Insights Into The Mechanism of Tau Polymerization And The Effects of Small Molecules

DISSERTATION

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ABSTRACT

Alzheimer’s disease and related tauopathies are characterized by the loss of neurons occurring in parallel with the formation of filamentous lesions composed of the tau protein. Though macroscopic analysis has provided insights into the development of tau lesions, the mechanism by which filaments assemble has remained unknown.

The first stage in clarifying the reaction pathway was to characterize the partially folded intermediate species and determine conditions which promote intermediate stabilization. Data indicate that intermediate species contain increased secondary and tertiary structure. Several planar aromatic dyes including Congo red, thiazin red, thioflavin S known to bind β-structure, were capable of triggering filament formation. They were also capable of dramatically reducing critical concentration and nucleation rate relative to other inducers.

In the presence of β sheet binding dyes assembly time courses were sigmoidal and reached plateau within 8 hours. Reaction kietics were utilized to estimate nucleus cluster size as well as several of the rate contstants governing the reaction. These estimates along with data from polymerization time courses were used to create a mathematical model of the tau polymerization reaction. These methods were further utilized to examine the effects of alternative splicing on tau polymerization. The effects of individual exons on both nucleation and elongation rates were determined through analysis of reaction kinetics.
Finally, the effects of a previously described inhibitor of tau polymerization, N744 were examined over a wide concentration range. Results revealed a biphasic effect with inhibitory activity at low dye concentrations followed first by relief of inhibition, and then enhancement. Greater than 50% inhibition was seen over only a narrow concentration range. Changes in activity parallel changes in dye aggregation state, with dimers predominant under inhibitory concentrations. In the presence of tau protein the dimerization constant for N744 was decreased over an order of magnitude relative to controls. These results suggest that ligand aggregation may play an important role in the development of effective fibrillization inhibitors.
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LIST OF ABBREVIATIONS

3R, three microtubule repeat containing isoform; 4R, four microtubule repeat containing isoform; AA, arachidonic acid; Aβ, amyloid beta peptide; AD, Alzheimer’s disease; ALS, amyotrophic lateral sclerosis; ANS, 8-anilino-1-naphthalenesulfonic acid; CBD, corticobasal degeneration; CC, critical concentration; CD, circular dichroism; CR, Congo Red; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EM, electron microscopy; FTDP, frontotemporal dementia with Parkinsonism; FTDP-17, frontotemporal dementia with Parkinsonism associated with chromosome 17; FTLD, frontotemporal lobar degeneration, formerly Pick’s disease; h, hours; K crit, critical concentration; k app, apparent rate constant; koff, reverse elongation constant; kon, forward elongation constant; LLS, laser light scattering; μM, micromolar; mM, millimolar; nm, nanometer; nM, nanomolar; PHF, paired helical filament; s, seconds; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy; TMV, tobacco mosaic virus; PSP, progressive supranuclear palsy; ThS, thioflavin S; TR, thiazin red
CHAPTER 1
1. INTRODUCTION

1.1 Tau and Tauopathies

Alzheimer’s disease (AD) is the most prevalent form of dementia in the United States, affecting as many as ten percent of those over 65 and half of those over 85. Recent census data indicates that as many as four million Americans suffer from the disease (Hebert, Scherr et al. 2003). Due to an aging population and changing demographics this figure is expected to rise to 11 to 16 million (Brookmeyer, Gray et al. 1998). Because patients typically live years after diagnosis, AD is also one of the most costly public health issues (Ernst and Hay 1994).

Defined on the basis of pathology, AD is characterized by a loss of neurons accompanied by the formation of two classes of proteinacious lesions: extracellular plaques composed of amyloid-β, as well as intracellular deposits of the microtubule associated protein tau. Formation of tau lesions, but not amyloid plaques, correlates with both cognitive decline and cell death (Gomez-Isla, Hollister et al. 1997; Giannakopoulos, Herrmann et al. 2003). In the case of AD, neuron loss is severe in even mild cases with advanced cases showing up to 90 percent cell death in affected areas (Gomez-Isla, Price et al. 1996). These tau lesions, classified as neurofibrillary tangles (NFTs), neuropil threads or dystrophic neurites, are also found in a host of other conditions including
frontotemporal dementia with Parkinsonism (FTDP), corticobasal degeneration (CBD), progressive supernuclear palsy (PSP), and frontotemporal lobar degeneration (formerly known as Pick’s disease). Closer examination with electron microscopy reveals that rather than amorphous aggregates tau forms highly ordered filaments that fill the cell (Kidd 1963). Within each cell filaments assume either a paired helical or straight morphology, with their relative prevalence dependent on disease (Yagishita, Itoh et al. 1981). Analysis using circular dichroism and x-ray diffraction show that at their core tau fibrils contain beta sheets orthogonal to the long axis of the filament (Berriman, Serpell et al. 2003; Barghorn, Davies et al. 2004; von Bergen, Barghorn et al. 2005). This characteristic cross-beta pattern has been found in multiple proteins involved in deposition diseases such as amyloid-β, α-synuclein, and huntingtin (Bates, Mangiarini et al. 1998; Serpell, Berriman et al. 2000; Serpell, Sunde et al. 2000; Stromer and Serpell 2005).

The tau protein is expressed as a six isoform ensemble owing to the alternative splicing of a single gene on chromosome 17 (Goedert, Spillantini et al. 1989). Inclusion or exclusion of exon 10 results in isoforms having either three or four microtubule binding repeats. Amino terminal exons 2 and 3 are also variably spliced with isoforms having one, both or neither. As a result, isoforms vary in length between 352 and 441 amino acids (Himmler, Drechsel et al. 1989). Splicing is developmentally regulated, with only the shortest isoform expressed in fetal brain, and all six in the adult (Kosik, Orecchio et al. 1989). In normal brain tissue tau is highly concentrated in axons bound to microtubules providing stability for the cytoskeleton, as well as contributing to the dynamic spacing of the microtubules (Harada, Oguchi et al. 1994). Experiments using
knockout animals suggest tau may also play a role in neurodevelopment (Takei, Teng et al. 2000; Dawson, Ferreira et al. 2001). When not bound to the microtubule surface the tau protein is highly soluble with little tertiary structure. This natively unfolded conformation is typical of amyloid forming proteins and may be partially responsible for their propensity to aggregate (Uversky and Fink 2004).

The role of tau fibrillization in disease progression, as marker or mediator, has been the subject of debate. Unlike the amyloid plaques, tau lesions appear in a highly stereotyped spatiotemporal pattern making them useful for staging Alzheimer’s disease (Braak and Braak 1991). Also, the spatial distribution of tau pathology could be used to differentiate between related disorders. Both of these facts demonstrate the usefulness of tau as a marker of disease progression but, it remains unknown whether tau polymerization has a causal relationship to neurodegeneration. However, several lines of evidence support a more central role for tau in AD. Protease resistant aggregates, termed “ghost tangles” that remain in the brains of tauopathy patients, mark the deaths of tangle bearing neurons. Mutations in the tau gene, whether intronic or exonic, are sufficient to cause pathology as evidenced by heritable forms of FTDP and FTLD (Hutton, Lendon et al. 1998; Spillantini, Crowther et al. 1998; Hasegawa, Smith et al. 1999; Rizzini, Goedert et al. 2000; Spillantini, Yoshida et al. 2000; Neumann, Schulz-Schaeffer et al. 2001; Hayashi, Toyoshima et al. 2002; Poorkaj, Muma et al. 2002; Rosso, van Herpen et al. 2002; Yoshida, Crowther et al. 2002; Grover, England et al. 2003; Hogg, Grujic et al. 2003; Kobayashi, Ota et al. 2003). Overexpression of human tau in animal models has also been shown to induce neurodegeneration (Ishihara, Hong et al. 1999; Spittaels, Vanden Haute et al. 1999; Hall, Lee et al. 2001). These findings have led to the development
of several hypotheses regarding the role of tau in Alzheimer’s disease. Tau pathology could potentially represent either a loss or toxic gain of function. The abolition of the tau-microtubule interaction through mutation or post translational modification could have detrimental effects on the cytoskeleton. For example, the loss of tau as a stabilizer could result in the dissociation of microtubules and therefore disruption of intracellular trafficking. Indeed, tau isolated from the brains of AD patients shows a reduced ability to bind microtubules. Phosphorylation of tau in sites occupied in disease in vitro can also reproduce this effect (Bramblett, Goedert et al. 1993). The second hypothesis states that the aggregation of tau into PHFs is toxic to the neurons. Neurons containing fibrillar pathology show evidence of signaling deficits not present in non-affected cells (Callahan and Coleman 1995). In addition, the binding of PHFs to proteasomes results in significant decreases in proteasome activity relative to unaffected individuals (Keck, Nitsch et al. 2003). Evidence suggesting a causal role for tau in disease progression highlights the importance of elucidating the mechanism by which lesions form.

1.2 Tau pathology in disease

As stated above, lesion formation in the brains of AD patients follows a stereotyped pattern (Braak and Braak 1991). In addition, the staging of neurofibrillary pathology has been described at the level of individual cells. Though they cannot provide data regarding the mechanism of lesion formation, these studies may provide information on the biochemical events occurring inside the cells.

The initial detectable stage in NFT formation is the appearance of “pretangles”, non-fibrillar, microtubule free tau either in the cytoplasm or associated with membrane
bound organelles (Bancher, Brunner et al. 1989; Mena, Edwards et al. 1996). Though these deposits react with antibodies to the tau protein they do not bind to β-sheet specific dyes such as thiazin red or thioflavin S (Galvan, David et al. 2001). This suggests that the dissociation from the microtubule surface occurs before conformational changes and increases in secondary structure. Posttranslational modifications such as phosphorylation have also been observed in “pretantal” tau. Phosphorylation can result in disruption of microtubule binding and may also increase the propensity of tau to polymerize. These findings suggest that one of the earliest events in disease is the abolition of the tau-microtubule interaction. Conformational change characterizes the second stage of lesion formation with the appearance of reactivity to dyes including the aforementioned ThS and TR. Though the optical properties of these dyes are not fully understood, they are thought to preferentially bind β-sheet structure (LeVine 1993; Friedhoff, Schneider et al. 1998; Krebs, Bromley et al. 2005). This does not necessarily indicate the presence of mature filaments since partially folded intermediates, including monomeric species, also react with these dyes (Khurana, Uversky et al. 2001; Kim, Randolph et al. 2003; Chirita, Congdon et al. 2005). Thioflavin positive species are often observed in association with intracellular membranes, suggesting that heterogeneous interactions may facilitate the conformational change (Mena, Edwards et al. 1996; Galvan, David et al. 2001). Further evidence for a tau-membrane interaction can be seen in biopsy tissue, where tau filaments have been observed in endwise association with membranes (Gray, Paula-Barbosa et al. 1987).

Following the conformational changes, amorphous tau deposits are replaced by mature filaments. Though neuropil threads contain predominantly straight filaments, in
later stage disease PHF morphology becomes prevalent (Perry, Kawai et al. 1991). PHFs isolated from authentic tissue have the appearance of “twisted ribbons” with a periodicity of 80 nm and a varying width of between 10-20 nm (Ksiezak-Reding and Wall 1994). Filaments average 300-600 nm while adopting an exponential length distribution (Chirita and Kuret 2004). Transmission electron micrographs reveal that each PFH is composed of two, three domain, c-shaped hemifilaments (Crowther 1991). It is possible to propagate these hemifilaments independently of one another by incubating PHF “seeds” with recombinant tau in the presence of arachidonic acid (King, Ahuja et al. 1999; King, Ghoshal et al. 2001). Mass per unit length data indicate that each cross beta sheet along the filament axis contains ~1.5 of these core units (Berriman, Serpell et al. 2003). Scanning electron microscopy has revealed that both straight filaments and PHFs have a similar mass per unit length and thus may represent different arrangements of the hemifilaments (King, Ghoshal et al. 2001).

Within the filaments themselves, tau molecules adopt a cross-beta pattern seen in other amyloid forming proteins (Berriman, Serpell et al. 2003; Barghorn, Davies et al. 2004; von Bergen, Barghorn et al. 2005). Extended beta strands run perpendicular to the long axis of the filament with hydrogen bonds between the strands forming a continuous beta sheet (Petkova, Ishii et al. 2002; Petkova, Leapman et al. 2005). This beta sheet structure forms the protease resistant core, found in authentic filaments, which spans an approximately 93 residue segment representing at least three of the microtubule binding repeats (Novak, Kabat et al. 1993). The number of residues making up the filament core is common to all six tau isoforms, indicating that though splicing affects polymerization kinetics, it may not affect the final structure of the fibrils.
Late stage disease is characterized by modification of existing filaments as well as increasing bulk tau levels. Once filament formation occurs, the tau protein becomes progressively modified in later stage disease. Levels of phosphorylation increase dramatically, as much as three fold, relative to non-filamentous tau (Kopke, Tung et al. 1993; Ksiezak-Reding and Wall 1994). Much of the phosphorylation occurs in ~30 sites divided into two regions on either side of the microtubule binding region. However, hyperphosphorylation likely represents greater occupancy rather than binding at a greater number of sites. Additional conformational changes, as demonstrated by changing reactivity to conformation specific antibodies Alz-50 and Tau-66, are also present in late stage tissue (Bondareff, Harrington et al. 1994; Garcia-Sierra, Ghoshal et al. 2003). PHFs isolated from end stage tissue also display increased resistance to chaotropic agents, indicating that filaments undergo covalent cross-linking (Iqbal, Zaidi et al. 1984). In contrast to earlier stages, in late stage disease bulk tau levels rise relative to age matched controls (Khatoon, Grundke-Iqbal et al. 1992).

Studies of macroscopic pathology provide valuable information regarding the timing of events in lesion formation, despite being silent on mechanism. Specifically, these studies indicate that the earliest event in tangle formation is the dissociation of tau from microtubules, its normal binding partner. This occurs before conformational changes and aggregation and also suggests that post translational modifications may influence the polymerization pathway.
1.3 Modeling tau polymerization in vitro

1.3.1 Methods

In order to obtain detailed information regarding the mechanism of tau filament formation without the complications of an in vivo system, in vitro modeling provides a means to study the reaction. The first issue in modeling polymerization in vitro is whether recombinant proteins recapitulate the properties of authentic protein. Non-tagged tau protein is typically purified using heat denaturing or cation exchange chromatography followed by gel filtration (Crowther, Olesen et al. 1994; Gustke, Trinczek et al. 1994; Paudel and Li 1999). In addition, the acid solubility of the tau protein has been leveraged for purification (Crowther, Olesen et al. 1994; Alonso, Zaidi et al. 2001). However, these methods utilize harsh conditions which may in fact alter the aggregation behavior of tau (Alonso, Zaidi et al. 2001). To combat this, recombinant protein containing a poly-histidine tag has been utilized. His-tagged tau presents similar hydrodynamic properties as tissue derived tau, as a random coil monomer with a 20:1 axial ratio. These findings hold regardless of isoform or presence of a poly-histidine affinity tag (Cleveland, Hwo et al. 1977; Barghorn, Davies et al. 2004; Chirita, Congdon et al. 2005). In addition, though the tag adds an additional positive charge, it does not appear to affect polymerization (King, Gamblin et al. 2000).

Difficulties in modeling the polymerization reaction arise due to the fact that tau protein does not readily assemble even at supersaturated concentrations. Spontaneous association can be achieved with some peptide fragments incubated at highly supersaturated (200 μM) concentrations (Wille, Drewes et al. 1992; Crowther, Olesen et al. 1994), but full length protein, whether recombinant or expressed in situ does not self
assemble (Schweers, Mandelkow et al. 1995; Arrasate, Perez et al. 1999; Perez, Arrasate et al. 2001; Ko, DeTure et al. 2002; Goux, Kopplin et al. 2004). Seeding the reaction with preformed PHFs or synthetic filaments has also proved inefficient (Friedhoff, von Bergen et al. 1998; King, Ahuja et al. 1999). In addition, extremes of pH and ionic strength are also capable of triggering filament formation; however, under physiological conditions tau polymerization is not supported (Crowther, Olesen et al. 1994). To counter this problem a variety of compounds have been used to induce tau polymerization. One of the earliest methods for inducing tau polymerization involved exposure of the protein to urea, resulting in filaments with dimensions similar to those found in authentic PHFs (Montejo de Garcini and Avila 1987; Montejo de Garcini, Carrascosa et al. 1988). Polyanions such as RNA and heparin sulfate are also capable of promoting polymerization of tau constructs under oxidizing conditions (Kampers, Friedhoff et al. 1996; Perez, Valpuesta et al. 1996). However, these compounds are inefficient fibrillization inducers in the presence of reducing agents such as DTT. In addition, full-length four-repeat isoforms do not polymerize efficiently in the presence of polyanions. This may result from the formation of intramolecular disulfide bonds in tau isoforms containing two cysteine residues. Another category of polymerization inducing compounds is the anionic surfactants. This category includes fatty acids, alkyl sulfate detergents, and anionic microspheres which present a negatively charged surface to the tau protein. Incubation with tau is sufficient to depress the critical micelle concentration for both arachidonic acid and alkyl detergents (Chirita, Necula et al. 2003). At fibrillization inducing concentrations both arachidonic acid and alkyl sulfates are in micellar form. In contrast to polyanions, anionic surfactants are efficient inducers of
polymerization in full-length tau under reducing conditions. Reactions can also be carried out at much lower tau concentrations (>2 μM) which more closely reflect conditions in vivo.

Once filament formation in vitro becomes possible, the issue of how to quantify the polymerization reaction arises. Generally, assay methods can be divided into solution and electron microscopy based categories. Solution based assays used to quantify tau fibrillization include static laser light scattering, fluorescence spectroscopy, and sedimentation. Static laser light scattering (LLS) provides a sensitive assay method capable of detecting smaller aggregates than either turbidity or optical density (Pallitto and Murphy 2001). Another advantage is that with LLS the scattered light is directly proportional to weight average mass at a zero scattering angle. This methodology has been used to study tau polymerization in the presence of both heparin and anionic surfactants (Gamblin, King et al. 2000; Luo, He et al. 2000). In both cases, following the addition of inducers, light scattering increased with time in a hyperbolic manner. Hyperbolic time course curves typically indicate spontaneous aggregation of monomers into small aggregates followed later by filament formation. However, it has been shown that tau protein leads to a decrease in the surfactant critical micelle concentration (Chirita, Necula et al. 2003). The immediate jump in light scattering is unrelated to filament formation and results from the initial formation of a tau-inducer complex. A reaction containing assembly incompetent protein capable of depressing the critical micelle concentration, run in parallel, can be used to correct for this phenomenon (Necula and Kuret 2004). This can include assembly-incompetent mutant tau proteins such as I277P/I308P or other molecules such as histones (von Bergen, Barghorn et al. 2001;
Necula and Kuret 2004; von Bergen, Li et al. 2005). Though this method can provide information on reaction kinetics, it cannot distinguish between amorphous aggregates and filaments or provide data on filament length distributions.

Fluorescence spectroscopy utilizes the Stokes shift which occurs when thioflavin dyes bind to the β sheet structure within amyloid filaments. Though this method can provide real time data with a large number of measurements, it has several disadvantages. For example, increased fluorescence does not necessarily indicate filament formation. Studies of prion protein intermediates revealed that cross-beta structure can be achieved within a protein monomer (Govaerts, Wille et al. 2004). Tau protein incubated with anionic surfactants produced immediate increases in thioflavin S reactivity with initial velocity directly proportional to tau concentration (Chirita, Congdon et al. 2005). This indicates that the species binding thioflavin S is monomeric. In addition, tau protein incubated below the critical concentration required for polymerization can produce dramatic increases in thioflavin S signaling in the absence of filaments. These findings indicate that results from studies using fluorescence to assay potential fibrillization inhibitors should be interpreted cautiously. Because ThS may react to intermediate species as well as mature polymers, decreases in fluorescence obtained with inhibitors may not, in fact, indicate decreased polymerization. Pharmacological studies using fluorescence may also be confounded due to competitive binding between thioflavin dyes and small molecule inhibitors (Necula, Chirita et al. 2005). Many described inhibitors are aromatic dyes or dye derivatives, some of which have extinction coefficients which may interfere with ThS fluorescence.
Finally, sedimentation assays have been utilized to quantify polymerization by separating soluble and insoluble protein through centrifugation or filtration (Chirita, Necula et al. 2003; Pickhardt, Gazova et al. 2005). However, these methods can not distinguish between well resolved filaments and amorphous aggregates, or indeed small soluble aggregates from monomers. This becomes problematic when reactions are carried out under conditions which support the formation of amorphous aggregates, such as inclusion of crude brain extracts. In these cases sedimentation assays can provide only a qualitative estimate of polymerization. In addition, because filaments isolated from authentic disease tissue are soluble in nonionic detergents such as sarkosyl, detergent extraction is often incorporated into PHF purification protocols. However, measurements of tau concentrations in sarkosyl soluble fractions again yield only qualitative data on fibrillization.

In contrast to solution based methods, electron microscopy involves directly viewing tau filaments. Polymerization reactions occur in solution but resultant reaction products are adsorbed onto hydrophobic formvar-coated grids prior to quantification. The assay is well characterized and presents several advantages over solution based methods. One of the most important advantages is the high specificity of the assay including the ability to differentiate between filaments and amorphous aggregates, assembly intermediates, and other non-filamentous material. This results in a high level of analytical sensitivity owing to the excellent signal to noise ratio. In addition, detection of both authentic (Kopke, Tung et al. 1993) and synthetic filaments (Necula and Kuret 2004) is linear over a large concentration range resulting in high levels of precision. The
assay is unaffected by the levels of inducers or pharmacological agents present, indicating that the assay is robust.

The ability to provide information on filament morphology and length distribution also represents a unique advantage of microscopy. Filament morphology can reflect several factors, including intermediate structure, and may therefore provide information on the assembly pathway. The issue of length distribution is of special significance. Distributions can be used to determine the phases of the reaction as well as distinguish between mechanisms of disaggregation (Chirita, Necula et al. 2004). For example, endwise depolymerization can be distinguished from random breakage by measuring the slope of the length distribution. This in turn can provide information about the elementary dissociation rate constant (Necula, Chirita et al. 2005).

Though electron microscopy provides multiple advantages over solution based methods, it does require that certain conditions be met in order to be effective. For example, the protein in question must form well resolved isolated filaments. Tau filaments are ideal due to their well-resolved, unbranched structure; however, other proteins involved in deposition diseases such as α-synuclein do not meet this criterion, thus limiting the utility of microscopy. In addition, unincorporated protein monomers can compete with filaments for binding to the grid, meaning that bulk protein concentration must be controlled for. However, adsorption is linear up to 1 μM protein concentration and fixed samples can be diluted, such that bulk tau concentrations do not interfere with adsorption (Necula and Kuret 2004). All experimental conditions may not be appropriate when utilizing electron microscopy. Inducers such as heparin produce fewer well defined filaments relative to anionic surfactants. When incubated at highly supersaturated
concentrations tau protein is also prone to formation of fractals rather than filaments. These aggregates are characterized by irregular shapes and may form when proteins in solution aggregate via hydrophobic or electrostatic interactions. Other conditions which promote filament clumping could also interfere with the assay. Finally, microscopy shares a disadvantage with centrifugation in its inability to detect small aggregates. However, when compared to time course data obtained from laser light scattering assays, estimates of lag time were within the standard error of the estimate.

