were monitored at different concentrations of SDS (Figures 4(a), (b)). The kinetic traces at each concentration of SDS were describable by a single exponential equation. The t= ∞ points of the kinetic folding traces fell on the equilibrium folding curve, indicating that the reaction had been monitored till completion (Figures 4(c), (d)). However, the extrapolated

fluorescence values at t = 0 were not those expected for the initial, disordered state, but were significantly higher (Figures 4(c), (d)). This indicated the transient population of a burst phase intermediate, IL_n which was distinct from both the U state and the final helical state (FL₅) with respect to its Trp fluorescence properties. The fluorescence values at t = 0 had a sigmoidal dependence on SDS concentration (Figures 4(c), (d)). This suggested the initial establishment of a pre-equilibrium between U and IL_n formed by the cooperative SDS binding-induced folding of U, before subsequent slower folding occurred to the



Figure 4. Kinetics of SDS-induced folding of tau-K18 as monitored by Trp fluorescence. SDS-induced folding was monitored by the change in intrinsic Trp fluorescence at 330 nm, upon excitation at 295 nm. Cys354 (a) and Cys316 (b) tau-K18 in native buffer were diluted into native buffer containing SDS, to a fixed concentration of 280 μ M. The kinetic traces of unlabelled and labelled protein are shown in green and purple, respectively. Each trace was normalized to a value of 1 for the fluorescence signal of the unlabelled protein in the absence of SDS. The solid lines through the data are fits to a single exponential equation. The solid and dashed horizontal lines indicate the fluorescence signals for protein in the absence of SDS and in the presence of SDS, respectively, where green lines represent the unlabelled protein. Panels c and d compare the kinetic amplitudes to the equilibrium amplitudes of folding of labelled and unlabelled Cys354 and Cys316 tau-K18, respectively. \circ , equilibrium folding curve; \blacktriangle , the t = 0 points; and \bullet , the t = 0 points; and \bullet , the t = ∞ points of the kinetic traces of folding induced by SDS for the labelled protein. The data has been normalized to a value of 1 for the fluorescence signal of the labelled protein. The data has been normalized to a value of 1 for the fluorescence by SDS for the labelled protein. The solid lines through the t = 0 points (\bigstar , \bigstar) are fits to equation 3.

final helical conformation. Hence, the $U \leftrightarrow IL_n$ transition was analysed according to a cooperative binding-induced folding mechanism. It was possible for folding from U to ILn to proceed through both the CS and IF pathways, and both the pathways would normally need to be taken into consideration while fitting the data. However, only the CS mechanism was considered because the PET-FCS experiments had indicated that a minor intermediate conformation, I, exists in equilibrium with U in the absence of SDS. Moreover, the equilibrium constant for the $U \leftrightarrow I$ step obtained from fitting the kinetic data to Scheme 2 (Eq. (3)), using this assumption, matched the value obtained from the PET-FCS experiment. The value obtained for n from the fit to equation 3, was 5, indicating that SDS binding occurred only during the formation of IL₅ and not later.

Model for SDS binding-induced folding of tau-K18. The rate constant of the observable phase in the sub-millisecond time domain was found to increase non-linearly with SDS concentration, and saturate at high SDS concentration (Figure 5(a)). This indicated that a concentration-independent conformational transition step ($IL_5 \leftrightarrow FL_5$) followed the concentration-dependent binding step. In summary, the model, deduced from the kinetic data, describes a cooperative binding-induced folding step which is complete within the dead time, and which leads to the formation of IL_5 . This is followed by a conformational transition ($IL_5 \leftrightarrow FL_5$) in the sub-millisecond time domain (Figure 6). It was also important for the equilibrium data to be explainable by the same model. Indeed, the product of the equilibrium constants of the individual steps on the pathway, $U \leftrightarrow I \leftrightarrow IL_5 \leftrightarrow FL_5$, which were obtained from the kinetic data, matched with the equilibrium constant of the overall $U \leftrightarrow FL_5$ transition, obtained from fitting the data in Figure 1 (see Data analysis).

