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Permalink

<https://escholarship.org/uc/item/5j52c1vw>

Journal

Journal of the American Society for Mass Spectrometry, 30(1)

ISSN

1044-0305

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Publication Date


2019

DOI

10.1007/s13361-018-2027-6

Peer reviewed

Native Top-Down Mass Spectrometry and Ion Mobility Spectrometry of the Interaction of Tau Protein with a Molecular Tweezer Assembly Modulator

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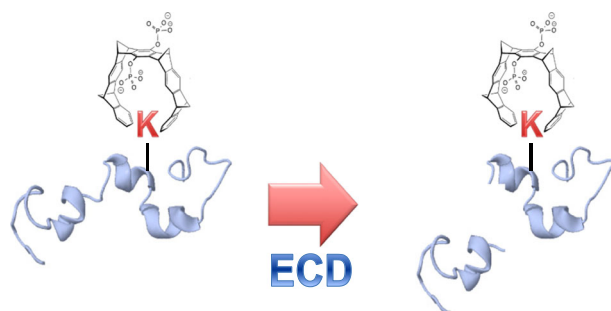
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Abstract. Native top-down mass spectrometry (MS) and ion mobility spectrometry (IMS) were applied to characterize the interaction of a molecular tweezer assembly modulator, CLR01, with tau, a protein believed to be involved in a number of neurodegenerative disorders, including Alzheimer's disease. The tweezer CLR01 has been shown to inhibit aggregation of amyloidogenic polypeptides without toxic side effects. ESI-MS spectra for different forms of tau protein (full-length, fragments,

phosphorylated, etc.) in the presence of CLR01 indicate a primary binding stoichiometry of 1:1. The relatively high charging of the protein measured from non-denaturing solutions is typical of intrinsically disordered proteins, such as tau. Top-down mass spectrometry using electron capture dissociation (ECD) is a tool used to determine not only the sites of post-translational modifications but also the binding site(s) of non-covalent interacting ligands to biomolecules. The intact protein and the protein-modulator complex were subjected to ECD-MS to obtain sequence information, map phosphorylation sites, and pinpoint the sites of inhibitor binding. The ESI-MS study of intact tau proteins indicates that top-down MS is amenable to the study of various tau isoforms and their post-translational modifications (PTMs). The ECD-MS data point to a CLR01 binding site in the microtubule-binding region of tau, spanning residues K294-K331, which includes a six-residue nucleating segment PHF6 (VQIVYK) implicated in aggregation. Furthermore, ion mobility experiments on the tau fragment in the presence of CLR01 and phosphorylated tau reveal a shift towards a more compact structure. The mass spectrometry study suggests a picture for the molecular mechanism of the modulation of protein-protein interactions in tau by CLR01.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13361-018-2027-6>) contains supplementary material, which is available to authorized users.

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Published online: 30 July 2018

Keywords: Electrospray ionization, Electron capture dissociation, Native mass spectrometry, Top-down mass spectrometry, Tau, Tweezer

Received: 29 April 2018/Revised: 3 July 2018/Accepted: 3 July 2018

Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder that manifests itself through loss of synaptic transmission and neuronal death. Most neurodegenerative diseases are defined by a single type of lesion. By contrast, the complex pathophysiology in AD includes a double proteinopathy characterized by aggregation of amyloid β -protein ($A\beta$) in amyloid plaques and hyperphosphorylated tau protein in neurofibrillary tangles (NFT) [1, 2]. Though insoluble NFTs are the hallmark of tau pathology, early forming, water-soluble tau oligomers are likely the most toxic species [3–5]. Interest in tau pathophysiology is not limited to AD but extends to other diseases collectively known as tauopathies, in which aberrant post-translational modifications and self-assembly of tau into neurotoxic species is a central underlying mechanism [6, 7].

Tau stabilizes microtubules and is expressed predominantly in the central and peripheral nervous systems with an abundant presence in axons. It consists of an N-terminal projection domain, a proline-rich region, a microtubule-binding repeat region, and a C-terminal domain. In human brain, alternative mRNA splicing of microtubule-associated protein tau (MAPT) transcripts on chromosome 17q21.31 results in 6 isoforms of the tau protein ranging from 352 to 441 amino acids in length [8, 9]. These isoforms differ depending on the number of 29-residue inserts and can be categorized depending on whether they contain three or four carboxy-terminal repeat domains (3R or 4R, respectively).

