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Protein and Peptide Gas-phase Structure Investigation Using Collision Cross Section Measurements and Hydrogen Deuterium Exchange

Mahdiar Khakinejad

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**Protein and Peptide Gas-phase Structure Investigation Using Collision
Cross Section Measurements and Hydrogen Deuterium Exchange**

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**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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phase hydrogen-deuterium exchange, molecular dynamics simulation,
collision cross section, conformational space sampling**

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ABSTRACT

Protein and Peptide Gas-phase Structure Investigation Using Collision Cross Section Measurements and Hydrogen Deuterium Exchange

Mahdiar Khakinejad

Protein and peptide gas-phase structure analysis provides the opportunity to study these species outside of their explicit environment where the interaction network with surrounding molecules makes the analysis difficult. Although gas-phase structure analysis offers a unique opportunity to study the intrinsic behavior of these biomolecules, proteins and peptides exhibit very low vapor pressures. Peptide and protein ions can be rendered in the gas-phase using electrospray ionization (ESI). There is a growing body of literature that shows proteins and peptides can maintain solution structures during the process of ESI and these structures can persist for a few hundred milliseconds.

Techniques for monitoring gas-phase protein and peptide ion structures are categorized as physical probes and chemical probes. Collision cross section (CCS) measurement, being a physical probe, is a powerful method to investigate gas-phase structure size; however, CCS values alone do not establish a one-to-one relation with structure (i.e., the CCS value is an orientationally averaged value). Here we propose the utility of gas-phase hydrogen deuterium exchange (HDX) as a second criterion of structure elucidation. The proposed approach includes extensive molecular dynamic (MD) simulations to sample biomolecular ion conformation space with the production of numerous, random *in-silico* structures. Subsequently a CCS can be calculated for these structures and theoretical CCS values are compared with experimental values to produce a pool of candidate structures. Utilizing a chemical reaction model based on the gas-phase HDX mechanism, the HDX kinetics behavior of these candidate structures are predicted and compared to experimental results to nominate the best *in-silico* structures which match (chemically and physically) with experimental observations.

For the predictive approach to succeed, an extensive technique and method development is essential. To combine CCS measurements and gas-phase HDX studies at the amino acid residue level, for the first time a drift tube is connected to a linear ion trap (LIT) with electron transfer dissociation (ETD) capability. In this manner CCS and per-residue deuterium uptake measurements for a model peptide carried out successfully. In this study, the gas-phase conformations of electrosprayed ions of the model peptide KKDDDDIHK have been examined. Using ion structures obtained from MD simulations and considering charge-site/exchange-site density the level of the maximum total deuterium uptake for the gas-phase ions is explained. Also a new hydrogen accessibility scoring (HAS) model that includes two distance calculations (charge site to carbonyl group and carbonyl group to exchange site) is applied to the *in-silico* structures to describe the expected HDX behavior for these structures. Further investigation to improve the accuracy of the model is accomplished by a “per-residue” HDX kinetics study of the model peptide. In this study, the ion residence time and the deuterium uptake

of each residue is measured at different partial pressures of D₂O. Subsequently the contribution of each residue to the overall HDX rate of the intact peptide ion is calculated. These rate contributions of the residues exhibit a better fit to HAS than their maximum deuterium uptake.

Proteins and peptides with very frequent acidic residue in their sequence provide very poor signal levels when employing positive polarity ESI. Also, the comparison of protonated and deprotonated ions of these biomolecules offers the potential to provide a better structural characterization. Per-residue deuterium uptake values resulting from collision-induced dissociation (CID) of the model peptide KKDDDDIHK were used to investigate the degree of hydrogen deuterium scrambling for deprotonated ions. Remarkably, limited isotopic scrambling was observed in this study of this small model peptide. This data and the per-residue deuterium uptake of the triply-protonated model peptide Acetyl-PAAAAKAAAAKAAAAK are exploited to propose a lemma to allocate protonation and deprotonation sites for peptide ions in the gas-phase. Insulin ions, as a small protein model system, are examined to investigate the relation of the maximum deuterium uptake value for each insulin chain to the exposed surface area of each insulin subunit. The results show that the methodology can be applied on the protein complexes to provide information about the exposed surface area of each subunit.

Dedication

I dedicate this work to my beloved wife and colleague, Samaneh. Without your wonderful help in Molecular Dynamics simulations, none of this could be possible. Thank you for your willingness to support my opinion and ideas in research, and your patience and support in life.

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List of Symbols and Abbreviations

PDB	Protein databank
NMR	Nuclear magnetic resonance spectroscopy
MS	Mass spectrometry
ESI	Electrospray ionization
MALDI	Matrix-assisted laser desorption ionization
IMS	Ion mobility spectrometry
CID	Collision-induced dissociation
HDX	Hydrogen Deuterium Exchange
v_d	Drift Velocity
d	Drift tube length
t_d	Drift time
E	Electric Field
K	Mobility
m_l	Ion mass
m_b	Buffer gas mass
z	Number of charge
e	Charge of electron
k_B	Boltzmann constant
T_{eff}	Effective temperature
N_b	Buffer gas number density
Ω	Collision cross section
FTICR	Fourier transform ion cyclotron resonance
ECD	Electron capture dissociation
ETD	Electron transfer dissociation
MD	Molecular dynamics
TWIG	Traveling wave ion guide
F	Ion funnel

G	Ion gates
IA	Ion activation
LTDQ	Linear trap quadrupole (linear ion trap)
HAS	Hydrogen accessibility scoring
NEC	Number of effective collisions
AGC	Active gain control
XIDTD	Extracted ion drift time distribution
RED	RESP ESP charge Derive
AMBER	Assisted Model Building with Energy Refinement
TM	Trajectory method
STP	Standard temperature and pressure
k	Reaction rate constant
t_i	Time increment
S_O	Carbonyl score
S_H	Hydrogen score
S_R	Residue score
d_{O-N}	Carbonyl charge distance
d_{H-O}	Carbonyl hydrogen distance
SP	Model peptide KKDDDDDDIHKIK
MEP	Molecular electrostatic potential
SAS	Solvent accessible surface areas
SES	Solvent excluded surface areas
S_{HDXS}	Hydrogen score (S_H)
$H_{Surface}$	Hydrogen surface
S_{Chain}	Chain score

1. Introduction: Protein and Peptide Gas-phase Structure Investigation Using Collision Cross-section measurement and hydrogen deuterium exchange

In recent decades, tremendous effort and progress have been made in the characterization of protein structure; the number of protein structures added to the protein databank (PDB) structural database has grown from ~142 in 1990 to over 9000 by 2013 [1]. Despite this progress, protein structure characterization is a challenging task because of limitations in the analytical methodologies used for structure characterization. Ideally, X-ray crystallography would produce high-resolution structural information for protein complexes; however, such structure determination not only requires the in vitro reconstitution of purified protein subunits but also the ability to crystallize the protein complex [2]. Although ground breaking studies in the determination of protein quaternary structure elucidation occurred in 2000 [3-5], molecular size yet limits the elucidation of such structure by X-ray crystallography [2]. Nuclear magnetic resonance spectroscopy (NMR) also suffers from the limitation of molecular size, although recent advances such as magic angle spinning NMR have enabled limited studies of protein subunit architecture [6]. More recently, advances in cryo electron microscopy have been used to study large protein complexes although challenges remain with regard to resolution, throughput, and instrumental parameter optimization (e.g., electron energies) [7, 8].

Mass spectrometry (MS) is a valuable tool for providing structural information for protein ions. Indeed sensitivity and speed advantages boost MS-

based techniques in the field of high-throughput structural proteomics [2, 9-12]. Mass spectrometry (MS) can provide information about the molecular weights and primary structure of proteins [13, 14]. However, obtaining information about three-dimensional shape by MS alone is challenging. The advent of soft ionization methods like electrospray ionization (ESI) [15], and matrix-assisted laser desorption ionization (MALDI) [13], provided the means to produce intact protein ions in the gas-phase. Early gas-phase studies employing a suitable fragmentation method revealed that protein sequencing was feasible in the gas phase [16]. Although sequencing can provide invaluable information about protein primary structure, protein function is often ultimately governed by higher order structure. Many studies were carried out to investigate the type of protein ion structure formed in the solvent-free environment. Early studies, after the introduction of ESI, revealed that under suitable ionization conditions, weak, non-covalent protein interactions could be maintained in the absence of solvent [17]. A study of the conformations formed from different ESI charge states using ion mobility spectrometry (IMS) suggested that secondary structural elements and tertiary structure could be retained essentially invoking an argument of solution conformation memory [18]. Later, after the observation of intact protein complexes in the gas-phase, the concept of native MS was introduced [11, 19]. Studies of ubiquitin indicated that different structures of the anhydrous protein are rooted in the same structure types in solution [20].

Anhydrous protein structure investigations can be accomplished by different methods and techniques which can be categorized as chemical probes

and physical probes [21]. Physical probes do not alter the chemical composition of the protein ions. Gas-phase dissociation techniques can act as a physical method for protein structure studies. Nearly 25 years ago, fragmentation patterns from collision-induced dissociation (CID) were correlated with protein structure [22]. Blackbody infrared radiative dissociation was shown to provide valuable information about the structures and thermodynamic properties of protein ions in the gas-phase [23]. Nearly 20 years ago, ion mobility spectrometry (IMS) was coupled with MS to provide structural information about the shapes of protein ions formed by electrospray ionization [24, 25]. The use of collision cross section (CCS) measurements as a physical probe of ion structure represented a significant advance in the study of protein ion structure. The CCS of a protein ion is directly related to its available surface for momentum transfer in collision events with a neutral buffer gas and, therefore, is related to protein structure [26]. Triple quadrupole instruments were originally used to measure CCS [27], however such measurements in triple quadrupole instruments can lead to large errors in CCS determination [28]. Ion mobility spectrometry (IMS) has been widely used for ion mobility measurements.

In chemical probe methods, protein ions undergo chemical reactions in which the reactivity of the ion with the reagent of interest can provide structural data. Chemical reactions for gas-phase protein structure studies can be divided into three categories [21]: 1) proton transfer reactions [29]; 2) isotopic hydrogen-deuterium exchange (HDX) [30]; and 3) molecular adduction of solvent by the bimolecular ion [31]. Among the different chemical probes, HDX has proven to be

one of the most useful methods and many structural investigations have been carried out using this technique [32-35].

1.1. Ion Mobility Spectrometry

Ion mobility spectrometry is attributed to all principle methods and instrumentation for ion structure characterization using direct or indirect measurement of their transit times through a supporting gas atmosphere in an electric field [36]. In traditional IMS, a swarm of ions is pulsed into a drift tube. A constant voltage drop on the cylindrical lenses which are separated by isolator spacers constructs a uniform field along the drift tube which pushes the ions through the neutral buffer gas. Under these conditions, ions travel across the drift tube with a constant velocity. From the measured drift time, the ion velocity can be calculated as:

$$v_d = \frac{d}{t_d} \quad \text{Equation 1.1}$$

In which v_d is the drift velocity, d is length of the drift tube and t_d is the drift time.

The drift velocity can be normalized to the electric field:

$$K = \frac{v_d}{E} \quad \text{Equation 1.2}$$

E is the electric field and K is the mobility of the ion. The mobility of a swarm of ions (with a defined structure) is a constant value depending upon the specific buffer gas employed, and its temperature and pressure provided that the ions are thermalized (within the low-field limit). Ions are thermalized when the energy gained in an ion-neutral impact is negligible in comparison to its thermal energy

[36]. The mobility of ions in a neutral gas under a constant electric field can be related to the intrinsic value of collision cross-section defined by:

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T_{eff})^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{1}{K} \frac{760}{P} \frac{T_{eff}}{273.2} \frac{1}{N_b} . \quad \text{Equation 1.3}$$

In equation 1.3, P , m_i and m_b are buffer gas pressure, ion and neutral gas masses, respectively. ze is the charge of the ions, k_B is Boltzmann's constant, T_{eff} is the effective temperature of the ions and the number density at STP is denoted by N_b [26]. CCS values can provide general information about stable gas-phase ion conformers and their relative compactness [37]; the experimental cross section values can be compared and matched with those calculated for nominal structures obtained from molecular dynamics (MD) simulations [24, 38, 39]. The Jarrold and Bowers research groups used IMS techniques to study carbon cluster ions and correlate CCS values to such structural information [40, 41]. Later, additional theoretical studies established an exact hard-sphere scattering method and the trajectory calculation method for determining the cross sections of trial structures [42, 43]. Trial structures for biomolecular ions could then be directly to experimentally determined mobilities. Jarold's group provided the mobcal software for obtaining CCS values for MD structures [44].

1.2. Hydrogen Deuterium exchange

HDX techniques have been widely used as a chemical probe for protein structure studies in the gas-phase [30, 32, 45-49]. The first experiments carried out in a Fourier transform ion cyclotron resonance mass spectrometer (FTICR), involved the careful study of changes in isotopic envelopes of protein ions which

indicated the presence of different, stable conformers for a protein ion *in-vacuo* [33]. The same methodology was applied to study gas-phase conformer transformations of cytochrome *c* ions [50]. A comprehensive mechanistic study performed by the Beauchamp group confirmed the structural information to be obtained from HDX data [45]. A combination of IMS and gas-phase HDX was introduced by the Clemmer group [30]. In this study, a partial pressure of D₂O gas was introduced into the drift tube buffer gas, and the gas-phase conformers of cytochrome *c* ions were selected and the maximum deuterium uptake levels of each were determined. In a separate study using HDX in a FTICR cell, the HDX kinetics behavior of singly-protonated bradykinin ions was studied [49]. In this study an ensemble of *in-silico* structures exhibiting a salt bridge and a HDX model were utilized to predict the collective HDX behavior for matching *in-silico* ions. The model could successfully predict the isotopic envelop and total deuterium uptake of the peptide ions under different D₂O exposure times. Further mechanistic investigations of gas-phase HDX involved the study of cytochrome *c* ions examined at different temperatures [32]. Lower kinetic rates at higher temperature were explained by the necessity of a long-lived reaction intermediate. To explain the different deuterium incorporation values at different temperatures, MD simulations were utilized. For this study the candidate *in-silico* structures from MD with matching CCS values (to experimental values) were used to interpret the HDX data. The lack of site-specific deuterium uptake values prevented a more complete structural characterization. The advent of electron capture dissociation [51] (ECD) and electron transfer dissociation [52] (ETD)

(non-ergodic methods of ion fragmentation) provides the opportunity of ascertaining per-residue levels of deuterium uptake for peptide and protein ions [53].

In recent studies, a drift tube coupled to a linear ion trap instrument equipped with ETD were exploited to measure CCS values and obtain per-residue deuterium uptake values [47, 48, 54, 55]. A pool of random, *in-silico* structures were produced using a cyclic simulated annealing approach. The CCS values for these theoretical structures were calculated and compared with experimental CCS values; structures with matching values were selected as candidate structures. A hydrogen accessibility scoring (HAS) algorithm was proposed to score each labile hydrogen on candidate ion structures, and, subsequently, per-residue deuterium uptake values were predicted by a number of effective collisions (NEC) model for these ion structures. The theoretical HDX behavior of candidate structures was employed as the second criterion of structure elucidation to yield a population value for each of these nominal structures within an ESI distribution.

The HDX kinetics and maximum deuterium uptake levels of deprotonated peptide and protein ions were reported recently for the first time [56-58]. In these studies, per-residue deuterium uptake values were exploited to assign deprotonation sites for a model peptide ion [57]. In addition to the HDX behavior analysis, MD simulations and CCS measurements were utilized to produce a pool of candidate structures. A HAS-NEC model was utilized to predict existing

gas-phase conformers. Using this approach it was proposed that the relative surface area exposure of protein complexes could be predicted [58].

1.3. References

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2. Combining Ion Mobility Spectrometry with Hydrogen-Deuterium Exchange and Top-Down MS for Peptide Ion Structure Analysis ¹

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2.1. Introduction

Over the last several decades, tremendous progress has been made in the characterization of the three-dimensional structures of proteins. Evidence of this progress is demonstrated by the fact that the number of protein structures added to the protein databank (PDB) structural database has grown from ~142 in 1990 to over 9000 by 2013 [1]. Despite this progress, protein structure characterization is inaccessible for a number of species in part due to limitations in the analytical methodologies used to obtain detailed structures. For example, many proteins do not readily form crystals amenable for x-ray diffraction studies and nuclear magnetic resonance spectroscopy is limited by the physical size of the proteins that can be studied [2, 3]. Structure characterization challenges are magnified for protein-protein complexes or other protein-ligand interactions.

The introduction of soft ionization techniques such as electrospray ionization (ESI) [4] and matrix-assisted laser desorption ionization (MALDI) [5, 6] enabled the characterization of gas-phase protein ions by mass spectrometry [7-9]. Shortly thereafter, much attention was directed at determining the structures formed by these ions and their degree of similarity to antecedent solution structures [10-26]. One early concern in the characterization of gas-phase protein ion structure was whether or not such ions undergo structural transformations affecting the ability to relate gas-phase and solution conformers [27].

Experiments combining ion mobility spectrometry (IMS) with mass spectrometry

(MS) showed that specific protein ion conformers undergo sizeable structural transformations over relatively short timescales [28, 29]. Other experiments demonstrated that, for select ions, solution conformers can persist for extended periods of time in the gas phase allowing structural characterization of solution relevant species [30-33].

One technique considered to hold potential for the characterization of protein ion conformations was the use of gas-phase hydrogen-deuterium exchange (HDX) combined with MS. Early work utilized HDX in FTICR instruments to demonstrate the presence of multiple conformations of protein ions based on the observation of different exchange rates [13, 26, 34]. Significant effort was also directed at deciphering the exchange mechanisms for different deuterating reagents [35, 36]. Despite early advances, the use of gas-phase HDX as a structural analysis tool gradually received less attention due to its inability to elucidate accessible and inaccessible sites. The advent of electron capture dissociation (ECD) [37] and electron transfer dissociation (ETD) [38] provided the ability to produce fragment ions without the accompanying problem of isotope scrambling encountered with collision-induced dissociation (CID) [39].

Here we report the use of gas-phase HDX and top-down MS characterization of specific peptide ion conformations. Ion conformers are selected for characterization according to their mobilities through a buffer gas containing He (2.50 Torr) and a small percentage of D₂O (0.06 Torr). In general, elongated conformers are observed to exhibit decreased exchange compared with more compact species. Ion structures obtained from molecular dynamics

(MD) experiments suggest that the increased density of charge sites and exchange sites for more compact conformers could result in increased exchange. The experiments are similar to IMS-HDX-MS experiments performed in the past in which the exchange levels of specific protein ions were determined at room temperature and as a function of drift gas temperature [40, 41]. Conceptually the study is also similar to that reported for the characterization of ubiquitin ions using a commercial traveling wave ion guide (TWIG) instrument [42-44]. Compared with the former and latter studies, distinguishing aspects of this work include the ability to locate exchange sites and link site accessibility to specific gas-phase structures, respectively.

2.2. Experimental

2.2.1. Model peptide selection.

Although the KKDDDDDIKLIK is similar to peptides used to examine HDX scrambling subsequent to solution-exchange experiments [45-47] several characteristics rendered it desirable for these experiments. First, the lysine residues at the C-terminal and N-terminal regions of the molecule provide electrosprayed ions of sufficient charge (+3 and +4) for higher-efficiency ETD analysis. Second the terminal region locations of the charge sites provide a model for testing the relative accessibility of hydrogens on interior amino acid residues. Third, the repetition of specific amino acid residues (aspartic acid and isoleucine) allows the determination of differences in residues having the same numbers of exchangeable hydrogens. Finally, the peptide is of intermediate size (length) providing a sufficient number of exchangeable hydrogens to distinguish

conformers as well as producing dominant conformations for given charge states and lower-intensity conformations (for triply-charged ions) that can be examined by IMS and HDX.

2.2.2. Sample preparation.

The synthetic peptide KKDDDDDIKK was purchased (Genscript Corp., Piscataway, NJ, USA) and used without further purification. Ultra-pure (chromatography grade) deionized water, acetonitrile, and formic acid (Fisher Scientific, Fair Lawn, New Jersey, USA) were used to generate stock and ESI solutions of the peptide. The stock peptide solution was prepared by dissolving 1.0 mg of peptide in 1.0 mL of ultra-pure water and was capped in a glass vial and maintained in a refrigerator (4 °C); no stock solution was used a week after preparation. ESI solutions (0.1 mg·mL⁻¹) were prepared daily by diluting 0.1 mL of stock solution with addition of 0.4 mL of water and 0.5 mL of acetonitrile. Then ~1 µL of formic acid was added to the solution. Samples were infused through a pulled-tip capillary using a flow rate of 300 nL·min⁻¹.

2.2.3. Ion mobility measurements.

The use of IMS for biopolymer analysis has an extensive history [14, 15, 22, 24, 48-50]; the development of IMS instrumentation [51-56], applications [53, 57-62] and theory [63-66] have been discussed in detail. Only a brief description of the recording of IMS-MS datasets using the current instrumentation is presented here. Figure 2.1 shows a schematic diagram of the drift tube, linear ion trap instrument used to conduct these experiments. The schematic notes the sequential positions of ion funnels (F), ion gates (G), and ion activation (IA)

regions. The ESI source, desolvation region, and drift tube were home built and the linear ion trap (LTQ Velos) was purchased (ThermoScientific, San Jose, CA, USA). The instrument configuration is similar to that reported previously [62, 67-69]. Peptide ions were generated by electrospraying a solution through a pulled-tip capillary. The capillary was biased at $\sim +2200$ V above the entrance aperture of the desolvation region (Figure 2.1). Ions were desolvated and focused into an “hour-glass” ion funnel [56], ion trap device (F1/IA1/G1 in Figure 2.1). Ions were trapped near the exit region of the first ion funnel (F1) and periodically pulsed into the drift tube. The home-built, 1-m-long drift tube was filled with He buffer gas (300 K) and supported a drift field of ~ 12 V $\cdot$ cm $^{-1}$. Ions separated in the drift tube based on differences in their mobilities through the buffer gas. Those of specific mobilities were selected via the second ion gate (G2 in Figure 2.1) for transmission into the linear ion trap for mass analysis.

Drift time (t_D) data were recorded using scanned delay times between the high-voltage pulse applied to the drift tube entrance gate (G1) and the mobility selection gate (G2). In the current instrument, G1 is a gridded lens that is separated from the last lens of F1 (Figure 2.1) by a teflon insulator (2 mm). G2 is a Tyndall gate consisting of two gridded lenses spaced apart by 3 mm and located directly in front of the second ion funnel (F2 in Figure 2.1). G1 prevented ions from entering the drift tube using a ~ 20 to 40 V bias relative to the last lens of the first funnel. Periodically (20 ms), a 150 μ s-long voltage pulse was applied to G1 to lower the voltage 20 V below the exit lens of the ion funnel. The application of this pulse was synchronized to the mobility selection pulse applied

to G2. Here a bias differential of ~ 10 V neutralizes all ions with the exception of those selected for transmission by the time delay between voltage pulses at G1 and G2. The timing of G1 and G2 was controlled by two four-channel digital/delay pulse generators (Stanford Research Systems, Sunnyvale, CA, USA). t_D delays between G1 and G2 were scanned from ~ 3 ms to ~ 15 ms (200 μ s increments) to encompass the entire t_D range of all ions produced by ESI. Data acquisition was accomplished by collecting a mass spectrum (0.5 min) for each t_D selection setting.