FRET reports on the structure of the intermediate. Figure 5(b) compares the average FRET efficiencies of IL_5 and FL_5 at a high (saturating) SDS concentration, for both Cys354 and Cys316 tau-K18. In the case of Cys354 tau-K18 (Figure 5(b)), the FRET efficiency of IL_5 was more than that of FL_5 indicating that the relative distance between R3 and R4 was more in FL_5 than in IL_5 . The FRET efficiency in IL_5 of Cys316 tau-K18, which monitors helix formation in R3, was only marginally more than that of the final state, indicating that IL_5 and FL_5 had very similar helix content in R3 (Figure 5(b)).

The FRET efficiency depends on both the distance separating donor and acceptor, R, and the Forster distance, R_0 (Eq. (6)). R_0 depends on several terms (Eq. (7)). Of these terms, the quantum yield of Trp fluorescence is higher is the folded form. The overlap integral, will also be different in the folded form because of the blue shift in the Trp fluorescence spectrum. The refractive index of the medium remains unperturbed. The value of κ^2 is assumed to be 2/3 in both the unfolded and folded states, since both states are monomeric and dynamic in nature. It is to be noted that since R₀ has a 1/6th power



Figure 5. FRET-monitored folding of tau-K18. (a) Dependence of the observed rate constant λ (\bigcirc , Cys354; \bigcirc , DANS-labelled Cys354; \triangle , Cys316; \triangle , DANS-labelled Cys316 tau-K18), of SDS binding-induced folding on SDS concentration. The error bars represent the standard deviations obtained from three independent experiments. The solid line through the data is a fit to equation 4. The values obtained for K_{UI}, K^l_D, k_f and k_u, from the fits of the data in panel a to equation 4, and to fits of the t = 0 data in Figure 4 to equation 3 are 3 ± 0.5 , $(1.8 \pm 0.6) \times 10^{11} \mu M^5$, $(18.5 \pm 1) \times 10^3 s^{-1}$ and $(6.1 \pm 0.47) \times 10^2 s^{-1}$, respectively. (b) Comparison of the FRET efficiency of the unfolded state (blue), burst phase intermediate (cyan) and the final state (grey) of Cys354 and Cys316 tau-K18 in 280 μ M SDS. The FRET efficiencies were calculated using equation 5. The error bars are standard deviations obtained from three independent experiments.



Figure 6. Model of tau-K18 folding induced by SDS binding. The model accounts quantitatively for the data shown in Figure 4. U and UL₅ represent the unbound and SDS-bound disordered states, respectively. I and IL₅ represent the unbound and SDS-bound intermediate states, respectively. FL₅ represents the SDS-bound folded state. The reaction occurring in the dead time, giving rise to the burst phase, is shown in the box. The U \leftrightarrow I \leftrightarrow IL₅ route represents a conformational selection mechanism. The alternate route, U \leftrightarrow UL₅ \leftrightarrow IL₅, which represents an induced fit mechanism, is assumed to not contribute significantly to the formation of IL₅.

dependence on the above factors (Eq. (7)), its value will not be affected significantly by small changes in their values. Thus, any change in the FRET efficiency can be attributed to a change R during the course of folding. Moreover, the different extents of change in FRET efficiency seen for Cys354 tau-K18 and Cys316 tau-K18, for both of whom Trp 328 was the FRET donor, would represent different extents of changes in R in the two cases.

The elongation rate of fibril formation is linearly dependent on the concentration of FL₅. Fibrilization of 20 µM wt tau-K18 was carried out at different concentrations of SDS, under conditions identical to those under which the SDS binding-induced folding reactions were carried out (Figure 7). The fibrilization process was monitored by the measurement of Thioflavin T (ThT) fluorescence emission at 475 nm, upon excitation at 440 nm, in a 96-well plate. ThT fluorescence is a widely used probe for amyloid fibril formation⁴². It should be noted here that tau-K18 did not appear to aggregate in the absence of SDS; or even at 30 and 70 μ M SDS concentrations (Figure 7(a)). At all higher SDS concentrations, the fibrilization process displayed sigmoidal kinetics, with a lag phase of \sim 45 min duration (Figure 7(a)). The kinetic traces of fibril formation, at all the SDS concentrations used, saturated at around the same ThT fluorescence intensity, indicating that, at each concentration of SDS, the same amount of monomers had been converted into fibril. However, the rate of conversion varied as a function of SDS concentration (Figure 7(a), (b)). The elongation rate of aggregation was determined by measuring the

slope of initial points of the elongation phase. The elongation rate was found to increase in a sigmoidal manner as a function of SDS concentration (Figure 7(b)). Figure 7(c) shows the dependence of the elongation rate on the population of FL₅. The linear dependence indicated a direct role of the FL₅ state in the aggregation of tau-K18.

