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Revealing Conformational Variants of Solution-Phase Intrinsically Disordered Tau Protein at the Single-Molecule Level

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Abstract

Intrinsically disordered proteins, such as tau protein, adopt a variety of conformations in solution, complicating solution-phase structural studies. We employ an anti-Brownian electrokinetic (ABEL) trap to prolong measurements of single tau proteins in solution. Once trapped, we record the fluorescence anisotropy to investigate the diversity of conformations sampled by the single molecules. A distribution of anisotropy values obtained from trapped tau protein is conspicuously bimodal while those obtained by trapping a globular protein or individual fluorophores are not. Time-resolved fluorescence anisotropy measurements are used to provide an explanation of the bimodal distribution as originating from a shift in the compaction of the two different families of conformations.

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Graphical Abstract

A microfluidic trap that cancels Brownian motion of individual molecules reveals solution-phase diversity in an intrinsically disordered protein (IDP) involved with Alzheimer's Disease.

Keywords

Single-molecule studies; Protein structures; Biophysics; Fluorescence spectroscopy; Microfluidics

Tau protein is an intrinsically disordered protein (IDP) that performs a vital role in the human central nervous system by regulating^[1] and stabilizing microtubules in the axons of neurons.^[2] Tau consists of an N-terminal projection domain, proline rich region, microtubule-binding region (MTBR), and C-terminal tail (Figure 1a).^[3] The MTBR consists of 3 or 4 imperfect repeats that have a strong affinity for microtubules.^[4] In the pathogenic form, however, this repeat region loses this affinity, adopts a β -sheet structure,^[5] and aggregates into paired helical and straight filaments associated with Alzheimer's disease^[6] or other types of filaments associated with other tauopathies.^[7] Pathogenic aggregation is also influenced in a complex manner by phosphorylation of tau by cellular kinases.^[8] Importantly, it has also been suggested that it is not the insoluble aggregates, but rather the soluble precursor oligomers that are the toxic species.^[9] These studies highlight that understanding the conformations of monomers and the earliest steps of aggregation are critical to understanding Alzheimer's disease etiology.

Tau protein is flexible and known to display conformational dynamics in solution. Circular dichroism measurements of tau monomer show a characteristic trough around 200 nm indicating a random coil structure.^[10] Electron paramagnetic resonance measurements confirm a high degree of mobility of residues in the MTBR and C-terminal end.^[11] Even though tau is an IDP, there is evidence that it is not completely devoid of structural motifs. Intramolecular distances – as determined by Förster resonance energy transfer (FRET) – are shorter than would be expected for a perfect random coil, leading to the proposed “paperclip” conformation.^[12] Detailed single-molecule (SM) FRET measurements further confirm the global compactness of the tau protein with the N- and C-termini in relatively close contact with the MTBR while proposing an “S-shaped” conformation.^[13] There is also evidence through the interaction of tau protein with microtubules and aggregation inducers that different regions of the protein adopt different conformations^[14] and different degrees of compaction.^[13] NMR techniques are also capable of reporting on intramolecular distances and, when combined with computational studies, imply that monomeric tau adopts at least 30 different conformers in solution.^[15]

Solution-phase SM measurements are a powerful tool for characterizing the heterogeneity of protein conformations, particularly when attachment to a surface is likely to induce artificial conformations.^[16] Solution-phase SM studies can report not only on different conformations

and assemblies,^[13, 17] but also the transition pathways,^[18] and kinetics of the conversions.^[19] These methods, however, examine freely diffusing proteins as they transiently pass through a near diffraction-limited confocal volume. Due to the limited time (and number of photons collected) during the molecule's residence in the excitation volume, measurements are often characterized by low signal-to-noise ratios, with histograms significantly broadened by shot noise.

Here, we prolong the measurement of the freely diffusing protein by employing an anti-Brownian electrokinetic (ABEL) trap. This microfluidic device tracks the position of a fluorescently labeled tau protein and provides real-time feedback voltages which induce an electrokinetic flow to push the protein back to center, allowing observation for multiple seconds without perturbative immobilization.^[16c, 20] These long observation times allow collection of orders of magnitude more photons, leading to histograms whose widths may be representative of distinct molecular populations, rather than a consequence of limited statistics.

In this work, we use the ABEL trap to perform the first prolonged single-molecule investigation on a monomeric IDP. We employ fluorescence anisotropy as a reporter for solution-phase conformations of individual tau proteins. Fluorescence anisotropy is a versatile tool for monitoring rotational dynamics of interacting biomolecules in solution.^[21] Anisotropy is a measure of the shift in polarization of emitted photons relative to excitation photons, providing a way of characterizing the extent and timescale of molecular reorientation during the nanosecond excited-state lifetime.