Figure 2.1. Schematic diagram of the instrument used in the current studies. The abbreviations F, G, and IA represent ion funnel, ion gate, and ion activation regions, respectively.

Buffer gas pressure was measured with a Baratron capacitance manometer (MKS, Andover, MA, USA). Prior to IMS measurements, the buffer gas was evacuated from the drift tube and the pressure gauge was zeroed at the base pressure. Collision cross sections (Ω) recorded for protein (five conformers of cytochrome *c*) and peptide ions (3 conformers of bradykinin) agreed ($1.5\% \pm 0.5\%$) with values reported previously [58, 70] ensuring that pressure, temperature, and electric field values were recorded accurately.

2.2.4. Mass spectrometry measurements.

Mass spectra were generated for all ions by setting the gates to pass all ions. In this operational mode, the drift tube acted to transmit all ions into the mass analyzer. The LTQ Velos (ThermoScientific, San Jose, CA, USA) mass analyzer scan parameters utilized a m/z range of 80 to 2000 as well as automatic gain control (AGC). The AGC threshold was 1×10^6 ions. For these analyses, sample injection times of 200 ms (5 microscans) were utilized. During t_D distribution generation, the ion gates in the drift tube were activated to transmit ions of a specific mobility into the linear trap. For these analyses, the AGC was disabled and a sample injection time of 200 ms (5 microscans) was also employed.

2.2.5. Generation of IMS-MS datasets and extracted ion drift time distributions (XIDTD).

To generate the two-dimensional IMS-MS datasets, each t_D -selected mass spectrum (.RAW file) was converted to a separate text file using the Xcalibur software suite (ThermoScientific, San Jose, CA, USA). All x and y data points (m/z values and intensities) were then associated with the respective mobility selection time (t_D) using software developed in house. The software was written in C++ using the Visual Studio 2007 software suite (Microsoft Corporation, Redmond, WA, USA). The executable was accessed through Windows 7.0 (Microsoft Corporation, Redmond, WA, USA) and the program functioned from the command prompt where user-defined parameters (intensity threshold, t_D start and increment, and m/z increment) were input. The program read in the data

points from the individual mass spectra and created a three-column array text file containing t_D , m/z , and intensity (i) values. Intensity filters were applied to control the size of the three-column array file. To generate XIDTDs for ions of specific m/z values [68], a separate program developed in house was used. This program was also written in C++ using the Visual Studio 2007 software suite (Microsoft Corporation, Redmond, WA, USA). The executable was accessed through Windows 7.0 (Microsoft Corporation, Redmond, WA, USA) and the program functioned from the command prompt where user-defined parameters (i threshold, t_D minimum and maximum values, and m/z minimum and maximum values) were input. The program integrates all intensities within the user-defined m/z range for each t_D window across the t_D range.

2.2.6. HDX experiments.

HDX in the gas phase was accomplished by introducing D₂O (Sigma Aldrich, St. Louis) into the drift tube buffer gas system. D₂O was subjected to several freezing and melting cycles under vacuum to remove contaminant gas. D₂O and He pressures were adjusted with leak valves (Granville Phillips, Longmont, CO, USA) and monitored using a Baratron capacitance manometer (MKS, Andover, MA, USA). For these experiments, the helium pressure was set at 2.50 ± 0.01 Torr. The partial pressure of D₂O was varied from 0.05 Torr to 0.32 Torr. Two-dimensional (2D) $t_D(m/z)$ distributions were initially recorded in pure He to allow the determination of accurate peptide ion collision cross sections. With the addition of D₂O, the XIDTDs were monitored to ensure no conformational transitions occurred; that is, the t_D distributions of the different conformers simply

shifted to longer t_{DS} as demonstrated previously [41]. Additionally, the deuterium for the different ions was recorded at different D₂O pressures. Uptake increased to maximum values at ~0.06 Torr partial pressure of D₂O and did not increase significantly (up to 0.32 Torr). Therefore, mobility selection and deuterium exchange site determination was conducted at ~0.06 Torr of D₂O to allow for increased measurement sensitivity. The partial pressures of the components of the buffer gas system were stable differing by less than ± 0.01 Torr. Upon removal of the D₂O, the total pressure quickly equilibrated to 2.50 Torr (pure He). Achieving the same base pressure coupled with the stability of the pressure of the He/D₂O mixture confirms that the partial pressures of D₂O and He remain constant during the measurement.

2.2.6. Peptide ion dissociation with ETD.

Residue specific deuterium uptake determination experiments were conducted using ETD characterization of mobility-selected conformations. To accomplish ion dissociation, the ETD source filament was set at 90°C and the reagent chamber pressure was increased to 20×10^{-5} Torr using N₂ gas. Anions were produced using Fluoranthrene reagent (ThermoScientific, San Jose, CA, USA). Precursor ions were isolated using variable window sizes centered on the isotopic distribution for the different ions. The ion injection time was maintained at 200 ms (5 microscans) for the ETD measurements. The m/z isolation window was variable and set to encompass the precursor ion isotopic envelope. Ion fragmentation mass spectra were generated by varying the total collection time (ETD-MS acquisition times of ~5 to ~20 minutes) for each mobility-selected (300

μ s wide) conformer. These times correspond to a maximum of ~6000 mobility selections to generate the mass spectrum.

2.2.7. Deuterium uptake levels.

The number of deuteriums incorporated into specific ions (precursor or fragment) was determined by subtracting the average m/z value for each c- and z-ion from the average m/z value of the same ion after undergoing HDX.

Average m/z values were determined using a simple algorithm developed in house. This program was also written in C++ using the Visual Studio 2007 software suite (Microsoft Corporation, Redmond, WA, USA). The executable was accessed through Windows 7.0 (Microsoft Corporation, Redmond, WA, USA) and the program functioned from the command prompt where each m/z point within a user-defined isotopic distribution was weighted by its i ; the i values and $m/z \cdot i$ products were summed and the average m/z was then computed by dividing by the products by the summed i values.

2.2.8. Molecular dynamics simulations.

MD experiments required the construction of $[M+3H]^{3+}$ and $[M+4H]^{4+}$ KKDDDDIIKLIK peptide ions. Charge sites for the different ions were selected based on relative gas-phase basicities of the amino acid residues and Coulomb Energy minimization. For quadruply-charged ions, the ϵ -nitrogens of K1, K2, K10, and K13 residues were protonated. For the triply-charged ions, the ϵ -nitrogens of K1, K10, and K13 residues were protonated. It is noted that the alternate charge site configurations (K1, K2, K10 and K2, K10, K13) were also tested for triply-charged ions and yielded ion structures that were typically 20% to 30% too large

for comparison to IMS data. For each charge state, two starting structures were utilized. The first structure represented an alpha helix by setting ϕ and ψ angles of -48 and -57. The second structure represented a linear/extended ion with ϕ and ψ angles of 135 and -140, respectively. These initial structures were optimized at a quantum chemical level of HF/6-31G(d) and used for calculating molecular electrostatic potential (MEP) values and charge fitting performed with the R.E.D. Server [71-76]. The AMBER force field FF96 [77] was employed to generate the initial extended structures for molecular dynamics. Simulated annealing was performed in a manner similar to that described previously [15, 78]. An energy-minimized conformer was subjected to simulated annealing for 40 ps using the AMBER12 package [79]. Here the conformer was subjected to dynamics at 1000 K. Then the temperature was gradually decreased to 50 K and the structure was subsequently energy-minimized. 1000 cycles of annealing were conducted for 40 ps each in this manner where the ending, energy-minimized structure from one cycle was subjected to dynamics at 1000 K for the next cycle. The 1000 cycles of annealing were carried out with no non-bonded cutoffs to generate 1000 candidate structures. Collision cross section calculations were performed for the 1000 conformers using the mobcal [80] software employing the trajectory method (TM) [81]. The total molecular energies of the 1000 conformers were plotted as a function of collision cross section. Representative low-energy structures with matching (<1% - near that of the experimental accuracy) collision cross sections were selected for comparisons to experimental data.

2.3. Results and Discussion

2.3.1. Gas-phase deuterium uptake.

Upon electrospraying the model peptide KKDDDDDIKK, the predominant species produced are the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions as shown in Figure 2.2. Because of the decreased intensity of $[M+2H]^{2+}$ ions (not shown in Figure 2.2) as well as the inability to obtain useful information regarding amino acid sites of deuterium uptake via ETD, characterization of doubly-charged ions by HDX-ETD-MS has not been pursued. Figure 2.2 also shows the uptake of deuterium for the quadruply- and triply-charged ions. Addition of D_2O to the drift tube, results in shifts of the m/z values of the various ions to higher values indicating the incorporation of deuterium. From the observed mass shifts, the deuterium uptake for the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions is determined to be 12.80 ± 0.01 and 15.32 ± 0.03 , respectively. The total number of exchangeable hydrogens on the neutral KKDDDDIKK peptide is 28 (4, 3, 2, 2, 2, 2, 1, 1, 3, 1, 1, and 4 for the respective residues). Thus, after accounting for the number of protons for each ion, these values represent ~40% and ~50%, respectively, of the total available hydrogens for exchange. Here we note that these initial assessments have been performed without employing mobility selection.

Therefore the determined uptake values are for ensembles of greater conformational variety for each ion.

Figure 2.2. Mass spectra obtained upon electrospraying the model peptide KKDDDDDIKK. The bottom and top traces represent conditions in which 0 and 0.08 Torr, respectively, of D_2O is added to the 2.50 Torr of He buffer gas. The ions characterized by IMS-HDX-MS are labeled in the bottom trace. The average m/z value for the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions (not mobility selected) are 390.96 and 520.97, respectively. The respective m/z values for the ions that have been subjected to HDX are 394.17 and 526.08.

In recording the deuterium uptake by different ions, it is instructive to consider past comparisons between HDX performed in a drift tube and that performed in a FTICR mass spectrometer [41]. For both types of studies, maximum exchange levels were determined upon reaching a pressure-reaction time product point in which increased numbers of collisions with D_2O do not result in appreciably higher exchange levels. For protein ions these exchange

levels were observed to occur at lower values for ion mobility studies compared to those recorded in a FTICR instrument [13, 34, 40, 41]. Even though the trap times of the FTICR experiments were significantly longer (10 to 100 ×) than the t_D s of various ions, the much higher pressure of D₂O used in drift tube studies ensured similar numbers of ion-neutral collisions. Therefore, the differences in exchange levels were largely attributed to longer timescale molecular fluctuations that were not sampled on the short timescale of the drift tube measurement. As with the previous drift tube studies [40, 41], it is expected that the observed deuterium uptake represents a “snapshot” of rapidly exchanging hydrogens for each conformation.

2.3.2. Peptide ion collision cross sections.

The XIDTDs obtained for the various ions can be used to determine collision cross sections using the expression [82]:

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N} \quad (2.1).$$

In Equation 2.1, k_B , T , and ze correspond to Boltzmann’s constant, the buffer gas temperature, and the ion charge, respectively. m_I and m_B are the mass of the ion and the mass of the buffer gas, respectively. The variables t_D , E , L , P , and N correspond to the measured t_D , electric field, length of the drift tube, pressure of the buffer gas and the neutral number density at standard temperature and pressure (STP), respectively. Figure 2.3 shows the collision cross section distributions for the ions that have been studied; the distribution is obtained by determining the corresponding collision cross section (Equation 2.1) for each t_D window obtained from the individual XIDTDs.

Figure 2.3. Collision cross section distributions (left panel) for the [M+4H]⁴⁺ (bottom trace) and [M+3H]³⁺ (top trace) ions. These distributions are produced by applying Equation 2.1 to each t_D window obtained from an XIDTD (see Experimental section). [M+3H]³⁺ ion conformers for which MS/MS information is shown are indicated by the letters “a” and “b”. Expanded regions of MS/MS spectra showing c_{12} ions for mobility-selected [M+4H]⁴⁺ (bottom trace) and [M+3H]³⁺ (top trace) ions are shown in the panel on the right.

The collision cross section distributions (Figure 2.3) show that the [M+4H]⁴⁺ ions produce the most elongated conformations with a dominant feature corresponding to a collision cross section of 418 Å². A separate, lower-intensity conformation is observed at 471 Å². For the [M+3H]³⁺ ions, a dominant conformation is observed at 340 Å². A shoulder is also observed with a maximum intensity at a collision cross section of 367 Å².

In examining the HDX characteristics of different ion conformations, the addition of D₂O to the drift buffer gas causes the ions to shift to longer t_D values

without significantly altering the proportion of ion conformations for each charge state. Figure 2.4 shows the resulting distribution for the triply-charged ions upon addition of ~ 0.06 Torr D_2O . For the HDX experiments, species with collision cross sections corresponding to the compact $[M+3H]^{3+}$ ions ($340 \text{ \AA}^2$), the partially-folded $[M+3H]^{3+}$ ions ($367 \text{ \AA}^2$), and the elongated $[M+4H]^{4+}$ ions are mobility-selected prior to ETD-MS analysis. A question arises regarding the conformational purity that can be obtained from the mobility selections. Figure 2.4 also shows the features that would be obtained by theoretical transport of single ion conformations [82] for compact $[M+3H]^{3+}$ ions ($340 \text{ \AA}^2$) and partially-folded $[M+3H]^{3+}$ ions ($367 \text{ \AA}^2$). Based on this analysis it is evident that little contamination of one selection by the other conformer is likely. That said, because the partially-folded conformer cannot be resolved from ions with overlapping mobilities, its selection includes a greater number of ions with different conformations. However, these species would all have larger collision cross sections than the compact $[M+3H]^{3+}$ ions ($340 \text{ \AA}^2$) and would be similar in size making it possible for comparisons to ion structures types obtained by molecular dynamics simulations (see below).

Figure 2.4. t_D distribution (solid trace) of the $[M+3H]^{3+}$ ions produced after D_2O has been introduced into the He buffer gas. The red arrow shows the t_D of the dominant feature in the He only data. Black arrows show the positions of the mobility-selections for the ETD-MS analysis of compact and partially-folded conformations. The small- and long-dashed line traces represent the transport of single conformations for ions that have the same mobilities as the compact and partially-folded ions, respectively.

2.3.3. Determining deuterium uptake per residue.

To determine the uptake differences of the specific amino acid residues for the various ion conformers, ETD experiments have been conducted in which only ions associated with specific conformers are dissociated. The full ETD spectra of the various ion conformations exposed to D_2O are shown in Figure 2.5 in brief, upon dissociation each conformer presents complete homologous c- and z-ion series. By examining the mass shifts for the c- and z-ions produced by ETD it is possible to determine differences in the uptake of deuteriums by the same amino acid residues for specific ion conformers.

Figure 2.5. Total deuterium uptake for singly-charged, complementary c- and z-type ions of compact $[M+3H]^{3+}$ ions.

Figure 2.3 shows an example of the isotopic distributions for the c_{12} ions produced from the dominant ion conformers for the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ ions

after HDX. For the quadruply-charged conformer having a collision cross section of $418 \text{ \AA}^2$, the average number of deuteriums incorporated into the c_{12} ion is 11.0 ± 0.1 (Figure 2.3). Deuterium incorporation by the more elongated quadruply-charged ions could not be determined due to low signal levels. In comparison the average numbers of incorporated deuteriums for the more compact of the $[M+3H]^{3+}$ ions is 13.58 ± 0.08 . The increased deuterium uptake for the triply-charged ions compared with the quadruply-charged ions is highlighted by the m/z shift in the isotopic distributions shown in Figure 2.3. The deuterium uptake for each of the observed c-ions (which comprised nearly a complete homologous series for each conformer) is reported in Table 2.1. Notably, the z-ion series shows very similar uptake levels for the same peptide regions as indicated in Figure 2.6.

Figure 2.6. Total deuterium uptake for singly-charged, complementary c- and z-type ions of compact $[M+3H]^{+3}$ ions.

Table 2.1. Deuterium uptake for each fragment ion produced by ETD of select precursor ion conformations.

Ion ^a	$\Omega(\text{\AA}^2)^b$	Fragment Ion ^c											
		C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	C ₉	C ₁₀	C ₁₁	C ₁₂
[M+4H] ⁴⁺	418±4	3.3±0.1	5.0±0.1	5.1±0.1	6.6±0.1	7.2±0.2	7.50±0.06	8.62±0.08	9.0±0.3	9.4±0.2	10.0±0.1	10.1±0.2	11.0±0.1
[M+3H] ³⁺	340±3	3.49±0.02	5.07±0.03	6.13±0.07	6.54±0.03	8.11±0.03	9.21±0.05	10.1±0.1	10.5±0.1	10.78±0.06	12.15±0.03	12.5±0.2	14.04±0.03
[M+3H] ³⁺	366±3	3.34±0.04	4.79±0.08	5.91±0.08	6.22±0.04	6.63±0.06	8.7±0.1	9.4±0.2	9.6±0.1	10.10±0.07	11.6±0.2	11.9±0.2	13.58±0.03

^aIons produced upon electrospraying the peptide KKDDDDDD.

^bCollision cross section as determined from XIDTDs (Figure3) using Equation 1.

^cFragment ions produced upon precursor ion dissociation. Deuterium uptake is recorded as the difference between the average mass of the indicated ion and the average mass of the same ion produced after HDX. Errors represent deviation about the mean.

To determine differences in accessible exchange sites associated with the various ions and ion conformations, it is instructive to determine the number of incorporated deuteriums for each amino acid residue. This is accomplished for the i^{th} residue by subtracting the deuterium uptake for the $c_{(i-1)}$ ion from the c_i ion. In this manner the deuterium uptake for the different amino acid residues can be ascertained and compared across all ions. The exceptions are the 1st and 13th residues for which the c_1 and z_1 ion deuterium uptake can be used, respectively. Figure 2.7 shows such a comparison for the elongated (418 Å²) [M+4H]⁴⁺ ions and the more compact (340 Å²) [M+3H]³⁺ ions. In comparison to the triply-charged ions, the quadruply-charged ions exhibit less uptake for a number of residues including D3, D5, D6, K10, and I12. For only one residue, D4, is an increase in uptake observed for the quadruply-charged ions relative to the triply-charged ions. The sum of the decreased uptake values for the D3, D5, D6, K10 and I12 residues is consistent with the observed difference in overall uptake between the conformers for these [M+4H]⁴⁺ and [M+3H]³⁺ ions (Figure 2.2).

The deuterium uptake by residue comparison of more compact (340 Å²) and more elongated (367 Å²) conformers of [M+3H]³⁺ ions is shown in Figure 2.8. In general the deuterium uptake levels are similar for the amino acid residues of the two conformers. Notable exceptions are the D5 and the D6 residues. For those respective residues, the compact and elongated conformers exhibit greater uptake compared one to another. The K13 residue is also observed to exhibit

increased uptake for the compact conformer. Of note in the comparison of the two $[M+3H]^{3+}$ conformers is the observation that the I12 residue incorporates more (>1 on average) deuteriums than the I8, I9, and I11 amino acid residues. Interestingly, the per-residue analysis can only show an increase of more than 1 deuterium for isoleucine if the donated species on formation of the c_{12} ions (ETD mechanism) [38, 83, 84] was more predominantly a deuterium. Finally, compared to all other aspartic acid residues of these two ion conformers, the D4 residue exhibits a relatively decreased level of uptake for both conformers.

Figure 2.7. Bar graph showing the deuterium uptake by each amino acid residue for $[M+4H]^{4+}$ (blue) and $[M+3H]^{3+}$ (red) ion conformers having collision cross sections of $418 \text{ \AA}^2$ and $340 \text{ \AA}^2$, respectively. Error bars represent propagation of the error (one standard deviation, $N = 3$) provided in Table 2.1. The total number of exchangeable hydrogens for each residue is included on the horizontal axis label (including protons on charge sites).

Figure 2.8. Bar graph showing the deuterium uptake by each amino acid residue for more elongated $[M+3H]^{3+}$ (blue) and more compact $[M+3H]^{3+}$ (red) ion conformers having collision cross sections of $387 \text{ \AA}^2$ and $340 \text{ \AA}^2$, respectively. Error bars represent propagation of the error (one standard deviation, $N = 3$) provided in Table 2.1. The total number of exchangeable hydrogens for each residue is included parenthetically on the horizontal axis label (including protons on charge sites).

An accurate per-residue analysis requires that no HD scrambling occur during the measurement. HD scrambling would result in a reappportioning of the deuterium label to individual residues based upon the statistical representation of the number of exchangeable sites [39, 46, 47]. Therefore, residues of the same type would not be expected to exhibit large differences in deuterium incorporation. This is not the case in the present data. As an example consider the case discussed above where one I residue exhibited significantly greater

deuterium uptake than the other three I residues. A more detailed comparison of the experimental deuterium uptake by residue to that expected from extensive scrambling is presented in the Figure 2.9.

Figure 2.9. Per-residue deuterium uptake plots showing the experimental uptake by the elongated $[M+4H]^{4+}$ ions having a collision cross section of $418 \text{ \AA}^2$ (diamond symbols) and the uptake expected for significant HD scrambling (square symbols).

2.3.4. Comparison to hypothetical ion structures from MD.

The simulated annealing investigations provided a number of ion structures for comparison to the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ ion conformers. Figure 2.10 shows representative, low-energy structures of compact and partially-folded (triply-charged) and elongated (quadruply-charged) ions.

Figure 2.10. Schematic representation of ion structures obtained from simulated annealing studies performed by MD. Low-energy structures having collision cross sections matching the experimentally observed conformational types (compact, partially folded, and elongated) are represented here. Ion charge state and collision cross section labels are provided for each structure.

The respective calculated collision cross sections for these ions are 337 Å², 367 Å², and 420 Å². Experimental values for the compact [M+3H]³⁺, partially-folded [M+3H]³⁺, and elongated [M+4H]⁴⁺ ions are 340 Å², 367 Å², and 418 Å², respectively.

To draw correlations between the computer-generated structures and the experimentally determined deuterium uptake levels, it is worthwhile to consider the mechanism for gas-phase HDX by D₂O. Exchange proceeds via a relay mechanism requiring a long-lived reaction intermediate involving interactions between the D₂O molecule and a charge site as well as a less basic site on the peptide ion [35]. Deuterium is incorporated by proton abstraction from the charge site and concomitant transfer of deuterium to the less basic site. Therefore, deuterium incorporation at a specific residue, to some degree, indicates accessibility to a charge site [36, 40].

Molecular dynamics studies of cytochrome *c* ions have shown that a highly-compact ion of nearly half the charge can allow access to more exchangeable hydrogens than a more elongated ion [40]. This is due to the positioning of the charges within closer proximity (including access resulting from molecular fluctuations of a highly dynamic species) to exchange sites. A similar charge-site/exchange-site density argument can be used to help explain the HDX behavior of the peptide ions presented here. Consider the compact [M+3H]³⁺ ion structure (337 Å²) shown in Figure 2.10. Noticeably, the charge sites are all observed to interact with electronegative groups on interior residues. For example, the K1, K10, and K13 residues are primarily charge solvated by the D4,

D7, and D3 residues, respectively. Accommodating this charge solvation requires that the molecule adopt a more globular conformation. The partially-folded $[M+3H]^{3+}$ ion structure ($367 \text{ \AA}^2$) shows that only K10 and K13 residues are charge solvated on the interior (D7 and D5 residues, respectively) while the K1 residue protrudes away toward the N-terminus. The elongated $[M+4H]^{4+}$ ion structure ($337 \text{ \AA}^2$) shows an arrangement of charge sites where the average distance between the charge sites is maximized to reduce Coulomb repulsion.