Discussion

The tau-K18 and tau-K19 isoforms had both been found to fold into a helical state in the presence of SDS micelles, lipid micelles, or a bilaver composed of negatively charged lipid molecules.^{30,31} The current study shows that SDS can also induce helix formation in both tau-K18 and full-length tau (hTau40), at concentrations where it is monomeric (the critical micellar concentration of SDS under the buffer conditions used is 2.3 mM.⁴³ Moreover, the extent of helix formation is very similar for both hTau40 and tau-K18. Tau-K18, the four-repeat MTBR-containing fragment of tau was chosen to study the mechanism of SDS binding-induced folding, because the sequence stretch in which helical structure is induced was known to be located within it.^{30,31,44} Helices had been shown to span \sim 15 residues in each of the repeats (Figure 1(b)). Presumably, the SDS binding-sites are also in the same region.

SDS binding-induced folding proceeds by a CS mechanism. In the equilibrium study of binding-induced folding, binding and folding appeared to occur in one, all-or-none step. Owing to the stochastic nature of the binding and folding events, it is, however, highly unlikely that both binding and folding occur in one step. The involvement of multiple steps became evident, when the kinetic studies revealed the transient accumulation of a burst phase intermediate, IL₅. It was not possible to determine whether the $U \leftrightarrow IL_5$ transition occurs by the CS or by the IF mechanism, because the rate constant of the formation of IL₅ was too fast to be measured even CFM. The two mechanisms can be bv distinguished only when the dependence of the observed rate constant on ligand concentration can be determined.45

Most IDPs have been found to follow an IF mechanism^{3,4,6,46} for folding, which is justified by invocation of a fly-casting mechanism.⁴⁷ The binding of a ligand to a disordered state is believed to be more feasible because of the greater capture radius of the disordered state. Minor populations of binding-competent species have, however, been shown by high-resolution probes to be in equilibrium with the major unfolded population in the case of some IDPs,^{48,49} suggesting that they might acquire structure *via* a CS mechanism.

In the case of tau too, single molecule anisotropy measurements had revealed a minor conformation, more compact than the U state, to be present in



Figure 7. Kinetics of SDS-induced aggregation of wt tau-K18. (a) ThT fluorescence monitored kinetics of fibril formation by 20 μ M tau-K18 at different SDS concentrations. The concentrations of SDS used were 0 (black open), 30 (black filled), 70 (red), 160 (orange), 180 (yellow), 210 (green), 280 (blue), 330 (indigo) and 360 (purple) μ M. The protein did not aggregate in 0, 30 and 70 μ M SDS. (b) The elongation rates of aggregation were calculated from the slopes of the initial 7–8 points of the elongation phase, and are plotted against SDS concentration. The error bars represent the spread in the data from three experiments. The line through the data is drawn by inspection only. (c) Plot of elongation rate of aggregation, determined from ThT binding *vs* fraction of FL₅. The data was fit to straight line equation.

equilibrium with the unstructured state.⁵⁰ Moreover, ensemble NMR measurements,39 and molecular dynamics simulations coupled to crosslinking experiments,⁴⁹ had shown the presence of residual structure in tau, which also suggests the presence of a structured state in equilibrium with U. The current study also shows, using PET-FCS measurements, that a 200-400 ns fluctuation occurs in the U state, representing transient sampling of a partially folded conformation, I. In addition, the sigmoidal transition from U to IL₅ indicates that folding occurs upon the cooperative binding of SDS. This suggests that the SDS binding-sites, present in the fragment, have long-range interactions with each other. Since I is more compact than U, the probability of the long-range interactions, across the repeat domains, is expected to be more in I. This makes folding by CS mechanism more plausible than an IF mechanism. Hence, a CS mechanism was used to analyze the data.