We investigate the full-length isoform of tau protein (htau40) containing 441 residues and weighing 45 kDa.^[22] For our studies, the naturally occurring cysteine residues at 291 and 322^[22] have been mutated to serine residues, and the tyrosine residue at 310 has been mutated to a cysteine (htau40-Y310C). The cysteine is then labeled by covalently linking an ATTO647N dye (Figure 1a), chosen for its high stability at the required high photon count rate.^[16c] This labeling position has been used previously without disturbing the structure or functionality of tau protein.^[12] We will refer to this dye-labeled mutant simply as htau40, unless otherwise noted.

To properly calibrate our method and gain meaningful insight into solution-phase conformations of htau40, we establish a baseline for comparison consisting of multiple control samples: free ATTO647N dye and microbial transglutaminase (MTG), a globular protein comparable in size (38 kDa) to htau40 with a single cysteine residue in the active site available for labeling (Figure 1b).^[23]

Upon activation of the ABEL trap, solution-phase molecules were trapped for an average of 3 seconds (Figure S1) and the steady-state anisotropy was determined (Figure 2). All protein samples were trapped in 25 % glycerol to slow the evaporation of the buffer during the experiment and to facilitate trapping by lowering the diffusion constant. Free ATTO647N dye was trapped in 50 % and 80 % glycerol in order to produce a comparable anisotropy to htau40 and MTG, respectively (Figure 3).

The fluorescence anisotropy of each molecule is indicative of the freedom of rotational motion of the attached ATTO647N dye. If the fluorophore can quickly explore a full range of angles, the steady-state anisotropy value will be near zero due to complete depolarization. If the molecule has a restricted range of motion, or if rotational motions are sluggish, higher values of anisotropy are expected.

After prolonged trapping of hundreds of individual molecules, we constructed anisotropy histograms for each sample (Figure 3). The histogram for htau40 displays a conspicuous bimodal distribution of anisotropy values (Figure 3a), while the distribution for MTG can be fit to a single, narrower Gaussian (Figure 3b). The differences in shape and center anisotropy values indicate a difference between the fluorescence-depolarizing motions of the two samples: the htau40 sample has a lower anisotropy value and bimodal distribution and thus more freedom of rotation and conformational heterogeneity. If the label on MTG were situated in a less constrained environment, a center anisotropy value closer to htau40 would be expected as the dye would likely have more rotational freedom, though a single peak would still be anticipated. The average anisotropy values for these two histograms agree with bulk samples, indicating that trapping does not influence protein rotational motions (Table S1).

In order to determine whether the wide, bimodal distribution is unique to htau40 or whether it is characteristic of any disordered protein, we induced disorder in MTG by denaturing it with 6 M guanidinium chloride (GdmCl). Upon denaturation of MTG, the anisotropy histogram shifts to a lower value despite the increase in viscosity, indicating that the fluorophore is able to rotate more freely, as expected, and also shows a single, broad, dominant peak in the anisotropy histogram (Figure 3d). When htau40 is trapped in the presence of 6 M GdmCl, the distribution merges into a single peak with an average anisotropy value between the two peaks in Figure 3a (Figure 3c).

Another important comparison is made between a protein – which has the potential for solution-phase heterogeneity – and samples such as free molecular ATTO647N which is not expected to have multiple conformations in solution (Figure 3e). Since the dye samples produced comparable number of photons per event, these samples were used to ascertain the narrowest width of anisotropy histogram that we can resolve at each anisotropy value due to instrument limitations or dye photophysics.^[24] Comparison of the widths of the protein histograms to the dye histograms at the same average anisotropy value suggests that while these processes contribute to the observed anisotropy, the protein heterogeneity makes the dominant contribution to the observed anisotropy distribution.

Though NMR techniques have found at least 30 solution-phase conformers,^[15] our single-molecule experiments indicate two underlying populations. We attribute these two underlying conformations to two families of related conformers and not simply two conformations. We also note that we saw no evidence of transitions between these families within the average trapping window, and thus these conformations are stable on the timescale of multiple seconds (SI). We do note that over an hour of trapping htau40, the average anisotropy slowly changes from favoring the lower anisotropy peak ($r_L = 0.17$) to

the upper anisotropy peak ($r_U = 0.21$) (Figure S2). This detail indicates that conversion between the states occurs over a timescale of tens of minutes.