By setting an exchange site accessibility threshold of $5 \text{ \AA}$ as used in previous studies [36, 40], a qualitative match to exchange levels for the three different structures is achieved. For example, the number of accessible hydrogens (excluding those on the charge sites –see below) for the compact $[M+3H]^{3+}$ ion structure ($337 \text{ \AA}^2$) is 8. For the partially-folded $[M+3H]^{3+}$ ion structure ($367 \text{ \AA}^2$) this number decreases to 7. The elongated $[M+4H]^{4+}$ ion structure ($337 \text{ \AA}^2$) exhibits the least number of accessible hydrogens with a value of 5. This ordering of exchange site accessibility scales with the experimentally determined maximum exchange levels of 15.5, 14.9, and 12.9 for the compact $[M+3H]^{3+}$, partially-folded $[M+3H]^{3+}$, and elongated $[M+4H]^{4+}$ ions.

Although the charge-site/exchange-site accessibility model provides a qualitative demonstration of increased accessibility with an overall decrease in ion size, there are limitations to its utility for structural analysis. The model would indicate that all hydrogens on the lysine charge sites would exchange. The experimental data show that this is not correct. For example, on average, the K10 residue of the elongated $[M+4H]^{4+}$ ion conformer only exchanges ~15% of all

hydrogens. The inability of the exchange distance method to model the observed HDX behavior can be further demonstrated with a per-residue comparison. Figure 2.11 shows the predicted uptake per residue for each ion structure using the 5 Å distance method for determining accessibility. Here the data has been scaled to the experimentally observed maximum exchange values. Also shown are the experimentally determined uptake values. In general, the model significantly overestimates the uptake at the K10 and K13 charge sites for all conformations. For the compact $[M+3H]^{3+}$ ions, the model significantly underestimates the uptake at the K2, D5, and I12 residues while overestimating that of the D3 residue. For the partially-folded $[M+3H]^{3+}$ ions, the model significantly underestimates the uptake of the D3, D6, and I12 residues while that of the I8 residue is significantly overestimated. Finally, for the elongated $[M+4H]^{4+}$ ions, the uptake for all lysine residues is significantly overestimated while essentially no accessibility is estimated for multiple residues (D3, D4, D6, I8, I9, I11, and I12). Therefore, although the model demonstrates the concept that increased charge-site/exchange-site density can indeed explain increased HDX levels for compact ions, a better model is required to explain the differences in uptake among the individual amino acid residues.

A separate model has been investigated to account for the decreased exchange for many of the lysine residues as well as the increased exchange on the interior of the molecule (especially the elongated $[M+4H]^{4+}$ ions). Here, two distances are used to make estimates of exchange site accessibility. The first distance is that from the charge site to less basic carbonyl sites (i.e., the location

where the original transfer of the deuterium occurs according to the relay mechanism) [35]. The next distance used is that from the accessible carbonyl oxygen to heteroatom hydrogens. A value of 7 Å has been used for both distance thresholds. Additionally, to consider the decreased flexibility of these carbonyl sites, a weighting equal to the inverse of the distance is applied to both distance factors. The calculation results using this model are also shown in Figure 2.11 for the three different ion conformers.

Figure 2.11. Dot plots showing the deuterium uptake per residue for IMS-HDX-MS experiments (solid symbols) and structure models (open symbols). Results for the 5 Å distance threshold are shown on the left where the top (A1), middle (B1), and bottom (C1) plots represent analyses for the compact $[M+3H]^{3+}$ ions, the partially-folded $[M+3H]^{3+}$ ions, and the elongated $[M+4H]^{4+}$ ions, respectively. Results for the two-distance threshold model are shown on the right where the top (A2), middle (B2), and bottom (C2) plots represent the same analyses as those on the left.

As with the first model, the exchange levels have been scaled using the experimentally determined maximum exchange value for each ion conformer.

The new model significantly improves the estimate of accessibility of the K10

residue for all conformers. Additionally the model better estimates the relative exchange values for the aspartic acid residues and isoleucine residues with the exception of the I12 residue. Other exceptions include the D4, D6, and D3 residues of the compact $[M+3H]^{3+}$, partially-folded $[M+3H]^{3+}$, and the elongated $[M+4H]^{4+}$ ions, respectively. A comparison of RMSD values for the two models shows the relative improvement in agreement for the second, two-distance model. RMSD values for the first and second models are 4.4 and 2.2 (compact $[M+3H]^{3+}$ ions), 3.3 and 2.4 (partially-folded $[M+3H]^{3+}$ ions), and 2.7 and 2.1 (elongated $[M+4H]^{4+}$ ions), respectively.

Comparisons such as that shown in Figure 2.1 reveal some of the challenges encountered in using molecular dynamics simulations to obtain structural information supporting IMS and HDX data. Because a simple distance threshold model cannot account for the observed uptake levels of individual amino acid residues, more extensive molecular dynamics simulations are required. Based on the preliminary comparisons presented here, several factors should be considered in the upcoming studies. First, distance thresholds such as those described above can be explored using MD. That is, different values can be examined using all structures from continuous MD (300 K) of a single structural type. Second, scaling factors can be introduced to account for facile deuterium transfer from the less basic site to other sites within the ion (e.g., differences in gas-phase basicity). Third, another factor representing collision frequency can be incorporated based on the frequency with which an exchange site satisfies the distance criteria above. Clearly many parameters can be tested

to obtain better comparisons between IMS-HDX-MS results and structures obtained from MD. One approach currently being pursued to aid this process is the determination of exchange rates of individual amino acid residues for specific ion conformations. This is accomplished by monitoring the per-residue uptake for conditions utilizing many different partial pressures of D₂O. These data may be used in the future to tailor the criteria (e.g., the facile deuterium transfer factor) for MD comparisons.

2.4. Conclusions

The gas-phase conformations of ions produced by electrospraying the model peptide KKDDDDDIKK have been examined by IMS coupled with gas-phase HDX-MS techniques. The various ion conformations comprising the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions are observed to exhibit different levels of total deuterium uptake. In general, more compact ions exhibit increased levels of exchange. Ion structures obtained from MD suggest that the increased HDX levels for compact structures could result from increased charge-state/exchange-site density even though there are fewer charges on the more compact ions. Comparisons to the computer generated structures also indicate that a simple model employing a charge-site/exchange-site distance threshold for exchange cannot account for the observed per-residue deuterium uptake levels. A new model that incorporates additional distance constraints is able to better capture the per-residue deuterium uptake and demonstrates that in the future a multi-factor model is necessary to merge structural information from IMS-HDX-MS experiments and MD simulation studies.

2.5. References

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3. Gas-phase hydrogen-deuterium exchange labeling of select peptide ion conformer types: a per-residue kinetics analysis ¹

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3.1. Introduction

Shortly after the advent of the soft ionization techniques of electrospray ionization (ESI) [1] and matrix-assisted laser desorption ionization (MALDI) [2, 3], gas-phase hydrogen deuterium exchange (HDX) techniques [4-7] were introduced for the characterization of protein ion structure. Early experiments demonstrated the presence of coexisting ion conformations as evidenced by multiple exchange rates and maximum levels of exchange [4]. Shortly later, the effects of ion heating on exchange rates and maximum exchange levels were investigated for individual charge states of cytochrome *c* ions [8, 9]. Additionally, experiments were designed to reveal the mechanism of HDX for a variety of deuterating agents and peptide ions [10]. Other experiments detailed HDX characterization of a variety of protein ions demonstrating the presence of multiple conformations as well as revealing factors that affect HDX of specific ions such as conformational rigidity [7]. Gas-phase HDX-MS experiments also extended to the characterization of other species including small organic molecules [11, 12], nucleotides [13-16], and peptides [17, 18].

The development of ion mobility spectrometry (IMS) techniques for distinguishing ion conformers of large biomolecular ions coincided with the early application of gas-phase HDX techniques [19, 20]. An early extension of the IMS studies was the combination of IMS and HDX characterization [21]. Here, the

HDX rates and maximum exchange levels were recorded for select ion conformation types (i.e., those distinguished by unique mobilities). These early studies demonstrated that compact protein ion conformers exchanged fewer hydrogens than more elongated ion conformers at room temperature presumably due to the inaccessibility of heteroatom hydrogens (exchange sites) resulting from the protein fold. However, temperature-dependent studies revealed increased levels of exchange for both conformer types such that at the highest temperatures studied, nearly complete exchange was observed for both [9]. Interestingly, for the elongated ions, protection of a number of sites was observed even at the highest temperatures utilized. The increased exchange level for the compact ions at elevated temperatures was explained as resulting from increased exchange-site accessibility by charge sites due to conformational fluctuation of these ion conformers.

Early IMS-HDX-MS studies sought to develop exchange models to refine the structural information obtained from the IMS measurements [5, 9]. For example, for bradykinin ions, molecular dynamics simulations were used to describe the observed exchange levels in terms of exchange site accessibility (to charge sites as well as to collisions with D₂O molecules) [5]. Subsequent experiments involving much larger cytochrome c ions utilized molecular dynamics (MD) simulations to demonstrate an exchange model that considered only the distance to charge sites [9]. For these studies, accessibility to D₂O was considered to be accounted for based on the fact that charge sites were located on exterior amino acid residues.

Despite the early progress in structure refinement, the IMS-HDX-MS approach received only limited application in part because of the inability to determine the exact location of deuterium incorporation. For example, ion fragmentation techniques such as collision-induced dissociation (CID), were implicated in the process of deuterium migration resulting in a scrambling of the structural label [22]. The advent of non-ergodic ion dissociation techniques such as electron capture dissociation (ECD) [23] and electron transfer dissociation (ETD) [24] presented the possibility of preserving the location of the structural label [25]. Early attempts at combining IMS-HDX-MS/MS approaches employed instrumentation that utilized a traveling wave ion guide (TWIG) apparatus [26]. These initial experiments revealed HDX protection for ubiquitin ions allowing the comparison to solution structures. A note of caution should be presented for the interpretation of gas-phase HDX data for experiments employing a TWIG device. Experiments [27] have shown that care should be taken in the selection of operational parameters as often the ions are heated during the separation process which should affect ion HDX rates and maximum exchange levels [9].

Recently we have demonstrated the use of drift tube IMS combined with HDX-MS/MS for the characterization of peptide ion conformers of the model peptide KKDDDDDIKK [28]. These experiments demonstrated the ability to determine the location of deuterium uptake for selected ion conformer types and yielded a model for HDX that can be utilized in conjunction with nominal structures obtained from MD to provide refined ion structural information. More recently, the IMS-HDX-MS approach has been used to characterize select

conformations of negatively-charged peptide and protein ions [29]. This work shows an ability to decipher the roles of site accessibility (to charge sites and D₂O) as well as conformational flexibility in the overall exchange process.

This work builds upon the IMS-HDX-MS experiments described above. Here, the concept of developing a model for gas-phase HDX is extended to include information obtained from kinetics studies. To do this, MS/MS experiments for peptide ion conformers are conducted at varying D₂O partial pressures in order to determine the rates of exchange of individual amino acid residues. We observe evidence for fast- and slow-exchanging processes for these residues as observed for the total exchange process of ion conformers. Conformer type- and residue-specific exchange rates are observed for a number of ions. This information has been used to improve our exchange-site accessibility model based upon comparisons to single structures (representing conformer type) obtained from MD yielding a 1.6 to 3.3 fold improvement in agreement (see below) between experiment and theory. Although timely kinetics experiments monitoring individual residue uptake as a function of reaction time have been performed in the past [18], to our knowledge, the work reported here presents the first HDX kinetics results for individual amino acids within a polypeptide chain for mobility-selected ion conformation types.

3.2. Experimental

3.2.1. Sample preparation.

The peptide KKDDDDDIKLIK (95% purity) was purchased (Genscript Corp., Piscataway, NJ, USA) and used without further purification. This peptide

was selected for these gas-phase HDX studies because of properties associated with charge site and exchange site locations as well as its ability to produce ions suitable for examination by ETD [28]. The ESI solution comprising $0.1 \text{ mg}\cdot\text{mL}^{-1}$ of the peptide in water:acetonitrile:formic acid (49.95:49.95:0.10 v/v/v) solution was prepared fresh from stock solution ($1.0 \text{ mg}\cdot\text{mL}^{-1}$ in pure water) daily. The samples were infused through pulled-tip capillary that was biased at +2200 V above the instrument entrance orifice. A flow rate of $300 \text{ nL}\cdot\text{min}^{-1}$ was used for these studies.

3.2.2. IMS-MS measurements.

Biomolecular ion structure analysis utilizing IMS has an extensive history [19, 20, 30-32]. The development of IMS instrumentation [20, 33-40], theory [41-45] and applications [46-50] is described in the literature. Here only a brief description of the IMS-MS instrument and its operation is presented. Figure 2.1 shows a schematic diagram of the instrument showing home-built components (ESI source, desolvation region, and drift tube) that have been coupled to a linear ion trap (LTQ Velos™, ThermoScientific, San Jose, CA, USA). The instrument configuration is similar to that described in previous reports [28, 29, 51]. Electrosprayed peptide ions were stored in a “hour-glass” ion funnel device [52] (Figure 2.1) where they were periodically (20 ms) pulsed into a 1-m-long drift tube filled with ~ 2.5 Torr of 300 K He buffer gas. A drift field of $\sim 10 \text{ V}\cdot\text{cm}^{-1}$ was employed for these studies. Using an ion gate positioned near the end of the uniform field in the drift tube (G2 in Figure 2.1), ions of select mobilities were transmitted into the linear ion trap for mass analysis. A Baratron capacitance

manometer (MKS, Andover, MA, USA) was used for buffer gas pressure determinations.

Two 4-channel digital delay pulse generators (Stanford Research Systems, Sunnyvale, CA, USA) were used to control the collection of mobility-resolved mass spectra. One generator was used to apply a high-voltage pulse to the first ion gate (G1 in Figure 2.1) and the second to apply a time-delayed pulse to G2. The first pulse initiates the drift time (t_D) measurement while the second pulse selects ions of given mobilities for transport into the mass spectrometer. t_D selection times were scanned from 5.7 to 9.0 ms using step increments of 100 μ s. Mass spectra were recorded for each delay time setting. To generate mass spectra for all ions, both gates were set to transmit operational modes. To collect the MS and MS/MS spectra the mass analyzer was scanned from 80 to 2000 and 140 to 2000 m/z , respectively. The latter scan range was automatically set by the LTQ Tune software suite (ThermoScientific, San Jose, CA, USA) upon selection of the precursor ions.

3.2.3. Generation of IMS-MS datasets and extracted ion drift time distributions (XIDTD) [51].

As described previously [28], each t_D -resolved mass spectrum was manually converted to a separate text file. An algorithm developed in house linked t_D values with each mass spectrum generating a three-column array text file of points with intensity (i) values above user-defined thresholds. Using a separate program developed in house, i values were integrated across user-defined m/z ranges at select t_D values. Plotting these integrated values as a function of t_D generated individual XIDTDs.

3.2.4. HDX measurements.

HDX measurements were accomplished as described previously [9, 21, 28, 29]. Briefly, after purifying (freeze/thaw cycles), D₂O was added to the He buffer gas using a leak valve (Granville Phillips, Longmont, CO, USA). The partial pressures of He and D₂O were monitored with the capacitance manometer. In the present studies the He pressure was set at 2.50 ± 0.01 Torr. The partial pressure of D₂O was varied from 0 to ~ 0.080 Torr. HDX rates are estimated considering pseudo first order kinetics [4, 9, 21]. The log of the number of remaining exchangeable hydrogens is plotted as a function of the product of D₂O partial pressure and ion t_D . The slopes of linear best fit lines to the data were used to determine HDX rates. To avoid discrepancies in HDX rates associated with transitions from fast- to slow-exchanging sites as well as HDX saturation, only data for a subset of the D₂O partial pressures were used in the rate calculation. Fast-exchanging hydrogens utilized data from D₂O partial pressures ranging from 0 to 0.008 Torr and slow-exchanging hydrogens employed values ranging from 0.015 to 0.060 Torr.

Admittedly, accurate measurement of partial pressure is difficult as the increased pressure from the added gas can affect the flow of the first gas. To verify D₂O partial pressure determinations, the rate constants of [M+7H]⁷⁺ and [M+8H]⁸⁺ cytochrome c ions have been determined to be 4.5 and 5.0×10^{-13} cm³·molecule⁻¹·s⁻¹. These values are in good agreement with those reported using FT-MS (5 and 7×10^{-13} cm³·molecule⁻¹·s⁻¹, respectively) [8] and IMS-MS measurements of [M+8H]⁸⁺ ions (between 4 and 7×10^{-13} cm³·molecule⁻¹·s⁻¹)

[21]. Therefore it is possible that the relatively small amount of added D₂O does not significantly alter the flow of He resulting from the 10³ pressure differential across its separate leak valve. Notably, the contributions of individual amino acid residues as described above are not as influenced by accurate partial pressure determinations as the kinetics data for the precursor ions and their fragment ions were collected sequentially ensuring exposure to the same D₂O partial pressures.

3.2.5. Peptide ion dissociation by ETD and residue deuterium uptake levels.

ETD characterization of mobility-selected conformer types was accomplished as described previously [28]. Precursor ions were isolated using varying *m/z* widths depending on their isotopic distribution. For ion dissociation, ion injection times of 200 ms (5 microscans) were utilized. Additionally, a total data collection time of 5 minutes was employed to generate the ETD mass spectrum. The average *m/z* value of fragment ions were compared before and after subjecting precursor ions to HDX to determine spectral shifts. Comparisons of the deuterium content of sequential fragment ions provided the uptake by individual amino acid residues [28]. The contributions of individual amino acid residues to overall conformer type HDX rate was determined by plotting the log of remaining HDX sites versus the product of D₂O partial pressure and *t_D*. The distinction here was that the number of remaining HDX sites was determined by subtracting the deuterium content of each individual residue at the separate D₂O partial pressure settings.

3.2.6. Modeling HDX kinetics and ion isotopic distribution.

An algorithm developed in house was used to determine the expected deuterium uptake by different precursor and fragment ions at specific partial pressures of D₂O. The results can be used to generate hypothetical isotopic distributions for precursor or fragment ions. In the calculation, the numbers of fast- and slow-exchanging hydrogens were first input for given ions. Next, the ion t_D , the time increment (t_i) for each calculation, and the rate constants for fast- and slow-exchanging (k_f and k_s , respectively) hydrogens were input. Values of k_f and k_s were determined at different D₂O pressures by multiplying the reported rates of exchange for each conformer type (Table 3.1 and Table 3.2) by the D₂O number density. In the following manner, the algorithm simulated the process of HDX as each individual ion within the population drifts through the drift tube. For a given ion at a given pressure of D₂O, at each t_i the fraction of exchange sites expected to have exchanged was calculated as:

$$1 - \frac{A}{A_0} \quad (3.1),$$

where

$$\frac{A}{A_0} = e^{-kt_i} \quad (3.2).$$

In Equation 3.1 the ratio A/A_0 represents the fraction of sites that remain (i.e., not exchanged). In Equation 3.2, k was either k_f or k_s depending upon which exchange site was under consideration. Next a random number was generated and a ratio of this random number to the total number of possible random numbers was computed. If the ratio were less than the fraction altered (Equation 3.1), then an exchange event for the given ion was recorded for a particular

exchange site. This process was repeated for all exchange sites as the ion was stepped through the entire t_D . Then these steps were repeated for a user-defined number of ions. Finally, the numbers of ions exhibiting various exchange levels were histogrammed and the output was saved as a text file.

3.2.7. Molecular dynamics simulations.

MD experiments were accomplished using a simulated annealing procedure described previously [20, 28]. A brief description is provided here. Using the AMBER12 package [53], representative structures of the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ peptide ions were first subjected to dynamics at 1000 K for 8 ps. Over 32 ps, the ion structures are cooled to 50 K and then energy minimized. One thousand energy-minimized structures were generated for the separate ions in an iterative fashion. For the 1000 conformers the mobcal [54] software employing the trajectory method (TM) [42] was used to obtain collision cross sections. Matching (<1% difference in collision cross section), low-energy ion structures were selected for comparisons to the experimental data.

3.2.8. Hydrogen accessibility scoring (HAS).

Each amino acid residue's score (S_R) was represented as the sum of the labile hydrogen scores (S_H) associated with a residue. These labile sites include the side-chain heteroatom hydrogens as well as the backbone amide hydrogen of the $i+1$ residue due to the location of the bond cleavage for c-ions. To calculate the hydrogen score, first, each oxygen on a carbonyl within 7Å of a charge site was scored (S_O) where the score was scaled as the inverse distance to the charge site (d_{O-N}). This was termed an HDX-active carbonyl oxygen

representing the initial site of deuterium incorporation [10]. In the case that the oxygen on the carbonyl was within this proximity (7Å) to multiple charge sites, the oxygen was scored as the sum of the inverse distances to the charge sites as shown in Equation 3:

$$S_O = \sum \frac{1}{d_{O-N}} \quad (3.3).$$

Second, each heteroatom hydrogen near (again within 7Å) the active carbonyl oxygen was scored as:

$$S_H = \sum \frac{S_O}{d_{H-O}} \quad (3.4).$$

In Equation 4, S_H is the hydrogen score and d_{H-O} is the distance between the hydrogen and the HDX active carbonyl. The residue score then was determined as:

$$S_R = \sum S_H \quad (3.5).$$

For comparison to the experimental results, the residue scores for the representative conformer types were normalized. The selection of parameters for this scoring procedure are discussed in greater detail below.

It is instructive to briefly consider the physical factors associated with the selection of the S_R parameters and the distances. First, the scaling of the S_O and S_H values with the inverse distances was intended to represent the decreased probability of exchange due to longer distances between the required peptide ion moieties forming the long-lived reaction intermediate [10] as well as the decreased conformational flexibility of the backbone carbonyl. The distance of 7 Å was selected because it provided the best fit to the experimental data (see discussion below). Second the summation within these two values was intended

to represent the increased likelihood of reaction intermediate formation for oxygens on carbonyls located near multiple charge sites (S_O score) and hydrogens located near multiple oxygens on “HDX-active” carbonyls (S_H score). Third, the amino acid residue score (S_R) contained the summation of hydrogens to reflect the relative contributions of individual residues to the exchange rate. Finally, to compare to experimental data, the S_R values were normalized to provide a value that can be directly compared to the normalized experimental rate contributions.

3.3. Results and Discussion

3.3.1. Peptide ion collision cross sections.

Figure 3.1 shows the two-dimensional (2D) IMS-MS dataset obtained upon electrospraying the model peptide. As shown in Figure 3.1, primarily the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ peptide ions are produced. For the $[M+3H]^{3+}$ ions, a dominant feature is observed at $t_D \sim 6.5$ ms while multiple unresolved features are observed at higher t_D values (~ 6.8 to ~ 7.2 ms). Of these unresolved features, the most abundant peak apex is observed at ~ 7.0 ms. The less abundant $[M+4H]^{4+}$ peptide ions yield two conformers having t_D values of ~ 5.9 ms and ~ 6.2 ms. For these studies, the HDX kinetics of individual amino acid residues have been determined for these four conformer types. Here we note that usage of the term conformer type. The HDX data suggest the presence of multiple conformers having similar mobilities (see discussion below). As in our previous studies, it is noted that, although the less mobile conformer types are not resolved from conformers of similar size, they are not contaminated by the dominant, high-

mobility ions. That is, the HDX data represent exchange for select conformer types distinguished by their separate t_D values.