Formation of helical state is driven by hydrophobic collapse induced by SDS. The final helical form, FL₅, which forms from IL₅, has its Trp 328 fluorescence intensity less than that of IL₅. This suggests that the region in and around Trp 328 (R3) in the wt protein is buried to a greater extent in IL₅ than in FL₅. Trp 328 could become buried either because of structural changes in the protein, or the burial could be in the SDS hydrocarbon tail, and it is difficult to between these possibilities. distinguish Nevertheless, since the FRET efficiency across the distance separating the R3 and R4 segments was more in IL_5 than in FL_5 (Figure 5(b)), the former possibility seems to be more probable. This suggests that R3 and R4 come close

together within the 37 μ s dead time, when the formation of IL₅ is complete. It is possible that the reduction in distance during the U to IL₅ transition could be a result of a hydrophobic collapse induced by the binding of the hydrophobic fatty acyl tail of SDS. A hydrophobic collapse leading to the unfavourable burial of H-bonding groups would facilitate the formation of H-bonds such as those found in helices.^{51,52} Cys316 tau-K18 was expected to monitor the formation of helical structure in the R3 segment. The observation that the FRET efficiency for the distance within the R3 segment is the same for IL₅ and FL₅ (Figure 5(b)), suggests that the R3 segment acquired a helical conformation in IL₅ itself.

It could be argued that the hyperfluorescent, burst phase species is the SDS-bound unfolded state (UL_n) , from which FL_n forms in the observable phase $(U \leftrightarrow UL_n \leftrightarrow FL_n)$, and the binding of SDS to U and its conversion to UL_n in the dead time causes the fluorescence intensity to increase. But the FRET efficiency of the burst phase product is different from that of U and thus of UL_n , making it very likely that the hyper-fluorescent, burst phase species is indeed a distinct state, IL_n .

α-helix to β-sheet transition during the aggregation of the helical conformation. The formation of neurofibrillary tangles (NFT) and paired helical filaments (PHF) in the brain by aggregation-prone proteins, such as tau and α-synuclein, is associated with neurodegenerative diseases. Tau is an IDP associated with many neurodegenerative diseases including Alzheimer's disease and Pick's disease.¹⁶ Purified PHF and NFT from the brains of patients with Alzheimer's disease⁵³ and cultured hippocampal neurons⁵⁴

have been found to be associated with membrane components. Interestingly; the aggregation of tau is also known to be induced by anionic lipid vesicles and fatty acids.^{55–58} Previously; a single molecule fluorescence resonance energy transfer (smFRET) study had identified a monomeric form of tau having a conformation distinct from the disordered form in the presence of another aggregation inducer, heparin.⁴⁰ In another study; using molecular dynamics simulations, it was found that prior to aggregation, the MTBR region of tau acquired helical structure.⁵⁹ This indicated that; like in the case of many other proteins,^{60–63} the folding and aggregation energy landscapes of tau are linked.

Phospholipids are an integral part of the membrane, and a phospholipid mimetic such as SDS is expected to simulate a membrane environment. It was therefore important to determine the nature of the aggregationcompetent conformation that was populated in the presence of SDS. Previous computational and experimental studies had shown the formation of transient monomeric, α -helical intermediates, in the case of IFABP,⁶⁴ α -synuclein,² A β ,⁶⁵ amylin⁶⁶ and EGFP⁶⁷ under aggregation conditions. These helical species could be only indirectly linked to aggregation.⁶⁷⁻⁶⁹ In this study, the elongation rate of fibril formation was found to be linearly dependent on the concentration of FL_5 (Figure 7(c)), suggesting a direct role of the FL₅ ensemble in the aggregation of tau. In the FL_5 ensemble, FL_5 has been shown to be in a rapid ($\tau \sim 1 \ \mu s$) equilibrium with the more compact conformation FL₅* (Figure 3(a)-(c), Table 1). At present it is not possible to determine which of these two members of the FL₅ ensemble, is the aggregation-competent conformation.

It has been shown that a compact conformation of tau, formed in the presence of model lipid membrane, can induce membrane disruption.⁷⁰ Membrane disruption is an important means by which amyloid aggregates of tau spread in the brain.⁷¹ In future studies, it will be important to determine whether intermediate states such as IL_5 and FL_5 , which have been shown in this study to directly form tau amyloid aggregates, could be directly involved in the spreading of neurodegenerative diseases.