1.3.2 Mechanism of assembly

Tau polymerization has been modeled as a homogeneous nucleation dependent process, similar to those seen in other cytoskeletal proteins such as tubulin or actin. The classical mechanism involves the spontaneous association of monomers to form an energetically unfavorable species termed the nucleus. Once this critical cluster size is achieved, nascent filaments grow exponentially, eventually reaching equilibrium where filament length remains constant and monomers associate and dissociate from filament ends. The unfavorable equilibrium for the nucleation reaction relative to that for elongation results in the characteristic features of the mechanism. First of these is a pre-equilibrium phase, or lag time, during which nucleation occurs. Second, the presence of a critical concentration, below which filament formation is not supported, also typifies this mechanism. Finally, nucleation dependent reactions support secondary nucleation through seeding with preformed nuclei and spontaneous polymer elongation.

Multiple proteins involved in deposition diseases such as α-synuclein, amyloid-β, and huntingtin assemble via a homogeneous nucleation dependent mechanism. However, this model is insufficient to account for the behavior of tau. Recombinant full length tau
proteins typically do not self associate at physiological concentrations, pH, ionic strength, and temperature (Wille, Drewes et al. 1992; Crowther, Olesen et al. 1994; Friedhoff, Schneider et al. 1998; King, Gamblin et al. 2000). When incubated at highly supersaturated concentrations (above 200 μM) truncated tau peptides and full length protein can overcome this barrier and form filaments (Crowther, Olesen et al. 1994; Perez, Hernandez et al. 2002). Overcoming charge repulsion may explain the need for high concentration of protein. This may also be due to fibrillization competing with off pathway folding. For example, tau isoforms with four microtubule binding repeats contain two cystiene residues, while those with three repeats have only one. Under oxidizing conditions four-repeat isoforms can form intramolecular disulfide bonds. These bonds result in assembly-incompetent “compact” tau monomers which represent a trapped side reaction. In contrast, three repeat isoforms can dimerize, a potentially important step in polymerization under oxidizing conditions. This may explain why maintenance of reducing conditions promotes fibrillization in tissue derived preparations (Wilson and Binder 1995). Moreover, solutions of purified recombinant tau protein do not efficiently respond to seeding in the absence of exogenous fibrillization inducers such as heparin or fatty acids (Friedhoff, von Bergen et al. 1998; King, Ahuja et al. 1999).

Kinetic and thermodynamic barriers to filament formation may be overcome through the addition of anionic condensing agents including anionic polymers (heparin, nucleic acids, etc) and anionic surfactants (fatty acids, alkyl sulfate detergents). Surfactants act in micellar form to promote polymerization through adsorption of tau onto the negatively charged surface. Adsorption as a key step in the polymerization process is emphasized by the finding that solid anionic microspheres also trigger filament
formation (Chirita and Kuret 2004). Addition of these inducers to solutions of tau protein produces an immediate increase in ThS fluorescence, indicating that the presence of anionic surfaces promotes an increase in secondary structure. Increases in fluorescence are time dependent, but do not display the lag phase seen using electron microscopy (Figure 1.1). These findings indicate that a species containing increased secondary structure, or intermediate, exists prior to filament formation (Chirita and Kuret 2004). A similar pattern is seen when polyanions such as heparin are used (Friedhoff, Schneider et al. 1998; Friedhoff, von Bergen et al. 1998; Chirita and Kuret 2004). ThS signal increases hyperbolically without a lag time, indicating that this intermediate, or a similar species containing increased secondary structure, is present in the polymerization pathway. Folding intermediates have been discovered in the polymerization pathway for a variety of amyloid forming proteins, including α-synuclein (Uversky, Li et al. 2001), insulin (Ahmad, Millett et al. 2003; Ahmad, Millett et al. 2004), prion (Gerber, Tahiri-Alaoui et al. 2007), and alpha-chymotrypsin (Pallares, Vendrell et al. 2004), and indeed may be a universal feature of amyloid fibril formation. Moreover, the natively unfolded conformation of the tau monomer in the absence of inducers may also be a prerequisite for amyloid formation. Because of its reactivity and metastability it was proposed that the tau intermediate is a partially folded molecule containing increased β sheet structure. We would therefore hypothesize that compounds or conditions which promote the formation of, or stabilize, this intermediate will trigger filament formation.

In our proposed model of filament formation, prior to the addition of inducer, the bulk of tau protein in solution exists as assembly incompetent monomers dominated by random coil conformation. This may explain why solutions of recombinant protein are
resistant to seeding. Formation of assembly competent intermediates precedes and is necessary for polymerization. These intermediates contain increased secondary structure with exposed β sheet hydrogen bonding edges, as well as solvent-exposed hydrophobic patches, making them prone to self association (Lansbury 1999). It is these intermediates which spontaneously associate to form nuclei and filaments (hypothesized mechanism outlined in Figure 1.2.).

As a result of the energy barrier between the intermediate and mature filaments, a pronounced quiescent period, or lag time, is observed. Lag times vary with the inducer used, though once nucleation is achieved, total filament length increases exponentially until reaching equilibrium. At equilibrium total filament length remains constant with protein monomers associating and dissociating from filament ends.

Reaction progress curves can be modeled using logistic functions as well as other sigmoidal functions such as Gompertz. These functions have been used to analyze biological growth in a variety of contexts from protein aggregation to forestry and actuarial tables. Each of these functions is characterized by a time constant, corresponding to the time of maximal growth, an apparent rate constant \( k_{\text{app}} \), describing the growth rate of the total filament population, and a carrying capacity, or final equilibrium position. In the absence of elementary rate constants, kinetic parameters can provide indirect information. For example, time constants can be used to estimate lag time and therefore provide a means for comparing nucleation rates (Chirita and Kuret 2004).

When using anionic surfactants, total filament length reaches equilibrium within 24 hours. At all points during the polymerization reaction, filament lengths assume a near
exponential distribution (Wilson and Binder 1995). Filaments isolated from authentic disease tissue show a similar pattern, providing support for the hypothesis that tau polymerization is nucleation-dependent. Incubation for longer time periods reveals a second equilibrium, as a portion of the filament population adopts the PHF morphology and mass per unit length seen in disease. However, though the concentration of filaments displaying the classical paired helical morphology rises, this shift does not significantly affect total filament length. Evolution of the PHF morphology may represent the annealing of “hemifilaments” generated by anionic surfactants similar to the lateral aggregation of amyloid-β fibrils (King, Gamblin et al. 2000; King, Ghoshal et al. 2001; Pallitto and Murphy 2001). As with other aspects of the polymerization process, conversion to PHF morphology is most likely influenced by bulk tau concentrations as well as isoform composition.

Finally, the carrying capacity, or equilibrium between filamentous and non-filamentous tau, which may include monomers, intermediates and soluble aggregates, provides information on the concentration dependence of filament formation. Protein incubated at varying concentration will yield different equilibrium positions. Plotting equilibrium position against protein concentration yields an estimate of critical concentration. This value represents both the minimal protein concentration required for polymerization as well as the concentration of unincorporated protein at equilibrium. It also reflects the stability of the tau filaments, with a lowering of critical concentration indicative of enhanced stability.
1.4 Unresolved Issues

Discrepancies observed between timecourse studies conducted using different assay methods indicate the presence of a β-sheet containing intermediate species in the reaction pathway. This may explain why even at high concentrations tau does not self assemble and why seeding is inefficient in the absence of inducers; tau must first assume an assembly competent conformation. We hypothesize that conditions stabilizing this intermediate will trigger filament formation.

Though tau aggregation is often modeled as a homogeneous nucleation dependent mechanism, this model does not fully explain the behavior of tau. The anionic surfactants utilized most likely induce polymerization through a heterogeneous mechanism, and thus preclude the application of classical nucleation theory. Recently discovered planar, aromatic β-sheet binding dyes induce filament formation at sub-micromolar tau concentrations without the need for micelle formation. The ability to induce polymerization at such low concentrations reduces the likelihood of observing non-ideal behavior. Also, because these molecules induce polymerization via a homogeneous mechanism, the application of well established kinetic treatments is possible. Use of these dyes, together with our well characterized electron microscopy assay, provides the means to study the polymerization reaction.

The critical nucleus cluster size in the tau polymerization reaction has been estimated using tau constructs comprising the microtubule binding region, aggregated in the presence of heparin (Friedhoff, von Bergen et al. 1998). Reaction progress was followed using ThS fluorescence and data was analyzed assuming a homogeneous nucleation mechanism. The maximal rate of ThS fluorescence rose linearly with
increasing protein and inducer concentrations and the nucleus size was estimated as 4-7 protein dimers (Friedhoff, von Bergen et al. 1998). However, the kinetic treatment used assumed that changes in velocity were due solely to nucleation rate, ignoring the contribution of filament extension rates. In addition, as mentioned previously, fluorescence assays may be confounded by the presence of partially folded intermediate species capable of binding ThS (Chirita and Kuret 2004). This estimate should therefore be regarded as an upper limit. In fact, the critical nucleus cluster does not necessarily have to be oligomeric. Evidence from studies utilizing poly-glutamine repeats demonstrated that nucleation can occur via a conformational change within a protein monomer (Chen, Ferrone et al. 2002). Herein we will estimate the nucleus size for the first time, under homogeneous nucleation conditions with full length, wild type tau incubated under physiological conditions and concentrations.

Another issue yet to be resolved is the effect of alternative splicing on tau polymerization. Within this area there are several unanswered questions. For example, intronic mutations affecting the ratio of isoforms containing three and four microtubule binding repeats are sufficient to cause disease. Non-human primates show differential susceptibility to development of tauopathies. Also, though fetal tau is phosphorylated to levels seen in authentic disease, it does not form lesions. Previous studies attempting to address these issues utilized static laser light scattering to assay the polymerization of the different tau isoforms (King, Gamblin et al. 2000). However, these experiments were carried out before it was known that the presence of tau protein results in depression of the critical micelle concentration. Because of this, these results are not corrected for the presence of inducer micelles, and the data should be interpreted with caution. Also, no
filaments were formed from either htau24 or htau23 making a full comparison among the isoforms impossible. As with reaction mechanism, small molecule inducers have made it possible to examine the effects of individual exons on tau polymerization.

Because tau has been implicated as a mediator of neurodegenerative disease, it is a potential target for hypothesis testing. As a random coil in solution, the tau protein presents an unattractive target for pharmacology. However, in disease tau monomers undergo conformational changes and form well ordered filaments displaying regular repeating morphology. These conformational changes may produce novel binding sites that could be utilized in the development of fibrillization agonists, contrast agents, and fibrillization inhibitors. Identification of fibrillization antagonists could allow researchers to examine the role of tau polymerization in development of pathology and potentially is the first step to the development of therapeutic agents.

Because many fibrillization agonists and antagonists are planar aromatic compounds with similar structures, the question of why they exert different effects becomes important. One potential answer is that compounds which act as fibrillization antagonists are more prone to self-aggregation. Thus, the inhibitory species may be oligomeric. Previously, this laboratory has described a small molecule inhibitor of tau polymerization N744 (Chirita, Necula et al. 2004; Necula, Chirita et al. 2005). Herein we will examine the dose response relationship of N744 and how aggregation affects its inhibitory activity.
1.5 Summary

Alzheimer’s disease and other tauopathies are characterized by the disappearance of neurons in concert with the appearance of proteinacious inclusions. This makes tau aggregates a powerful marker and potential mediator of disease. Though the tau protein exists as a random coil in solution, it nonetheless is capable of forming well ordered filaments. The mechanism by which natively unfolded tau protein assembles remains unknown. Previous studies have indicated that a partially folded intermediate species exists in the polymerization pathway. It has also been hypothesized that the tau protein assembles via a nucleation dependent mechanism. Here we will test these hypotheses in vitro under near physiological conditions using full length recombinant protein. A clarification of the fibrillization mechanism may provide clues to the stressors affecting the cells as well as potentially aid in the development of contrast agents and polymerization antagonists. There also exist multiple unanswered questions regarding tau alternative splicing. For example, an alteration in the ratio of isoforms containing three or four microtubule binding repeats is sufficient to cause disease. Finally, tau pharmacology is also poorly understood. Because tau filaments display regular morphology with accompanying conformational changes, it is reasonable to hypothesize that these changes result in the formation of novel binding sites. Herein we will examine the effects of a previously described fibrillization antagonist over a large concentration range. The data presented here represent significant findings regarding the mechanism of tau polymerization, consequences of alternative splicing, and the effects of small molecules on tau fibrillization.
1.6. Figures

Figure 1.1 A β-sheet containing species is present prior to filament formation.

Tau polymerization time courses were followed using either electron microscopy (left axis) or ThS fluorescence (right axis). For electron microscopy 4 μM htau40 was incubated under standard conditions at 37° C for up to 24 hours in the presence of carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM). Data points represent the average total filament length ± standard deviation (SD) from 5-10 negatives. Samples assayed with utilizing fluorescence were prepared utilizing the same concentrations of tau and microspheres as those assayed by EM, with the inclusion of 10 μM ThS in the reaction mixture. Before filaments become visible by EM, significant fluorescence signal can already be detected. This suggests that a non-fibrillar species capable of binding thioflavin S is present prior to polymerization. Because ThS is known to bind β-sheets, it was hypothesized that the addition of exogenous inducers triggered a conformational change in the tau protein resulting in an increase in β-sheet content.
We hypothesize that the tau protein assembles via a modified nucleation dependent mechanism. The bulk of tau in solution exists as an assembly in competent monomer (Ux). Conformational change is achieved either spontaneously, in which case it may be stabilized by β sheet binding dyes, or through the addition of exogenous inducers including anionic surfactants (arachidonic acid, alkyl sulfate detergents, anionic microspheres, etc) or polyanions (heparin, RNA, etc.). The conformational change results in the formation of the partially folded intermediate species (I) and potentially multiple other assembly competent species (Uc). Assembly competent species contain increased secondary structure and are capable of binding β-sheet selective molecules such as ThS. These intermediates then spontaneously aggregate until the critical nucleus size is reached. Once nucleation occurs the energetically favorable extension reaction can proceed. Each of these steps can potentially be modified by posttranslational modifications, alternative splicing and mutations. Data presented herein will address issues of reaction mechanism, nucleus cluster size and elementary rate constants for the nucleation and elongation reaction.

Figure 1.2. Hypothesized mechanism of tau fibrillization.

We hypothesize that the tau protein assembles via a modified nucleation dependent mechanism. The bulk of tau in solution exists as an assembly in competent monomer (Ux). Conformational change is achieved either spontaneously, in which case it may be stabilized by β sheet binding dyes, or through the addition of exogenous inducers including anionic surfactants (arachidonic acid, alkyl sulfate detergents, anionic microspheres, etc) or polyanions (heparin, RNA, etc.). The conformational change results in the formation of the partially folded intermediate species (I) and potentially multiple other assembly competent species (Uc). Assembly competent species contain increased secondary structure and are capable of binding β-sheet selective molecules such as ThS. These intermediates then spontaneously aggregate until the critical nucleus size is reached. Once nucleation occurs the energetically favorable extension reaction can proceed. Each of these steps can potentially be modified by posttranslational modifications, alternative splicing and mutations. Data presented herein will address issues of reaction mechanism, nucleus cluster size and elementary rate constants for the nucleation and elongation reaction.
CHAPTER 2

2. TRIGGERS OF FULL LENGTH TAU AGGREGATION: A ROLE FOR PARTIALLY FOLDED INTERMEDIATES

2.1 Introduction

Tau is a microtubule associated protein implicated in the progression of AD and other tauopathic neurodegenerative diseases (Buee, Bussiere et al. 2000). It purifies from normal brain tissue as an ensemble of alternatively spliced products with monomeric quaternary structure (Cleveland, Hwo et al. 1977). In disease, however, tau protein accumulates in lesions composed of fibrillar aggregates displaying the cross-beta sheet diffraction pattern of “amyloid” (Berriman, Serpell et al. 2003). \textit{In vitro}, tau can be induced to assemble with sigmoidal kinetics consistent with a nucleation-dependent process (Chirita, Necula et al. 2004; Necula and Kuret 2004). This behavior is typically ascribed to the requirement for a necessary but scarce species in the reaction pathway termed the “nucleus” (Ferrone, Ivanova et al. 2002). Once formed however, subsequent lengthening of the nascent filament by stepwise addition of protomeric species is energetically favorable and follows in a reaction termed elongation. The favorable elongation reaction relative to the less favorable nucleation reaction results in the characteristic features of this mechanism, including a pre-equilibrium phase.
characterized by a lag time during which nucleation proceeds, the existence of a critical protomer concentration, below which filament formation is not supported, and seeding behavior, whereby addition of preformed nuclei or filaments surmounts the energy barrier of nucleation. Experience with recombinant tau constructs suggests, however, that classic nucleation-elongation theory as elaborated for spontaneous association (i.e., homogeneous nucleation) is insufficient to completely account for the aggregation behavior of tau. For example, recombinant full-length tau produced in vitro or through expression in situ does not spontaneously assemble as predicted by theory, even at high levels of supersaturation (Crowther, Olesen et al. 1994; Friedhoff, Schneider et al. 1998; King, Ahuja et al. 1999). Moreover, the predicted seeding behavior is inefficient in the absence of exogenous fibrillization promoters (Friedhoff, von Bergen et al. 1998; King, Ahuja et al. 1999).

These data suggest that molecular events in addition to those underlying classic nucleation-elongation behavior are responsible for triggering fibrillization of full-length tau isoforms. Because tau is posttranslationally modified in disease, abnormal phosphorylation, glycation, and/or oxidation reactions have all been suggested to play triggering roles. Experience with phosphorylation mimicry mutants in the context of purified recombinant preparations indicates, however, that although modifications can promote fibrillization, the mechanism is limited to enhancement of the elongation reaction through a lowering of the critical concentration for assembly (Necula and Kuret 2004). Similar results were found for glycated recombinant tau preparations (Necula and Kuret 2004). Moreover, oxidation of tau into disulfide-bonded dimers can accelerate fibrillization without triggering it (Barghorn and Mandelkow 2002), and both N- and C-
terminal proteolytic truncations are later-stage events in disease temporally unrelated to the triggering reaction (Horowitz, Patterson et al. 2004). In fact, the ultrastructure of tau filaments in AD tissue suggests that the trigger may not involve spontaneous self-association at all. In end stage disease, individual PHFs appear in endwise association with membranes, consistent with surface-mediated nucleation and unidirectional extension from stable tau-membrane complexes (Gray, Paula-Barbosa et al. 1987). These data are consistent with a heterogeneous nucleation mechanism, where association of tau with other intracellular components triggers aggregation. This reaction can be mimicked in vitro by contacting purified preparations of full-length recombinant tau protein with polyanions or anionic micelles, vesicles, or microspheres, with fibrillization proceeding over a period of hours (Perez, Hernandez et al. 2002; Chirita, Necula et al. 2003; Chirita and Kuret 2004). Immediately after contact, and well before filament formation, species of tau is present that can be detected by ThS, a non-covalent probe of β-sheet structure (Chirita and Kuret 2004). We have termed these species “intermediates” because of the timing of their appearance during the reaction time course and have proposed that they are the true substrates for the nucleation reaction. This interpretation predicts that tau fibrillization is triggered by conditions that support the adoption of assembly-competent conformations.

Here we test this hypothesis using full-length recombinant tau protein and anionic inducers of assembly. The data indicate that the stabilization of an intermediate folding state characterized by increased β-sheet structure is sufficient to trigger the fibrillization of full-length protein.
2.2 Materials and Methods

2.2.1 Materials. Recombinant His-tagged htau40 was prepared as described previously. (Carmel, Mager et al. 1996; Necula, Chirita et al. 2003) AA (Cayman Chemicals, Ann Arbor, MD) was dissolved in ethanol and stored at -80°C until used. Glutaraldehyde, uranyl acetate, and 300 mesh carbon-coated copper grids were from Electron Microscopy Sciences (Ft. Washington, PA). Stock solutions of thiazin red (TCI America, Portland, OR) and ThS (Sigma, St.Louis, MO) were prepared in water, whereas Congo red (City Chemical, West Haven, CT) and ANS (Sigma, St.Louis, MO) were prepared in DMSO and ethanol, respectively. Carboxylate-conjugated polystyrene microspheres (90 nm diameter, molecular area = 12 Å²/eq) were from Bangs Laboratories, Inc. (Fishers, IN).

2.2.2 Fibrillization assays. Under standard conditions, htau40 was incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) for up to 24 h in the presence or absence of fibrillization inducers (AA, carboxylate modified microspheres, ThS, Congo Red, thiazin red, or ANS). For analysis by EM, aliquots were removed, treated with 2% glutaraldehyde (final concentration), mounted on formvar/carbon-coated 300 mesh grids, and negatively stained with 2% uranyl acetate as described previously (King, Ahuja et al. 1999; Chirita, Necula et al. 2003). Random images were viewed in a Phillips CM 12 transmission electron microscope operated at 65 kV, captured on film at 8,000 – 60,000-fold magnification, digitized, and imported into Optimas 6.5.1 for quantification of filament lengths and numbers (King, Ahuja et al. 1999). Individual filaments ≥50 nm in length were counted manually.
2.2.3 ThS fluorescence measurements. Tau was aggregated at 37°C as described above except that the reaction contained 10 μM ThS. Resultant changes in fluorescence were monitored at $\lambda_{ex} = 440$ nm and $\lambda_{em} = 495$ nm in a FlexStation plate reader (Molecular Devices, Sunnydale, CA) operated at sensitivity 10, high PMT using black-matrix, clear-bottom 96 well isoplates (Wallac, Turku, Finland) and sealed with transparent foil (NUNC, Denmark).

