2020

Protein/peptide characterization using mass spectrometry and molecular dynamics simulations

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**Protein/peptide characterization using mass spectrometry and molecular
dynamics simulations**

Ahmad Kiani Karanji

Dissertation submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
In
Chemistry

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2020

Key Terms: protein/peptide, molecular dynamics simulation, mass spectrometry,
hydrogen deuterium exchange (HDX), Huntingtin, electron transfer dissociation, Nt17,
lipid vesicle, ion mobility, collision cross section, blue proteorhodopsin,

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Abstract

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Ahmad Kiani Karanji

Mass spectrometry (MS) based-techniques and molecular dynamics (MD) simulations have been used to characterize protein/peptide structure as well as their interactions with lipid vesicles and detergents. Chapter 1 introduces an introduction to the concepts and tools that were used in this work. In Chapter 2, the dominant gas-phase conformer of $[M+3H]^{3+}$ ions of the model peptide Acetyl-PSSSSKSSSSKSSSSKSSSSK are examined with ion mobility spectrometry (IMS), gas-phase hydrogen deuterium exchange (HDX), and mass spectrometry (MS) techniques. This section furthers the development of a protein structural prediction tool by providing information about gas-phase ion conformers of two model peptides having different solution conformational states. In Chapter 3, interactions between the first 17 amino acid residues (Nt17 peptide) of the Huntingtin protein (htt) with lipid vesicles comprised of two subclasses of phospholipids are examined using electrospray ionization - mass spectrometry (ESI-MS) and MD simulations. Notably, the aggregation of Htt results in Huntington's disease (HD). Chapter 4 presents a MD simulations study of proteorhodopsin (PR) oligomers in different membrane environments. Proteorhodopsins are transmembrane proteins and members of the microbial rhodopsin family which act as a proton pump in the bioenergetics processes in bacteria. Finally, Chapter 5 discusses future directions for utilizing experimental and computational methods to obtain information about protein/peptide structure. Overall, the combination of MS-based techniques and MD simulations is shown to provide unique and enhanced structural information for intractable protein/peptide species.

Dedication

I dedicate this work to my lovely and caring wife, Marziyeh! I am lucky to have you as my companion; I could not go through this journey without you. And to my daughter, Melika! I want you to know that you are always my first concern. Also, to my parents Maman and Baba, your love is always inspiring for me, and my brothers Mohammad, Rasoul, and Mostafa and my sister Fatemeh. I missed all of you the entire time.

Acknowledgements

I must thank my research advisors Dr. Stephen J. Valentine and Dr. Blake Mertz for all the life and research skills that they taught me. These two men taught me how to best approach the research subjects; I applied the same critical thinking approach to the other aspects of my life. I would like to express my thanks to all my committee members: Dr. Stephen J. Valentine, Dr. Blake Mertz, Dr. Brian V. Popp, Dr. Fabian Goulay, and Dr. Mark McLaughlin for serving on my committee as well as Dr. Peng Li and Dr. Justin Legleiter for helping me. Also, I would like to thank a long list of outstanding friends and colleagues with whom I worked including: Dr. Hossein Maleki, Dr. Sadegh Faramarzi, Kushani Attanayake, Sandra N. Majuta, Daud Sharif, Anthony DeBastiani, Kinkini Jayasundara, Madison Pursell, Samira Hajian, Maryssa Beasley, Dr. Jim Arndt, Dr. Mahdiar Khakinejad, Dr. Samaneh Kondalaji, Dr. Chitrak Gupta, Nandhini Ranganathan, Chong Li, Eric Sefah, Corey Cleavenger, Violetta Burns Casamayor, Nick Frazee, and Kyle Billings.

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List of Symbols and Abbreviations

vd	Drift velocity
IMS-MS	Ion mobility spectrometry mass spectrometry
MD	Molecular dynamics
K	Mobility
E	Electric field
L	Length of drift tube
t_b	Drift time
N	The number density of the buffer gas
m	Ion mass
M	Mass of the buffer gas
e	Elemental charge
k_B	Boltzmann distribution constant
T	Temperature
z	Ion charge
Ω	Collision cross section
P	Pressure
Htt	Huntingtin
Nt17	The first 17 amino acid of Huntingtin
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry MS ⁿ Multistage tandem mass spectrometry
ETD	Electron transfer dissociation
CID	Collision induced dissociation
ESI	Electrospray ionization
LIT	Linear ion trap
LTQ	Linear trap quadrupole (same meaning as LIT)
CCS	Collision cross section
DDM	n-Dodecyl-β-D-Maltoside
DPC	Dodecylphosphocholine
DHPC	1,2-dihexanoyl-sn-glycero-3-phosphocholine
LB	Lipid bilayer
POPE	1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phosphoethanolamine
POPG	1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phosphoglycerol
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
D₂O	Deuterium oxide
PR	Proteorhodopsin
MeOH	Methanol
NH₄OH	Ammonium hydroxide
NMR	Nuclear magnetic resonance spectroscopy
LC-MS	Liquid chromatography mass spectrometry

GC	Gas chromatography
GC-MS	Mass spectrometry-Gas chromatography
LC	Liquid chromatography
RMSD	Root mean square of the deviation
HAS	Hydrogen accessibility scoring
PDB	Protein data bank
VMD	Visual Molecular Dynamics
NAMD	Nanoscale Molecular Dynamics

1. Introduction: Tools Used for protein/peptide structure characterization and identification of protein-lipid interactions: Electron Transfer Dissociation (CID), Ion Mobility Spectrometry-Mass Spectrometry (IMS-MS), Molecular Dynamics (MD) Simulations, and Hydrogen Deuterium Exchange

Protein/peptide characterization

Proteins are biopolymers containing sequences of amino acids bound together by peptide bonds. These large biomolecules are involved in many biological activities such as energy conversions, and chemical transportation ¹⁻³. The 3D structural characterization of proteins is an important pre-requisite for studying the function of these large biomolecules in biological media ⁴⁻⁵. An error in the protein structure may lead to disease; for example; misfolding of Huntingtin protein (htt) can lead to aggregation and the formation of amyloid fibrils in neurons ⁶.

Historically, protein characterization has been conducted with spectroscopic methods such as nuclear magnetic resonance (NMR) ⁷⁻¹⁴, fluorescence ¹⁵⁻¹⁸ and circular dichroism (CD) ^{12, 19-20}, UV-based methods ²¹⁻²² and Raman spectroscopy ²³. Along with these powerful analytical techniques high resolution methods such as x-ray crystallography ²⁴⁻²⁶ and cryo-electron microscopy ²⁷⁻³⁰ can be used to study protein structure and their interactions. Although these traditional techniques are widely used, they suffer from some limitations such as difficulties in sample preparation ³¹. It is worth mentioning that there is a limitation in crystalizing some of the proteins ³². In addition, many of the membrane proteins are not soluble in water because of their hydrophobic nature; therefore it can be difficult to study such structures using NMR ⁹. Furthermore, some globular proteins tend to aggregate and create big systems which are difficult to study using NMR (~15 KDa limitation) ³¹. Another limitation can be that some of

the protein interactions are transient and cannot be captured using these traditional techniques³³. Finally, it should be noted that an increasing number of studies is beginning to show the presence of multiple, coexisting structures under native conditions³⁴⁻³⁶. Some of these limitations can be addressed with mass spectrometry (MS) and mass spectrometry-based techniques such as hydrogen deuterium exchange (HDX). Several advantages include those associated with analysis speed and sensitivity as well as an ability to “freeze out” coexisting structures³⁷⁻⁴⁰.

Early MS-based studies of proteins involved studies of the conformational properties and the folding pathways of native-like proteins using electrospray ionization-mass spectrometry (ESI-MS)⁴¹⁻⁴⁵. These studies were often performed to obtain information about the mechanism of action of these important biomolecules. Shortly later, gas-phase separation techniques were introduced as a tool to characterize protein structure⁴⁶⁻⁴⁹. In the gas phase, protein ion structure is not stabilized through hydrogen bonding with the solvent molecules^{46, 50-51}. Ion mobility spectrometry-mass spectrometry (IMS-MS) was introduced to effectively increase the resolution of the MS data and reveal different gas-phase structures corresponding to co-existing protein conformations in solution^{48, 52}. For gas-phase ion structure characterization, IMS is an appropriate separation technique because of its high compatibility with mass spectrometry measurements⁵³⁻⁵⁶. Indeed, IMS-MS measurements were used early on to suggest that many protein ions could remain in their solution conformational state for hundreds of milliseconds which is sufficiently long enough to study with ion mobility instruments⁵⁷⁻⁵⁹. Other aspects of MS-based studies include the theoretical investigations of the ionization processes of protein⁶⁰⁻⁶¹, and labeling techniques^{31, 37, 62-63}.

The combination of experimental techniques such as MS with molecular dynamics (MD) simulations can provide valuable information regarding protein structures as well as their

interactions with other chemical species ⁶⁴⁻⁶⁷. For example, MS can be used to provide information about the contact sites of a protein interacting with other proteins or with other biological compounds, as well as finding the structured/unstructured and rigid/dynamic regions of a protein followed by relating this information to the higher-order structure ³¹. The atomistic view of MD simulations enables scientists to have a better understanding of the chemistry of these molecules.

IMS-MS

Ion mobility spectrometry (IMS) is a gas-phase ion separation technique that has frequently been used to study the structure of biomolecular ions ⁴⁹. The high compatibility of this separation technique with MS makes it available in many newly designed commercial instrumentations ⁶⁸⁻⁶⁹. In this technique the separation is realized according to the ion's charge and overall size. IMS instrumentation consists of a drift tube (a pressurized enclosure) with electrostatic lenses (Figure 1.1) ⁷⁰. The first step in an IMS measurement scheme is to ionize the molecules thereby transferring the solution-phase molecules to the gas phase as ions. To detect the molecules using a mass analyzer requires that the molecules are charged. Therefore, an ionization technique to be used in such experiments should provide these two requirements (volatilization and ionization) ⁶⁰. The ions originating from the source are then introduced to a drift tube such as that shown in Figure 1.1 and subsequently separated according to their size-to-charge ratios. The drift tube can be coupled with different mass analyzers such as the LTQ mass spectrometer for mass analysis and ion detection ⁷¹.

Figure 1. 1 Schematic diagram of an IMS-MS instrument used in the study of protein and peptide ion structures. Shown is the electrospray ionization source, the source desolvation region, the ion funnel (F1), ion activation (IA), and ion gate (G) regions. Also shown are the drift tube lenses. Finally, the linear ion trap mass spectrometer is also shown. Figure adapted with permission from ⁷⁰.

An ion's transit time inside the drift tube is referred to as the drift time (t_D). This t_D value can be used to calculate the collision cross-section (CCS) of the ion ⁷². The CCS can be loosely described as the projection of the orientationally-averaged ion or the average area swept through the buffer gas. CCS values can be calculated using the equation below.

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N} \quad \text{Equation 1. 1}$$

In Equation 1.1, k_B is the Boltzmann's constant, T is the buffer gas temperature, the term $\left[\frac{1}{m_I} + \frac{1}{m_B} \right]$ is the reduced mass, E is the electric field, and L is the length of the drift tube.

Hydrogen Deuterium Exchange (HDX)

One of the commonly used MS-based techniques in the study of protein structure is that of hydrogen deuterium exchange (HDX). Traditionally performed in solution, HDX is based on the replacement of accessible amide hydrogens with deuteriums along the protein backbone ^{1, 73-74}. Areas in which exchange does not occur can then be ascertained using MS measurements and such areas can be suggested to be involved in important interactions such as the hydrogen bonding networks associated with secondary structural elements (a helices and b sheets) ^{63, 75-79}. HDX experiments are possible because accessible protons on the protein are

constantly exchanging with the solvent protons; this process is not detectable by MS because no mass change occurs ¹. To carry out HDX experiments the sample is reacted with a deuterated solvent such as D₂O. Depending on the structure of the protein, some of the backbone amide hydrogens will exchange with deuteriums. The HDX reaction can be quenched after a certain period of time by lowering the pH and temperature ⁸⁰. Factors that affect the amount of deuterium incorporation include the solution temperature and pH and the tertiary structure of the protein ⁸¹. Lowering the pH to a point (~3.0) ⁸⁰ will result in decreased HDX rates. Thereafter, HDX rates increase and deuterium incorporation is increased. Complete HDX can happen on the ms time scale for random coil portions of proteins, but it will take minutes to hours for the parts of the protein involved in establishing secondary structure. Therefore, protection information can be correlated to higher order structure of proteins ⁸¹.

Different methods can be utilized to detect the incorporated deuteriums in the protein structure. One approach is to digest the protein to obtain smaller peptides using an enzyme in the solution phase. This is followed by cold liquid chromatography (LC) to separate the peptides and detect them using subsequent mass analysis ⁸²⁻⁸⁵. Another approach is to utilize the intact protein ions and fragment them in the gas phase to determine where the deuteriums were incorporated. In either case, the gas-phase dissociation technique to be used should be non-ergodic in order to avoid the mobilization and scrambling of the deuterium label ⁸⁶⁻⁸⁸.

More recently, gas-phase HDX has been introduced as a means to elucidate protein/peptide ion structure ^{62-63, 89-91}. Here, the gas-phase protein ions are subjected to collisions with a deuterating reagent gas (typically ND₃ and D₂O). As with solution-phase experiments, reaction occurs for accessible hydrogens. A notable difference is that accessible hydrogens are those that are not buried (accessible to collisions) or are accessible to original incorporation sites ^{1, 75, 78}. Therefore, the determination of

incorporation site is directly related to structural features such as surface accessibility as well as accessibility to charge sites. These features have been successfully modeled to provide an estimation of the overall ion structure ⁹²⁻⁹³.

Electron Transfer Dissociation (ETD)

As mentioned above, HDX is a powerful method to study the structures of biomolecules. Additionally, HDX can be performed in the solution phase using liquid chromatography-mass spectrometry (LC-MS) ⁹⁴ and online HDX ⁹⁵, or in the gas phase using drift tube ion mobility spectrometry ⁹⁶[more refs including Clemmer]. There are two main methods of protein characterization in the fields of structural and comparative proteomics. Introducing the peptides resulting from enzymatic cleavage in the solution phase to the mass spectrometer is known as the bottom-up approach ⁷⁹⁻⁸⁰. Conversely, transferring the intact protein into the mass spectrometer and using an appropriate gas-phase dissociation method is known as the top-down approach⁸⁷⁻⁸⁸. The former approach is the most commonly used method to detect deuterium exchange in proteins, but it suffers from some limitations. One of the limitations for bottom-up HDX is the deuterium back exchange with hydrogens ⁹⁷. This can occur to some extent during the separation step and for this reason the HPLC separation is maintained at cold temperatures. Additionally, it can occur in the gas phase before the de-solvated ions are analyzed by the mass spectrometer. Another limitation can be the lack of complete sequence coverage using a bottom-up approach for some proteins ⁹⁸⁻⁹⁹. To overcome these limitations, the top-down approach can be utilized to determine the sites of deuterium incorporation. Top-down proteomics can result in a per-residue level of resolution ¹⁰⁰. That said, there are only few dissociation methods that can be used effectively in the top-down approach. The main problem with conventional gas-phase fragmentation methods like collision-Induced dissociation (CID) and higher-energy collisional dissociation (HCD) is that these methods elevate the internal

energy of the biomolecular ions ¹. This excess amount of energy increases the mobilization of exchangeable hydrogens which causes the protons (and deuterons) to move across the peptide backbone and to other heteroatom sites. In the case of HDX this will randomize the distribution of deuterium across the biomolecule. This process is called HDX scrambling ⁹⁴. Strategies to overcome this problem are to use faster fragmentation techniques such as electron transfer dissociation (ETD) ¹⁰¹⁻¹⁰³. Here, fragmentation happens after the molecule reacts with the ETD reagent ions (typically radical anions). For ETD, a highly-charged ion (triply charged or quadropoly charged) is often required.

MD Simulations

Molecular dynamics (MD) simulations is based on Newton's second law ¹⁰⁴. Basically, it is possible to calculate the position of the system at $t+\Delta t$ and write a trajectory for the system using Newton's second law of motion (Equation 1.3) when the acceleration of the system, the initial position at time t , and the position at time $t-\Delta t$ are available as demonstrated in Equation 1.2 and 1.3.

$$r(t + \delta t) \approx 2r(t) - r(t - \delta t) + a(t)\delta t^2 \quad \text{Equation 1. 2}$$

$$m_i \frac{d^2 \vec{r}_i}{dt^2} = \vec{F}_i = -\vec{\nabla} U(\vec{R}) \quad \text{Equation 1. 3}$$

In these equations, m_i is the mass, and $\vec{r}_i$ is the position for each atom and $\vec{F}_i$ is the applied force on that atom. Also $U(\vec{R})$ is the potential energy which is defined as:

$$U(\vec{r}_1, \vec{r}_2, \vec{r}_3, \dots \vec{r}_N) = U(\vec{R}) \quad \text{Equation 1. 4}$$

To write a trajectory it is essential to calculate the acceleration of an atom. Using equation 1.3 the acceleration (a) of each atom can be calculated. In order to calculate a , a knowledge of the forces exerted upon it is required. And finally, to acquire the forces, it is necessary to define energy functions $U(\vec{r}_1)$ for each particle. These energy functions are the sum of bonded and nonbonded terms. The bonded terms are usually defined for bonds

(Equation 1.5), angles, dihedrals angles, and improper angles (Equation 1.6). Non-bonded interactions consist of Lenard-Jones potentials and charge-charge potentials (Equation 1.7).

$$U(\vec{R}) = U_{bonded} + U_{nonbonded} \quad \text{Equation 1. 5}$$

$$U_{bonded} = U_{bond} + U_{angle} + U_{dihedral} + U_{improper} \quad \text{Equation 1. 6}$$

$$U_{nonbonded} = U_{Vander Waals} + U_{Electrostatics} \quad \text{Equation 1. 7}$$

For each of the U_{bonded} terms the definition is:

$$U_{bond} = \sum k_i^{bond} (r_i - r_0)^2 \quad \text{Equation 1. 8,}$$

$$U_{angle} = \sum k_i^{angle} (\theta_i - \theta_0)^2 \quad \text{Equation 1. 9, and}$$

$$U_{dihedral} = \sum k_i^{dihedral} [1 + \cos(n_i \phi_i + \delta_i)] \quad \text{Equation 1. 10,}$$

in which k_i^{bond} is the bond energy constant, r_i is the distance between any pair of atoms, r_0 is the equilibrium distance between any pair of atoms, k_i^{angle} is the angular energy constant, θ_i is the angle between three atoms bond together, θ_0 is the equilibrium angle between three atoms bond together, $k_i^{dihedral}$ dihedral energy constant, n_i is multiplicity, ϕ_i is the dihedral angle between 4 atoms, and δ_i is the angle where the potential passes through its minimum value.

