Published in final edited form as:

Biochemistry. 2017 November 07; 56(44): 5900–5909. doi:10.1021/acs.biochem.7b00653.

Dimerization and Long Range Repulsion Established by Both Terminals of the Microtubule Associated Protein Tau

Zachary J. Donhauser^{*}, Jared T. Saunders[†], Dennis S. D'Urso[‡], and Teresa A. Garrett

Department of Chemistry, Vassar College, Poughkeepsie, New York 12601

Abstract

Tau is a microtubule associated protein found in neuronal axons that has several well-known functions, such as promoting microtubule polymerization, stabilizing microtubules against depolymerization, and spatially organizing microtubules in axons. Two contrasting models have been previously described to explain tau's ability to organize the spacing between microtubules: complementary dimerization of the projection domains of taus on adjacent microtubules, or, tau's projection domain acting as a polyelectrolyte brush. In the present study, atomic force microscopy was used to interrogate intermolecular interactions between layers of tau protein immobilized on mica substrates and on silicon nitride atomic force microscope tips. On these surfaces, tau adopts an orientation comparable to that when bound to microtubules, with the basic microtubule binding domain immobilized and the acidic domains extending into solution. Force distance curves collected with atomic force microscopy reveal that full-length human tau, when assembled into dense surface-bound layers, can participate in attractive electrostatic interactions consistent with the previously-reported dimerization model. However, modulating the ionic strength of the surrounding solution can change the structure of these layers to produce purely repulsive interactions consistent with a polyelectrolyte brush structure, thus providing biophysical evidence to support both the zipper and brush models. Further, a pair of projection domain deletion mutants were examined in order to investigate whether the projection domain of the protein is essential for the dimerization and brush models. Force-distance curves collected on layers of these proteins demonstrate that the C-terminal can play a role analogous to that of the projection domain.

Graphical Abstract

^{*}Corresponding author: Prof. Zachary Donhauser, Department of Chemistry, Vassar College, 124 Raymond Ave, Poughkeepsie, New York 12601, Telephone: (845) 437-5739; FAX: (845) 437-5747; zadonhauser@vassar.edu.

[†]Present address: Department of Molecular Biology, Princeton University, 119 Lewis Thomas Laboratory, Washington Rd., Princeton, NJ 08544

[‡]Present address: 1025 Spruce St., Philadelphia, PA 19107

Supporting Information

The Supporting Information (PDF) contains the methods and data of a turbidity assay of microtubules copolymerized with tau constructs.

The authors declare no conflict of interest.

INTRODUCTION

Tau is a neuronal microtubule associated protein (MAP), necessary for proper development of the central nervous system. It has several well-established functions, which include promoting microtubule polymerization, stabilizing them against depolymerization, and controlling the spacing between microtubules in axons.^{1, 2} In addition, tau has been the focus of significant recent attention because of its connection with a variety of neurodegenerative disorders such as Alzheimer's disease, frontotemporal dementia and parkinsonism linked to chromosome,17 and Pick's disease.³ These disorders have pathologies characterized by aberrant aggregation of tau, so interest in the protein has increased in part because these tau abnormalities have been considered as a target for drugs and therapies for these diseases.^{4, 5}

In solution, tau is a natively unstructured protein, so it is typical to describe the protein as containing discrete regions delimited by enzymatic cleavage sites, with specific known functions, and/or with characteristic amino acid content.⁶ The tau domains that are of interest in the present work (and highlighted in Figure 1) are the projection domain (in full length human tau, amino acids 1–151), the proline rich domains P1 (151–198) and P2 (198–244), the four repeats of the microtubule binding domain (244–369), the so-called 'fifth repeat' flanking the microtubule binding domain (369–400), and the C-terminal (400–441). The projection domain comprises a high density of acidic residues, such that it bears a significant negative charge at physiological pH and above. When tau is bound to microtubules, this region of the protein extends away from the microtubule surface. The proline rich region is delineated into two domains (P1 and P2) by a chymotrypsin cleavage site at S198, which coincidentally cleaves the protein into a C-terminal fragment (198–441) that binds microtubules and an N-terminal fragment (1–197) that does not. Both the P1 and P2 domains comprise 23% proline. The microtubule binding domain (residues 244–369) is very basic, interacting with microtubules in part through electrostatic interactions with the acidic microtubule surface. While the majority of the C-terminal end of the tau is basic, a second acidic region of the protein is found in the terminal 41 amino acids.

Tau's structure has been investigated with a variety of approaches such as IR spectroscopy, CD spectroscopy, and electron microscopy,⁷ chemical denaturation,⁸ and single molecule fluorescence and modeling,⁹ which support the conclusion that tau is intrinsically disordered when in solution. However, some evidence has been found to indicate that tau adopts transient secondary structure through intramolecular interaction between the projection domain, the C-terminal 'tail' and the microtubule binding domain. Combined FRET and EPR measurements indicated transient interactions between the C- and N-terminals of the protein with the microtubule binding domain,⁸ resulting in a paperclip-like conformation of the protein. In another set of experiments, aggregation of tau that normally occurs in solution through interactions between microtubule binding domains was promoted by enzymatic cleavage of the C-terminal domain¹⁰ and inhibited by the presence of the C-terminal fragment.¹¹ This study supports a model with an important interaction between the C-terminal and the microtubule binding domain.