To gain insight into the molecular motions responsible for the observed heterogeneity, bulk time-resolved fluorescence anisotropy measurements were performed (Figure 4). The free dye samples show monoexponential anisotropy decays, as expected, due to their lack of available conformations (Figure S3). Anisotropy decays for htau40 samples, on the other hand, display triexponential behavior with an initial fast (ϕ_f) decay followed by an intermediate (ϕ_i) and slow (ϕ_s) decay. The timescales of the decays were used to assign their likely originating motions. We have assigned the fastest component to dye rotations about their flexible linkers and the intermediate component to a segmental motion of the protein.^[25] The slowest component could either be a different segmental motion for a larger domain or a global motion around the shorter axis of a non-spherical object. Global motions corresponding to motion around longer axes of the IDP are too long to be observed here (SI).

MTG samples, on the other hand, show biexponential decay behavior. The short decay for MTG is also likely due to the rotation of the dye around the linker, though it may also contain contributions from segmental motion of a short loop. The absence of an intermediate decay is consistent with the more defined structure of MTG, with the longer component ascribed to global motion.

The protein anisotropy decays are fit to equations describing wobbling-in-a-cone or cone-in-a-cone behavior with one or two local motions, where the critical parameters include cone half-angles (Θ) and rotational correlation times (ϕ), Figure 4 inset.^[26] Critically, the Θ s of the fast components corresponding to local dye motion are quite similar for the two proteins (29° for ATTO647N attached to htau40 and 30° when attached to MTG) even as the average timescales of these motions are very different ($\phi_f = 0.25$ ns for htau40 and 1.9 ns for MTG) (Table S3). The significantly slower motion in MTG is expected due to the restrictive environment of the active site, which may preclude large amplitude dye motions that contribute to anisotropy decay.^[27] In contrast, the dye attached to htau40 exhibits some limitations in motion compared to an untethered dye, but is substantially freer than in MTG.

Segmental motions of the tau protein are likely responsible for the intermediate component in the anisotropy decay as IDPs have been identified via time-resolved anisotropy to have high backbone flexibility that may stiffen upon binding to a partner.^[25] The intermediate timescale dynamics corresponding to the segmental motion of htau40 was found to have an average correlation time of $\phi_i = 5.3$ ns and a cone of half-angle $\Theta_i = 30^\circ$. The existence of this intermediate segmental motion is unique to the IDP. MTG does not display an intermediate timescale motion, and beyond the fast decay, only showed one slow motion, $\phi_s = 90$ ns, likely originating from the global motion of the molecule. The long timescale motion of htau40 exhibits $\phi_s = 118$ ns.

Using the single-molecule and time-resolved data presented in Figures 3 and 4, we can understand the origins of the bimodal anisotropy distribution for htau40. That a bimodal distribution is observed only in htau40 and not the other samples suggests root causes

specific to the IDP. One possibility is that hydrophobic interactions between the dye and protein result in this bimodal distribution.^[28] However, several pieces of evidence suggest such interactions are not the origin. First, molecular dynamics simulations have shown that fluorescent dyes generally track the motions of their proximal residues.^[27] Second, when hydrolyzed ATTO647N is mixed with unlabeled htau40 (mutant with no cysteine residues), the bulk anisotropy of the dye is not altered, indicative of an absence of strong hydrophobic interactions (SI). Third, when replacing ATTO647N with the less-hydrophobic ATTO633 (SI), the time-resolved anisotropy shows that both have three qualitatively similar component decays (Figure S4). Fourth, and perhaps most compelling, though the ATTO633-labeled htau40 does not show two conspicuous peaks (Figure S5), the distribution of anisotropies is broad and asymmetric, and analysis of the underlying rotational correlation times reveals a bimodal distribution for the ATTO633-labeled sample that is strikingly similar to the bimodal distribution for the ATTO647N-labeled sample (Figure S5) and both dye-labeled sample distributions imply two populations (Figure S6). Thus, even as the specific distribution of observed anisotropies is sensitive to the hydrophobicity of the dye (SI), the existence of two underlying populations of protein conformations is confirmed by observation of two populations of reduced correlation times for both labels and is thus independent of the label and intrinsic to the protein.