For the $U_{nonbonded}$ terms the definition is:

$$U_{Vander Waals} = \sum_{i=1} \sum_{j \neq i} 4\varepsilon_{ij} [(\frac{\sigma_{ij}}{r_{ij}})^{12} - (\frac{\sigma_{ij}}{r_{ij}})^6] \quad \text{Equation 1. 11, and}$$

$$U_{Electrostatics} = \sum_{i=1} \sum_{j \neq i} \frac{q_i q_j}{\varepsilon r_{ij}} \quad \text{Equation 1. 12,}$$

in which ε_{ij} is the depth of the potential well, σ_{ij} is the finite distance at which the inter-atomic potential is zero, r_{ij} is the distance between two atoms, q_i and q_j are atomic charges, and ε is Coulomb's constant.

One of the applications of MD simulations in the field of mass spectrometry is to study the behavior of molecules during the final stages of the ESI process ⁶⁰. An advantage of this application arises because of the time scale over which the MD simulations can now be

performed as well as the fact that they provide an atomistic view that cannot be obtained in any other fashion. This is the subject of Chapter 5 in this report.

Area per lipid

One of the concepts that is widely used in this document is the change of lipid composition along with the tracking of the interaction of peptides with such membrane systems. The lipid composition in eukaryotic membranes consists primarily of phosphatidylcholine (PC)¹⁰⁵. This lipid type occupies more than 50% of the phospholipids in almost all eukaryotic membranes. In an aqueous environment, phosphatidylcholine self-organizes spontaneously and creates a lipid bilayer in which the polar headgroups face the polar solution and the nonpolar tails face each other. Some of the PC lipids are saturated and therefore their tails are straighter. A saturated PC has a low area-per-lipid value because of this ordered structure (Figure 2). DMPC is one of the PC lipids that has a low area-per-lipid value because of the completely saturated tail. Most PC lipids have one or two cis-unsaturated fatty acyl chain(s). This causes an increase in the area-per-lipid value for the bilayer. Another way to increase the area-per-lipid value is to introduce methyl groups in the tail such as 1,2-di-O-phytanyl-sn-glycero-3-phosphocholine (PhPC). Figure 2 summarizes the area per lipid phenomenon.

Figure 1. 2 Area per lipid Phenomenon. Shown are the lipid structures (left) for DMPC, DOPC, and PhPC. The bilayer formed by each lipid system is illustrated in front of each lipid. The area-per-lipid values for DMPC, DOPC and PhPC are 59.0 Å² [93], 67.4 Å² [94], and 80.6 Å² [20] respectively at 30 °C.

Structure Studies

In Chapter 2 a comparison between two synthetic peptides that differ significantly in their native structure in the solution phase is performed using IMS, HDX, and MD simulations. For a serine containing peptide (Acetyl-PSSSSKSSSSKSSSSKSSSSK), the [M+3H]³⁺ ions are observed predominantly as a relatively compact conformer type. Upon subjecting these ions to electron transfer dissociation (ETD), the level of protection for each amino acid residue in the peptide sequence has been assessed. The overall per-residue deuterium uptake is observed to be relatively more efficient for the neutral residues than for the model peptide Acetyl-PAAAAKAAAAKAAAAKAAAAK. In comparison, the N-terminal and C-terminal regions of the serine peptide show greater relative protection compared with interior residues. Molecular dynamics (MD) simulations are used to generate candidate structures for collision cross section and HDX reactivity matching. Hydrogen accessibility scoring (HAS) for select structural candidates from MD simulations is used to suggest conformer types that could contribute to the observed HDX patterns. The results are discussed with respect to recent studies employing extensive MD simulations of gas-phase structure establishment of a peptide system.

In Chapter 3 of this report, the interactions of the first 17 amino acid residues (Nt17 peptide) of the Huntingtin protein (htt) with lipid vesicles is investigated ¹⁰⁶. Huntington's disease (HD) is a neurodegenerative disease ^{3, 107} caused by the expansion of a polyglutamine-coding CAG repeat in the HTT gene ¹⁰⁸⁻¹¹². The first 17 amino acid residues of htt (Nt17 of htt) are thought to play an important role in the protein's function; Nt17 is one of two membrane-binding domains in htt. The Nt17 peptide is intrinsically disordered in

solution and it undergoes a conformational shift from unstructured to an amphipathic α -helix in the presence of a membrane^{6, 111, 113-114}. This conformational change makes the Nt17 peptide a lipid-binding domain for htt¹¹⁵. It is believed that the amphipathic characteristic of the peptide causes the more hydrophilic residues to face toward the polar solvent and the more hydrophobic residues to face toward the lipid vesicles¹¹⁶. The composition of the lipid vesicle plays an important role in this interaction as the peptide displays greater affinity towards phosphatidylcholine (PC) lipid vesicles^{111, 117-118}. Overall, the peptide is shown to have a greater propensity to interact with vesicles of phosphatidylcholine (PC) rather than phosphatidylethanolamine (PE) lipids. Mass spectra show an increase in lipid-bound peptide adducts where the ordering of the number of such specie is 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) > 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) > 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphoethanolamine (POPE). MD simulations suggest that the compactness of the bilayer plays a role in governing peptide interactions. The peptide shows greater disruption of the DOPC bilayer order at the surface to interact with the hydrophobic tails of the lipid molecules via hydrophobic residues. Conversely, the POPE vesicle remains ordered and lipids display transient interactions with the peptide through the formation of hydrogen bonds with hydrophilic residues. The POPC system displays intermediate behavior regarding the degree of peptide-membrane interaction. Finally, the simulations suggest a helix stabilizing effect resulting from the interactions between hydrophobic residues and the lipid tails of the DOPC bilayer.

In Chapter 4, the stability and the role of important residues in oligomeric forms of PR in a POPE:POPG lipid bilayer and different detergent systems is investigated. Several residues play a role in the ion pumping process from the cytoplasmic side to the extracellular side including E108 which acts as a proton donor, the retinol, D97 which acts

as a proton acceptor, and E142 which is the proton releaser. In the dark state where no photon is illuminated, all these residues are in their ground state¹¹⁹⁻¹²⁰. It has been suggested that the native state of the protein is a pentameric/hexameric form because these oligomeric forms are dominant for many detergent/lipid conditions. The protein obtains structural stability upon forming noncovalent interactions between the monomers in the oligomeric form. In this work molecular dynamics (MD) simulations are utilized to characterize the effect of different membrane environments on the stability of PR in the dark state. Dodecylphosphocholine (DPC), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), and n-Dodecyl- β -D-Maltoside (DDM) detergent systems along with 1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phosphoethanolamine (POPE) and 1-Hexadecanoyl-2-(9Z-Octadecenoyl)-sn-Glycero-3-Phosphoglycerol (POPG) lipid bilayer systems are used to study the pentameric/hexameric forms of PR.

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2. Comparison of Peptide Ion Conformers Arising from Non-Helical and Helical Peptides Using Ion Mobility Spectrometry and Gas-Phase Hydrogen/Deuterium Exchange

Reprinted with permission from Journal of American Society for Mass Spectrometry: Comparison of Peptide Ion Conformers Arising from Non-Helical and Helical Peptides Using Ion Mobility Spectrometry and Gas-Phase Hydrogen/Deuterium Exchange. Ahmad Kiani Karanji, Mahdiar Khakinejad, Samaneh Ghassabi Kondalaji, Sandra N. Majuta, Kushani Attanayake, Stephen J. Valentine. J. Am. Soc. Mass Spectrom. 2018, 29, 12, 2402–2412.

Introduction

Since the discovery of the structure of the α -helix in polypeptides, ¹ significant research has been devoted to determining factors that affect the formation of elements of secondary structure and in particular those effecting helix formation ². Initial efforts, boosted by the expanding available protein structure data, focused on the relationship between the presence of α -helices and the frequency of occurrence of specific amino acid residues ³⁻⁷. Shortly later, Chou and Fasman proposed a method for predicting this secondary structure element using amino acid sequence information ⁸. Over the next several decades, interest in helix structure prediction progressed to the point where a relatively high degree of accuracy was obtained ⁹⁻¹⁰. This progress coincided with efforts to refine helix propensities of the individual amino acid residues ^{2, 11-12} and the concept has even been extended to structure destabilization imparted by D-amino acid residues ¹³⁻¹⁴.

The rapid increase in the number of experimentally-determined protein structures over the last three decades ¹⁵⁻¹⁶ has significantly enabled protein structure prediction capabilities. Perhaps, the simplest conception is to use sequence homology based on comparisons to known structures to infer structural elements for proteins of interest ¹⁷⁻¹⁹. Over the years other computational techniques have advanced significantly and protein structure prediction efforts can currently be grouped into those using ab initio methods (or ab initio guided approaches) and

template-based methods (fold recognition and threading, comparative modelling) ²⁰. Currently, protein structure prediction remains a highly active and rapidly evolving area of research ²¹⁻²³.

A challenge in the characterization of polypeptide structure are species that have been designated as intrinsically disordered ²⁴⁻²⁶. Although efforts have been developed for predicting unstructured regions of proteins ²⁷⁻²⁸, recent developments regarding the nature of species classified as intrinsically disordered have called into question whether or not structural assessments are possible. One area of interest is the study of peptide structure. For example, the peptide bradykinin was examined by NMR many years ago and only a small portion of the molecule was assigned to a β -turn structural type ²⁹. Recent experiments that employed ion mobility spectrometry (IMS) combined with mass spectrometry (MS) have shed some light on the inability of solution-phase measurements to determine prevailing structural motifs for peptides; for bradykinin, these experiments revealed the presence of multiple, co-existing solution structures ³⁰⁻³¹. Although the exact relationship of gas-phase structures to antecedent solution states is still under investigation, such efforts suggested that gas-phase analyses could provide insight into the various structures of polypeptides that exist in solution. Interestingly, extending the approach has suggested the presence of multiple protein structures for species for which well-defined native conformations predominate ³².

Results from the early IMS-MS experiments lead to questions regarding the degree to which elements of secondary structure are preserved in the gas-phase for such dynamic systems and whether or not solution structures can be derived from high-quality gas-phase structures. To study this, recent experiments have examined the conformations of $[M+3H]^{3+}$ ions of the model peptide Acetyl-PAAAAKAAAAKAAAAK (hereafter referred to as the A-containing peptide) using a combination of IMS-MS and gas-phase hydrogen/deuterium exchange (HDX) measurements and molecular modeling techniques ³³⁻³⁵. This peptide was selected because of its relatively large propensity to form a helix in solution ^{2, 36-37}. In general,

studies suggested that the presence of multiple conformer types of varying degree of helicity can contribute to the $[M+3H]^{3+}$ ion conformers that were studied³³. In many respects, this work was similar in nature to early efforts aimed at designing gas-phase peptide ion structure³⁸ as well as studies that have characterized peptide structural motifs with gas-phase HDX³⁹⁻⁴⁰.

The present work examines the $[M+3H]^{3+}$ ions of the model peptide Acetyl-PSSSSKSSSSKSSSSKSSSSK (hereafter referred to as the S-containing peptide). This peptide has been designed to exhibit less helical propensity in solution². Indeed, because the hydroxyl oxygen of serine (S) can hydrogen bond with the carbonyl oxygen of the 3rd residue, S is known to destabilize α -helix structure⁴¹. Additionally, regions of high S density are associated with helix-breaking structure⁴². The model peptide was also designed because of the potential to experience a different process of gas-phase conformation establishment when compared with the previously studied A-containing peptide. Previous work has suggested that the non-polar A residues may strongly associate with the a polar vacuum environment during the final stages of ESI^{35, 43}; it may be expected that the S residues will more strongly favor solvent interaction during the final stages of ESI. These differences offer the opportunity to compare the ion structure types formed by very different solution conformers and possibly by different processes of gas-phase conformer establishment.

Overall, ion collision cross section values are nearly the same compared with the A-containing peptide. However, because the S-containing peptide has a larger molecular weight, one may conclude the overall structure is more compact. Additionally, there are significant differences in the relative accessibility of exchange sites of terminal and middle amino acid residues with the latter being more accessible to the S-containing peptide. These characteristics are discussed below using structures obtained from simulated annealing to help provide insight into relative accessibility. By providing information about gas-phase ion conformer establishment from different solution states, these efforts may help to enhance protein structure

prediction tools in the future. Finally, this work adds to the growing repertoire of studies that employ IMS-MS, gas-phase HDX, and/or MD simulation techniques for peptide/protein ion structure characterization⁴⁴⁻⁵³.

Experimental

Sample Preparation. The model peptide acetyl-PSSSSKSSSSKSSSSKSSSSK (97.3% purity) was purchased from GenScript (Piscataway, NJ, USA) and used without further purification. To prepare the 1 mg/mL stock solution, the peptide was dissolved in the appropriate amount of Milli-Q water. The stock solution was then diluted 10 fold with a 100 mM solution of ammonium acetate in water to prepare the ESI solutions. This solution was infused (0.5 μ L/min) through a pulled-tipped capillary which was biased $\sim +2200$ V relative to the entrance of the hybrid IMS-MS instrument.

IMS-HDX-MS Measurements. For the ion mobility measurements conducted here, a dual-gate drift tube similar to others described in the literature was used⁵⁴⁻⁵⁵. The details of the drift time (t_D) and MS measurements have been described in detail previously⁵⁶⁻⁵⁷. Briefly, for this study, ions generated by ESI were periodically (50 Hz) pulsed into a ~ 1 -m-long drift tube that was filled with ~ 2.5 Torr of 300 K helium buffer gas. For the dual ion gate approach, the second gate was set to transmit at specific delay times in order to record mobility-resolved mass spectra. This delay time was scanned (200 μ s increments) across the mobility distribution of the $[M+3H]^{3+}$ ions. Mass spectra were recorded by a linear ion trap (LTQ Velos; ThermoScientific, San Jose, CA, USA) instrument. A mass-to-charge (m/z) range of 400 to 1000 was employed. For each t_D increment mass spectra were recorded for 0.5 min. For mobility selection, the second ion gate was set to transmit for 200 μ s.

The HDX measurements conducted here are modeled after foundational work in which the reactivities of different biomolecular ions were examined upon reaction with gaseous deuterating reagent⁵⁸⁻⁶⁵. For HDX measurements, ~0.02 Torr of D₂O was added to the buffer gas. With the addition of D₂O, the t_D distributions shifted to longer times as shown in Figure 2.1. Therefore, for the tandem MS (MS/MS) measurements, the delay times were scanned across the t_D distributions obtained when employing D₂O to determine the mobility selection time corresponding with the dominant, compact conformer type for the [M+3H]³⁺ ions. As it was desired to examine the HDX behavior of the dominant, most compact ion conformer type, mobility selection was initiated 200 μ s prior to the peak maximum time point in order to minimize contamination from larger ions (Figure 2.1).

Figure 2. $1\ t_D$ distributions for the $[M+3H]^{3+}$ ions of the model peptide Acetyl-PSSSSKSSSSKSSSSKSSSSK.

The bottom and top traces show the distributions obtained in the absence of D_2O and after the introduction of ~ 0.02 Torr D_2O . For both distributions, a delay step time of $200\ \mu s$ was used and each mobility-resolved spectrum was collected for 0.5 min. Integration of the isotopic envelope for the $[M+3H]^{3+}$ ions at each delay time setting provided the intensity values for the distributions. The distributions have been normalized to show relative intensities. The red bar in the top trace shows the typical mobility selection time and width.

Peptide Ion Dissociation by ETD and Per-Residue Deuterium Uptake Calculations. The MS/MS analyses described here have also been influenced by foundational work demonstrating the utility of MS/MS to determine deuterium uptake from gas- and solution-phase experiments ⁶⁶⁻⁶⁸. Briefly, mobility-selected $[M+3H]^{3+}$ ions were subjected to electron transfer dissociation (ETD) ⁶⁹ to determine the per-residue deuterium incorporation as described previously ^{56, 70}. To perform ETD measurements, mobility-selected ions were isolated by m/z and the ion injection time was maintained at 200 ms (5 microscans). Because ETD did not generate a number of lower m/z ions, the deuterium uptake value for each residue was obtained using both z- and c-type ions. The per-residue deuterium uptake values for the fragment ions were obtained by first subtracting the average mass of the fragment ions obtained in the absence of D₂O from the average mass of those generated upon using D₂O reagent gas. For the triplicate measurements, the amount of deuterium incorporated in each residue was calculated by subtracting the deuterium uptake values of the appropriate adjacent fragment ions. Here it is noted that mass spectrometer conditions including all focusing optics were maintained at conditions that have been shown to not induce deuterium scrambling ⁷¹.