Tau is a natively unfolded or intrinsically disordered protein (IDP). However, this does not altogether eliminate global order. Single-molecule Förster resonance energy transfer (FRET) has shown long-range N- and C-terminal contacts, as well as contacts between both termini and repeat regions, suggesting tau has an S-shaped fold [10]. Full-length tau assembles into filaments via its repeats, with the N- and C-terminal regions forming a fuzzy coat [11, 12]. Recombinant tau filaments, as well as those derived from the human brain, contain a cross- β structure core of ~ 90 amino acids, characteristic of amyloid fibrils [13]. This core includes the microtubule-binding region (MTBR) region. Two important nucleating sequences within the MTBR have been identified, namely PHF6 (VQIVYK) and PHF6* (VQIINK) [14].

In the brain, tau undergoes various post-translational modifications (PTMs) including phosphorylation, acetylation, methylation, glycosylation, isomerization, O-GlcNAcylation, nitration, sumoylation, ubiquitination, as well as truncation [7]. Several PTMs have been considered to play a key role in tau aggregation [15]. Among these, phosphorylation is considered to be an important modification with 85 putative sites

located on tau. Abnormal phosphorylation (at non-physiological sites) is a key characteristic of NFTs and in AD; it is thought to precede tau aggregation [16]. Tau phosphorylation is inversely related to its ability to bind microtubules and filamentous tau is abnormally phosphorylated [17]. But whether phosphorylation is a cause or a by-product of aggregation remains to be determined. To date, there is no strong evidence linking changes in kinase or phosphatase activity to human tauopathies. Although aggregated tau is heavily phosphorylated in the human brain, not all phosphorylated tau is aggregated. In fact, there is evidence to suggest that highly phosphorylated tau is associated with neuroplasticity, a reversible process that is not associated with the formation of filaments [18]. However, there is recent evidence linking full-length tau hyperphosphorylation to conformational shifts and amyloidogenic propensity.

Non-covalent interactions between molecules possessing aromatic rings (π - π and CH- π) are generally acknowledged to be important in the formation of ordered biological systems [19–21]. Therefore, a host molecule that is designed to exploit aromatic interactions for targeted recognition of certain amino acids in relevant peptide regions could be of significant therapeutic interest. The approach of applying synthetic compounds known as molecular tweezers (MT) to inhibit abnormal self-assembly of proteins via combination of hydrophobic and electrostatic interactions has been shown to be effective against amyloidogenic proteins [22, 23]. The name “molecular tweezer” was first used in the 1990s to describe a class of U-shaped molecules with rigid aromatic sidewalls. The U-shaped cavity, in principle, could accommodate a sterically favorable functional group on a target molecule [24].

Contrary to the many artificial receptor molecules that have been developed for amino acids with little to no selectivity to specific residues, the MT compound CLR01 shows selectivity towards lysine, and to lesser extent arginine residues. CLR01 binds preferentially with high on-off rate to Lys residues on unstructured proteins and remodels their assembly into non-toxic, non-amyloidogenic structures. It acts as a unique nanochaperone, which rather than assisting in the folding of the native structure, prevents formation of toxic oligomers and aggregates. A derivative compound, CLR03, that shares the polar bridgehead structure but lacks the hydrophobic arms was designed as a negative control [25]. The mechanism of MT inhibition is based on competing electrostatic and hydrophobic interactions, which are essential for abnormal self-assembly and aggregation. Furthermore, low-binding affinity and high on-off rate allow MT to disrupt abnormal self-assembly without interfering with normal protein function [22, 23].

ESI-MS and IMS for Structural Analysis of Tau and Tau/CLR01 Complex

Since the early 1990s, electrospray ionization mass spectrometry (ESI-MS) has been used for the measurement of non-covalent protein-protein and protein-ligand complexes [26–28]. Through its soft ionization process, “native” ESI [29] generally does not result in molecular fragmentation and can preserve weakly bound complexes and ligands, from salts and solvent molecules to metal cofactors and protein aggregates. While lacking the direct three-dimensional structural information provided by X-ray crystallography and NMR, ESI-MS can nevertheless provide valuable stoichiometry and heterogeneity information. Thus, ESI-MS can reveal a specific picture of the protein quaternary structure [30].