It has been suggested that during protein folding, short helical segments are formed, which unite to form a highly helical, globular intermediate, from which different secondary structures arise.72 Indeed, transient *a*-helical intermediates have been seen to form during the folding of several β-sheetrich proteins, including β -lactoglobulin,⁷³ as well as the src,74 fyn75 and PI3K75 SH3 domains. In the case of amyloid fibril formation too, α -helix to β sheet conversion occurs.^{76–78} In the case of aggregation leading to fibril formation, stabilization of inter-molecular interactions relative to short-range. intra-molecular interactions could result in *a*-helix to β-sheet conversion. It is known that helices are stabilized by hydrophobic, packing interactions, arising from the tertiary structure, as evident from the fact that sequence segments which form helices in proteins do not fold to a helices in isolation.75 Hydrophobic amino acid residues are typically scarce in an IDP such as tau, compared to in a globular protein. In the folded state of tau-K18, helices are formed in the four repeats, but there appear to be a lack of long-range interactions to stabilize them. Consequently, the formation of fibrils with cross- β -sheet structure would be favoured.

Conclusion

In this study, PET-FCS experiments have shown that in the unfolded state in the absence of any SDS, tau-K18 exists in a rapid (200-400 ns) equilibrium with а compact intermediate conformation, I. Upon the addition of sub-micellar concentrations of SDS, helical structure is induced in the protein, 5 molecules of SDS have been shown to bind to 1 molecule of tau-K18 within the dead time of a microsecond mixing set-up. The binding of SDS induces helical structure in I. SDSbound I folds to the final helical state in the 200-400 µs time domain. The final helical state is a monomeric conformation from which amyloid fibril formation commences.

Materials and methods

Buffers and reagents. All the reagents used in this study were of the highest purity grade, from

Table 1 Parameters determined from the ACFs obtained from PET-FCS experiments.

	Cys354 tau-K18		Cys316 tau-K18	
	-SDS	+360 μM SDS	-SDS	+360 μM SDS
K _f	0.17 ± 0.02	0.11 ± 0.01	0.39 ± 0.04	0.3 ± 0.03
τ _f (μs)	0.39 ± 0.02	1.27 ± 0.15	0.23 ± 0.04	0.73 ± 0.03
Ks	0.1 ± 0.01	0.09 ± 0.01	0.23 ± 0.02	0.08 ± 0.01
τ _s (μS)	76 ± 9	100 ± 11	70 ± 5	27 ± 4.3
τ _D (μ s)	463 ± 22	630 ± 40	423 ± 23	585 ± 53

The parameters were obtained by fitting the data in Figure 3 for Cys354 and Cys316 tau-K18 to equation 8. τ_f and τ_s represents the time constants of relaxation of fast and slow phase, respectively. K_f and K_s respectively represent the amplitudes of those phases. τ_D is the diffusion time. The errors represent the spread in data, calculated from three independent experiments.

Sigma. 10% SDS of ultra-high purity was obtained from the United States Biochemical Corporation. 25 mM Tris-HCl, 50 mM NaCl buffer (pH 7.3) was used as the native buffer, and the folding buffer contained different concentrations of SDS in the native buffer. All the experiments were carried out at 25 °C.

Protein purification, IAEDANS and Atto 655 labelling. Protein purification was carried out according to the protocol described previously.² The purity was found to be > 95%, as checked by ESI-MS. The protein concentration was determined by the BCA assay (Thermo Scientific). Labelling of the proteins by IAEDANS and Atto 655 was carried out according to the protocol provided by Molecular Probes. The mass of the protein increased by 306 and 650 Da, respectively, upon labelling by IAE-DANS and Atto 655. The concentration of DANSlabelled protein was measured by the BCA assay, and the concentration of Atto 655-labelled protein was determined from its absorbance at 663 nm, using a molar extinction coefficient of 1.25×10^5 $M^{-1}cm^{-1}$.

CD spectra and fluorescence emission spectra. Far UV-CD spectra were acquired using a Jasco J-815 spectropolarimeter. The CD signal was collected from 200 to 250 nm, with a bandwidth of 1 nm, in a 2 mm cuvette. Fluorescence emission spectra were acquired using a Fluoromax 3 (Horiba) spectrofluorimeter, with the excitation wavelength set at 295 nm. The excitation slit width was 1 nm, and the fluorescence emission signal was collected from 305 to 430 nm, with the emission slit width set at 10 nm.

Equilibrium SDS binding-induced folding experiments. Equilibrium experiments were carried out using the Fluoromax-3 (fluorescence) and Jasco J-815 spectropolarimeter (far UV-CD), with cuvettes of 1 cm and 2 mm path length, respectively. The CD signal was collected at 222 nm.