Molecular Dynamics (MD) Simulations. Details regarding the *in-vacuo* MD simulations can also be found in the prior, related work ⁷². Briefly, the initial structures of the $[M+3H]^{3+}$ ions for simulated annealing were generated using the Visual Molecular Dynamics (VMD). The CHARMM36 force field was employed for structural parameterization. All of the parameters were defined in this force field. The CNEU patch in the top_all36_prot topology file were used to protonate the C-terminal K residue resulting in a +1 formal charge. The LSN patch was used to protonate the more N-terminal K residues. Acetyl groups were attached to the N-terminus portion of the P residue by using the ACP patch. 5000 steps of geometry and energy minimization were carried out using the Nanoscale Molecular Dynamics (NAMD) software suite for the $[M+3H]^{3+}$ peptide ions with charge arrangements of K(6)-K(11)-K(16), K(6)-K(11)-K(21),

K(6)-K(16)-K(21) and K(11)-K(16)-K(21). Then, the structures were subjected to cyclic simulated annealing (SA) for conformational space sampling. For SA runs (timestep of 1 fs), the Berendsen temperature coupling algorithm was used. For the SA algorithm, the temperature of the *in-vacuo* system was suddenly increased to 1010 K over 10 steps. The heated structure was maintained at that temperature for 40 ps and then cooled to a lower temperature (10 K) over two different timescales (40 and 1200 ps). The annealed structures were subsequently energy minimized to 0 K to generate candidate structures. The energy minimized structure of each cycle was imported as the starting point for the next heating-cooling cycle. Each minimized structure was gradually heated to 300 K and equilibrated. The structures then undergo 5 ns of MD simulations with no non-bonded cutoffs for long-range interactions while the temperature was constant. The Mobcal software suite ⁷³ was used to calculate theoretical collision cross-section values using the trajectory method ⁷⁴.

Hydrogen Accessibility Scoring (HAS) and HDX Kinetics Modeling. HAS scores were calculated for *in-silico* structures with matching collision cross sections. Two candidate structures which contained CCS values matching experimental measurements were selected for discussion. The selection was based on a first-pass examination of HAS scores for which one ion structure displayed increased proximity of charge sites to middle residues while the other displayed less access to these residues (see discussion below) as is suggested to be required by the “relay” mechanism for HDX ⁷⁵. Hydrogen accessibility was computed as described previously ⁷⁶ where each carbonyl site was scored based on its relative access to charge sites on the hypothetical peptide ions. Then the distances between exchange sites and these scored carbonyls were used to obtain relative theoretical reactivities of each exchange site which were summed for individual residues to provide a residue score. Finally, the residue scores were scaled based on relative values of surface accessibility using the solvent accessibility surface area (SASA) approach in the VMD software suite. Using these residue scores, the deuterium incorporation

was estimated for $[M+3H]^{3+}$ ions using an HDX kinetics model described previously⁵⁶. Briefly, a population of ions (1000) was stepped (1 μ s) through a mobility separation ($\sim$ 10 ms) and the exchange nature of each site was computed at each step based on the contribution of the residue to the overall rate of exchange for the ions. After stepping all ions through the mobility separation time, the HDX simulation algorithm determined the amount of deuterium incorporation for each residue; this theoretical uptake value is compared to the experimental results below.

Results and Discussion

Peptide Ion Collision Cross Sections. Summing the spectral counts for the $[M+3H]^{3+}$ peptide ions at each mobility selection allows the generation of the t_D distribution shown in Figure 2.1 (bottom trace). The distribution is dominated by a feature having a t_D of $\sim$ 8.5 ms. A shoulder, indicating the presence of larger ion conformer types is also observed. With the addition of $\sim$ 0.02 Torr of D_2O , the entire distribution shifts to longer times. The dominant peak is observed to have a t_D of $\sim$ 9.3 ms representing a shift of $\sim$ 0.8 ms. Again, a shoulder of similar intensity is observed at longer t_D values. The shift in the shoulder feature is also observed to be of the same magnitude. That the distribution is similar in the presence and absence of D_2O suggests that the HDX process does not result in structural transformations and it is therefore possible to monitor the reactivities of select ion conformer types. This is similar to results that have been presented for protein ions⁶⁰.

From the ion t_D distribution, it is possible to determine the collision cross section of the $[M+3H]^{3+}$ ions. Collision cross sections are determined using Equation 1⁷⁷:

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N}$$

Equation 2. 1

In Equation 1, ze and k_B are the charge of the ion and Boltzmann's constant, respectively. The variables m_i and m_B are the mass of the ion and the mass of the buffer gas, respectively. E , L , T , and P represent the electric field, the length of the drift tube, and the temperature and pressure of the buffer gas, respectively. Finally, N is the neutral number density at standard temperature and pressure (STP) conditions. Using Equation 1, the $^{\text{He}}\Omega_{\text{DT}}$ (DT = linear drift tube) is determined to be $424 \text{ \AA}^2$ and $464 \text{ \AA}^2$ for the prominent feature and shoulder, respectively. These values are larger but similar to those determined for more compact and partially-unfolded A-containing peptide conformers ($417 \text{ \AA}^2$ and $438 \text{ \AA}^2$)^{34, 76}.

A goal in examining the model peptide ions is to begin to determine the degree of similarity in solution- and gas-phase peptide structure in order to better understand ion conformer establishment. A number of studies suggest that a high degree of structure preservation is observed for relatively small proteins⁷⁸⁻⁷⁹. The fate of even smaller peptide ions is less clear. As mentioned above, comparisons of the data reported here for the S-containing peptide are made with those obtained from previous studies of the A-containing peptide because of expected differences in predominant solution conformers. Indeed, CD spectroscopy of the A-containing peptide suggests a high-degree of helical character as indicated by the negative bands at ~ 207 and 223 nm as well as the positive band at $\sim 190 \text{ nm}$ shown in Figure S1 in the Supplementary Information section (Appendix A). The CD spectrum for the S-containing peptide (Figure S1 in the Supplementary Information section (Appendix A)) shows a very clear difference in predominant solution structure for this peptide where the features indicating α -helix are clearly missing and a broad band at lower wavelengths is indicative of more random coil character.

Because of these differences in solution structure, it is of interest to consider the compact ion conformers for both the S- and A-containing peptides. Notably, the collision cross section of the S-containing peptide is only $\sim 1.7\%$ larger than that of the A-containing peptide ion (see above).

This occurs even though the molecular weight of the S-containing peptide is ~14.1% larger than that of the comparative peptide. Thus, the overall gas-phase “packing” of the S-containing peptide is more extensive than that of the A-containing peptide. This increased atom density for the S-containing peptide ions offers the opportunity to determine whether or not distinctive HDX behavior can be observed that would be consistent with such ion structures. Thus for the S-containing peptide, the residue specific deuterium content was examined in detail; the deuterium content was then compared to that of the A-containing peptide and the results are discussed below. Here it is noted that it was not possible to compare the more elongated conformer of the $[M+3H]^{3+}$ ions as the signal level for the mobility-selected ions was insufficient to obtain ion fragmentation spectra.

Per-residue Deuterium Uptake. Upon introduction of D_2O to the drift tube, the m/z of the $[M+3H]^{3+}$ ions shifted to higher values ($m/z \sim 695.4$) indicating the incorporation of 20.1 deuteriums (19.9 ± 0.9 , on average). Overall there are 48 exchangeable hydrogens (including the three protonation sites). Thus, ~41% of the exchangeable hydrogens are observed to undergo exchange. This is similar to the level of deuterium incorporation observed for the A-containing peptide ⁷⁶. Therefore, it is useful to consider the amount of deuterium incorporation occurring at the neutral S and A residues. Notably, because the ETD approach used here does not differentiate between side-chain and backbone sites, the question of relative accessibility of different site types for the S residues cannot currently be answered. However, a semi-quantitative comparison of residue accessibility can still be achieved based on the per-residue deuterium content.

To determine the S-residue deuterium content for the gas-phase HDX studies, the more compact $[M+3H]^{3+}$ precursor ions were mobility selected and subjected to ETD both before and after introduction of the D_2O reagent gas. Figure 2.2a shows the fragmentation spectrum produced for the unlabeled ions. Two strong features are observed at $m/z \sim 688.7$ and

$m/z \sim 1032.5$ and correspond to the $[M+3H]^{3+}$ precursor ions and the charge-reduced, doubly-charged ions. Overall, a general lack of low- m/z ions is observed in the fragmentation spectrum; dataset features for the z_4 through z_{20} and the c_6 through c_{20} fragment ions are observed. Observed ions used in deuterium content calculations with their associated m/z values are provided in Table S1 in the Supplementary Information section (Appendix A). Because many lower m/z fragment ions are not observed, the deuterium uptake values for individual amino acid residues have been determined using a combination of c and z ions. There are two advantages in using both c and z ions to obtain the uptake values. The first is that nearly complete sequence coverage can be obtained and the second is that a double check of deuterium uptake is provided especially for dataset features of lower intensity.

After undergoing gas-phase HDX, the fragment ions produced by ETD broaden due to an increased number of isotopologues and shift to higher m/z values as shown in Figure 2.2b. In the case of the z ions, by subtracting the z_{i-1} ion from the z_i , the deuterium uptake value for the residue numbered $N - i - 1$ can be determined for which N is the number of total peptide residues (21 in this case). For the c ions, by measuring the difference between the c_{i-1} and the c_i ions, the deuterium content of the residue number i can be calculated. For example, the average m/z for the peak corresponding to z_{15} ions is 1432.68 and the same peak when D_2O is introduced is m/z 1445.40. Thus the deuterium content is calculated to be 12.72. Another example in Figure 2.2 is the c_6 ions. In this case the average m/z is 633.70 and the corresponding average m/z in the presence of D_2O is 638.78. Here the deuterium content is 5.08. Table S1 in the Supplementary Information section (Appendix A) provides the deuterium uptake values for the amino acid residues obtained from the z and c ion fragments.

Figure 2. 2 Ion fragmentation spectra obtained upon ETD of the $[M+3H]^{3+}$ ions of the model peptide Acetyl-PSSSSKSSSSKSSSSKSSSSK.

a) Shows a typical ion fragmentation spectrum for ions that are not exposed to D_2O in the drift tube. b) Shows a typical ion fragmentation spectrum for ions that have been exposed to D_2O in the drift tube. Several c and z ions are labeled with arrows indicating their location in both spectra.

To provide further insight into the determination of deuterium uptake, it is instructive to examine expanded regions of the mass spectra shown in Figure 2.2. Figure S2 in the Supplementary Information section (Appendix A) shows expanded regions for three fragment ions. In general, as the fragment ion mass increases, an increased shift to higher m/z is observed and a wider isotopic distribution is encountered for ions produced after HDX (Figure S2 in the Supplementary Information section (Appendix A)). For fragment ions produced after HDX, the average m/z value is computed by locating the dataset feature edges (typically $\leq 20\%$ peak height) and then computing the weighted average of the isotope peaks between the edges. The average m/z of the fragment ion produced prior to introduction of D_2O is then subtracted from this value to obtain the overall m/z shift resulting from deuterium incorporation.

S- and A-residue deuterium content comparison. To investigate whether the per-residue deuterium content reveals differences in relative residue accessibility between the S- and A-containing peptides, the per-residue deuterium content is here compared. As mentioned above, one comparison that is readily available is that of overall HDX efficiency for these residueus. For the S-containing peptide, on average, the number of deuteriums incorporated for all S residues is ~ 9.4 ($\sim 28.5\%$ of the 33 total available). For the A-containing peptide, a value of $\sim 19.5\%$ efficiency (~ 3.3 of 17 available sites), on average, is obtained. The higher efficiency could be reflective of the more compact nature of the S-containing peptide as more exchange sites are in closer proximity to charge sites and deuterium incorporation sites as required by the reaction mechanism⁷⁵.

For the reactivity comparisons at the per-residue level, a question arises as to how the evaluation should be performed. For example, the S-containing peptide contains 16 more exchangeable hydrogens due to the side-chain site on the S residues when compared with the A-containing peptide ions. Therefore a direct comparison of per-residue deuterium content may not provide the simplest representation of the relative accessibility. Thus the S and A deuterium

content values are scaled according to their fractional values of the total deuterium incorporated into the neutral residues. K residues are not included in this analysis because, considering the HDX mechanism, different charge site configurations could contribute to the observed differences. Here, rather the interest is in comparing the average differences in accessibility to different regions of the peptide through the use of the S and A residues.

Figure 2.3 shows the comparison of the deuterium content of the S and A residues on the respective peptide ions. For the A-containing peptide, only four residues show significantly greater accessibility; these are the A4, A5, A19 and A20 residues. For the S-containing peptide, the residues showing significantly increased accessibility are the S2, S9, S10, S13, S15, and S17 residues. Overall, the results suggest a greater accessibility to middle residues for the S-containing peptide ion. This could result from increased charge solvation by the S residues as discussed below. Notably, the relative per-residue deuterium uptake values shown in Figure 2.3 exhibit different levels of precision (also shown as independent values in Table S1). This appears to primarily result from variability in determining the peak centers of low abundance fragment ions. For example, fragment ions used to determine the deuterium content for S3 and S18 residues are ~40% lower in intensity relative to those used for the S13 and S19 residues. That said, the error levels are in line with those reported previously^{72, 76, 80-81}.

Figure 2. 3 Bar graph showing the fraction of deuterium for S (red bars) and A (blue bars) residues obtained from MS/MS analysis of mobility selected ions.

Fractional values are calculated as the ratio of the deuterium content of the specified residue to the total deuterium content from neutral residues for the respective peptides. Error bars are obtained by propagating the error (one standard deviation) of the individual deuterium uptake values.

Comparison to candidate structures from MD simulations. The HDX behavior of the S-containing peptide was further considered using MD simulations. To find the best structural type (or types) exhibiting similar exchange site accessibility to the gas-phase conformer type, simulated annealing was conducted for $[M + 3H]^{3+}$ peptide ions using two different charge configurations as well as two different simulation times (see Experimental section). As shown in Figure S3 in the Supplementary Information section (Appendix A), the collision cross sections obtained from MD simulations are, for the most part, significantly smaller than the experimental value. An examination of some of these structures, reveals extremely compact ion conformer types with significant charge solvation of the charge sites by the serine residues as shown in Figure S4 (Supplementary Information section (Appendix A)). Indeed, as an indicator of how the MD simulations captured such charge solvation, consider that only 71 of 8000 structures were observed to have collision cross sections within $\pm 2\%$ of the experimental value for the compact $[M+3H]^{3+}$ ions.

To consider which of the structural types with matching collision cross sections best represented the experimental data, HDX kinetics modeling was employed (see Experimental section above). Notably, this work does not include the very extensive MD trajectory modeling that was performed previously for the A-containing peptide^{34-35, 76}. Rather, here the MD simulations are only performed to provide an idea of conformer types that could account for the observed HDX accessibility⁷². From HAS scoring and kinetics modeling of the 71 structures, several conformer types appeared to exhibit significant charge solvation by middle residues. Based on the best ability to match the experimental results (see below), one of these ion structures was selected for discussion. This ion structure is shown in Figure 2.4C.

Figure 2. 4 The comparison of the experimental and theoretical fraction deuterium incorporated values.

Panel A shows the comparison of the experimental fraction deuterium incorporated values (blue bars) and the theoretical fraction deuterium incorporated values (red bars) for the ion conformation shown in panel C of the model peptide Acetyl-PSSSSKSSSSKSSSSKSSSSK. Panel B shows the same comparison for the ion conformation shown in panel D. In panels C and D, several residues are labeled to demonstrate their relative accessibility for HDX (see text for details).

The agreement between experimental and theoretical (HDX kinetics modeling) relative deuterium content is shown as a bar graph in Figure 2.4A. A number of trends are shown to agree between the experimental and theoretical values. Here we note that the theory favors sites that are in relative proximity to backbone carbonyls that are in relative proximity to charge sites. A trend captured by the theory for the first ion conformer is the relatively low levels of uptake for S3 and S4 and an increase in uptake for S5 and S7. This is followed by a slight decrease for S8 and then increases for S9 and S10. The model slightly overestimates the deuterium content for S12 to S14. This is followed by relatively good agreement between S15 and S17. The worst agreement is observed at the C-terminal and N-terminal ends for the S2, S18, and S20 residues. Overall, the average percent difference between experiment and theory is observed to be ~27.5% across all S residues.

Figure 2.4D shows a separate ion conformer for which increased accessibility is observed for the terminal peptide portions relative to middle residues. For example, for this ion conformer there is an increase in estimated deuterium content for the N-terminal residues S3, S4, and S5 with an increase also observed for the C-terminal residues S18, S19, and S20. These increases are offset by decreases in theoretical deuterium content for residues S7 to S14 as well as S17. For this structure, the average percent difference between experiment and theory is ~41.4%.

The differences in the quality of HDX reactivity matching for the ion candidate structures can be described via the HAS scores and the structure images shown in Figure 2.4. For the ion structure providing the best HDX reactivity matching, the charge site assignments are K6, K16, and K21 while the charge site assignments for the second ion structure are K6, K11, and K21. One noticeable difference in the structures is that the former ion conformer with its charge arrangement exhibits less charge density. Indeed, the relative Coulomb energy of the former ion conformer is only 69% that of the latter. Thus a more even dispersion of charge throughout

the ion conformer is observed. For the first ion conformer (Figure 2.4C), the relative increase in theoretical deuterium content would be elevated for the S17 residue due to its hydrogens proximity to the S13 (2.9 Å), S14 (2.1 Å), and S17 (3.6 Å) carbonyls. These carbonyls are highly scored because they are in close proximity to the K21 charge site (all ≤ 1 Å) and the S13 carbonyl is also within 2.5 Å of the K16 charge site. The S15 residue has a large theoretical deuterium uptake as well due to its hydrogens proximity to the K11 (1.9 Å), S12 (3.0 Å), and S14 (3.1 Å) carbonyls. The K11 and S12 carbonyls are scored highly because they are within 3 Å of the K16 and K21 charge sites. In comparison, only one scored carbonyl is positioned at ≤ 4 Å from the S19 hydrogens. The increased theoretical deuterium uptake for the S10 to the S14 sites results from the fact that these residues fall between the K6 and K16 charge sites and there is a significant compaction of this portion of the ion such that exchange sites are in close proximity to a greater number of carbonyl sites. For example, the S10 residue has hydrogens that are within 1.8 Å and 2.9 Å of scored carbonyls on the S4 and S7 residues. Additionally, labile hydrogens are ≤ 4 Å of scored carbonyls on the S8 and S9 residues. These scored carbonyls are in closer proximity to the K6 charge site. Finally, although the S2, S3, and S4 residues are in relative proximity to the K6 charge site, there is a decreased number of close carbonyl sites. For example, for hydrogens on the S2 residue, only the S2 carbonyl is within ≤ 4 Å.