Contrary to the more commonly employed “bottom-up” approach, whereby proteins are enzymatically digested followed by tandem spectrometry, “top-down” MS has the potential to retain labile PTMs and ligands generally lost in the bottom-up approach [31–35]. Moreover, tandem MS/MS using electron capture dissociation (ECD) can better preserve labile PTMs often lost during collisionally activated dissociation [36].

Native top-down MS can also reveal the specific site(s) of non-covalent ligand binding and the location of surface residues [31, 37–41]. ECD fragmentation dissociates covalent backbone bonds but preserves non-covalently bound ligands. We have effectively implemented ECD-MS to study the interaction of various aggregation-prone proteins with molecular tweezer CLR01. Our top-down ESI-ECD-MS/MS approach was used to identify the binding sites of CLR01 on A β protein [42] and α -synuclein (AS), the aggregation-prone pathogenic species in Parkinson’s disease [43]. Most recently, using native ESI-MS for the analysis of the 17-residue N-terminal fragment (N17) of Huntingtin protein (Htt), implicated in the pathology of Huntington’s disease, we observed a 1:1 N17:CLR01 complex with an estimated K_d of 20 μ M. In addition, ECD-MS/MS revealed Lys9 as the most probable site of CLR01 binding to N17 [44].

In this study, we employed native top-down ESI-MS and ion mobility MS to gain structural insight into tau and the tau/CLR01 complex. We sought to determine tau/CLR01 binding stoichiometry and well as map site(s) of CLR01 binding. Because phosphorylation is implicated in tau’s propensity to aggregate, we analyzed phosphorylated tau and its CLR01 binding properties.

Experimental

Materials

The expression and purification of 0N3R, PHP-0N3R, and some of the 2N4R tau used for this study was previously published [42]. Some 2N4R tau, 4R fragment, the C291A fragment, and its phosphorylated form were from Unité de Glycobiologie Structurale et Fonctionnelle, CNRS, Université

Lille; the expression and purification protocols are detailed in the Supplemental Materials. The proteins stored in 10 mM HEPES, 50 mM (NH₄)₂SO₄, 2 mM TCEP, pH 7.5 were buffer exchanged into 200 mM ammonium acetate, pH 6.8. Tau/CLR01 complexes were prepared at 1:1, 1:2, and 1:5 ratios, respectively. CLR01 and CLR03 were prepared and purified as described previously [45].

Mass Spectrometry

Fourier transform ion cyclotron resonance (FT-ICR) MS experiments were performed using a 15-Tesla Bruker Solarix system with an infinity ICR cell. Protein solutions were loaded into metal-coated borosilicate capillaries (Au/Pd-coated, 1- μ m i.d.; Thermo Fisher Scientific, West Palm Beach, FL, USA) and sprayed at a flow rate of 10–40 nL/min through a nano-ESI source. The ESI capillary voltage was set to 0.9 kV. The time-of-flight was 1 ms. ECD experiments were performed on isolated precursor ions (10 m/z isolation window) with an ECD pulse length of 0.03 s, ECD bias of 1.5 V, and ECD lens of 10 V. Up to 400 scans were averaged for each spectrum, and all spectra were externally calibrated with cesium iodide. MS/MS spectra were initially interpreted with the aid of ProSight Light (Northwestern University), in-house software, and verified manually using Protein Prospector (v 5.20.0; University of California, San Francisco).

Ion Mobility Spectrometry

Ion mobility (IM) experiments were performed on a Waters Synapt G2-Si quadrupole time-of-flight (QTOF) system equipped with a travelling wave ion mobility separation (TWIMS) module. All ion mobility spectra were acquired in positive-ion mode. Capillary voltage and cone voltage were set to 1.2 kV and 30 V, respectively. Up to 300 scans were averaged for each acquisition.

Results and Discussion

Top-Down MS of Tau and Tau/CLR01 Complex

ESI-MS spectra for intact tau proteins acquired in aqueous pH 6.8 solutions show a high degree of charging (Figure 1). The intrinsically disordered nature of the protein appears to enable a high degree of charging that is more typical of globular proteins measured from denaturing solution conditions [46].