Figure 3.1. Two-dimensional (2D) $t_D, m/z$ dataset for ions produced upon electrospraying the model peptide. The data are plotted on a raised-relief color map. Here the data have been normalized. The $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions are labeled. Asterisks (*) denote dataset features that were mobility selected to obtain conformer specific kinetics information as well as kinetics information for single amino acid residues.

XIDTDs obtained for the various ions can be used to determine collision cross sections using the expression [41]:

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N} \quad (3.6).$$

In Equation 3.6, the constants N , and k_B correspond to the buffer gas neutral number density at STP and Boltzmann's constant. ze , m_I and m_B are the ion's charge, the mass of the ion and the mass of the buffer gas, respectively. The terms T , E , L , and P correspond to the buffer gas temperature, the electric field, the length of the drift tube, and the buffer gas pressure, respectively. For the

[M+3H]³⁺ peptide ion conformers the high- and low-mobility features have collision cross sections of 340 Å² and 367 Å², respectively. The high- and low-mobility features for the [M+4H]⁴⁺ peptide ions have collision cross sections of 418 Å² and 446 Å², respectively. For given charge states, ions exhibiting comparatively smaller collision cross sections are termed more compact conformer types and those with larger collision cross sections are termed more elongated or more diffuse conformer types.

3.3.2. Determining HDX kinetics.

For each conformer type, to calculate the number of remaining exchange sites at each D₂O partial pressure setting, it is necessary to know the total number of accessible sites for each conformer. This requires that the experiment reach saturation conditions – the point at which “exchangeable” hydrogens have reacted. This term is noted in quotations as the data demonstrate that some accessible hydrogens reacting at very slow rates may not necessarily undergo complete exchange (see discussion of the I8 residue below).

Figure 3.2. a) Total deuterium uptake as a function of the product of D₂O partial pressure and t_D . This data was recorded for the compact $[M+3H]^{3+}$ ion conformer type (see Figure 1) of the model peptide. These data were obtained at varying D₂O partial pressures (0, 0.003, 0.005, 0.008, 0.010, 0.015, 0.035, 0.045, 0.060, and 0.080 Torr). b) Plot of the natural log of the number of remaining exchangeable hydrogens as a function of the product of D₂O partial pressure and t_D for compact $[M+3H]^{3+}$ ions. Linear fits of fast- and slow-exchanging hydrogens are shown. Data points representing transitions between fast- and slow-exchanging hydrogens as well as saturation conditions are shown as solid circles; they are not used in HDX rate determinations. Rate constants obtained from the slopes of the linear fits are provided in Table 3.1 and Table 3.2.

Figure 3.2a shows the total deuterium uptake of the more compact conformer type of the $[M+3H]^{3+}$ ions at each D₂O partial pressure setting. The number of incorporated deuteriums increases rapidly at low partial pressure settings and begins to plateau at ~15 incorporated deuteriums at higher pressures. The maximum HDX level obtained from the weighted isotopic distribution for the more compact $[M+3H]^{3+}$ ion conformer type is 15.4 obtained at a D₂O partial pressure of ~0.08 Torr. The maximum HDX level for the more

elongated $[M+3H]^{3+}$ and the more compact and elongated $[M+4H]^{4+}$ ion conformer types are 15.1, 13.4, and 13.7, respectively. These values are in good agreement with those reported earlier for the common ion conformer types [28]. The number of remaining hydrogens is simply the maximum exchange level minus the deuterium incorporation level at a given D_2O partial pressure setting.

Figure 3.3. a) Total deuterium uptake as a function of the product of D_2O partial pressure and t_D . These data were recorded for the more elongated $[M+3H]^{3+}$ ion conformer type (see Figure 3.2) of the model peptide. The data were obtained at varying D_2O partial pressures (0, 0.003, 0.005, 0.008, 0.010, 0.015, 0.035, 0.045, 0.060, and 0.080 Torr). b) Plot of the natural log of the number of remaining exchangeable hydrogens as a function of the product of D_2O partial pressure and t_D for the respective $[M+3H]^{3+}$ ions. Linear fits of fast- and slow-exchanging hydrogens are shown. Data points representing transitions between fast- and slow-exchanging hydrogens as well as saturation conditions are shown as solid circles; they are not used in HDX rate determinations. Rate constants obtained from the slopes of the linear fits are provided in Table 3.1 and Table 3.2 in the manuscript.

As mentioned above, to obtain a rate constant the logarithm of the remaining accessible sites versus the product of t_d and the partial pressure of

D₂O is plotted as shown in Figure 3.2b for the more compact, triply-charged ions.

Kinetics data for the other conformer types are shown in Figures 3. 3 to 3.5.

Figure 3.4. a) Total deuterium uptake as a function of the product of D₂O partial pressure and t_D . These data were recorded for the more compact $[M+4H]^{4+}$ ion conformer type (see Figure 3.2) of the model peptide. The data were obtained at varying D₂O partial pressures (0, 0.003, 0.005, 0.008, 0.010, 0.015, 0.035, 0.045, 0.060, and 0.080 Torr). b) Plot of the natural log of the number of remaining exchangeable hydrogens as a function of the product of D₂O partial pressure and t_D for the respective $[M+4H]^{4+}$ ions. Linear fits of fast- and slow-exchanging hydrogens are shown. Data points representing transitions between fast- and slow-exchanging hydrogens as well as saturation conditions are shown as solid circles; they are not used in HDX rate determinations. Rate constants obtained from the slopes of the linear fits are provided in Table 3.1 and Table 3.2 in the manuscript.

In general, all conformer types exhibited two linear regions in the kinetics plots corresponding to fast and slow HDX. As expected, for all conformer types, the onset of the second linear (slow) area occurs just prior to saturation conditions. The rate constants obtained from the kinetics plots for each ion conformer type are provided in Tables 3.1 and 3.2. It is noted that for the

reported kinetics data, all correlation coefficients from the plots are ≥ 0.9 .

Generally, the rate constants for fast exchange are usually one to two orders of magnitude larger than the slow exchange values. Because the slow-exchange rate is not determined for many amino acid residues, the comparisons below are focused on the fast-exchange process (i.e., rate constants obtained from exchange data for the first 4 D₂O pressure values).

Figure 3.5. a) Total deuterium uptake as a function of the product of D₂O partial pressure and t_D . These data were recorded for the more elongated $[M+4H]^{4+}$ ion conformer type (see Figure 3.2 in the manuscript) of the model peptide. The data were obtained at varying D₂O partial pressures (0, 0.003, 0.005, 0.008, 0.010, 0.015, 0.035, 0.045, 0.060, and 0.080 Torr). b) Plot of the natural log of the number of remaining exchangeable hydrogens as a function of the product of D₂O partial pressure and t_D for the respective $[M+4H]^{4+}$ ions. Linear fits of fast- and slow-exchanging hydrogens are shown. Data points representing transitions between fast- and slow-exchanging hydrogens as well as saturation conditions are shown as solid circles; they are not used in HDX rate determinations. Rate constants obtained from the slopes of the linear fits are provided in Table 3.1 and Table 3.2 in the manuscript.

As described in further detail below, each exchange site should exhibit a characteristic rate of reaction that is dependent on its accessibility to, for example, D₂O, charge sites, and the original site of deuterium incorporation [5, 9, 10, 28]. That groups of heteroatom hydrogens appear to exhibit two measurably separate HDX rates is intriguing. It is therefore instructive to consider the origin of the fast- and slow-exchange rates. Consider the more compact [M+3H]³⁺ ions (340 Å²). At ~0.005 Torr D₂O, these ions experience 5500 collisions (collision frequency × *t_D*), on average, with D₂O molecules leading to an exchange of ~4 hydrogens (i.e., 4 effective collisions per ion). This represents an effective collision percentage of ~0.07% for fast-exchanging hydrogens. For slow-exchanging hydrogens, considering deuterium uptake from ~0.02 to ~0.05 Torr of D₂O an effective collision percentage of ~0.004% (corresponding to ~2 deuteriums) is determined. Despite the distinct nature of each site, from this comparison, one possible origin for fast- and slow-exchanging sites becomes evident. The fast-exchanging sites may be comprised of those in relative proximity to charge sites while the slow-exchanging sites may represent those that are less accessible to charge sites. Such a scenario would lead to increased effective collisions for the fast-exchange sites. Indeed, molecular dynamics simulations used as a semi-quantitative description of deuterium uptake data from IMS-HDX experiments for cytochrome *c* ions suggest that sites more distant to charge sites would require many more collisions with D₂O as they come into proximity of charge sites less frequently during transit in the drift tube [9].

Table 1. Percent contribution to gas-phase HDX rates by individual amino acid residues for fast-exchanging sites.

Table 1. Percent contribution to gas-phase HDX rates by individual amino acid residues for fast-exchanging sites.														
Ion Type	Rate Constant ^a	Percent Contribution to Exchange Rate ^b												
		K(1)	K(2)	D(3)	D(4)	D(5)	D(6)	D(7)	I(8)	I(9)	K(10)	I(11)	I(12)	K(13) ^c
[M+3H] ³⁺ (Q=343 Å ²)	2.8±0.1 ^d	22.0±1.0	8.0±1.0	6.6±0.6	5.7±0.5	6.7±0.4	5.5±0.3	5.1±0.3	0.1±0.1	0.6±0.3	6.7±0.6	0.2±0.1	6.6±0.6	24.0±2.0
[M+3H] ³⁺ (Q=369 Å ²)	2.4±0.1	22.0±1.0	8.0±1.0	7.0±1.0	6.3±0.5	6.0±1.0	6.0±1.0	4.7±0.3	0.3±0.1	0.5±0.1	6.4±0.1	0.2±0.1	5.8±2.0	26.0±2.0
[M+4H] ⁴⁺ (Q=418 Å ²)	2.1±0.2	24.2±0.1	18.0±4.0	5.1±0.4	5.0±0.4	5.0±1.0	3.6±0.3	6.0±1.0	4.0±1.0	2.0±1.0	4.0±2.0	1.7±0.4	5.5±0.5	15.0±1.0
[M+4H] ⁴⁺ (Q=446 Å ²)	2.1±0.1	23.0±2.0	15.0±1.0	5.0±1.0	6.0±1.0	5.0±1.0	4.1±0.2	4.0±1.0	4.0±0.4	3.0±1.0	2.1±0.1	4.0±1.0	4.2±0.1	19.0±2.0

^a Estimated rate constants reported as value / 10⁻¹³ cm³·molecule⁻¹·s⁻¹

^b Percent contribution to the overall exchange rate for each amino acid residue (residue rate / conformer type rate · 100%).

^c Because the z1 ion was not observed, the K13 contribution was determined by subtracting the average mass for the c12 ion from the mass of the respective Mⁿ⁺ precursor ion at each D₂O partial pressure.

^d Reported errors are obtained from the standard error of the kinetics plot slopes provided by the LINEST function in Excel.

Table 2. Percent contribution to gas-phase HDX rates by individual amino acid residues for slow-exchanging sites.

Table 2. Percent contribution to gas-phase HDX rates by individual amino acid residues for slow-exchanging sites.														
Ion Type	Rate Constant ^a	Percent Contribution to Exchange Rate ^b												
		K(1)	K(2)	D(3)	D(4)	D(5)	D(6)	D(7)	I(8)	I(9)	K(10)	I(11)	I(12)	K(13) ^c
[M+3H] ³⁺ (Q=343 Å ²)	0.12±0.01 ^d	4.9±0.1	27.0±3.0	5.1±0.5	NA	6.5±0.5	6.4±0.5	NA	NA	NA	9.0±1.0	0.8±0.1	NA	5.0±0.5
[M+3H] ³⁺ (Q=369 Å ²)	0.10±0.01	19.0±1.0	7.0±1.0	0.6±0.1	NA	4.8±0.1	5.0±1.0	0.8±0.1	NA	NA	7.7±0.1	NA	NA	7.0±0.3
[M+4H] ⁴⁺ (Q=418 Å ²)	0.09±0.01	16.5±0.1	12.0±2.0	5.0±0.4	NA	4.7±0.5	10.0±1.0	NA	NA	NA	8.0±5.0	NA	NA	10.0±0.4
[M+4H] ⁴⁺ (Q=446 Å ²)	0.11±0.01	15.0±1.0	7.0±0.3	5.0±1.0	NA	3.0±1.0	4.8±0.2	3.0±1.0	NA	NA	7.3±0.1	1.0±0.2	NA	6.0±0.5

^a Estimated rate constants reported as value / 10⁻¹³ cm³·molecule⁻¹·s⁻¹.

^b Percent contribution to the overall exchange rate for each amino acid residue (residue rate / conformer type rate · 100%).

^c Because the z1 ion was not observed, the K13 contribution was determined by subtracting the average mass for the c12 ion from the mass of the respective W⁺ precursor ion at each D₂O partial pressure.

^d Reported errors are obtained from the standard error of the kinetics plot slopes provided by the UNEST function in Excel.

In the model presented, these distant sites are observed to exchange in the onset to, and within, the plateau region of the simulated deuterium incorporation curve.

From Table 3.1, it is evident that $[M+3H]^{3+}$ ions exhibit faster exchange kinetics than the $[M+4H]^{4+}$ ions. Because the proposed relay mechanism requires a long-lived reaction intermediate, a structure that provides a suitable distance between the charge site and less basic sites such as backbone carbonyls [5, 9, 10] is necessary for exchange. For the $[M+4H]^{4+}$ ions, Coulombic repulsion results in conformational rigidity which makes it both more difficult to locate charge sites near carbonyls and maintain the intermediate form for an extended time period [7, 9, 28, 29]. In summary, the charge site cannot readily access some interior residues. Conversely for the compact $[M+3H]^{3+}$ ions which contain more Coulombic repulsion than more diffuse $[M+3H]^{3+}$ ions, the compact structure type provides suitable distances for the relay mechanism effectively mitigating decreased conformational flexibility.

3.3.3. Determining percent contribution to HDX rate of individual amino acid residues.

To perform kinetic studies for individual amino acid residues, ETD experiments are conducted on each mobility-selected conformer type to determine residue deuterium uptake levels at each partial pressure of D_2O . Figure 3.6 shows c_2 and c_{12} ions for compact $[M+3H]^{3+}$ ion conformer types at different partial pressures of D_2O . Here the isotopic distributions for each ion fragment shift to higher m/z values with partial pressure of D_2O . As before, this

shift is observed to be the greatest at the lower partial pressures of D₂O and reaches plateau values at the highest pressures [18]. To compute the per-residue deuterium content, the deuterium content of the c_i residue is subtracted from that of the c_{i+1} residue; this difference comprises the deuterium content of the side-chain of the i^{th} residue and the backbone amide of the $i^{\text{th}}+1$ residue. As with the conformer type kinetics determinations (Figure 3.2), the log of remaining hydrogens (computed using the residue deuterium content) is plotted as a function of the product of t_D and partial pressure of D₂O.

Figure 3.6. Expanded regions of mass spectra for the c_2 (panel a) and c_{12} (panel b) fragment ions of the compact $[M+3H]^{3+}$ ion conformer type (340 Å²) obtained under conditions of different partial pressures of D₂O. For these data, partial pressures of D₂O were set to 0.003, 0.005, 0.008, 0.01, 0.035, 0.045, and 0.060 torr in order of bottom-to-top mass spectral traces (both panels). The average mass values from these ions (and all c-ions) are used to compute the per-residue deuterium incorporation at different D₂O partial pressures (see Experimental section).

Figure 3.7 shows that fast- and slow-HDX rates are observed for the K1 residues of the four ion conformer types. The relative contributions of individual amino acid residues to HDX rate can be computed by ratioing the individual residue rates by the ion conformer type HDX rate. These values, expressed as percentages, are listed in Table 3.1 (fast exchanging) and Table 3.2 (slow exchanging). The residue contributions reported in Tables 3.1 and 3.2 are distinguished according to deuterium uptake within the fast- and slow-exchange regions (D_2O partial pressure settings – Figure 3.2B) of the intact ion conformer types. As a consequence, even though the contributions of some residues are comparatively small (e.g., I8), they are included in the fast-exchanging category because the values were determined using the first 4 D_2O partial pressure settings (including zero). That is, this type of measurement reveals the contributions of individual residues to those sites that are categorized as exchanging rapidly (or slowly – Table 3.2) according to the precursor ion data (Figure 3.2B).

Notably, the residue contributions in Table 3.1 do not sum to exactly 100%. This is due in part to the fact that they are determined independently for each amino acid residue; each measurement has an associated error (reported in Tables 3.1 and 3.2). It is important to note that in all cases, the sum of the contributions in Table 3.1 is less than 100%. One possible explanation for this observation is that the multiply-charged precursor ions exhibit a greater number of exchangeable sites due to the attached protons. Therefore, the formation of c-

type fragment ions may result in a loss of deuterium content thereby lowering the calculated HDX rates.

Some general trends are observed with respect to the relative HDX rates of the individual amino acids reported in Table 3.1. For example, a comparison of the relative HDX rates (or residue contributions – Table 3.1) of the K1 and K2

Figure 3.7. Plots of the natural log of the number of remaining exchangeable hydrogens as a function of the product of D_2O partial pressure and t_D for the first K residue of the four different ion conformers. The top traces show the data for compact $[\text{M}+3\text{H}]^{3+}$ (panel a) and $[\text{M}+4\text{H}]^{4+}$ (panel c) ions and the bottom traces show data for diffuse $[\text{M}+3\text{H}]^{3+}$ (panel b) and $[\text{M}+4\text{H}]^{4+}$ (panel d) ion conformer types. Linear fits of fast- and slow-exchanging hydrogens are shown. Varying D_2O partial pressures (0, 0.003, 0.005, 0.008, 0.010, 0.015, 0.035, 0.045, 0.060, and 0.080 Torr) were utilized in the collection of this data. Data points representing transitions between fast- and slow-exchanging hydrogens as well as saturation conditions are shown as solid circles; they are not used in HDX rate determinations. The relative contributions of each amino acid residue to the overall conformer HDX rate is determined as outlined in the Experimental section and reported in Table 3.1 and Table 3.2.

residues of the different conformer types shows a dependence on charge state. For the $[M+3H]^{3+}$ ion conformer types, the K1 hydrogens exchange at $\sim 2.7\times$ the rate of the K2 residue while for the $[M+4H]^{4+}$ ion conformer types, the exchange rates are closer with K1 exchanging 1.3 to 1.4 fold faster. One possible explanation for this observation is that the K2 residue is not protonated for the former ions whereas it is likely to be protonated for quadruply-charged ions. Here the enhanced incorporation on the charged residue would result from the fact that this residue forms part of the long-lived reaction intermediate for gas-phase exchange [10].

A separate comparison between the different ions shows that, for the most part, the D residues exhibit larger relative HDX rates (1.31 ± 0.14 fold) for the $[M+3H]^{3+}$ ions (both conformer types) compared with the $[M+4H]^{4+}$ ions. An exception is the D7 residue where no difference is observed. It is also notable that the D6 residue on the more compact of the quadruply-charged ion types exhibits the smallest relative HDX rate. One explanation that is consistent with these results is that conformational rigidity resulting from Coulomb repulsion of charge sites on the quadruply-charged ions renders this interior site less accessible to exchange via the relay mechanism. This decreased accessibility for the elongated conformer type of quadruply-charged ions has been proposed previously in comparisons of maximum HDX levels for different amino acid residues [28]. Such an explanation can be used to describe the relative rates of all D residues for the various ion conformer types suggesting limited access to charge sites (e.g., compared to the K residues).

For the I8 and I9 residues, increased HDX rate contributions are observed for the quadruply-charged ions (~4 to ~40 fold). However, for the K10 residue, a ~1.5 to ~3 fold decrease in HDX rate contribution is observed for the quadruply-charged ions. The reverse trend is observed for the I11 residue where much larger relative HDX rates are observed for the quadruply-charged ions (~9 to ~18 fold). One possible explanation for these observations is that the more elongated nature of these $[M+4H]^{4+}$ ions coupled with the increased charge number renders these interior I8 and I9 residues more accessible to exchange. The decreased HDX rate contribution for the K10 residue itself could necessarily arise from a decreased accessibility of the charge site to the original site of deuterium incorporation perhaps due to conformational rigidity resulting from increased Coulomb repulsion.

For the I residues, the largest relative HDX rates are observed for the I12 residues for all ion conformer types. Here a note of caution is presented. In a previous report, it was noted that the I12 residue exhibited a maximum HDX level greater than 1 deuterium [28]. This was the highest exchange level of all isoleucine residues. The increased exchange level of the I12 residue was attributed to more frequent transfer of a deuterium onto this particular amino acid residue during the ETD process [24]. In this manner, the increased exchange rate of the I12 residue could be more reflective of the rapid HDX rates of the neighboring K13 charge site [28].

3.3.4. Saturation conditions: considerations for single amino acid residues.

It is instructive to consider the fraction of all ions from a single ion conformer that have achieved complete exchange at various pressures of D₂O. For this analysis we consider two HDX site types: fast- and slow-exchanging hydrogens. First, the number of ion-D₂O collisions required for exchange of a single site of each type is determined. For example, this number of collisions can be obtained by dividing the total number of ion-D₂O collisions (using the ion's collision frequency) by the total number of exchanges. For fast-exchanging sites, this is determined from the first or second D₂O pressure setting (0.003 to 0.005 Torr). For fast-exchanging hydrogens of the compact [M+3H]³⁺ ion conformer type, the number of required collisions is ~1000 and for slow-exchanging hydrogens it is ~23000. The number of effective collisions would then be the total number of ion-D₂O collisions divided by the number of required collisions for one exchange. Using these values, it is possible to estimate the fraction of ions from a hypothetical population that have exchanged all fast-exchanging sites or all slow-exchanging sites as a function of the number of effective collisions. Note that the ratio of effective collisions to collisions is entirely dependent upon the HDX rates.

To model deuterium uptake behavior for single exchange sites within unique ion conformers as a function of effective ion-D₂O collisions, an algorithm (C++) has been developed to simulate the uptake phenomena. To model m peptide ions with n labile hydrogens an integer array ($m \times n$) is defined, and all of the array elements are set to zero. Each deuterium uptake phenomenon (one

effective collision – see above) is defined by changing the value of one random array element to 1 (effectively imposing pseudo-first order kinetics). The program allows the user to define the ratio of collisions to effective collisions. After a predefined number of collisions, all of the array elements are summed according to the given ions and the proportion of the ions that have exchanged a given site(s) is determined. A do/while loop allows for a change in the number of collisions with D₂O, and the uptake results are saved to a text file.

Figure 3.8. Plot of the ion population fraction simulated to undergo complete exchange of all fast- and slow-exchanging hydrogens (blue and red traces, respectively) as a function of the number of effective collisions. The respective numbers of hydrogens used in the simulation are 13 and 3. The vertical dashed line shows the number of effective collisions at the highest D₂O pressures used in the current study. The horizontal dashed line shows the HDX level corresponding to exchange of all sites for all ions used in the simulation. One thousand ions have been used in each of the simulations.