The fluorescence signal was collected at 330 nm, with a slit width of 10 nm. An excitation wavelength of 295 nm, with a slit width of 1 nm was used. The concentration of the protein used in the experiments was in the range of 2.5 to 5 μ M.

Continuous flow mixing experiments. All kinetic experiments in the sub-millisecond time regime were carried out using a custom-built continuous flow mixing (CFM) set up,⁴¹ having a dead time of $37 \pm 5 \,\mu$ s. A Ti Sapphire laser (Mai Tai, Spectra physics) was used as the excitation source. A wavelength of 295 nm was obtained from a primary wavelength of 885 nm, by the use of flexible second and third harmonic generators (GWU, Spectra physics). The fluorescence emission signal was collected at 330 nm using a bandpass filter (Asahi Spectra) placed in front of a CCD camera (Synapse, Horiba Jobin Yvon). Protein and buffer solutions were mixed at a flow rate of 7 ml/min, in

the ratio 3:4, to achieve a final SDS concentration in the range of 140 to 360 μ M.

PET-FCS experiments. PET-FCS experiments were carried out as described previously using a Micro Time-200 confocal microscope from Picoquant.³⁸ The pinhole used was 100 μ m in diameter. At each concentration of SDS, the data acquisition time was 20–30 min. In the case of FCS measurements with protein carried out in the presence of 6 M urea, it was necessary to correct for the change in viscosity. The correction factor was determined by carrying out FCS measurements with just the free dye in 6 M urea.

Aggregation reactions. The aggregation reactions were carried out by the addition of buffers having SDS concentrations ranging from 160 to $360 \,\mu$ M. The protein concentration was fixed at $20 \,\mu$ M, and the reaction was carried out in a 96 well plate and monitored at 25 °C in a Fluoroskan (Thermo Scientific) in the presence of $32 \,\mu$ M ThT.²⁴

Data analysis

 $U+nL\leftrightarrow FL_n$

Calculation of relative fluorescence values in sub-millisecond kinetic measurements. The illumination of the channel in the CFM setup was not uniform. Hence, the fluorescence signal during folding was normalized to the signal of the unfolded protein. The relative fluorescence signal (S_{rel}) was calculated as, S_{rel} = $\frac{(S_f - S_{sb})}{(S_{up} - S_{nb})}$ Here, S_f, S_{up}, S_{sb} and S_{nb} are the fluorescence

Here, S_f , S_{up} , S_{sb} and S_{nb} are the fluorescence signals during folding, of unfolded protein, of buffer containing SDS, and of native buffer, respectively.

Analysis of ligand binding-induced folding data.

Equilibrium data. Far-UV CD and fluorescencemonitored equilibrium data were analysed according to a two-state, all-or-none binding model, in which the unfolded (U) state and ligand (L) are in equilibrium with a fully ligand-bound state, FL_n :

Scheme 1

 $K = \frac{|U||L|^n}{|FL_n|}$ is the equilibrium dissociation constant associated with the binding reaction. Hence, the observed signal (CD or fluorescence), y_{obs} , in the presence of ligand, is given by,

$$yz_{obs} = y_0 + \frac{(y_b + m.[L] - y_0).[L]^n}{[L]^n + K}$$
(1)

Here, y_0 represents the signal in the absence of the ligand, which does not have any dependence on [L]. y_b and m are the intercept and slope, respectively, of the bound protein zbaseline. n is the number of ligand binding-sites, and [L] is the concentration of ligand.

The fraction of the protein bound by ligand, f_b , is given by,

$$f_{b} = \frac{\left[L\right]^{n}}{\left[L\right]^{n} + K}$$
(2)

Kinetic data. The following minimal mechanism was used to analyse the data



In this mechanism, an intermediate, I, is present in equilibrium with U, in the absence of any ligand, L. n molecules of L bind to one molecule of protein to form the ligand-bound intermediate IL_n. IL_n can form from U by an induced fit (IF) pathway, $U \leftrightarrow UL_n \leftrightarrow IL_n$, and/or by a conformational selection (CS) route, $U \leftrightarrow I \leftrightarrow IL_n$. IL_n is assumed to be the only conformation competent to fold further to the folded state, FL_n. Thus, $K = K_{UI}.K_{PI}^{I}=K_{D}^{U}.K_{UI}^{L}.K_{FI}$