A 4R-tau and a 4R-repeat fragment (residues 257–359; 11 kDa) were examined for binding to CLR01 (sequences listed in Figure S1). Previously, using thioflavin-T fluorescence and electron microscopy, CLR01 was shown to inhibit 0N3R-tau aggregation (induced by arachidonic acid) completely at 1:1 M ratio [42]. The addition of CLR01 (structure; see Figure S2) to the tau proteins resulted in non-covalently bound 1:1 protein/CLR01 complexes, which appear as distinct distributions with characteristic shifts to higher m/z values for each charge state (Figure 2). A relatively low fraction of the tau

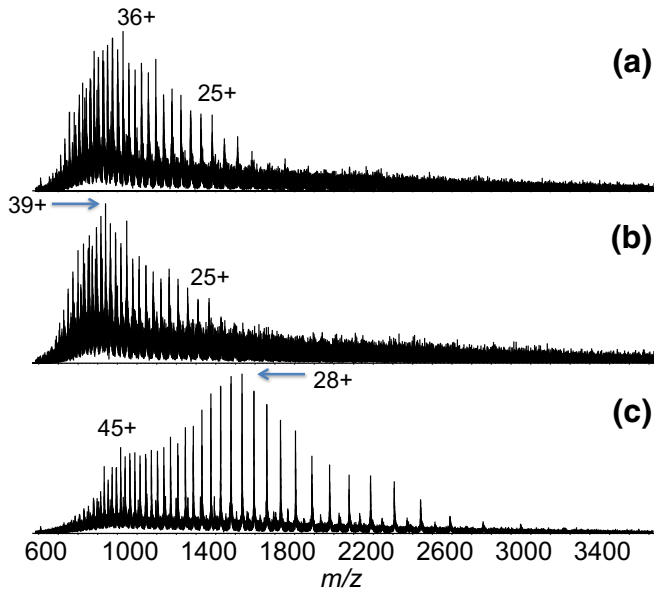


Figure 1. ESI mass spectra of (a) pseudohyperphosphorylated PHP-3R-tau (37.1 kDa), (b) 3R-tau (36.8 kDa), and (c) 4R-tau (45.8 kDa) from pH 6.8 aqueous solutions

proteins were found to bind to CLR01, which is consistent with the micromolar-range K_d binding constants found for CLR01 binding to other proteins examined previously [43]. The 4R-fragment tau spectra show a bimodal charge state distribution that is not observed in the CLR01-bound distribution (Figure 2D). This may be an indication that CLR01 binding stabilizes tau by shifting it towards a single conformation (vide infra).

The intact 4R-fragment tau and 4R-fragment tau/CLR01 complex were further subjected to native top-down MS with ECD to obtain sequence information and pinpoint the site(s) of inhibitor binding (Figure 3). For the 10+ charge state of the intact 4R-fragment tau, both N- and C-termini showed extensive fragmentation, but formation of ECD product ions from the core region spanning the microtubule-binding domain was poor in comparison

(Figure 3, left panel). This could be due to the presence of a disulfide bond, as there are two Cys-residues in this core region. But despite the limited fragmentation in the microtubule-binding region, up to six fragments were observed between the two cysteines.

Top-down ECD-MS of the 10+-charged holo-form showed CLR01-bound c- and z-product ions (Figure 3, right panel, marked in red) that point towards the core of the tau fragment as the likely region of ligand binding. This is the part of the microtubule-binding domain that contains the six-residue nucleating segment PHF6 (VQIVYK) that forms a characteristic cross- β steric zipper. From the molecular tweezer CLR01's bias towards binding to lysine residues, the top-down mass spectra suggests CLR01 to be bound to the K294-K331 region, which includes lysine K294, K298, K311, K317, K321, and K331, and is a known microtubule-binding region. This is consistent with solution-state NMR studies on tau and CLR01 binding, which show the greatest perturbation in the NMR spectra at resonances for K321 and K331 (data not shown).

Because CLR01 binding has been shown to disrupt tau oligomerization, and the VQIVYK segment has been shown as a key component of the seeding process for fibrilization, it may be reasonable to infer that CLR01's inhibitory effect is related to its binding to lysines at or near the VQIVYK region, i.e., K290, K311, K317, K321, and K331.