Figure 3.8 shows a comparison of fast- and slow-exchanging sites. Based on the experimental uptake data (Figure 3.2 in the manuscript) for the compact $[M+3H]^{3+}$ ion conformer type, a total of 13 sites and 3 sites are used to represent fast- and slow-exchanging hydrogens, respectively. For the fast-exchanging sites, the fraction of ions that undergo complete exchange increases rapidly up to about 30,000 effective collisions. Then the rate of increase in this fraction of ions slows and begins to plateau at ~50,000 effective collisions. It is noted that this number is representative of the maximum D₂O pressure used in these studies. Here ~95% of the ion population exhibits exchange of all sites. However, it is noted that full exchange (all ions) of these sites is not achieved until ~150,000 effective collisions. For the slow-exchanging sites, the increase in the fraction of ions that have undergone full exchange is much slower as a function of the number of effective collisions. At the highest pressures used in the kinetics determinations (0.06 Torr D₂O), only ~12% of the ion population exhibits complete exchange. It is noted that under the current experimental conditions, it is not possible to operate at significantly higher pressures of D₂O due to decreased sensitivity and elevated pressures in the ion trap mass spectrometer. Therefore, it is expected that many of the accessible slow-exchanging sites and even a portion of the accessible fast-exchanging sites for given ion conformers do not undergo exchange. That said, because the exchange levels are observed to plateau at higher D₂O pressures (e.g., Figure 3.2a in the manuscript) and indeed higher pressures would only increase HDX values by a small percentage, it is noted that the current study is informative in that HDX levels (and ultimately

HDX rates – see manuscript) provide meaningful information regarding the relative accessibility of specific exchange sites.

The HDX simulation presented above can be used to describe the exchange behavior of single amino acid residues. Figure 3.9 shows the average deuterium uptake estimated for a single I residue as a function of D₂O partial pressure multiplied by t_D . Based on the kinetics data for the I8 residue (Table 3.1 in the manuscript) in the compact [M+3H]³⁺ ion conformer type, the lone exchange site is modeled as a very slow-exchanging site (lower effective collisions to overall collisions ratio).

Figure 3.9. Plot of the average deuterium uptake of a population of ions containing 1 slow-exchanging site as a function of the product of the D₂O partial pressure multiplied by t_D . The x-axis value is obtained from a fit of total collisions with D₂O versus the pressure-time product from experimental data. For this simulation, the relative contribution to HDX rate by the I8 residue in the compact [M+3H]³⁺ ion conformer type (Table 3.1 in the manuscript) has been considered to determine the number of effective collisions at each D₂O partial pressure setting. One thousand ions have been used in this simulation.

In Figure 3.9, the y-axis is labeled as the average deuterium uptake. Because the computation only considers a site containing one hydrogen, the fraction of the ion population that is observed to exchange (Figure 3.8) is equal to the average deuterium uptake for this site. It is noted that if the simulation were performed for an amino acid residue with more than one fast exchange site, then the average deuterium uptake would be computed by weighting each uptake level by the number of ions exhibiting that degree of exchange.

Figure 3.10. Plot of the average deuterium uptake of a population of ions containing 1 fast-exchanging site (blue trace) and 1 slow-exchanging site (red trace) as a function of the product of the D_2O partial pressure multiplied by t_D . The x-axis value is obtained from a fit of total collisions with D_2O versus the pressure-time product from experimental data. Also shown is the sum of the two HDX profiles (green trace) to represent the deuterium uptake of a single amino acid residue containing 2 exchange sites such as the D6 residue in the peptide ion. One thousand ions have been used in each of the simulations.

In addition to the I8 residue, a simulation has been performed for the D6 residue within the same conformer type. For this simulation two collision ratios are utilized. These are obtained from the fast- and slow-exchange kinetics data (D6) in Tables 3.1 and 3.2. Figure 8 shows the total uptake level estimated for the D6 residue (the summation of the fast- and slow-exchanging sites). Note that at the highest D₂O partial pressures used in this study, the HDX level is estimated to be 1.15 which is near the experimentally determined value of 1.2±0.1.

3.3.5. ETD spectra and ion conformer considerations.

It is noteworthy that the isotopic distributions of the c₂ and c₁₂ ions (Figure 3.6) do not become more narrow under saturation conditions as may be expected for single ion conformations [5]. It is therefore instructive to consider factors that influence the relative exchange rates. For example, the I8 residue is reported to present a 0.1% contribution to the total rate constant for the compact [M+3H]³⁺ ion conformer type. This indicates that the I8 residue accounts for ~0.0013 of the total deuterium uptake (0.1% of the roughly 13 fast-exchanging hydrogens). A question arises as to the meaning of this number. Does this contribution arise from a population of ions containing multiple ion conformers in which one (or more) low abundance ion conformer is able to undergo exchange at the I8 residue while the dominant ion conformer(s) is not? Or, could this number simply reflect the statistical uptake distribution of a population of ions from a single conformer? That is, exchange at the single site of the I8 residue occurs at such a slow rate so that only a small portion of the total ion population

incorporates a single deuterium. A HDX simulation (Figure 3.8) using the determined effective collisions for the I8 residue results in ~96% and ~4% of the total ion population that have exchanged 0 and 1 deuterium, respectively, upon exposure to 0.01 Torr partial pressure D₂O. Uptake at this residue would thus account for ~0.3% of the nearly 13 fast-exchanging hydrogens ($0.04 \text{ deuteriums} \div 13 \text{ deuteriums} \times 100\%$). Because the experimentally-determined rate constant contribution is even lower than this number, it can be suggested the latter explanation can account for the observed deuterium uptake at the I8 residue. However, such an explanation does not discount the presence of other ion conformations within the mobility selection (see below) as such species could exhibit similarly slow HDX rates for the I8 residue. Notably, at 6-fold higher partial pressure D₂O (0.06 Torr), the same simulation results in ~85% and ~15% of the total ion population that have exchanged 0 and 1 deuterium, respectively. Therefore, according to the model, the deuterium uptake attributed to the I8 residue over this 6-fold range in D₂O partial pressure would be difficult to distinguish in the ion conformer type HDX profile (Figure 3.2a).

One of the implications of the treatment of the I8 residue data is that, a portion of the isotope peak broadening at elevated D₂O partial pressures can be attributed to sites with slow to intermediate HDX rates. That said, it is instructive to consider the possibility that multiple ion conformations exhibiting unique exchange site accessibilities could also contribute to the broad isotope distributions. HDX simulations have also been conducted to determine the relative width of an isotopic distribution that would be obtained for c₁₂ ions arising

from a single ion conformer using the experimentally determined rates of exchange. The simulation results are shown in Figure 3.11.

Figure 3.11. a) Plot of the total deuterium uptake as a function of the product of the D₂O partial pressure multiplied by t_D for a hypothetical population of $[\text{M}+3\text{H}]^{3+}$ ions. The simulations (see Experimental section) presented here are for calculations of a single ion conformer. A total of 3000 ions have been used in the simulation at each D₂O partial pressure. b) The black trace shows a plot of the isotopic distribution for c_{12} ions obtained upon exchange of the compact $[\text{M}+3\text{H}]^{3+}$ ion conformer type followed by MS/MS analysis by ETD. These experimental data were obtained at a D₂O partial pressure of 0.06 Torr. The red trace shows the isotopic distribution obtained from the kinetics simulation of a single ion conformer described above. 3000 ions have been used in the simulation.

In summary, the simulation distribution is nearly 60% narrower than the experimental distribution for the c_{12} ions. One likely explanation for this is that the mobility selection results in multiple ion conformers that exhibit unique exchange site accessibilities. It is noted that the numbers of fast- and slow-exchanging hydrogens would to some degree be similar for the various conformers otherwise the separation of conformer-specific isotopic distributions would be observed [4]. Because of the inability to distinguish the exact number of unique ion conformers present in each mobility selection, it is important to note that the HDX data reported here only represent the average uptake of a number of ion conformers. For this reason, the mobility-selected ions are referred to throughout this manuscript as conformational types. It can be argued that such nomenclature was previously established in FTICR studies [4, 8] where broad isotopic distributions were observed under saturation conditions yet only single conformation types were reported. It is also noted that such a treatment of the data is useful for comparisons with nominal structures obtained from molecular dynamics simulations (see below) as these structures also are used as being representative of specific structural types (e.g., relative degree of compactness).

3.3.6. Ion conformation type and per residue HDX kinetics.

Having presented possible HDX scenarios that could explain the observed differences in exchange rate, it is important to note that these descriptions are cursory and used here only to present plausible explanations. Clearly, previous experiments have indicated that a number of factors including conformational rigidity, D_2O accessibility, and charge site and exchange site accessibility affect

the overall exchange process [5, 7, 9, 28]. To better examine some of these factors, ion structures obtained from MD are considered. Figure 3.12 shows ion structures obtained upon performing simulated annealing for the triply- and quadruply-charged ions. These structures are here used to develop a model to help describe the observed exchange rates for the amino acid residues. It is noted that, in these initial stages of developing an HDX model, single structures representing conformational types or families are used.

Figure 3.12. Schematic representation of ion structures obtained from simulated annealing studies performed by MD. Low-energy structures having collision cross sections matching ($\pm 1\%$) the experimentally observed conformational types (compact, partially-folded, and elongated) are represented here. Ion charge state and collision cross section labels are provided for each structure.

In developing the HDX model with the use of single structures, it is useful to reconsider the generally accepted relay mechanism for peptide ion HDX using D₂O as a deuterating agent [10]. Figure 3.13 shows a schematic for the HDX mechanism with D₂O. In the first step of HDX, a proton from a charged, basic site transfers to D₂O with simultaneous transfer of a deuteron to a less basic carbonyl site [5, 10]. A second step is required for deuterium incorporation at specific sites. Here the basic amino acid residue abstracts a proton from an available exchange site (or the incorporated deuteron itself) with transfer of the deuteron from the carbonyl site to the exchange site. Therefore, for the purposes of the HDX model investigated here (see Experimental section), deuterium incorporation at a specific exchange site requires accessibility of that site to a carbonyl site that in turn has accessibility to a charge site. Additionally, for a carbonyl to be accessible it should be located on the peptide surface and also the distance from the charge site should be suitable for intermediate formation (previous models have used 4 Å and 5 Å) [5, 9]. As an initial formulation of the HDX model presented here, for the studied peptide it is presumed that all carbonyls are on the surface and that they have the same exposure to D₂O. Additionally a distance threshold of 7 Å has been selected (iterative optimization)

for d_{O-N} and d_{H-O} parameters (Equations 3.3 and 3.4, respectively) using the single ion structures obtained from MD.

Figure 3.13. a) Schematic of the long-lived reaction intermediate proposed by the mechanism of HDX with D_2O [10]. From such a structure a proton transfers from the charged, basic site to a D_2O molecule and simultaneously a deuteron from D_2O transfers to a neighboring carbonyl group. b) Schematic showing the second step of deuterium incorporation which includes transfer of a proton to the basic site with transfer of a deuteron from the carbonyl group to the proton donor site (HDX exchange site).

Figure 3.14 shows the comparison of the normalized theoretical HDX rate contributions by individual amino acid residues and the normalized experimental values. It is instructive to consider the quality of the fit of the new HDX rate model. For this description, results for the comparison with the compact $[M+3H]^{3+}$ ion conformer type are considered. Here the agreement between the experiment and theory are quite good. For example, the model captures the relative HDX rates of all K residues. Additionally, the model displays the correct magnitudes of change for the D residues compared with the I8 and I9 residues.

Figure 3.14. Plots comparing the residue hydrogen accessibility scores with the relative exchange rates for the individual amino acid residues. Solid squares show the normalized value of contribution of each residue to the fast rate constant of the various ion conformers. Open diamonds show the normalized values of the residue scores. Panels a and b show the comparison for the compact and partially-folded $[M+3H]^{3+}$ ions, respectively. Panels c and d show the comparison for the elongated and the most diffuse $[M+4H]^{4+}$ ions, respectively.

The exception to this comparison is the D7 residue where the model underestimates the HDX rate contribution. The model then correctly captures the increased and decreased HDX rate contributions for the K10 and I11 residues. The model significantly under predicts the HDX rate contribution for the I12 residue; however, as mentioned above and observed previously, it is likely that the experimental HDX rate is affected by fast deuterium incorporation on a charge site and subsequent deuterium transfer during the ETD process. To

contextualize the performance of the HDX rate model, it is noted that it outperforms a prior model for representing maximum HDX levels for this same peptide [28]. Prior RMSD (experimental versus theoretical) values are larger compared to those for data presented in Figure 3.14 by 1.6, 3.3, and 1.6 fold for the more compact and more elongated $[M+3H]^{3+}$ and the more compact $[M+4H]^{4+}$ ions, respectively.

That the HDX model captures the experimental trends so well having been developed from single ion structures (as opposed to multiple structures from MD), suggests that, to a large degree, these conformational types may indeed represent the conformations comprising the gas-phase ions. Admittedly, one of the goals of IMS-HDX-MS studies is to utilize the combined mobility and reactivity information to better confirm structural candidates from MD. Here, the utility of the IMS-HDX measurement should be noted. The mobility information provides a restraint on conformational size that may help to guide MD simulations to provide information about the variety of conformations that could produce the observed HDX results. In that regard, future efforts can be directed at improving the HDX model. For example, it may be possible to better capture the HDX rate contribution of the K10 residue for the different ion conformer types. That is, future MD work may not only focus on the role of conformational fluctuation but also the presence of multiple conformers of similar size to improve the HDX model. This should be relatively straightforward to implement where distance thresholds are monitored by MD for a series of low-energy conformers. Indeed, the consideration of many structures may begin to also provide clues

about the slow-exchanging process (Table 3.2). Additionally, energetic factors can be considered such as the apparent gas-phase basicities [55] of different exchange sites, sites of initial incorporation, and charge sites. Another factor that should be considered is salt bridge formation [56]. Previous IMS-HDX-MS work showed that the potential for salt bridge formation had less effect upon observed HDX levels of peptide anions compared with charge site location and conformational rigidity arising from Coulomb repulsion and disulfide bonds [29]. That said, the use of IMS-HDX with MD where multiple structures are utilized may also reveal the influence of structural rigidity due to salt bridge formation. These factors are currently being investigated for a number of small peptide ion systems using IMS-HDX-MS experiments and MD studies.

Figure 3.14 shows a comparison of fast- and slow-exchanging sites. Based on the experimental uptake data (Figure 3.2 in the manuscript) for the compact $[M+3H]^{3+}$ ion conformer type, a total of 13 sites and 3 sites are used to represent fast- and slow-exchanging hydrogens, respectively. For the fast-exchanging sites, the fraction of ions that undergo complete exchange increases rapidly up to about 30,000 effective collisions. Then the rate of increase in this fraction of ions slows and begins to plateau at ~50,000 effective collisions. It is noted that this number is representative of the maximum D_2O pressure used in these studies. Here ~95% of the ion population exhibits exchange of all sites. However, it is noted that full exchange (all ions) of these sites is not achieved until ~150,000 effective collisions. For the slow-exchanging sites, the increase in the fraction of ions that have undergone full exchange is much slower as a function of the

number of effective collisions. At the highest pressures used in the kinetics determinations (0.06 Torr D₂O), only ~12% of the ion population exhibits complete exchange. It is noted that under the current experimental conditions, it is not possible to operate at significantly higher pressures of D₂O due to decreased sensitivity and elevated pressures in the ion trap mass spectrometer. Therefore, it is expected that many of the accessible slow-exchanging sites and even a portion of the accessible fast-exchanging sites for given ion conformers do not undergo exchange. That said, because the exchange levels are observed to plateau at higher D₂O pressures (e.g., Figure 3.2a in the manuscript) and indeed higher pressures would only increase HDX values by a small percentage, it is noted that the current study is informative in that HDX levels (and ultimately HDX rates – see manuscript) provide meaningful information regarding the relative accessibility of specific exchange sites.

The HDX simulation presented above can be used to describe the exchange behavior of single amino acid residues. Figure 3.9 shows the average deuterium uptake estimated for a single I residue as a function of D₂O partial pressure multiplied by t_D . Based on the kinetics data for the I8 residue (Table 3.1 in the manuscript) in the compact [M+3H]³⁺ ion conformer type, the lone exchange site is modeled as a very slow-exchanging site (lower effective collisions to overall collisions ratio). In Figure 3.9, the y-axis is labeled as the average deuterium uptake. Because the computation only considers a site containing one hydrogen, the fraction of the ion population that is observed to exchange (Figure 3.9) is equal to the average deuterium uptake for this site. It is

noted that if the simulation were performed for an amino acid residue with more than one fast exchange site, then the average deuterium uptake would be computed by weighting each uptake level by the number of ions exhibiting that degree of exchange. In addition to the I8 residue, a simulation has been performed for the D6 residue within the same conformer type. For this simulation two collision ratios are utilized. These are obtained from the fast- and slow-exchange kinetics data (D6) in Tables 3.1 and 3.2. Figure 3.10 shows the total uptake level estimated for the D6 residue (the summation of the fast- and slow-exchanging sites). Note that at the highest D₂O partial pressures used in this study, the HDX level is estimated to be 1.15 which is near the experimentally determined value of 1.2 ± 0.1 .

3.4. Conclusions

Combined IMS-HDX-MS experiments have been used to determine the relative rates of exchange of individual amino acid residues within ion conformational types. To our knowledge this is the first report of kinetics data for individual amino acid residues within a mobility-selected peptide ion. Several trends associated with charge state are observed. In general HDX rate contributions of D residues are slightly greater for $[M+3H]^{3+}$ ions compared with those for $[M+4H]^{4+}$ ions. Decreased conformational flexibility of the more-highly charged ions may account for this as the charge sites have less access to interior D residues. The K1 and K2 residues also exhibit charge state dependent HDX rate contributions. For the $[M+3H]^{3+}$ ions, the K1 exchanges at more than double the rate of the K2 residue; in comparison, these residues exhibit more similar

exchange rates for the $[M+4H]^{4+}$ ion conformers. Finally, differences in HDX rate contributions of the I8 and I9 residues suggests increased access to these residues by charge sites (or initial sites of deuterium incorporation) for the more elongated, quadruply-charged ions.

A HDX model has been developed using single ion structures from MD. This model takes into account the distance between the charge site and the initial site of deuterium incorporation (all oxygens on carbonyls for this model) as well as the distance between the carbonyl oxygen and the heteroatom exchange site. Distance thresholds of 7Å have been used and the score values (hydrogen score and carbonyl oxygen score) are scaled as the inverse of the distance. The model improves the approximation of the experimental data by a factors of 1.6 to 3.3. Additionally, the model lays the foundation for future studies that consider molecular fluctuations and energetic barriers to exchange.

3.5. References

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4. Ion Mobility Spectrometry-Hydrogen Deuterium Exchange Mass Spectrometry of Anions: Part 2. Assigning Charge Sites and Assessing Hydrogen-Deuterium Scrambling¹

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4.1. Introduction

The first experiments that combined gas-phase hydrogen deuterium exchange (HDX) and mass spectrometry (MS) as a structural probe for biomolecular ions were conducted more than 20 years ago [1-4]. Shortly later, ion mobility spectrometry (IMS) was combined with HDX-MS to study the reactivities of ions of select mobilities [5, 6]. These studies revealed the maximum HDX levels and rates of exchange for protein and peptide ions and even determined the HDX reactivities of some ions at elevated temperatures [7]. Additionally it was demonstrated that IMS-HDX-MS data could be used with molecular dynamics (MD) simulations to provide additional conformational information in the form of hydrogen accessibility [6, 7].

Despite these early demonstrations, IMS-HDX-MS studies were not pursued to a significant degree for nearly ten years [8]. This may be attributed to several factors. First, the limitations of MD to obtain reliable structures for large, gas-phase ions hindered the ability to test the various hydrogen accessibility models. Relatedly, the models utilized several speculative assumptions such as charge site locations as well as threshold distances between accessible hydrogens to charge sites to obtain qualitative matches to HDX levels. Therefore, effectively testing such assumptions requires access to accurate

structures obtained from MD. Finally, at the time, site-specific deuterium uptake information to aid model development could not be obtained due to problems associated with hydrogen-deuterium (HD) scrambling during the ion fragmentation process [9, 10].

The advent of non-ergodic ion fragmentation techniques such as electron capture dissociation (ECD) [11] and electron transfer dissociation (ETD) [12] presented the means to determine the location of deuterium incorporation [13, 14]. Shortly later, HDX-ETD-MS characterization was coupled with ion mobility separations using a traveling wave ion guide (TWIG) instrument [15]. Indeed, using this approach researchers reported the deuterium uptake for specific ion conformers that were representative of solution conformers for ubiquitin ions. More recently, Rand and coworkers developed a rapid method for gas-phase HDX enabling the analysis of proteins and peptides distinguished by condensed-phase separations [16].

Recently, we reported the combination of a constant-field drift tube with a linear ion trap to perform IMS-HDX-MS/MS experiments [17, 18]. In these studies, dual ion gating allowed the probing of site-specific deuterium uptake for select ion conformations. That is, the gating of mobility-selected ions is conducted prior to their fragmentation (ETD) which is performed in a linear ion trap mass spectrometer. The HDX data was used to develop a model which employed site-specific deuterium uptake data to confirm mobility-matched ion structures obtained from molecular dynamics simulations [17]. Later, site-specific HDX kinetics information was used to improve the overall efficacy of the model [18]. Notably, other important studies have used the deuterium uptake kinetics

data for mechanistic studies of ion fragmentation [19]. One limitation of these experiments is that linear ion traps employing ETD are largely configured to only allow the fragmentation of positively-charged ions. This complicates studies seeking to determine the accessibility of hydrogens on protein ions for many species. For example, a significant number of proteins contain a disproportionately larger number of acidic residues than basic residues.

One consideration in the study of negatively-charged peptide and protein ions is whether or not collisional activation can be used more effectively (compared to positive ion studies) to ascertain the location of deuterium uptake in IMS-HDX-MS/MS experiments. As part of this consideration it is necessary to briefly review the mechanism of HD scrambling which can be described by the “mobile proton” model of peptide ion dissociation [20-23]. For positively-charged ions, collisional activation leads to intramolecular proton transfer where the locations of protons dictate the types of fragment ions produced. This phenomenon can describe HD scrambling because deuterons can be mobilized as well as protons leading to a shuffling of the deuterium: hydrogen content of specific amino acid residues. The extent of HD scrambling depends not only upon the effective temperature of the ions but also the differences in energy levels of different charge configurations [24]. For example, the energy required for the transfer of a proton from a protonated lysine to a backbone amide is significantly less than that of a neutral lysine or a salt-bridge. The consideration of such differences between positively-charged and negatively-charged ions served as a motivation for the study presented here.

The experiments described below evaluate the utility of IMS-HDX-MS/MS for the elucidation of structural details for negatively-charged peptide ions. Despite the possibility of partial scrambling (assumed to be backbone amide hydrogens) observed for the peptide ions, site-specific deuterium uptake information can be retained. This uptake information can be used to elucidate structural details such as the location of deprotonation sites as well as the relative accessibilities of hydrogens to those charge sites on the peptide ion structures. Notably, this study extends on previous negative-ion experiments revealing effects of the protein fold as well as charge location on hydrogen site accessibility [25]. This work also lays the foundation for the research described in the accompanying study (chapter 5) where the limited HD scrambling associated with anions is considered as an enabling phenomenon in the study of protein complex structures.