For the ion conformation presenting decreased agreement in theoretical and experimental deuterium content (Figure 2.4), the decrease at S2 results from the placement of this residue further away from charge sites. This is evident from the fact that the only carbonyl sites within 4 Å of any hydrogen on the S2 residue are the N-terminal acetyl group and the P1 residue and both of these carbonyls are not highly scored due to their distance to charge sites. Decreases in middle residues S9 and S10 can be seen to result from the placement of these residues further away from charge sites and scored carbonyl sites. For example, hydrogens on the S10

residue only encounter the S8 carbonyl at a distance that is ≤ 4 Å. Finally, the increased theoretical deuterium content at the C-terminal residues (S18, S19, and S20) results from the fact that these residues are in close proximity to the K6 and K21 charge sites as well as a number of carbonyl sites. For example, the hydrogens on the S19 residue are within 1.5 Å and 2.5 Å of the scored carbonyls on the S18 and S19 residues. These carbonyls are scored highly as they are 0.5 Å and 1.7 Å from the K21 charge site. Additionally, the latter carbonyl is also within 5.7 Å of the K6 and K11 residues. As shown in Figure 2.4, the structure exhibiting the decreased HDX reactivity match appears to position the S4 and S5 residues such that they would not exhibit greater accessibility as determined by the HAS approach. However, upon rotation of the ion structure, it becomes apparent that the exchangeable hydrogens on the S4 and S5 residues are near a number of highly scored carbonyl sites as shown in Figure S5 in the Supplementary Information section (Appendix A). Indeed, the hydrogens on these residues are ≤ 2 Å from the N-terminal acetyl group carbonyl and the P1 carbonyl.

It is instructive to consider the type of ion structure that would be required to result in increased exchange of the S2 and S20 residues as is observed experimentally (Figure 2.4). One consideration is that more compact structures in which the N-terminal and C-terminal ends turn back toward the middle residues would provide increased HAS scores for these residues. Figure S6 in the Supplementary Information section (Appendix A) shows a compact ion structure ($^{\text{He}}\Omega_{\text{DT}} = 363.0 \text{ Å}^2$) for which HAS scoring and HDX kinetics modeling was performed. The relative deuterium incorporation levels for several residues are also shown in Figure S6 (Appendix A). Notably, the value for the S20 residue is the largest of all residues and that of S2 is among the largest values as well. Additionally, the deuterium incorporation values for the neighboring S4 and S18 residues are among the lowest. These results are similar to what is observed experimentally for N- and C-terminal residues (Figure 2.3 and Figure 2.4). The high value for the S2 residue results from the fact that a N-terminal bend positions this residue such

that the S2 backbone hydrogen is in relatively close proximity of the acetyl carbonyl (3.3 Å), the P1 carbonyl (3.1 Å), and the S2 carbonyl (2.1 Å). These carbonyl sites also have relatively high scores as they are located near the K6 charge site. The side-chain hydrogen of S2 is located near (1.7 Å) the S10 carbonyl which is one of the highest scoring carbonyl sites being near multiple charge sites. In contrast, the backbone hydrogen and the side-chain hydrogen of the S4 residue are not within 3 and 4 Å, respectively, of any scored carbonyl. For the S20 residue, hydrogens are in close proximity to the highly scored carbonyls on S19 (3.1 Å) and S8 (2.0 Å). The S18 residue hydrogens are not within 3 Å of any scored carbonyl.

It is necessary to present a note of caution regarding the comparisons between theoretical and experimental deuterium uptake here. Such comparisons are not intended to be definitive as they do not consider ion conformer dynamics or the possible contribution by multiple ion conformers with different charge site configurations. As mentioned above, the failings of the approach are especially obvious in the inability of either ion structure to account for the increased deuterium content of the S2 and S20 residues observed experimentally. The improved matching of HAS scores with experimental values for these residues when using a far too compact ion structure is intriguing. For example, such analysis may suggest that multiple ion conformer types comprise the mobility selection and a portion of these contain bends at either the N-terminal or the C-terminal end with the other end extended (in order to exhibit the correct cross section). That said, the comparisons are useful insofar as they demonstrate conformer types exhibiting specific charge solvation that could account for the observed differences in relative deuterium content of specific peptide regions. Additionally, such comparisons represent the foundational work that will be extended to exhaustive MD simulations that do account for conformer dynamics as well as the presence of different conformer types. In future works, such simulations will be conducted in the gas and solution

phases as was performed for the A-containing peptide ³⁴⁻³⁵ and the results will be compared with regard to the preferred types of structure formed for the different ions.

Conclusions

IMS-HDX-MS/MS measurements coupled with MD simulations have been used to gain insight into the behavior of $[M+3H]^{3+}$ ions of the model peptide Acetyl-PSSSSKSSSSKSSSSKSSSSK. From the IMS measurements a dominant, compact ion conformer is observed. In comparison to the same ions for the model peptide Acetyl-PAAAAKAAAAKAAAAKAAAAK, the S-containing peptide is observed to be relatively more compact. This may be somewhat expected as CD spectroscopy suggests that the former peptide exhibits more random coil nature while the A-containing peptide exhibits significantly more α -helical character. In addition to, and likely attributed to, the increased compactness, the S-containing peptide shows increased accessibility of interior residues to gas-phase HDX. MD simulations suggest that ion conformers demonstrating specific, relatively disperse charge solvation by interior residues could account for the observed HDX behavior of the $[M+3H]^{3+}$ ions.

The disparity in hypothetical and experimental deuterium uptake suggests the need for highly extensive MD simulations to better understand the contributions of ion structure dynamics as well as contributions from multiple ion conformers. This limitation was also encountered for the A-containing peptide where exhaustive gas-phase and ESI droplet simulations have been conducted to better fit the experimental data ³⁴⁻³⁵. Thus, in order to gain insight into the ion structures resulting from very different solution structures, it will be necessary, in future studies, to conduct these same exhaustive analyses where a convergence between gas-phase ion structures and those emerging from ESI droplets is obtained. With these structures it will be possible to compare the major differences in structures arising from the S- and A-containing peptides. The comparison of such predominant structures could provide information about the

degree of preservation of secondary structure for these smaller biomolecules and could thus help with our understanding of the process of gas-phase ion structure establishment.

Acknowledgments

We are grateful for financial support from the National Institutes of Health (R01GM114494).

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3. Investigating the interactions of the first 17 amino acid residues of Huntingtin with lipid vesicles using mass spectrometry and molecular dynamics

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Introduction

The majority of cell membrane volume in biological systems is occupied by lipids and proteins. The interactions between such components within complex systems have been studied for years¹⁻⁵. Protein/peptide interaction with the membrane at its surface is an important topic in many fields of study including drug delivery, cell-cell recognition, signal transduction, as well as the study of disease processes⁶⁻¹⁶.

Peptides that interact with the plasma membrane through processes of traversal, fusion, or residing directly at the membrane surface interface are termed membrane active¹⁷. It has been suggested that the manner in which the peptide locates within the membrane is highly dependent on the membrane lipid composition¹⁸. Broadly categorized, the interactions have been described as the “barrel-stave” and “carpet” mechanisms¹⁷. The former process involves the insertion of the peptide into the membrane core while the latter involves binding at the surface primarily through interactions with the polar head groups.

Recently a special type of peptide-membrane interaction has come under increased examination. This involves peptides that form amphipathic helices. In general amphipathic helices are polypeptide regions that form a helix upon interacting with hydrophilic/hydrophobic interfaces.¹⁹ A characteristic of such protein regions is the regular incorporation of non-polar amino acid residues at every third or fourth position. This allows the helix to form in which the

non-polar face interacts with the lipid constituents of the membrane while the polar face is positioned in the aqueous environment. This anchors the protein to the membrane and orients the helical region parallel to the surface¹⁹.

Huntington's disease (HD) is one of several neurological conditions characterized by Huntingtin protein (htt) fibril formation in brain tissue. Fibril formation is caused by a polyglutamine (polyQ) expansion occurring in the N-terminal region of the htt²⁰. Studies have shown that the first 17 amino acid residues (Nt17) as well as a downstream proline-rich region of htt play important roles in protein stability as well as sub-cellular localization²¹⁻²⁵. The Nt17 peptide is suggested to play an important role in fibril formation²⁶ in which the self-association of the peptide favorably positions the expanded polyQ tracts for aggregation. The subcellular location of htt, necessitates its interaction with the lipid membrane bilayer and is mediated by posttranslational modifications of Nt17 residues²⁷⁻²⁹. Additionally, the interaction of Nt17 with the lipid membrane bilayer has been shown to be an important factor in protein aggregation in HD^{14, 30-32} where Nt17 can participate in lipid-binding as well as protein-protein interactions.

The location of hydrophobic residues (L4, L7, A10/F11, L14, and F17) within the primary sequence of the Nt17 peptide suggests a propensity to form an amphipathic helix at the lipid membrane surface. This is supported by recent solid-state NMR studies which suggest that the peptide lies horizontally along the membrane surface³³. A recent study by Mousseau *et al.* examined effects of the Nt17 peptide on a phospholipid bilayer³⁴. The per-residue helix propensity in the presence of the POPC lipid bilayer was compared with the NMR structure. Overall general agreement was achieved for the two models; however, the MD study suggested that residue numbers 1–4 are more ordered because they are less accessible to the solvent.

Here we present studies of the interaction of Nt17 with the different lipid bilayer systems [1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphoethanolamine (POPE)]

using native mass spectrometry (MS) ³⁵ and molecular dynamics (MD) simulations. MS reveals that Nt17 has a different propensity for interaction with vesicles of different lipid compositions. The data suggest that the level of peptide interaction is DOPC > POPC > POPE. Modeling these peptide-lipid systems using MD simulations also supports this interpretation of the MS data and helps to provide a molecular view of the tendency of the peptide to form non-specific interactions with lipids.

In general, the combined study presented here is representative of a growing body of work that utilizes native MS with a goal of elucidating protein interactions with lipid membranes³⁶⁻⁴⁰ as well as the effect of such interactions upon biomolecular structure ⁴¹. Additionally, the work becomes part of a larger body of studies aimed at determining the mechanisms of aggregation for pathologically-relevant proteins. ^{26, 42-52 53}

Experimental

Reagents. The Nt17 peptide (>90% purity) was synthesized by GenScript (Piscataway, NJ, USA) and used without further purification. The peptide sequence is: MATLEKLMKAFESLKSF. The peptide was dissolved in ACS/USP absolute ethanol (Pharmco, Brookfield, CT) at a concentration of 1 mg/mL to make stock solution. The powder lipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphoethanolamine (POPE)) were obtained from Avanti Polar Lipids (Alabaster, AL) and dissolved in HPLC grade chloroform (Fisher Scientific, Fair Lawn, NJ) to yield a 10 mg/mL stock solution for each lipid.

Vesicle Capture-Freeze-Drying (VCFD). Details about sample preparation using the vesicle production method are provided elsewhere ⁴¹. Briefly, 100 μ L of the stock solution (~1200 μ M) for each lipid were dried using a speedvac (Thermo Scientific Speedvac)

maintained under nitrogen gas flow. The dried samples were rehydrated in 1 ml of HPLC grade water with 10 mM ammonium acetate buffer and sonicated for 30 minutes. Then, the samples were subjected to 10 freeze-thaw cycles using liquid nitrogen and a $\sim 50^{\circ}\text{C}$ water bath. Subsequently, 100 μL of the stock solution of the Nt17 peptide ($\sim 100\ \mu\text{M}$) was added to each lipid solution. For all samples, lipids were maintained at a final concentration that was ~ 10 times greater than the Nt17 peptide. The peptide/lipid complex solutions were stored over night at 37°C for equilibration. Notably, a limitation of this study is that the physiological concentration of Nt17-containing protein is largely unknown although it has been estimated to be $0.15\ \mu\text{M}$ in the cytosol of neural cells⁵⁴. Experiments here were designed with higher peptide concentrations ($10\ \mu\text{M}$) to enhance the level of peptide-membrane interactions as well as to abbreviate the timescale of such interactions as well as peptide-peptide interactions. Notably this approach is used to study htt aggregation using THT⁵⁵ and PDA assays⁵⁶ and to perform CD spectroscopy¹⁴ and AFM⁵⁷ and MS³⁸ measurements.

Electrospray-Mass Spectrometry (ESI-MS). All of the experiments were carried out using a Q Exactive Hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA). The experiments were conducted using the commercial HESI source available with the MS instrument. For both ionization sources MS spectra were recorded in positive ion mode over a mass-to-charge (m/z) range of 500 to 4000. The samples were infused at a rate of $10\ \mu\text{L}/\text{min}$ using a heated syringe and infusion line to ensure that the POPE vesicles did not undergo a phase transition. The needle was biased at 3,500 V relative to the instrument inlet. The instrument parameters used for ESI were: 450°C for the capillary inlet temperature, 80 V for the S-lens assembly, 400 ms for the maximum injecting time, 1×10^6 for the AGC, and 70,000 for the MS resolution. Each spectrum was recorded in triplicate for 180 seconds each to ensure data collection reproducibility. To analyze the MS data, the Xcalibur 2.2 software suite (Thermo Scientific) was used.

A limited number of experiments were performed on a linear ion trap mass spectrometer (LTQ, Thermo Fisher, San Jose, CA) to examine product ions upon isolation of lipidated species (see below). For these experiments, the commercial HESI source was utilized and mass spectra were recorded in positive ion mode using a m/z range of 900 to 2000 m/z). The same flow rate and needle bias parameters were used for these experiments. Precursor ions were isolated using a 10 m/z window and no collisional activation was employed.

Molecular Dynamics (MD) Simulations. For MD studies, two types of models were used. The first involved initiation with a linear structure to represent the initial interaction between the peptide and the membrane surface. Because full helix formation at the surface of the membrane appeared to be an unreachable event because of the long timescale, MD simulations were also performed in which the peptide was forced to be helical and placed directly above the membrane. Notably the NMR structure suggests that it is helical at the surface³³. For the latter simulations, the goal was to determine the degree of amphipathic helix interactions with the different lipid membranes.

For the initial random coil simulation of the Nt17 peptide, the fully linear structure was built in Avogadro⁵⁸ and subjected to 10 ns of simulation in pure water. The output was used as an input file for the Nt17-lipid bilayer simulation. For the helix model, the Nt17 peptide pdb structure (2LD2) was obtained from the protein data bank website³³ and used as the initial input file for the MD simulations. The CHARMM-GUI server (www.charmm-gui.org) was used to create the lipid bilayer system⁵⁹. The peptide was placed above the bilayer in the z axis and solvated (explicit model) with water. All the simulations were performed in triplicate. The M1 residue at the N-terminus as well as the lysine residues (K6, K9, K15) were protonated. The carboxylic acid of F17 at the C-terminus and the two glutamic acid residues (E5, E12) were deprotonated. This provided a net charge of +1 for the modeled peptides. The nano-scale molecular dynamics (NAMD 2.13) software⁶⁰ was utilized to perform minimization and

equilibration of the system based on the CHARMM-GUI protocol and CHARMM c36 force field for lipids and proteins ⁶¹. The protocol consists of 1950 ps minimization and equilibration process in which restraints from the peptide backbone, lipids, water and ion molecules are gradually removed in six steps ⁶². Parameters for all-atom MD simulations using the periodic boundary condition were: NPT ensemble, 2 fs for the timestep, 310 K for the temperature and 12 Å for the cutoff. The visual MD (VMD) suite was used to visualize and analyze the simulation data. The GridMAT-MD script was used to calculate the theoretical area per lipid ⁶³.

Results and Discussion

Peptide-lipid interactions examined with native MS. Studies have shown that the presence of the Nt17 flanking sequence in the htt protein is important for membrane interactions ^{14, 21, 30}. This association has been suggested to serve to anchor huntingtin protein (htt) to the membrane ⁶⁴. To investigate the peptide interactions with DOPC lipid, mass spectra were recorded for the sample in which the peptide was incubated with the DOPC vesicles. For these studies, experiments were conducted in duplicate.

Figure 3.1 shows a typical mass spectrum for the DOPC-peptide system. ESI produces ions of several different charge state for the Nt17-lipid system. In the mass spectrum, the doubly-charged monomer peptide ions ($[M+2H]^{2+}$ at $m/z \sim 988$) predominate. Dimer, trimer and tetramer ions for the Nt17 peptide are also observed at $m/z \sim 1317$, 1482, 1580, respectively. These species are of significantly lower abundance (Figure 3.1).

Figure 3. 1 Full range mass spectrum of the peptide-DOPC system.

For labeling purposes, a large red circle represents a single Nt17 peptide, a green triangle represents a single DOPC lipid molecule. Insets show expanded regions for a number of peptide-lipid complex ions. Ion identities are shown as symbol representations. Reproduced with permission from the Journal of Mass Spectrometry: Investigating the interactions of the first 17 amino acid residues of Huntingtin with lipid vesicles using mass spectrometry and molecular dynamics. Kiani Karanji, A., Beasley, M., Sharif, D., Ranjbaran A., Legleiter, J. and Valentine, S. J. Journal of Mass Spectrometry 55 (1), e4470. Copyright © 2019 John Wiley & Sons, Ltd.