2.2.4 Circular dichroism. Samples were prepared for CD by desalting (Bio-Rad Econo-Pac 10DG column) into 100 mM NaClO$_4$, 10 mM H$_3$BO$_3$ (pH 7.4). These buffer components were employed because of their transparency at far UV wavelengths (Schmid, Pridmore et al. 1997). Spectra were collected (186-260 nm) at 25°C with an AVIV model 202 CD spectrometer and a quartz cuvette with 1 mm path length. Four repetitive scans (4 s integration time, 1 nm step size, and 1 mm bandwidth) were recorded, averaged, and corrected for buffer only blank without additional filtering or smoothing. Raw CD signals (in millidegrees) were converted to mean residue molar ellipticity $[\theta]_M$ (with units of degree·cm$^2$/dmol) using the formula $[\theta]_M = [\theta]_{obs}/(10 \cdot l \cdot c \cdot n)$ where $[\theta]_{obs}$ is the observed ellipticity in millidegrees, $l$ is the length in centimeters, $c$ is the molar concentration of protein, and $n$ is the number of residues in the protein (Woody, Sugeta et al. 1996). Secondary structure composition of $\alpha$–helix (normal and distorted), $\beta$-strand (normal and distorted), turns and random coil were estimated from net spectra using CONTIN/LL (Provencher and Glockner 1981), CDSSTR (Krishnamurthy and Johnson 2004), and SELCON3 (Sreerama, Venyaminov et al. 1999) algorithms. Resultant estimates were averaged and presented ± S.D (Sreerama and Woody 2000).
2.2.5 Hydrodynamic Analysis. Analytical size-exclusion chromatography was performed (4°C) on a 24-mL (10 x 300 mm) Superose 6 10/300GL column equilibrated with buffer (10 mM HEPES, pH 7.0, 100 mM NaCl) and operated at .5 mL/min. Standards (Potschka 1987) included throglobulin (8.0 nm), equine apoferritin (6.2 nm), yeast alcohol dehydrogenase (4.6 nm), and bovine serum albumin (3.6 nm). Hydrodynamic radii were estimated from partition coefficients as described previously (Vancura, O'Connor et al. 1993).

Velocity sedimentation measurements were performed in linear 4-20% sucrose gradients containing 10 mM HEPES, pH 7.4, and 150 mM NaCl. Standards included bovine serum albumin (4.3 s), bovine erythrocyte carbonic anhydrase (3.2 s) myoglobin (1.9 s), and cytochrome C (1.6 s). After centrifugation (200,000g for 20 h at 4°C), gradients were fractioned and sedimentation coefficients were estimated as described previously (Vancura, O'Connor et al. 1993). Native molecular weight was calculated from hydrodynamic parameters as described previously (Siegel and Monty 1966) using a partial specific volume calibrated for tau amino acid sequence (Perkins 1986). Theoretical hydrodynamic radii for tau in naïve, molten globule, pre-molten globule, random coil, and denatured states were calculated from molecular weight using the empirical equations deduced by Tcherkasskaya et al (Tcherkasskaya, Davidson et al. 2003). Observed (i.e., apparent) frictional ratios \((f/f_0)_{obs}\) were calculated from hydrodynamic radii \(r_{hyd}\) by the equation (Siegel and Monty 1966):

\[
(f/f_0)_{obs} = r_{hyd}/[(3vM/4\pi N)^{1/3}]
\]

where \(v\) is the partial specific volume calculated as described above, \(M\) is the molecular weight and \(N\) is Avogadro’s number.
2.2.6 Analytical methods. Sigmoidal reaction progress curves were fit to a 3-parameter Gompertz function as described previously (Necula and Kuret 2004). Lag times, defined as the time when the tangent to the point of maximum polymerization rate intersects the abscissa of the sigmoidal curve (Evans, Berger et al. 1995), were calculated from the resultant Gompertz parameters (Necula and Kuret 2004).

Hyperbolic reaction progress curves were fit to the polynomial series (Philo and Selwyn 1973):

\[ y = \alpha + \beta t + \gamma t^2 + \ldots + \zeta t^6 \quad (2) \]

where \( y \) is fluorescence intensity at time \( t \), and the coefficient \( \beta \) approximates the initial velocity.

Concentration-response data were fit to a three parameter log normal function:

\[
y = ae^{\left( -0.5 \left( \ln \frac{x}{x_0} \right)^2 \right)} \quad (3)
\]

where \( y \) is the amount of product at ligand concentration \( x \), \( a \) is the maximum amount of product appearing at optimal concentration \( x_0 \), and \( b \) is a constant.

Nucleation statistics were fit to a Poisson distribution:

\[
P(n) = \frac{e^{-\lambda} \lambda^n}{n!} \quad (4)
\]

where \( P(n) \) is the probability of a microsphere randomly nucleating \( n \) filaments where the average number of filaments per microspheres is \( \lambda \).
2.3 Results.

2.3.1 Recombinant tau protein is an extended monomer in solution.

Authentic tissue derived tau proteins are asymmetric monomers when free in solution (Cleveland, Hwo et al. 1977). Conversely, it has been claimed that recombinant tau forms dimers at high concentration (Barghorn and Mandelkow 2002) in a reaction that is further favored by the presence of a poly-His tag (Yao, Tomoo et al. 2003). Dimerization has been postulated as a key pre-nucleation step in tau aggregation (Barghorn and Mandelkow 2002). In view of these conflicting claims, recombinant htau40 was subjected to hydrodynamic analysis using a combination of gel filtration chromatography and velocity sedimentation. Results show that under the near-physiological buffer conditions used for fibrillization experiments, recombinant htau40 is an asymmetric monomer with hydrophobic properties similar to authentic tau. (Table 2.1.)

Empirical formulae relating hydrodynamic radius to chain length have been derived for different folding states (Tcherkasskaya, Davidson et al. 2003). The utility of these equations for comparing proteins of differing chain lengths can be increased by converting hydrodynamic radii into frictional ratios \( f/f_0 \) as described in Experimental procedures. Comparison of the experimentally determined \( f/f_0 \) value for recombinant poly-His-tagged human tau40 with these theoretical predictions indicated that the preparation consists of a monomer in random coil conformational state at the start of the aggregation pathway, consistent with the behavior of tissue-derived porcine tau (Cleveland, Hwo et al. 1977) and recombinant non-his-tagged htau40 (Paudel 1997). (Figure 2.1)
2.3.2 Surface-mediated intermediate formation is first order with respect to tau concentration.

Monomers progress to polymer through an intermediate stage is characterized by an ability to bind ThS, a fluorescent probe of β sheet structure (Chirita and Kuret 2004). Because ThS fluorescence appeared immediately during the fibrillization lag time and also below the critical concentration of filament formation as assessed by electron microscopy and laser light scattering measurements, it was assumed to directly reflect the concentration of non-filamentous species. To further characterize these species, the time course of ThS fluorescence resulting from incubation of monomeric htau40 in the presence of carboxylated microspheres was followed in real time. ThS fluorescence appeared immediately upon contacting the microspheres and then increased hyperbolically as a function of time. In contrast, preincubation of tau with microspheres prior to the addition of probe resulted in immediate formation of ThS fluorescence, whereas incubation of tau with ThS alone (at low micromolar concentration) yielded only modest fluorescence (data not shown). These data confirmed that intermediates formed after contacting the microsphere surface and were not induced by the presence of low micromolar concentrations of probe.

Previously, intermediate formation was shown to be first order with respect to microsphere concentration (Chirita and Kuret 2004). To determine reaction order with respect to tau protein, the initial velocity of ThS fluorescence production was measured as a function of tau concentration. (Figure 2.2A) Because initial velocities were estimated during fibrillization lag time, the rate of appearance of ThS fluorescence was assumed to reflect the formation of pre-fibrillar species. Replots of this data in double log format
revealed a slope of 0.90 ± 0.02, indicating that the appearance of ThS fluorescence is first order with respect to protein concentration. (Figure 2.2B) These data suggest that the intermediate corresponds to a conformation of monomer. It further suggests that aggregation of tau into supramolecular complexes occurs after intermediate formation.

Nucleation-elongation reactions are characterized by a critical concentration, above which all additional protomers incorporate into filaments (Zhao and Moore 2003). It is the highest protein monomer concentration that does not support fibrillization, and therefore corresponds to the abscissa intercept of the tau concentration dependence curve. To determine the relationship between intermediate formation and filament critical concentration, the monomer concentration dependence of htau40 fibrillization and intermediate formation was determined in the presence of carboxylated microspheres using EM and ThS fluorescence assays, respectively. Fibrillization was directly proportional to tau concentration with an abscissa intercept of 2.06 ± 0.10 μM. This value is consistent with estimates made by laser light scattering methods (Zhao and Moore 2003; Necula and Kuret 2004; Necula and Kuret 2004) in the presence of anionic surfactants and indicates that the final equilibrium between filament ends and unincorporated tau is similar in the presence of diverse anionic inducers. The relationship between final ThS fluorescence intensity at equilibrium and tau concentration below the critical concentration for fibrillization was also linear under these conditions. In contrast to filament formation, however, the curve intersected the abscissa within one standard error of the estimate of the origin (0.03 ± 0.05 μM) indicating that intermediate formation did not have a critical concentration. (Figure 2.3) These data are consistent with the ThS–
reactive intermediate being a monomer that does not require a minimal concentration for formation.

2.3.3 Intermediate secondary structure.

The ability of the tau intermediate to bind ThS probe suggests that it contains increased β-sheet structure relative to natively unfolded recombinant starting material. To test this prediction, htau40 was incubated in the presence or absence of arachidonic acid inducer for 20 min and then subjected to circular dichroism spectroscopy. The analysis was conducted at the critical concentration and within fibrillization lag time to ensure that mature filaments did not contribute to circular dichroism signal. In the absence of arachidonic acid, full-length htau40 yielded a CD spectrum typical of unfolded proteins with a broad minimum of elipticity centered at 195 nm and a shoulder at 220 nm. Molar elipticities at 200 and 222 nm were -13538 and -3236 respectively, consistent with theoretical studies showing that the coiled state can accommodate substantial local secondary structure (Fitzkee and Rose 2004). The presence of arachidonic acid led to an increase in the \([\theta]_{222}/[\theta]_{200}\) ratio, consistent with intermediate formation being accompanied by an increase in secondary structure and a loss in random coil (Figure 2.4A). The final elipticity values at 222 and 200 nm were characteristic of the pre-molten globule folding state (Uversky and Fink 2004). The amounts of secondary structure were estimated by fitting CD spectra to three independent algorithms as described in Experimental procedures. In the absence of inducer, htau40 adopted 5.5 ± 1.4% alpha helix, 25.0 ± 3.9% beta strand and 69.4 ± 3.6% random coil/turn conformation. These values were generally similar to those found at room temperature for bovine tau isolated without harsh heat or acid treatments (Ruben, Ciardelli et al. 1997). Inducer converted an
additional 7.1 ± 1.2 % of residues to beta conformation at the expense of random coil/turn conformation (Figure 2.4B). Because the proportion of the preparation in partially folded conformation is unknown, this increase must be considered a lower limit. Apparent increase in alpha helix content were not significant at p< 0.05. These data suggest that the tau intermediate is a partially folded monomer enriched in β-sheet content relative to the starting condition.

2.3.4 Intermediate tertiary structure.

In addition to its secondary structure, the pre-molten globule state is characterized by a partially collapsed structure with a loosely packed hydrophobic core. To confirm that the intermediate had partially folded character, htau40 was incubated in the presence or absence of arachidonic acid inducer for 24 h and then examined for an ability to bind ANS, a fluorescent probe of surface-exposed hydrophobic patches (Semisotnov, Rodionova et al. 1991; Chaffotte, Guijarro et al. 1997; Schonbrunn, Eschenburg et al. 2000). The analysis was conducted below the critical concentration to ensure that filaments did not contribute to ANS fluorescence. ANS in buffer alone fluoresced weakly at an optimum wavelength of $\lambda_{em} = 500$ nm. In contrast, ANS in the presence of intermediates (prepared by incubation of htau40 with AA inducer) fluoresced brightly with a blue-shifted optimum of $\lambda_{em} = 487$ nm (Figure 2.5). The increase in fluorescence intensity was not fully recapitulated by either tau protein alone or by AA micelles formed from incubation with protamine, indicating that the enhanced fluorescent signal observed in the presence of tau and AA came primarily from binding to altered conformations of tau. Taken together, the above data suggest that anionic inducers stabilize monomeric tau
in a partially folded, β-sheet enriched conformation, and that this conformation precedes the polymerization reaction.

### 2.3.5 Intermediate stabilization triggers tau fibrillization.

We hypothesize that formation of the intermediate conformation is necessary and sufficient for triggering fibrillization. If so, then conditions that stabilize the intermediate should induce tau fibrillation. To test this prediction, htau40 was incubated with increasing concentrations of ThS, a dye that binds tightly ($K_D \sim 1\mu M$) to the intermediate conformation (King, Ahuja et al. 1999; Chirita and Kuret 2004). Other dyes known to interact with β-sheet structure were investigated as well, including Congo Red (Klunk, Pettegrew et al. 1989) and thiazin red (Mena, Edwards et al. 1995). Linkage theory (Wyman and Gill, 1990) predicts that rising dye concentrations drive tau monomer equilibrium toward the intermediate conformation by mass action, and then toward the filamentous state through positive polysteric linkage with the self-association reaction (linkage refers to the influence of ligand binding upon other equilibria including aggregation reactions and phase transitions). Results confirmed that Congo Red, thiazin red, and ThS were all capable of inducing tau fibrillization independently of anionic inducers. Dye-induced filaments differed from anion-induced filaments in morphology, growing as twisted ribbons with maximum widths of $25 \pm 4$ nm, minimum widths of $11 \pm 2$ nm, and half periodicity of $170 \pm 39$ nm ($n = 42$) during time periods $\leq 24$ h. In contrast, straight filaments formed over identical time periods with arachidonic acid had a consistent width of $13 \pm 2$ nm ($n = 45$) and no twist. Moreover, dye-induced filaments were greater in number and shorter in length than arachidonic acid suggesting that dyes were especially efficacious inducers of nucleation. Although capable of binding the
partially folded structure of the intermediate, ANS did not induce fibrillization at concentrations up to 500 μM, indicating that the linkage reaction was selective for planar aromatic dyes known to bind to β-sheet structure (Figure 2.6). Congo Red was the most potent and efficacious inducer tested, with concentrations as low as 10 μM yielding large numbers of filaments. ThS treatment also lead to large numbers of small filaments, but its potency was ten-fold lower than Congo Red. Thiazin red was the least efficacious ligand tested, yielding few filaments of relatively long length at optimal concentrations. Nonetheless, these filaments were most amenable to experimentation (because they were fewer and longer in length) and so thiazin red was used for detailed characterization experiments described below.

To quantify the potency of the reaction htau40 was incubated with varying concentrations of thiazin red and assayed for fibrillization using quantitative EM. Inducer activity followed a biphasic log-normal concentration response curve with an optimum potency of 114 ± 2 mM and 115 ± 3 μM in the presence of 1 and 2 μM htau40 respectively (Figure 2.7). Congo Red followed a similar profile, although the optimum appeared ~10 fold lower (data not shown). These data indicate that, unlike anionic inducers, dye-mediated fibrillization reactions do not depend on tau/inducer ratios.

**2.3.6 Intermediate stabilization promotes filament nucleation.**

To determine whether thiazin red influenced filament nucleation rates, the time course of htau40 fibrillization was followed by EM over a period of up to 24 h in the presence of 50 and 100 μM thiazin red at 37°C and no agitation. When incubated at htau40 concentrations ≤ 1 μM, reaction progress curves were sigmoidal with clear lag, exponential growth, and equilibrium phases. As thiazin red concentrations were raised
from 50 to 100 μM in the presence of 0.8 μM htau40, lag times decreased from 0.73 ± 0.09 h to 0.30 ± 0.04 h (Figure 2.8). These data suggest that acceleration of tau fibrillization with dye inducers results from increased rates of filament nucleation.

To confirm this observation, tau fibrillization induced by anionic microspheres was examined in the presence and absence of thiazin red. Nucleation events were counted as the number of filaments formed on the surface of each microsphere. In the absence of thiazin red ~80% of beads had no filaments, ~20% had one filament, and a small percentage had 2 filaments. Beads with three or more filaments were never observed (n =100). The data fit a Poisson distribution (averaging 0.19 filaments/microsphere) indicating that nucleation events were random and independent of each other. Addition of thiazin red to 50 μM concentration shifted the Poisson distribution so that the average microsphere contained 1.95 filaments. Further increases in thiazin red concentration to 100 μM raised the average still higher, to 2.17 filaments/microsphere (Figure 2.9). Together these data suggest that intermediate stabilization had a large positive effect on nucleation rate, and that this effect was additive with anionic inducers.

2.3.7 Effects of intermediate stabilization on critical concentration.

In the presence of thiazin red, htau40 fibrillized at submicromolar concentrations, far below the critical concentration estimated in the presence of anionic inducers. To clarify the issue, critical concentrations were estimated as a function of thiazin red concentration. Results show extremely low critical concentrations of 0.20 ± 0.07 and 0.21 ± 0.05 at 50 and 100 μM thiazin red respectively (Figure 2.10). These data indicate that thiazin red treatment led to critical concentrations 10-fold lower than did anionic
inducers, and the presence of dyes influenced post-nuclear equilibria in addition to nucleation rate and filament morphology.

2.3.8 Thiazin red induces fibrillization in monomeric form.

Although anionic inducers such as arachidonic acid are monomers below 200 mM in aqueous solution, the presence of protein such as tau triggers the rapid formation of micellar aggregates in which fibrillization inducing activity resides (Chirita, Necula et al. 2003). Planar aromatic dyes also self-assemble to form supramolecular aggregates at high concentration (Stopa, Gorny et al. 1998; Stopa, Piekarska et al. 2003), suggesting that formation of anionic micelles may also mediate the dye inducer activity. To test this hypothesis, the absorbance properties of thiazin red were measured in the presence or absence of tau protein. Dye aggregation is accompanied by characteristic shifts in absorbance optima depending on the structure of the aggregate formed. In methanol, where dye aggregation is not supported, thiazin red absorbance was characterized by a major peak centered at 514 nm and a shoulder at 548 nm (Figure 2.11). This pattern is characteristic of hydrazone tautomers of 1-naphthol-2phenylazo derivatives such as thiazin red. The pattern did not change significantly in the presence of aqueous buffer with or without tau protein. These data suggest that thiazin red does not form detectable supramolecular complexes under assay conditions, and that the active form of the dye is a planar monomer.
2.4 Discussion.

2.4.1 Triggers of tau fibrillization.

Isolated tau proteins have the hydrodynamic properties of random coil monomers in solution, consistent with their equilibrium conformation being “natively unfolded”. Despite the lack of organized tertiary structure, tau monomers do not assemble spontaneously under physiological conditions over experimentally tractable time periods. The barrier to aggregation can be overcome \textit{in vitro} through truncation of the tau molecule to short peptides within the microtubule binding region (the region the modulates fibrillization \textit{in vivo}) Indeed, peptide fragments ranging from as small as two amino acids (Goux, Kopplin et al. 2004) to as large as four full repeats (Schweers, Mandelkow et al. 1995; Arrasate, Perez et al. 1999; Perez, Hernandez et al. 2002) aggregate spontaneously to form amyloid filaments over a period of days when incubated at high concentrations (>150 μM). Spontaneous assembly becomes progressively less efficient as the length of tau increases toward full-length isoforms (Schweers, Mandelkow et al. 1995), eventually requiring extremes of ionic strength, pH, temperature, and tau concentrations (Crowther, Olesen et al. 1994). At near physiological buffer conditions and tau concentrations (1-10 μM), aggregation of full-length isoforms becomes still less favorable and virtually undetectable over a period of weeks (King, Ahuja et al. 1999). These data suggest that the rate limiting barrier for aggregation of full-length tau isoforms is conformational in nature.

Although truncation reduces the conformational barrier to aggregation, the macroscopic pathway observed in authentic tissue indicates that the initial steps of aggregation involve primarily full-length tau isoforms (Horowitz, Patterson et al. 2004).
What triggers fibrillization of full-length tau under physiological conditions? In the case of anionic inducers it appears to be stabilization of monomeric intermediates characterized by increased β-structure (as detected by circular dichroism and binding of the fluorescent probe ThS), and a partially collapsed tertiary structure (as detected by binding of the fluorescent probe ANS) resembling the pre-molten globule state. Similar states mediate the fibrillization of α-synuclein and amylin, suggesting a commonality in the mechanism of fibrillization of natively unfolded proteins. However, not all intermediates may be thioflavin dye reactive, and not all reactive intermediates are monomers. For example, aggregation of transthyretin monomer initiated by shifting supersaturated solutions to acidic pH yields immediate formation of thioflavin dye reactivity as found here for tau protein (Hurshman, White et al. 2004). In the case of transthyretin, however, thioflavin dye reactivity appeared in tandem with protein oligomers rather than monomers. Consistent with the reactive species being multimeric, the initial velocity of thioflavin dye fluorescence increased as a power function of protein concentration. These data indicate that the intermediates involved in amyloid formation may be structurally diverse.

In the current work, tau assembly intermediates appeared in the presence of anionic surfactants and microspheres, but it is likely that other tau fibrillization inducers act similarly. For example, treatment of tau with the polyanions inducer heparin modulates its ability to serve as a substrate for protein kinases, indicating a change in conformation upon binding (Paudel and Li 1999). In addition solvent additives such as urea that induce fibrillization of full-length tau proteins (Montejo de Garcini and Avila 1987; Montejo de Garcini, Carrascosa et al. 1988) also modulate the formation of
assembly-competent conformations of insulin (Ahmad, Millett et al. 2004). In both cases activity resides with urea but not guanidine. Together these data are consistent with the stabilization of assembly-competent intermediate conformations being intimately connected with triggering of fibrillization.

The conversion from natively unfolded tau to assembly competent intermediate has the characteristics of an allosteric transition. Initially, natively unfolded htau40 is an extended monomer containing ~25% of residue in beta strand conformation. In the presence of anionic surfactant inducer, htau40 adopts a condensed conformation containing additional beta conformation. In addition to anionic surfactants, tau fibrillization can be induced by planar aromatic ligands complementary to \( \beta \)-sheet structure such as Congo Red, thiazin red and ThS. In contrast, the non-planar anionic aromatic dye ANS, which has affinity for condensed folding states including pre-molten globule but not \( \beta \)-sheet structure, did not induce tau fibrillization even at concentrations up to 500 \( \mu \)M. These data suggest that the formation of additional \( \beta \)-sheet structure in the intermediate is a key requirement in triggering nucleation. It cannot be excluded, however, that certain structural elements interfere with nucleation and are resolved by intermediate formation.

### 2.4.2 The tau aggregation pathway.

Once \( \beta \)-sheet enriched intermediates are populated, tau aggregates spontaneously in a reaction characterized by a lag time and critical concentration. In terms of aggregation kinetics, intermediate formation leads to shorter lag times reflecting increased rates of nucleation. Because intermediate formation is rapid and
intramolecular, oligomerization (including dimer formation) appears to be a secondary reaction potentially related to nucleation.