4.2. Experimental

4.2.1. Sample preparation.

The synthetic peptide (SP) KKDDDDDIKK and a model peptide (MP) Ace-PAAAKAAAKAAAKAAAK (Genscript Corp., Piscataway, NJ, USA) were purchased (each >90% purity) and used without further purification. Ultra-pure (chromatography-grade) deionized water, acetonitrile, ammonium hydroxide, and glacial acetic acid (Fisher Scientific, Fair Lawn, NJ, USA) were used to generate stock and ESI solutions of the peptide. The stock solutions consisted of 1.0 mg of peptide in 1.0 mL of ultra-pure water. These solutions were capped in glass vials and subsequently maintained in a refrigerator (4 °C). Sample solutions (0.1 mg·mL⁻¹) for ESI were prepared fresh immediately prior to

analysis by diluting 0.1 mL of stock solution with the addition of 0.4 mL of ammonium hydroxide and 0.5 mL of acetonitrile. For the limited positive-ion experiments, for P5, 0.49 mL of stock solution was combined with 0.5 mL of acetonitrile and 0.01 mL of glacial acetic acid. For MP solutions, 0.01 mL of glacial acetic acid was added to 0.99 mL of stock solution. ESI solutions were infused ($300 \text{ nL} \cdot \text{min}^{-1}$) through a pulled-tip capillary ($50 \text{ }\mu\text{m}$ ID) and a bias of -2200 V relative to the instrument entrance orifice was applied to the solution. A bias of +2200 V as used for positive-ion studies.

4.2.2 Ion mobility measurements.

The history of the application of IMS for the characterization of biomolecules is well documented in the literature. Detailed discussions of the development of IMS instrumentation [26-44], theory [45-56] and applications [57-61] have been presented elsewhere. The hybrid instrument combining a constant-field drift tube with a linear ion trap mass spectrometer has also been described previously [17, 18, 25]. A brief description of the instrument and data collection and analysis is presented here.

Electrosprayed peptide ions were trapped in an ion funnel trap [62, 63] in a differentially-pumped desolvation chamber and periodically (typically 50 Hz) pulsed into the drift tube. The drift tube was filled with He buffer gas (300 K) and the lenses supported an electric field of $\sim 10 \text{ V} \cdot \text{cm}^{-1}$. Ions separated in the drift tube according to differences in their mobilities through the buffer gas. The second ion gate was activated at specific time delays in order to select ions of specific mobilities for transmission into the linear ion trap (LTQ Velos, ThermoScientific, San Jose, CA, USA) for mass analysis. Delay times between

these two gates (front and back of the drift tube) were scanned to generate mobility-resolved MS datasets.

4.2.3. Mass spectrometry measurements and the generation of IMS-MS datasets.

Total ion mass spectra were generated by setting both drift tube gates to transmit all ions into the mass analyzer. The linear ion trap scan parameters included a m/z range of 80 to 2000 as well as an automatic gain control (AGC) threshold setting of 1×10^6 ions. For these analyses, sample injection times of 200 ms (5 microscans) were utilized. During drift time (t_D) selections, the ion gates in the drift tube were activated as mentioned above. Data acquisition was achieved by collecting a single mass spectrum (0.5 min) for each t_D selection setting. During ion gating, the AGC was disabled and a sample injection time of 200 ms (5 microscans) was also employed. Two-dimensional (2D) IMS-MS datasets were generated as described previously [17]. Briefly, an algorithm developed in house associated each t_D selection time with its resultant mass spectrum to create a three-column array file with t_D , m/z , and intensity (i) information. From this text file, extracted ion drift time distributions (XIDTDs) [64] and extracted mass spectra could be obtained using user defined thresholds as described previously [17, 18, 25].

4.2.4. HDX measurements.

Determining the amount of deuterium incorporated into specific peptide ion conformer types using the IMS-MS instrument here has been described in detail previously [17, 18, 25]. Only a brief description is provided here. To accomplish gas-phase HDX, D₂O (>99%, Sigma Aldrich, St. Louis, MO, USA)

was added to the He buffer gas in the drift tube using a separate leak valve (Granville Philips, Longmont, CO, USA) and monitored using a capacitance manometer (Baratron®, MKS, Andover, MA, USA). For these experiments, the He and D₂O pressures were typically set at ~2.50 and ~0.04 Torr, respectively. t_D distributions were first recorded in pure He to allow the determination of accurate collision cross sections from XIDTDs. The number of deuteriums incorporated into specific ion conformer types were determined by subtracting average m/z values of ions passing through the pure He buffer gas from those obtained after passing through a He:D₂O mixture in the drift tube.

4.2.5. Molecular dynamics simulations.

MD experiments required the creation of the [M-3H]³⁻ and [M-2H]²⁻ SP ions. The charge sites for these ions were selected considering ion collision cross sections obtained for MD structures, gas-phase acidities and Coulomb energies, and deuterium uptake and MS/MS data as described here and in the Results and Discussion section. For [M-3H]³⁻ SP ions, the ε-oxygens on residues D4, and D7, and the C-terminus were deprotonated while for [M-2H]²⁻ ions, the ε-oxygens on residues D4 and D6 were deprotonated. Alternate charge site configurations were also tested for both doubly- and triply-charged SP ions; however, these structures typically exhibited collision cross sections that were 20% to 30% larger than experimentally determined values.

Some amino acid residues were not predefined in the amber force field. For appropriate force field calculations for these residues within the SP molecule, two initial, hypothetical structures were utilized. The first structure represented an alpha helix where the φ and ψ bond angles were set to the respective values of -

48 and -57. The second structure was an extended conformer where ϕ and ψ angles of 135 and -140, respectively, were used. Each initial structure was optimized at a quantum chemical level of HF/6-31G(d) and used for calculating molecular electrostatic potential (MEP) values and charge fitting performed with the R.E.D. Server [65-70]. To generate the initial structures for MD, the AMBER force field FF12SB was employed. The method utilized for simulated annealing was similar to that described previously to generate 1000 candidate structures [32, 71]. The collision cross sections of the 1000 conformers were calculated using the mobcal [72] software employing the trajectory method (TM) [50]. The potential energies of the 1000 ion conformers obtained from the simulated annealing runs were plotted as a function of collision cross section for each peptide ion. Matching (<2% difference in collision cross section), low-energy ion structures were selected for comparisons to experimental data.

4.2.6. Hydrogen accessibility scoring.

Deuterium uptake predictions were performed for the SP ions using a modified hydrogen accessibility scoring model that has been described in detail previously [17, 18]. In the previous model, the distances between charge sites and heteroatom exchange sites were determined using nominal ion structures obtained from MD. These exchange sites were then scored based on a summation of the inverse distances to charge sites. Next the summation of the hydrogen scores for given amino acid residues yielded a residue score. The residue scores were then normalized such that their sum was equal to the experimental deuterium uptake values for the different ion conformers.

For the experiments reported here, these hydrogen accessibility scores served as scaling factors for an “effective” ion-neutral collision model that simulates HDX according to pseudo first order kinetics [18]. As described previously, the ratio of the total number of collisions to the number of effective collisions (resulting in incorporation of deuterium) was estimated based on experimental measurements of ion conformer uptake at low D₂O partial pressures. The hydrogen accessibility scores were then used to scale this ratio for individual amino acid residues. Next, using these scaled ratios, a monte carlo simulation imposing pseudo first order kinetics for exchange was performed in which the amount of deuterium incorporated for each residue was calculated for a population of ions [18]. Average theoretical uptake values were then compared to the experimentally determined values for each residue to provide an indicator of the suitability of the ion structure obtained from MD.

4.3. Results and Discussion

4.3.1. Peptide ion collision cross sections.

Collision cross sections for the dominant features in the t_D separation have been determined for all ions of the two samples using the expression [48]:

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N} \quad (4.1).$$

In Equation 4.1, ze and k_B correspond to the charge of the ion and Boltzmann’s constant, respectively. The variables m_I and m_B correspond to the mass of the ion and the mass of the buffer gas, respectively. E , L , T , and P correspond to the electric field, length of the drift tube, and the temperature and pressure of the

buffer gas, respectively. N represents the neutral number density at standard temperature and pressure (STP) conditions.

Figure 4.1 shows a three-dimensional (3D) raised-relief plot of electrosprayed SP ions. The most intense features in this dataset are $[M-2H]^{2-}$ ions. These ions are categorized as two conformer types having t_D values of 7.8 ms ($\Omega = 279 \pm 2 \text{ \AA}^2$) and 9.1 ms ($\Omega = 315 \pm 2 \text{ \AA}^2$). The more intense (and more diffuse conformer) has an unresolved shoulder at a t_D of 8.8 ms. Two dataset features not visible in Figure 4.1 correspond to very low intensity $[M-3H]^{3-}$ ions. From an XIDTD, the triply-charged ions are shown to exhibit a dominant, diffuse conformer type ($\Omega = 362 \pm 2 \text{ \AA}^2$) and a less abundant more compact conformer type ($\Omega = 340 \pm 2 \text{ \AA}^2$) as well as several more diffuse ions of considerably lower abundance. Figure 4.1 also shows the presence of several multimeric species. Because these ions have the same t_D values, it is not possible to determine whether or not such species are produced by dissociation of higher-order multimers. In support of this argument is the fact that calculated cross sections for these ions are significantly larger than those determined for many other multimeric peptide ions [73]. Additionally such multimeric species have been

observed previously using this hybrid instrument [25]. Because of this uncertainty, these species are not discussed further.

Figure 4.1. Three-dimensional (t_D , m/z , i) raised-relief plot produced upon electrospraying a solution of the SP peptide. Dominant ions in the distribution are labeled.

4.3.2. CID of the dominant SP ion conformers and per-residue deuterium uptake.

Figure 4.2 shows the mass spectrum obtained upon CID of the more diffuse $[M-2H]^{2-}$ SP ions. Previous studies have shown that CID of deprotonated peptide ions results in a variety of fragment ions including water loss species as well as internal fragments [74, 75]. Because of this property of anions as well as the loss of ion signal due to mobility selection, the acquisition of the data for the CID mass spectrum requires a significant time. Indeed the data represented by the mass spectrum shown in Figure 4.2 was collected for 1 hour. Among the many different fragment ions produced by CID, a number of ions from which deuterium uptake calculations can be pursued (labeled in Figure 4.2). From this analysis, no ion can be availed to determine the deuterium uptake at the K1 residue. The first ion used is the c_2 ion, so here the term “K1+K2” is used to represent the first two residues of the SP peptide.

Figure 4.2. CID mass spectrum obtained upon collisional activation of $[M-2H]^{2-}$ SP ions. Fragment ions used for deuterium uptake calculations are labeled.

As mentioned above, collisional activation of precursor ions can cause extensive HD scrambling. One previous study revealed that the use of high-energy CID resulted in near complete HD scrambling for backbone amide sites on deprotonated peptides [10]. There are two major differences between the study reported here and the previous experiments. First, in the former studies high-energy CID was employed while here, low-energy CID has been used. Second, gas-phase HDX involves not only backbone amide sites but also side-chain sites and, to date, no direct study showing the degree of isotopic

scrambling involving side chain sites for deprotonated peptides has been conducted.

Figure 4.3. Bar graphs showing the predicted (red) and measured (blue) deuterium uptake for each residue after CID. Predicted values assume 100% HD scrambling. Panel a and panel b show the comparison for the $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP ions, respectively.

A comparison of the experimental and theoretical (representing 100% HD scrambling) deuterium content per residue in $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP ions is shown in Figure 4.3. Here the per-residue values associated with complete HD scrambling are computed based on the apportioning of the total deuterium content according to the fractional number of exchangeable sites on given residues. For the more diffuse $[M-2H]^{2-}$ ions a statistically significant difference in deuterium content is observed for residues K1+K2, D3, D4, D5, D6, and D7. Indeed only the I8 residue exhibits the same deuterium content value as that

expected for complete HD scrambling. Figure 4.3 also shows the comparison that is obtained for $[M-3H]^{3-}$ SP ions. Here residues D3, D4, and to a lesser extent D6 show a disparity when compared to 100% HD scrambling while data for the residues K1+K2 and D5 are consistent with complete scrambling. That said, the deuterium content values of these residues are also consistent with those obtained from a hydrogen accessibility model indicating the expected gas-phase deuterium uptake for a representative ion conformer type (see discussion below).

Having noted the inability to completely eliminate the possibility of HD scrambling for the $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP ions it is necessary to consider the comparison of side-chain and backbone amide sites. The anion CID mechanism [76] and HD scrambling [10] studies for deprotonated peptides suggest that extensive scrambling on the peptide backbone occurs. That is, because the CID mechanism requires movement of protons along the peptide backbone, the effective ion temperature for CID would render near complete backbone scrambling. The deuterium content of the diffuse $[M-2H]^{2-}$ and $[M-3H]^{3-}$ ions shown in Figure 4.3 does not discount backbone scrambling; that is, the disparity in the comparison of the experimental results with that expected for 100% HD scrambling could result entirely (or primarily) from side-chain label retention. Furthermore, recent IMS-HDX-MS/MS studies show extensive side-chain deuterium uptake relative to backbone deuterium incorporation [18, 77].

4.3.3. Deprotonation sites on the $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP peptide ions.

For accurate MD results, it is necessary to know the location of the deprotonation sites. For the SP peptide, there are 6 possible residues that can

represent these sites corresponding to 15 and 20 (for $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP ions, respectively) possible arrangements of charges. It may be argued that, for MD simulations, one should consider the apparent gas phase acidities of different residues [78, 79]; however, estimating apparent gas phase acidities requires a knowledge of the distance between charge sites [78] which requires candidate structures from MD. Even knowing the distance between potential charge sites may not be sufficient to accurately estimate correct locations as the most stable ion structure can dictate unique charge arrangements [80].

Here we propose the use of HDX-MS/MS data to find the most likely deprotonation sites for the $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP ions. Figure 4.3 shows the per-residue deuterium content after ion fragmentation of the diffuse $[M-2H]^{2-}$ ions. It can be argued that D residues with a deuterium content greater than 1 should not be assigned as charge sites because they contain more than one exchangeable hydrogen. This leaves 3 possible charge site locations (residues D4 and D6 and the C-terminus) as well as three different charge site configurations. All three configurations were analyzed by MD and the charge arrangements which included the C-terminus yielded structures that were 20% larger (collision cross sections) than the experimental values. Therefore, the D4 and D6 residues were assigned as the deprotonation sites for the $[M-2H]^{2-}$ SP ions. Estimating the deprotonation sites for the $[M-3H]^{3-}$ SP ions using the same approach is straightforward. Only the D4 residue exhibits a deuterium content that is less than 1 (Figure 4.3); therefore, a plausible charge site arrangement consists of residues D4 and D7 and the C-terminus. As with the doubly-charged ions other charge configurations were also modeled and found to lead to

structures with generally larger collision cross sections than observed experimentally.

4.3.4. Hydrogen accessibility scoring of SP ions and expected deuterium uptake values.

A question arising from the HDX measurements is whether or not the results for the more diffuse $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP ions allows the elucidation of structural information. To accomplish this, the deuterium content values can be compared to expected values obtained from a hydrogen scoring model that is applied to nominal ion structures obtained from MD. Figure 4.4 shows two ion structures for the diffuse $[M-2H]^{2-}$ SP ions having collision cross sections that match the experimentally determined values. The first ion structure contains deprotonated D4 and D6 residues while the second ion structure contains a deprotonated D3 residue and the C-terminus. A description of the various interactions within the two structures is provided here. For the ion structure with the charge site assignment based on the MS/MS data (Figure 4.4a), the oxygens on the negatively-charged D4 residue are observed to interact with hydrogens on the side-chains of the D3 and D5 residues. The deprotonated side chain of the D6 residue is observed to be charge solvated by the I9-K13 peptide backbone. The second ion structure (Figure 4.4b) exhibits less extensive backbone amide solvation at the C-terminal end. For this structure, the oxygens of the D3 residue interact with the side chain hydrogen of the D5 residue and the backbone amide

of the D6 residue. The oxygens on the deprotonated C-terminus interact with the hydrogen on the D6 side chain and the K10 backbone amide.

Figure 4.4. Nominal $[M-2H]^{2-}$ SP ion structures obtained from MD for two different charge site configurations. Both structures exhibit matching collision cross section with the more diffuse ($\Omega=315 \text{ \AA}^2$) $[M-2H]^{2-}$ SP ions. Panel a shows a resultant low-energy structure from MD in which the D4 and D6 residues serve as deprotonation sites while in panel b a structure with deprotonated D3 and C-terminus sites is shown.

The structures obtained from the MD can be used to test the charge site configurations intimated by the MS/MS data. Figure 4.5 compares the experimentally determined uptake values with theoretical uptake values for individual residues computed using the two $[M-2H]^{2-}$ ion structures (Figure 4.4) from MD and the hydrogen accessibility model. For the ion structure for which the charge site placement suggested by the MS/MS data was utilized, there is generally better agreement with the experiment. For example, the D3, D6, and I8 residues all show a dramatic improvement in the comparison of theory and

experiment for this ion structure (Figure 4.4a). For all other residues there is no significant difference in predictive capabilities between the two structures.

Figure 4.5. Bar graphs comparing predicted (red) and experimental (blue) per-residue deuterium uptake values for diffuse $[M-2H]^{2-}$ SP ions. The theoretical uptake values in each panel are calculated using the structures from MD exhibiting different charge site arrangements (Figure 1). Panel a shows the comparison for the ion structure in which the deprotonation sites are selected as the D4 and D6 residues. Panel b shows the comparison for the ion structure for which the D3 and C-terminus are used as charge sites.

Having noted the superior match with the $[M-2H]^{2-}$ ion structure shown in Figure 4.4a, it is instructive to evaluate some of the differences observed between the experimental and theoretical deuterium uptake values (Figure 4.5a). As mentioned above, complete HD scrambling could occur on the backbone of the peptide ion while that on the side chains is limited. To consider the effect of complete backbone scrambling, consider the residues with only 1 exchangeable

hydrogen (residues D4, D6, and I8). All of these residues have a hydrogen accessibility score that is greater than the average deuterium content. It appears that HD scrambling along the backbone may result in a decrease in deuterium content for all three of these residues. That is, even though these sites are highly accessible to charge sites, HD scrambling results in the movement of incorporated deuteriums to other regions of the peptide.

Having shown the improved agreement (hydrogen accessibility and deuterium content) for the $[M-2H]^{2-}$ ion structure with the charge locations intimated by the MS/MS data, it is useful to consider the structural information afforded by the per-residue analysis. The D5 residue shows the highest deuterium content value per exchange site (1.7/2) in Figure 4.5a. Such extensive deuterium content can be ascribed to the location of the D5 residue. That is, the D4 and D6 flanking residues are considered to be charge sites. Therefore, the D5 hydrogens would be more accessible to complex formation involving the charge sites that would be required for exchange via the relay mechanism [81]. Indeed the hydrogen accessibility model also suggests that a higher deuterium content value should be obtained for the D5 residue. Although residues K1+K2 show a relatively large deuterium content, the ratio of deuterium per exchange site (3/7) is relatively low (Figure 4.5a). Low deuterium uptake for these residues shows decreased accessibility to the more distal charge sites. Again, this is reflected in the hydrogen accessibility score (3.2). In a separate example, the D3 and D7 residues show about the same deuterium uptake values which is reflective of their being similarly positioned to single charge sites (the D4 and D6 residues, respectively). The relative deuterium uptake values for the D3 and D7

residues are also accurately captured in the hydrogen accessibility model even showing a slight increase from the former to the latter residue as determined experimentally (Figure 4.5a).

Figure 4.6. Nominal $[M-2H]^{2-}$ SP ion structures obtained from MD for two different charge site configurations. Both structures exhibit matching collision cross section with the more diffuse ($\Omega=362 \text{ \AA}^2$) $[M-3H]^{3-}$ SP ions. Panel a shows a resultant low-energy structure from MD in which the D4 and D7 residues and the C-terminus serve as deprotonation sites while in panel b a structure with deprotonated D3, D5, and D7 residues is shown.

Figure 4.6 shows the two ion structures for the diffuse $[M-3H]^{3-}$ SP ions having matching collision cross sections. The charge site locations for the first and second ion structures are the D4 and D7 residues and the C-terminus and the D3, D5, and D7 residues, respectively. Although these ion structures provide similar collision cross sections, a notable difference is observed. For the first structure with charge placements based on the MS/MS data, the D4 and D7 residues are charge solvated by neighboring D residues as well as the N-

terminus. The C-terminus charge protrudes away from the ion. For the second structure, the D5 and D7 residues show a significantly increased degree of charge solvation by the C-terminal residues.

Figure 4.7. Bar graphs comparing predicted (red) and experimental (blue) per-residue deuterium uptake values for diffuse $[M-3H]^3-$ SP ions. The theoretical uptake values in each panel are calculated using the structures from MD exhibiting different charge site arrangements (Figure 3). Panel a shows the comparison for the ion structure in which the deprotonation sites are selected as the D4 and D7 residues as well as the C-terminus. Panel b shows the comparison for the ion structure for which the D3, D5, and D7 residues are used as charge sites.

Figure 4.7 shows the comparison of the hydrogen accessibility scores with the experimental deuterium uptake values for the two $[M-3H]^3-$ SP ion structures obtained from MD. As with the $[M-2H]^2-$ ions, the agreement between experiment and theory is improved for the structure (Figure 4.6a) for which the charge site location is based upon the MS/MS data (see above). This improvement is

observed in the comparison of the D3, D4, and D5 residues. Indeed, only a slight decrease in predictive capability is observed for the K1+K2 region. Interestingly, the location of the charge site at the D4 residue does not appear to impart an enhancement in the deuterium uptake at neighboring D residues with the exception perhaps of the D3 residue.

Figure 4.8. Bar graphs comparing predicted (red) and experimental (blue) per-residue deuterium uptake for the more diffuse $[M+3H]^{3+}$ SP ions. The charge site arrangement shown in panel a is the K1, K10, and K13 residues. For panel b the charge site arrangement is the K1, K2, and K13 residues.

4.3.5. Extension of charge site localization to positively-charged ions.

Although this proof of concept study shows an advantage for IMS-HDX-MS/MS in determining the locations of charges for negatively charged peptide ions, it is desirable to test the applicability to other types of ions. Here we compare results for positively-charged peptide ions. Prior IMS-HDX-MS/MS (via

ETD) studies of $[M+3H]^{3+}$ SP ions have provided candidate structures from MD which show good agreement between experimental and theoretical deuterium uptake values. For the matching ion structure, the K1, K10, and K13 residues are protonated. Figure 4.8a shows that the agreement between the hydrogen accessibility method [18] and the experimental deuterium uptake is very good. Indeed the theory captures the relative changes in deuterium uptake between residues remarkably well with the exception of the K10 and I12 residues. For a separation ion structure of matching collision cross section having protonation sites of K1, K2, and K10, the agreement is markedly decreased. Indeed the only residues that do show agreement between the experiment and the theory are the D7, I8, and I11 residues.