In addition to the ions described above, ions comprised of peptide-lipid complexes are also observed. These range in size from peptide monomer to tetramer ions. A complete list of the observed protein-lipid complex ions is presented in Table 3.1. Notably, monomer peptide ions with differing numbers of lipid adducts are observed. These range in size from 1 to 7 attached lipids. This relatively high number of adducts could, to some degree, indicate the propensity of the lipid to interact with this peptide (see discussion below). In addition to these ions, peptide-lipid complexes which include one lipid attached to dimer and trimer species are observed at m/z ~1578 and 1678, respectively, Figure 3.1). Table 3.1 shows the multimeric peptide-lipid ions that have been observed in these studies. In general, the number of separate ions as well as the number of attached lipids decreases with higher order peptide multimer. For example, >1 lipid adduct is observed for all the peptide multimers except for the tetramer where only 1 lipid is attached (Table 3.1).

Ion ^a	m/z ^b	Charge state	Ion ^a	m/z ^b	Charge state	Ion ^a	m/z ^b	Charge state
Nt17 + 1DOPC	2760.64	1	Nt17 + 1POPC	2734.63	1	Nt17 + 1POPE	2692.59	1
Nt17 + 1DOPC	1380.82	2	Nt17 + 1POPC	1367.81	2	Nt17 + 1POPE	1346.79	2
1Nt17 + 2DOPC	3546.24	1	Nt17 + 2POPC	1747.61	2	Nt17 + 2POPE	1705.57	2
Nt17 + 2DOPC	1773.62	2	Nt17 + 3POPC	2127.40	2	Nt17 + 3POPE	2064.34	2
Nt17 + 3DOPC	2166.42	2	Nt17 + 4POPC	2507.2	2	Nt17 + 4POPE	2423.12	2
Nt17 + 4DOPC	2559.22	2	Nt17 + 5POPC	2886.99	2	Nt17 + 5POPE	2781.89	2
Nt17 + 5DOPC	2952.02	2	Nt17 + 6POPC	3266.79	2	2Nt17 + 1POPE	2333.81	2
Nt17 + 6DOPC	3344.82	2	2Nt17 + 1POPC	2354.83	2	2Nt17 + 1POPE	1556.21	3
Nt17 + 7DOPC	3737.62	2	2Nt17 + 1POPC	1570.22	3	2Nt17 + 2POPE	1795.39	3
2Nt17 + 1DOPC	2367.84	2	2Nt17 + 2POPC	1823.42	3	2Nt17 + 3POPE	2034.57	3
2Nt17 + 1DOPC	1578.89	3	2Nt17 + 3POPC	2076.61	3			
2Nt17 + 2DOPC	2760.84	2	2Nt17 + 4POPC	2329.81	3			
2Nt17 + 2DOPC	1840.76	3	2Nt17 + 5POPC	2583.01	3			
2Nt17 + 3DOPC	3153.44	2	3Nt17 + 1POPC	2228.23	3			
2Nt17 + 3DOPC	2102.62	3	3Nt17 + 2POPC	2481.43	3			
2Nt17 + 4DOPC	3546.24	2	3Nt17 + 1POPC	1671.42	4			
2Nt17 + 4DOPC	2364.49	3	3Nt17 + 2POPC	1861.32	4			
2Nt17 + 5DOPC	3939.04	2	4Nt17 + 1POPC	2164.93	4			
2Nt17 + 5DOPC	2626.36	3						
2Nt17 + 5DOPC	1970.02	4						
2Nt17 + 6DOPC	2888.22	3						
2Nt17 + 7DOPC	3150.09	3						
2Nt17 + 8DOPC	3411.96	3						
3Nt17 + 1DOPC	3354.86	2						
3Nt17 + 1DOPC	2236.90	3						
3Nt17 + 1DOPC	1677.93	4						
3Nt17 + 2DOPC	2498.77	3						
3Nt17 + 2DOPC	1874.33	4						
3Nt17 + 3DOPC	2070.73	4						
3Nt17 + 4DOPC	3022.50	3						
3Nt17 + 4DOPC	2267.13	4						
3Nt17 + 5DOPC	3284.37	3						
3Nt17 + 5DOPC	2463.53	4						
3Nt17 + 6DOPC	2659.93	4						
3Nt17 + 7DOPC	2856.33	4						
3Nt17 + 8DOPC	3052.73	4						
4Nt17 + 1DOPC	2894.92	3						
4Nt17 + 1DOPC	2171.44	4						

^a The following average masses has been used to assign the peaks: 1974.04 for Nt17 peptide, 785.60 for DOPC lipid and 717.54 for POPE lipid.

Table 3. 1 Assigned compounds and their corresponding mass to charge ratio

Lipid preference of Nt17. The affinity of amphipathic α -helices for lipid bilayers has been shown to be dependent on the physicochemical characteristics of the membrane ⁶⁵⁻⁶⁷. One characteristic showing importance is the packing defects of the membrane. Beyer *et al.* have suggested that a small radius of curvature is essential for increasing peptide-membrane interaction ⁶⁸. In this study it was suggested that peptide binding to a membrane depends on the presence of defects in the membrane structure at the membrane-water interface. These short-lived defects create temporary spacing between the lipid head groups which allows greater interaction between the lipid and the peptide. To investigate this phenomenon further by MS, experiments were also conducted using DOPC, POPC, and POPE lipid systems. All are zwitterionic lipids; however, the DOPC lipid contains a much bulkier head group which is expected to increase the overall area per lipid ⁶⁹. Additionally, DOPC and POPE systems have different lipid tails, that can impact their fluidity at given temperatures. DOPC, POPC, and POPE have respective phase transition temperatures of -17, -2 and 25 °C. Thus the DOPC bilayer system is more fluid under experimental conditions (~37 °C) which can more readily create short-lived defects in the exposed membrane leaflet.

By comparing the mass spectral results from the Nt17-DOPC (Figure 3.1), and Nt-17-POPC and Nt17-POPE (Figure S1 in the Supporting Information section (Appendix B)) systems, it may be possible to obtain some information about differences in types of interaction between the peptide and the respective lipid vesicles. The assigned peaks for the peptide-PC and peptide-PE complex ions are listed in Table 1. From Table 3.1, it is readily apparent that the most lipid adducts are observed in the DOPC system. For example, 1 Nt17 peptide is observed with up to 7 attached DOPC lipids. Conversely, in the POPE system, only up to 5 lipid adducts are observed for the monomer peptide. In comparison, this number is 6 for the POPC system.

The greater propensity of the peptide to bind with DOPC extends to the higher-order oligomers of Nt17. For the DOPC system up to 8 lipid adducts are observed with peptide

dimers while this limit is 5 for POPC and 3 for POPE systems. There was no mass spectral feature corresponding to the peptide trimer and tetramer containing POPE lipid adduct but the peak for tetramer with one lipid adduct can be seen at $m/z \sim 2171.44$ for the DOPC system. Overall, 38 lipid adduct species are observed for the DOPC vesicle system while only 10 related peaks are observed for the POPE vesicle system suggesting the possibility of a different interaction type for the former lipid vesicles. Notably, the phenomenon of increased lipid adducts in the DOPC system is not a result of the relative number of peptide monomers or multimers believed to form in solution as these are observed in similar abundances for all peptide-lipid systems. Together these results suggest that differences in interaction between the Nt17 peptide individual lipid vesicles may be responsible for differences in the mass spectra.

Co-mixing Nt17 peptide and lipids without vesicle formation. To investigate the role of the ESI process on the numbers of Nt17 peptide ions with lipid adducts, experiments were performed in which the peptide and lipid were co-mixed and, after a similar incubation time, electrosprayed into the mass spectrometer. More specifically, the Nt17 peptide and lipids were mixed without any preparation (without sonication and freeze drying) for 5 hours in 10 mM ammonium acetate buffer. The assigned mass spectral peaks for these datasets are shown in bold in Table 1. Compared to the experiments in which lipid vesicles were generated before adding the peptide, in all cases, co-mixing peptide and lipid showed a significantly decreased number of assigned species. Only 4 ions ($[\text{Nt17}+1\text{DOPC}+\text{H}]^+$, $[\text{Nt17}+1\text{DOPC}+2\text{H}]^{2+}$, $[\text{Nt17}+2\text{DOPC}+2\text{H}]^{2+}$, and $[\text{Nt17}+3\text{DOPC}+2\text{H}]^{2+}$ at m/z 2760.64, 1380.82, 1773.62, and 2166.42 relatively) were assigned for the Nt17-DOPC system Table 3.1. For the Nt17-POPC and for the Nt17-POPE systems, only 6 and 3 species were observed, respectively. This indicates that vesicle formation is an important step to detect elevated peptide-lipid interactions. It also supports the premise that the mass spectral peak diversity is an indication of solution interaction as opposed to only processes occurring in the ESI droplets.

As a final consideration for co-mixing experiments, it is noted that only 2 of 13 assigned peaks are for Nt17 dimers with lipid adducts. This could suggest that Nt17 requires a membrane interface to form increased numbers of oligomeric species and this is revealed in the MS data. This would be consistent with the concept of mode of action for amphipathic helices as well as proposed mechanisms of oligomer formation for *htt*³³. That said, caution must be used in interpreting the mass spectral data as the dataset features could arise in another manner. For example, an increased degree of interaction with the lipid membrane could translate into a large population of adsorbed monomer. During the ESI process, this increased likelihood of being located at the membrane surface could result in an increase in peptide numbers within ESI progeny droplets resulting in multimeric ions. Therefore, no conclusions can be drawn with regard to the degree of membrane-induced oligomerization. To disentangle the routes of formation for such a heterogeneous mixture of oligomeric species is the subject of future, more extensive experiments employing a greater number of lipid systems as well as gas-phase separations and reactivity studies^{26, 70-73}. This process will also be examined by MD simulations where multiple peptides are examined with the membrane systems. This preliminary study does, however, show that vesicle formation is required to produce a greater number of oligomer ions with lipids attached in the mass spectrum.

Consideration of peptide-lipid ion intensities. It is instructive to consider the ion intensities with regard to the degree of interaction between peptides and lipid vesicles. Figure S2 in the Supporting Information section (Appendix B) shows the relative intensities of the various ions observed for the three separate lipid systems. Notably, the ions with PE adducts are of higher intensity especially for those species containing 1 lipid adduct. The question arises as to the origin of this higher intensity in light of the observation that a greater number of diverse lipidated species is observed for DOPC and POPC systems. In other words, is this value reflective of greater interaction overall (independent of type) at the membrane surface? Here, a

note of caution is raised based on a few observations. First, separate experiments have been performed on a LTQ mass spectrometer in which the $[\text{Nt17+L+2H}]^{2+}$ (where L is DOPC or POPE) ions were isolated in the linear ion trap. Immediately upon isolation, the DOPC-containing ions are observed to dissociate to some degree to yield $[\text{Nt17+H}]^+$ ions. This did not occur for the POPE-containing ions as shown in Figure S3 in the Supporting Information section (Appendix B). Based on this observation, it is possible that many ions with differently-oriented lipid adducts are very fragile and do not survive to the point of mass detection. Additionally, this could extend to the oligomeric species where a greater number of unique ions is observed for DOPC (Table 3.1). Due to such differences, it may not be possible to be fully determine whether or not ion intensity affords a clear indication of the degree of surface interaction. The results are presented here as being of interest and worthy of further MS and molecular modeling studies.

Molecular dynamics (MD) simulations: random coil interaction. To consider initial interactions between the Nt17 peptide and the lipid membrane, MD simulations were performed in which the initial peptide structure was random coil (see Experimental Section). To ensure that the proper parameters had been utilized for the simulations, the area per lipid was determined as a function of MD trajectory time. These results are shown for the DOPC, POPC, and POPE lipid systems in Figure 3.2. The average area per lipid values for the respective lipid systems are 68.6, 65.1, 57.3 Å² (Figure 3.2A and Supplementary Table 1 (Appendix B)) This is consistent with the ordering and magnitudes of the reported areas per lipid and transition temperatures for DOPC ⁷⁴, POPC⁷⁵, and POPE ⁶⁹.

Figure 3. 2 Area per lipid calculations of the 100-member lipid bilayer for the DOPC, POPC and POPE and relative helical propensity for Nt17 peptide in the presence of these lipid vesicles.

A) Theoretical area per lipid calculations of the 100-member lipid bilayer for the DOPC, POPC and POPE. The linear relationship shows that the average area per lipid number for each DOPC, POPC, and POPE are 68.59, 65.12, 57.26 respectively. B) the relative degree of peptide helicity as a function of simulation time for all three lipid systems. Here the initial structure for the Nt17 peptide was completely random coil. Reproduced with permission from the Journal of Mass Spectrometry: Investigating the interactions of the first 17 amino acid residues of Huntingtin with lipid vesicles using mass spectrometry and molecular dynamics. Kiani Karanji, A., Beasley, M., Sharif, D., Ranjbaran A., Legleiter, J. and Valentine, S. J. Journal of Mass Spectrometry 55 (1), e4470. Copyright © 2019 John Wiley & Sons, Ltd.

One measurement that provides some insight into interaction differences with the random coil peptide is the degree of helicity within the peptide as a function of MD trajectory time. Figure 3.2B shows the results for the three different lipid systems. Of note is the increased degree of helicity achieved for the peptide in the DOPC system simulation. There are two major times in which extensive helix formation is observed occurring at ~100 and ~470 ns. It is instructive to observe how helix formation proceeds near these time points. Figure 3.3 shows this for the first time point region. In both instances, the Nt17 peptide is observed to interact with the membrane via the first 2 to 3 amino acid residues. Over the next few ns in the simulation, a single turn to two helical turns is observed. This rapidly propagates to extensive helical formation (~50% in both cases) with concomitant detachment from the membrane surface. Notably, the degree of helix formation for the POPC and POPE lipid systems does not occur to the degree observed in the two instances for DOPC.

Figure 3. 3 Schematic representations of random coil Nt17 peptide interacting with the DOPC bilayer.

A) The Nt17 peptide in solution having no contact with the bilayer. B) The peptide interacting with the bilayer surface after 93 ns simulation time. C) DOPC bilayer inducing some peptide helical formation. D) The Nt17 peptide leaves the bilayer surface with ~65% helicity at a time of 120 ns. The peptides and DOPC lipid bilayer are shown with the “new cartoon” and “surf” drawing models, respectively. Reproduced with permission from the Journal of Mass Spectrometry: Investigating the interactions of the first 17 amino acid residues of Huntingtin with lipid vesicles using mass spectrometry and molecular dynamics. Kiani Karanji, A., Beasley, M., Sharif, D., Ranjbaran A., Legleiter, J. and Valentine, S. J. Journal of Mass Spectrometry 55 (1), e4470. Copyright © 2019 John Wiley & Sons, Ltd.

Molecular dynamics (MD) simulations: helix-membrane compatibility. Although the simulations described above present an intriguing possibility regarding differences in helix initiation roles by different membrane types, it is possible that they do not fully represent the interactions expected for a system in which the peptide has been incubated extensively in the presence of the lipid vesicles. Because experimental evidence suggests that, at the membrane surface, the peptide is helical, MD simulations have also been performed in which the fully helical peptide is placed at the membrane surface. This was performed as the computational time required to observe full helix formation while at the membrane surface may be prohibitively long according to the random coil simulations.

For the helix-membrane studies, the peptide was placed equidistant (29 Å – center of the bilayer to peptide center of mass) above the three different lipid bilayer head groups. Figure 3.4A shows a heat map representing the distance between each residue in the peptide and the center of the lipid bilayer across the simulation time. For the peptide-DOPC system, the MD simulations suggest that the N-terminus section of the peptide helps to maintain the entire peptide at the surface of the bilayer. This occurs during the first 50 nanoseconds of the simulation. The process initiates with the N-terminal residues over this relatively short timescale. Specifically, the first three amino acid residues (M1, A2, and T3), are the first to locate closer to the lipid membrane (Figure 3.4A).

Figure 3. 4 Heat maps showing the distance between α -carbons of each residue on the Nt17 peptide to the center of the bilayer as a function of MD simulation time.

Panels A, B and B show the results for the DOPC, POPC and the POPE bilayer systems, respectively. The legend in the figure shows the distance ($\text{\AA}$) represented by each color. Reproduced with permission from the Journal of Mass Spectrometry: Investigating the interactions of the first 17 amino acid residues of Huntingtin with lipid vesicles using mass spectrometry and molecular dynamics. Kiani Karanji, A., Beasley, M., Sharif, D., Ranjbaran A., Legleiter, J. and Valentine, S. J. Journal of Mass Spectrometry 55 (1), e4470. Copyright © 2019 John Wiley & Sons, Ltd.

This is followed by positioning successive residues (moving toward the C-terminus) within increased proximity of the lipid membrane. Notably, the first three residues are not associated with the helical character of the peptide obtained from the protein databank. Figure 3.4A also provides information regarding the relative orientation of the peptide with respect to the DOPC lipid membrane. After 50 nanoseconds, distinct bands are observed in the heat map. This reveals increased proximity for specific regions of the peptide. For example, residues 1 through 4, 7 and 8, 10 and 11, and 14 and 15 are in much closer proximity to the lipid membrane. This result is consistent with the suggested structure and topology of the Nt17 peptide interacting with a lipid membrane from solid-state NMR (ssNMR) experiments ³³.