One technique that has been successful in interrogating the structure and forces present in microtubule-tau complexes is small-angle X-ray scattering. Using an approach where the force between microtubule-tau complexes was modulated using osmotic pressure, Chung *et al* demonstrated that at high coverages, taus with long projection domains could stabilize well-spaced microtubules up even under high pressure, pointing to a brush-like structure for bound tau.¹² However, under extreme pressures that forced microtubules to bundle closely, evidence for tau-tau crosslinking was observed. In a follow-up study,¹³ the same group demonstrated that the strongly anionic character of tau's projection domain was the primary component of a repulsive barrier that prevented close approach of neighboring microtubules. In addition, using C-terminal deletion mutants, they demonstrated that the C-terminal domain can play an analogous role to the projection domain in controlling interactions between tau-microtubule complexes.

An additional important tool for the study of tau aggregation and intermolecular interactions is atomic force microscopy, which has been applied using a variety of experimental strategies. Feinstein and coworkers used a combination of biochemical assays and AFM imaging of chemically crosslinked microtubule-tau complexes to show that oligomerization of tau may be induced by its interactions with microtubules.¹⁴ Barrantes *et al.* used a combination of AFM imaging and force spectroscopy to follow the formation of tau aggregates over long time periods, and observed the formation of disease-like tau fibrils both in the presence and absence of aggregation-inducers.¹⁵ Similarly, Müller and coworkers also observed the formation of tau fibrils and characterized them using high resolution imaging¹⁶ to reveal helical structures, which were interrogated by force spectroscopy and surrounded by a "fuzzy coat".¹⁷ The "fuzzy coat" surrounding the fibrils had two distinct layers which were attributed to the projection and C-terminal domains protruding from a fibril core composed of microtubule binding domains. The same group also investigated intramolecular interactions in pro- and anti-aggregant tau mutants using force 'pulling' experiments, and found evidence for some folding in the microtubule binding domain, and evidence for nonspecific electrostatic interactions between the termini and the microtubule binding domain.^{17, 18}

Complementing these AFM experiments are a pair of earlier studies that examined longer range and intermolecular interactions between the projection domains of MAPs and tau when immobilized on solid mica substrates.^{19, 20} Mukhopadhyay and Hoh used mica as a substrate to immobilize a mixture of MAPs that included MAP2a/b and tau, and interrogated these layers with AFM. Their results were consistent with immobilization of microtubule binding domains along with the formation of a polyelectrolyte brush consisting of net-negatively charged MAP projection domains protruding from the surface into the surrounding solution. In this model, the polymer brush creates a long-range repulsive force that is primarily entropic in origin.^{19, 21} In a similar study, Israelachvili and coworkers used a surface force apparatus to investigate interactions between tau layers immobilized on mica surfaces.²⁰ When tau was immobilized on two opposing mica surfaces a long range repulsive interaction was observed, followed by an attractive jump-to-contact. These results were described in terms of an “electrostatic zipper” model whereby tau forms complementary dimers in solution and when immobilized on surfaces. These dimers were proposed to result from a symmetric electrostatic interaction between the negatively-charged N-terminal projection domains and the positively charged proline-rich domains of dimer partners, and the results were used to support a cross-bridge model for controlled spacing and bundling of microtubules.

In both of these studies, mica served as a particularly suitable adsorption substrate for MAPs not only because of its ease of preparation and atomic scale flatness, but more importantly because of its ability to mimic microtubules in binding to MAPs. Microtubules and mica have similar surface charge densities ($\sim 1 \text{ e}^-/\text{nm}^2$),^{19, 20, 22} which support relatively strong electrostatically-based immobilization of the positively-charged microtubule binding domains while allowing the negatively-charged projection domain to protrude into solution.

The present work describes AFM experiments related to earlier studies^{19, 20} that investigate tau immobilized on mica but that examine more closely the roles of both the projection domain and the C-terminal in determining tau-tau intermolecular interactions. Work on the three tau constructs shown in Figure 1 is reported: FL-htau40, full length 441 amino acid human tau; (1–150), a projection domain deletion mutant with the first 150 N-terminal amino acids removed but P1 and P2 intact; and (1–197), a projection domain deletion mutant with the first 197 amino acids removed, lacking P1 but with P2 intact. Our experiments also examined the behavior of the protein at different solution conditions, described schematically in Figure 2. At low ionic strength conditions, we found that the N-terminal projection domain participates in long-range repulsive and attractive electrostatic interactions consistent with an “electrostatic zipper” model,²⁰ but we also found that the negatively-charged C-terminal domain can mediate similar interactions. At ionic strength closer to physiological conditions, we did not observe evidence for attractive electrostatic interactions between tau projection domains or evidence for tau dimerization, but did find data consistent with the projection domain extended from the surface to create a polymer brush. Our data are consistent with a model where both the N-terminal and C-terminal domains each establish polyelectrolyte brushes when immobilized on a solid support, and that only under certain restricted conditions could we observe specific attractive intermolecular interactions that would be necessary for dimerization of the protein.