This interpretation of the data presented in Figure 3 is further supported by the ECD-MS fragmentation pattern of the 4R-repeat domain fragment C291A (Figure S3), where a cysteine substitution to alanine eliminated the possibility of the disulfide bond and resulted in considerable fragmentation in that region. Fragmentation patterns of the CLR01-bound 17+ charge state point to CLR01 binding to lysines spanning the VQIINK and VQIVYK regions (lysines K294, K298, K311, K317, K321) (Figure S4), similar to the proposed binding sites for the 10+ charge state of the 4R-tau fragment (Figure 3). Certainly, CLR01 binds to many Lys residues. The fragmentation map (Figure S4)

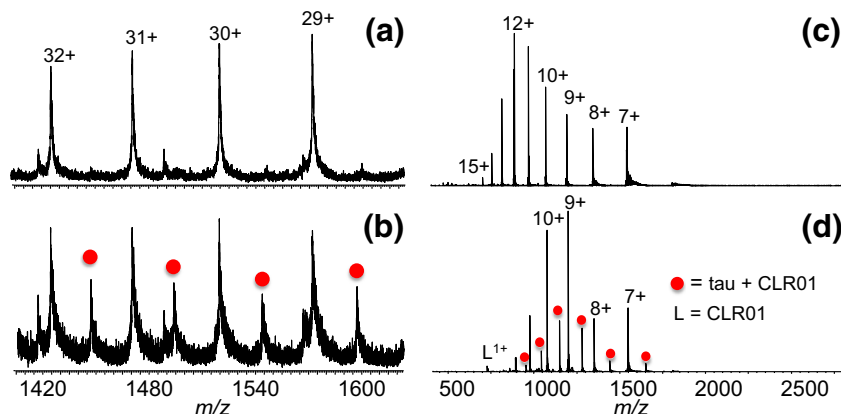


Figure 2. (a) ESI-MS of 4R tau, (b) CLR01 + 4R tau, (c) 4R-repeat domain fragment, and (d) CLR01 + 4R-repeat domain fragment. (Red circles represent peaks for the 1:1 protein/ CLR01 complex)

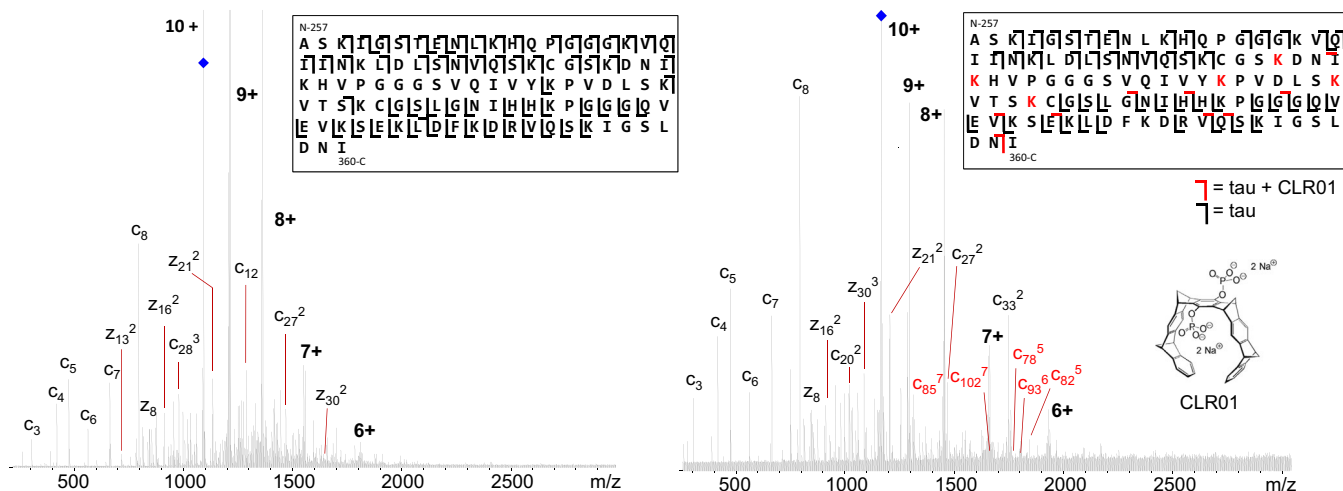


Figure 3. ECD-MS of 10+ charge state of 4R-repeat domain fragment. Fragmentation pattern in the control (left) vs ligand-bound state (right) indicates CLR01 binding in the MTBR region, spanning lysines K294-K331

shows evidence for CLR01 binding to other Lys residues outside the proposed binding region (microtubule-binding domain), but their relative abundance compared to product ions containing lysine residues in the proposed binding region is lower. The fact that CLR01 binds to the microtubule-binding domain that is prone to aggregation is a significant result of this study.