The MD simulations for the POPC lipid system (Figure 3.4B) show different behavior compared with the DOPC results. One similarity is that initial interaction at the N-terminus is observed for residues 1 and 2 after 50 ns. This, however, is shorter lived for the POPC system as it disappears after 50 ns. By 150 ns this initial interaction is replaced by primary interaction at residues 7 and 8 which persists for the remainder of the simulation. In a sense, this interaction anchors, to some degree, the peptide and allows other interactions to eventually occur. For example, the interaction for residue 1 and 2 is re-established at ~220 ns. Subsequent interactions for residues 3 and 4, 11, and 14 and 15 are established by ~330, 460,

and 490 ns, respectively. The end result is the similar observance of bands associated with hydrophobic residues showing increased interaction; however, this association occurs at a much longer time point than that for the DOPC system.

The MD simulations for the POPE lipid membrane reveal a markedly different behavior regarding peptide-lipid interaction as shown in Figure 3.4C. As noted by the heat map, very little of the peptide is ever located within 20 Å of the lipid membrane. As with the DOPC model, the first three residues are located closest to the lipid membrane across the simulation time frame. Indeed, periodically, the M1 residue is the only residue that locates within 20 Å of the lipid membrane. Similar to the DOPC system, specific bands in the heat map (Figure 3.4C) reveal peptide residues exhibiting relatively increased proximity to the POPE lipid membrane; residues 1 and 2, 5 and 6, 9 and 10, 12 and 13, and 16 and 17 are clearly located in increased proximity to the membrane across the simulation timescale. The clear differences between the modeled membrane systems are: 1) the residue numbers that are in the closest proximity; and, 2) the relative degree of proximity (observed to be much greater for the DOPC modeling).

Molecular Dynamics (MD) Simulations: peptide orientation. It is instructive to consider the origin of the bands presented in the heat maps in Figure 3.4. Notably, for all systems, the positioning of residues in closer proximity to the lipid membrane is ~3 to 4 residues apart. This distance in peptide primary sequence suggests a connection to helical structure. Additionally, the fact that different residues interact with the different lipid membranes suggests membrane-guided lipid orienting.

Because the Nt17 region is known to form an amphipathic helix ⁷⁶⁻⁷⁷, it is instructive to examine the characteristics of the amino acid residues exhibiting greater lipid interaction. Figure 3.5A represents the nature of the peptide-lipid interaction for the DOPC system. Notably, because the same residues are involved for the POPC system (Figure 3.4), only the DOPC system is discussed here. As indicated by the heat map (Figure 3.4A), the peptide is noticeably

close to the center of the bilayer. This can be observed pictorially in Figure 3.5A where the Nt17 peptide is located exactly at the membrane surface. At this moment in the simulation, the A2, L4, L7, F11 and L14 residues exhibit the greatest interaction with DOPC lipid molecules which matches with results from the previously performed ssNMR study ³³. Indeed, the general interaction is between these hydrophobic residues and the lipid tails (Figure 3.5C). These interactions necessarily cause the more hydrophilic residues of the amphipathic helix to extend into the aqueous environment. This result is in very good agreement with previous simulations using this lipid system ³⁴.

Figure 3.5B shows that the Nt17 peptide interacts more distantly with the POPE membrane surface. Figure 3.5C presents the interaction type between the peptide and one POPE lipid. This shows a simulation frame in which the peptide is located near its closest overall proximity to the lipid bilayer. Notably, the protonated K9 and K15 residues are shown to interact with the zwitterionic head groups. Indeed, the residues that exhibit the closest proximity to the lipid membrane across the dynamics run (Figure 3.4C) are the hydrophilic K6, K9, S13, and S16 residues. It is noted that the S16 residue resides next to the protonated K15 residue. Overall, the peptide-lipid interactions are dominated by hydrophilic residues for the POPE system.

Figure 3. 5 Molecular representations of the position/orientation of the Nt17 peptide on the surface of the DOPC lipid bilayer, and the POPE lipid bilayer.

(A) the DOPC lipid bilayer, and (B) the POPE lipid bilayer. The peptides are shown with the ribbon rendering of the polypeptide backbone. Hydrophobic residues interacting with the DOPC lipids are rendered with the “licorice” drawing method and hydrophilic residues are represented by the “CPK” model. The lipid bilayer is depicted as an electron cloud rendering and the lipid head groups are emphasized. Panels A and B show results for the MD simulations for the DOPC and POPE lipid systems, respectively. Note that the DOPC system requires visualization

from a top-down perspective because a side-on visualization does not reveal the peptide which is essentially interspersed within the lipid head groups. Schematic representations of the Nt17 peptide interacting with lipid molecules of the DOPC and POPE lipid systems are provided in panels C and D, respectively. The peptides are shown with the ribbon rendering of the polypeptide backbone. Hydrophobic residues interacting with the DOPC lipids are rendered with the “licorice” drawing method and hydrophilic residues are represented by the “CPK” model. Interacting residues are labeled. A distance measurement is used to represent a hydrogen bond in the POPE system. Reproduced with permission from the Journal of Mass Spectrometry: Investigating the interactions of the first 17 amino acid residues of Huntingtin with lipid vesicles using mass spectrometry and molecular dynamics. Kiani Karanji, A., Beasley, M., Sharif, D., Ranjbaran A., Legleiter, J. and Valentine, S. J. Journal of Mass Spectrometry 55 (1), e4470. Copyright © 2019 John Wiley & Sons, Ltd.

A question that arises is what is the net effect of the interactions described above on overall peptide orientation at the lipid membrane? Figure 3.5 helps to illustrate the net effect of these interactions. Overall, for the DOPC lipid system, the insertion of the hydrophobic residues to some degree anchors the peptide right at the lipid surface (i.e., among the head groups) as shown in Figure 3.5A. Therefore, there is greater disruption of the lipid bilayer at this location. Conversely, for the POPE system, the peptide is maintained at a distance from the lipid bilayer surface due to hydrogen bond interactions between charged residues and the head group constituents (Figure 3.5B). Therefore, the anchoring of the peptide occurs at several Å distance from the bilayer surface and the membrane integrity is largely preserved. It is important to note that the relative peptide orientation derived from the MD simulations (Figures 3.4 and 3.5) strongly supports the mass spectral data (Figure 3.1 and Table 3.1) discussed above. That is, the increased interaction with a greater number of lipid molecules for the DOPC system would suggest the observance of a greater number of lipid adducts ³⁶ as is observed in the mass spectra. Furthermore, the greater difficulty to establish the strongly adsorbed peptide for the

POPC system (Figure 3.4B) could indicate the intermediate number of lipid adducts associated with this peptide-lipid system.

MD simulations: potential origin of interaction differences. Because the DOPC and the POPE lipids both contain zwitterionic head groups, it is instructive to consider differences that could account for the different predominant interactions observed with the MD simulations. One possibility is that the higher order of the POPE lipid bilayer disallows the interactions by the hydrophobic residues as are observed for the DOPC system. Here it is proposed that the POPE lipids can make strong intermolecular H-bonds between the phosphate group on one lipid and the carbonyl oxygen atom of an adjacent lipid resulting in a higher ordering of the POPE bilayer. In contrast, the terminal methyl groups of the DOPC system disrupt such hydrogen bonding networks ⁷⁸ and, therefore, the head group spacing increases. As mentioned above, studies have hypothesized that the interaction between amphipathic α -helices and membranes are influenced by short-lived defects created by membrane dynamics ^{67-68, 79}. Thus, when a membrane is more fluid there will be more defects, and the peptide will have more opportunity to explore those defects leading to increased interactions with the lipid tails. Additionally, greater spacing between the headgroups resulting from membrane curvature may also lead to a higher propensity of peptide binding. ³⁰. Based on these hypotheses, it is instructive to compare the results with the average area per lipid calculations (Figure 3.2A). This analysis shows increasing numbers of lipidated species with increasing area per lipid. This supports the idea that the higher area per lipid value for the DOPC bilayer system could also provide more short-lived defects for the peptide interaction. The greater disorder (spacing) as well as the more fluid characteristics of the DOPC system would thus allow for increased interaction between the lipid tails and the hydrophobic residues as observed in Figure 3.5C.

MD simulations: peptide helical persistence. As mentioned above, the formation of an amphipathic α -helix is hallmark of the Nt17 tract ⁸⁰⁻⁸⁴ in htt. Notably, it has previously been

proposed that lipid interactions with the Nt17 amphipathic helix could occur precisely as suggested by the DOPC MD simulations. It has also been proposed that helix-helix interactions are required to form early-stage oligomers that ultimately lead to protein aggregation associated with HD ⁸¹. It has also been suggested that peptide-lipid interaction could ultimately induce helix formation ⁸⁵. Therefore, it could be argued that suitable peptide-lipid interactions could favor the persistence of the amphipathic helix. The time-dependent helical character of the modeled Nt17 peptide has been assessed for the DOPC and POPE lipid systems investigated here. Figure S4 in the Supporting Information section (Appendix B) shows the helical content of the Nt17 peptide across the two different simulations. For the DOPC simulations, the peptide begins as relatively helical (~65%). The helicity is almost immediately disrupted within the first few nanoseconds and is reestablished at the 68% level for the first 70 ns before stabilizing at ~75% for the remainder of the simulation. The initial collapse and the lower helicity correlate with the initial interaction of the N-terminal region with the lipid bilayer (Figure 3.3A). The relatively stable region (sustained helicity) corresponds with increased interaction with the lipid. In comparison, for the POPE system, no initial collapse is evident, however, a greater number of collapse events is observed across the entire simulation. Additionally, the overall average level of helicity trends downward across the simulation and is ultimately observed to be well below that for the DOPC simulation. These observations are the same for the replicate analysis.

Conclusions

Electrospray ionization - mass spectrometry (ESI-MS) and molecular dynamics (MD) simulations have been used to study the lipid interaction properties of the first 17 amino acid residues of Huntingtin protein (htt). In this study, phosphatidylcholine (PC), the most common lipid in the eukaryotic membrane, as well as phosphatidylethanolamine (PE) lipids were used.

From mass spectra data, a noticeable difference was observed in the number and type of peptide-lipid complex ions. The MS spectra indicate that the Nt17 peptide yields more adducts with the DOPC lipid in both monomeric and multimeric ion forms in comparison to the POPC and POPE lipids. MD simulations provide atomic insight into these interactions. The simulation showed that the residues of the Nt17 peptide were generally closer to the center of the DOPC bilayer throughout the simulation. The amphipathic peptide interacts with the DOPC bilayer with hydrophobic residues. However, when the POPE lipid bilayer was examined, the hydrophilic residues interact with the lipid headgroups. Further analysis utilizing the simulation data reveals that the area per lipid may play an important role in the Nt17-lipid interactions. The increased surface area for the DOPC bilayer results in a longer lasting interaction as the Nt17 peptide is imbedded within the lipid headgroups resulting in more effective interaction with the hydrophobic tails. The simulation also suggests that the increased interaction between the Nt17 peptide and the DOPC lipid bilayer leads to greater stabilization of helicity for the peptide. Conversely, as the peptide increases aqueous interactions as opposed to the lipid bilayer, it loses some helical character over time. As the Nt17 peptide is a lipid-binding domain, its interactions with the membrane can lead to the formation of helix bundles at the bilayer surface. Understanding the nature of these interactions is vital because they can trigger the aggregation process and ultimately result in disease.

Acknowledgments

We are grateful for financial support from the National Institutes of Health (R01GM114494). We are also grateful for stimulating conversations regarding molecular dynamics simulations with Professor Blake Mertz of West Virginia University.

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4. Molecular dynamics simulations of Proteorhodopsin oligomers in lipid bilayer and micellar systems

Introduction

Proteorhodopsins (PRs) are microbial membrane proteins which exist as an oligomer (usually pentamer or hexamer) in their native form ¹. PRs are found in bacteria and they function as a proton pump. The ion pumping triggers with a photon absorbed by retinal and a series of events drive the proton to the outside of the bacteria (Figure 4.1) ². The process starts with the retinal conformational change from all-*trans* isomer to 13-*cis* upon the photon absorption. This causes the formation of a photointermediate state for the protein which is known as the K state. In the next step of the photocycle, D97 receives the proton from the protonated Schiff base (PSB), resulting in the formation of M state (Figure 4.1 and Figure 4.2). The PSB linkage is a result of the covalent binding between retinal and a lysine amino acid. A proton is transferred from the proton-donor E108 to the deprotonated SB, this step forms the N state. The proton lost by E108 is replaced by a proton from solvent. This state is known as the O state (Figure 4.1) ³. As H75 has a hydrogen bond with the D97, this interaction tunes the pKa value for the proton acceptor ⁴.

Figure 4. 1 Proteorhodopsin photocycle.

The photocycle starts with isomerization of the retinal molecule from all *trans* to 13-*cis* upon the absorption of a photon. The result of this photocycle is the transfer of a proton from the bacteria's cytoplasm to the periplasm.

PRs can be classified as green proteorhodopsin (GPR) ⁵ or blue proteorhodopsin (BPR) according to the maximum light absorption ⁶. PRs can form oligomeric states in order to increase the protein stability ^{3-4, 7-8}. This oligomerization includes pentameric and hexameric forms of the protein ⁹⁻¹¹ (Figure 4.2). In the pentameric form, protein monomers such E50-R51 and D52-R51 salt bridges are specifically important for the protein stabilization ⁷. In the hexameric form the corresponding residues in the BPR are E32-R33-S34-D35 in PDB 4JQ6. Maciejko, J. *et al.* have suggested that the presence of S34 will result in some degree of disruption in the R33-D35 salt bridge in the hexameric form therefore the pentameric oligomer is

more favorable for the BPR ⁷. The stability of a proteorhodopsin is also influenced by the membrane composition ^{8, 12}. For example, the surface charge effect of the lipid bilayer on the recruitment as well as the orientation of PR in a detergent-mediated reconstitution procedure has been investigated by Noy and co-workers ¹². The effect of membrane system on the stability of oligomeric states of PR is also a subject of debate. It has been possible to capture the oligomeric PR within DDM ¹⁰, and DPC ⁸ micelles as well as various lipid bilayer systems ^{7, 138}.

Figure 4. 2 Pentameric form of Proteorhodopsin.

The monomers are labeled as A, B, C, D and E. The direction of the proton migration from cytoplasm to periplasm is shown with a red arrow. The proton donor (E108), retinal, proton acceptor (D97), and protein releaser (E142) residues are assigned only for the monomer A of the pentamer. The monomer A is made transparent for better representation of the residues.

Our fundamental understanding of PR's structure and function is still not complete. The remaining questions include:

1. How are the dynamic interactions between the monomers of an oligomeric PR?
2. What is the relation between the structural changes induced by variation of membrane environments on the PR interactions?

Molecular dynamics (MD) simulations can provide an atomistic insight into these events to reveal the details of the membrane composition effect on the photocycle as well as structural stability of PR.

Here we used Gaussian accelerated molecular dynamics (GaMD) ¹⁴⁻¹⁵ simulations to investigate the effect of three detergent systems, *n*-dodecylphosphocholine (DPC), 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), and *n*-dodecyl- β -D-maltoside (DDM) as well as a bilayer system 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE): 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) on the structure of PR in its oligomeric forms. Detergents and lipids can be classified as harsh and mild according to their ability to denature proteins ¹⁶⁻¹⁷. In general, charged detergents with smaller head groups and shorter tails are classified as harsher detergents ¹⁸. The important residues for oligomerization have also been monitored to study the oligomeric stability of PR.

Computational Methods

For the starting structure, the X-ray crystal structure of BPR from HOT75 and Med12 in the dark state (PDB 4KLY and 4JQ6) ⁴ were used. Missing loops were modeled using MODELLER ¹⁹ based on the NMR structure of GPR (PDB 2L6X) ²⁰. The protonation states of the important residues in the dark states are as follows: D97 deprotonated, E108 protonated, E142 protonated, H75 neutral with HSE protonated, all other titratable residues were in conventional protonation states.

Details of the simulations can be found in **Table 1**. The CHARMM-GUI server (www.charmm-gui.org) was used to create surfactant corona and lipid bilayer system around the protein ²¹. In all cases we make sure that all the hydrophobic area of the protein is covered by detergents/lipid bilayer. The ionic strength of 100 mM NaCl was used in all the simulations.

All the systems were minimized for 1 ps, then heated up through 4 steps for 0.5 ns with 10 kcal/mol constraints on the protein backbone. The minimization and heating were done with NAMD 2.13 ²² using CHARMM c36 force field for detergents/lipids and proteins ²³. After minimization, all the simulations were subjected to conventional molecular dynamics (cMD) simulation followed by Gaussian accelerated MD simulations implemented in NAMD ¹⁵ with GPU acceleration and 2 fs time step. GaMD is a method that applies harmonic boost potential to enhance sampling by lowering the energy barriers in the 3D energy landscape of a biomolecule ^{14-15, 24}. In GaMD there is no need to predefine collective variables (CVs) thus unconstrained enhanced sampling is achievable ²⁵. Smoothing the potential energy allows the MD simulation to cover the processing time of a biological system much faster. For example, a few hundred of nanosecond of a MD has been suggested to cover the biological processes that are in the millisecond time scale for membrane protein simulations ^{14, 26-28}.

For the GaMD simulations, a dual boost potential was applied, to both the total potential energy and the dihedral terms. NPT ensemble was used for all the cMD and GaMD simulations at 310 K and 1 bar using a Langevin thermostat and a Berendsen barostat. Particle mesh Ewald (PME) summation was used to calculate the electrostatic interactions ²⁹. For non-bonded interactions, a force switching of 10 Å and a cutoff of 12 Å were utilized. Visual Molecular Dynamics (VMD) ²² and LOOS ³⁰ were used to visualize and analyze the data.

Table 4. 1 Simulation method and run times for all membrane systems.