Furthermore, the projection domain is not essential for establishing the putative “electrostatic zipper” dimerization that was described previously.²⁰

MATERIALS AND METHODS

Cloning and protein purification -

FL-htau40 and two N-terminal deletion constructs were PCR amplified using the cDNA encoding 4R human tau 40 cloned in the pRK172 plasmid (a generous gift from Dr. Linda Amos) and the following primers (Eurofins MWG Operon): a single C-terminal primer [5' GGTGGTGAATTCTCACAACCCTGCTTGCCAGGG 3'] and three unique N-terminal primers [5' TAGTAGCATATGGCTGACCCCGCCAGGAGTTCG 3' (FL-htau40); 5' TAATTACATATGATC-GCCACACCGCGGGGAGCAGCCC 3' ((1–150)); 5' TAATAACATATGAGCAGCCCGGCTC-CCCAGGCACTCCC 3' ((1–197))]. The C-terminal and N-terminal primers introduce BamHI and NdeI sites, respectively (underlined). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Purified PCR products and pET23 were digested with BamHI and NdeI (New England Biolabs). Digested pET23 was treated with calf intestinal phosphatase (CIP) (New England BioLabs). PCR products and linearized pET23 were resolved on a 1% TAE agarose gel and then excised from the gel and extracted using a QIAquick Gel Extraction Kit (Qiagen). Each tau PCR construct was ligated into the pET23 vector using T4 DNA ligase (New England BioLabs). Ligation reactions were transformed into One Shot® XL1 Blue cells (Invitrogen) and Transformants selected for by growth on LB containing 100 µg/mL ampicillin (LB-amp) agar overnight at 37 °C. Individual colonies were cultured overnight at 37°C with shaking at 225 rpm in 3 mL LB-amp liquid media. Plasmids were purified from the overnight cultures using a MiniPrep kit (Qiagen), digested with BamHI and NdeI to verify presence of the insert. All tau constructs were confirmed by sequencing at Molecular Cloning Labs.

Plasmids were transformed into BL21(DE3) cells (Stratagene) and individual colonies were selected for growth overnight in 3 mL LB-amp at 37°C and then used to start 1 L culture at OD₆₀₀ = 0.01. Cultures were grown to OD₆₀₀ = 0.4, at which time protein expression was induced with 0.5 mM IPTG and cultured for an additional 3 hours. Cells were collected by centrifugation at 5000 × g, 4 °C for 20', washed with acetate buffer (50 mM sodium acetate pH 5.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT), collected again by centrifugation as described above, then re-suspended in 10 mL acetate buffer and stored at –80°C.

Re-suspended cultures were lysed twice using a French pressure cell at 18000 psi. The resulting lysate was centrifuged at 100,000 × g and 4°C for 15 minutes to remove membranes and un-lysed cells. The membrane-free supernatant was immersed in a boiling water bath for 5 minutes, placed on ice for at least 30 seconds, and spun at 100,000 × g at 4 °C for 15 min to remove precipitated proteins. Typically, ~8 mL of heat-treated supernatant was purified at a flow rate of 1 mL/min using a 5 mL HiTrap SP ion exchange column (GE Healthcare) equilibrated in acetate buffer, with proteins separated on a 0-0.5 M NaCl gradient over 50 mL. Tau eluted at ~0.3 M NaCl. Fractions containing tau were identified using SDS-PAGE pooled and buffer exchanged using Econo-Pac® size exclusion chromatography columns (Biorad) into BRB-8 (0.1 mM MgCl₂, 0.1mM EGTA, 8 mM K-PIPES, pH 6.85). Tau-containing fractions identified using SDS-PAGE were pooled, snap

frozen in liquid nitrogen, lyophilized, and stored in a desiccator at -20°C until use. Prior to use, lyophilized samples were reconstituted with distilled deionized water in one tenth of their original volume. Tau protein concentration was analyzed using the Pierce BCA Protein Assay Kit (Thermo Scientific), using bovine serum albumin (BSA) as the standard.

Atomic force microscopy -

A Nanoscope Multimode IIIA microscope (Bruker, Santa Barbara, CA) was used for all atomic force microscopy experiments. Triangular silicon nitride cantilevers (MLCT “D”, Bruker, Santa Barbara, CA) with nominal spring constants of 0.04 N/m were cleaned with a 30 minute exposure in a custom-built UV-ozone chamber containing an ozone-producing mercury grid lamp (BHK, Inc, Ontario, CA) for 30 minutes. 10 L aliquots of reconstituted tau were diluted with 90 L 1mM K-PIPES (pH 7.2) pipetted onto a freshly-cleaved mica surface (Electron Microscopy Sciences, Hatfield, PA) in a glass fluid cell (approximate volume 50 μL) with the AFM tip and incubated for 30 minutes. Following incubation, the fluid cell was purged with 5–10 mL of 1 mM K-PIPES to remove any weakly adsorbed or unbound protein.