Here, KUI is the equilibrium constant of unfolding of I, K_{UI}^L is the equilibrium constant of unfolding of IL, K_D^L and K_D^U are the equilibrium constants of dissociation of L from IL_n and UL_n, respectively. $K_{FI} \stackrel{L}{=} \left(=\frac{k_u}{k}\right)$ is the equilibrium constant of the $IL_n \leftrightarrow FL_n$ transition. Since a burst phase change in fluorescence was observed to take place within the 37 µs dead time of continuous flow mixing, it was assumed that IL_n forms within the dead time. It is assumed that only the CS route (see above) leads to the formation of IL_n, and that a preequilibrium is established between U, I and IL_n within the dead time of mixing, before folding occurs to FL_n. If the fraction of U, I and IL_n present at pre-equilibrium are f_u , f_l and f_{lLn} , with $f_u + f_l + f_{lLn} = 1$, then the burst phase signal, y_{obs}^{bp} , is given by,

$$\begin{split} &y_{obs}^{op} = y_{U}.t_{U} + y_{I}.t_{I} + y_{ILn}.t_{ILn} \\ &= \frac{y_{u}.K_{UI}.K_{D}^{I} + y_{I}.(K_{D}^{I} + [L]^{n})}{K_{UI}.K_{D}^{I} + K_{D}^{I} + [L]^{n}} \end{split} \tag{3}$$

and the observed rate constant of folding to FL_n is given by:

$$k_{obs} = k_u + \frac{k_f.[L]^n}{K_{UI}.K_D^l + K_D^l + [L]^n} \tag{4}$$

Calculation of FRET efficiency. The FRET efficiency (E) was calculated as:

$$E = 1 - \frac{F_{DA}}{F_{D}}$$
(5)

Here, F_{DA} and F_D are the fluorescence intensities of the donor in the presence (DANS-labelled protein) and absence (unlabelled protein) of the acceptor, respectively. The dependence of E on the distance separating the donor and acceptor, R, is given by:

$$\mathsf{E} = \frac{1}{1 + \left(\frac{\mathsf{R}}{\mathsf{B}_{\mathsf{c}}}\right)^6} \tag{6}$$

 R_0 , the distance at which the FRET efficiency is 0.5, is given by:

$$\mathsf{R}_{0} = 0.211.(\kappa^{2}.q.\eta^{-4}.J(\lambda))^{\frac{1}{6}} \tag{7}$$

 κ^2 is the orientation factor and has a value of 2/3 when the donor and acceptor are oriented randomly with respect to each other. q is the quantum yield, η is the refractive index of the medium, and J(λ) is the overlap integral between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. 37

Fitting of the autocorrelation function (ACF) derived from PET-FCS. The data for Attolabelled Cys316 and Cys354 tau-K18 were fitted to the following equation:

$$G(t) = \frac{1 + K_{f.}e^{-\frac{t}{\tau_{f}}} + K_{s.}e^{-\frac{t}{\tau_{s}}}}{N.(1 + \frac{t}{\tau_{D}})}$$

$$(8)$$

where τ_D represent the diffusion time, and N represents the number of molecules in the confocal volume. τ_s and τ_f represent the slow and fast time constants, respectively, of processes giving rise to the exponential components, and K_s and K_f are their amplitudes.

The data for the Trp-less protein variants were fitted to the following equation:

$$G(t) = \frac{1 + K_{s}.e^{-\frac{t}{\tau_{s}}}}{N.(1 + \frac{t}{\tau_{s}})}$$
(9)

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CRediT authorship contribution statement

Sreemantee Sen: Conceptualization, Methodology, Software, Data curation. Harish Kumar: Conceptualization, Methodology, Software. Jayant B. Udgaonkar: Conceptualization, Supervision, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021. 167254.

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Tau; PET-FCS; Microsecond mixing; Conformational selection; Amyloid fibril

Abbreviations:

IDP, intrinsically disordered protein; SDS, sodium dodecyl sulfate; CMC, critical micellar concentration; CD, circular dichroism; PET-FCS, photoinduced electron transfer coupled to fluorescence correlation spectroscopy; CS, conformational selection; IF, induced fit; CFM, continuous flow mixing; L, ligand; MTBR, microtubule binding repeat; R_H, hydrodynamic radius; FRET, fluorescence resonance energy transfer; PHF, paired helical filament; NFT, neurofibrillary tangle

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