Membrane	Oligomer	# atoms	# detergents or lipids	lipid (detergent) per monomer	Waters / lipid (detergent)	cMD (ns)	GaMD (ns)	Total (ns)
DPC	P	191917	380	76	50141/380=132	77	320	397
	P	191917	380	76	132	99	328	429
	H	326958	380	63	94050/380=247	445	403	848
	H	326958	380	63	247	140	342	482
DDM	P	209823	350	70	54381/350=155	54	320	374
	P	209823	350	70	155	57	346	403
	H	357141	400	66	101029/400=252	253	527	780
	H	357141	400	66	252	362	306	668
DHPC	P	196881	350	70	50655/350=144.7	52	345	397
	P	196881	350	70	144.7	83	345	428
	H	341878	380	63	97120/380=255	414	308	722
	H	341878	380	63	255	84	313	397
POPE:POPG	P	244212	827	165	40644/827=49	53	311	364
	P	244212	827	165	49	52	323	375
	D52A-P	244202	827	165	49	37	117	154
	R51A-P	244142	827	165	49	37	117	154
	H	289929	808	134	55613/808=69	353	1165	1518
	H	289929	808	134	69	314	1019	1333
	D52A-H	289905	808	134	69	33	102	135
	R51A-H	289833	808	134	69	33	103	136

For the simplicity, H and P were used as hexamer and pentamer respectively. Since each system were simulated in two copies, we were labeling them by 1 and 2. In many cases terms like LB-H1 were used which means the lipid bilayer-hexamer system and copy 1 was the

subject. As another example DDM-P2 means the environment is DDM, the protein is in pentameric form and the copy 2 was discussed.

Results and Discussion

Stability of PR oligomeric complexes. intra-protein contacts often lead to a more stable protein ³¹. In the case of PR, it is suggested that forming oligomeric structure is intrinsic ³² because the protein reconstitutes in different membrane environments ³³⁻³⁴. Protein packing is also been suggested as another mechanism by which proteins gain stability ³⁵⁻³⁸. For example, Fleming and co-workers showed that membrane proteins have higher packing values than soluble protein, indicating the helix-packing is an important factor for the stability of membrane proteins ³⁸. This packing is mainly due to the transmembrane (TM) helix–helix interfaces. This tool could be used to measure the efficiency of packing between monomers in an oligomeric complex. In the present work, the pentameric and hexameric forms of PR were used in different membrane environments (lipid bilayer, DDM, DHPC and DPC) in the dark state. To investigate the oligomeric state stability of PR in different membrane systems, the packing score ³⁹ analysis was performed. The hypothesis is that denser TM bundle enabling PR to proceed more efficiently through photocycle. This can be explained by assuming that a closer interaction between the key residues helps the photocycle to perform faster by stabilizing the protein more efficiently. Figure 4.3 shows the packing score probability of hexameric and pentameric forms of PR scaled according to the environment composition. This figure shows that the protein is generally stable in the timeframe and all the environments that this study was performed in.

Figure 4. 3 Packing score as measured for all oligomeric systems.

LB = lipid bilayer; DDM = dodecyl maltoside; DHPC: diC7-PC; DPC: dodecylphosphocholine. H=Hexamer and P=Pentamer. The number 1 and 2 after each system represents the two copy runs for each system. A-B = monomer A – monomer B, B-C = monomer B – monomer C, C-D = monomer C – monomer D, D-E = monomer D – monomer E, E-F = monomer E – monomer F, F-A = monomer F – monomer A. Worth to mention that in the pentameric system the magenta color is for E-A = monomer E – monomer A. There is no F-A interface in the pentameric systems.

However, hexameric form of the protein in the lipid bilayer is the only system that has 2 symmetric less-packed interfaces in both the copy runs. In LB-H1 system, the interfaces between C-D and F-A monomers are less packed in comparison to the other four interfaces. For LB-H2, there are a relatively less packing at the interfaces between B-C and E-F monomers. It can be inferred that according to the simulation data in the case of the dissociation of the hexameric system, there will be 2 trimer oligomers in the lipid bilayer environment. For the PR-DHPC system, the pentameric form of the protein tend to be more uniformly grouped than

hexamers. Nevertheless, the hexameric form have higher (more efficient) packing in the PR-DPC system.

To further analyze the oligomeric tightness of PR protein, the distance between the center of masses of the adjacent heptameric TM bundles was measured. Figure 4.4 shows that there is a clear increase in the average distance (about 2-4 Å) from hexameric to pentameric oligomer of PR. These results suggest that overall hexameric system pack more tightly than pentamers. This in general tracks the trends of the packing scores. Notably, for the lipid bilayer systems, the less packed interfaces have longer center of mass distances (C-D and F-A interfaces in LB-H1 and B-C and E-F interfaces in LB-H2). In another example, the F-A interface in the DDM-H2 system has the lowest packing score, that appears to have the longest center of mass distance. The same pattern can be applied for the D-E interfaces in DDM-P2 and DPC-P2 systems. It is essential to mention that the center of mass distance is not necessarily equivalent to the packing score. While the distance shows how far the center of masses are, the packing score reflects the short-distance interfaces (such as salt bridges) which by definition of packing score is a sum over a very short-ranged function ($\frac{1}{r^6}$). As it is suggested by Glaubitz et al. ⁷ the beltlike salt-bridges (D52-R51 and E50-R51), that are located at the top part of the cytoplasmic-interacting site of PR, stabilize the oligomeric forms of PR. While the packing score analysis reflects the short distance interactions the movements of the rest of the protein causes the center of mass distances different from the packing score plots.

Figure 4. 4 Monomer-monomer distances between helix bundles: lipid bilayer vs. detergents.

Cross protomer interactions. As mentioned before E50-R51-D52 salt bridges play an important role in the oligomerization and stability of PR oligomers. It has been suggested that breaking the E50-R51 salt-bridge causes a “monomer switch”⁷ for PR. Figure 4.5 shows the distance probability of the interaction at this interface. The distance distribution is generally more stable for the hexamer than pentamer in all the four studied systems. The salt bridge distance is ~4 Å, however, in some cases the salt bridge is broken and the distribution shifts to 6-7 Å. For instance, the interface between monomer E and F and B-C in LB-H2 system is broken at some point. The probability of breaking this interaction is also noticeable in some pentamer systems such as in DDM-P and DPC-P.

Figure 4. 5 Salt bridge distances between E50-R51.

Another important salt bridge is between D52 and R51. Figure 4.6 shows the distribution of this interaction for the PR protein in different environments. Compared to the E50-R51 salt bridge, D52-R51 interaction is more stable. Except for the LB-H system in which there is a notable higher distance for the other systems only a small tail was observed. Furthermore, a shorter distance between the D52-R51 than E50-R51 was observed especially for the pentameric systems.

Generally, it is essential to know which salt bridge is stronger in the PR to control the monomer-oligomer population. A desired population of monomer can be obtained by mutating the correct residue in a pentameric or hexameric systems in different environments. This work can guide through new mutations in order to control the monomer-oligomer populations.

It worth mentioning that the salt bridges are fundamentally different in hexamer and pentamer forms of PR. While in the pentameric system form the sequence is regular E50-R51-D52, in the hexameric form there is an additional residue (serine) between R51 and D52 which affects the

stability of the salt bridge triad in the hexamer. This is most likely why there is a longer distance between D52-R51 in hexamer compare to the same distance in pentamer.

Also, a 2D plot relation between the R51 χ_1 dihedral angle and D52-R51 and E50-R51 distances shows that weaker packing from detergent environments allows E50-R51-D52 to interact more closely, specifically in that ± 60 -degree orientation. As such, DHPC systems are the only ones that noticeably sample R51 dihedral angle at ± 60 that are combined with short enough distance to form a salt bridge. In comparison to hexameric system in DHPC (Figure S4.3 (Appendix C)), the pentameric system in DHPC (Figure S4.4 (Appendix C)) demonstrate a definite shift in the population of the R51 dihedral which causes this system to have much larger percentages of simulation time in ± 60 deg orientation, coupled with tighter binding to E50/D52. Part of this is attributable to the additional residue in the hexamer system (as mentioned before). In this case, membrane environment is not as important, rather the oligomeric form and the position of salt bridge triad is critical. (compare Figure S4.3 (Appendix C) and Figure S4. 4 (Appendix C)).

To further investigate the effect of E50-R51-D52 salt-bridges on the protein packing score the mutation of D52 and R51 to alanine were performed separately for both the oligomer systems in the lipid bilayer environment. Figure S4. 1 (Appendix C) represents the packing score analysis for the wildtype PR in the lipid bilayer system versus mutated version of the protein in the same environment. The analysis reveals that the mutation of either D52 or R51 leads to weaker oligomeric bundle packing. This is more pronounced weakening of the interface for R51A. The center of mass analysis shows that the mutations lead to slight increase in hexamers but have no effect or decrease in pentameric systems (Figure S4. 2 (Appendix C)).

Figure 4. 6 Salt bridge distances between D52-R51.

Other than the two well known cross protomer interactions in the PR oligomers, the salt bridge between D52 and K59 at the adjacent monomers are also important. Figure 4.7 depicts a strong indications between D52-K59 salt bridge. This interaction is more of a factor in stabilization of the hexamer vs. pentamer, regardless of membrane environment. In all lipid bilayer and detergent environments a close interaction between D52-K59 is centered around $<4 \text{ \AA}$ for hexameric systems that is much more stable than the same interaction for the pentameric systems in which the distribution is more expanded. In most of the cases in the pentamer systems there is at least one interface that are close to each other (have a distance $<4 \text{ \AA}$) but this not consistent for the other interfaces. For example the interface between monomers D-E in DDM-P, DHPC-P, and DPC-P is close but the other interfaces are not as close as the interface between D and E.

Figure 4. 7 Salt bridge distances between D52-K59.

W34-H75 interaction. Because of the effect of the H75 on the pKa value of the proton acceptor D97 investigating the hydrogen bond between W34 and H75 is important ⁴⁰⁻⁴¹. The H75 distance analysis reveals that while there is a distribution of distances from 2.5-7.5 Å, regardless of the membrane system for H75-D97 interaction (Figure S4. 5 (Appendix C)), the W34-H75 distance is much more pronounced at extremely short distances at <2.5 Å (Figure 4. 8). Also, for all the studied systems there is a peak that is centered ~4 Å which is still a strong interaction between W34 and H75. In some cases, in some of the monomers the distance increases to >5 Å (Figure 4. 6). Furthermore, the χ_1 dihedral angle analysis of H75 reveals that this residue adopts 2 conformations in the pentamer (-60 and 180) versus one conformation in the hexamer (-60), but not directly related to interactions with either D97 or W34 (Figure S4. 6 (Appendix C) and Figure S4. 7 (Appendix C)). As it is suggested by Luecke and coworkers

W34D mutation causes a drastic alteration in the BPR function⁴ the close interaction of W34 and H75 is playing an important role in the photochemical activity of PR.

Figure 4. 8 Hydrogen bond between W34 and H75.

Retinal binding pocket. One initial step to help with photoisomerization is to transfer a H⁺ from the protonated schiff base (PSB) on the retinal to the proton acceptor D97. The pKa value of the PSB is affected by the localized changes in the binding pocket. Two important residues that the PSB is interacting with are D97 and D227 that according to the x-ray structure of PR the D227 is in closer interaction with the PSB⁴. D227 is important because retinal photoisomerization selectivity is controlled by this residue⁴². Also, it is been shown that D227 has a critical role in modulating the pKa value for the PSB and therefore the rate of the photocycle of proteorhodopsin⁴³. Figure 4. 9 and Figure 4. 10 show that in the hexamer system

D227 is stabilizing the PSB in the all-trans conformation, while D97 is free to fluctuate. A close interaction ($>4 \text{ \AA}$) between the NZ atoms of the D227 with the NZ atom at the retinal in all the hexamer systems is shown in Figure 4. 9. The same analysis for the distance between D97 and retinal in Figure 4. 10 shows a broad distribution of probabilities for the D97-retinal distance in the hexamer systems. This is indicating that only after isomerization the D97 residue is close enough to the PSB to facilitate proton transfer characteristic of the K to M transition.

Conversely in the pentameric system, D97 is (potentially) bridged with water molecule to PSB, allowing it to be more stable (Figure 4. 10). In contrast, D227 is more distant, but only for a subset of monomers (Figure 4. 9).

Figure 4. 9 Retinal binding pocket: Schiff base to D227.

Figure 4. 10 Retinal binding pocket: Schiff base to D97.

Conclusion

Molecular dynamics simulations were used to study proteorhodopsin protein in its oligomeric (pentameric and hexameric) form in four different membrane environments. To study the stability of PR the packing score and salt bridge analysis were performed. The packing score and center of chain distance analysis showed that the PR is stable in both the pentameric and hexameric form in all four membrane systems in the simulation time scale. The distance analysis of important salt bridges also reveals that D52-R51 is especially important in the pentameric system. Another salt bridge that is obviously strong in the hexameric system and less pronounced in the pentameric system was the D52-K59 interaction. The W34 interactions were also studied to investigate the importance of the W34-H75 interaction in the photochemical

activity of proteorhodopsin. Finally, the interaction between the PSB and close aspartic acid residues were studied.

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5. Future Directions: protein characterization using mass spectrometry and molecular dynamics simulations

Determine the solution structure of pHLIP in state I

pHLIP is a negatively-charged, membrane-active peptide which potentially can be used to deliver drugs to the cellular environment ¹. Ongoing research is directed at determining the structure, interactions, and functional properties of this peptide. Researchers have divided the functional properties into three states ²: in state one the peptide is in solution and has no secondary structure; in state 2 the peptide is at the surface of a lipid bilayer and can bind to it under basic conditions while the structure is still random coil; and, finally, for the state 3, by changing the pH to slightly acidic conditions which introduces positive charge to the acidic residues, pHLIP folds into an α -helix and penetrates the bilayer ³. The pHLIP structure in the solution phase (state I) is not completely understood. Identifying the helical region(s) of the pHLIP peptide at physiological and low pH is a crucial step to garner a better understanding of the peptide structure in state I. This could lead to more effective pHLIP targeting for tumor cells ⁴⁻⁶.

Different analytical tools such as circular dichroism (CD), NMR and fluorescence spectroscopy as well as other techniques like MD simulations have been utilized to characterize pHLIP behavior in these states ^{1, 5, 7-9}. Online, solution hydrogen-deuterium exchange (HDX) can be used to identify the pHLIP structure when it is not interacting with lipids ¹⁰. This approach can provide the amount of deuterium incorporation for each residue within the peptide. The amino acids that are more accessible (less involved in hydrogen bonding) to solution exchange will experience more deuterium uptake. That is, HDX can reveal the secondary structure of the peptide in solution at the amino acid residue level. This is accomplished with native MS and tandem mass spectrometry (MS/MS) employing electron transfer dissociation (ETD) ¹¹⁻¹².

Ion mobility spectrometry (IMS) coupled with gas-phase HDX (IMS-HDX) can also be used to determine the structural properties of the peptide ¹³⁻¹⁵. Drift time measurements using IMS can provide information about the number of unique solution conformations that the peptide exhibits. By using ion mobility spectrometry- ion mobility spectrometry (IMS-IMS) we can select a specific conformer in the first IMS and introduce it to the second IMS in order to perform HDX. Recently, it has been shown that peptide ions in non-helical states have distinguished deuterium uptake compared to helical states using gas phase IMS and HDX ¹⁶. Thus, a per-residue analysis such as that proposed here could reveal the location of helical portions of proteins/peptides. These techniques can be used in conjunction with the solution-phase HDX and MD simulations to reveal the location of helical turns in the pHLIP peptide as has been accomplished for model peptides ¹⁷⁻²².

To confirm the structural changes when the pH is reduced from 7 to 2.5, CD spectra were recorded for the wild type pHLIP peptide at the different pH values mentioned above. Fig. 5.1 shows that the helical propensity for the peptide increases from ~2% to 10% when the pH is reduced from 7 to 2.5. Finding the residues that are responsible for this helix formation is important. HDX coupled with an enzymatic digestion method can also be used to identify the helix portion ^{14, 23-24}. Here, the ability of each fragment to uptake deuterium is calculated upon digestion of the peptide with the enzyme pepsin.

Figure 5. 1 CD spectrum of pHLIP recorded at two different pH levels.

Figure 5.2 shows the setup that can be used in order to perform online, solution-phase hydrogen deuterium exchange ¹⁰. In this setup the pHLIP peptide will interact with D₂O for a select period of time based on solvent flow rates and capillary diameters. Subsequently the analyte solution will undergo online digestion using the enzyme pepsin. The digestion will also be conducted for a select period of time. The pHLIP:D₂O ratio will be set to be 5:95. The flow rate for the pepsin is typically maintained at the flow rate for the peptide solution. Additionally, kinetics studies will be performed by changing the length of the reaction capillary and the flow rates of the solutions. For example, by changing the flow rate for the peptide solution to 2 μ L/min (Figure 5.2) and the flow rate for the D₂O solution to 38 μ L/min, the HDX reaction time will increase to 10 seconds.

Figure 5. 2 Online hydrogen deuterium exchange apparatus. The peptide solution and water mixing occurs at T_1 and HDX occurs along L . By changing this length, different exchange rates can be measured.