Typically, force curves were collected in 3×3 arrays, with adjacent curves offset laterally by 50 nm. Collection of 9-curve arrays was repeated at different locations on the surface, and arrays were often separated by a purge with 1 mM K-PIPES. The speed of approach and retraction was typically 400–500 nm/s, and within this range the approach speed did not have an effect on the measured tip-surface forces. The procedure was repeated as many times as needed to observe the reproducible curves reported here. New buffers could also be introduced between arrays with a 5–10 mL purge through the fluid cell.

Force curves were analyzed using custom scripts in MATLAB. Each force-distance curve was assigned a ‘contact point’, which was the intersection of a line fit to the zero force region of the curve and a line fit to the steepest (linear) part of the curve in the high force region (typically ~ 3 nN). Cantilever sensitivity values were obtained from the slope of the second fit line. For averaging of force curves, all curves in a 9-curve array were aligned to a common ‘contact point’. Average force-distance curves were transformed into force-separation curves by subtracting the cantilever deflection in nm from the sample z-position at each point in the curve. Using this method, any points along the line fit to the steepest part of the force curve are assigned a tip-sample distance of zero, although we note that absolute distances determined with this method cannot be assigned unambiguously.²³

Control experiments with other proteins were conducted using whole MAP fraction (Cytoskeleton, Denver, CO) or BSA (Sigma-Aldrich, St. Louis, MO). These proteins were diluted to 0.3–1 mg/ml in 1 mM PIPES and used as described above.

Protein Quantitation on Silicon Nitride and Mica -

Solutions of tau were incubated on freshly-cleaved mica or UV-ozone cleaned silicon nitride (University Wafer, South Boston, MA) in an area that was well-defined using a hydrophobic PAP pen (Sigma-Aldrich, St. Louis, MO). After incubating for 30 minutes, samples were rinsed with either 1mM K-PIPES or 100mM NaCl in 1mM K-PIPES to mimic AFM solution exchange conditions. After drying the rinsed surfaces under a gentle stream of N_2 ,

tau constructs bound to mica in each sample were removed from the surface using a solubilization buffer (2% SDS in 10 mM CAPS, pH 10), and concentrations in the lift-off buffer were determined using SDS-PAGE gel densitometry with tau standards or micro-BCA assays (Pierce) with BSA as a standard.

Microtubule Bundling Assay-

Qualitative assessment of extent of polymerization, number of microtubules, and amount of microtubule bundling promoted by the tau constructs was conducted using optical microscopy. Microtubules were co-polymerized with each tau protein by incubating 6.9 μM rhodamine-labeled tubulin (Cytoskeleton, Inc.), 3.4 mM MgCl_2 , 0.9 mM GTP, 4% DMSO, 8 μM tau, and 10 μM taxol in BRB-80 at 37°C for 40 min. Samples were imaged using a Nikon Eclipse E400 epifluorescence microscope with a 100x Nikon Plan Fluor Objective (1.25 NA) at room temperature. Images were acquired with a SPOT Insight Model 18.2 Color Mosaic Camera and SPOT imaging software. Adobe Photoshop was used to optimize image contrast and brightness for publication.

RESULTS AND DISCUSSION

In order compare the behavior of tau in our assay to that of other proteins, we collected a series of control force-distance curves in 1 mM K-PIPES on bare mica, immobilized BSA and immobilized MAPs. For a quantitative assessment of the range of repulsive interactions between the AFM tip and these surfaces, we fit regions of the force-distance (F - d) curves to the exponential decay function

$$F = Ae^{-d/\lambda},$$

with the decay length, λ , and the amplitude, A , as the fitting parameters. This has been shown to be a good approximation for the interactions between a parabolic AFM tip and a flat surface,²⁴ and for the interactions between an AFM tip and a polymer-coated surface^{25, 26} at intermediate distances. In each of the force curves presented in Figure 3 through Figure 6, the curves representing tip-sample approach are presented and each is an average of a spatial array of nine curves. The shading represents ± 1 standard deviation for the set of 9 curves.

Representative curves from the control surfaces are shown in Figure 3. On mica (Figure 3A), we observed a roughly exponential decay in force from the surface with a decay length of 6.7 ± 1.3 nm ($N = 5$ sets of 9 curves). This is consistent with previous work,²⁷ and results from the interactions between the electrostatic double layers of the charged surfaces of the mica substrate and silicon nitride tip. Freshly prepared mica surfaces have a well-known structure that takes on a net negative charge in most solutions due to the solubilization of surface ions that are exposed during mica cleavage. The silicon nitride tips were prepared for imaging by cleaning them in a strongly oxidizing environment, which likely results in a layer of oxide on the surface which, in these solutions, has a similar negative charge to that of mica.^{24, 28} Figure 3B shows an average force-distance curve for BSA immobilized on mica. The force decays exponentially as a function of distance, but with an extended decay