We can examine the role of the five parameters – ϕ_f , ϕ_i , ϕ_s , θ_f , θ_i – that could cause the observed partitioning into two anisotropy populations, with an upper anisotropy, $r_U = 0.21$, and a lower anisotropy, $r_L = 0.17$ (two peaks in Figure 3a), observed in our single-molecule experiments. For each parameter, we examine how reasonable it is for the average value from the bulk anisotropy to be distributed among an upper and lower population needed to generate the two steady-state anisotropy peaks. Partitioning of the fast component of the decay (0.25 ns) could cause the population centered at $r_U = 0.21$ if the upper value, $\phi_{f,U}$, were 0.69 ns, but not even a value of 0 ns for the lower value, $\phi_{f,L}$, could account for the peak at $r_L = 0.17$. Thus, partitioning in ϕ_f cannot account for the observed single-molecule behavior. Similarly, ϕ_i and ϕ_s would have to change dramatically to account for the bimodal distribution of htau40, with ϕ_i partitioning from 5.3 ns into lower and upper values of 1.1 ns and 10.5 ns and ϕ_s partitioning from 118 ns into values of 11.3 ns and over 1 μ s. This spread is extremely large, as even attachment to binding partners and consequent increase in rigidity only results in slowing of segmental motions by a factor of six,^[25] suggesting an alternative explanation is necessary.

A change in cone angle is thus a more likely origin of partitioning. A split from 29° for the cone half-angle of the dye motion (θ_f) into 27° and 35° could cause this partitioning. Alternatively, the 30° cone half-angle of the protein segmental motion (θ_i) could split into 22° and 49° in order to account for this observation. Thus, even as very large changes in timescale of rotational decay are required for partitioning, even subtle changes in θ_f could more reasonably achieve the same effect, making fluorescence polarization anisotropy essentially an amplifier of a small conformational change. Consequently, we ascribe the partitioning of steady-state anisotropies into two solution-phase populations with different cone angles, though we are unable to decipher which one is the root cause.

Since htau40 is an IDP, it is expected to have a variety of conformations, yet we illustrate through single-molecule fluorescence anisotropy that these solution-phase conformations partition into two families of stable conformers. These families are distinguished by a change in cone angle explored by the fluorescent probe and proximal protein segment, suggesting a more compact family and a less compact family. That these families were not observed in freely-diffusing single-molecule FRET experiments^[13] suggests that the conformational changes are fairly subtle, highlighting two notions: 1) that anisotropy and FRET are complementary techniques that may be sensitive to different structural differences and 2) that a critical benefit of higher photon counts due to a prolonged trapping time is a reduced contribution of shot noise, allowing resolution of smaller structural differences.

One possible interpretation of our finding is that one family may be more prone to pathological aggregation, and thus a preferred intermediate. Less compact structures, including those induced by binding to heparin^[13] or caused by mutations,^[29] expose the MTBR region, facilitating inter-molecular contacts. Alternatively, the Alz-50 configuration, recognized by Alz-50 antibodies, has the N-terminus blocking the MTBR region. This spatial arrangement is observed in tau paired helical filaments, but is believed to be a precursor to aggregation.^[30] Observation of a more compact solution-phase structure of tau may also be related to the solution-phase presence of this species. Additional experiments to further characterize these solution-phase conformers are ongoing and will be necessary to definitively determine the nature and biological role of these species. However, use of a new single-molecule method for IDPs to identify and begin to characterize these families of monomer conformations is a critical step toward understanding the first stages of the htau40 aggregation pathway that leads to disease.

There is an outstanding need for a molecular understanding of the earliest steps of tau conformational change and aggregation, even as substantial progress is being made understanding later steps in the process for tau^[6b, 19d] and other IDPs.^[19c] Our approach can potentially satisfy this need – both for tau and other IDPs – by providing increased observation time, which has allowed us to differentiate between two families of long-lived conformations adopted by tau protein in solution by their degree of compaction. Further, fluorescence polarization anisotropy can be combined with other complementary probes to maximize information content,^[17c, 31] all while trapping molecules in solution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structures of (a) htau40 with cysteine residue labeled with ATTO647N dye and (b) microbial transglutaminase (MTG) based on crystal structure 1IU4 from reference 23.

Figure 2.

Representative traces for molecules in the ABEL trap, with feedback voltages off (left) and on (right). (a) htau40-ATTO647N, (b) MTG-ATTO647N, and (c) MTG-ATTO647N denatured with 6 M guanidinium chloride (GdmCl) were trapped in solutions with 25 % glycerol. (d) Hydrolyzed ATTO647N was trapped in buffer with 50 % glycerol. Change points in the trapping data are shown in red (left axis) and anisotropy is shown in blue (right axis).

Figure 3.

Histograms of single-molecule anisotropy values (black) fit with Gaussian curves (multiple colors). μ is the average anisotropy and σ is the standard deviation. Samples for plots (a) – (d) were prepared in a buffer with 25 % glycerol.

Figure 4. Time-resolved anisotropy of htau40-ATTO647N (magenta) and MTG (purple). Anisotropy parameters are given in Table S3. Samples were measured in a buffer which contained 25 % glycerol.