Determine the effect of membrane composition on permeation of pHLIP in membrane

For proteins that insert into the membrane, many contain mostly short peptide sequences that can penetrate the membrane and transport cargo or disrupt the function of the membrane upon insertion²⁵⁻²⁷. Studying these species and the method of insertion is important because it may permit, for example, the selection and optimization of drug delivery peptides based on their binding energies to the membrane. pH (low) insertion peptide (pHLIP) is a cell-active peptide. It is known that pHLIP inserts into the lipid bilayer membrane at $\text{pH} \sim 6.1$ while a pH value around that associated with cancer cells is ~ 6.8 . Therefore, there is a need to develop strategies to facilitate pHLIP insertion at higher pH values. A larger area per lipid might aid membrane-active

peptide insertion. Differences in the lipid systems could play a role in the degree and type of peptide-lipid interactions. Membranes also have a capture and stabilization effect on the peptide. However, the insertion ability of this peptide requires the presence of both the membrane and a decreased pH value. The increase in area per lipid might reveal a threshold size for “turning on” peptide-lipid interaction (and thus insertion). That is, the peptide may be able to insert at a higher pH just by changing the area per lipid value of the membrane. The structural details of pHLIP (as a membrane-active peptide) including interactions with different lipid systems can be examined with native MS and MD simulations. Peptide-lipid bilayer interactions can be examined at neutral pH to study the effect of membrane composition. To obtain experimental information about the role of membrane composition on the lipid-binding ability of pHLIP, the insertion ability as a function of membrane (completely saturated as with DMPC to high area per lipid such as 4ME 16:0 PC) can be investigated. It is expected that the peptide will insert more readily into the membrane with larger area per lipid. A better understanding of the conformational changes of the membrane-active peptide as the lipid system changes will provide a better understanding of how this peptide functions.

A recent NMR study shows that pHLIP in state II, when it is at the surface of a bilayer, exhibits an interaction with the POPC headgroups⁸. The study was performed at three different pH values. It was shown that, depending on the pH, there is a distinct difference between the ability of pHLIP to get absorbed at the lipid surface. It was demonstrated that the peptide penetrates deeper into the membrane at pH ~6.4. As a membrane with higher area per lipid value provides more spacing for the peptide to insert, it is interesting to study the interactions between the peptide with other PC lipids using native MS. Figure 5.3 represents the mass spectrum for the interaction between pHLIP and POPC lipid vesicle.

Figure 5. 3 The mass spectrum (ESI-MS) for a pHLIP-POPC system. Different monomeric and multimeric ions are labeled (see legend for details).

As mentioned above, because cancer cells exist in a slightly acidic medium, pHLIP can differentiate between a cancer cell and healthy cells ². Therefore, pHLIP insertion into a membrane thus makes it a good candidate for targeted therapy ²⁸. That said, initial peptide-lipid interactions can represent an essential step associated with a variety of membrane-active peptide functions or they can represent other serious disorders resulting from oligomerization of a non-penetrating peptide ²⁹⁻³⁵. As such, many other physiological conditions are directly related to initial interactions between the peptide's residues and the hydrophilic head group or the hydrophobic tail of the membrane. Providing detailed knowledge of the peptide-lipid membrane interactions can ultimately result in the development of new treatment strategies for different diseases.

The strange case of leptin; an electrospray ionization study

Ionizing molecules is an obvious prerequisite step for mass spectrometry (MS) experiments ³⁶. Due to the softness of the method, the ability of providing multiply-charged ions, and the ease of coupling with analytical instruments, the most common technique to accomplish the ionization step in commercial MS devices is electrospray ionization (ESI) ³⁷. The importance of studying the mechanism of the ESI process is in intelligently designing better experiments using ESI-MS as well as for the construction of improved MS instrumentation. In ESI, a solution is infused through a needle that is biased relative to the mass spectrometer inlet. The applied electric field causes a destabilization of the liquid meniscus and the formation of a Taylor cone ³⁶⁻³⁹. This generates a stream that will disintegrate into micrometer-sized droplets with excess charge. These droplets will then undergo a series of solvent evaporation and fission events until the naked analyte ion is introduced into the mass spectrometer. The process of evaporation-fission can happen several times up to a point such that the radius is small enough (<10 nm) for the molecule to leave the droplet ³⁷. It is possible to obtain different populations of ions having different numbers of protons attached and creating characteristic charge state distributions in the mass spectrum.

For small ions (low molecular weight such as sodium), the proposed mechanism is the ion evaporation model (IEM) ⁴⁰⁻⁴¹. In this mechanism small ions can eject from a droplet in the final stages of the ESI process. Conversely, for high molecular weight systems such as proteins, two mechanisms have been proposed including: the charged residue model (CRM) ⁴²⁻⁴⁴ and the chain ejection model (CEM) ³⁶.

Notably, the charge state distribution (CSD) of proteins in the mass spectrum is significantly different for folded and unfolded proteins which suggests that the ionization process is different for these two states; CEM is suggested for proteins in their folded state and the CRM is for unfolded proteins ⁴⁵. In CEM hydrophobic and electrostatic forces drive the protein to the

surface of the droplet and when the protein is expelled from the droplet, some charges at the surface of the droplet will be transferred to the protein ⁴⁵. In CRM, the evaporation continues until the solvent is completely evaporated and the remaining charge is transferred to the protein ^{44, 46}. For proteins with a mixture of secondary structure and unstructured regions, the mechanism might be different. For example, leptin is a globular protein which is responsible for normalizing body weight ⁴⁷⁻⁴⁹. A recently acquired ESI-MS spectrum for this protein is shown in Figure 5.4 (B). Here the protein was electrosprayed using native MS solvent conditions. Noticeably the spectrum consists of two regions: one region with low CSD centered at +8 and a second region with high CSD centered at +11. For comparison purposes, a spectrum for the globular protein myoglobin conducted under the same conditions as those for the leptin protein was collected (Figure 5.4). For myoglobin, the overall number of separate charge states is reduced, and they are centered around +8. In addition, the signal level for the leptin protein was about an order of magnitude larger than that of myoglobin. Taken together, this indicates that the ionization method could be very different for leptin; notably, as a compact globular protein, myoglobin may be expected to follow the traditional CRM process. Such a difference may result from the unstructured region of the leptin protein which results in the bi-model CSD. Analyzing the crystal structure of leptin ⁴⁷ reveals that 35% of the residues comprise random coil regions which mostly range from Ser25 to Gly38 (14 residues) and Leu39 to Thr 50 (11 residues) and Ser95 to Leu107 (12 residues). This unstructured region comprises almost an entire face of the protein. The same analysis for myoglobin ⁵⁰ shows that only 20% of the residues are in the random coil regions and the longest stretch of random coil residues ranges from Phe43 to Thr 51 (9 residues).

To further investigate the electrospray ionization behavior of these two proteins we utilized molecular dynamics (MD) simulations. Most of the mechanistic studies for the last stages of the ESI process have been performed by MD simulations. Two water droplet systems

with ~4.5 nm radius and a protein in their center have been initially modeled. The droplet charge for each system is assigned to be lower than the Rayleigh limit. Figure 5.5 depicts the movements of each protein within its respective droplet. The MD simulations show that the leptin protein tends to present near the surface of the droplet while the Myoglobin remains closer to the droplet center. The snapshots of these movements show that the random coil regions of the leptin protein play an important role in driving the protein to the droplet surface. Clearly, repeated studies must be conducted to conclude that such behavior is affected by these regions and this is not just Brownian motion. Additionally, a longer simulation time is required to see the ejection of the protein from the droplet. Konermann and co-workers have already shown that for a completely denatured protein the ejection happens in <10 ns time frame ⁴⁵. A difference is the overall size of the water droplet. The larger size was selected to better track the interaction with the droplet surface over time.

Figure 5. 4 Mass spectra for for A) Myoglobin and B) leptin generated by native MS. Here the proteins were electrosprayed from buffered (100 mM ammonium acetate) solutions. Charge states are labeled for each protein. Insets show the structures of the two different proteins. Note the long, unstructured region for the protein leptin which comprises an entire face of the protein.

Figure 5. 5 The distance profile of the center of mass for myoglobin and leptin proteins to the water droplet center of mass. The distance to the droplet surface is provided. The two different traces are labeled for the respective proteins.

Modeling the CRM behavior of proteins will require a substantially a longer time ^{39, 51-52}. Future studies should be conducted in which the droplet drying is allowed to proceed to the point of ion ejection or ion formation via CRM. From multiple analyses, it may be possible to deduce whether or not it will be possible to form leptin ions via the CEM process. Other experiments can be conducted in which proteins of varying degrees of unstructured region are examined to observe whether or not the CEM holds for those of higher unstructured state. To these studies,

ion mobility spectrometry investigations can be added. For example, with IMS it will be possible to determine whether or not the protein ions exhibit a greater change in conformational size as evidenced by comparisons of ion cross sections with those calculated for the native state using the crystal structure coordinates. These same experiments can also be conducted for the similar proteins with long unstructured regions comprised of >30% random coil residues.

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Appendix A

Figure Captions

Figure S2. 1. CD spectroscopy of both model peptides PAAAAKAAAAKAAAAKAAAAK and PSSSSKSSSSKSSSSKSSSSK. The alanine sequence is showing a high-degree of helical character as indicated by the negative bands at ~207 and 223 nm as well as the positive band at ~190 nm. For the serine sequence peptide, the helicity is missing and a broad band at lower wavelengths is indicative of more random coil character.

Figure S2. 2. Conformational space sampling of 40-ps and 1200-ps SA for each conformer arrangement. The structures with the collision cross section within $\pm 2\%$ are in red dash line section.

Figure S2. 3. An example of very compact ion conformer type with charge configuration of K6_K11_K21 and theoretical CCS value of $348 \text{ \AA}^2$ Calculated by Mobcal software. This structure has a significant charge solvation of the charge sites by the serine residues.

Figure S2. 4. Schematic of S4 and S5 is explaining the increase in the predicted deuterium content for the conformer with high accessibility at the terminal peptide portions rather than the interior residues. These residues are close to the carbonyl groups showed by stars.

Figure S2. 5. A) One structure with a wrong CCS value but showing a high D incorporation value for S20. Hydroxyl and amine groups are bending back to the structure while for S18 these functional groups are facing outside. B) Visualizing the same structure on another view is showing that S2 is bending inside the structure and S4 is bending outside.

Table Captions

Table S2. 1. Deuterium uptake values calculation for serine residues in the model peptide.

Figure S2. 1 CD spectroscopy of both model peptides PAAAAKAAAAKAAAAKAAAAK and PSSSSKSSSSKSSSSKSSSSK.

Figure S2. 2 Conformational space sampling of 40-ps and 1200-ps SA for each conformer arrangement.

Figure S2. 3 An example of very compact ion conformer type with charge configuration of K6_K11_K21 and theoretical CCS value of 348 Å² Calculated by Mobcal software.

Figure S2. 4 The increase in the predicted deuterium content for the conformer with high accessibility at the terminal peptide portions rather than the interior residues.

Figure S2. 5 An example of a structure with a wrong CCS value but showing a high D incorporation value for S20.

Residue Number	Fragments	m/z ^a	m/z D ₂ O	D uptake ^b
S2	Z ₁₉	1821.88	1839.86	0.79
S3	Z ₁₈	1734.80	1752.52	0.26
S4	Z ₁₇	1647.72	1665.13	0.31
S5	Z ₁₆	1560.64	1577.47	0.58
S7	Z ₁₄	1345.39	1357.91	0.58
S8	Z ₁₃	1258.31	1270.36	0.47
S9	Z ₁₂	1171.23	1182.63	0.65
S10	Z ₁₁	1084.15	1094.79	0.76
S12	Z ₉	868.90	876.53	0.55
S13	Z ₈	781.82	788.94	0.51
S15	Z ₆	607.66	613.65	0.76
S7	C ₇	720.76	726.59	0.75
S8	C ₈	807.84	814.11	0.44
S9	C ₉	894.91	901.83	0.64
S10	C ₁₀	981.99	989.67	0.76
S12	C ₁₂	1197.25	1207.91	0.56
S13	C ₁₃	1284.33	1295.47	0.48
S14	C ₁₄	1371.40	1383.14	0.59
S15	C ₁₅	1458.48	1470.92	0.70
S17	C ₁₇	1673.74	1689.80	0.60
S18	C ₁₈	1760.82	1777.09	0.21
S19	C ₁₉	1847.89	1864.65	0.48
S20	C ₂₀	1934.97	1952.82	1.09
S2	Z ₁₉	1821.88	1839.82	0.95
S3	Z ₁₈	1734.80	1752.50	0.24
S4	Z ₁₇	1647.72	1665.16	0.26
S5	Z ₁₆	1560.64	1577.51	0.57
S7	Z ₁₄	1345.39	1358.06	0.78
S8	Z ₁₃	1258.31	1270.42	0.56
S9	Z ₁₂	1171.23	1182.71	0.63
S10	Z ₁₁	1084.15	1094.84	0.79
S12	Z ₉	868.90	876.53	0.58
S13	Z ₈	781.82	789.00	0.45
S15	Z ₆	607.66	613.62	0.81
S7	C ₇	720.76	726.40	0.67
S8	C ₈	807.84	813.97	0.49
S9	C ₉	894.91	901.62	0.57
S10	C ₁₀	981.99	989.59	0.89
S12	C ₁₂	1197.25	1208.01	0.67
S13	C ₁₃	1284.33	1295.54	0.45
S14	C ₁₄	1371.40	1383.10	0.48
S15	C ₁₅	1458.48	1470.88	0.70
S17	C ₁₇	1673.74	1689.94	0.69

Residue Number	Fragments	m/z^a	m/z D₂O	D uptake^b
S18	C₁₈	1760.82	1777.26	0.24
S19	C₁₉	1847.89	1864.83	0.49
S20	C₂₀	1934.97	1952.80	0.89
S2	Z₁₉	1821.88	1838.79	0.82
S3	Z₁₈	1734.80	1751.25	0.46
S4	Z₁₇	1647.72	1663.77	0.40
S5	Z₁₆	1560.64	1576.02	0.67
S7	Z₁₄	1345.39	1356.59	0.71
S8	Z₁₃	1258.31	1269.03	0.48
S9	Z₁₂	1171.23	1181.28	0.67
S10	Z₁₁	1084.15	1093.51	0.69
S12	Z₉	868.90	875.23	0.56
S13	Z₈	781.82	787.71	0.44
S15	Z₆	607.66	612.50	1.05
S7	C₇	720.76	724.95	0.67
S9	C₉	894.91	900.16	0.58
S10	C₁₀	981.99	987.95	0.71
S13	C₁₃	1284.33	1293.91	0.50
S14	C₁₄	1371.40	1381.42	0.43
S15	C₁₅	1458.48	1469.14	0.64
S17	C₁₇	1673.74	1688.66	0.77
S18	C₁₈	1760.82	1776.17	0.43
S19	C₁₉	1847.89	1863.71	0.46
S20	C₂₀	1934.97	1951.83	1.04

Table S2. 1 Deuterium uptake values calculation for serine residues in the model peptide.

m/z^a recorded for lipid sequence without D₂O

D uptake^b calculated for each residue

Appendix B

Figure S3. 1. Mass spectrum showing the ions obtained upon ESI of the Nt17-POPC and Nt17-POPE vesicle systems (compare with Figure 1 in the manuscript). For labeling purposes, a large red circle represents a single Nt17 peptide, a green triangle represents a single lipid molecule. Insets show expanded regions in which specific ions would be present. Notably the Nt17+7POPC, Nt17+6POPE and Nt17+7POPE ion species are not observed although their counterparts for the DOPC system are present in relatively high abundance (Table 1 in the manuscript).

Figure S3. 2. Relative intensities of lipid-bound peptide peaks in mass spectra for Nt17-DOPC (A) Nt17-POPC (B) and Nt17-POPE (C) systems. In each case $[\text{Nt17}+2\text{H}]^{2+}$ has been chosen as the reference for scaling (ratioing) the intensities. Lipids bound to monomer, dimer, trimer and tetramer are shown in blue, orange, yellow and gray, respectively.

Figure S3. 3. Isolation of $[\text{Nt17}+1\text{L}+2\text{H}]^{2+}$ ions for DOPC (A) and POPE (B) in the linear ion trap mass spectrometer. For labeling purposes, a large red circle represents a single Nt17 peptide and a green triangle represents a single lipid molecule.

Figure S3. 4. The relative degree of peptide helicity as a function of simulation time for the three lipid systems. Here the initial structures for the Nt17 peptide exhibit ~70% helicity.

Table S1. Structural properties of the modeled lipid membranes.

Lipid bilayer	Area per lipid from Experiment ($\text{\AA}^2$)	Calculated average area per lipid ($\text{\AA}^2$)	Calculated Thickness (nm)	Calculated radius of gyration
DOPC	67.4 ⁵³	68.59 $\pm$ 1.02	38.63 $\pm$ 0.03	27.42 $\pm$ 0.01
POPC	64.3 ⁵⁴	65.12 $\pm$ 1.01	39.03 $\pm$ 0.15	26.91 $\pm$ 0.03
POPE	56.6 ⁵⁵	57.26 $\pm$ 1.01	42.27 $\pm$ 0.45	25.75 $\pm$ 0.16

Table S3. 1 Structural properties of the modeled lipid membranes.

Figure S3. 1 Mass spectrum showing the ions obtained upon ESI of the Nt17-POPC and Nt17-POPE vesicle systems.

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Figure S3. 2 Relative intensities of lipid-bound peptide peaks in mass spectra for Nt17-lipid vesicles.

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Figure S3. 3 Isolation of $[Nt17+1L+2H]^{2+}$ ions for DOPC (A) and POPE (B) in the linear ion trap mass spectrometer.

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Figure S3. 4 The relative degree of peptide helicity as a function of simulation time for the three lipid systems.

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Appendix C

Figure S4. 1 R51 dihedral angle in DHPC-H. A) D52-R51 distance, B) E50-R51 distance.

Figure S4. 2 R51 dihedral angle in DHPC-P. A) D52-R51 distance, B) E50-R51 distance.

Figure S4. 3 Packing score for WT vs mutations.

Figure S4. 4 Distance between the center of monomers, WT vs mutations.

Figure S4. 5 Distance between H75 and D97.

Figure S4. 6 H75 dihedral angle. A) H75-D97 B) W34-H75 distances in LB-Pentamer system.

Figure S4. 7 H75 dihedral angle. A) H75-D97 B) W34-H75 distances in LB-Hexamers system.