length of 9.4 ± 0.4 nm ($N=3$), resulting from the presence of the protein layer on the surface, and in good agreement with the dimensions of the globular BSA protein. As depicted in Figure 3C, adsorption of a solution of MAPs on the mica surface results in a marked increase in the magnitude and range of forces observed. Long range repulsive interactions produced by the MAPs, increase the decay length to 36.6 ± 3.2 nm ($N=9$), with repulsive forces measured at distances beyond 100 nm from the surface. This is consistent with previously published results¹⁹ and indicates that MAPs adsorb on mica and the silicon nitride tip with charged domains projecting from the surface to create a polyelectrolyte brush. Taken together, the curves in Figure 3 provide a useful set of benchmarks for interpreting force-distance curves of the tau constructs on mica.

When FL-htau40 is immobilized on mica, the force-distance curves displayed more complex behavior, shown in Figure 4. Initially, when probed in low ionic strength buffer (Figure 4A), a weak repulsive force appears when the tip-surface distance is >60 nm and rises until the surfaces are separated by ~ 40 nm. At this point, the repulsion between the surfaces decreases to a local minimum when the surfaces are separated by ~ 30 nm, indicating the presence of more attractive (or fewer repulsive) interactions. As the tip and surface are brought into closer contact, the force rises again as the protein layers are under compression. The shape and reproducibility of these curves reveal the presence of a structured protein layer on the surfaces. More specifically, the decrease in force at intermediate distance could be caused by favorable electrostatic bridging interactions between the mica surface and the tip, made possible by complementary dimerization of tau on one or both of the surfaces. In this interpretation, the low ionic strength conditions used in these experiments permits tau dimers in solution and on the surface to be held together by an “electrostatic zipper” between oppositely-charged domains in dimer pairs. This results in the adsorption of a tau multilayer on the surface (represented in the top panel of Figure 2), with some positively charged regions of the tau protein extending into solution. As the surfaces approach each other, there is an opportunity for cross-bridging when the layers interdigitate and the exposed microtubule binding domains interact with the opposing surface. This is qualitatively similar to observations made of FL-htau40 in symmetric surface force apparatus experiments.²⁰

When higher ionic strength buffer (100 mM NaCl in 1 mM K-PIPES) is introduced into the system, there are two significant changes in the force distance curves measured with the AFM (Figure 4B): 1) The range of tip-surface interactions decreases, such repulsive force can only be measured to a distance of <40 nm, and 2) the force increases monotonically as the tip-surface distance decreases (Figure 2, middle panel). The decrease in tau layer thickness under high ionic strength conditions is unsurprising as it is likely due to partial collapse of the tau polyelectrolyte brush in the salt solution because of charge screening on the polymer backbone. In addition to affecting tau layer thickness, the purge with higher salt concentration also disrupts tau dimer or multilayer pairing, releasing tau not directly bound to the mica or nitride surfaces, and eliminating opportunities for bridging interactions and the local minimum observed in Figure 4A.

As a third step in this experiment, we removed the high ionic strength buffer and returned to the initial solution conditions by purging with 1 mM K-PIPES and repeated the force-distance measurements. As seen in Figure 4C, in curves taken under these conditions, the tau

layers do not return to their initial state. This observation, combined with our measurement of a decrease of tau surface density during high salt washes (described below), points to a disruption of the tau multilayers that were initially present at low ionic strength. Higher salt concentration used in the intermediate purge is enough to disrupt the “electrostatic zipper” dimerization interactions and release the multilayer into solution during rinsing, while tau directly bound to the mica surface remains. In spite of the release of the multilayer structure, long range forces are still observed, with repulsive force measured at distances >50 nm. This observation is consistent with tau acting as a polyelectrolyte brush with projection domains extending away from the surface (represented in the bottom panel of Figure 2). When these tau curves are compared to BSA, a globular protein of similar molecular weight (~ 66 kDa, compared to ~ 46 kDa for FL-htau40), the range of repulsive interaction is markedly longer for tau, indicating a extended tau conformation perpendicular to the surface. Additionally, these FL-htau40 force curves are qualitatively similar to those of the whole MAP fraction (Figure 3C) in that they show ‘soft’ contact, where the force changes very gradually with distance, between the tip and surface occurring over very long range. The much longer range forces produced by the MAPs are expected because the MAP fraction is primarily made up of higher molecular weight MAPs, such as MAP1 and MAP2. Large isoforms MAP2, for example, can have projection domains exceeding 1000 amino acids, and in experiments examining the spacing of microtubule-MAP complexes in vivo, the projection domain of MAP2C results in spacing between microtubules about three times larger than tau.²⁹