How does intermediate stabilization promote filament nucleation? Hypothetical models of amyloid fibrils constructed from fiber diffraction patterns provide potential clues. Intramolecular refolding models (Perez, Hernandez et al. 2002), where amino acid residues of a single protein protomer form parallel and cylindrical $\beta$-sheets, are consistent with monomeric nucleus cluster sizes determined from nucleation rates (Gamblin, Berry et al. 2003). In this case, the rate-limiting intramolecular conformational change is necessary and sufficient for the elongation reaction to proceed spontaneously. In other words, intramolecular refolding is coincident with nucleation. In contrast, the intermolecular association of $\beta$-sheets predicted by the zipper-spine models (Ivanova, Sawaya et al. 2004) suggests that nucleation of this structure involves protein oligomerization to form a stable unit before elongation is supported. Because the tau nucleus is oligomeric (Friedhoff, von Bergen et al. 1998), tau filaments are better modeled by the zipper-spine model than the intramolecular folding model put forth for proteins containing poly-L-glutamine tracts. We propose that tau nucleation corresponds to an oligomerization reaction involving the face-to-face packing of beta sheet segments orthogonal to the fiber axis (Diaz-Avalos, Long et al. 2003; Ivanova, Sawaya et al. 2004). Seen in this way, one major role of intermediate formation is to prearrange $\beta$-sheet structure for incorporation into the oligomeric nucleus. Once nucleated, the nascent filament lengthens by addition of protomers parallel to the filament axis. Tissue-derived tau filaments achieve a final $\beta$-sheet content of $57 \pm 7\%$ (Berriman, Serpell et al. 2003). This value, which is an average from mixed isoforms and presumably overestimated
owing to limited proteolysis (Horowitz, Patterson et al. 2004), suggests that β-structure extends far beyond the ~90 amino acid residue core of paired helical filaments (Novak, Kabat et al. 1993). These data are consistent with the progression from natively unfolded full-length monomer to intermediate and finally filament conformation being characterized by continuously increasing β-sheet content.

*In vitro*, tau filament morphology varies with isoform (Hasegawa, Crowther et al. 1997) and primary structure (Perez, Hernandez et al. 2002). The data presented herein indicates that morphology also depends on the nature of the inducer. Anionic surfactants and microspheres induce primarily untwisted 13 nm wide filaments from htau40 over early time periods and these transition to twisted filaments with the mass per unit length of paired helical filaments over a period of days (King, Ghoshal et al. 2001). In contrast, thiazin red induced twisted ribbons with a maximal width of 25 nm from the earliest time points. The factors influencing amyloid filament morphology are not fully understood. In the case of β2-microglobulin (Perez, Hernandez et al. 2002) and immunoglobulin light chain variable domain (Khurana, Gillespie et al. 2001), filament morphology is linked to the conformational properties of the precursor state. Differences in tau filament morphology in the presence of different inducers may reflect similar considerations. In fact, it is conceivable that nucleation rate, filament morphology, and critical concentration all depend on the nature of the fibrillization inducer, owing in part to the differences in the fine structure of the intermediate species they stabilize. Alternatively, dyes may incorporate into filaments and thereby influence morphology at other stages of assembly. Because tau filaments develop over a period of decades (Morsch, Simon et al. 1999), the final conformation of filaments *in vivo* (i.e., paired helical filaments) may
reflect free energy considerations (Turner, Briehl et al. 2003) rather than just the nature of the inducer or structure of the assembly intermediate.

2.4.3 Triggers and enhancers.

What triggers fibrillization of full-length tau isoforms in sporadic disease? The endwise associations of tau filaments with intracellular membranes in biopsy specimens of AD tissue (Gray, Paula-Barbosa et al. 1987) suggest that anionic surfaces may present pathophysiologically relevant sources of triggering activity. But as shown here, planar aromatic dyes also can trigger the fibrillization reaction, perhaps mimicking the activity of naturally occurring small molecules (Santa-Maria, Hernandez et al. 2004). Indeed, small molecule metabolites can induce the fibrillization of proteins other than tau (Goldbaum, Oppermann et al. 2003), suggesting that additional sources of exogenous triggering may exist. Moreover, tissue derived tau contains multiple posttranslational modifications, and these too may trigger the aggregation reaction. For example, phospho-tau isolated from AD brain spontaneously aggregates in vitro in a reaction that is antagonized by phosphatase treatment (Alonso, Zaidi et al. 2001). However, these experiments require pretreatment with urea, a known modulator of intermediate structure. (Ahmad, Millett et al. 2004) In fact, both phosphorylation and glycation enhance tau fibrillization at the elongation step (Necula and Kuret 2004). Acting in this way, enhancers stabilize filaments and increase the driving force for nucleation without necessarily triggering the reaction. In terms of the zipper-spine model, triggers and enhancers are predicted to differentially affect the equilibria orthogonal and parallel to the filament axis, respectively. It is likely, therefore, that alternative splicing, mutation, and posttranslational modifications will have differential effects on the nucleation and
extension phases of the tau aggregation reaction. It will be useful to characterize these modulators at the level of intermediate formation.

2.4.4 Pharmacological considerations.

Tau filament formation is a robust marker of degeneration, and so is an attractive agent for premortem diagnostic development. Small molecule ligands capable of binding amyloid conformation with high affinity may be useful for this purpose (Zhuang, Kung et al. 2003). The data presented here, however, suggest that reagents such as these must be used with care owing to their ability to drive the fibrillization reaction. The high concentrations (>25 μM) required for dyes such as ThS to drive fibrillization suggest the existence of a preequilibrium, where binding to natively unfolded tau is slow because few binding sites are prearranged. It will be important to determine whether binding affinity for filaments can be separated from affinity for intermediate states, or whether linkage with the nucleation reaction can be severed.

2.4.5 Conclusions.

Protein aggregation is controlled by conformational and colloidal stability in solution, either of which can be rate limiting for amyloid formation depending on conditions (Ferrari, Hoerndli et al. 2003). Despite being natively unfolded, similar considerations apply to tau protein. Under physiological conditions, the equilibrium conformation of full-length htau40 is not assembly competent, and so the fibrillization reaction is triggered by those agents or mutations that stabilize assembly competent conformations. Triggering activity is characterized by greatly accelerated nucleation rates. In contrast, posttranslational modifications that stabilize the filamentous state by promoting the elongation reaction act as enhancers. These are characterized by decreases
in critical concentration without acceleration of the nucleation reaction. Evolving models of amyloid structure suggest a physical basis to these observations.
2.5 Tables

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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<tbody>
<tr>
<td>Hydrodynamic Radius (nm)</td>
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</tr>
<tr>
<td>Sedimentation coefficient (s_{20,w})</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Native molecular mass (Da)(^a)</td>
<td>45,000 ± 8,000</td>
</tr>
<tr>
<td>Native molecular mass (Da)(^b)</td>
<td>47,881</td>
</tr>
<tr>
<td>Native structure</td>
<td>monomer</td>
</tr>
<tr>
<td>((f/f_0)_{\text{obs}})</td>
<td>2.32 ± 0.39</td>
</tr>
<tr>
<td>((f/f_0)_{\text{shape}})</td>
<td>1.95 ± 0.33(^c)</td>
</tr>
<tr>
<td>Axial ratio (prolate ellipsoid)</td>
<td>~19:1</td>
</tr>
</tbody>
</table>

\(^a\)Estimated from hydrodynamic parameters  
\(^b\)Estimated by mass spectroscopy  
\(^c\)Corrected for hydration assuming 0.5 g water/g protein

Table 2.1: Recombinant htau40 physical properties
2.6 Figures.

Figure 2.1. Tau protein is a monomer with random coil character.

The \(\frac{f}{f_0}\)\(_{\text{obs}}\) (observes frictional ration) estimated for recombinant poly-His-tagged htau40 by hydrodynamic methods (○; Table 2.1) was compared to theoretical values (■) for the protein in six folding states (1, native; 2, molten globule; 3, pre-molten globule; 4, random coil; 5, denatured in 8 M urea; and 6, denatured in guanidine HCl) calculated on the basis of empirical correlations between hydrodynamic radius and chain length. (Tcherkasskaya, Davidson et al. 2003) Each folding state is depicted as a category arbitrarily placed at equal intervals on the abscissa, whereas the solid line is drawn solely to aid visualization. The relative errors for calculated \(\frac{f}{f_0}\)\(_{\text{obs}}\) values are reportedly \(\leq 0\%\). (Tcherkasskaya, Davidson et al. 2003) The hydrodynamic behavior of the tau preparation used throughout this work was characteristic of a monomer in random coil state.
Figure 2.2. Intermediate formation is first order with respect to tau concentration.

(A) The time course of ThS fluorescence formation resulting from incubation (37°C) of htau40 at 0.5 (□), 1 (■), 2 (○), and 4 μM (●) in the presence of carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM) was followed by fluorescence spectroscopy. Each point represents a fluorescence measurement at time $t$, whereas solid lines represent best fit of the data points to a sixth power polynomial series. The rate and extent of ThS fluorescence increased with increasing tau concentrations. (B) Replot of the initial velocities ± SE estimated from the progress curves shown in panel A versus tau concentrations in double logarithmic format, where the line represents best fit of the data points to a linear regression. The slope of the replot being (0.90 ± 0.02) was consistent with the initial velocity of ThS signal generation being directly proportional to tau concentration under these experimental conditions.
Carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM) were incubated (24 h at 37 °C) with varying concentrations of htau40 (0-4 μM) in the presence of ThS and then assayed for fibrillization by EM (○) and intermediate formation by ThS fluorescence (●). Each data point represents the mean ± SD of triplicate determinations whereas the solid lines represent best fit of the data points to a linear regression. In the case of fibrillization, the regression line intercepted the abscissa at 2.06 ± 0.10 μM htau40, indicating the existence of a critical concentration. In contrast, ThS fluorescence was detectable below the critical concentration of fibrillization, with the regression line intersecting the abscissa at the origin. Whereas fibrillization was characterized by a critical concentration, appearance of ThS fluorescence was not.

Figure 2.3. Critical concentration for tau filament formation and intermediate formation in the presence of anionic microspheres.
Figure 2.4. Intermediate formation is accompanied by changes in secondary structure.

Htau40 (2.0 μM) was incubated (20 min at 25°C) in 100 mM sodium perchlorate, 20 mM boric acid, pH 7.4 in the presence (●) or absence (○) of 50 μM arachidonic acid inducer, then immediately subjected to far-UV CD spectroscopy (37°C). (A) Intermediate formation was accompanied by an increase in [θ]_{195} and a decrease in [θ]_{220}, consistent with the formation of secondary structure. (B) CD spectra were individually fit by CDSTTR, SELCON3, and CONTIN/LL algorithms to calculate estimates of total α-helical, β-strand and random coil/turn conformation. The three estimates were then averaged and plotted ± SD for htau40 in the presence (black bars) or absence (white bars) of AA inducer. Intermediate formation was accompanied by increases in β-conformation and decreases in random coil/turn conformation. *, p< 0.05, comparing two populations.
Figure 2.5. Effects of intermediate formation on ANS fluorescence spectra.

Reactions containing assembly buffer and no additions (●), 1.9 μM htau40 (■), 1.9 μM htau40 and 50 μM AA inducer (□), or 0.8 μM protamine and 50 μM AA inducer (○) were prepared. After incubation (24 hours at 25°C), aliquots were removed, treated with 20 μM ANS (final concentration), and immediately subjected to fluorescence spectroscopy (λ<sub>ex</sub> = 350 nm, λ<sub>em</sub> = 400-600 nm). ANS in buffer alone fluoresced weakly at an optimum wavelength of λ<sub>em</sub> = 500 nm (●). In contrast, intermediates prepared by incubation of htau40 with AA inducer fluoresced brightly with a blue shifted optimum of λ<sub>em</sub> 487 nm (□). Neither tau without inducer (■) nor AA micelles formed in the presence of protamine (○) were capable of recapitulating the ANS fluorescence enhancement observed in the presence of htau40 with AA inducer, indicating that ANS binds preferentially to tau intermediates.
Figure 2.6. Planar aromatic dyes trigger tau fibrillization.

Htau40 (4 μM) was incubated (24 hours at 37°C) under standard conditions with (A), 25 μM Congo Red (CR); (B), 100 μM Thiazin red (TR); (C), 100 μM ThS; (D) 75 μM AA (3h at 37°C); or (E) 100 μM ANS. Planar aromatic dyes with known ability to bind β-sheet structure such as Congo Red, Thiazin red, and ThS induced the formation of tau filaments with twisted ribbon morphology (see Figure 2.9 for higher magnification image) clearly distinct from the straight filaments induced by AA. In contrast, the aromatic dye ANS, which binds hydrophobic clusters in the partially folded intermediate, did not have inducer ability when tested in the range of 5-500 μM. These data indicate that dyes complementary to β-sheet structure induce tau fibrillization, and that the filament morphology observed reflects the fibrillization inducer employed. Bar = 100 nm.
Figure 2.7. Potency of thiazin red dependent tau fibrillization.

The dependence of tau fibrillization equilibrium on thiazin red concentration (1-1000 μM) was determined in the presence of 1 (○) or 2 μM (●) htau40 after 24 hour incubation at 37°C by quantitative electron microscopy. Each data point represents the mean ± SD of triplicate determinations, whereas solid lines represent best fit to a log-normal distribution. The concentration response curves were biphasic, with an optimum centered on approximately 114 μM.
Figure 2.8. Thiazin red modulates tau nucleation rate.

The time course (37°C) of htau40 (0.8 μM) fibrillization in the presence of 50 (○) or 100 (●) μM thiazin red was determined by EM. Each data point represents total filament length/field ($\Gamma_f$) as a function of time whereas each sigmoid curve represents the best fit of the data points to a three-parameter Gompertz function. Lag times calculated as described in Analytical methods were 0.73 ± 0.09h and 0.30 ± 0.04h for 50 and 100 μM thiazin red, respectively. The concentration dependence of lag time was consistent with thiazin red increasing the rate of tau filament nucleation.
Figure 2.9. Thiazin red increases frequency of heterogeneous nucleation.

Carboxylate-substituted polystyrene microspheres (90 nm diameter, 11.6 Å²/eq molecular area; 124 pM) were incubated (24 hours at 37°C) with htau40 (4 μM) in the (A), absence or (B), presence of 50 μM thiazin red then visualized by electron microscopy. In the absence of thiazin red, microspheres induced formation of straight filaments, with frequency rarely exceeding one nucleation event per bead. In contrast, the presence of thiazin red increased nucleation frequency, so that multiple filaments per bead were a common occurrence. Note the change in filament morphology to twisted ribbon in the presence of thiazin red. (C), Statistical distributions of the number of nucleation events per bead were then calculated for reactions conducted in the presence of 0, 50, and 100 μM thiazin red (TR). Each bar represents the number of nucleations (filaments) per bead normalized as percent frequency, whereas each solid line represents best fit of the data to the Poisson distribution. Thiazin red increased nucleation frequency so that more multiple filaments per bead were observed. Bar = 100 nm.
Figure 2.10. Critical concentration of tau fibrillization in the presence of thiazin red.

The dependence of fibrillization equilibrium on tau concentration (0.1-2 μM) was determined in the presence of 50 (○) or 100 μM (●) thiazin red after 24 hours incubation at 37°C by quantitative electron microscopy. Each data point represents the mean ± SD of triplicate determinations, whereas the solid lines represent best fit to a linear regression. Critical concentrations determined from the abscissa intercepts of regression lines were 0.02 ± 0.07 at 50 and 0.21 ± 0.05 at 100 μM thiazin red respectively.
Figure 2.11. Thiazin red induces tau fibrillization in monomeric form.

Thiazin red was dissolved in either methanol (●), assembly buffer (○), or assembly buffer containing 4 μM htau40 (■), incubated for 24 hours at 37°C, and finally subjected to absorbance scans in the range of 400-600 nm. All curves showed an absorbance maximum centered on 514 nm with a shoulder at 548 nm, suggesting that thiazin red did not aggregate into supermolecular complexes under standard assay conditions.
CHAPTER 3

3. TAU FIBRILLIZATION OCCURS VIA A PSEUDOHOMOGENEOUS NUCLEATION MECHANISM: THE IMPORTANCE OF DIMERIZATION AND AN ESTIMATE OF ELEMENTARY RATE CONSTANTS

3.1 Introduction.

Under normal conditions, the microtubule associated protein tau is highly concentrated in axons contributing to the stability and dynamic spacing of the cytoskeleton. However, in Alzheimer’s disease and other tauopathies, the binding partnership between tau and microtubules is abolished and insoluble filamentous aggregates form, which fill the neuronal cell bodies and processes. These filaments display the parallel, in register cross-beta structure common to amyloid forming proteins (Kidd 1963; Yagishita, Itoh et al. 1981; Barghorn, Davies et al. 2004). Development of neurofibrillary lesions in AD follows a highly stereotyped pattern (Braak and Braak 1991) and correlates with both neurodegeneration and cognitive decline (Gomez-Isla, Hollister et al. 1997). As a result, tau fibrillization is a potent marker for disease related events at the molecular and cellular levels, regardless of its role as a potential mediator of degeneration. Though lesion formation has been well studied at the macroscopic level, the tau polymerization pathway remains elusive. Because Alzheimer’s disease is primarily sporadic with significant environmental contributions to relative risk, understanding the mechanism through which filaments assemble may provide molecular clues as to the initiating stresses affecting neurons in early stage disease.
Protein aggregation can occur via a number of distinct mechanisms. Like many other proteins involved in deposition diseases including amyloid-β (Lomakin, Chung et al. 1996), α-synuclein (Wood, Wypych et al. 1999) and poly-L-glutamine repeats (Chen, Ferrone et al. 2002), tau is thought to polymerize through a nucleation-elongation mechanism (Friedhoff, von Bergen et al. 1998). In this mechanism, reaction rate is limited by the formation of a thermodynamically unstable species termed the nucleus. Once this critical nucleus cluster size is reached, however, subsequent additions to the nascent filament end are energetically favorable and elongation proceeds efficiently. The classical homogeneous nucleation mechanism is characterized by the spontaneous aggregation of monomers, with a lag time followed by exponential polymer growth, a critical monomer concentration below which polymer formation is not supported, and the ability of preformed seeds to overcome the initial lag time. Additionally, homogeneous nucleation results in a near-Poisson distribution of filament lengths at early time points and a shift towards exponential distributions at equilibrium.

Application of homogeneous nucleation theory to tau aggregation has been hindered, however, by the inability of full-length tau to spontaneously associate even at highly supersaturated concentrations (King, Ahuja et al. 1999). This issue can be overcome through the addition of polyanions such as heparin (Goedert, Jakes et al. 1996) or micelle forming anionic surfactants such as arachidonic acid (Chirita, Necula et al. 2003). These compounds appear to induce polymerization through a heterogeneous nucleation mechanism, where the exogenous substance promotes nucleation on its surface. Addition of anionic inducers also increases the efficiency of seeding reactions, indicating that they promote conformational changes in full-length protein in addition to
facilitating nucleation (Friedhoff, von Bergen et al. 1998; King, Ahuja et al. 1999). As a result of the complex nature of these interactions, use of anionic macromolecules for detailed characterization of tau polymerization has been problematic.

Recently, we demonstrated that the kinetic barrier to spontaneous polymerization in full-length can be overcome through the addition of small molecule agonists. Planar aromatic dyes such as thiazin red, Congo red, and ThS, which bind β-sheet structure with high affinity, are capable of stabilizing assembly competent conformations and therefore drive aggregation. These agents are capable of triggering fibrillization at submicromolar concentrations, in the absence of mutations, truncations, or the need to form large tau-inducer complexes (Chirita, Congdon et al. 2005). These findings suggest that one of the rate limiting steps in tau aggregation is the adoption of a partially folded, β-sheet enriched conformation. Nucleation represents a subsequent bottleneck in the reaction, and as yet the nature of the nucleus remains unclear for full-length protein.

Further difficulties arise when other potential polymerization mechanisms are taken into account. For example, in addition to homogeneous and heterogeneous nucleation, protein filaments may also assemble via downhill polymerization or secondary nucleation. Downhill polymerization represents the simplest potential reaction mechanism. In this mechanism nuclei do not exist in thermodynamic equilibrium with monomer, but rather are formed by slow stepwise addition of monomers or oligomers. (Librizzi and Rischel 2005) This mechanism had been proposed for at least one amyloid forming protein, transthyretin (Hurshman, White et al. 2004), as well as tubulin (Flyvbjerg, Jobs et al. 1996). Secondary nucleation represents another alternative to the classical nucleation-elongation mechanism in which protein fibrils are capable of
promoting nucleation of subsequent fibrils through budding, branching or filament breakage. Hemoglobin (Ferrone, Ivanova et al. 2002) and insulin (Librizzi and Rischel 2005), another amyloid forming protein, are thought to assemble primarily through secondary nucleation once the first fibrils are formed. Because alternative mechanisms exist, methods to differentiate between them must be utilized. To this end, analysis of polymerization kinetics provides a solution. For example, in the case of downhill polymerization, initial reaction rate is dependent on the number of slow steps required for nucleus formation. As a result of this when plotted against time squared the increase in polymer mass will be described by a power function, the exponent of which will be equal to the number of slow aggregation steps (Flyvbjerg, Jobs et al. 1996). In the case of homogeneous nucleation, however, polymer mass will be directly proportional to time squared and initial velocity plots will be linear (Librizzi and Rischel 2005). In the case of secondary nucleation, different concentrations of protein produce different exponent values (Librizzi and Rischel 2005). One can also utilize length distribution to differentiate between reaction mechanisms. In the case of secondary nucleation, both the average filament length and slope of the length distribution should remain constant over time (Briehl, Mann et al. 1990). However, theory predicts different behavior in the case of homogeneous nucleation. The mode height of the distribution is predicted to drop as a function of time and also experience a transient shift. Finally, as the reaction progresses, distributions will become skewed towards longer lengths (Hall and Minton 2004).

Here we utilize thiazin red to characterize the aggregation pathway for full-length tau under near physiological buffer conditions and reducing environment. Data indicate that under these conditions, tau assembly can be approximated by a homogeneous
nucleation mechanism with monomer addition. Through the application of homogeneous nucleation theory to tau polymerization we have provided the first estimates of nucleus cluster size for full-length tau, as well as a measure of elementary rate constants for nucleation and elongation. These data indicate which steps are rate limiting in the reaction and must be overcome for polymerization to occur.

3.2 Materials and Methods.

3.2.1. Materials. Recombinant histidine tagged human tau40 was prepared as previously described. Glutaraldehyde, uranyl acetate, and 300 mesh formvar carbon-coated electron microscope grids were purchased from Electron Microscopy Sciences (Ft. Washington, PA) Thiazin red was obtained from TCI America (Portland, OR). The mathematical simulation program JACOBIAN was generously provided by Numerica Technology (Cambridge, MA).

3.2.2. Electron microscopy and STEM. Htau40 (0.4μM, 0.5μM, 0.6μM, 0.8μM & 1 μM) was incubated at 37 °C without agitation in assembly buffer consisting of 10mM HEPES, 100mM NaCl, and 5mM dithiothreitol (pH 7.4). Thiazin red (100μM) was used to induce polymerization. Aliquots were withdrawn at specific time points and prepared for electron microscopy and filaments quantified as previously described (King, Ahuja et al. 1999; Chirita, Necula et al. 2003). Length distributions for each concentration and time point were also determined. Ten-nanometer length ranges or “bins” were established and the total number of filaments in each of these bins was determined.

In addition to time course data dissociation rates were also determined using electron microscopy. Recombinant htau40 (1.6μM) was incubated as described above for
24 hours. Following incubation the reaction mixture was diluted 10 fold in assembly buffer. Aliquots were withdrawn at specific time points up to five hours post dilution. Samples were fixed and stained in the same manner as the time course samples and total filament length determined.