Overall, the tau protein sequences have an unusually stable core region that appears to be stable to ECD dissociation. Fragmentation appears to be limited to the flexible termini regions (Figure 3). Other intrinsically disordered proteins, such as α -synuclein, allow for much higher yields for top-down MS, up to 90% sequence coverage [47]. The data shown for the 103-residue 4R-repeat domain fragment shows 61% sequence coverage (62 interresidue cleavages out of 102 total interresidue bonds) for the apo-form. The 4R-repeat domain C291A fragment (Figures S3, S4, and S5) is 30% longer (133 residues). Besides being a longer sequence, it is unclear why sequence coverage is significantly lower (less than 40%) compared to the data shown in Figure 3. Sequence coverage is difficult to predict, as many factors are likely to contribute to top-down MS efficiency. However, the native top-down MS data is consistent with the subsequent IM-MS data that shows the compaction of the protein upon CLR01 binding (vide infra). Sequence coverage drops from 61 to 46% with CLR01 binding.

Because tau phosphorylation is directly related to its ability to bind microtubules, we tested our native top-down MS methods to map phosphorylation sites on a synthetic phosphorylated 4R-tau fragment C291A. CLR01 binding to phosphorylated tau was also tested. In the case of tau, it is particularly interesting to determine how phosphorylation affects the action of the negatively charged CLR01. ESI-MS shows singly and doubly phosphorylated 4R-tau fragment C291A, and a 1:1 complex formed between both singly and doubly phosphorylated 4R-fragment

C291A and CLR01 (Figure 4). ECD-MS of the singly phosphorylated protein established the primary phosphorylation site to be S235 (Figure S5), one of several serine/threonine sites found in endogenous forms of tau to be phosphorylated. T231 is another site expected to be phosphorylated. From the holo- c_{234} product ion, the fragmentation map suggests T231 to be phosphorylated also. However, the holo- c_{237} product is seven times more abundant than holo- c_{234} , suggesting that T231 is a secondary phosphorylation site to S235. (Unfortunately, the signal intensity of the doubly phosphorylated and CLR01-bound C291A 4R-fragment tau were not sufficient for isolation and fragmentation by ECD-MS with our instrument system.)

Ion Mobility MS of Tau and Tau/CLR01

The Bowers group reported the use of ion mobility coupled to mass spectrometry (IM-MS) to study the effect of

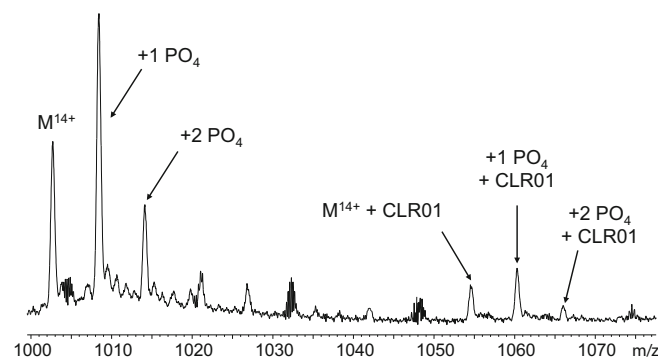


Figure 4. Partial ESI mass spectrum of phosphorylated 4R-repeat domain fragment C291A showing multiple phosphorylation events in the unmodified and CLR01-bound forms CLR01 added to a 1:5 (tau/CLR01 ratio)

CLR01 binding to A β [48]. IM-MS can separate gas phase protein aggregates and protein-ligand complexes based on their size and shape. Ions of different sizes and shapes will experience different mobilities and drift times as they travel through a chamber filled with an inert gas under a low electric field [48–50]. Ion drift time is a direct consequence of its size and shape or collisional cross-section (CCS). Folded or more compact species will therefore experience shorter drift times. This feature enables the separation of conformers having the same m/z , oligomers, proteins with various PTMs, and proteins bound to ligands. The Bowers study revealed a CLR01-mediated compaction of A β dimers and tetramers, eliminating higher-order oligomers [48]. Their work prompted us to apply IM-MS to investigate the effects of CLR01 binding on the structure of tau.