To more carefully examine the role of tau’s projection domain in determining the tau-tau interactions that were observed, the same experiments were conducted with (1–197), the tau construct lacking the N-terminal projection domain. For this truncated construct, very similar behavior to FL-htau40 was observed, but over shorter range. In the initial low ionic strength condition (Figure 5A), we again observed longer-range repulsion, a medium range attractive minimum, and then short range steric repulsion. Because this tau construct lacks the projection domain, the observed behavior cannot be explained on the basis of an N-terminal to proline-rich domain “electrostatic zipper” interaction that was previously postulated.²⁰ However, complementary, electrostatically-based dimerization is still possible between the negatively charged C-terminal (amino acids 400–441), and the positively charged region flanking the microtubule binding domain (amino acids 369–400). This type of interaction would produce a multilayer on the mica surface that exposes microtubule binding domains to the solution, which are capable of cross-bridging between the mica surface and AFM tip to create an attractive region in the force curves. Analogous to FL-htau40, we find that force-distance curves suggesting cross-bridging are eliminated when 100 mM NaCl is introduced into the system (Figure 5B). When the system is restored with the low ionic strength buffer (Figure 5C), the force curves display long range repulsive force and less attraction at medium range. This is likely due to the partial removal of a multilayer that was present initially. Together the results in Figure 5A–C demonstrate that the “electrostatic zipper” effect is not unique to FL-htau40 and that it can be present in constructs completely lacking the projection domain. These results also indicate that in some circumstances the behavior of the negatively-charged C-terminal mimics that of the projection domain, with an ability to form a polyelectrolyte brush and to participate in the somewhat specific intermolecular interactions required for dimerization.

The third tau construct we examined, (1–150), lacks the full projection domain, but does contain the full proline rich region (amino acids 151–244) adjacent to the microtubule binding domain. For this construct, we did not consistently observe the long range repulsive interaction that was observed for the other two constructs (Figure 6). Instead, when the mica surface and silicon nitride tip were coated in (1–150) layers, the force-distance curves were characterized by an attractive force at short range (<20 nm), and very steep steric repulsion compared to FL-htau40 and (1–197). Introduction of 100 mM NaCl reduced the strength of the interaction between the tau layers, indicating that it is primarily electrostatic in nature. When the 1 mM Pipes solution was re-introduced into the system, the strength of the attractive interaction increased. The curves point to the presence a condensed protein layer that is capable of some cross-bridging interactions, or to charge neutralization of the mica and/or nitride surfaces. In the case of (1–150), there is no evidence that the C-terminal is acting as a polyelectrolyte brush as it was in (1–197). Because (1–197) differs from (1–150) only by the absence of the P1 domain, the data in Figure 6 suggests a role for P1 in interacting with the C-terminal domain that prevents formation of a polyelectrolyte brush and the formation of complementary dimers.

To aid in interpretation of the force curves, the surface densities of tau constructs on mica and silicon nitride were measured under conditions analogous to the AFM experiments. Surface densities were estimated by removing the proteins from the surface, and quantitating the amount using SDS-PAGE densitometry or microBCA assays. The results were qualitatively similar for each construct. After incubation of tau in 1 mM PIPES on the surfaces and rinsing with the same buffer, measured surface densities were 0.5–1.0 $\mu\text{g}/\text{cm}^2$. This value is comparable to that previously reported and is indicative of a multilayer of protein.^{19, 20} After rinsing with 100 mM NaCl in 1 mM PIPES, we found that the surface densities decreased to the range of 0.2–0.5 g/cm^2 .

The AFM data presented here is useful in elucidating the function of tau's projection domain, identifying putative tau-tau interactions, and in identifying functions of the C-terminal tail of tau.

One important role of tau is to organize microtubules by controlling the spacing between them in axons, and several models have already been proposed to explain this function. Relevant to the present work, Hoh and coworkers suggested that when tau is bound to microtubules, the projection domain is unstructured and subject to Brownian motion. The motion, combined with steric repulsion for other proteins near the projecting tau creates a zone of 'entropic exclusion' surrounding the microtubule.^{19, 30} In another model proposed by Israelachvili and coworkers, taus bound to neighboring microtubules are able to pair by establishing an "electrostatic zipper" via overlap of adjacent tau projection domains.²⁰ In this model, overlap between the negatively charged projection domain and positively charged proline-rich region of adjacent taus occurs with some degree of specificity and allows for very well-controlled spacing of microtubules.

In our AFM analysis of FL-htau40, we observe the formation of multilayers and evidence for complementary dimerization of the protein, which is in good agreement with the "electrostatic zipper" model. However, in order to induce dimerization, very low ionic

strength conditions were used here and in the previously published work.²⁰ We find that under conditions more comparable to physiological ionic strength, tau dimers dissociate and the microtubule binding domain remains associated with the mica surface, our proxy for the surface of a microtubule. In addition, even at high ionic strength and in the absence of the tau multilayer, the projection domain still provides a repulsive force between adjacent surfaces that is relatively long range. This data supports the view that the spacing between microtubules can be controlled by entropic exclusion and that specific intermolecular interactions between taus are not specifically required. The thickness of this entropic exclusion zone is affected by the ionic strength of the surrounding solution (see Figure 4B compared to Figure 4C) which could be further controlled *in vivo* via alternative splicing of the projection domain^{20, 31} or phosphorylation.^{19, 32} Furthermore, complementary dimerization of tau is not a feature specific to the projection domain, as the data presented here indicate that it can also occur in the C-terminal region of the protein. These data corroborate an earlier study that determined that the C-terminal can play a role equivalent to that projection domain.¹³