To determine mass per unit length human tau 40 (8μM) was incubated as described above for 24 hours. Samples were then gel filtered through S500 beads to remove unincorporated protein monomers. Filtered samples were then flash frozen on dry ice and sent to Brookhaven National Laboratory (Upton, NY) for imaging. Mass per unit length was measured for filaments and tobacco mosaic virus (TMV) using the program PC Mass 29. Known values for TMV used to calculate that of the tau filaments as described in (Ksiezak-Reding and Wall 2005).

3.2.3. Analytical methods. In order to determine nucleus cluster size, two established kinetic treatments were employed. First, time course data was fit to the homogeneous nucleation kinetic model of Ferrone (Ferrone 1999):

$$\Delta = \frac{1}{2} (k_n)^2 K_n c^{(n+2)} t^2$$  (5)

where \( \Delta \) is proportional to total filament mass at time \( t \), \( k_+ \) is the elongation rate, \( K_n \) is a constant describing the monomer-nucleus equilibrium, \( c \) is the bulk tau concentration, and \( n \) is the number of molecules in the nucleus. Thus plots of \( \Delta \) as a function of \( t^2 \) yield curves with slope:

$$slope = \frac{1}{2} (k_+)^2 K_n c^{(n+2)}$$  (6)

and so:

$$\log(slope) = \log(\frac{1}{2} k_+ K_n) + (n + 2) \log(c)$$  (7)
Thus slopes of replots of log(slope) vs. log(c) yield an estimate of (n+2).

Data from the first 30% of the time course was plotted against time squared and fit to linear curves. Logs of the resultant slopes were then plotted against the log of protein concentration and the data again fit to a linear curve.

Second, the concentration dependence of lag time was analyzed by the method of Oosawa (Oosawa and Asakura 1975) as extended by Hofrichter and Eaton (Eaton and Hofrichter 1978). Time course data for each concentration was fit to a logistic function (Kuret, Congdon et al. 2005). Lag times were estimated from the x-axis intercept of a line tangent to the inflection point of the sigmoidal reaction progress curve. Plots of log lag time vs. log of protein concentration yield straight lines of slope (n+1)/2.

Data from dissociation time courses was plotted with natural log of total filament length against time in seconds as previously described (Kristofferson, Karr et al. 1980; Necula, Chirita et al. 2005). Because length distribution for tau filaments is near Poisson, we are able to apply the treatment for determining the dissociation constant developed by Kristofferson (Kristofferson, Karr et al. 1980). A linear function was fit to the points. The slope of this line, $k_{app}$, was then used to calculate the reverse elongation constant $k_{off}$:

$$k_{off} = \frac{L_0 \times k_{app}}{3.9 \times [ends]} \quad (8)$$

where $L_0$ is equal to the total filament length at time 0, and [ends] represents the total number of filaments. Data from the STEM analysis provides the value for number of monomers per nanometer, 3.9. The resulting $k_{off}$ was then used to calculate the forward elongation constant $k_{on}$:
where \( K_{\text{crit}} \) is the critical protein concentration. These calculated results were then incorporated into the mathematical model.

In order to model tau polymerization, data was first converted from total filament length to molar concentration using the estimated critical concentration. Because critical concentration represents the amount of monomer remaining unincorporated into filaments at equilibrium, it was assumed that all additional material had aggregated. The ratio of monomer to protomer at equilibrium was first calculated for each protein concentration. This ratio was then used to calculate protomer concentration for each individual time point.

The reaction was then modeled based on the approach of Wegner and Engel (Wegner and Engel 1975) with the addition of an activation step representing the formation of the partially folded intermediate. Monomer concentration, \( C_1 \) in the reaction was estimated as:

\[
C_1 = C_{\text{total}} - F \quad (10)
\]

where \( C_{\text{total}} \) is the total protein concentration and \( F \) is filament length. Activation, or intra-molecular refolding was modeled as:

\[
\frac{dC_p}{dt} = k_1c_0 - k_2c_1 \quad (11)
\]

where \( C_p \) is the concentration of protomer, \( c_0 \) is the concentration of unfolded assembly incompetent monomers, \( c_1 \) is the concentration of intermediate and \( k_1 \) and \( k_2 \) are respectively the rates of folding and unfolding of the intermediate. The nucleation step
was modeled as:

\[ \frac{dC_n}{dt} = k_{n+}(c_1)^n - k_{n-}(c_2) \] (12)

where \( n \) is the nucleus cluster size, \( c_1 \) is assembly competent monomer concentration, \( k_{n+} \) and \( k_{n-} \) are the forward and reverse nucleation rates, and \( c_2 \) is the concentration of dimer.

Finally, change in filament elongation was modeled as:

\[ \frac{dF}{dt} = (k_{on} \times c_1 - k_{off}) \times C_p \] (13)

where \( F \) is total filament length. The nucleation constants were adjusted by hand until simulated curves fit the experimental data before being optimized.

Time dependent changes in length distribution were also estimated using constants obtained through mathematical simulation. Utilizing these values, frequency distributions were estimated for single points and as a function of time, and compared to experimental data. By comparing simulated values to experimental results, the accuracy of the simulation can be assessed.

3.3 Results.

3.3.1. Assembly competent tau protein polymerizes with nucleation dependent kinetics. Recombinant tau 40 (0.4\( \mu \)M, 0.5\( \mu \)M, 0.6\( \mu \)M, 0.8\( \mu \)M & 1\( \mu \)M) was incubated with 100\( \mu \)M thiazin red for 24 hours under physiological pH and ionic strength. Reaction time courses were followed using quantitative electron microscopy and the resultant curves fitted to a Gompertz function. The resultant reaction curves have the sigmoidal shape typical of nucleation dependent reactions, with clear lag times, followed by exponential phases in which total filament length increases rapidly, and equilibrium
where total filament length remains constant (Figure 3.1). Both initial reaction velocity and equilibrium position were concentration dependent.

3.3.2 **Tau assembles via a dimeric nucleus.** Data from time course progress curves was subjected to two kinetic treatments to obtain an estimate of nucleus cluster size. The initial 30% of the reaction progress curves were replotted against time squared and fit to linear functions (Figure 3.2.A). The slopes of these fits were determined and were plotted against protein concentration in a double log format as specified by Ferrone (Ferrone 1999). Again the results were fitted to a linear function (Figure 3.2.B). Two was subtracted from the resultant slope and an estimated nucleus cluster of 1.7 molecules was obtained.

Because filament numbers are low at the initial time points and measurements are therefore prone to error a second kinetic treatment was used to confirm the previous results. The log of lag time was plotted against the log of protein concentration to yield a slope of 1.2 (Figure 3.3). This was then multiplied by two to yield an estimated nucleus cluster size of 1.4 tau molecules (Oosawa and Asakura 1975; Eaton and Hofrichter 1978). These results indicate that the nucleus cluster size is small, possibly dimeric. However, the possibility that the rate limiting step is intramolecular cannot be completely ruled out.

3.3.3 **Tau filaments contain ~2 molecules per β-sheet.** In order to calculate the dissociation constant $k_{\text{off}}$ from the apparent rate constant $k_{\text{app}}$ we determined the mass per unit length of tau filaments created *in vitro*. Human tau 40 (8μM) was incubated with 100μM thiazin red for 24 hours before being flash frozen on dry ice and sent to Brookhaven National Laboratory for STEM analysis. One hundred fifty one
measurements of tau filaments, and 200 of TMV were taken from the STEM images. Mean mass per unit length of TMV was calculated as 145 ± 7 kDa/nm with a value of 205 ± 38 for tau filaments. The known value of 131 kDa/nm for TMV was used to correct the values resulting in an estimated mass per unit length of 185 ± 34 for tau. This value was then used to calculate a value of 3.85 ± 0.71 molecules per nm or, 1.81 ± 0.33 molecules per β sheet (Figure 3.4). This value agrees with previously published results (von Bergen, Barghorn et al. 2006).

3.3.4 Mathematical simulation and estimation of rate constants. Critical protein concentration, $K_{\text{crit}}$ was measured by plotting total filament length at equilibrium against protein concentration. Data was fit to a linear function with the x-intercept equal to $K_{\text{crit}}$ which was found to be 0.22 ± 0.01 μM (Figure 3.5.A). The reverse elongation constant $k_{\text{off}}$ was determined using data from a dissociation time course. Recombinant protein (1.6 μM) was incubated with 100 μM thiazin red for 24 hours and then diluted ten fold (Figure 3.5.B). Filament decay was measured with electron microscopy and $k_{\text{off}}$ was calculated using the formula described above. The data yielded a $k_{\text{off}}$ of 0.02 s⁻¹. This was then divided by the critical concentration to give a $k_{\text{on}}$ of $10^5$ M⁻¹s⁻¹. Using the JACOBIAN program the nucleation constant was estimated as 5 mM. Finally, the activation constant was estimated as ~0.1. Simulated progress curves were then overlaid with experimental data.

3.3.5 Length distributions support a nucleation dependent mechanism. In addition to total length filament length distributions were determined for each concentration and time point. Five length distributions (15 min, 30 min, 90 min, 7 hours, and 24 hours) taken from the 1 μM timecourse were plotted and compared to the pattern expected
assuming homogeneous nucleation. At the earliest time point the mode fell into the 21-30 nm length range. By 90 minutes this value had shifted to the 31-40 nm range and remained there up to and including the seven hour time point. However, though total filament length did not continue to increase, by 24 hours the mode had returned to its original value (Figure 3.6A). This behavior is consistent with that expected in nucleation dependent processes (Hall and Minton 2004). In addition, average filament length and the slope of the distribution were calculated and plotted against time. Average filament length increased with time accompanied by a concurrent decrease in distribution slope. (Figure 3.6B) This pattern is inconsistent with secondary nucleation and thus allows this mechanism to be ruled out.

Simulated length distributions match the shape a skewness of the data as well as the time dependent drop in mode height and spreading of the data. (Figure 3.7) However, because the shortest filaments are difficult to measure, data from the 1-10 nm length range is missing.

### 3.4 Discussion

The data collected support the hypothesis that small molecule fibrillization agonists act via a pseudo-homogeneous nucleation mechanism (summarized in Figure 3.8). Because this mechanism approximates true homogeneous nucleation, we are able to capitalize on a solid theoretical background. These results, together with previous data, suggest that there are at least three rate limiting steps in the tau fibrillization reaction. The first is the disruption of the binding partnership between tau and microtubules. Following this, tau must undergo a conformational change and assume an assembly
competent conformation. Finally, dimerization, and thus nucleation, represents the final rate limiting step which must be overcome.

One of the earliest events in disease, and a rate limiting step in the polymerization reaction, is the abolition of the tau-microtubule binding partnership (Bancher, Brunner et al. 1989). In order for filament formation to occur, a pool of soluble tau must be readily available in the cytoplasm.

Because dissociation of tau from the microtubule surface increases the free concentration in the cytoplasm, it has been suggested that the law of mass action can explain filament formation (Singleton, Myers et al. 2004). However, incubation of tau in vitro, even at supersaturated concentrations, does not produce filaments in an experimentally tractable time frame. This indicates that a more complex mechanism is required to explain tau fibrillization. In our proposed mechanism, the majority of tau monomers in solution exist in assembly incompetent conformations. Any modification, conditions, or mutations that support formation of assembly competent conformations act as triggers of polymerization. This may include pH or ionic strength which decreases colloidal stability as well as exogenous inducers such as anionic surfactants, polyanions, or β sheet binding dyes. In disease, association with intracellular membranes may also promote conformational change. Finally, FTDP-17 mutation ΔK280 has been shown to spontaneously polymerize in vitro (Barghorn, Zheng-Fischhofer et al. 2000).

Once this conformational barrier is overcome, however, polymerization follows with nucleation dependent kinetics. A second category of modulators is capable of stabilizing filaments, as well as fostering monomer association. Thus, these modulators affect nucleation and elongation rates, without necessarily producing the original
conformational change. These enhancers may include phosphorylation, glycation, truncation, mutation, and alternative splicing. The exponential growth phase is then followed by equilibrium, in which tau monomers associate and dissociate from filament ends (Carulla, Caddy et al. 2005). This mechanism differs from the more complex mechanisms such as heterogeneous nucleation, requiring the interaction with exogenous substrates, and secondary nucleation, such as branching or filament breakage. It may be because experiments were carried out at very low protein concentrations, where nonideality is minimized, that the pseudohomogeneous behavior was observable. However, this does not rule out the possibility of other more complicated scenarios occurring at higher protein concentrations.

In contrast to previous estimates of nucleus cluster size the present study was conducted using full length protein incubated under physiological conditions and at low protein concentration. In previous studies, polymerization was induced using heparin, and therefore heterogeneous nucleation which may result in off pathway products (Friedhoff, von Bergen et al. 1998). The model used also assumed dimer addition following nucleation. In addition, the fluorescence based methods used to quantify filament polymerization are subject to interference from β sheet containing intermediate species (Chirita and Kuret 2004). The present results also represent the first obtained under reducing conditions, which better reflect early stage disease.

Experimental findings emphasize the importance of dimerization in the polymerization reaction. The rate limiting step to aggregation is dimer formation, with its significance further supported by the mass per unit length findings. It is tempting to speculate that dimer formation represents monomer association occurring orthogonal to
the long filament axis. Indeed face to face packing of β sheets is seen in amyloid β fibrils. In addition, mutations in amyloid β which foster intermolecular interactions perpendicular to the filament axis increase nucleation rates in vitro (Petkova, Yau et al. 2006). Amyloid fibrils formed from the yeast protein Sup35 contain a parallel in register β sheet spine, similar to that seen in Aβ (Nelson, Sawaya et al. 2005). Side chains protruding from these β sheets interdigitate to exclude water, forming a steric zipper. Monomers forming this dry interface make up the stable structural unit of cross beta spine. Formation of this unit is the rate limiting step in the polymerization reaction, as both conformational changes and additional stacking occur much more rapidly. A similar mechanism may be at work in tau. Assembly competent intermediates contain increased β sheet structure and hydrophobic interactions between secondary structural elements may form the basis for tau nucleation (Chirita, Congdon et al. 2005). However, the tau dimer may form via a distinct mechanism.

Events occurring in disease tissue may also result in enhancement of dimerization and thus polymerization and lesion formation. The mechanism presented here represents a base from which to explore the roles of post translational modifications, mutations and alternative splicing on tau polymerization. For example, in AD tissue tau protein is heavily and abnormally phosphorylated achieving a stoichiometry four fold above age matched controls (Kopke, Tung et al. 1993). Phosphorylation potentially affects each of the rate limiting steps in the polymerization process. It is implicated early in disease in abolition of the tau-microtubule interaction (Bramblett, Goedert et al. 1993; Li, Yin et al. 2004). Because phosphorylation results in a lowered isoelectric point as well as a reduced net charge, it would be predicted to cause reduced solubility and therefore promote
aggregation (Chiti, Stefani et al. 2003). Phosphorylation with neuronal cdc-2 like protein kinase (NCLK) has been found to promote dimerization in both recombinant and brain derived human tau (Paudel 1997). Facilitation of dimerization and thus nucleation may be a potential mechanism by which phosphorylation leads to tau polymerization in vivo. In addition to nucleation, phosphorylation is also capable of modifying elongation rate. Experiments using site specific pseudophosphorylation mutant tau demonstrate that addition of negative charges stabilizes filament ends resulting in a reduction of critical concentration (Necula and Kuret 2004). Results suggest that site specificity is essential for phosphorylation, meaning that changes in polymerization do not simply reflect changes in colloidal stability.

Another potential enhancer of filament formation, oxidation, can also enhance dimer formation via dityrosine and disulfide linkages. Possession of ApoE allele ε4, a gene involved in antioxidant activity, is a risk factor for development of AD (Saunders, Schmader et al. 1993). Other risk factors including brain injury, stroke, hypertension, poor diet, and lack of exercise are also associated with increased levels of oxidative stress (Guo, Cupples et al. 2000; Friedland, Fritsch et al. 2001; Luchsinger, Tang et al. 2002; Honig, Tang et al. 2003). In contrast, consumption of anti-oxidants as well as the use of nonsteroidal anti-inflammatory medications are protective (in t' Veld, Ruitenberg et al. 2001; Engelhart, Geerlings et al. 2002). Post mortem examination reveals patients suffering from mild cognitive impairment (MCI) and AD show increased levels of oxidation of both lipids and nucleic acid (Keller, Schmitt et al. 2005; Migliore, Fontana et al. 2005). In addition, oxidation also results in activation of protein kinases such as GSK-3, which may further promote protein aggregation. Increases in levels of oxidation
precede the appearance of NFTs and the increased oxidation may play a role in tau polymerization through the formation of intermolecular bonds.

Formation of oxidative dimers is a key rate limiting step in the polymerization of \( \alpha \)-synuclein and has been proposed as a necessary event in tau fibrillization (Friedhoff, von Bergen et al. 1998; Krishnan, Chi et al. 2003). In addition, dityrosine bond formation has been observed in amyloid \( \beta \) isolated from post mortem AD tissue (Nagano, Huang et al. 2004). Polyalanine peptides incubated in vitro under oxidizing conditions, also show a propensity for dityrosine bond formation (Giri, Bhattacharyya et al. 2007). In the case of amyloid-\( \beta \), dimerization was triggered by the pro-inflammatory enzyme COX-2. Similar to phosphorylation, promotion of dimerization may explain the role of inflammation in the brains of AD patients.

Disulfide bond formation has been proposed as a necessary step in paired helical filament (PHF) formation with cross linked tau dimers aggregating to form filaments (Schweers, Mandelkow et al. 1995). Though the importance of disulfide bond formation in vitro is controversial, in the context of disease oxidative stress and disulfide bonds between molecules may play an important role in tau aggregation. Disulfide bond formation, and differences in the mechanism of dimer formation, may also be responsible for the differential involvement of tau isoforms seen in disease (Buee, Bussiere et al. 2000). For example, three repeat isoforms contain only one cysteine residue, thus disulfide bonds will only form between monomers. Therefore, disulfide bond formation could enhance the rate of dimerization in three repeat isoforms.

Alternative splicing of tau exons 2, 3, and 10 results in the six isoform ensemble seen in adult brain and represents another plausible enhancer of filament formation.
Inclusion of the third microtubule binding repeat, exon 10, results in increased total filament mass at equilibrium (King, Gamblin et al. 2000). In addition, intronic mutations which shift isoform ratios are sufficient to cause disease, specifically genetic forms of FTDP, indicating that isoform structure influences its propensity for polymerization (Brandt, Hundelt et al. 2005). Decreases in colloidal stability, which would promote nucleation, or stabilization of mature filaments may explain these findings.

Additionally, certain naturally occurring FTDP-17 coding region mutations may also function as enhancers. Several of these mutant proteins show an increased ability to polymerize in vitro (Arrasate, Perez et al. 1999; Goedert, Jakes et al. 1999; Barghorn, Zheng-Fischhofer et al. 2000). Changes in net protein charge, or increased ability to support secondary structure could underlie these findings (von Bergen, Barghorn et al. 2001).

Other posttranslational modifications, such as glycation and truncation, seen in disease also affect elongation rates. Glycated residues, as well as advanced glycation end products (AGEs) have been found in authentic PHFs isolated from Alzheimer tissue (Ledesma, Bonay et al. 1995; Ko, Ko et al. 1999). In vitro, glycation has been shown to result in increased filament mass at equilibrium due to a decreased critical concentration required for tau polymerization (Ledesma, Medina et al. 1996; Necula and Kuret 2004). Similarly, truncation of the tau protein has also been shown to lower the critical concentration (Yin and Kuret 2006).

In disease one would expect that multiple mechanisms triggering conformational change, as well as enhancing filament formation, to be occurring simultaneously. These may act synergistically to polymerize and stabilize mature filaments. The aim of the
current study is to elucidate the mechanism by which tau protein assembles and to identify key rate limiting steps in the reaction. It is the first to provide an estimation of nucleus cluster size using full length protein under physiological conditions as well as estimates of individual elementary rate constants. The data emphasize the role of dimerization as a key event in the fibrillization pathway consistent with findings regarding other amyloid forming proteins.
Recombinant full-length human tau 40 (1\(\mu\)M (\(^{\Delta}\)), 0.8\(\mu\)M (\(^{\Box}\)), 0.6\(\mu\)M (\(^{\bullet}\)), 0.5\(\mu\)M (\(^{\circ}\)), and 0.4\(\mu\)M (\(^{\bullet}\))) was incubated in assembly buffer at 37\(^{\circ}\) C for 24 hours. Fibrillization was induced with 100\(\mu\)M thiazin red. Aliquots were removed at indicated time points, fixed with glutaraldehyde, and adsorbed for 1 minute onto formvar/carbon coated grids. Samples were then negatively stained with 2% uranyl acetate. Filaments were counted manually using Optimas6.5. Data points represent average total filament length from multiple negatives, and solid lines represent fits to mathematical simulations.
Figure 3.2. Initial velocity plots indicate a dimeric nucleus.

(A) Htau40 was incubated at 0.4 (▲), 0.5 (□), 0.6 (■), 0.8 (○) and 1 µM (●) for up to 24 hours (37°C) in the presence of 100 µM thiazin red. The time course of polymerization was followed using quantitative electron microscopy. Data from the first 30% of the reaction were plotted against time squared (h²). Each point represents the average total filament length ± SD, whereas solid lines represent best fit to a linear regression. (B) Slopes of each linear regression were determined and plotted against total tau concentration in double log format. Data points represent the slopes of the initial velocity plots ± SE and the solid line represents best fit to a standard linear regression. The slope of the resultant regression was determined in accordance with the kinetic treatment (Ferrone 1999) described in analytical methods (two was subtracted from the value). This yields an estimate of critical nucleus size of two molecules.
Figure 3.3. Lag time is concentration dependent.

Lag time was determined for each of the five tau concentrations (0.4-1 μM) using the fit of the time course data to a logistic function. Each data point represents the calculated lag times, with the solid line a best fit to a linear regression. In order to determine nucleus cluster size, the slope of the regression is equal to (n+1)/2 where n is the nucleus cluster size. (Oosawa and Asakura 1975; Eaton and Hofrichter 1978) Nucleus cluster size was estimated as 1.4 monomers. This further indicates that the cluster size is small. However this does not completely rule out the possibility that the rate limiting step is intramolecular.
Figure 3.4. Tau filaments contain ~1.8 molecules per beta sheet.