To further demonstrate the utility of the IMS-HDX-MS/MS in assigning charge sites in positive ion mode, separate experiments were performed for peptide ions formed by electrospraying the MP peptide (Ace-PAAAAKAAAAKAAAAK). As with the positively charged SP ions, the MS/MS analysis is performed by ETD in order to preserve the location of the deuterium label. After HDX, the more compact ($\Omega=417 \text{ \AA}^2$) $[M+3H]^{3+}$ MP ions were mobility selected and subjected to ETD. The per-residue deuterium content for the compact conformer is shown in Figure 4.9.

Selecting 3 charge locations from 4 available sites results in 4 possible charge site configurations. The observation of the c_6 and z_1 ions in the ETD spectra suggests that the K6 and K21 residues are protonation sites. The question that arises is whether the K11 or the K16 residue is the remaining protonation site. The potential charge arrangements (residues K6, K11, K21 and

K6, K16, K21) were used in MD. Figure 4.9a shows the predicted deuterium uptake pattern for the structure using residues K6, K11, and K21 as protonation sites. A comparison with experimental deuterium uptake values shows good agreement with data for this charge site assignment with the exception of residues A17, A18, and A20. For residues K6, K11, and K16, a smaller deviation is observed (<0.3).

Figure 4.9. Bar graphs comparing predicted (red) and experimental (blue) per-residue deuterium uptake for the more diffuse $[M+3H]^{3+}$ MP ions. The charge site arrangement shown in panel a is the K6, K16, and K21 residues. For panel b the charge site arrangement is the K6, K11, and K21 residues.

Figure 4.9b compares the predicted deuterium uptake pattern for the structure with the K6, K16, and K21 residue charge site arrangement with the experimental values. Residues K11, K16, and K21 exhibit a significant deviation (>0.7) from

the experimental deuterium uptake values. Therefore, these data suggest that the appropriate charge site assignment is residues K6, K11, and K16.

4.4. Conclusions

The deuterium uptake levels have been recorded for select ion conformations of negatively-charged peptide ions. CID of $[M-2H]^{2-}$ and $[M-3H]^{3-}$ SP peptide ions suggests only a limited degree of HD scrambling that is primarily relegated to backbone amide sites; label retention is observed for side-chain sites. One advantage of the limited HD scrambling resulting from collisional activation in negative ion mode is the ability to locate charge sites based on comparisons of per-residue deuterium incorporation. These charge site determinations aid the production of candidate ion structures from MD which are necessary to obtain useful structural information from IMS-HDX data in the form of hydrogen site accessibility. Although several drawbacks exist for these negative ion analyses including the potential for backbone HD scrambling and the production of more complicated ion fragmentation spectra, these proof-of-concept experiments show that the mapping of charge sites and exchange sites with CID is possible for negatively-charged ions. Additionally, the approach can be extended to positively-charged peptide ions to confirm the locations of assigned protonation sites for MD.

4.5. References.

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5. Ion Mobility Spectrometry-Hydrogen Deuterium Exchange Mass Spectrometry of Anions: Part 3. Estimating Surface Area Exposure by Deuterium Uptake¹

¹ Khakinejad, M., et al., Ion Mobility Spectrometry-Hydrogen Deuterium Exchange Mass Spectrometry of Anions: Part 3. Estimating Surface Area Exposure by Deuterium Uptake. Journal of the American Society for Mass Spectrometry, 2016. 27(3): p. 462-473.

5.1. Introduction

Over the last 20 years, the elucidation of protein structures has proceeded at an accelerating rate [1]. Because of the importance of protein complexes in performing specific biological functions, over recent years increased attention has been given to the development of techniques for quaternary structure determination. Ideally, X-ray crystallography would be used to provide high-resolution structural information for protein complexes; however, structural studies require the in vitro reconstitution of purified protein subunits as well as the ability to crystallize the protein complex [2]. Although ground breaking studies in the determination of protein quaternary structure occurred in 2000 [3-5], there are yet limitations in the elucidation of such structures by X-ray crystallography [2]. Nuclear magnetic resonance spectroscopy (NMR) also suffers from a limitation associated with molecular size, however, recent advances such as magic angle spinning NMR have enabled its limited application to the study of protein subunit architecture [6]. More recently, impressive advances in cryoelectron microscopy have allowed the study of large protein complexes although challenges remain with regard to resolution, throughput, and instrumental parameter optimization (e.g., electron energies) [7, 8].

To overcome some of the challenges associated with traditional methods of structure characterization, mass spectrometry (MS) techniques have been

developed. A major area of research involves chemical modification of the proteins of interest followed by MS characterization of the intact protein (top-down approach) or proteolytic fragments (bottom-up approach). One strategy that is used extensively is solution hydrogen-deuterium exchange (HDX). For typical experiments, a protein is temporarily subjected to exchange (hydrogens for deuteriums – exchange in, or deuteriums for hydrogens – exchange out) followed by reaction quenching, enzymatic digestion, and liquid chromatography (LC) – tandem mass spectrometry (MS/MS) [9, 10]. Several challenges arise from this approach including digestion efficiency and peptide identification (especially for samples producing complex peptide mixtures) as well as comparing the data with *in-silico* structures [9-12]. Another approach involves the use of chemical cross-linking of amino acid residues that are in three-dimensional (3D) proximity followed by enzymatic digestion and LC-MS/MS to identify neighboring sites [13, 14]. Similar to HDX studies, peptide ion identification presents a challenge to such an approach. Finally, more recently, a number of studies have sought to introduce other chemical modifications into proteins in solution followed by MS analysis [15-17]. Although promising, such techniques experience some of the same challenges of the HDX LC-MS/MS approaches and have largely been limited to smaller protein analysis.

During the mid 1990s, researchers presented the idea of native MS whereby the composition of ions of non-covalent protein complexes could be analyzed by electrospray ionization (ESI) – MS [18]. Studies in this area have proceeded rapidly over the last two decades allowing the determination of protein

complex masses and stoichiometries [18, 19]. It is recognized that the size of ions that can be interrogated by the approach is nearly limitless extending to the tens of megadaltons [20-22]. Additionally, collisional activation can be used to provide some information regarding individual protein subunits [23]. One breakthrough approach has been the use of surface-induced dissociation (SID) which allows the preservation of the charge distribution within the product ions [24]. Although native MS provides advantages in terms of sensitivity, throughput, and characterization breadth, challenges associated with sample purity as well as the requirement of unique MS instrumentation and operational conditions exist.

As an expansion of native MS studies, seminal experiments have demonstrated the utility of incorporating ion mobility spectrometry (IMS) measurements. This addition presents shape information for protein complex and aggregate ions in the form of collision cross section (CCS) measurements [25-27]. Although the concept of comparisons of CCS values for biomolecular ions was established nearly a decade earlier [28-31], theoretical approaches for the generation of trial structures for extremely large ions have proceeded at a rapid pace [32]. One notable advantage of the IMS-MS approach for protein complex studies is that collisional activation can be used to study protein complex, subcomplex, and subunit ions [23, 33, 34]. A limitation of IMS-MS studies is that only general information regarding ion shape is obtained. The extraction of higher-resolution structural information requires the ability to obtain relevant comparisons to accurate ion structures as may be obtained by molecular

dynamics (MD) simulations. This not only presents challenges with obtaining accurate structures themselves but also with the ability to calculate reliable CCS for many trial structures [32].

One approach intended to provide additional structural information is the incorporation of gas-phase HDX with IMS-MS analysis. The relative degree of exchange site accessibility has been assessed for different protein ion conformations [35, 36]. Additionally, HDX site accessibility models have been presented for ion structures obtained from MD [36, 37]. A limitation of the approach is that a detailed description of accessible exchange sites was not possible due to the potential for hydrogen-deuterium (HD) scrambling upon precursor ion fragmentation by collision-induced dissociation (CID) [38]. The introduction of non-ergodic ion fragmentation techniques has provided a means for preserving the location of the deuterium uptake label [39]. Indeed, recent studies have demonstrated that per-residue deuterium uptake can be determined for specific ion conformers as distinguished by IMS [40, 41].

In chapter 4, IMS-HDX-MS/MS has been performed on peptide anions whereupon precursor ion CID, the locations of charge sites can be determined. Additionally, the study accompanying this work (chapter4) shows some ability to use CID as an ion activation approach for the study of deuterium incorporation within large peptide anions. In this study, bovine insulin is used as a model system to examine the degree of label retention for a multi-chain protein system. In these proof-of-principle studies we find limited HD scrambling up to the separation of the peptide chains. Finally, models to estimate the relative exposed

surface area of different insulin sub-chains are presented. The results are discussed with respect to the applicability of such approaches for protein complex structure characterization.

5.2. Experimental

5.2.1. Sample preparation.

Much of the experimental details is presented in the study accompanying (chapter 4) this work. Here only a brief description of the experimental parameters is presented. The synthetic peptide having the sequence of KKDDDDDIKK and bovine insulin (each >90% purity) were purchased (Genscript, Piscataway, NJ 08854; and Sigma Aldrich, St. Louis, MO, USA, respectively) and used without further purification. From stock solutions (1.0 mg·mL⁻¹), ESI solutions consisting of 10 mM Ammonium hydroxide:acetonitrile (1:1) were prepared such that the final analyte concentration was 0.1 mg·mL⁻¹. Infused samples (300 nL·min⁻¹) were electrosprayed into the hybrid IMS-MS instrument [41-43] using a bias of -2200 V.

5.2.2. IMS-MS measurements.

Comprehensive descriptions of IMS instrumentation and applications exist in the literature [25, 44-50]. The new IMS-MS instrument has also been described previously [41, 42] as well as in chapter 4. Using a dual ion gate drift tube configuration, electrosprayed anions were periodically pulsed into a drift tube with the first ion gate and mobility selected with the second ion gate. For a given mobility selection, MS and MS/MS spectra were recorded with the linear ion trap (LIT) mass spectrometer (LTQ Velos, ThermoScientific, San Jose, CA,

USA). By scanning the delay times between the front and back ion gate, mass spectral characterization of the entire drift time (t_D) distribution was accomplished. For all mobility-resolved MS datasets, the LIT scan parameters employed a m/z range of 80 to 2000. Sample injection times of 200 ms (5 microscans) were utilized. t_D -selected precursor ion mass spectra were collected for 0.5 min each. Two-dimensional (2D) IMS-MS datasets were generated using an algorithm that created a three-column array file associating m/z and intensity (i) information with each t_D selection.

5.2.3. HDX measurements.

D₂O (>99%, Sigma Aldrich, St. Louis, MO, USA) was introduced into the He buffer gas using a separate leak valve (Granville Philips, Longmont, CO, USA); a D₂O partial pressure setting of 0.04 Torr was used for these studies. Here we note that in previous studies of insulin anions, higher partial pressures of D₂O were used to obtain maximum HDX levels [42]. A lower pressure is used here to allow the consideration of only the fast exchanging hydrogens [43]. That is, these sites are the most amenable to modeling (exhibiting the same HDX rate) by the HDX accessibility method (see below). t_D , m/z distributions were recorded in pure He and subsequently in the He:D₂O mixture. For specific ion conformers the number of incorporated deuteriums was determined from the difference in precursor ion average mass before and after exposure to D₂O. The deuterium content of chain A and chain B fragment ions was also obtained by comparing the m/z values of these ions formed upon CID of precursor ions before and after undergoing HDX.

Because previous experiments have shown that multiple ion conformers can exist for given mobility selections [43], it is useful to consider the effect of the width of the mobility selection. We have increased the gate width up to 1 ms for the ion conformer types studied and observe no significant difference in the deuterium content of the chain A and chain B fragment ions. For these experiments we have utilized selection widths of 200 μ s to be consistent with the narrowest range of ion conformers possible for the current IMS-MS instrumentation.

5.2.4. Molecular dynamics simulations.

MD experiments required the creation of the $[M-4H]^{4-}$ and $[M-5H]^{5-}$ insulin ion starting structures. Charge site location considerations were the same as those described in the chapter 4. Indeed the ion structures for the KKDDDDDIKK anions are the same as reported therein. For bovine insulin, there are 6 acidic residues with one being adjacent to an arginine residue (E21 and R22 on chain B). This site was assumed to form a salt bridge and so, for the $[M-5H]^{5-}$ insulin ions, all remaining acidic sites were deprotonated. For $[M-4H]^{4-}$ insulin ions, Coulomb energy considerations suggest that two negative charges be placed on chain A with the other two being placed on chain B which has 3 acidic sites (E4, E17, C-terminus). The intrinsic gas-phase acidity of the E residue is much larger than the C-terminus [51], therefore the E4 and E17 residues were used as charge sites on chain B. Additionally, alternative charge placements for MD resulted in structures that were too large for comparison with

the IMS data and the HDX prediction data matched the above-mentioned charge site configuration.

Starting ion structures for simulated annealing were obtained as described here. Chain A and chain B ions were created as linear species. Each ion was heated separately to 1000 K and subsequently cooled and energy minimized. Low-energy structures for each peptide ion that exhibited similar distances between cysteine residues (as assessed by intra-chain disulfide bonds) were selected and joined with disulfide bonds to form the insulin precursor ion starting structures. Simulated annealing was performed as described previously [41, 43] and low-energy structures with matching CCSs for the $[M-4H]^{4-}$ and $[M-5H]^{5-}$ ions were used for the hydrogen accessibility scoring and surface area assessment. The CCS values for 1000 conformers from simulated annealing were calculated using the mobcal [52] software employing the trajectory method (TM) [53]. Here, the MSMS program was used to calculate the solvent accessible (SAS) and solvent excluded (SES) surface areas [54]. To calculate the SES and SAS values, a probe sphere radius of 1.4 Å and a triangulation density of 1.0 vertex/Å² were used.

5.2.5. Estimating peptide chain deuterium content under 100% HD scrambling conditions.

To estimate the expected deuterium content for 100% HD scrambling, the total number of labile hydrogens was first calculated. Per-site 100% HD scrambling values were calculated by dividing the total uptake by the number of labile hydrogens. The per-site values were then be summed for each chain to

estimate the expected deuterium content. As an example, consider a dimer peptide ion that contains 30 labile hydrogens and experiences, on average, an increase in mass of ~15 Da. Under 100% scrambling conditions, the per-site deuterium content would be ~0.5. These values can then be summed for each peptide chain according to their distinctive numbers of labile hydrogens to determine expected uptake values for individual peptide chains under 100% HD scrambling conditions. This method is the same as the generally accepted method presented by Jorgenson and coworkers [39].

5.2.6. HDX accessibility scoring.

The model for scoring relative hydrogen accessibility was based on the generally accepted “relay” mechanism for HDX as presented by Beauchamp and coworkers and Chan and Enke [55, 56]. That is, the current model is similar to other models that considered threshold distances between charge sites and exchange sites; the current model also considers distances between exchange sites and the site of initial deuterium incorporation. Briefly, deuterium uptake predictions were performed for the sub-chains of bovine insulin using a modified method described previously [57, 58]. As before, the distances between charge sites and exchange sites were determined using nominal ion structures obtained from MD. Heteroatom exchange sites were then scored (S_{HDXS}) based on a summation of the inverse distances according to Equation 5.1.

$$S_{HDXS} = \sum \frac{1}{d_{O-H}} \quad (5.1)$$

In Equation 5.11, d_{O-H} represents the distance between the exchange site and a deprotonated carboxylic acid site. Although, the accessibility treatment presented

by Equation 5.1 describes the relative accessibility of exchange sites to charge sites, the effects of protein fold protection are not taken into account. To investigate exchange site protection from the protein fold, two different methods of chain scoring were used, which are discussed comprehensively below. Insulin sub-chain scores (representing relative uptake capability) obtained from the S_{HDXS} values modified by the protein fold effects (see below) were then normalized to the total deuterium uptake of the precursor ions and compared to the experimental values.

5.3. Results and Discussion

5.3.1. Peptide and protein ion collision cross sections.

Figure 5.1 shows the 3D raised relief plot for insulin ions indicating t_D and m/z values for different conformer types. A dominant feature comprising $[M-5H]^{5-}$ insulin ions is observed at a t_D of 9.5 ms corresponding to a relatively diffuse conformer type having a CCS of $808 \pm 5 \text{ \AA}^2$. Here the term diffuse refers to larger ion structures within a given charge state. Also observed for these ions are some unresolved lower mobility features. At m/z 1432 $[M-4H]^{4-}$ insulin ions exhibit two major conformer types; one appears at a t_D of 9.6ms ($\Omega = 676 \pm 3 \text{ \AA}^2$) and the other is observed at a t_D of 10.3ms ($\Omega = 779 \pm 3 \text{ \AA}^2$). $[M-3H]^{3-}$ insulin ions show the lowest intensities and the most compact structures.

Figure 5.1. Three-dimensional (t_D , m/z , i) raised-relief plot produced upon electrospraying a solution of bovine insulin. Dominant ions in the distribution are labeled.

The most compact $[M-3H]^{3-}$ insulin ions appear at a t_D of 9.8 ms ($\Omega = 528 \pm 2 \text{ \AA}^2$) and are observed to be more intense than the more diffuse triply-charged ions appearing at a t_D of 10.8ms ($\Omega = 595 \pm 2 \text{ \AA}^2$) as shown in Figure 5.2. It is instructive to consider the charge state dependence of collision cross sections for the insulin system. For the $[M-5H]^{5-}$ ions only one diffuse conformer type exists which is very similar to the more diffuse $[M-4H]^{4-}$ ions (808 $\text{\AA}^2$ versus 779 $\text{\AA}^2$, respectively). This small increase in cross section – from the latter to the former ions – can be attributed to the minor structural change of ion elongation resulting from greater Coulomb repulsion. The more compact $[M-4H]^{4-}$ ions are intermediate in size compared to the compact $[M-3H]^{3-}$ ions and the diffuse conformer types, which shows a structural transition effected by Coulomb repulsion similar to that described previously for protein ions [59].

Figure 5.2. t_D distribution of $[M-3H]^{3+}$ insulin ions. The most compact $[M-3H]^{3+}$ ions appear at $t_D \sim 9.8$ ms ($\Omega = 528 \pm 2 \text{ \AA}^2$) and are observed to be more intense than the more diffuse triply-charged ions [$t_D \sim 10.8$ ms ($\Omega = 595 \pm 2 \text{ \AA}^2$)].

Collision cross sections for the dominant features in Figure 5.1 have been calculated using Equation 5.2 [60].

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[\frac{1}{m_I} + \frac{1}{m_B} \right]^{1/2} \frac{t_D E}{L} \frac{760}{P} \frac{T}{273.2} \frac{1}{N} \quad (5.2)$$

In Equation 5.2, ze and k_B correspond to the charge of the ion and Boltzmann's constant, respectively and m_I and m_B correspond to the mass of the ion and the mass of the buffer gas, respectively. The variables E , L , T , and P correspond to the electric field, length of the drift tube, and the temperature and pressure of the buffer gas, respectively. N is neutral number density under standard temperature and pressure (STP) conditions.

5.3.2. Insulin as a model system for determining subunit HD scrambling.

In the study of protein complex structure, a knowledge of the relative amount of exposed surface area of each subunit is desirable. That is, such information can provide insight about the overall morphology and the arrangement of subunits in a protein complex. Therefore, measurements of total deuterium uptake by each subunit may provide a method for estimating the relative exposed surface area of subunits. For such experiments, HDX-MS/MS data are useful only if no extensive inter-subunit HD scrambling occurs upon dissociation of the precursor ions. That is, dissociation must occur at an earlier time point than that of extensive proton mobilization between subunits. Insulin was chosen for this study because the single polypeptide chain A is strongly tethered to the separate peptide chain B by 2 disulfide bonds. The insulin system therefore, can be considered a worst-case scenario for small protein complex ions. For comparison purposes, Table 5.1 shows the full dissociation normalized collision energies for a series of related peptide dimers of similar size to the insulin system studied here. Notably, the values are nearly two-fold smaller than those required to dissociate the insulin ions. The small protein was also selected because of the limited m/z range of the LIT mass spectrometer.

Admittedly the ESI solution used in these studies does not resemble native MS conditions. The home-built IMS-MS instrument is significantly less sensitive in negative ion mode. Therefore, the ESI solvent composition for protein and peptide anions was selected based on its ability to provide the largest ion signal levels. Under such conditions appreciable amounts of multimer ions are

not observed for larger peptides. For this reason disulfide bonded insulin was selected for the study. That said, MD shows that ion structures matching the IMS and HDX data are sufficiently compact to indicate comparatively close proximity of sub chains [61].

Table 5.1. Normalized collision energies required for complete dissociation of peptide ion dimers [2M+3H]³⁺

sequence	collision cross section (Å ²)	normalized collision energy
MATLEKLMKAFESLKSF	608	20
	667	18
	683	17
MATLEQLMKAFESLKSF	613	34
	650	26
	677	24
	709	22
MATLEKLMQAFESLKSF	656	24
	709	23
MATLEKLMKAFESLQSF	613	28
	698	23
MATLEQLMQAFESLKSF	634	23
	656	23
	689	21
MATLEQLMKAFESLQSF	667	22
	688	22
MATLEQLMQAFESLQSQ	592	23
	645	23
	677	23

5.3.3. CID of insulin anions to assess hydrogen-deuterium scrambling.

Figure 5.3 shows the CID spectra for the more diffuse [M-4H]⁴⁻ and [M-5H]⁵⁻ insulin ions. In Figure 5.3a, an increase in the ion activation energy to a normalized collision energy (instrument parameter) of 30, results in three separate features in the mass spectrum. The remaining precursor ions are observed at *m/z* 1432. More intense features are observed at *m/z* 1167 and *m/z* 1698. These features are assigned to doubly-charged chain A and doubly-charged chain B fragment ions, respectively. Here we note that multiple studies have indicated different types of ions formed by dissociation of disulfide-bonded peptide and protein anions. Some experiments have shown retention or loss of

both sulfur atoms by one peptide ion fragment [62-64]. Our data is identical to protein anion studies in which the disulfide bond is cleaved [64].

Figure 5.3. CID spectra of the more diffuse $[M-4H]^{4-}$ (panel a) and the $[M-5H]^{5-}$ (panel b) insulin ions. The major peaks in panel a, from left to right, are: doubly-charged chain A, quadrupoly-charged insulin, and doubly-charged chain B. In panel b, the major peaks, from left to right, are: triply-charged chain A, an unassigned fragment ion, quintuply-charged insulin, and doubly-charged chain B. The blue trace represents data collected in the absence of D_2O in drift tube, while the red trace represents data collected using a D_2O partial pressure of 0.04 Torr.

After exposing the diffuse $[M-4H]^{4-}$ ions to D_2O (partial pressure of ~0.04 Torr) and subsequently fragmenting the precursor ions using the same activation parameters, a nearly identical mass spectrum is produced. That is, the relative intensities of the precursor ions and doubly-charged chain A and chain B ions are similar to those observed prior to the introduction of D_2O into the drift tube. A difference is that the ions observed after HDX are shifted to higher m/z values

indicating deuterium incorporation by the precursor ions. The $[M-5H]^{5-}$ insulin ions exhibit similar behavior under CID conditions with the exception that another unassigned fragment ion peak near the precursor ion is observed as shown in Figure 5.3b. The deuterium content for each peptide chain is calculated by subtracting the m/z of the fragment ion produced from precursor ions not subjected to HDX from the m/z of the same fragment ion produced after HDX.