In the AFM measurements of tau-tau interactions reported here and elsewhere, when tau constructs were immobilized on mica and the AFM tip, they were assumed to adopt conformations similar to tau bound to microtubules. Previous studies have proposed that mica is a reasonable proxy for microtubules because of the similarity in charge density between the mica surface and the surface of microtubules, and the ability to observe polymer brush-like behavior from the protein, presumably from the projection domain protruding from the surface.^{19, 20} The AFM data presented here supports the conclusion that there are greater intermolecular attractions between N-terminal truncated taus compared to the full-length protein. To provide additional evidence that the tau-tau interactions measured in these types of AFM experiments are representative of the interactions of tau when bound to microtubules, we conducted fluorescence microscopy of tau-microtubule complexes for our three constructs (Figure 7). These images appear to show significant bundling for microtubule- (1–150) complexes (Figure 7C), which is consistent with the attractive interaction observed with AFM. Microtubule- (1–197) complexes also appear to display limited bundling (Figure 7D), consistent with AFM data that shows a slightly more attractive interaction compared to FL-htau40. Our observations of FL-htau40-microtubule complexes (Figure 7B) showed no/negligible bundling of microtubules *in vitro*, consistent with the purely repulsive interaction observed for FL-htau40 at high ionic strength. A comparison of Figure 7B (FL-htau40) and Figure 7C/D (N-terminal truncated mutants) shows that there is a clear difference in microtubule morphology that is consistent with microtubule bundling for the mutants. Similar observations of bundling using fluorescence microscopy have been made previously.⁶ We also observed increased light scattering from microtubule samples polymerized with N-terminal truncated taus (Figure S1, Supporting Information), which is consistent with microtubule bundling. In these measurements, higher-order bundles of microtubules cause an increase in turbidity compared to samples of well-dispersed microtubules.^{6,33}

Microtubule bundling from N-terminal truncation mutants is well-described in the literature. For example, Fauquant *et al.* studied a mutant “F4” similar to our mutant (1–197) which showed bundling of microtubules in electron microscopy and light scattering measurements.

³⁴ Similarly, a study of a series of truncated tau mutants using multiple experimental approaches found that microtubule bundles formed when tubulin was assembled with N- and C-terminal truncated taus.³⁵ Very similar tau mutants were studied by Gustke *et al.*, who observed microtubule bundling and noted that bundles observed in electron microscopy also corresponded to high optical density in light scattering measurements and observable microtubule bundles in fluorescence microscopy.⁶

An additional important area of tau research is in understanding aggregation and the formation of pathological tau fibrils. Muller and coworkers found that for tau fibrils formed through interactions between microtubule binding domains, both the projection domain and the C-terminal extend outward from the fibril core to create a two-layer polyelectrolyte brush or “fuzzy coat”.¹⁷ In these assemblies, the outer layer of the fuzzy coat arises from the projection domain, and the inner layer arises from the shorter C-terminal. The AFM data presented here for FL-htau40 and (1–197) support the view that both the projection domain and the C-terminal act as polyelectrolyte brushes that could establish a two-layer ‘fuzzy coat’. In particular force curves collected on (1–197) are consistent with the C-terminal acting as a polyelectrolyte brush, while force curves collected on FL-htau40 demonstrate that the projection domain establishes a thicker brush. The ability of the projection domain and the C-terminal to establish a layered polyelectrolyte brush could have an impact on aggregation of tau fibrils, and on tau’s ability to stabilize and organize microtubules.

The force curves shown in Figures 3 through 6 were collected during tip-sample approach, there is no ‘jump-to-contact’ that is common in AFM force curves, even though flexible cantilevers were used in these experiments (nominally, $k = 0.04$ N/m). The absence of a jump-to-contact indicates that the gradient of the attractive surface forces with respect to distance do not exceed the cantilever spring constant. Even on the steepest attractive (negative) part of the (1–150) curves, the slope does not exceed -0.02 N/m. Because of the low forces involved here, we were able to use soft, sensitive cantilevers to capture small changes in force, but also avoid instabilities in the approach force curves. However, upon retraction (data not shown) there were typically several such instabilities in the force curves, characteristic of force pulling experiments,¹⁸ and indicating the formation of new, stronger intermolecular tau-tau or tau-surface interactions when the tau on the tip and the tau on the surface were in intimate contact. We observed these in all constructs and solution conditions tested. The advantage of focusing on approach curves is that we are able to observe weaker, long range forces established by the tau layers that are obscured by the stronger attractive interactions that occur after intimate contact between the AFM tip and the mica surface. The possibility exists that the stronger intermolecular interactions observed during retraction could disrupt the structure of the tau layer because of repeated, fast pulling. However, we did not generally observe changes in force curves after repeated pulling in the same region of the surface. Rather, curves collected in same area of the surface were quite reproducible. This may be because the tip-surface contact area contains tens or hundreds of molecules such that the disruption of a few molecules by pulling does not significantly affect the AFM measurements.