(A) Htau40 (8 μM) was incubated in assembly buffer without agitation, in the presence of 100 μM thiazin red, for 24 hours at 37°C. Samples were then gel filtered through S500 beads, flash frozen, and sent for STEM analysis. One hundred fifty one measurements of tau filaments (τ) were taken from the recorded images. Tobacco mosaic virus (TMV) was used as a calibrant. (B) Mass per unit length measurements were obtained for tau filaments and TMV. Bars represent mass per unit frequencies for tau (light grey) and TMV (dark grey). A total of 151 measurements were taken for tau and 200 for TMV. The average mass per unit length for TMV 145 ± 7 kDa/nm, while tau filaments averaged 205 ± 38. Known values for TMV were then used to correct the data, resulting in a mass per unit length of 185 ± 34. Using these results, and the known molecular weight of the tau protein, a value of 3.85 ± 0.71 molecules per nm or 1.81 ± 0.33 molecules per β sheet was obtained.
Figure 3.5. Critical concentration and dissociation time course provide estimates of elongation rate constants.

In order to estimate the elongation rate constants critical concentration and the dissociation rate constant were determined. (A), Htau40 (0.4 - 1 μM) was incubated under standard conditions for 24 hours at 37°C. Polymerization time course were followed using EM and the total filament length at equilibrium was plotted against total tau concentration. Data points are the average of multiple negatives ± SD and the solid line represents best fit to a linear function. Critical concentration was determined to be 0.22 ± 0.01 μM. (B), Htau40 (1.6 μM) was incubated under standard conditions for 24 hours at 37°C. Following this the mixture was diluted 10-fold in assembly buffer, such that the bulk tau level was below the critical concentration. Each data point represents the average total filament length ± SD for multiple images, whereas the solid line is best fit to a linear curve. The slope of this line was then used to calculate the dissociation rate constant $k_{off}$ using Equation 8. The dissociation constant was calculated as 3.3 ± 0.3 x10^{-3} s^{-1}. Using the critical concentration, dissociation constant, and Equation 9, the association rate constant $k_{on}$ was determined as 1.2 x 10^4 M^{-1}. 
Figure 3.6. Length distributions further support a nucleation dependent mechanism.

(A) Frequency based length distributions were determined for each reaction time point. Bins represent 10 nm length ranges. The numbers of filaments in each length range were counted and the distributions obtained for several time points (▲ 15 min, ▲ 30 min, ▼ 90 min, ▼ 7 hours, and X 24 hours) were plotted. At longer time points distribution broadens and becomes skewed towards longer filament lengths. In addition, the mode shifts to the right at intermediate time points before returning to its original position. These results are consistent with established theory and provide further evidence in support of a nucleation dependent mechanism. (B) Average filament length (●) and slope of the length distribution (○) were determined for each time point in the 1 μM Htau40 time course. Average length increased in a time dependent manner with a concurrent decrease in slope. Because these results are inconsistent with secondary nucleation, this potential mechanism can be ruled out.
Figure 3.7 Simulated length distributions based on estimated rate constants.

Rate constants obtained through mathematical simulation were utilized to estimate time dependent changes in frequency based length distributions. (A) (C) Graph shows changes in length distribution as a function of time for Htau40 (1 μM (A), 0.4 mM (C)) incubated at 37°C based on rate constants obtained through simulation. Estimated distributions show a rapid decrease in mode height as well as a spreading of the distribution consistent with experimental results. (B) (D) Length distributions for individual time points show that estimated distributions match the skewness and shape of the experimental data. Bars represent the number of filaments in each length range while solid lines are estimated distributions.
Figure 3.8. Hypothetical mechanism for tau polymerization and identification of rate limiting steps.

The scheme represents our proposed mechanism and rate constants used to model tau filament formation in vitro. Under normal conditions tau exists as a random coil, assembly incompetent monomer (Uₓ). A conformational change to an assembly competent state is required for polymerization. This can be achieved through the use of exogenous inducers including anionic surfactants or β sheet binding dyes. Once this assembly competent species (Uₓ) is populated, monomers spontaneously associate and dimerize to form the unstable tau nucleus (N). Following nucleation, extension occurs through further addition of assembly competent monomers to the filament (F) ends.

Experimental data indicate that at least three rate limiting steps exist in the tau polymerization pathway. The first of these is dissociation from the microtubule surface. Once the tau protein is free in the cytoplasm, it must undergo a conformational change to assume a partially folded, assembly competent form. Following population of the intermediate species, dimerization represents the final barrier that must be overcome for filament polymerization to proceed. Mathematical simulation of reaction progress curves has produced the first estimates of elementary rate constants for the nucleation and elongation reaction. Results show that nucleation and elongation have similar association rates. However, the dissociation rate for nucleation is much more rapid. This model provides a base from which to explore the roles of mutations, alternative splicing, and post translational modifications on the polymerization process.
CHAPTER 4

4. ISOFORM STRUCTURE INFLUENCES TAU AGGREGATION

4.1 Introduction

A host of neurodegenerative diseases feature insoluble filamentous aggregates of microtubule associated protein (MAP) tau, termed neurofibrillary tangles (NFT), as a pathological hallmark. The molecular pathogenesis of these conditions including Alzheimer’s disease, frontotemporal lobar degeneration (FTLD, formerly Pick’s disease), progressive supernuclear palsy (PSP), corticobasal degeneration (CBD), and frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), collectively termed tauopathies, is poorly understood. Alternative splicing of three exons of a single gene on chromosome 17 results in the six isoform ensemble found in the adult human brain. Inclusion or exclusion of exon 10 results in isoforms containing either three or four imperfect C-terminal microtubule binding repeats. Of the six human tau isoforms, three contain three repeats (3R isoforms) while the other three contain four (4R isoforms). The splice variants also differ in having 0, 1 or 2 N-terminal inserts owing to the alternative splicing of exons 2 and 3 (Himmler, Drechsel et al. 1989). The physical differences between the six isoforms are summarized in Figure 4.1.
Alternative splicing of the tau gene is developmentally regulated with only the shortest form expressed in fetal nervous tissue (Kosik, Orecchio et al. 1989). However, in the adult brain both 3R and 4R isoforms are equally expressed. This ratio may be essential for maintaining neuronal health. Though mutations in the tau gene itself have not been found in AD, recent evidence suggests that a shift toward overexpression of 4R isoforms occurs in affected brain regions (Yasojima, McGeer et al. 1999; Ginsberg, Che et al. 2006). In the case of FTDP-17, mutations which alter the splicing of the tau mRNA are sufficient to cause disease (Hutton, Lendon et al. 1998; Gao, Memmott et al. 2000). The majority of these splicing mutations are associated with an increase in 4R/3R ratio, although the total tau levels appear to stay the same. Several of the missense mutations found in FTDP-17 are also capable of shifting the isoform ratio with the majority of those favoring the inclusion of exon 10 (Brandt, Hundelt et al. 2005). However, at least one naturally occurring exonic mutation appears to shift expression towards three repeat isoforms (van Swieten, Bronner et al. 2007). Changes in relative isoform expression can also be seen in other tauopathies such as PSP and CBD (Lee, Goedert et al. 2001; Gibb, de Silva et al. 2004). Though the majority of tauopathies appear to involve an increase in 4R isoforms, rare 3R tauopathies do exist (Buee, Bussiere et al. 2000).

Discovery of pathogenic mutations has given rise to attempts to create animal models of tauopathies. This has led to the development of several lines of transgenic animals that over-express a single tau isoform (3R or 4R), a mutant protein, or protein kinases thought to be upstream in the development of hyperphosphorylated tau. However, none of these models has been completely successful in recapitulating the disease phenotype seen in humans (Hutton, Lewis et al. 2001). Transgenic mice
expressing human 4R tau isoforms develop motor disturbances, specifically in tasks involving balance. Histopathologically, these mice display dilated axons containing neurofilaments and dystrophic neurites that stained with a variety of antibodies to hyperphosphorylated tau, including AT8, AT180, and PHF1 (Probst, Gotz et al. 2000). Overexpressing 3R isoforms has also been shown to give rise to partial FTDP-17 pathology in a mouse model (Ishihara, Hong et al. 1999). However, both of these models overexpress tau to the extent that they do not correspond to the physiological tau levels in the disease. In addition, transgenic lines that overexpresses an exon 10 mutant tau (P301L) which is implicated in FTDP-17 in humans, develops motor and behavioral deficits with early hind limb dysfunction followed by dystonic posture, docility, reduced weight, and decreased vocalization. These mice show profound neuronal loss in spinal cord and tau positive neurofibrillary tangles throughout the brain (Lewis, McGowan et al. 2000). Findings such as these further indicate that specific isoform expression may be a key factor in the development of pathology.

Though these findings suggest that isoform expression may play a role in the pathogenesis of neurodegenerative diseases, several unanswered questions remain. Hyperphosphorylation is believed to be the most plausible driving factor for tau fibrillization, and tau aggregates found in disease are highly phosphorylated. Yet fetal tau, despite being hyperphosphorylated to levels comparable to those seen in disease, is not prone to aggregation (Kenessey and Yen 1993). Some primates such as chimpanzees are highly resistant to the development of tau pathology (Holzer, Craxton et al. 2004), whereas others such as baboons (Schultz, Dehghani et al. 2000; Schultz, Hubbard et al. 2000), rhesus monkeys (Hartig, Klein et al. 2000) and lemurs (Giannakopoulos, Silhol et
al. 1997; Hartig, Klein et al. 2000) have been shown to develop tau bearing lesions. Neurons also display selective vulnerability in disease, with pyramidal neurons preferentially affected in AD, PSP, and CBD, while in other conditions such as FTLD (formerly Pick’s disease) the granule cells of the dentate gyrus display high densities of aggregate containing neurons (Hof, Bouras et al. 1994). Also, of the two known haplotypes of the tau gene, the H1 haplotype contains a polymorphism in the intron between exons 9 and 10 which shifts the ratio towards 4R isoforms (Baker, Litvan et al. 1999). This haplotype in turn is associated with the development of PSP. We hypothesize that this increase in the 4R/3R isoform ratio is the direct cause of pathology seen in FTDP-17. We hypothesize that lesion formation in disease is related to the structure and hence varying solubility of the different tau isoforms. To test this hypothesis, using our well established in vitro model, we examined the polymerization kinetics of all six human tau isoforms, thus delineating the effects of the presence or absence of the C-terminal repeats and N-terminal inserts on aggregation.

4.2 Methods

4.2.1 Materials. Recombinant poly-his tagged tau isoforms (htau40, 34, 24, 39, 37, 23) were prepared as described previously. Thiazin red was obtained from TCI America (Portland, OR) and stock solutions were prepared in distilled water. Glutaraldehyde, uranyl acetate, and formvar-coated 300 mesh copper grids were purchased from Electron Microscopy Sciences (Ft.Washington, PA).
4.2.2 Fibrillization assays. All tau isoforms were incubated without agitation in assembly buffer containing 10mM HEPES (pH 7.4), 100mM NaCl, and 5 mM Dithiothreitol (DTT) for up to 24 hours at 37°C. Thiazin red (100 μM) was added to the reaction mixture to induce polymerization. In preparation for electron microscopy, aliquots were removed, fixed with glutaraldehyde (2% total volume), and adsorbed onto formvar-coated grids for 1 minute. Samples were then negatively stained with 2% uranyl acetate. Grids were viewed in an FEI Tecnai G2 BIO Twin TEM operated at 80 kV. Digital images were captured and total filament length was determined using either Optimas 6.5 or Image J. All filaments 10 nm or longer were used for analysis and results were calculated as total filament length per field ± the standard deviation.

4.2.3 Determination of critical concentration. Varying concentrations of each tau isoform were added to reaction mixtures containing buffer, 5 mM DTT, and 100 μM thiazin red from serially diluted stocks. Samples were incubated at 37°C for 24 hours, allowing the reactions to come to equilibrium. Aliquots were then prepared for electron microscopy and images collected as described above. Total filament length per field ± the standard deviation was plotted as a function of tau concentration. Data was fit to a linear function and the abscissa intercept, or critical concentration, was determined for each isoform.

4.2.4 Determinations of k_{off} and k_{on}. A reaction mixture, including 100 μM thiazin red was prepared as described above for each tau isoform and incubated at 37°C for 24 hours. After incubation the mixtures were diluted ten-fold such that the concentration of tau in each sample was below the critical concentration. One hundred μL aliquots were withdrawn each hour between one and five hours post-dilution. Samples were fixed,
mounted, and stained as described and images were collected. Total filament length and total filament number were determined for each isoform and time point. Data from the dissociation time courses was plotted as natural log of total filament length against time in seconds. The data was fit to a linear function was fit the data and the slope of each resultant line was determined. This value represents the apparent rate constant, $k_{\text{app}}$. This figure can then be used to calculate the actual dissociation constant $k_{\text{off}}$ using Equation 8 as described above. Using this value and the critical concentrations obtained for each isoform, the association rate constant, $k_{\text{on}}$, was determined using Equation 9.

4.2.5 Time courses. Fibrillization time courses were carried out at a constant supersaturation, 0.3 μM above the critical concentration, for each isoform. Reactions were incubated for 24 hours with aliquots withdrawn at specific time points (15, 30, 45, 60, 90, 120, 180, 240, 300, 420 min and 24h), fixed with 2% glutaraldehyde, and mounted on grids for electron microscopy. Random images were collected from each time point and isoform with total filament length determined as described above. Time courses were fit to Gompertz functions and lag times determined as described previously.

4.2.6 Isoform dominance. Recombinant human tau40 and 23, the longest and shortest isoforms respectively, were incubated at ratios of 100/0, 75/25, 50/50, and 25/75 %. Each of these mixtures was incubated at varying bulk tau levels. Isoform mixtures were incubated in assembly buffer, including 100 μM thiazin red, for 24 hours. Samples were prepared for electron microscopy as described above and total filament length for each sample was determined. Resultant total length was then plotted against bulk tau concentration and the critical concentration for each isoform ratio was calculated ± the
standard deviation as described above. These values were then compared to the expected results assuming no isoform dominance.

4.2.7 Pairwise comparisons. To fully delineate the effects of individual exons on aggregation propensity, off and on rate pairwise comparisons were made between isoforms differing in any one exon. Comparisons were expressed as a ratio (R) between critical concentrations, and on or off rates of the isoforms. Pairwise comparisons were made between 23/24, 37/34 and 39/40 for exon 10, 23/37 and 24/34 for exon 2, 37/39 and 34/40 for exon 3.

4.2.8 Statistics. To determine the significance of these results a “Z” test was performed using the program JMP 6. The standard error of the mean was used along with R and the value 1.00 to calculate the “Z score” or the “Z stat” which was then used to estimate the p value. This provides an estimate of how the R value is different than 1.00 which was considered to be as no effect. A p value of less than 0.05 was considered to be significant.

4.3 Results

4.3.1 Alternatively spliced exons affect critical concentration. Utilizing thiazin red, all tau isoforms were induced to form filaments. The critical concentration was determined for each and pairwise comparisons were performed. Critical concentration reflects both the minimal concentration which will support filament formation and the total amount of unincorporated monomer in solution at equilibrium. Therefore, a lowered critical concentration indicates that a greater proportion of the bulk protein is incorporated into filaments. From lowest to highest, the rank order of isoform critical concentrations is
htau34, 40, 24, 37, 39, and 23 (Figure 4.2.A). All isoforms containing four microtubule binding repeats have critical concentrations below those with three. Statistical analysis revealed that inclusion of exon 10 lowers the critical concentration approximately six fold ($p<0.001$). Thus, isoforms containing exon ten aggregate, on average, at six fold lower concentrations than those without (Figure 4.2.B). Similarly, exon 2 exerts its effect by decreasing the critical concentration two fold ($p<0.001$). In contrast, inclusion of exon 3 raised the critical concentration by approximately 30% ($p<0.001$). This result indicates that the presence of exon 3 exerts a destabilizing influence on the filaments.

4.3.2 Inclusion of exon 10 results in lowered dissociation rates. Variations in critical concentration result from differences in the elementary rate constants, $k_{off}$ and $k_{on}$. Dissociation rates were determined for each individual isoform by plotting total filament length post-dilution as a function of time. This yields a pseudo zero order curve representing the apparent rate constant $k_{app}$. This value was then used to calculate $k_{off}$ using Equation 8. Dissociation rates reflect the relative stability of the filaments. Consistent with critical concentration results, 4R isoforms have a three fold lower $k_{off}$ than the 3R isoforms ($p<0.005$), indicating that the presence of exon 10 greatly stabilizes mature filaments (Figure 4.3.A). Addition of exons 2 and 3 did not result in a significant change in the rate of dissociation.

4.3.3 Exons 2 and 10 double the rate of association. The association rate constant, $k_{on}$, was calculated using Equation 9, the $k_{off}$, and $K_{crit}$ values for each isoform with the associated error statistically propagated (Figure 4.3.B). Again utilizing pairwise comparisons, the presence of exon 10 was found to increase the rate of association, on average, two fold ($p<0.05$). Addition of exon 2 increases $k_{on}$ by two fold, thus explaining
its affect on critical concentration. Inclusion of exon 3 resulted in an approximately 30% decrease in association rate; however it was not statistically significant for one of the two pairwise comparisons.

4.3.4 Isoform structure influences nucleation rate. All isoforms were incubated at constant supersaturation (critical concentration + 0.3μM) and the polymerization time course was followed by electron microscopy (Figure 4.4). The lag time was calculated from the curve by finding the x intercept of a line drawn tangent to the point of inflection. In all cases isoforms containing four microtubule binding repeats had shorter lag times than those with three (Figure 4.5.A). Analysis of lag time provides an indirect indication of nucleation rate. As stated above, nucleation represents the formation of an energetically unfavorable species and one of the rate limiting steps in the polymerization process. Utilizing pairwise comparisons, it was determined that inclusion of exon 10 results in a nearly four fold decrease in lag time, with exons 2 and 3 showing no significant effect (Figure 4.5.B).

4.3.5 Htau40 exerts partial dominance over htau23. The longest human tau isoform, htau40, was incubated with htau23, the shortest, at varying ratios and then assayed for critical concentration. Results were plotted as percentage of htau40 against 1/K_{crit} and compared to those expected assuming no dominance (Figure 4.6). The data shows that when htau40 makes up the majority of bulk tau (75-50%) in the reaction critical concentration is lowered relative to the expected values. These findings indicate that more assembly prone splice variants may play a role in recruiting other isoforms into lesions.
4.4 Discussion.

The ultimate cause of tau aggregation in Alzheimer’s disease and other tauopathies remains elusive. Despite the existence of transgenic models, none have yet been able to fully reiterate the complete pathology. Several hypotheses have been put forth to explain the behavior of tau. For example, the law of mass action has been proposed to explain tau aggregation, as well as that of other proteins like amyloid-β and α-synuclein (Singleton, Myers et al. 2004). This theory postulates that increased protein concentrations will result in faster aggregation. However, this is inadequate as even supersaturated conditions do not result in tau fibrillization \textit{in vitro}. Hence, we hypothesize that tau must assume an assembly competent confirmation prior to filament formation. Once a population of assembly competent monomers accumulates, however, tau assembles via a nucleation dependent pathway with concentration-dependent polymerization rates. Together, these phenomena account for much but not all of tau kinetics. Indeed other factors such as posttranslational modifications and oxidative dimer formation may also play a role.

Another possible factor influencing tau aggregation is the isoelectric point and thus solubility of the protein (Chiti, Stefani et al. 2003). However, as with the law of mass action, changes in solubility are insufficient to account for the behavior of tau. In fact, results confirm that isoform structure plays a greater role in aggregation behavior of tau than does charge and isoelectric point. For example, addition of exon 10 increases the charge of the tau molecule and would therefore be expected to increase its solubility when, in actuality, it dramatically lowers the critical concentration and augments the nucleation rate. This effect is not merely an artifact of supersaturation as each isoform
was incubated at the same level above its critical concentration. Addition of exon 2 lowers the critical concentration regardless of the presence or absence of exon 10. It also decreases the nucleation rate, although this effect is diminished in the presence of exon 10. Exon 3 exerts small negative effects on the critical concentration and the nucleation rate in both 3R and 4R backgrounds.

A group of intronic mutations clustered around the splice donor site of exon 10 are sufficient to cause FTDP-17 (Hutton, Lendon et al. 1998; Hutton 2000). Though these mutations do not alter the protein itself, they were found to increase production of 4R relative to 3R isoforms (Spillantini, Goedert et al. 1997; Hutton, Lendon et al. 1998). Some exonic mutations have also been discovered that alter the ratio of splice variants (Brandt, Hundelt et al. 2005). Our results demonstrate that increases in 4R isoforms would favor tau fibrillization in disease via decreases in the critical concentration required for polymerization. Tau maturation is observed to be similar in glia, and hence these principles apply to glial deposits as well (Muller, Heinrich et al. 1997). Also, two haplotypes (H1 and H2) have been defined for the tau gene, which are described by eight single nucleotide polymorphisms (Baker, Litvan et al. 1999). Homozygosity for the more common H1 haplotype results in a predisposition to progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). The H1 haplotype is also linked with increases in the proportion of four repeat isoforms (Myers, Pittman et al. 2006). The presence of multiple haplotypes may explain the differential susceptibility of primates to tauopathies. Chimpanzees are resistant to the development of tau related pathology despite possessing identical amino acid sequences to the human tau gene (Gearing, Rebeck et al. 1994; Holzer, Craxton et al. 2004). Interestingly, it was found that
chimpanzees and gorillas have the gene sequence coding for the H2 haplotype. This results in chimpanzees and gorillas expressing a higher proportion of RNA transcripts lacking exon 10 (Holzer, Craxton et al. 2004). Resistance to tau related pathology in chimpanzees is due to over-expression of exon 10 lacking (3R) isoforms with higher critical concentrations, and thus a lower aggregation potential. These results also demonstrate explicitly how the H1 haplotype predisposes carriers to PSP and CBD.

One of the major hypotheses regarding tau is that polymerization is the result of hyperphosphorylation. In late stage disease, filamentous tau is phosphorylated at a stoichiometry significantly greater than that of normal tau (Ksiezak-Reding, Liu et al. 1992; Kopke, Tung et al. 1993). However, fetal brain tissue contains tau phosphorylated at nearly the same level, and at many of the same sites as PHF tau, but does not form tangles (Brion, Smith et al. 1993; Kenessey and Yen 1993). Again, these findings can be explained by the differential ability of tau isoforms to aggregate. Alternative splicing of tau is developmentally regulated with only the shortest isoform, htau23, expressed prenatally (Takuma, Arawaka et al. 2003). Studies using phosphorylation mimicry mutants demonstrate that phosphorylation can lower tau’s critical concentration (Necula and Kuret 2004). However, our results show that its critical concentration is sufficiently high to create a barrier to aggregation that is not easily surmounted at physiological tau concentrations, even in the presence of phosphorylation.