Ion mobility spectra of 4R-tau fragment were acquired under native solution conditions. There were significant differences in ion mobility profiles between the free and CLR01-bound 4R-tau fragment for the 9+ and 8+ charge states (Figure 5). A shift towards a more compact form and a decrease in the elongated form is promoted upon CLR01 binding. This is more readily observed for the 7+ charged protein (Figure 6). The more elongated conformer represented by the peak with a drift time of ca. 10 ms decreased in relative abundance with an increase in the more compact conformer (drift time ca. 7.5 ms) upon CLR01 binding. A small amount of a doubly CLR01 bound form is also present; this effect towards a more compact form is even more pronounced for this doubly bound form. A similar relationship between tau compaction and charge state was also observed for the singly CLR01-bound C291A 4R-tau fragment, especially for the lower charge states (Figure S6). The TWIMS resolving power for these tau proteins is similar to our TWIMS measurements of another intrinsically disordered protein, α -synuclein [47, 51], and a recent TWIMS study of tau proteins [52].

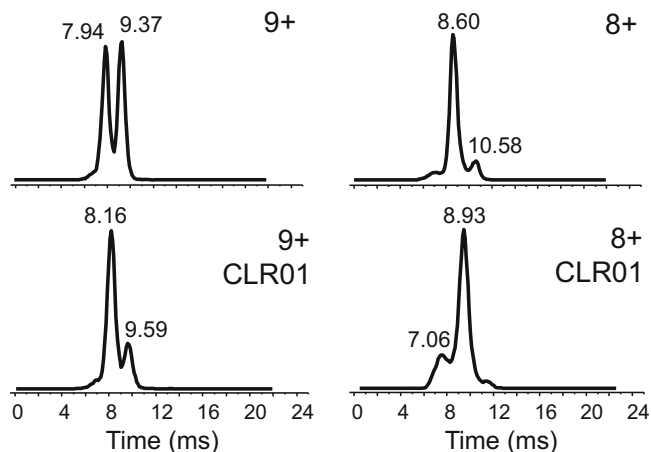


Figure 5. IM-MS of 9+ (left) and 8+ (right) charge states of 4R-repeat domain fragment with and without CLR01 (40 μ M tau/CLR01 in a 1:1 ratio)

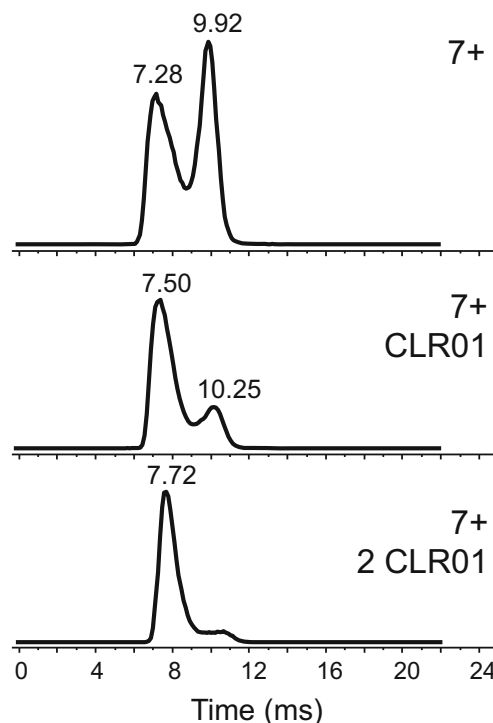


Figure 6. IM-MS of 7+ charge states of unbound 4R-repeat domain tau fragment (top), singly bound (middle) and doubly bound fragment (bottom) with CLR01 (40 μ M tau/CLR01 in a 1:1 ratio)

Similar shifts towards more compact structures were also observed for the phosphorylated C291A 4R-tau fragment. Singly and doubly phosphorylated species showed little to no difference in drift time compared to the unmodified version. The binding of CLR01 resulted in the appearance of more compact structures. For example, for the 13+ charge state, there were marked differences in overall peak shape and drift time between the unmodified and doubly phosphorylated tau in the presence of CLR01 (Figure 7).

It is not completely clear what influence phosphorylation has on phosphoprotein gas phase structures. From work reported by Scrivens [53] and Clemmer [54], phosphorylation of *peptides* results in compaction of peptide conformations from intramolecular interactions with neighboring residues and the peptide backbone. Whether this behavior extends to larger phosphoprotein systems is unclear. Ion pairing, e.g., salt bridges, could indeed affect gas phase structures [55]. The data shown in Figure 7 for singly and doubly phosphorylated tau does not indicate a *significant* difference in gas phase structure with phosphorylation. But CLR01-binding drives protein compaction for the phosphorylated proteins even more so compared to the non-phosphorylated versions.