Through the use of AFM force spectroscopy, evidence has been presented here to support the two popular models of tau-tau interactions that control microtubule spacing, and reveals that careful control over environmental conditions effects what type of intermolecular interactions predominate in our measurements. Under conditions favorable for strong electrostatic intermolecular interactions (low ionic strength, relatively high protein concentration in solution) we have found evidence for complementary dimerization of tau, supporting the “electrostatic zipper” model. Under conditions where tau is immobilized on the surface and higher concentrations of salt are used to disrupt complementary electrostatic dimerization, we find that tau can adopt structure more like a polyelectrolyte brush. So indeed both models may be contributing to tau’s physiological functions, with the dominant model being condition-dependent. Notably, the data collected here also indicate that the complex interactions between taus that have historically been attributed to the projection domain alone are not dictated solely by this region of the protein. Indeed the complementary dimerization, (aka the “electrostatic zipper” effect) does not require the projection domain and can take place through other regions of the protein. We postulate that the negatively-charged C-terminal tail of the tau protein, can behave in a similar fashion as the N-terminal projection domain, including projecting from the surface, and participating in dimer formation. As the methods presented are useful in assessing tau-tau intermolecular interactions, they may also provide a novel approach to examine tau’s behavior in under conditions more relevant to neurodegenerative disorders, for example when phosphorylated or with a truncated C-terminal domain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding information

Funding from NIH Grant #1R15GM083256-01 and the Phoebe H. Beadle Science Fund are most gratefully acknowledged.

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FIGURE 1.

The three tau constructs used in this experiment, with some domains delineated. The projection domain is indicated in blue, the proline rich region (P1 and P2) is indicated in gray, the four repeats of the microtubule binding domain (R1–R4) are indicated in red, the so-called “fifth repeat”, or flanking domain, is indicated in pink, and the C-terminal domain is indicated green. Above FL-htau40 are the amino acid numbers demarcating domains. Bottom: Charge distribution for FL-htau40 at pH 7.2. In this diagram, the charge is averaged over 10 amino acids.

FIGURE 2.

Schematic representation of the tip and mica substrate setup after incubating with tau and purging with fresh buffer. Top: Initially, the system is incubated with protein in 1 mM PIPES pH 7.2 and rinsed with the same buffer prior to AFM measurements, which results in deposition of tau multilayers on the tip and surface. Middle: In a second step, the system is purged with 100 mM NaCl in 1 mM PIPES, pH 7.2, which releases the tau multilayer and leaves tau directly bound to the surfaces. Bottom: In a final step, the solution is returned to initial conditions via purging with 1 mM PIPES, allowing the surface-bound tau to adopt a brush-like structure.

FIGURE 3.

Control force curves. Each curve represents an average of 9 individual curves taken around the same local area on the surface, and the shading represents ± 1 SD in the force. Force curves were taken in 1 mM PIPES on **A)** bare mica; **B)** bovine serum albumin, and; **C)** whole MAP fraction.

FIGURE 4.

Force curves collected when FL-htau40 is immobilized on the mica surface and AFM tip. Each curve represents an average of 9 individual curves taken around the same local area on the surface, and the shading represents ± 1 SD in the force. Force curves were obtained in **A**) 1 mM K-Pipes; **B**) the same location after purging the fluid cell with 100 mM NaCl in 1 mM K-Pipes, and; **C**) the same location after re-purging the fluid cell with 1 mM K-Pipes (0 mM NaCl).

FIGURE 5.

Force curves collected when (1–197) is immobilized on the mica surface and AFM tip. Each curve represents an average of 9 individual curves taken around the same local area on the surface, and the shading represents ± 1 SD in the force. Force curves were obtained in **A**) 1 mM K-Pipes; **B**) the same location after purging the fluid cell with 100 mM NaCl in 1 mM K-Pipes, and; **C**) the same location after re-purging the fluid cell with 1 mM K-Pipes (0 mM NaCl).

FIGURE 6.

Force curves collected when (1–150) is immobilized on the mica surface and AFM tip. Each curve represents an average of 9 individual curves taken around the same local area on the surface, and the shading represents ± 1 SD in the force. Force curves were obtained in **A**) 1 mM K-Pipes; **B**) the same location after purging the fluid cell with 100 mM NaCl in 1 mM K-Pipes, and; **C**) the same location after re-purging the fluid cell with 1 mM K-Pipes (0 mM NaCl).

FIGURE 7.

Optical micrographs of rhodamine-labeled microtubules co-polymerized with tau. The tau construct used is indicated in the upper left corner of each image, A. is a tau-free control, and B. contains FL-htau40. Noticeable bundling is observed for microtubules polymerized with both: C. $\Delta(1-150)$, and D. $\Delta(1-197)$. Some of the structures that appear to be bundles of microtubules are highlighted in the yellow boxes. In each image, the scale bar is 20 μm .