Another unexplained aspect of disease is the selective vulnerability of specific cell populations. One of the areas involved in the genesis of tau pathology very early in the course of disease is the hippocampus. Specifically, the pyramidal neurons of the CA 1 and CA2 regions of the hippocampus typically degenerate in AD and FTDP-17
whereas the granule cells remain unaffected. Granule cells express 3R isoforms alone whereas the pyramidal cells express both 3R and 4R isoforms (Goedert, Spillantini et al. 1989; Yasojima, McGeer et al. 1999). However in FTLD, a disorder characterized by aggregates of 3R isoforms, granule cells are highly affected (Hof, Bouras et al. 1994). Our results indicate that isoform expression may be a key factor in the vulnerability of neurons to the development of pathology in specific conditions. This does not, however, explain why 3R isoforms, which are far less assembly prone, aggregate in FTLD. A potential answer for this phenomenon is oxidative stress. It may be that in FTLD there is a greater level of oxidation leading to the formation of intermolecular disulfide bonds. Because 4R tau has two sulphhydryl groups, an intramolecular disulfide bond is possible which would produce and assembly incompetent species, whereas 3R tau contains only one sulphhydryl moiety and thus forms intermolecular disulfide bridges. This would results in dimerization, and may in turn promote nucleation and further polymerization.

Our results suggest development of mouse models that feature expression of human 4R2N or 4R1N repeats at normal physiological level, as they would represent a better model for NFT formation. Models involving the 3R isoforms could be developed the tau pathology, but they may require a system overexpressing the human tau.

These findings indicate that potentially three classes of tauopathy exist differentiated on the basis of isoform involvement. Class 1 would include disorders in which all six tau isoforms are involved. This would include: AD, Down’s syndrome, ALS, Neiman-Pick type C, postencephalitic parkinsonism, and normal aging. Class II disorders would comprise those involving predominately four repeat isoforms, including PSP, CBD, and genetic forms of FTDP-17 in which intronic or exonic mutations result in
a relative increase in 4R isoforms. Progressive supranuclear palsy is a degenerative neurological disorder characterized by ataxia, slowing or inability to generate saccadic eye movements, and axial rigidity. PSP is relatively rare in the United States, with an estimated incidence 1.1 per 100,000, and though the majority of cases are sporadic, a few known pedigrees exist. The disorder is much more common on the islands of Guam and Guadeloupe, located in the Pacific and French West Indies respectively. In both cases the prevalence of the disease has been linked to dietary factors and possibly an interaction with genetics. Corticobasal degeneration is another rare sporadic tauopathy with a slowly progressing late onset course. Finally, Class III disorders are those conditions in which 3R isoforms make up the majority of pathology. In this case only FTLD, a rare disorder characterized by progressive dementia and personality degeneration, and one naturally occurring FTDP-17 mutation ΔK280 have been identified.

4.6. Conclusions

The results presented answer several unresolved issues regarding the effects of tau alternative splicing. In the context of disease, these findings explain how a shift in isoform ratios is sufficient to cause disease, and the selective vulnerability of neurons. In addition, they also provide a rationale for observations from normal tissue, including why fetal tau does not polymerize. Results show that inclusion of exon 10 results in significant decreases in critical concentration, filament dissociation rate, and lag time. Exon 2 also exerts a positive effect making isoforms containing it more assembly prone, while exon 3 produces a small negative effect. However, these effects are not dependent on changes in solubility or pH.
In the adult human brain, alternative splicing of a single gene on chromosome 17 results in the observed six isoform ensemble. Isoforms can be divided into two major groups containing either three or four microtubule binding repeats. Further differences result from the inclusion or exclusion of amino terminal exons two and three.
Figure 4.2. Alternative splicing affects critical concentration.

All six tau isoforms (htau34 (●), htau40 (▲), htau24 (■), htau37 (○), htau39 (Δ), htau23 (□)) were incubated at varying concentrations under standard conditions at 37°C for 24 hours in the presence of 100 μM thiazin red. (A) Total filament length was plotted against bulk protein concentration and fit to a linear regression. The abscissa intercept was determined ± SE. Data points represent an average total length of multiple images whereas the solid lines are best fit to a linear function. (B) Pairwise comparisons between isoforms differing in only one exon were made to determine the effects of individual exons on critical concentration. Both exons 2 and 10 lowered critical concentrations two and six fold respectively ($p<0.001$). Exon 3 exerts a smaller, but significant ($p<0.001$) 30% increase in critical concentration. The results are not dependent on changes in pH or isoelectric point.
Figure 4.3. Alternative splicing affects on and off rates.

Individual isoforms were incubated under standard conditions for 24 hours at 37°C. Following incubation samples were diluted ten fold, such that bulk protein levels were below the critical concentration. (A) Depolymerization time courses were followed with quantitative electron microscopy with total filament length plotted against time and fit to a linear function. The slope of the resultant line yielded the $k_{\text{app}}$, or apparent dissociation rate constant. This was then converted into the actual dissociation constant, $k_{\text{off}}$, using Equation 8. Bars represent values calculated for $k_{\text{off}} \pm \text{SE}$. Each of the four repeat isoforms show reduced dissociation rates relative to three repeat splice variants. Pairwise comparisons revealed that the presence of exon 10 produced a three fold reduction in $k_{\text{off}}$ ($p<0.005$). Exons 2 and 3 did not produce significant differences. (B) Association rate was calculated using the critical concentration and dissociation constants for each isoform (Equation 9). Both exons 2 and 10 resulted in a two fold increase in association rate ($p<0.05$). Exon 3 appeared to lower association rate ~30% but this finding was not significant in one of the two pairwise comparisons.
Figure 4.4. Time course of polymerization of all human tau isoforms.

Individual tau isoforms (htau40 (●), htau34 (■), htau24 (▲), htau39 (○), htau37 (□) and htau23 (Δ)) were incubated at a constant supersaturation (critical concentration + 0.3 μM) for 24 hours at 37°C under standard conditions in the presence of 100 μM thiazin red. Aliquots were removed at indicated time points and total filament length determined. Data points represent the average of multiple negatives, whereas solid lines represent best fit of the data to a three parameter Gompertz function. Curves are normalized for protein concentration. In all cases, isoforms containing three microtubule binding repeats produced longer lag times than those with four.
Figure 4.5. Isoform lag times and the effects of alternatively spliced exons.

Analysis of lag times provides information regarding the nucleation rates of individual isoforms and the effects of individual exons. (A) Lag times for each isoform were determined ± SE based on the fit of experimental data to a three parameter Gompertz function. Again, three repeat isoforms have greater lag times than those with four repeats indication that alternative splicing affects the nucleation reaction as well as elongation. (B) Pairwise comparisons revealed that inclusion of exon 10 results in a four fold decrease in lag time, and thus a positive effect on nucleation rate. Pairwise comparisons of exons 2 and 3 showed no significant effects.
Figure 4.6. Tau isoforms display partial dominance in polymerization.

To better understand how the presence of more assembly prone isoforms affects less assembly prone isoforms, htau40 and htau23 were incubated together at varying relative percentages and total bulk protein concentrations. Samples were incubated for 24 hours at 37°C under standard conditions in the presence of 100 μM thiazin red. These isoform mixtures (■) were assayed for critical concentration and compared to values expected assuming no effect (♦). When a majority of the bulk tau concentration (50-75%) is made up of htau40, critical concentration is reduced relative to expected results. These data suggest that in addition to reduced critical concentration and lag times, isoforms containing four microtubule binding repeats may also be able to directly recruit less assembly prone isoforms.
CHAPTER 5

5. POTENCY OF A TAU FIBRILLIZATION INHIBITOR DEPENDS ON ITS AGGREGATION STATE

5.1 Introduction.

In multiple neurodegenerative diseases including Alzheimer’s, Parkinson’s, and Huntington’s, protein misfolding and aggregation are characteristic events (Dobson 2003). Formation of fibrillar lesions correlates spatially and temporally with disease progression indicating that protein polymers may be directly linked with cytotoxicity (Caughey and Lansbury 2003). Because of this, protein aggregation presents an attractive target for intervention. Small molecule polymerization inhibitors including azo dyes, heterocycles, and phenothiazines have been investigated as potential therapeutic agents (Demaimay, Harper et al. 1998; Heiser, Scherzinger et al. 2000; Rudyk, Knaggs et al. 2003; Sellarajah, Lekishvili et al. 2004). However, interactions between these compounds and self-associating proteins are complex, with inhibitor molecules capable of binding both high affinity sites along the filament axis (Jin, Claborn et al. 2003; Krebs, Bromley et al. 2005) as well as partially folded monomeric intermediates and oligomers (Khurana, Gillespie et al. 2001; Kim, Randolph et al. 2003; Masuda, Suzuki et al. 2006). As a result, a single compound may inhibit or induce polymerization depending on the species it binds (Heiser, Scherzinger et al. 2000; Rudyk, Vasiljevic et al. 2000; Kim,
Randolph et al. 2003; Chirita, Congdon et al. 2005). In addition, even with a single binding partner inhibition and promotion of polymerization can predominate under different concentrations, leading to complex dose response relationships. For example, incubation of Congo Red with brain slice preparations derived from Huntington’s disease mouse models results in a triphasic dose response curve. Partial antagonist activity can be seen at submicromolar concentrations. However, as concentrations of Congo Red rise, inhibition is lost and the dye begins to promote polymerization. Finally, at the highest concentrations tested, Congo Red did not appear to either promote or inhibit huntingtin aggregation (Smith, Portier et al. 2001). These results demonstrate the importance of testing potential therapeutic compounds over a wide range of concentration regimes, as well as assessing the mechanisms through which these compounds exert their effects.

Recently, we have examined the effects of aromatic heterocycles on the polymerization of tau, the major component of neurofibrillary lesions in Alzheimer’s disease and other related disorders (Chirita, Necula et al. 2004; Necula, Chirita et al. 2005). In disease, the tau protein transitions from natively unfolded monomers to well ordered filaments in which beta sheets stack orthogonal to the long filament axis (Margittai and Langen 2004). When incubated under physiological concentrations in vitro, exogenous fibrillization inducers are required to promote polymerization in experimentally tractable time periods (King, Ahuja et al. 1999). These exogenous compounds include anionic micelle forming agents and anionic microspheres, which provide a surface capable of triggering heterogeneous nucleation (Chirita, Necula et al. 2003; Chirita and Kuret 2004; Chirita, Congdon et al. 2005). At low concentrations neither Congo Red nor benzothiazole derivatives such as ThS are capable of modulating
tau polymerization. In contrast, at higher concentrations these dyes facilitate filament nucleation in the presence of poly-anions and are capable of triggering aggregation independently (Chirita, Congdon et al. 2005). This activity may be owing to an ability to bind and organize β-sheet structure. Alternatively, cyanine dye N744 has been shown to inhibit tau polymerization at substochiometric concentrations (Chirita, Necula et al. 2004). We hypothesized that dye aggregation is a contributing factor to the differential activity of aromatic heterocycles. Aggregation is a common phenomenon in many structural dye classes and is influenced by pH, temperature, ionic strength, and solvent polarity (Murakami 2002). These dyes aggregate via a nucleation dependent mechanism similar to proteins such as tau and Aβ (Saijo, Isshiki et al. 1995). In solution, primary nucleation is homogeneous when dye molecules are incubated alone. However, dye polymerization can be influenced by exogenous factors including silver halide salts (Herz 1977), duplex DNA (Wang, Silva et al. 2000), and proteins containing β-sheet structure (Hermel, Holtje et al. 1995; Hermel, Schmahl et al. 1999). In the presence of each of these substrates absorbance spectra vary suggesting that exogenous compounds may affect dye nucleation and molecular organization.

Here we examine the concentration dependence of N744, a cyanine dye, on the modulation of tau aggregation under near physiological conditions in vitro. Results show a complex dose response relationship similar to that seen using Congo Red in a biological model of Huntington’s disease, suggesting a mechanistic basis for this effect.
5.2 Material and Methods.

5.2.1 Materials. Recombinant wild type htau40 and htau40\textsuperscript{T212E}, a pseudophosphorylation mutant, were prepared as previously described. (Carmel, Mager et al. 1996; Necula and Kuret 2004) Mixed histones (type II-A from calf thymus) were obtained from Sigma (St.Louis, MO). Stock solutions of alkyl sulfate detergent C\textsubscript{18}H\textsubscript{37}SO\textsubscript{4}Na (Research Plus, Boyonne, NJ) were prepared in 1:1 H\textsubscript{2}O/isopropanol. Glutaraldehyde, 300 mesh carbon-coated copper grids, and uranyl acetate were purchased from Electron Microscopy Sciences (Ft. Washington, PA) N744 was custom synthesized by deCODE Genetics (Lemont, IL) and stock solutions were prepared in DMSO. Carboxylate-conjugated polystyrene microspheres (90 nm diameter, molecular area =12 Å\textsuperscript{2}/eq) were obtained from Bangs Laboratories, Inc (Fishers, IN).

5.2.2 Aggregation assays. Protein preparations were incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) in the presence or absence of both fibrillization inducers and N744. Control reactions were normalized for DMSO content with the final concentration limited to 2% in all reactions.

Samples were prepared for electron microscopy by removing aliquots at designated time points which were then fixed with 2% glutaraldehyde, adsorbed onto formvar/carbon-coated 300 mesh grids, and negatively stained with uranyl acetate (2% final volume) (Chirita, Necula et al. 2003). Images were viewed in a Phillips CM-12 transmission electron microscope operated at 65 kV. Random images were captured on film at 8,000- 60,000 fold magnification, digitized, and imported into Optimas 6.5 for filament quantification. Individual filament lengths were determined manually.
5.2.3 Critical concentration determination. Htau40 at varying concentrations (2-9 μM) was incubated at room temperature (22°C) for 26 hours without agitation in the presence of alkyl sulfate detergent C_{18}H_{37}SO_{4}Na. Samples were analyzed by static laser light scattering as described previously, and net scattering was plotted against tau concentration. Data was fit to a linear function and critical concentration was estimated from the abscissa intercept.

5.2.4 Dye aggregation. Cyanine dye N744 was incubated at 37°C for 1 hour at varying concentrations (.2 – 30 μM) in the presence of either tau or mixed histones. Following incubation, samples were subjected to absorbance spectroscopy (Varian Cary 50 Bio) over a 450-700 nm wavelength range. Dye monomer concentration (C_{m}) was estimated as described previously (West, Lovell et al. 1970; Necula, Chirita et al. 2005). The N744 extinction coefficient was established in methanol, then used to estimate monomer concentrations in aqueous solutions after deconvolution of absorbance data into Gaussian peaks using Beer-Lambert law:

\[ A = \varepsilon C_m l \]  

(14)

where \( A \) is absorbance, \( \varepsilon \) is the extinction coefficient, and \( l \) is path length. Dimer (C_{d}) concentration was estimated from total dye concentration (C_{t}) using the formula:

\[ C_d = (C_t - C_m)/2 \]  

(15)

This value was then used to estimates the dimerization constant \( K_{\text{dim}} \) (West, Lovell et al. 1970; Necula, Chirita et al. 2005):

\[ K_{\text{dim}} = C_m^2/C_d \]  

(16)

Values were derived from at least three spectra and expressed ± SD.
5.2.5 Analytical methods. In order to determine the volume of tau adsorbed onto the microsphere surface ($V_{\text{shell}}$), the diameters of microspheres incubated with or without tau protein were determined. Using these data $V_{\text{shell}}$ was determined using the formula:

$$V_{\text{shell}} = \frac{4}{3}\pi(r^3 - r_0^3) \quad (17)$$

where $r_0$ is the average microsphere radius in the absence of tau protein and $r$ is the average radius of microspheres incubated with tau. Values are given as ± SD of multiple measurements.

5.3 Results.

5.3.1 N744 activity is biphasic. At substoichiometric concentrations N744, a thiacarbocyanine dye, inhibits full-length Htau40 polymerization (Chirita, Necula et al. 2004; Necula, Chirita et al. 2005). (Figure 1 gives N744 structure) To further characterize its effect on tau aggregation, N744 was incubated over a broad concentration range with anionic surfactant inducer $C_{18}H_{37}SO_4Na$ under near physiological conditions and bulk tau concentrations (4 μM). This inducer was chosen because it can be used in both laser light scattering and electron microscopy assays (Necula and Kuret 2004; Necula and Kuret 2004). Consistent with previous findings, data showed that N744 inhibits tau aggregation with an IC$_{50}$ of ~300 nm under standard conditions (Chirita, Necula et al. 2004; Necula, Chirita et al. 2005). However, as concentrations of N744 surpassed 10 μM, inhibition is relieved with a revised IC$_{50}$ estimate of ~13 μM. At still higher concentrations, N744 was capable of enhancing the tau polymerization reaction to a level greater than that found without any inhibitor present. (Figure 5.2) These data suggest, that a complex dose response relationship exists where under different
concentration regimes N744 may act as either an inhibitor or enhancer of tau polymerization. This effect creates a limited concentration range over which the drug is effective at reducing tau polymerization with an IC50 ratio of ~43 between the different phases.

To confirm these results, the effect of N744 on Htau40 polymerization in the presence of anionic microspheres was examined. Unlike other anionic surfactants, microspheres do not undergo micellization or other potentially relevant structural changes (Chirita and Kuret 2004). Similar to results with C_{18}H_{37}SO_{4}Na, N744 depressed tau polymerization in the presence of microspheres up to a concentration of 3 μM. Once concentrations rise above 10 μM, however, inhibition is relieved with a large number of short filaments becoming visible by EM. (Figure 5.3) These data confirm that the concentration effect seen with N744 is not unique to micellar inducers.

5.3.2 Mechanism. In low concentration regimes, N744 inhibits tau polymerization by increasing the critical concentration required for fibrillization. This value represents the lowest protein concentration capable of supporting filament growth. Effects on critical concentration may occur through the stabilization of off-pathway species. For example, α-synuclein aggregation inhibitors have been shown to stabilize protofilaments, an assembly intermediate, and thus prevent their incorporation into mature fibrils (Conway, Rochet et al. 2001). Multiple studies of fibrillization antagonists of α-synuclein and amyloid-β have produced similar findings (Li, Zhu et al. 2004; Zhu, Rajamani et al. 2004; Masuda, Suzuki et al. 2006). Based on these data, we would predict that the effects on critical concentration would reverse at high dye concentrations. To test this hypothesis, critical concentration was estimated in the presence of up to 20 μM N744.
Critical concentration increased up to an optimal N744 concentration of ~4 μM, then began to decrease as N744 concentration rose further. (Figure 5.4) These data indicate that modulation of critical concentration is consistent with the ability of N744 to inhibit filament formation.

Another potential explanation for the behavior of N744 is that at high concentrations it becomes capable of directly inducing tau polymerization similar to Thiazin red, Congo Red and ThS (Chirita, Congdon et al. 2005). To test this hypothesis, full length htau40 was incubated under standard conditions with varying concentrations of N744 in the absence of other inducers. Samples were examined using electron microscopy; however, N744 did not induce aggregation at concentrations up to 100 μM (data not shown). These data demonstrate that the increases in filament mass seen at high dye concentrations are not the result of direct triggering of polymerization.

An additional possibility is that inhibitory activity decreases as a result of formation of higher order dye complexes. Thiacarbocyanine dyes are capable of self-association in both aqueous solutions and on solid surfaces due to strong dispersion forces between their planar faces (Herz 1977). Dye aggregation and variations in quaternary structure result in shifts in absorbance spectra, depending on the aggregates formed. Hypsochromatic shifts to shorter wavelengths are referred to as H-bands and consist of small aggregates but may reach larger sizes in the presence of substrates (Maskasky 1991). Bathochromic shifts, or J-bands, differ from H-bands by the angle of slippage between the planes of successive molecules (Necula, Chirita et al. 2005). In both cases the dye monomers aggregate in parallel plane-to-plane. (Figure 5.5 shows structures of dye aggregates)
To examine the aggregation of N744, absorbance spectra were recorded as a function of dye concentrations under various conditions. In pure methanol, N744 does not aggregate and absorbance appeared as a major band at 568 nm with a weak vibrational shoulder at 547 nm. This data was used to calibrate monomer concentration. Absorbance patterns were then examined under conditions known to promote aggregation. Surfactant micelles alone are capable of influencing dye aggregation, and to control for this N744 absorbance was measured in reactions where tau was replaced with mixed histones. Histone concentrations were chosen on the basis of Corrin-Harkins plots (Chirita, Necula et al. 2004) to yield the same critical micelle concentration as tau protein (Necula and Kuret 2004). Thus histone containing reaction serves as a control for non-specific micelle mediated dye aggregation. Under these conditions, N744 incubated at low bulk concentrations (200-600 nm) produced absorbance spectra dominated by monomer centered at 585 ± 2 nm. The bathochromic shift in absorbance at the monomer peak in the presence of alkyl sulfate detergents, relative to methanol, is consistent with established behavior (Mishra, Behera et al. 2000). As bulk concentrations of N744 increase however, the relative intensity of the monomer band decreased as the intensity of H-band aggregates rose. (Figure 5.6A) The concentration of monomer as a function of bulk N744 was determined by absorbance spectroscopy and used to calculate the concentration of aggregated dye protomers. Monomer concentration was plotted against protomer concentration on a double log plot and fit to a linear function, the slope of which was determined to be 2.4 ± 0.4. (Figure 5.7) This indicates that dimer is the predominant aggregate species formed in this concentration range. Using equations 14 and 15, the equilibrium dissociation constant (K_{dim}) for dimerization was calculated as
2.2 ± 0.5 μM. These results are similar to the value obtained by incubating N744 in assembly buffer alone (2.1 ± 0.4 μM). In contrast, as N744 concentration continue to rise above 600 nm, broad H-bands appear at shorter wavelengths and the slope of the double log plot steepens. These data indicate that higher order aggregates form as bulk concentrations of N744 increase. This pattern is consistent with previous studies of thiacarbocyanine dyes in the presence of alkyl sulfate detergent micelles (Chibisov, Prokhorenko et al. 1999).

This pattern is modified once tau protein is added to the reaction mixture. Even at concentration as low as 200 nM levels of dye monomer, appearing as a peak at 592 ± 1 nm, were greatly reduced in the presence of tau. (Figure 5.6B) This suggests that the presence of tau protein was sufficient to trigger significantly greater levels of dye aggregation than those seen with detergent alone. A double log plot of monomer against protomer concentration was created and, again data was fit to a linear function. The slope of the resultant line was determined as 2.0 ± 0.1, indicating that dimers were the primary aggregate formed in the 200-800 nM concentration range. (Figure 5.7) Absorbance spectra showed a major dimer band at ~540 nm, and the K_{dim} was estimated as 0.15 ± 0.01 μM, significantly lower than that observed with micelles alone. These data indicate that the presence of tau in combination with anionic micelles supported dye dimer formation an order of magnitude below that seen in buffer, tau, or micelles alone. Further increases in bulk N744 concentration resulted in the appearance of a third absorbance peak centered at ~513 nm, with a concurrent relative decrease in the monomer and dimer peaks. These data indicate that at higher bulk N744 concentrations, above 1 μM, higher order aggregates are formed at the expense of monomer and dimer. At the highest dye
concentrations, 30 μM, absorbance spectra are dominated by H-aggregate with monomer and dimer peaks becoming indistinct. This suggests that the loss of inhibitory activity at higher N744 concentrations may be a result of sequestration of dye into higher order aggregates.