Overall, the IM-MS analysis of the 4R-tau fragment showed that CLR01 binding reduces or eliminates longer drift-time species, suggesting that CLR01 binding shifts tau fragment conformation towards a more compact structure. This structural remodeling may point to a mechanism of CLR01-mediated inhibition of aggregation.

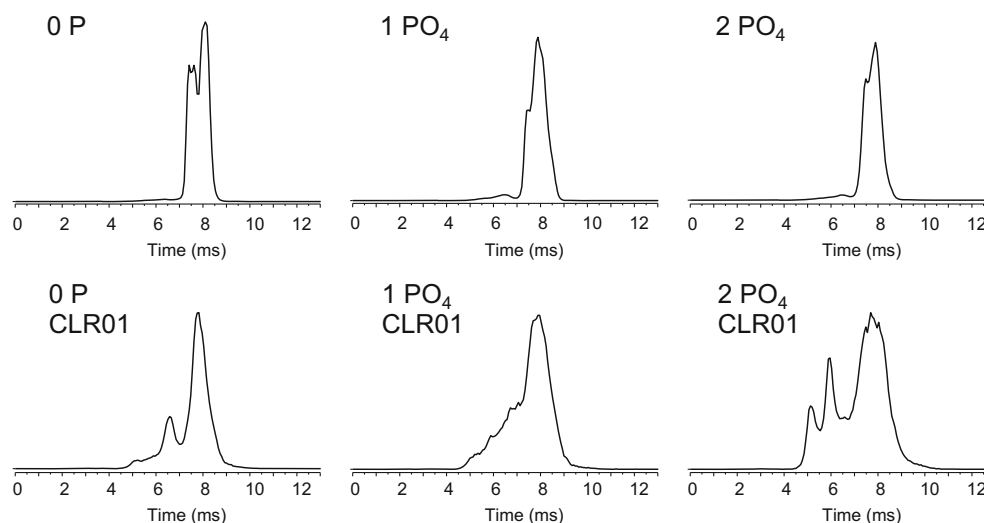


Figure 7. IM-MS of 13+ charge state of zero-, mono- and di-phosphorylated 4R-tau C291A fragment with and without CLR01 bound. (1:1 protein:ligand complex)

Conclusions

Data from native MS cast CLR01-mediated inhibition of tau aggregation in a more structurally informative light, with some insight into the mechanism of inhibition. ECD-MS data point towards the microtubule-binding domain as the likely site of CLR01 binding, which agrees with CLR01 stabilization of tau function. The microtubule-binding region also spans the two six-residue nucleating segments PHF6 (VQIVYK) and PHF6* (VQIINK) shown to be essential for seeding. It seems likely that the disruption of aggregation would involve CLR01 binding at or near these residues. From a mechanistic point of view, it also appears that inhibition of tau aggregation via CLR01 occurs through some form of structural remodeling and compaction of the protein. Similar results were obtained with A β and CLR01, where direct remodeling of A β by CLR01 disrupted the formation of dimers and tetramers, preventing higher-order oligomers from forming [48]. Future experiments will aim to identify how sequence alternations in various tau isoforms and tau PTMs affect CLR01 binding. These findings once again highlight the unique features of assembly modulator CLR01 and its tremendous therapeutic potential.

Acknowledgments

The authors would like to thank Dr. Hong Nguyen (UCLA) for help with data processing and Dr. Jing Di (UCLA) for providing the tau samples and sample information.

Funding Information

Support from the US National Institutes of Health (R01GM103479, S10RR028893, S10OD018504 to J.A.L.; RF1AG054000, R01AG050721 to G.B.), the Development and Promotion of Science and Technology Talents Project (DPST) and Royal Thai Government (to P.W.), the

Rachadapisek Sompot Fund, Chulalongkorn University (to P.W.), and the US Department of Energy (DE-FC02-02ER63421 to J.A.L.) are gratefully acknowledged. A.B. and M.E. were supported by CRC1093 and EH 100/19-1 from Deutsche Forschungsgemeinschaft.

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