McLafferty and coworkers first presented the concern of deuterium scrambling within collisionally-activated organic compound ions [65]. Since that time, a number of researchers have demonstrated HD scrambling for collisionally-activated biomolecular ions [38, 39, 66, 67]. Although some studies have demonstrated 100% scrambling including studies of peptide anions [67, 68], others have suggested that scrambling is less significant and indeed some structural information can be preserved from HDX experiments [38, 66]. The peptide chain deuterium content for the insulin fragment ions has been compared to that expected from 100% HD scrambling (see Experimental Section). The results of this comparison are shown in Figure 5.4. Notably, there is a significant difference between the deuterium content values expected for 100% HD scrambling versus the experimentally determined values. Therefore, the data are consistent with the occurrence of insulin chain separation prior to significant proton mobilization leading to 100% HD scrambling.

Figure 5.4. Bar graph showing the comparisons of deuterium uptake for the insulin chain ions. The blue and red bars represent the experimentally measured deuterium uptake and the deuterium uptake that would be observed upon 100% HD scrambling, respectively. The yellow bars show the expected deuterium uptake values using the hydrogen accessibility scoring for all of labile hydrogens (see Experimental section). Panels a and b represent data for the more diffuse $[M-4H]^4-$ and $[M-5H]^5-$ insulin ions, respectively. The error bars represent one standard deviation obtained from three separate measurements of mobility-selected ions.

Studies have shown that MS instrument parameters such as collisional activation time can affect the degree of HD scrambling [69]. To support the claim of limited (or no) HD scrambling under these conditions, we have subjected the $[M-4H]^4-$ insulin ions to increased CID activation times. Under these conditions, dissociation of the chain A and chain B peptides shows a shift toward increased inter-chain HD scrambling as shown in Figure 5.5. It is also instructive to note that the HDX values were determined using average m/z values of the isotopic envelopes.

Figure 5.5. Effect of CID parameters on inter-chain HD scrambling. The more diffuse $[M-4H]^{4+}$ insulin ions have been used in this experiment. Data collected using a normalized collision energy of 35 and an activation time of 10 ms is represented by the blue bar graphs while values of 25 and 50 ms, respectively, are represented by red bar graphs. The theoretical 100% HD scrambling values are also shown (green bar graphs).

To compare the behavior of deprotonated insulin and protonated insulin identical (MS instrument parameters) experiments have been repeated for positively-charged insulin ions. Figure 5.6 and Figure 5.7 show the results for diffuse $[M+4H]^{4+}$ ($\Omega = 720 \text{ \AA}^2$) and $[M+5H]^{5+}$ ($\Omega = 818 \text{ \AA}^2$) insulin ions, respectively. A normalized collision energy increase from 20 to 25 results in backbone cleavage and no intact chain A and chain B fragment ions are observed at higher CID energies. Such results are indicative of more extensive proton mobilization for these insulin ions compared with the deprotonated species.

Figure 5.6. CID spectra of $[M+4H]^{4+}$ insulin ions. A normalized collision energy (instrument parameter) was set at 20 and 25 for the left and right spectra, respectively. No intact chain A and chain B fragment ions were observed.

Indeed for the deprotonated insulin ions, the separation of chain A and chain B requires an even larger normalized collision energy of 30. Here we present a note of caution. The energy distribution of the ion population during the CID process is broad and these comparisons are thus only presented as semiquantitative assessments; more precise comparisons can be obtained by utilizing other ion dissociation techniques such as SID [70].

Figure 5.7. CID spectra of $[M+5H]^{5+}$ insulin ions. A normalized collision energy (instrument parameter) was set at 20 and 25 for the left and right spectra, respectively. No intact chain A and chain B fragment ions were observed.

It is well established that the various fragments produced by CID of peptide ions results from the mobility of available protons. During collisional activation of isotopically labeled peptide ions, this mobility results in the scrambling of the deuterium label. Therefore, the relatively limited HD scrambling observed between chain A and chain B fragment ions may result from what is here termed as limited proton mobilization for these deprotonated species. That is, for such ions the source of protons is more limited than for protonated ions. For example, there is only one proton source for the $[M-5H]^{5-}$ insulin ions (a neutral carboxylic acid group). In comparison, $[M+5H]^{5+}$ ions would have all

protonated basic residues as well as six neutral carboxylic acid residues. Additionally, it should be noted that the proton from the carboxylic acid side chains have been termed “locally” mobile due to the observation of limited fragment ion types resulting from its translocation [71-73]. Therefore the conditions of fewer available protons overall and the limited degree of mobility of some may account for the observed, limited inter-chain scrambling.

Figure 5.8. Panels a and b contain nominal structures obtained by MD for the more diffuse [M-4H]⁴⁺ and [M-5H]⁵⁺ insulin ions, respectively. The upper portions of the panels show detailed structures and the lower portions show the overall surface accessible morphology. In these schematic depictions, chain A and chain B are colored blue and red, respectively. Collision cross sections for the nominal structures are shown above each panel.

5.3.4. Hydrogen accessibility scoring combined with protein fold considerations.

Figure 5.4 reveals the disparity in deuterium uptake by the different insulin peptide chains. For the more diffuse $[M-4H]^{4-}$ insulin ions, chain B uptakes a considerably larger number (2.5 fold) of deuterium compared with chain A. Although chain B has a greater number of labile hydrogens than chain A (52 versus 42 – red bars in Figure 5.4), this difference alone cannot account for the observed deuterium within the different peptide chains. Using a nominal ion structure (conformer type) obtained from MD (shown in Figure 5.8) along with a hydrogen accessibility scoring method (see Experimental section), the deuterium content ratio (chain B:chain A) shouldn't be greater than 1.7 fold (yellow bars Figure 5.4). Therefore, it becomes necessary to consider other factors that may result in the greater protection of exchange sites on chain A.

Figure 5.9. Collision cross section values versus maximum deuterium uptake for different gas-phase conformers of a)ubiquitin and b)bovine cytochrome c anions [42].

Figure 5.9 shows a near linear relationship between the maximum deuterium uptake level and CCS of different ubiquitin and cytochrome *c* anions as reported in a recent study [42]. A question that arises is what accounts for the increased uptake levels with increased CCS. For the data shown in Figure 5.9, ions with larger collision cross sections typically have a greater number of deprotonation sites. Additionally, the more diffuse species should have a greater number of exchange sites exposed to D₂O. These two effects could account for the large increases in deuterium uptake with CCS for the ubiquitin and cytochrome *c* ions. That said, the largest ubiquitin ions exhibit a greater charge density than the smallest cytochrome *c* ions of similar size and yet they exhibit lower levels of deuterium uptake. Previously it has been suggested that this relative level of protection for the ubiquitin ions results from the fact that a disproportionate number of hydrogens are buried compared with the more exposed sites on cytochrome *c* ions.[42] It is also noted here that increased ion temperature can enhance the accessibility of exchange sites for species of higher charge density due to more “effective” (i.e., the formation of a critical reaction complex) conformational fluctuations [37].

The effects of protein fold protection are expected to decrease dramatically with decreased ion size. For example, for small peptides typically the more diffuse species experience less deuterium uptake [41]. Considering that no residue is completely buried within the peptide, the maximum deuterium uptake levels have been shown to depend primarily on distance from charge sites (e.g, S_{HDXS} values) [41]. In contrast, protein ions may have a considerable

number of residues buried in the interior. For example, cytochrome c has more than 200 labile hydrogens of which less than 40% on average are accessible at buffer gas temperatures of 300 K [37, 42]. Because the charge density doesn't change significantly with CCS, it may be argued that surface exposure has an increasing impact on hydrogen accessibility for these larger protein ions (even for elongated species). An extension of this concept is that quaternary structure may have a more pronounced effect upon relative deuterium uptake by subunits allowing the extraction of structural information in the form of assigning relative (periphery versus interior) positions.

It is therefore instructive to consider the effect of surface exposure for the insulin ions studied here. The ion structures exhibiting the lowest energies and having matching collision cross sections with the measured values for the more diffuse $[M-4H]^{4-}$ and $[M-5H]^{5-}$ insulin ions are shown in Figure 5.8a and Figure 5.8b, respectively. Exposed regions of chain A and chain B are also shown in Figure 5.8. To model the expected deuterium content in chain A and chain B fragment ions while accounting for the effects of protein fold protection, two different approaches have been considered. In the first approach, the S_{HDXS} value (Equation 5.1) of each hetero atom site is scaled by that site's surface accessibility ($H_{surface}$) as estimated from SES values. The chain accessibility scores (S_{chain}) are then computed using Equation 5.3.

$$S_{chain} = \sum(S_{HDXS} \times H_{surface}) \quad (5.3)$$

By scaling the sum of the S_{chain} values to the total deuterium uptake of the precursor ions, comparisons to experimental deuterium content can be made as

shown in Figure 5.10. Overall the agreement between experiment and theory is quite good for both the $[M-4H]^{4-}$ and the $[M-5H]^{5-}$ ions. The effect of incorporating the surface accessibility parameter is illustrated in Figure 5.4 where the usage of S_{HDXS} alone does not accurately model the experimental data.

Figure 5.10. Bar graph showing the comparisons of deuterium uptake for the insulin chain ions. The blue bars represent the experimentally measured deuterium uptake. The green bars show the expected deuterium uptake values using hydrogen accessibility scoring for all labile hydrogens scaled by surface exposure values (see Discussion section for details). The black bars demonstrate the expected hydrogen uptake values using the product of exposed area for each chain and their hydrogen accessibility score. Panels a and b contain data for the more diffuse $[M-4H]^{4-}$ and $[M-5H]^{5-}$ insulin ions, respectively. The error bars represent one standard deviation obtained from three separate measurements of mobility-selected ions.

Figures 5.11 and 5.12 show the effect of collision cross section of MD structures on the ability to match deuterium uptake levels. In the example, a larger collision cross section for $[M-4H]^{4-}$ ions results in deuterium uptake values that are significantly larger and smaller for peptide chain A and chain B, respectively.

Although the first approach provides a good estimate of the deuterium uptake level for each chain, it doesn't take into account the total exposed surface

$$\Omega=966 \text{ \AA}^2$$

Figure 5.11. An example of an incorrectly assigned MD structure with a larger (~24%) collision cross section value than the experimentally determined value.

area of each chain directly. It is instructive to evaluate a method that incorporates the complete exposed area of each peptide chain. One motivation for using the exposed area of the entire chain is to consider an approach where an estimated exposed surface area can be calculated directly from experimental uptake data. Here the chain surface area exposure ($surface_{chain}$) is computed from the sum of the SES values for the individual amino acid residues comprising a given chain. Thus, the new S_{chain} values are determined from,

$$S_{chain} = surface_{chain} \times \sum S_{HDXS} \quad (4).$$

Figure 5.12. Bar graph showing the effect of an incorrect assignment of ion structure on the theoretical deuterium uptake values of the insulin peptide chains. The blue bars show the experimental deuterium uptake values while the reds show the calculated uptake values using the incorrectly assigned structure shown in Figure 5.8.

Again the S_{chain} values can be scaled to the experimental deuterium uptake values and comparisons can be made to the experimental data as shown in Figure 5.10. The values are in good agreement with the experimental deuterium content. However, the agreement is not as good as that obtained when using the individual $H_{surface}$ values. Here we note that the good agreement of the former surface accessibility model with the experimental results further supports the idea that only limited HD scrambling occurs up to peptide chain dissociation for these experimental conditions. Although we cannot rule out the possibility of greater HD scrambling that is not accounted for because of the hypothetical structures obtained from MD, previous experiments have shown that conformational types

obtained from MD do accurately account for deuterium uptake using accessibility modeling[41, 43]. Additionally, we note that surface accessibility and charge site accessibility have been combined in a previous model to account for deuterium uptake by peptide ions [36].

Having incorporated $surface_{chain}$ into the expression for theoretical deuterium uptake, it is useful to consider whether or not experimental values can be related to $surface_{chain}$ without using S_{HDXS} . More interestingly, the question is whether or not the relative surface exposure for subunits in ions of protein complexes can be determined from deuterium uptake values alone. To accomplish such a comparison, another assumption is necessary. Here we consider the average S_{HDXS} values ($\bar{S}_{HDXS}$) for both chains as given by:

$$\bar{S}_{HDXS} = \frac{1}{n} \sum S_{HDXS} \quad (5.5).$$

In Equation 5.5, the variable n represents the total number of exchangeable hydrogens for a given peptide chain. The assumption to be made is that $\bar{S}_{HDXS}(\text{chain A})$ is equal to $\bar{S}_{HDXS}(\text{chain B})$. Using this relationship and rearranging Equation 5 followed by substitution into Equation 5.4, it is possible to derive the following ratio.

$$\frac{S_{chainA}}{S_{chainB}} = \frac{surface_{chainA} \times n_A}{surface_{chainB} \times n_B} \quad (5.6)$$

Here the values of n_a and n_b correspond to the total number of exchangeable hydrogens on chain A and chain B, respectively.

5.3.5. Estimating sub-chain surface area from deuterium uptake measurements.

From Equation 6 it is possible to express the fraction of surface area of each chain according to the manner shown in Equation 5.7.

$$\frac{S_{chainA}}{S_{insulin}} = \frac{surface_{chainA} \times n_A}{Surface_{insulin} \times n_{total}} \quad (5.7)$$

For the purposes of this comparison, in Equation 5.7, S_{chainA} and $S_{insulin}$ would correspond to the deuterium uptake values of chain A and precursor ions, respectively. Knowing values for n_A and n_B (and by extension n_{total}), allows the solving of the fractional representation of $surface_{chainA}$ and $surface_{chainB}$. These can be compared to the fractions obtained from the model structures. Notably, the model structures are here considered as relatively accurate representations of only ion conformation type based on the analysis described above and depicted in Figure 5.10. Figure 5.13 shows the results of the calculation using Equation 5.7.

Figure 5.13. Bar graph showing the ratio of the exposed surface area for insulin sub-chains to the total exposed surface area of insulin. Red and blue bars compare results for the solvent excluded surface area (MD) and the calculation using the experimental deuterium uptake values (Equation 5.6), respectively. Panels a and b represent values for the more diffuse [M-4H]⁴⁺ and [M-5H]⁵⁺ insulin ions, respectively.

Although the relative surface areas of chain A and chain B are in good agreement with those for the matching structure for the $[M-5H]^{5-}$ ions, the model does not agree with the structure for the more diffuse $[M-4H]^{4-}$ ions. In Figure 5.4, the greater difference between the 100% HD scrambling and the S_{HDXS} models for the $[M-4H]^{4-}$ ions suggests that the assumption that the accessibility of all hydrogens is the same (represented by $\bar{S}_{HDXS}$) is less supported for these ions. That is, the $[M-5H]^{5-}$ ions may possess a more even distribution of charges than the $[M-4H]^{4-}$ ions and the assumption that is made to generate Equation 5.6 is less valid for the latter ions. Notably, for protein complexes, the assumption could be more valid in that the hydrogens of the same subunit within a multiple-copy complex may be the same.

A limitation in the comparison in Figure 5.13 is the inability to estimate an error for the assumption regarding hydrogen accessibility that is necessary for formulating Equation 5.6. Therefore, the comparison only provides an estimation of the degree of dissimilarity in surface area assessment. It should be noted that the disagreement observed for the $[M-4H]^{4-}$ ions may result from the fact that the average accessibility of hydrogens is more different between chain A and chain B compared to the $[M-5H]^{5-}$ ions. Thus the latter ions would represent a more uniform charge distribution. That said, we cannot rule out the possibility that a more accurate structure (or subset of structures to reflect conformer dynamics) from MD could provide better agreement for the former ions. Having noted the limitation, it is instructive to consider the potential applicability of the approach. Consider the possibility that $\bar{S}_{HDXS}$ could be scaled according to a particular

protein system (e.g., different exchange site accessibilities due to differences in charge site density). Figure 5.9 suggests that it may be possible to estimate surface area using characteristic $\bar{S}_{HDXS}$ for the different subunits within a protein complex.

5.3.6. Comparison of protonated and deprotonated SP deuterium uptake patterns.

One reason for the selection of the KKDDDDDDIIKIIK peptide for this study is the opportunity to study sequence effects on hydrogen accessibility. One comparison that can be performed is that of hydrogen accessibility for anions versus cations. The latter species are expected to have charges located on the terminal K residues, however, the anions should have deprotonation sites in the middle of the peptide. Comparing the deuterium uptake patterns of the protonated and deprotonated peptide ions is interesting because hydrogen accessibility in the respective polarity modes should be reversed. Unfortunately ETD data for $[M+2H]^{2+}$ peptide ions is inefficient and the signal level for the compact $[M-3H]^{3-}$ ions is insufficient for CID analysis. For this reason, comparisons of species of similar size (CCS) but not necessarily of the same charge state have been performed. That said, to some degree, the hydrogen accessibility model results in a data normalization that allows the comparison of species of different charge state and opposite polarity.

Figure 5.14a shows the per-residue deuterium uptake comparison of compact $[M+3H]^{3+}$ and diffuse $[M-2H]^{2-}$ KKDDDDDDIIKIIK ions. It is also noted that

Figure 5.14. Bar graph showing the comparison of deuterium uptake for positively (red)- and negatively (blue)- charged KKDDDDDIKIIK peptide ions. Panel a compares deuterium uptake for the more compact ($\Omega = 340 \text{ \AA}^2$) [M+3H]³⁺ KKDDDDDIKIIK peptide ions (red bars) and the more diffuse ($\Omega = 315 \text{ \AA}^2$) [M-2H]²⁻ KKDDDDDIKIIK peptide ions (blue bars); panel b shows the same comparison for the diffuse ($\Omega = 367 \text{ \AA}^2$) [M+3H]³⁺ KKDDDDDIKIIK ions (red bars) with the diffuse ($\Omega = 362 \text{ \AA}^2$) [M-3H]³⁻ KKDDDDDIKIIK ions (blue bars). The error bars represent one standard deviation obtained from three separate measurements of mobility-selected ions.

these ions have relatively similar collision cross sections ($340 \text{ \AA}^2$ versus $315 \text{ \AA}^2$, respectively) allowing a rough assessment of conformer type. A cursory comparison of deuterium uptake shows that the overall trend is as expected according to charge site locations. On the protonated peptide, the deuterium uptake at terminal K residues is more pronounced; on the deprotonated peptide, the deuterium uptake at interior D residues is increased. Exceptions are the D4 and D6 residues for which the protonated peptide ion has two labile hydrogens while the deprotonated peptide ions have only one; additionally, the possibility for partial HD scrambling may exacerbate this difference in deuterium uptake for the

D4 and D6 residues. This same trend is observed in the comparison of the more diffuse $[M-3H]^{3-}$ and the more diffuse $[M+3H]^{3+}$ KKDDDDDDIIKIIK ions shown in Figure 5.14b.

5.3.7. The complementary nature of positive and negative polarity HDX.

As mentioned above, a purpose of this work is to consider the utility of gas-phase HDX as a chemical probe to characterize the surfaces of subunits in protein complexes providing information about complex morphology. One advantage in using D_2O as the deuterating reagent compared with other compounds (e.g., ND_3) is the possibility to also determine charge site locations for gas-phase ions based upon deuterium uptake “patches” as described above and depicted in Figure 5.15. That is residues in closer proximity to charge sites will generally exhibit higher deuterium uptake levels and faster HDX kinetics [37]. Although deuterium uptake may occur on residues that are fairly distant to charge sites due to conformational fluctuations (“breathing”), residues within $\sim 7\text{\AA}$ of a charge site exhibit more pronounced HDX activity [41]. Therefore, for considerations of exposed surface area, the surface residues that are within $7\text{\AA}$ of a charge site can be considered accessible and the summation of the surface area presented by such residues can represent the total surface accessible by HDX.

To obtain a better understanding of sites that are protected in subunit interface regions, it would be necessary to map the HDX accessible surface as completely as possible. Figure 5.15 shows the HDX accessible surface for a nominal structure for $[M-5H]^{5-}$ insulin ions according to the type of HDX

experiment to be conducted. Notably, a large portion of the total surface area is not accessible (within 7 Å of a positive or negative site). That said, the combination of negative ion analysis does provide a significant enhancement with regard to HDX accessible surface area. For example, for the structure shown in Figure 5.15, each polarity alone can probe less than 40% of the total surface area; together both polarities can probe about 60% of the surface area. That is, there is an increase in surface area coverage of ~50% by performing HDX in positive- and negative-ion modes. A note of caution is presented. The relatively large inaccessible area should be considered cautiously. That is, a single MD structure does not provide information about which areas become accessible as a result of conformational fluctuations over the relatively long timescale of the mobility measurement.

These experiments that utilize D₂O could be supplemented with those using ND₃ as the gas-phase reagent. Because ND₃ does not require the formation of a long-lived reaction intermediate bridging two ion locations as with D₂O [37], it is suggested to be a more direct probe of hydrogen accessibility [74, 75]. Recently foundational experiments have demonstrated the use of ND₃ in HDX experiments in which ETD is used to locate sites of deuterium incorporation [40, 76]. Here, and in the manuscript accompanying the work (chapter 4), D₂O has been used because of location constraints in the HDX process. That is, the requirement that exchange occurs near charge sites provides evidence for the location of charge sites (an important consideration in MD analysis) as well as the ability to check for inter-chain scrambling using a hydrogen accessibility scoring model described

here. Future experiments would benefit from combined HDX experiments using ND₃ and D₂O. Here it will be possible to gain information about potential charge site locations as well as to increase the surface area that can be characterized.

- Area accessible by negative polarity analysis
- Area accessible by positive polarity analysis
- Area accessible for both polarities
- Inaccessible area

Figure. 5.15. Surface accessible morphological representation for the nominal structure obtained by MD for the more diffuse [M-5H]⁵⁻ insulin ions. Surface HDX accessibility (see Experimental section) areas for negatively- and positively-charged ions are color coded. The inset legend provides the color code.

5.4. Conclusions

Studies with bovine insulin ions demonstrated one advantage of HDX analysis in negative ion mode. The lack of peptide bond cleavage and HD scrambling between chain A and chain B peptides upon dissociation of insulin ions suggests that the analysis of anions may, to some degree allow the

determination of deuterium incorporation for subunit ions within protein complexes. That said, such a result may be more suitable for an ion dissociation technique with rapid energy deposition such as SID [77]. Additionally, the potential for decreased proton mobilization upon CID of negatively-charged ions may allow the generation of the intact peptide chain ions as opposed to the induction of backbone fragmentation which challenges spectral interpretation as well as the ability to conduct multi-stage tandem mass spectrometry (MS^n) of the individual peptide chain ions.

This work also shows that the deuterium uptake for each peptide chain correlates with its exposed surface area as shown by two different approaches. Although somewhat limited in the precision of the estimation of exposed surface area for some ions, values for each chain can be estimated without using nominal structures from MD for some ions. This preliminary work still is of benefit for combined IMS-MS experiments. As an example consider that MD can determine numerous morphologies with matching collision cross sections. The approach described here can be used to increase the overall selectivity of structure comparisons in order to obtain relevant MD structures.

Finally, although the HDX accessibility model analysis shows that relatively large portions of protein surfaces can be probed by HDX for select anion conformers, the fraction of the surface area that can be studied by HDX is increased significantly if complementary cation studies are conducted. That is, the different accessibility regions (with D_2O as the deuterating reagent) for protonated and deprotonated peptides provides an opportunity for mapping a much larger portion

of the ion's surface. Such an enhancement may be valuable in the future for elucidating protein-protein interaction regions for protein complex ions as well as charge site locations.

5.5. References

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