However, though these findings provide a reason for the loss of inhibitory activity they do not explain the observed enhanced fibrillization seen at high dye concentrations. Because N744 does not directly trigger aggregation, we hypothesized that dye aggregation may work to increase the concentration of tau available for incorporation into filaments. When utilizing anionic surfactant inducers tau protein distributes into three pools, soluble protein, protein adsorbed onto the micelle or microsphere surface, and tau incorporated into filaments. N744 aggregates may be capable of shifting tau protein from one pool to another, most likely between the micelle/microsphere bound to the soluble fraction. To test this hypothesis, the volume of tau coating the surface of anionic microspheres was measured in the presence of varying concentrations of N744. By electron microscopy, microspheres incubated in the presence of tau appear to have a rough coat of protein. In order to measure the volume of this protein coat, the diameters of beads incubated with or without tau protein in varying concentrations of N744 were measured. In the absence of both tau and N744 beads were roughly spherical with an average diameter of 55 ± 2 nm. Addition of N744 up to a concentration of 30 μM did not alter these values. Addition of tau resulted in increased diameters as the beads became coated with negatively stained material. (Figure 5.8) The net volume difference between the two populations, or $V_{shell}$, was determined as a function of N744 concentration. Low concentrations of N744 produced only modest effects on shell volume. However, as
N744 concentrations rose above 1 μM, the amounts of material bound to the microsphere surface gradually declined. These data suggest that N744 aggregates are capable of competing with soluble tau for binding to the microsphere surface. By doing so the proportion of tau available for incorporation into filaments increases therefore leading to greater filament mass at equilibrium.

5.3.3. Effect of posttranslational modification. In disease, tau protein becomes heavily postranslationally modified including by extensive phosphorylation. (Buee, Bussiere et al. 2000) Previous studies have shown that incorporation of site specific negative charges can promote fibrillization by releasing tau from the microtubules (Biernat, Mandelkow et al. 1992; Bramblett, Goedert et al. 1993) and reducing the critical concentration required for tau polymerization (Necula and Kuret 2004). This effect can decrease the potency of polymerization inhibitors such as N744. To determine the effects of phosphorylation on the biphasic activity of N744, assembly of a phosphorylation mimicry mutant, Htau40T212E, in the presence of N744 was examined (Figure 5.2). The mutant protein was incubated under standard conditions in the presence of C18H37SO4Na and increasing concentrations of N744. This particular mutant was chosen because it produces the greatest effect on critical concentration relative to wild type tau. Htau40T212E also binds N744 with a similar potency to wild type thus eliminating binding affinity as a potential variable (Necula and Kuret 2004; Necula, Chirita et al. 2005). Under near physiological conditions, N744 inhibited polymerization of the mutant protein with an IC50 of ~600 nm, demonstrating the N744 potency is influenced by filament stabilizing modifications, such as phosphorylation. As with the wild type protein, inhibition was relieved as N744 concentrations rose. Higher dye concentrations led to a retracing of the IC50 to ~10 μM.
bulk N744. This retracing occurred at a lower bulk N744 concentration compared to wild type tau. The ratio of the IC$_{50}$ values obtained using mutant tau was $\sim$17, less than half that of wild type protein. These data show that the polymerization promoting effects of pseudophosphorylation affect both phases of N744 activity resulting in a narrowing of the effective concentration range.

5.4 Discussion. These results suggest that aggregation state can greatly influence the ability of cyanine dyes to modulate tau filament formation. Further, they show that the presence of exogenous macromolecules can exert a great effect on dye polymerization even at submicromolar concentrations. Together these findings indicate that dye aggregation is a potential source of complexity for pharmacological modulation of protein polymerization.

These findings are significant in several respects. First, they predict that dye aggregation is an important factor regulating activity in both the inhibitory and disinhibitory phases. During the inhibitory phase, a common observation among protein fibrillization inhibitors is that concentration effect curves are abnormally steep (Heiser, Scherzinger et al. 2000; Heiser, Engemann et al. 2002; Zhang, Smith et al. 2005). The steepness of the curves may result from high protein concentrations relative to ligand binding affinity. This then drives reactions into Goldstein’s Zone C, where concentration effects appear abnormally steep due to the fact that inhibition is directly proportional to ligand concentration (Goldstein 1944). However, because protein polymerization is complex and may involve the formation of intermediate species, ligands are presented with multiple potential binding partners (Uversky and Fink 2004). This raises the
possibility that steep concentration-effect relationships result from a form of cooperativity at low binding partner concentrations. We propose that the modestly steep concentration-effect curve for N744 inhibition reflects cooperativity due to dye dimerization. This hypothesis is supported by data showing time dependent dimer formation under physiological conditions in the presence of tau protein. In the presence of both tau protein and anionic micelles (conditions which trigger conformational changes in the tau protein) N744 dimers are greatly stabilized such that the dimerization constant, $K_{\text{dim}}$, decreases ten fold relative to $K_{\text{dim}}$ in the presence of either tau or micelles alone. More significantly, the dimerization constant drops below the IC$_{50}$ for inhibition of fibrillization. The anionic micelles have been shown to stabilize assembly competent, $\beta$-sheet enriched tau species (Chirita, Congdon et al. 2005). Because some cyanine dyes bind selectively to $\beta$-sheet structure, it is possible that this intermediate tau species could serve as a template for N744 dimerization (Hermel, Holtje et al. 1995; Sabate and Estelrich 2003). At low dye concentrations, interaction with this template promotes N744 dimerization. However, monomers or other small dye oligomers visible in the absorbance spectra may be the inhibitory species. An examination of the structure activity relationships between cyanine dyes may aid in identification of the inhibitory species. In addition, bis-cyclo-cyanines which undergo intramolecular folding to mimic face to face dimer formation will allow us to test the hypothesis that dimerization results in inhibitory activity (Herz 1974).

These results are also significant when considering the design and interpretation of fibrillization inhibitors in biological systems. Development of neurofibrillary lesions correlates with both cell loss and cognitive decline (Gomez-Isla, Hollister et al. 1997;
Ghoshal, Garcia-Sierra et al. 2002). Though the role of tau polymerization in disease is a subject of debate, filaments may be directly toxic to the cells that contain them. However, in some transgenic animal models, simple over-expression of wild type human tau protein is sufficient to cause neurodegeneration (Terwel, Lasrado et al. 2005). Because many biological models rely on over-expression of wild type or mutant tau protein, determining the source of toxicity in these models is difficult (Santacruz, Lewis et al. 2005). In authentic disease tau expression is not upregulated, which makes resolving the issue of toxicity a crucial one for developing future treatment strategies (Hyman, Augustinack et al. 2005). Development of selective fibrillization inhibitors may help to clarify these issues. Results from the present study suggest that appropriate dosing will be of importance for assessing compounds such as N744. Post-translational modifications including phosphorylation and C-terminal truncation, as well as supersaturated protein concentration, antagonize the effects of cyanines on critical concentration and diminish their potency (Necula and Kuret 2004; Yin and Kuret 2006). Concurrently, higher order dye aggregation may further constrict the therapeutic index to a narrow window of effective concentrations. As a result, even under optimal dosing conditions, fibrillization antagonists may provide only partial inhibition. A similar phenomenon may occur in models of Huntington’s disease treated with Congo Red. Congo Red is capable of inhibiting huntingtin polymerization with a monophasic and steep concentration-effect relationship \textit{in vitro} (Heiser, Scherzinger et al. 2000). This pattern becomes much more complex in biological models, with dose response relationships similar to those seen here emerging (Smith, Portier et al. 2001). Congo Red and other \(\beta\)-sheet binding dyes are capable of forming disordered micelles at millimolar
concentrations in addition to smaller aggregates at lower, pharmacologically relevant concentrations (Stopa, Gorny et al. 1998; Stopa, Gorny et al. 1998). For example, though the dimerization constant for Congo Red has been estimated as ~20 μM in aqueous solutions, this can vary in the presence of macromolecules (Yasunaga and Nishikawa 1972). Formation of larger H-aggregates of N744 also depends on the molecular environment, as none are observed at dye concentrations of up to 5 μM in buffer alone (Necula, Chirita et al. 2005). However, in the presence of anionic surfactant micelles drives extensive N744 aggregation at those same concentrations. These results demonstrate that though compounds may be well behaved in vitro, complex dose response relationships may emerge in biological systems due to heterogeneous nucleation.

Finally, results indicate that structural analysis studies must be carefully interpreted to account for the aggregation propensities of ligands such as N744. As both X-ray crystallography and NMR rely on high ligand concentrations for analysis, structures obtained may reflect higher order aggregates rather than the inhibitory species.

To summarize, cyanine dye N744 exhibits a complex dose response relationship in vitro with a limited range of concentrations at which it is an effective polymerization inhibitor. Dye aggregation may be an important factor mediating both inhibitory and disinhibitory phases of the concentration-effect curve. This suggests that ligand aggregation may be responsible for complex dose response data obtained from protein polymerization inhibitors in biological models of disease.
5.5. Figures

Figure 5.1. Structure of cyanine dye N744.
Figure 5.2. N744 displays biphasic concentration dependence.

Wild-type htau40 (●) and pseudophosphorylation mutant htau40T212E (○) were each incubated (4 h at 22°C) without agitation at 4 μM concentration in assembly buffer containing C_{18}H_{37}SO_{4}Na inducer (50 μM) and various concentrations of N744. Aliquots were then removed, stained with uranyl acetate, and viewed by electron microscopy as described in Materials and Methods. Each point represents total length of all filaments expressed as a normalized percentage of filament lengths measured in the presence of DMSO vehicle alone (triplicate determination ± S.D.), whereas the solid lines are drawn solely to aid visualization. Under these conditions, the effects of N744 on fibrillization of both htau40 and htau40T212E followed a biphasic concentration dependence.
Figure 5.3. N744 action in the presence of anionic microsphere inducer.

Wild-type htau40 (4 μM) was incubated (24 h at 37°C) in assembly buffer containing carboxylate-substituted polystyrene microspheres (124 pM) and varying concentrations of N744 (0, 1, 3, 10, or 30 μM as labeled in upper left corner of each panel). Aliquots were then removed and visualized by electron microscopy. In the absence of N744, tau formed filaments extending from the microsphere surface. Increasing concentrations of N744 initially decreased tau filament formation, but further increases relieved inhibition. These data suggest that the biphasic nature of N744 concentration dependence is not unique to surfactant inducers. Bar = 500 nm.
Figure 5.4. N744 modulates the critical concentration for fibrillization.

The critical concentration ($K_{\text{crit}}$) of wild-type htau40 fibrillization was estimated as a function of bulk N744 concentration (0, 1, 4, 7, 20 μM) by static laser light scattering spectroscopy as described in Materials and Methods. The ability of N744 to modulate critical concentration displayed a biphasic concentration dependence, with maximal activity apparent at ~4 μM N744.
Figure 5.5. Structures for N744 aggregates.

N744 and other thiacarbocyanine dye monomers are capable of aggregation at their planar surfaces. Changes in absorbance spectra depend on the aggregates formed and the angle of slippage between the planes of successive molecules. As shown, dye dimers have a slippage angle of 90°. Formation of H-bands, hypsochromatic shifts to shorter wavelengths, and J bands, or bathochromic shifts, result from the formation of larger dye aggregates. H and J aggregates differ in having slippage angles of greater, or less than 32° respectively.
Figure 5.6. N744 absorbance spectra.

Varying concentrations of N744 (0.2 – 30 μM) were incubated (1 h at 37°C) in assembly buffer containing C\textsubscript{18}H\textsubscript{37}SO\textsubscript{4}Na inducer (50 μM) and either (A) mixed histones or (B) htau40, subjected to absorbance spectroscopy, then plotted as extinction coefficient (ε; normalized to monomer band intensity in methanol) versus wavelength. The positions of N744 absorbance bands corresponding to monomer (M), dimer (D), H-aggregate (H), and J-aggregate (J) are shown at the top of each panel. The dotted line in the top panel depicts the absorbance of monomeric N744 in neat methanol solvent. Note: ordinate scales differ in the two panels.
Concentrations of N744 in monomeric ($C_m$) and aggregate ($C_t-C_m$) forms were calculated after deconvolution of the absorbance spectra shown in Fig. 5. Data points correspond to spectra collected in the presence of either htau40 (○) or mixed histones (●), with the associated errors corresponding to the S.E. of the estimate of the Gaussian fit of spectral data. Each line represents best fit of the data points to a linear regression. In the presence of htau40, data points corresponding to the bulk N744 concentration range of 0.2 – 0.8 μM fell on a single regression line with slope 2.0 ± 0.1. In the presence of histones, the regression slope was 2.4 ± 0.4 in the bulk N744 concentration range of 0.2 – 0.6 μM, but much higher in the range 0.6 – 1 μM (dotted line). These data indicate that dimerization is the principal aggregation reaction in the sub-micromolar concentration regime.
Carboxylate-substituted polystyrene microspheres (124 pM) were incubated (24 hours at 37°C) in assembly buffer containing htau40 (4 μM) and varying concentrations of N744. Aliquots were then visualized by transmission electron microscopy. (A) In the absence of tau, microspheres appeared as smooth-surfaced, roughly spherical objects without any associated filamentous material. (B) In the presence of tau protein, microspheres developed rough surfaces and appeared enlarged relative to beads incubated in the absence of protein, with 10 - 20% having associated filaments. The additional volume resulting from the increase in diameter ($V_{\text{shell}}$) was calculated using equation 4. (C) Increasing concentrations of N744 decreased the apparent shell volume, consistent with release of bound tau from the microsphere surface. Bar = 100 nm.
Alzheimer’s disease is both a devastating and costly illness, and with changing demographics, specifically the increase in the elderly population, represents a growing health concern. The intracellular lesions that define AD and related tauopathies correlate with neuron loss and cognitive decline, thus making them a powerful marker of disease progression. Tau aggregates may also be directly toxic to neurons, further highlighting the importance of understanding tau polymerization. Previous studies have been limited by the use of tau constructs, non-physiological conditions, and the fact that recombinant full-length protein is resistant to spontaneous association. The results presented herein represent significant advances in the understanding of tau polymerization and tau pharmacology.

Herein we have clarified many of the unresolved issues related to tau polymerization. The discovery of a partially folded intermediate species in the reaction pathway provides an explanation as to why tau is refractory to polymerization. Additionally, we have identified new ways to induce polymerization through the stabilization of these intermediates. With this improved understanding of the reaction mechanism, a detailed analysis of reaction kinetics became possible. Because the partially folded intermediates are the true substrate for nucleation, and inducers which
stabilize them trigger spontaneous assembly, we were able to apply classical nucleation theory to tau polymerization. This in turn allowed an estimation of the critical nucleus cluster size and elementary rate constants, the first for full length tau protein. We were also able to utilize these inducers to trigger polymerization in all six tau isoforms. This allowed the effects of individual exons on both the nucleation and extension phases to be quantified, something which had not been possible previously. Results from those studies reveal how mutations which affect isoform ratios cause lesion formation and resolve other poorly understood phenomena. For example, the difference in critical concentration between isoforms can provide a rationale for why fetal tau does not polymerize despite being highly phosphorylated.

In addition to a better understanding of the reaction mechanism, the data presented here also contribute to the understanding of tau pharmacology. Though planar aromatic compounds have been shown to both induce and antagonize polymerization in tau and other amyloid forming proteins, it was not known why compounds with similar structures should produce opposite effects. Our data suggest that the key difference is the ability of the compounds to self-assemble, which differentiates the agonists and inhibitors. This idea will be further developed in future studies. Pharmacology also provides an opportunity for hypothesis testing and can be used to explore issues such as whether tau aggregates contribute directly to cell death.

Herein we have presented data regarding polymerization of all six human tau isoforms and the effects of alternatively spliced exons. These data have relevance for the study of FTDP-17, in which mutations in the tau gene are sufficient to trigger lesion formation. These mutations are dominant and result in changes in personality followed
by cognitive decline. As discussed above, many of these mutations are intronic and exert their affects by altering the isoform ratio. However, intronic mutations are not the only causes of FTDP-17. To date over 20 disease causing mutations have been discovered in the coding region of the tau gene (Brandt, Hundelt et al. 2005; Goedert and Jakes 2005). Many of the exonic mutations are found in the microtubule binding region, the section of the tau molecule which makes up the core of PHFs, though some occur in the amino and carboxy terminals (Brandt, Hundelt et al. 2005; Goedert and Jakes 2005).

Mutations in the tau gene may promote filament formation in several ways. First, mutations have been found to reduce the affinity of tau for microtubules as shown through a reduced ability to promote microtubule assembly (Hasegawa, Smith et al. 1998; Hasegawa, Smith et al. 1999; Barghorn, Zheng-Fischhofer et al. 2000; Rizzini, Goedert et al. 2000; Neumann, Schulz-Schaeffer et al. 2001; Hayashi, Toyoshima et al. 2002; Poorkaj, Muma et al. 2002; Yoshida, Crowther et al. 2002; Grover, England et al. 2003; Hogg, Grujic et al. 2003; Kobayashi, Ota et al. 2003). Because mutant tau does not bind microtubules as effectively compared to wild type protein, mutations may result in an excess of tau in the cytoplasm. Additionally, mutant proteins may be more prone to phosphorylation than wild type tau. Studies have shown that FTDP-17 mutants became hyperphosphorylated faster and to a greater extent than wild type protein and are poor substrates for protein phosphatases (Goedert, Satumtira et al. 2000; Barghorn, Davies et al. 2004). Mutations may also affect the kinetics of the assembly reaction. Reduction in the critical protein concentration would result in the greater amount of polymerization seen relative to wild type tau. An increased nucleation rate could also result in the greater propensity for filament formation seen in FTDP-17 mutants.
To address these issues, a selection of mutant proteins which have not been shown to alter splicing will be analyzed using a variety of techniques to assay changes in reaction kinetics and adoption of secondary structure relative to wild type tau. As with the individual tau isoforms, the critical concentrations, elongation rate constants and lag times will be compared utilizing thiazin red inducer and assayed with the well characterized electron microscopy assay. Assembly of mutant proteins has previously been assayed using sedimentation, fluorescence, electron microscopy, and laser light scattering (Arrasate, Perez et al. 1999; Goedert, Jakes et al. 1999; Nacharaju, Lewis et al. 1999; Li, von Bergen et al. 2002; Barghorn, Davies et al. 2004; Brandt, Hundelt et al. 2005). However, none of these previous investigations have quantified findings with respect to changes in critical protein concentration, lag time, or rate constants. Results are often descriptive, stating only that mutants resulted in greater filament mass at equilibrium. Others rely on fluorescence-based assays, which, as stated above, may be confounded by the presence of beta sheet containing nonfibrillar species (Barghorn, Zheng-Fischhofer et al. 2000; Li, von Bergen et al. 2002). Light scattering results were obtained before the discovery of reduced critical micelle concentration in the presence of tau (Gamblin, King et al. 2000). In addition, artificial truncated constructs are often used which have limited relevance to disease (Barghorn, Zheng-Fischhofer et al. 2000; Li, von Bergen et al. 2002). With respect to secondary structure, we have previously shown that even small changes can be detected in full-length his-tagged proteins with circular dichroism (Chirita, Congdon et al. 2005). Therefore, detecting changes in adoption of secondary structure relative to wild type protein should be possible. In addition to revealing how coding region mutations cause disease, by utilizing proteins with
mutations occurring in different exons results may provide insights into the relative importance of different regions in polymerization.

In addition to an examination of the effects of mutations on tau polymerization, further studies of tau pharmacology may provide a better understanding of the properties which make an effective inhibitor and whether inhibition of fibrillization is necessary to arrest disease progression. Experimental data have shown that the thiacarbocyanine dye N744 displays a complex dose response relationship in the presence of tau protein and indicate that dye dimers may be the inhibitory species. Inhibition was reversed at higher N744 concentrations as the dye molecules formed larger aggregates, indicated by the appearance of H bands in the absorbance spectra. Results suggest that if dimerization could be encouraged, while larger aggregate formation prevented, one could obtain potent inhibition of fibrillization without the disinhibitory phase. To test this hypothesis, we will examine the activity of a bis-cyclo-cyanine. This molecule can mimic dimerization through intramolecular folding and does not form larger aggregates at higher dye concentrations (Herz 1974). In addition, we will examine the structure activity relationship of multiple cyanine dyes of similar structure to N744. Compounds will be tested for inhibitory activity and dye aggregation in the presence of tau. If our hypothesis is correct, and dimer formation is key to inhibiting polymerization, then these findings could influence the development of novel therapeutic compounds.

Finally, though neurofibrillary lesion formation has been studied extensively at the macroscopic level and correlations between lesion formation and neurodegeneration exist, it remains uncertain whether lesions cause neuronal death (Arriagada, Growdon et al. 1992; Gomez-Isla, Hollister et al. 1997). We will examine whether compounds that
have been successful in preventing degeneration are inhibitors of filament formation \textit{in vitro}. Several potential antagonists of tau filament formation belonging to different classes have been described, including anthroquinones, polyphenols, and porphyrins (Pickhardt, Gazova et al. 2005; Taniguchi, Suzuki et al. 2005). Many of these compounds were chosen for further study based on their ability to inhibit thioflavin S fluorescence. However, as stated above thioflavin S fluorescence does not necessarily indicate the presence of filamentous tau, thus these compounds may not actually inhibit filament formation (Chirita and Kuret 2004). These compounds may also prevent thioflavin binding without interfering with filament growth. In addition, inhibitory activity may result from the instability of the compounds under oxidizing conditions. Many of the compounds contain quinone structures, which when oxidized may be capable of forming covalent bonds with the tau protein (Zhu, Rajamani et al. 2004). This behavior has been observed with baicalein, a polyphenol. Baicalein was capable of inhibiting \(\alpha\)-synuclein fibrillization through formation of a Schiff base (Zhu, Rajamani et al. 2004). A similar process may be occurring when these chemicals are incubated with tau. Because this process is irreversible, these drugs may have limited utility. Covalent modification may prevent filament formation but it may also interfere with normal protein function.

Previously, ligands capable of preventing degeneration in \textit{in vivo} models of disease have been described (Hall, Lee et al. 2002). Overexpression of wild type human tau in lamprey neurons resulted in the formation of filaments consisting of hyperphosphorylated tau protein. The pattern of degeneration found in these cells mirrors that which has been observed in disease tissue, beginning with distal processes and
progressing towards the cell body (Hall, Lee et al. 2001). This progression can be retarded by the addition of a small molecule inhibitor, termed N3, to the water in which the fish live. A compound with a similar structure, N4, has also been tested but does not appear to slow degeneration (Hall, Lee et al. 2002). Though these compounds have been tested \textit{in vivo}, they have not been assayed for their ability to inhibit filament formation \textit{in vitro}. We hypothesize that tau fibrillization plays a central role in disease progress, and that inhibition, or disaggregation of filaments will result in a reduction of degeneration. To test this, the ability of N3 and N4 to inhibit tau polymerization \textit{in vitro} under near physiological conditions will be examined. These proposed future studies will build on the foundation established by the findings described above and will serve to further increase understanding of tau biology.
LIST OF REFERENCES


